THE CURRENT STATUS OF
LIQUID SCINTILLATION
COUNTING
THE CURRENT STATUS OF LIQUID SCINTILLATION COUNTING

Edited by

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with 48 contributors

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To Janet, April, and Daggy
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In 1957 a symposium was held at Northwestern University which brought together then-current information on the theory and practice of liquid scintillation counting. The proceedings, edited by Bell and Hayes,* were invaluable to the considerable number of laboratory workers interested in applying this novel technique to radiochemical analysis. Now the technique is no longer novel. There have been many major advances since—in instrumentation, in the understanding of the scintillation phenomenon, in sample preparation, and in data handling—but no major effort, in the meantime, at gathering them together. An ever increasing number of investigators, particularly in chemistry, the life sciences, and medicine, thus find it difficult to obtain important information concerning the best methods of measuring radioisotopes by scintillation counting.

The lack of a convenient reference source, which I bemoaned in conversations with several friends at Beckman Instruments Inc. several years ago, evolved into a plan for another symposium.

With the generous financial (and moral) support of Beckman Instruments Inc. and the New England Nuclear Corporation, and the sponsorship of the Massachusetts Institute of Technology and its Department of Nutrition and Food Science, I organized an International Symposium on the Current Status of Liquid Scintillation Counting, which was held at M.I.T. from March 31 to April 3, 1969. This volume is based on the proceedings of the symposium and is intended to answer the needs of the neophyte as well as the experienced user of liquid scintillation counters.

The book is divided into sections, each introduced by a short editorial note which will, I hope, be of particular service to readers who seek, with this book, to gain an introduction to the theory and practice of liquid scintillation counting:

I. Basic Mechanisms of Liquid Scintillation (Chapters 1–3)

New developments of the past several years in the basic physics of the liquid scintillation process are intercalated with an explicit general treatment of the subject. The choices of scintillator solutes and solvents should be more straightforward after consideration of the theoretical and practical effects of such a choice on the scintillation process.

II. Uses of the Scintillation Counter (Chapters 4–12)

The history of instrument development provides an important insight into the logic of the counter. Some practical aspects of

counting statistics and counting error are reviewed, and these cannot be overemphasized. The discussion of double isotope counting, low background counting, and flow monitoring are thorough and up-to-date. Cerenkov counting of $\beta$-radioactivity in the absence of scintillation solvent or solute is a new and potentially extremely useful application, as is the use of the liquid scintillation counter for luminescence measurements.

III. The Labeled Sample (Chapters 13–27)
These chapters are of an eminently practical nature, dealing with how one handles different sorts of samples before and after placing them in scintillation vials. Much of this information has not appeared in print elsewhere.

IV. Quenching (Chapters 28–31)
The methods of quench correction are thoroughly reviewed in these chapters. Much of the information on differences between chemical and color quenching, on external standardization, and on compensation for quenching is presented here for the first time.

V. Chemiluminescence (Chapters 32–34)
There has been relatively little in the scintillation counting literature on this very important subject. It is discussed here from both theoretical and practical vantage points.

VI. Data Handling (Chapters 35–37)
Examples of the three general approaches to automatic handling of counting data and appropriate source references are given.

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The International Symposium on the Current Status of Liquid Scintillation Counting and this volume, derived from it, were subsidized by Beckman Instruments Inc. and the New England Nuclear Corporation. They agreed upon the current need for such a volume and generously supported its conception, gestation, and parturition.

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I would like to thank certain individuals here without dismissing my debts of gratitude to many others, without whom neither the Symposium nor this volume would have come to pass:

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SECTION I
BASIC MECHANISMS OF LIQUID SCINTILLATION

In considering how β-radioactivity is measured by a liquid scintillation counter, it is appropriate to divide the operation of the instrument into consecutive stages, as suggested by Birks.¹

1. Scintillator and β-interaction

A negatively charged electron (β-particle) arising from isotopic decay in a labeled sample collides with a scintillator molecule—in liquid scintillation counting, this is usually an aromatic solute (see Chapter 3) dissolved in an organic solvent (see Chapter 2). The energy of the β-particle is transmitted to the electrons and nuclei it collides with in the scintillation solution; loss of energy by a radiative process (bremsstrahlung) is of little importance with the low-energy β-emitters for which liquid scintillation counting is the most suitable method of measurement. The effect of the absorption of β-energy in such collisions on the mean path of a β can be simply derived from data on β-attenuation in air, according to Cross.² The basic physics of β-particle interactions was not considered a province of this volume, although Klein³ delivered an excellent summary at the Symposium. The reader is urged to consult Birks¹ and Rohrer⁴ for excellent brief discussions and Evans⁵ or Lipkin⁶ for more extensive presentations.

2. The Scintillation Process

The modes of conversion of absorbed β-energy to luminescence photons which are then measurable by a liquid scintillation counter are dealt with at some length in Chapters 1–3. In Chapter 1, Birks reviews the considerable advances in the understanding of energy transfer from β to scintillator solvent and solute and between solvent and solute since the publication of his authoritative monograph¹ six years ago.

In Chapter 2, Laustriat, Voltz, and Klein review the effects on scintillation efficiency of different scintillator solvents. They extend the discussion in Chapter 1 of solvent-solute energy transfer, but point out that there is some disagreement between Birks and themselves as to whether the energy transfer is collisional or a result of a long-range dipole-dipole transfer.

In Chapter 3, Horrocks reviews the basis for choice of a scintillator solute and successfully weaves together both theory and practical information of the sort he presented several years ago in the proceedings of a symposium on organic scintillators.

An occasional reader may be impatient with the theory presented in this section, but he is misled by his haste if he avoids learning what the fundamental considerations in
selecting proper counting conditions are. If he finds the terminology uncomfortable, reading Chapters 2 and 3 before the concise summary in Chapter 1 may be helpful, as will the references recommended above. In addition, Parker's recent book on photoluminescence provides lucid additional background on the basis of light emission.

3. Transmission of Photons to the Counter

Light emitted by scintillator molecules must then reach the photomultiplier cathode. The influence of solvent and solutes on the efficiency of this process (the fluorescence yield) is reviewed, not only in Chapters 1–3 but also in Section IV in discussions of "color quenching."

4. Scintillation Counter Performance

Photons are absorbed at the photomultiplier tube cathode. Photoelectrons are emitted, collected at the first dynode, and then multiplied by succeeding dynodes in the photomultiplier tube. The basic considerations in liquid scintillation counter performance and design have been summarized by Birks and in Rapkin's review of the recent history of scintillation counters (Chapter 4).

REFERENCES

THE BASIC COMPONENTS of an organic liquid scintillator are an aromatic solvent, X (e.g., toluene), and an aromatic fluorescent primary solute, Y (e.g., PPO, PBD), of molar concentration $[Y] \sim 10^{-2} M$. The $\beta$-particle which initiates the scintillation dissipates its energy in X, and a fraction of this is converted into the fluorescence of Y. To this binary solution is commonly added an aromatic fluorescent secondary solute, Z (e.g., POPOP), of molar concentration $[Z] \sim 10^{-3} M$, whose function is to shift the fluorescence spectrum to longer wavelengths.

A liquid scintillator in contact with air also contains a molar concentration $[O_2] \sim 2 \times 10^{-3} M$ of dissolved oxygen, $O_2$, unless this is expelled by nitrogen bubbling, or by other means. The assay of a radioactive specimen by the internal scintillation counting technique involves the introduction into the scintillator of a molar concentration $[Q]$ of an impurity, $Q$, consisting of the specimen and any solubilizing, dispersing, bleaching, diluting or other agents that may be used for its incorporation. The scintillator process in the pure scintillator $XYZ$ will be considered initially, and the effect of $O_2$ and $Q$ will be discussed later.

Let us first outline the sequence of events in a scintillation counter, before considering those in the scintillator in more detail. A fraction $S$ of the energy $W$ of a $\beta$-particle dissipated in the scintillator is converted into $P$ fluorescence photons of mean energy $E$, so that

$$P = \frac{S}{E}W \quad \text{photons (1)}$$

The scintillation is detected by one or more photomultipliers. A fraction $G$ of the $P$ photons fall on a photocathode of mean photoelectric quantum efficiency $C$ which emits

$$N (=GCP) = \frac{GCS}{E}W \quad \text{photoelectrons (2)}$$

into the photomultiplier dynode system. Secondary electron multiplication in the dynode chain by a gain factor of $M$ results in an anode scintillation pulse amplitude of

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The use of a scintillation counter as a \( \beta \)-spectrometer depends on proportionality between \( L \) and \( W \), so that the other parameters in Equation (3) need to be independent of \( L \) and \( W \). The instrumental energy resolution \( R \) is mainly determined by statistical fluctuations in \( N (\pm N^{1/2}) \), and it approximates to

\[ R = N^{1/2}/N = N^{-1/2} \]

Most of the \( \beta \)-particle energy is dissipated thermally and does not yield fluorescence, so that the scintillation efficiency \( S \) is relatively low. Taking typical values of \( S = 0.03 \), \( E = 3 \) eV, a \( \beta \)-particle of \( W = 10 \) keV produces \( P = 100 \) photons. A typical good bialkali photocathode has \( C = 0.25 \), so that if all the photons impinge on it (\( G = 1 \)), they yield \( N = 25 \) photoelectrons, giving an energy resolution \( R = (25)^{-1/2} = 20 \) per cent. (In a typical two-tube coincidence instrument, \( G \approx 0.5 \) for each photocathode, and \( R \) is increased.) Any inefficiency or quenching in the scintillator reduces \( S \), causing a decrease in the pulse amplitude \( L \) and an increase in the resolution \( R \).

The conversion of the energy of an ionizing particle into fluorescence involves a number of consecutive, competing and parallel processes which are known collectively as the scintillation process. The constituent processes can be conveniently divided into two groups: the radiation physical processes, or primary processes, by which the particle energy is converted into the electronic excitation energy of molecular species or otherwise dissipated; and the photophysical processes, or secondary processes, by which the electronic excitation energy is converted into fluorescence or otherwise dissipated. There is more detailed knowledge of the photophysical processes since these have been studied independently using nonionizing ultraviolet radiation for excitation.

PHOTOPHYSICS OF AROMATIC MOLECULES

Aromatic molecules are characterized by benzenoid and/or heterocyclic ring structures containing unsaturated double bonds. The molecular shape is determined mainly by the bonding \( \sigma \)-electrons which link the constituent atoms. Each single bond is formed by a pair of \( \sigma \)-electrons. The second bond of each unsaturated double bond corresponds to a pair of \( \pi \)-electrons. The \( \pi \)-electrons are much less tightly bound to their parent carbon atoms than are the localized \( \sigma \)-electrons. They are mobile within the molecular framework, and they form \( \pi \)-electron molecular orbital systems. These \( \pi \)-electron systems are responsible for the near ultraviolet absorption spectra and for the fluorescence and scintillation properties of aromatic molecules.

The unexcited ground state of the \( \pi \)-electron system in an aromatic molecule is a singlet state, \( S_0 \), in which the electron spins are paired. The system has two series of excited states: the singlet states \( S_1, S_2, \ldots, S_n \), which together with \( S_0 \) comprise the singlet manifold; and the triplet states \( T_1, T_2, \ldots, T_n \), which comprise the triplet manifold. Each series of states increases in energy up to the ionization potential. The triplet states are lower in energy than the corresponding singlet states (Hund's rule). The following terminology is used to describe molecules of \( X \) in the various states:

<table>
<thead>
<tr>
<th>State</th>
<th>Molecule</th>
</tr>
</thead>
<tbody>
<tr>
<td>Singlet</td>
<td>( ^1X )</td>
</tr>
<tr>
<td>( S_0 )</td>
<td>( ^1X^* )</td>
</tr>
<tr>
<td>( S_1 )</td>
<td>( ^1X^{**} )</td>
</tr>
<tr>
<td>( S_0(n &gt; 1) )</td>
<td>( ^3X^* )</td>
</tr>
<tr>
<td>( ^3X^{**} )</td>
<td></td>
</tr>
</tbody>
</table>

and the molecules of \( Y \) and \( Z \) are described in a similar manner.

Following excitation into \( ^1X^{**} \) or \( ^3X^{**} \), four types of intramolecular transition can occur between neighboring states of the \( \pi \)-electron system: (1) Fluorescence is a spin-allowed radiative transition between states of the same multiplicity \( (S_1 \rightarrow S_0) \). (2) Phosphorescence is a spin-forbidden radiative transition between states of different multiplicity \( (T_1 \rightarrow S_0) \). In a radiative transition the
difference in energy of the initial and final states is emitted as a photon. (3) Internal conversion is a radiationless transition between states of the same multiplicity (e.g., $S_2 \rightarrow S_1$, $T_2 \rightarrow T_1$). (4) Intersystem crossing is a radiationless transition between states of different multiplicity (e.g., $S_1 \rightarrow T_1$, $T_1 \rightarrow S_0$). A radiationless transition occurs from the initial state into a vibrationally-excited level of the final state of the same energy. After the transition the excess vibrational energy is dissipated rapidly ($\sim 10^{-13}$ s) to the environment, and the excited molecule undergoes thermal relaxation.

The main photophysical processes that occur in an aromatic molecule after excitation are as follows (Fig. 1):

(I) $S_n \rightarrow S_{n-1}$ internal conversion
(II) $S_1 \rightarrow S_0$ fluorescence
(III) $S_1 \rightarrow S_0$ internal conversion
(IV) $S_1 \rightarrow T_1$ intersystem crossing
(V) $T_n \rightarrow T_{n-1}$ internal conversion
(VI) $T_1 \rightarrow S_0$ phosphorescence
(VII) $T_1 \rightarrow S_0$ intersystem crossing.

(I) and (V) are sufficiently rapid ($\sim 10^{-11}$ to $10^{-12}$ s) that radiative transitions from $S_n$ and $T_n$ are not normally observed (Kasha's rule). The phosphorescence (VI) is too weak in intensity to be observed in solutions of normal viscosity at room temperature.

In benzene and its alkyl derivatives there is cumulative evidence for an additional process (VIII) $S_n \rightarrow S_0$ internal conversion which competes with the $S_n \rightarrow S_{n-1}$ internal conversion (I) and which is believed to occur via an unstable stereo-isomeric state.²

**Excimers**

There is another important process that occurs in fluid solutions. The collision of an excited molecule $^1X^*$ with an unexcited molecule $^1X$,

$$^1X^* + ^1X \rightleftharpoons ^1D^*$$

results in the formation of an excited dimer, $^1D^*$, which is dissociated in the ground state and is known as an excimer.³ Excimer formation occurs in benzene, toluene and other aromatic solvents⁴ and in some scintillator solutes (e.g., PPO) at high concentrations.⁵

The dissociated ground state, first excited singlet state and higher excited singlet states of an excimer are referred to as $^1X$, $^1D^*$ and $^1D^{**}$, respectively. The excimer triplet states, $^3D^*$ and $^3D^{**}$, are normally dissociated. $^1D^* \rightarrow ^1D^{**}$ absorption transitions have been identified in the nanosecond pulse radiolysis of benzene and toluene.⁶,⁷ $^1D^{**}$ can also be produced⁸ by the process

$$^1X^{**} + ^1X \rightleftharpoons ^1D^{**}$$

The photophysical processes leading to the formation, dissociation and fluorescence of excimers are as follows (see Fig. 2):

IX $^1D^{**}$ formation from $^1X^{**}$ and $^1X$
X $^1D^{**}$ dissociation into $^1X^{**}$ and $^1X$
XI $^1D^{**}$ dissociation into $^3X^{**}$ and $^1X$
XII $^1D^{**}$ dissociation into $2^1X$
Fig. 2

The term, P-type (pyrene-type) delayed fluorescence, is used to distinguish it from E-type (eosin-type) delayed fluorescence which occurs in dye solutions. The latter process, which is due to thermal activation of $T_1$ to a vibrationally excited level isoenergetic with $S_1$ and intersystem crossing thereto, does not occur in scintillator molecules because of the relatively large energy gap.

**The Scintillation Process**

The scintillation process in a liquid scintillator solution $XYZ$ can be divided into the following steps: (a) the radiation physical processes in the solvent which yield $1X^{**}$, $1D^{**}$ and $3X^{**}$; (b) the internal conversion processes by which these are converted into $1X$, $1D^*$ and $X^*$; (c) the solvent excitation migration and solvent-solute energy transfer process by which $IX^*$ and $1X$ are converted into $1^*$ (and $1Z^*$); (d) the solute-solute energy transfer process by which $1^*$ is converted into $1Z^*$; and (e) the solute fluorescence of $1Z^*$ and/or $1^*$. The scintillation emission which originates from $1X^{**}$ and $1D^{**}$ by this sequence of events occurs within a few nanoseconds (1 ns

(XIII) $1D^{**} \rightarrow 1D^*$ internal conversion
(XIV) $1D^*$ formation from $1X^*$ and $1X$
(XV) $1D^*$ dissociation into $1X^*$ and $1X$
(XVI) $1D^* \rightarrow 21X$ excimer fluorescence
(XVII) $1D^*$ dissociation into $3X^*$ and $1X$
(XVIII) $1D^*$ dissociation into $21X$

The spectrum of the excimer fluorescence (XVI) is structureless, because it occurs in a dissociated state (21X), and it is at longer wavelengths than the structured molecular fluorescence spectrum (11).

**Triplet-Triplet Interaction**

There is a further collisional process which occurs in fluid solutions

\[ 3X^* + 3X^* \rightleftharpoons 1X^* + 1D^* \]  

yielding $1X^*$ and $1D^*$. The resultant $1X^*$ and $1D^*$ fluorescence is described as P-type delayed fluorescence. Thus, we have two additional processes:

(XIX) $3X^* - 3X^*$ interaction yielding $1X^*$ and $1X$

(XX) $3X^* - 3X^*$ interaction yielding $1D^*$
PHYSICS OF THE LIQUID SCINTILLATION PROCESS

= 10^{-9} s), and it is known as the fast scintillation component. The scintillation emission which originates from $X^* \rightarrow 3X^*$ interaction (XIX, XX), yielding $1X^*$ and $1D^*$, occurs over a few microseconds (1 \mu s = 10^{-6} s) and constitutes the delayed or slow scintillation component. The relative intensities of the fast and slow components depend on the specific ionization of the ionizing radiation, and this forms the basis of the pulse shape discrimination (PSD) technique, which is used to distinguish different types of radiations, e.g., neutrons and \gamma-rays. With $\beta$-particle excitation the intensity of the slow component is relatively small, and it is quenched by dissolved O$_2$. The subsequent discussion will be confined to processes leading to or competing with the fast scintillation component.

RADIATION PHYSICAL PROCESSES

The energy $W$ of an ionizing particle passing through a solvent $X$ is dissipated by excitation into \pi-electronic states ($1X^{**}, 3X^{**}$) or \sigma-electronic states ($\sigma^{**}$), or by ionization yielding positive molecular ions ($2X^+$) and slow electrons ($e^-$) or positive and negative free radicals ($F^+, F^-$) by the following processes:

(A) $1X + W \rightarrow 1X^{**}$
(B) $1X + W \rightarrow 3X^{**}$
(C) $1X + W \rightarrow \sigma^{**}$
(D) $1X + W \rightarrow 3X^+ + e^-$
(E) $1X + W \rightarrow F^+ + F^-$

Only about 14 per cent of the electrons are \pi-electrons, so that (C) is much more probable than (A) and (B). The total excitation probability (A, B, C) is similar in magnitude to the total ionization probability (D, E).

The slow electrons (\delta-rays) from (D) can produce secondary excitation and ionization (A, B, C, D, E) and they can also lead to the following processes:

(F) $2X^* + e^- \rightarrow 1X^{**}$
(G) $2X^* + e^- \rightarrow 3X^{**}$
(H) $2X^* + e^- \rightarrow \sigma^{**}$

(J) $1X + e^- \rightarrow 2X^-$
(K) $F^+ + e^- \rightarrow F$

The molecular ions produced by (D) and (J) can lead to the further processes:

(L) $1X^* + 3X^- \rightarrow 1D^{**}$
(M) $3X^* + 3X^- \rightarrow 3D^{**}$
(N) $1X^* + 3X^- \rightarrow 2D^+$
(O) $2D^+ + e^- \rightarrow 1D^{**}$
(P) $2D^+ + e^- \rightarrow 3D^{**}$

In any process in which singlet and triplet excited species are alternative products, (AB), (FG), (LM), (OP), they are produced in the approximate ratio of 1:3, corresponding to the multiplicity weighting factor.

Processes (A), (F), (L) and (O) yield $1X^{**}$ and $1D^{**}$ which contribute to the fast scintillation component. Processes (B), (G), (M) and (P) yield $3X^{**}$ and $3D^{**}$ which contribute to the slow scintillation component. Processes (C), (E), (H) and (K) are “wasteful” processes in which the energy is dissipated thermally or results in radiation damage. Not more than 10 per cent of the \beta-particle energy yields $1X^{**}$ and $1D^{**}$.

The dimer cations ($2D^+$) of benzene, toluene, mesitylene and other aromatic hydrocarbons have been observed as radiation products in low-temperature solutions. Under normal \beta-irradiation, the scintillation spectra of benzene and toluene are identical with their photofluorescence spectra and include components of $1M^*$ and $1D^*$. Irradiation of pure benzene, toluene and other aromatic liquids at room temperature with a very intense electron beam produces a scintillation emission which originates solely from the excimer $1D^*$. The molecular ($1X^{**}, 1X^*$) excitation is completely quenched by the high ionization quenching produced by the intense electron beam. These results demonstrate the importance of excimers in the solvent scintillation process, a factor which was overlooked in earlier work on the subject.

INTERNAL CONVERSION

In an aromatic solvent the internal conversion of $1X^{**}$ and $1D^{**}$ to $1X^*$ and $1D^*$ in-
volves the complex set of competing processes shown in Fig. 2. Because of this, the photo-
fluorescence quantum yield of benzene, toluene, p-xylene and other aromatic liquids de-
pends on the excitation wavelength.8 If the aromatic liquid is diluted with cyclohexane,
it also depends on the concentration, since this influences the probability of excimer for-
mation. A kinetic analysis of the experimental data in terms of the processes shown in Fig. 2 satisfactorily accounts for the behavior.8

The $^1X^*\text{**}$ energy dissipation process (VIII) competes with the $^1X^*\text{**} \rightarrow ^1X^*$ internal conversion (I) and reduces its quantum efficiency to $q_{XX}$. The $^1D^*\text{**}$ energy dissipation processes (XI) and (XII) compete with $^1D^*\text{**} \rightarrow ^1D^*$ internal conversion (XIII) and reduce its quantum efficiency to $q_{DD}$. The experimental values of $q_{XX}$ and $q_{DD}$, which depend on the excitation wavelength $\lambda_E$, are listed in Table 1 for benzene, toluene and p-xylene.8

The overall quantum efficiency of internal conversion is given by

$$q_{IC} = x'\, q_{XX} + d'\, q_{DD} = q_{XX} - d'\, (q_{XX} - q_{DD})$$

(8)

where $x'$ and $d'$ ($= 1 - x'$) are the fraction of higher excited species in the $^1X^*\text{**}$ and $^1D^*\text{**}$ states, respectively. In the absence of experimental data on $x'$ and $d'$, we shall assume that $x' \simeq x$, $d' \simeq d$, where $x$ and $d$ are the fractions of excited species in the $^1X^*$ and $^1D^*$ states, respectively. $x$ and $d$ are known from spectroscopic studies4 and are given in Table 2. The estimated values of $q_{IC}$ thus obtained are listed in Table 1.

The energy spectrum $F(E)$ of $^1X^*\text{**}$ and $^1D^*\text{**}$ produced by the radiation physical processes extends over a range of energy $E = \hbar c / \lambda_E$ from $S_1$ up to the ionization potential $I$, which corresponds to $\lambda_E \simeq 140$ nm. The mean quantum efficiency of internal conversion is given by

$$q_{IC} = \int_{S_1}^I F(E) q_{IC}(E) dE$$

(9)

where $F(E)$ is the fraction of $^1X^*\text{**}$ and $^1D^*\text{**}$ of energy $E$. The spectrum of $^1X^*\text{**}$ produced by direct excitation (A) will approximate to the absorption spectrum of $^1X^*$, but it is not possible to assess either the energy spectra or relative fractions of $^1X^*\text{**}$ and $^1D^*\text{**}$ produced by the recombination processes (F), (L) and (O). It is of interest to note that the values of $q_{IC}$ (Table 1) increase in the order, benzene, toluene, p-xylene. This is the same order as the scintillation efficiencies of solutions containing a given solute (e.g., 3 g l$^{-1}$ PPO) in these three solvents.

### SOLVENT EXCITATION MIGRATION AND SOLVENT-SOLUTE ENERGY TRANSFER

The excited solvent molecules ($^1X^*$) and excimers ($^1D^*$) are in dynamic equilibrium

$$^1X^* + ^1X^* \rightleftharpoons ^1D^*$$

(5)

With such a system in dilute solution in an inert solvent (e.g., cyclohexane) of viscosity $\eta$, the rate $k_{DX}[^1X]$ of the forward reaction is diffusion-controlled,3 so that

$$k_{DX} = \frac{8pRT}{3000\eta}$$

(10)

where $p$ is the reaction probability per collision, $R$ is the gas constant and $T$ the absolute temperature. The rate of the backward reaction

$$k_{XD} = k_{DX}/K_e$$

(11)

### Table 1.—Quantum Efficiencies of Internal Conversion

<table>
<thead>
<tr>
<th>$\lambda_E$ (nm)</th>
<th>$q_{XX}$</th>
<th>Toluene</th>
<th>p-Xylene</th>
</tr>
</thead>
<tbody>
<tr>
<td>195</td>
<td>0.28</td>
<td>0.48</td>
<td>0.39</td>
</tr>
<tr>
<td>200</td>
<td>0.31</td>
<td>0.56</td>
<td>0.45</td>
</tr>
<tr>
<td>205</td>
<td>0.35</td>
<td>0.56</td>
<td>0.47</td>
</tr>
<tr>
<td>210</td>
<td>0.44</td>
<td>0.62</td>
<td>0.54</td>
</tr>
<tr>
<td>220</td>
<td>0.44</td>
<td>0.62</td>
<td>0.54</td>
</tr>
</tbody>
</table>
PHYSICS OF THE LIQUID SCINTILLATION PROCESS

is determined by $k_{DX}$ and by the molar equilibrium constant of the reaction

$$K_e = \frac{[1D^*]}{[1X^*][1X]}$$  \hspace{1cm} (12)

In an aromatic solvent, where the molar concentration $[1X] \sim 10 \text{ M}$, an excited molecule $[1X^*]$ can interact rapidly with any of its unexcited neighbors $1X$ to yield $1D^*$. The rate $k_{DX}[1X]$ of excimer formation is determined by the molecular collision rate, which exceeds the rate of a diffusion-controlled process shown in Eq. (10). Since $K_e$ from Eq. (12) remains constant, the excimer dissociation rate $k_{XD}$ increases with $k_{DX}$.

Rapid excimer formation and dissociation provide the mechanism proposed by Birks and Conte\textsuperscript{14} for the efficient migration of the solvent excitation energy

$$1X^* + 1X_B \rightarrow 1D_{AB}^* \rightarrow 1X_A + 1X_B^*, \text{etc.} \hspace{1cm} (13)$$

where suffixes $A$ and $B$ refer to different solvent molecules. A sequence of processes as in Eq. (13) provides a random walk process by which excitation energy migrates between different solvent molecules. The migration coefficient is

$$\Lambda = \frac{\bar{a}^2}{6t}$$  \hspace{1cm} (14)

where $\bar{a}$ is the rms displacement and $t$ is the mean time from the formation of $1X^*$ to the dissociation of $1D^*$. $t$ is given by

$$1/t = 1/k_{DX}[1X] + 1/k_{XD}$$

$$= k_{XD}d$$  \hspace{1cm} (15)

where

$$d = \frac{K_e[1X]}{1 + K_e[1X]}$$  \hspace{1cm} (16)

is the fraction of excited solvent species in the $1D^*$ state. $x (= 1 - d)$ is the fraction in the $1X^*$ state. The values of the different parameters for four aromatic solvents\textsuperscript{14} are listed in Table 2.

If $k_X$ is the sum of the rates of processes (II), (III) and (IV) competing for the $1X^*$ excitation energy, and $k_D$ is the sum of processes (XVI), (XVII) and (XVIII) competing for the $1D^*$ excitation energy, the lifetime $\tau_{OX}$ of the solvent excitation energy in the absence of a solute is given by\textsuperscript{14}

$$1/\tau_{OX} = k_{OX} = xk_X + dk_D$$  \hspace{1cm} (17)

The introduction of a molar concentration $[Y]$ of a fluorescent solute, or $[Q]$ of an impurity, increases $k_{OX}$ to

$$(k_X)_Y = k_{OX} + k_{XY}[Y]$$  \hspace{1cm} (18)

$$(k_X)_Q = k_{OX} + k_{QX}[Q]$$  \hspace{1cm} (19)

where $k_{XY}$ and $k_{QX}$ are the rate parameters of energy transfer to $Y$ and quenching by $Q$, respectively.

Certain quenchers (oxygen, carbon tetrabromide, biacetyl) quench at every collision ($p = 1$) with an excited solvent molecule. The experimental values of $k_{QX}$ for these quenchers, and of $k_{XY}$ for various fluorescent solutes, are listed in Table 3.\textsuperscript{14} Within the experimental error, the values of $k_{QX}$ and $k_{XY}$ are identical and independent of the nature of $Y$. We have concluded that the solvent-solute energy transfer occurs by a collisional process and not by a long-range dipole-dipole transfer process as suggested by other authors. (See Reference 15 and Chapter 2.)

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**Table 2.—Properties of Alkyl Benzene Solvents\textsuperscript{14}**

<table>
<thead>
<tr>
<th></th>
<th>Benzene</th>
<th>Toluene</th>
<th>p-Xylene</th>
<th>Mesitylene</th>
</tr>
</thead>
<tbody>
<tr>
<td>$[1X]$</td>
<td>11.2 M</td>
<td>9.7 M</td>
<td>8.1 M</td>
<td>7.2 M</td>
</tr>
<tr>
<td>$K_e$ (M$^{-1}$)</td>
<td>0.12</td>
<td>0.055</td>
<td>0.02</td>
<td>0.018</td>
</tr>
<tr>
<td>$\Delta$ (10$^{-6}$ cm$^2$ s$^{-1}$)</td>
<td>6.8</td>
<td>7.5</td>
<td>9.9</td>
<td>7.3</td>
</tr>
<tr>
<td>$\bar{a}$ (10$^{-8}$ cm)</td>
<td>3.3</td>
<td>3.6</td>
<td>4.2</td>
<td>4.3</td>
</tr>
<tr>
<td>$x$</td>
<td>0.43</td>
<td>0.63</td>
<td>0.86</td>
<td>0.89</td>
</tr>
<tr>
<td>$d$</td>
<td>0.57</td>
<td>0.37</td>
<td>0.14</td>
<td>0.11</td>
</tr>
<tr>
<td>$k_{XD}$ (10$^{12}$ s$^{-1}$)</td>
<td>6.5</td>
<td>9.2</td>
<td>24</td>
<td>22</td>
</tr>
<tr>
<td>$k_{DX}$ (10$^{10}$ M$^{-1}$ s$^{-1}$)</td>
<td>7.8</td>
<td>5.1</td>
<td>4.9</td>
<td>3.9</td>
</tr>
</tbody>
</table>
Table 3.—Rate Parameters of Energy Transfer ($k_{XY}$) and Quenching ($k_{QX}$) of Alkyl Benzenes
(Units: 10$^8$ M$^{-1}$ s$^{-1}$)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Solute</th>
<th>Benzene</th>
<th>Toluene</th>
<th>p-Xylene</th>
<th>Mesitylene</th>
</tr>
</thead>
<tbody>
<tr>
<td>$k_{XY}$</td>
<td>PPO</td>
<td>48 (2)*</td>
<td>56 (2)</td>
<td>62 (2)</td>
<td>51 (1)</td>
</tr>
<tr>
<td></td>
<td>p-Terphenyl</td>
<td>50 (2)</td>
<td>55 (2)</td>
<td>47 (1)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>PBD</td>
<td>55 (2)</td>
<td>63 (2)</td>
<td>54 (1)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Anthracene</td>
<td>—</td>
<td>52 (1)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Ignight</td>
<td>—</td>
<td>47 (1)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td>$k_{QX}$</td>
<td>DPA</td>
<td>34 (1)</td>
<td>55 (1)</td>
<td>61 (1)</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Blacetyl</td>
<td>—</td>
<td>55 (1)</td>
<td>—</td>
<td>—</td>
</tr>
<tr>
<td></td>
<td>Oxygen</td>
<td>57 (2)</td>
<td>56 (2)</td>
<td>63 (2)</td>
<td>54 (1)</td>
</tr>
<tr>
<td></td>
<td>CBr$_4$</td>
<td>—</td>
<td>55 (1)</td>
<td>—</td>
<td>—</td>
</tr>
</tbody>
</table>

* Values listed are mean of number of independent measurements indicated in parentheses. See Reference 14 for details.

The rate parameter of the solvent-solute energy transfer is given by

$$k_{XY} = \frac{4\pi N(A + D_Y)R}{1000} (M^{-1} s^{-1}) \quad (20)$$

where $N$ is Avogadro’s number, $R$ is the collisional interaction distance, and $D_Y$ is the diffusion coefficient of $Y$. Equation (20) differs slightly from one previously proposed$^{14}$ in that $D_X$, the diffusion coefficient of $X$, is omitted, since the diffusional motion of the solvent molecules is already included in $A$. This amendment necessitates a slight modification of some of the parameters listed in Table 2, but the changes are within the limits of experimental error.

The quantum efficiency of solvent-solute energy transfer is given by$^1$

$$f_{XY} = \frac{k_{XY}[YY]}{k_{OXY} + k_{XY}[YY]} \quad (21)$$

The fluorescence quantum efficiency $q_{OY}$ and fluorescence lifetime $\tau_{OY}$ of $^{1}Y^{*}$, in the absence of $[Z]$, are given by

$$q_{OY} = \frac{k_{FY}}{k_{FY} + k_{II}} \quad (22)$$

$$1/\tau_{OY} = k_{OY} = k_{FY} + k_{II} \quad (23)$$

where $k_{FY}$ is the rate of fluorescence (II) and $k_{II}$ is the rate of the competing radiationless transitions (III) and (IV). For an efficient primary solute (e.g., p-terphenyl, PPO, PBD, butyl PBD), $q_{OY} \geq 0.8$ ns and $\tau_{OY} \leq 2$ ns.

Some reduction in the fluorescence quantum yield $\Phi_{OY}$ may occur at high concentrations due to concentration quenching, resulting from solute excimer formation (XIV). The scintillation efficiency $S$ is proportional to the product of $f_{XY}$, which increases with $[Y]$, and $\Phi_{OY}$, which decreases with increase in $[Y]$, so that $S$ may pass through a broad maximum at an optimum concentration $[Y]$, which is not usually critical.$^1$

**SOLUTE-SOLUTE ENERGY TRANSFER**

The function of the secondary solute $Z$ is to shift the fluorescence spectrum to longer wavelengths. The two original purposes were (a) to minimize self-absorption by the solvent in large volume scintillators, and (b) to match the fluorescence spectrum more closely to the photocathode spectral response. (a) is unnecessary with the small volume (~20 ml) scintillators used in internal liquid scintillation counting. The fluorescence maxima of PPO and PBD are at 380–390 nm, and those of POPOP and dimethyl POPOP are at 440–450 nm. The original S 11 photocathodes had a peak sensitivity at 440–450 nm, so that the addition of a secondary solute was advantageous. Current bialkali photocathodes have a peak sensitivity at 440–450 nm, which matches the fluorescence spectrum of PPO and PBD, so that the addition of a secondary solute to obtain spectral matching (b) is no longer necessary with these primary solutes. There are three other circumstances in which the use of a secondary solute may be advan-
tageous: (c) when the primary solute fluorescence is at shorter wavelengths (e.g., p-terphenyl, 350–360 nm), (d) when the scintillator vial is only partially transparent to the primary solute fluorescence, and (e) when the specimen Q itself introduces self-absorption (color quenching).

There are three processes by which the excitation energy may be transferred to 1Z*: (1) Direct solvent-solute (1X* → 1Z*) transfer by a process analogous to (1X* → ly*) transfer. 1X* and 1Z* compete for the solvent excitation, and the relative probabilities of transfer to the two species are in the ratio of their concentrations [1Y]/[1Z]. Since typically [1Y] ≈ 10 [1Z], about 10 per cent of the solvent excitation energy is transferred directly to 1Z.*

(2) Radiative (1Y* → 1Z*) transfer in which the 1Y* fluorescence is absorbed by 1Z before it escapes from the solution. The probability of escape through a thickness d (cm) is

\[ \frac{I}{I_0} = 10^{-e_0 [1Z]d} \]  

(25)

where \( e_0 \) is the molar extinction coefficient of 1Z averaged over the 1Y* fluorescence spectrum. For POPOP, \( e_0 \approx 3 \times 10^4 \) over the PPO fluorescence spectrum, so that if [1Z] = 1.33 × 10^{-4} M, 99 per cent of the PPO fluorescence is absorbed by POPOP in a 5 mm thickness. (3) Radiationless (1Y* → Z*) transfer by a long-range dipole-dipole interaction process becomes effective at higher values of [1Z], and it competes directly with the 1Y* fluorescence and with the radiative transfer. The competition between radiative and radiationless solute-solute transfer has been studied previously and described elsewhere.\(^1\)

**OXYGEN QUENCHING**

The dissolved oxygen commonly present in a liquid scintillator reduces the scintillation efficiency by about 20 per cent. Oxygen can quench excited molecules at various stages of the scintillation process.

(1) The rate parameter \( k_{OZ} \) of O₂ quenching of the solvent 1X* excitation is the same as that of solvent-solute energy transfer \( k_{XY} \) (Table 3). Hence \([O₂] \) reduces the quantum efficiency of solvent-solute energy transfer (Eq. 21) by a factor

\[ \frac{(f_{x})_o}{f_{x}} = \frac{k_{OX} + k_{RX} [1Y]}{k_{OX} + k_{RX} ([1Y] + [O₂])} \]  

(26)

At high [1Y], when \( k_{RX} [1Y] \gg k_{OX}, \)

\[ \frac{(f_{x})_o}{f_{x}} \approx \frac{[1Y]}{[1Y] + [O₂]} \]  

(26a)

This type of quenching can be reduced by an increase of [1Y], the molar concentration of primary solute, or by a decrease in [O₂].

(2) The rate parameter of O₂ quenching of the primary solute excitation 1Y* is \( k_{QY} \approx 3 \times 10^{16} \) M⁻¹ s⁻¹. The 1Y* fluorescence quantum efficiency is reduced in the presence of [O₂] by a factor

\[ \frac{(q_{oY})_o}{q_{oY}} = \frac{k_{OY}}{k_{OY} + k_{QY}[O₂]} \]  

(27)

This type of quenching can be reduced by an increase in \( k_{OY} \) (i.e., a decrease in the 1Y* fluorescence lifetime \( \tau_{oY} \)) or by a decrease in [O₂]. For [O₂] = 2 × 10⁻⁵ M, and for \( \tau_{oY} = 2 \) ns, \( k_{oY} = 5 \times 10^8 \) s⁻¹, \( (q_{oY})_o/q_{oY} = 0.89 \); and for \( \tau_{oY} = 1 \) ns, \( k_{oY} = 10^9 \) s⁻¹, \( (q_{oY})_o/q_{oY} = 0.94 \).

(3) The fluorescence of the secondary solute 1Z* is quenched in a similar manner. The quenching is reduced by a decrease in the 1Z* fluorescence lifetime or by a decrease in [O₂].

(4) Oxygen is an electron acceptor, and it can therefore compete with the radiation physical processes (F), (G), (J), (O) and (P), which yield \( \pi \)-electronic excited species.

(5) Oxygen quenches the triplet state 3X* with a rate parameter of \( k_{QT} \approx 4 \times 10^9 \) M⁻¹ s⁻¹, by the process

\[ 3X* + O₂ \rightarrow 1X + 1O₂* \]  

(28)

which yields singlet-excited oxygen 1O₂*. The latter is chemically reactive and may lead to peroxidation or chemiluminescence. The quenching of 3X* inhibits the triplet-triplet interaction processes (Eq. 7) which yield the slow scintillation component.
In many ways the presence of dissolved oxygen in a liquid scintillator is undesirable, and it should be eliminated if possible.

**Impurity Quenching**

The introduction of the specimen $[Q]$ reduces the scintillation efficiency by processes similar to those described for $O_2$. This impurity quenching is often erroneously described as chemical quenching, although the quenching processes are normally physical and do not involve any chemical reaction. The impurity quenching rate parameters are usually much less than for oxygen, and they depend critically on the nature of the impurity. Compounds containing atoms of high atomic number (heavy atoms) such as Br and I are among the more effective quenchers.

In addition to impurity quenching, the specimen may absorb the primary fluorescence and thereby reduce the photon collection efficiency $G$. This effect, which is known as color quenching, can often be reduced by bleaching or other chemical treatment of the specimen prior to its introduction or by the use of a relatively high concentration $[Z]$ of secondary solute to compete with the absorption. Subsequent chapters will deal with these practical questions in more detail.

**References**

In the preparation of liquid scintillators, choice of the solvent is most important because of its fundamental double role in the scintillation process, which is to convert the kinetic energy of particles into electronic excitation energy and to transfer the latter to the dissolved fluor. In absence of impurity, the scintillation yield strongly depends upon molecular properties of the solvent which pertain to these two main requirements of energy conversion and transmission. This "intrinsic" yield may also be reduced by foreign substances present as solvent impurities or added as samples to be counted. The magnitude of this quenching effect, consisting of radiationless deactivation of excited molecules, again depends upon the solvent since it affects the two energy transfers which take place in the scintillator solution. Many earlier studies on liquid scintillators (1949–1955) have given recognition to the importance of the solvent in light output. Their general conclusions, now quite well known, can be summarized as follows: (1) the best solvents are the aromatic ones, in the order of increasing efficiency. The scale is: benzene < toluene < p-xylene;\(^2\) (2) aliphatic solvents (cyclohexane, dioxane, ...) may present, at high fluor concentration, about half the efficiency of aromatics.\(^4\)\(^5\) Dioxane, is of particular interest because its complete miscibility with water allows introduction of aqueous samples;\(^6\) (3) secondary solvents, when added to liquid scintillators, may act as simple diluents (e.g., alcohols) or as quenchers (e.g., carbon tetrachloride);\(^2\)\(^5\) toluene-methanol mixtures, which also accept water, may be advantageously used to count small quantities of aqueous samples;\(^7\) (4) addition of naphthalene (mostly in the case of dioxane-based solutions) greatly enhances scintillation efficiency.\(^8\)

Since 1955, no important discovery leading to a notable improvement in the scintillation yield has been reported. During this period, a great deal of our work has been devoted to elementary mechanisms of processes taking place in irradiated organic materials, leading
to a better understanding of the participation of the solvent to the scintillation process. Our aim in this paper is to outline results of recent investigations concerning energy transfer and quenching in scintillator solvents, in the hope that better knowledge of fundamental phenomena may bring about a more efficient utilization of liquid scintillators. In the interests of simplicity, we shall neglect all processes giving rise to delayed emission. These have recently been reviewed and are of minor importance in scintillation counting of radioactive samples.

**Transfer of Solvent Excitation Energy**

The overall energy transfer process includes a sequence of events that are best examined separately. We shall therefore begin this brief review with a somewhat more detailed discussion of the nature of the solvent excited states involved in the mechanism of light emission than that in Chapter 1. Then we shall consider the main possible processes for energy diffusion within the solvent before trapping by the solute (fluor or quencher). This analysis will finally allow some general conclusions about the influence of solvent on the scintillation yield.

**Nature of Solvent Excited States Involved in Transfer Process**

Along their paths, charged particles interact with molecules, mostly by means of “optical” collisions, the cross sections of which are approximately proportional to the square of the dipole moment of the induced molecular electronic transitions. Such interactions then result in excitation levels for which optical transitions from the ground state $S_0$ are most allowed: to high energy singlet states $S_n$ (excited or superexcited) lying near the ionization threshold. Because the mean lifetime $\theta$ of the $S_n$ states is known to be very short ($\approx 10^{-12}$ s), it is often considered as being only subject to intramolecular processes such as ionization, isomerization, dissociation into free radicals or, most important in the case of scintillation, internal conversion to a lowest energy singlet state, $S_1$. The transition (shown in Fig. 1) can be given an efficiency: $\beta_s$. It has recently been shown however, that, due to the magnitude of its transition dipole moment, the probability for interaction of the $S_n$ state with a ground state of a neighboring molecule is significant and is not negligible.

**Intermolecular** processes therefore must also be taken into account. Among such interactions between adjacent molecules 1 and 2, one is of particular interest:

$$S_n(1) + S_0(2) \rightarrow S_0(1) + S_n(2) \quad \text{(Process A)}$$

since it may give rise within the solvent to an energy migration via high energy levels, similar to the migration occurring via lowest energy excited states:

$$S_1(1) + S_0(2) \rightarrow S_0(1) + S_1(2) \quad \text{(Process B)}$$

An ionized state $S^+$, produced by a $\beta$-particle may also participate in energy transfer. Such a mechanism, which has been proposed by Hamill, cannot at the present time be readily distinguished from those referred to as Process A. Henceforth $S^+$ will be implicitly included in Process A. Experimental results supporting the existence of Process B in aromatic solvents proposed by Furst and Kallmann, have been obtained by Voltz et al. in a study of energy transfer and quenching in various liquid scintillators excited by UV light. Evidence for Process A was given by Kallmann-Oster and Klein in a series of experiments dealing with radiodissociation of chloroform dissolved in benzene or its alkyl-derivatives. In aliphatic solvents only, Process A can be invoked to account for the relatively high scintillation yield of solutions of fluor in cyclohexane, since it has been recognized that no energy transfer occurs when lowest energy states of this solvent are excited by means of light in the far UV.

Since there is experimental evidence for energy transfer from both $S_1$ and $S_n$ solvent states, it seems appropriate to examine in
Fig. 1.—Excitation energy levels of solvent and fluor. Schematic representation of energy transfer via lowest and highest excited states of the solvent.

further detail the molecular processes involved in each phenomenon.

Energy Transfer via Solvent $S_1$ States

Energy transfer from lowest energy excited states $S_1$ of the solvent to a corresponding level $F_1$ of the fluorescent solute may be represented by the reaction

$$S_1 + F_0 \rightarrow S_0 + F_1$$

where $F_0$ denotes the ground state of fluor molecules. Since the rate constant $k_t$ of this reaction is generally found to be greater than those characterizing diffusion-controlled reactions, it is now generally admitted that an additional process (often referred to as "migration") contributes to the transfer of energy within the solvent before the excitation of solute molecules. Energy transfer therefore proceeds via two successive steps (motion and trapping), which must be analyzed separately (Fig. 1).

Energy motion. Solvent molecules excited to the $S_1$ state ($M^*$) may interact with neighbor molecules in their ground state ($M$) according to Process B, illustrated here by

$$M_1^* + M_2 \rightarrow M_1 + M_2^*$$

On the other hand, since the work of Ivanova, it is now recognized that molecular interaction between $M_1^*$ and $M_2$ may also give rise to an excimer $D^*$ which is very unstable and which rapidly ($\approx 10^{-11}$ s) dissociates by back reaction:

$$M_1^* + M_2 \rightleftharpoons D^*$$  (Process C)

Birks has proposed that such a process is important in energy migration. In recent work, Voltz and Klein have given a description of energy migration which encompasses diffusion and processes B and C.
Their description, based on an analysis of elementary phenomena involved in diffusion and in excimer formation and dissociation, may be summarized as follows.

Once formed on its lowest vibrational level $D_{v0}$ (point A on the bimolecular energy diagram in Fig. 2), the excited dimer is submitted to thermal collisions with neighbor molecules and may then be rapidly raised ($\approx 10^{-11}$ s) into one of the higher vibrational levels $D_{vn}$ whose lifetime is short ($\approx 10^{-12}$ s). If the vibronic energy of $D_{vn}$ is equal to or greater than the electronic energy of the monomer $S_1$ state (point B in the diagram), the excimer can transfer its excitation energy by resonance to a neighboring molecule because the energy requirement is then achieved. The excimer may also dissociate into $M^* + M$ because monomers can then escape the potential well (Fig. 2). The first of these two processes is analogous to Process B and is responsible for energy migration. The second is nothing but an elementary step of brownian motion. If $P_M$ and $P_D$ are the probabilities for a monomer and an excimer configuration, respectively, $D_M$ and $D_D$ being the diffusion coefficients of these two molecular entities and $\lambda$ the diffusion coefficient describing resonant migration, the overall motion of the $S_1$ excitation energy into the solvent will be characterized by a total diffusion coefficient $D^*$ given by

$$D^* = P_M(D_M + \lambda) + P_DD_D$$  \hspace{1cm} (1)

The probabilities $P_M$ and $P_D$ are related to the equilibrium constant $K_e$ of Process C by

$$P_M = (1 + K_e[M])^{-1}$$  \hspace{1cm} (2)
$$P_D = K_e[M] \cdot (1 + K_e[M])^{-1}$$  \hspace{1cm} (3)

and can be experimentally determined since $K_e$ is obtained from fluorescence data.\textsuperscript{12,22} As $D^*$ may in addition be deduced from energy transfer efficiency measurements,\textsuperscript{12} it is pos-

---

**Fig. 2.** —Potential energy of a system of two solvent molecules as a function of the intermolecular distance (2a) and lowest energy levels of an isolated molecule (2b). Schematic representation of both processes of diffusion and migration occurring from a solvent excimer.
Influence of the Solvent on Scintillation Yield

Possible to evaluate $A$. Values obtained for $P_M$, $P_D$ and $A$ in aromatic solvents are given in Table 1.

**Energy Trapping by Solutes (Fluors or Quenchers).** The main problem about this last step of energy transfer is deciding whether it consists of a short-range ("collisional"; see Chapter 1) or a long-ranged (resonant) process. (For practical considerations see Chapter 3.) To give this question a reliable answer, a series of comparative experiments has been performed by Volts et al.\(^2_7\) in which the trapping rate constant $k_t$ was determined for a number of degassed solutions of different solutes (fluors or quenchers such as carbon tetrachloride) in different aromatic solvents. Under UV excitation where only $S_1$ states can be involved, two general observations emerged from the set of results: (1) The rate constant $k_t$ is always greater for a fluor than for a quencher (in which case the jump is obviously collisional). (2) For a given solvent and different fluoros, the change in the rate constant value is correlated with the variation of $R_0$, the critical distance of dipole-dipole interaction between excited solvent and ground state solute molecules. The first observation strongly supports occurrence of a long-range energy transmission to the fluor; the second suggests that this transmission is due to a Förster-type of mechanism.

**Transfer Efficiency.** The quantum efficiency $\epsilon$ for the bimolecular transfer process is related to fluor molar concentration $[F]$ by

$$\epsilon = \frac{\tau k_t[F]}{1 + \tau k_t[F]} \quad \text{(4)}$$

where $\tau$ is the mean lifetime of the solvent $S_1$ state.\(^{3_8}\) For current liquid scintillators based on aromatic solvents, typical mean values for $\tau$ and $k_t$ are $35 \times 10^{-9}$ s\(^*\) and $50 \times 10^9$ M\(^{-1}\) s\(^{-1}\),\(^\dagger\) respectively, the product of these two quantities ranging around 1700 M\(^{-1}\). It therefore follows that transfer efficiencies through $S_1$ states tend toward unity for fluor concentration as low as $10^{-2}$ M.

**Energy Transfer via Solvent $S_n$ States**

We have already mentioned that a transfer process from solvent to solute through high energy $S_n$ states was theoretically possible and had been experimentally verified. Most of the experimental work along this line has been performed with aromatic solvents containing chloroform and carbon tetrachloride as energy traps. Chloroform especially is an excellent solute with which to study energy transfer from higher excited levels, since it has been shown\(^1_2,17\) not to accept excitation energy from the lower excited states of aromatic molecules but to dissociate when the solution is irradiated by high energy particles, indicating energy migration and trapping of primary solvent higher energy states. To discuss the overall process, we shall briefly consider these two successive steps.

**Energy Migration.** Due to the magnitude of oscillator strengths of $S_n$ states ($\ell \approx 1$), the dipole-dipole interaction energy between excited and unexcited adjacent molecules is high ($\approx 1000$ cm\(^{-1}\)) and the coupling strength must be qualified as "strong" in the Förster classification.\(^3_9\) It then follows that migration by process A (above) must be much more rapid for $S_n$ states than for $S_1$ states, in which the interaction energy is about one thousand times smaller and the coupling strength "very weak." The average time of localization of energy at a given molecular site has been estimated to be of the order of $4 \times 10^{-15}$ s.\(^10\) It is therefore unlikely that excimer formation can take place in such short a time and thus neither Process C nor diffusion contributes significantly to $S_n$ migration which is a purely resonant process and must be described in terms of delocalized excitons, as with crystals.\(^1_0\)

**Energy Trapping by Solutes (Quenchers and Fluors).** Quenching by impurities often involves the formation of a charge transfer complex between the aromatic excited molecules and the quencher. The trapping of high excitation energy by impurities such as chloroform and carbon tetrachloride is therefore
likely to take place through molecular encoun-
ters. Fluorescent solutes can also trap high energy excitation, as has been shown by Weinreb\textsuperscript{17} and, indirectly, by Kallmann-Oster.\textsuperscript{18} The solvent-solute interaction is certainly more complex for fluors than for quenchers: first, because of the importance of the \( S_n \leftrightarrow S_0 \) transition dipole moments, the transfer reaction

\[
S_n + F_0 \rightarrow S_0 + F_n
\]

(where \( F_n \) denotes solute higher singlet states (see Fig. 2) is likely to occur at a distance greater than molecular dimensions; second, because back processes such as

\[
F_n + S_0 \rightarrow F_0 + S_n
\]

(where \( S_n \) represents solvent energy levels lower than primary \( S_n \) energy) also take place and compete with intramolecular conversion to the lowest excited state \( F_1 \), of the fluor (\( F_n \rightarrow F_1 \) transition). Additional experimental data are necessary for a precise understanding of energy trapping by fluorescent solutes.

**Transfer Efficiency.** Quantum efficiency \( E \) for solvent-solute energy transfer via higher levels may be expressed by a relation similar to that applying to lowest excited states:

\[
E = \frac{\Theta K_t[F]}{1 + \Theta K_t[F]}
\]

where \( \Theta \) is the mean life time of the solvent \( S_n \) states, \( K_t \) the rate constant of the transfer reaction, and \([F]\) the solute (fluor or quencher) molar concentration. Direct measurement of the efficiency \( E \) requires the excitation of \( S_n \) states with far UV radiation.\textsuperscript{17} Values of the product \( \Theta K_t \) may also be obtained by other methods. In the case of chloroform or carbon tetrachloride aromatic solvent systems, a study of the sensitized \( \gamma \)-ray-induced radiodissociation of the quencher has yielded a \( \Theta K_t \approx 60 \text{ M}^{-1} \text{ s}^{-1} \). For fluoro-aliphatic solvent systems such as PPD-cyclohexane solution, the analysis of the pulse-height versus concentration yields \( \Theta K_t \approx 100 \text{ M}^{-1} \).

These values, which are more than one order of magnitude less than \( \tau K_t \) values obtained for the \( S_1 \rightarrow F_1 \) transfer in benzene and alkyl derivatives, indicate that the efficiency of the transfer process via high energy states becomes important (\( E \approx 0.8-0.9 \)) for solute concentrations of \( 10^{-3} \text{ M} \). If we assume \( 0 \) to be of the order of \( 10^{-12} \text{ s} \) and use the expression \( K_t \) as a function of the diffusion coefficient \( \Delta_n \) of excitation energy through \( S_n \) states,\textsuperscript{19} then

\[
K_t = 4\pi \frac{N}{1000} \Delta_n R
\]

\((N \text{ is the Avogadro number and } R \text{ a bimolecular interaction distance). When } R \approx 5 \text{ Å} \text{ in case of collisional quenching, as in benzene-chloroform solutions, } \Delta_n \text{ is found as expected, to be of the order of } 10^{-1} \text{ cm}^2 \text{ s}^{-1}, \text{ a value much greater than } \Delta \text{ for energy migration via } S_1 \text{ states in aromatic solvents (} \Delta \approx 10^{-4} \text{ cm}^2 \text{ s}^{-1} \).}

**Influence of Solvent on Intrinsic Scintillation Yield**

The intrinsic scintillation yield is defined as the number of photons emitted per unit energy absorbed in an impurity free scintillator. For convenience, this quantity may be expressed in terms of \( G \) (photons), the number of quanta released per energy loss of 100 eV by a nuclear particle. If \( G(S_n) \) and \( G(S_0) = G(S_n) \cdot \beta_s \) are the radiochemical yields for the production of \( S_n \) and \( S_1 \) solvent states, respectively,

\[
G(\text{photons}) = G(S_n) [E \cdot \beta_f + \beta_s \cdot e \cdot \rho_f] \quad (6)
\]

where \( \rho_f \) and \( \beta_f \) are the fluorescence and the internal conversion (\( F_n^* \rightarrow F_1 \) transition) quantum yields of fluor. With the exception of \( \rho_f \) and \( e \), the quantities included in Equation (6) are not accurately known, although available information allows some general conclusions.

In aromatic solvents where both types of energy transfer are in evidence, the internal conversion efficiency \( \beta_s \) may be significantly less than unity; in a pure solvent \( [F] = 0 \). Indeed, \( \beta_s(\phi) \) has been found to be 0.35, 0.50
and 0.65 for benzene, toluene and p-xylene, respectively. In the presence of a solute, values of \( \beta_s \) are still lower according to

\[
\beta_s = \frac{\beta_s(o)}{1 + \Theta K_d [F]} \tag{7}
\]

In polycyclic fluor molecules, however, the internal conversion efficiency \( \beta_F \) is likely to approach unity, as with anthracene and p-terphenyl. If we take as mean typical values \( \beta_s(o) \approx 0.5, \beta_F \approx 1, \Theta K_e \approx 60 M^{-1} \) and \( \tau k_e \approx 1500 M^{-1} \), it also becomes evident that the relative importance of the two possible transfer mechanisms strongly depends on the concentration of fluor: (1) at low fluor concentration ([F] \( \approx 10^{-2} M \)), use of the above values leads to

\[
\frac{\beta_s \cdot \epsilon}{E \cdot \beta_F} \approx 5
\]

meaning that the principal migration of excitation energy within the solvent is via lowest-energy \( S_1 \) states; (2) for intermediate concentrations ([F] \( \approx 10^{-2} M \)) commonly used in liquid scintillators, both possible routes may be of the same importance, since

\[
\frac{\beta_s \cdot \epsilon}{E \cdot \beta_F} \approx 0.8
\]

and (3) at high concentrations ([F] > \( 10^{-1} M \)), where both transfer efficiencies \( E \) and \( \epsilon \) tend toward unity,

\[
\frac{\beta_s \cdot \epsilon}{E \cdot \beta_F} \approx 0.1
\]

and energy transfer via solvent \( S_n \) states should therefore be predominant.

For different aromatic solvents which are similar molecules such as benzene and its alkyl derivatives, neither \( G(S_n) \) nor \( E \) differs significantly. According to Eq. (6), the scintillation yield should depend upon the value of \( \beta_s \) and should vary: benzene < toluene < p-xylene. Experimental data indeed support this view (see Table 1 and Refs. 2 and 3).

### QUENCHING OF SOLVENT EXCITATION ENERGY

Every compound acting as a fluorescence quencher also affects the luminescence yield of organic scintillators by inducing radiationless deactivation of excited molecules of both solvent and fluor. This effect has been recognized for a long time and has most often been noted with dissolved oxygen. If one excepts compounds with absorption bands which overlap the emission spectra of excited species, (in which case the decrease in emission intensity is merely due to trivial absorption or resonance energy transfer to the impurity), the mechanisms of quenching are still uncertain. There is no physical criterion yet available which allows one to predict the quenching effect of added substances.

In recent work, we have shown that the interaction between excited \( (M^*) \) and impurity \( (Q) \) molecules is likely to give rise to a transient excited charge transfer complex \([\text{CT-exciplex} (M^+Q^-)^k]\) where donor is the excited molecule and acceptor the impurity. Such an excited complex, once formed, may

<table>
<thead>
<tr>
<th>Table 1.—Characteristic Parameters of Scintillator Solvents</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solvent</td>
</tr>
<tr>
<td>----------------</td>
</tr>
<tr>
<td>Benzene</td>
</tr>
<tr>
<td>Toluene</td>
</tr>
<tr>
<td>p-Xylene</td>
</tr>
</tbody>
</table>

\( P_M \) and \( P_D \) are the probabilities for the excitation energy \( (S_1 \) state) to be localized in a monomer and an excimer configuration, respectively. \( \Lambda \) is the diffusion coefficient characterizing the migration process via \( S_1 \) states.
BASIC MECHANISMS OF LIQUID SCINTILLATION

undergo radiationless deactivation through different processes: (1) intersystem crossing to the triplet state of the aromatic molecule; (2) transition to the ground state; or (3) dissociation into free radicals. In the present discussion, the point of interest is not the final step but the mechanism of the initial one, since the quenching probability may be nothing but the complex formation probability. Due to the importance of quenching phenomena in liquid scintillation counting, we shall briefly summarize data showing that CT-exciplex formation is involved (in many cases at least) in fluorescence quenching, and we shall then examine how such a process occurs in liquid scintillator solvents during energy migration. (See also Chapter 3 regarding the influence of exciplex formation.)

**Intervention of Charge Transfer Complexes in Fluorescence Quenching**

A charge transfer complex may be roughly considered as a bimolecular association with an electrostatic binding energy resulting from the passage of an electron from one molecule (donor) to the other (acceptor). The formation probability of such a complex is therefore greater the lower the ionization potential $I_a$ of the donor and the higher the electron affinity $E_a$ of the acceptor. If the coulombic interaction energy between the two ions is denoted by $E_c$, the electronic potential energy $E_{CT}$ of the complex is given by:

$$E_{CT} = I_d - E_A - E_c$$  \hspace{1cm} (8)

If the quenching reaction is initiated by a complex (exciplex) formation, it must be written as:

$$M^* + Q \rightarrow (M^+Q^-)^* \rightarrow M + Q$$

and the quenching rate constant $k_Q$ must be the same as the rate constant $k_e$ for complex formation. If we consider the theory of transient activated complexes in chemical reactions and take into account the fact that in the present case the donor molecule already possesses the energy $E_s$ of its first excited state $S_1$, the rate constant should then be given by:

$$k_Q = k_e = K \exp \left( \frac{-E_{CT}}{kT} \right) \exp \left( \frac{-E_s}{kT} \right)$$  \hspace{1cm} (9)

where $k$ is the Boltzmann’s constant and $K$, a term including various parameters especially depend on kinetic energies of the different molecular entities. From Equation (8), this relation may be written in the form:

$$k_Q = K \exp \left( \frac{-I_d - E_s}{kT} \right) \exp \left( \frac{E_A}{kT} \right) \exp \left( \frac{-E_c}{kT} \right)$$  \hspace{1cm} (10)

The quantity $(I_d - E_s)$ may be considered as the ionization energy $I_d^*$ of excited molecules.

To verify Eq. (10), we have determined the quenching rate constant $k_Q$ for numerous systems consisting of aromatic solvents or fluor as donors, and chloroform, carbon

<table>
<thead>
<tr>
<th>Solvent</th>
<th>Quencher</th>
<th>$E_s$ (eV)</th>
<th>$I_d$ (eV)</th>
<th>$I_d^*$ (eV)</th>
<th>$E_A$ (eV)</th>
<th>$E_c$ (eV)</th>
<th>$k_Q$ (M$^{-1}$ s$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Benzene</td>
<td>CCl$_4$</td>
<td>1.2 x 10$^3$</td>
<td>4.5 x 10$^9$</td>
<td>0.5 x 10$^8$</td>
<td>$&lt;10^7$</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Toluene</td>
<td>CHCl$_3$</td>
<td>8.8</td>
<td>4.6</td>
<td>8 x 10$^9$</td>
<td>1 x 10$^9$</td>
<td>$&lt;10^7$</td>
<td></td>
</tr>
<tr>
<td>p-Xylene</td>
<td>CHCl$_3$</td>
<td>8.5</td>
<td>4.5</td>
<td>9.1 x 10$^9$</td>
<td>4 x 10$^6$</td>
<td>$&lt;10^7$</td>
<td></td>
</tr>
</tbody>
</table>

Parameters characterizing each constituent are (for quenchers): $E_A$, electron affinity relative to that of chloromethane; (for solvents): $I_d$, ionization energy (eV) and $E_s$, excitation energy of the first excited singlet state (eV), $I_d^* = I_d - E_s$. 

Table 2.—Quenching Rate Constants $k_Q$ (1 M$^{-1}$ s$^{-1}$) for Various Solvent-Quencher Systems in Cyclohexane
INFLUENCE OF THE SOLVENT ON SCINTILLATION YIELD

Fig. 3.—Molecular excitation energy levels and ionization energies of ground state ($I_d$) and excited ($I_d^*$) molecules.

tetrachloride or aliphatic quenchers as acceptors. For each system, the quantities $I_d$ and $E_{S1}$ which characterize the donor, and the reduction potential $E_{1/2}$ of the acceptor ($E_{1/2}$ is linearly related to electron affinity) have also been measured. Results were as expected: (1) for a given excited molecule (a given donor) the quenching rate constant $k_0$ increases with increasing electron affinity (or potential reduction) of the quencher (Table 2 and Fig. 4); (2) for a given quencher (a given acceptor), $k_0$ increases with decreasing donor ionization energy $I_d^*$ (Table 2); (3) the linear relationship between log $k_0$ and $E_{1/2}$ is observed for series of like compounds (Fig. 4).

Quenching of Excitation Energy Transferred via Solvent $S_1$ States

The implication of the above results regarding the trapping by impurities of energy transfer via solvent $S_1$ states is straightforward. For a given solvent [for a given value of $(I_d - E_{S1})$], the amount of quenching will be more important the greater the electron affinity of the impurity (see Fig. 4). This explains in particular the high quenching efficiency of carbon tetrachloride and the lesser effect (about 100 times smaller) of chloroform, since the latter has a much lower electron affinity. It may also account for the very important quenching effect of molecular oxygen, since this gas is known to be an efficient electron scavenger. For a given quencher, the quenching efficiency depends on the ionization energy $I_d^*$ of excited solvent molecules. In this respect, scintillators based on benzene are less subject to quenching than those based on toluene or $p$-xylene, since $I_d^*$ is lower for the first solvent than for the other two (see Table 1). The well-known observation that the addition of naphthalene as a second solvent to scintillators, especially to
those based on dioxane, reduces the quenching effect of additives,\(^9\) may partly be explained by the fact that energy then migrates via naphthalene \(S_1\) states, which are of lower energy than those of the first solvent and therefore less subject to complex formation (since a lower value of \(E_{S1}\) brings about a greater value of \(I_d\)).\(^{10}\)

\[\text{Fig. 4.—Variation of the quenching rate constant } k_Q \text{ of toluene as a function of the polarographic reduction potential } E_{1/2} \text{ of the quencher. Experimental points correspond to the following compounds: Dots—nitromethane (1), tetranitromethane (2), methylthiomethane (3), methylidithiomethane (4), isothiocyanic ethyl ester (5), X’s—mono-, di and trichloroacetic ethyl ester (6, 7, 8), chloro- and bromopropyne (9, 10), ethyl cyanide, chloride, bromide and iodide (11, 12, 13, 14).}\]

Quenching of Excitation Energy Transferred via Solvent \(S_n\) States

According to the charge transfer complex mechanism of quenching, high energy \(S_n\) states should be more efficiently deactivated by impurities than lower ones, since their ionization potential \(I_d^{*} = I_d - E_{S_n}\) is smaller. Experimental data support this view since, as we have already mentioned, \(S_n\) states produced by \(\gamma\) rays in aromatic solvents are deactivated by chloroform (which then dissociates), while UV-induced \(S_1\) states are not affected by this compound. The sensitized dissociation of carbon tetrachloride in benzene is more important when solvent \(S_n\) states are produced (\(\gamma\)-ray excitation) than when \(S_1\) states only are created (UV excitation).\(^{12}\)

The specific action of chloroform on the higher energy levels can be used to test the participation of these levels in solvent-fluor energy transfer. This method has been utilized by Kallmann-Oster in the case of aromatics. Results shown in Fig. 5 indicate that highly excited states of dioxane and cyclohexane also are involved in the emission process, since important quenching effects of chloroform are observed in scintillators based on these solvents.

Information on the nature of the high energy levels participating in energy transfer may be obtained from observations of the action of alcohols on scintillation yield. Figure 6 shows that in concentrated scintillators based on toluene and dioxane, where \(S_n\) states are involved in the transfer process, the addition of ethanol and methanol has a small influence, merely attributable to dilution, while in the case of cyclohexane, a notable quenching effect is observed. This indicates the occurrence of a particular type of energy transfer, which, in this solvent, could involve an ionized state \(S^+\), the alcohol behaving as a trap for the positive charges.\(^{17}\)

The fact that high energy states are generally more subject to quenching than lower energy states seems to explain why purity of the solvent has such a considerable influence.

\(^{10}\)In the case of dioxane, another cause of the increase in scintillation yield resulting from naphthalene addition is obviously the better transfer efficiency of naphthalene \(S_1\) states due to their longer lifetime.
INFLUENCE OF THE SOLVENT ON SCINTILLATION YIELD

**Fig. 5.**—Effect of chloroform on the scintillation yield of solutions of diphenyl-2,5-oxadiazole (PPD) (30 g/l). \( V_0 \) and \( V \) are the pulse heights obtained with \(^{137}\)Ba conversion electrons, in the absence and presence of chloroform, respectively.

**Fig. 6.**—Effect of ethanol and methanol on the scintillation yield of solutions of PPD. \( V_0 \) and \( V \) are the pulse heights in the absence and presence of alcohol, respectively.

on scintillation yield in the case of dioxane. It has been shown that purification of dioxane may double the scintillator efficiency, whereas purification of aromatics (in which energy can be transferred by low-lying excited levels) most often results in a very small increase (5–10%) of the light output.\(^{38}\)

**CONCLUSION**

Investigations performed during the last ten years on liquid scintillator solvents have not led to major improvements in the light emission yield but have contributed to a better understanding of the elementary mechanisms taking place within solvents during the scintillation process. Molecular properties required for efficient solvents are now more clearly understood, even if numerous questions, especially pertaining to energy transfer via high energy levels, are still to be elucidated. At the present time, it seems that no spectacular increase of the scintillation yield is to be expected in the near future. On one hand, high energy states of the solvent (through which the intrinsic transfer efficiency could eventually be improved) are very sensitive to quenching effects of added substances. On the other, the most commonly used aromatic solvents already display excellent transfer properties because of the stability of the lower energy excited state of their \( \pi \) electron system.
REFERENCES

1. Birks, J. B.: Chapter 1, this volume.
D. L. Horrocks

The use of organic liquids for the conversion of the energy of ionizing radiation into photons became practical when it was demonstrated that certain organic molecules, when dissolved in certain liquids at moderate concentrations (3-10 g/l), could efficiently convert the excitation of the liquid into emission in the near ultraviolet and visible regions. That shift made it possible to measure the scintillation efficiently with commercially available multiplier phototubes.

In this paper some of the properties of these dissolved organic molecules, "fluors" or "solute," will be discussed in relation to their usefulness in liquid scintillation counting. Many of the theoretically interpreted processes, such as the yield of intersystem crossing, delayed emission, and triplet-triplet interactions, have been discussed in the preceding chapters. Unless they might help to clarify an interpretation of the practical application of the solutes to liquid scintillation counting, they will not be our concern here.

Any discussion of scintillator solutes has to include a comparison of their relative (or if possible, the absolute) efficiencies or their fluorescence quantum yield, \( \Phi \) (defined as the fraction of excited molecules which return to the ground state by the process of fluorescence). There are several processes competing for the energy of the excited molecule. A few of these are:

1. \( ^1A^* \rightarrow A + h\nu \) fluorescence
2. \( ^1A^* \rightarrow A + \text{en} \) internal conversion
3. \( ^1A^* \rightarrow ^3A^* + \text{en} \) intersystem crossing
4. \( ^1A^* + A \rightarrow 2A + \text{en} \) self-quenching
5. \( ^1A^* + A \rightarrow ^1A_2^* + \text{en} \) excimer formation
6. \( ^1A^* + Q \rightarrow A + Q + \text{en} \) quenching

where \( ^1A^\ast, ^3A^\ast, A \) are the first excited singlet, first excited triplet, and ground states, respectively, of the solute molecule; \( Q \) is a quencher molecule; "en" is radiationless energy release; and \( ^1A_2^\ast \) is the first excited singlet state of the excimer of the solute.

The physics of these processes (presented with somewhat different notation) is extensively reviewed in Chapters 1 and 2.
FLUORESCENCE QUANTUM YIELD

Relative fluorescence quantum yields can be determined by measurement of the fluorescence spectra of solutes irradiated with a known number of quanta at a known absorption efficiency. Under the experimental condition of a proper choice of wavelength of excitation quanta, it is assumed that each photon absorbed produces an excited solute molecule. Measuring the fluorescence spectra, integrating the areas under the intensity-wavelength curves and comparing the areas will give a measure of the relative fluorescence quantum yield: rel. \( \Phi \). The absolute fluorescence quantum yield, \( \Phi \), is usually then obtained by comparison with the integrated fluorescence spectrum of a solute with a known absolute fluorescence quantum yield.\(^3\) If there is a large difference between wavelength distributions, a correction for the wavelength response of the photomultiplier tube must be made. There may also have to be corrections for such things as self-absorption, geometry of sample and other similar effects. Table I lists the \( \Phi \)'s of several solvents of Chapter 2 and solutes.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Abbreviation or Name</th>
<th>Average ( \lambda ), ( \mu )</th>
<th>Fluorescence Quantum Yield, ( \Phi )</th>
<th>Decay Time in Cyclohexane, nsec</th>
</tr>
</thead>
<tbody>
<tr>
<td>( \text{O} )</td>
<td>benzene</td>
<td>280</td>
<td>0.07</td>
<td>29</td>
</tr>
<tr>
<td>( \text{O-CH}_3 )</td>
<td>toluene</td>
<td>285</td>
<td>0.17</td>
<td>34</td>
</tr>
<tr>
<td>( \text{H}_3\text{C-O-CH}_3 )</td>
<td>p-xylene</td>
<td>290</td>
<td>0.43</td>
<td>30</td>
</tr>
<tr>
<td>( \text{O-O-O} )</td>
<td>naphthalene</td>
<td>335</td>
<td>0.23</td>
<td>96</td>
</tr>
<tr>
<td>( \text{O-O-O} )</td>
<td>p-terphenyl</td>
<td>340</td>
<td>0.93</td>
<td>1.0</td>
</tr>
<tr>
<td>( \text{N-N} )</td>
<td>PBD</td>
<td>370</td>
<td>0.83</td>
<td>1.4</td>
</tr>
<tr>
<td>( \text{N-N} )</td>
<td>( \alpha )-NPO</td>
<td>405</td>
<td>0.94</td>
<td>2.1</td>
</tr>
<tr>
<td>( \text{N-N} )</td>
<td>POPOP</td>
<td>415</td>
<td>0.93</td>
<td>1.5</td>
</tr>
<tr>
<td>( \text{H}_3\text{N} )</td>
<td>( \text{Me}_2)-POPOP</td>
<td>425</td>
<td>0.93</td>
<td>1.5</td>
</tr>
<tr>
<td>( \text{O} )</td>
<td>PPO</td>
<td>370</td>
<td>1.00</td>
<td>1.4</td>
</tr>
</tbody>
</table>

*From I. B. Berlman, "Handbook of Fluorescence Spectra of Aromatic Molecules" (Academic Press, New York, 1965). The \( \Phi \) values are based on 9,10-diphenyl anthracene as 1.00.

**Dilute solutions of the solute in cyclohexane.
The fluorescence quantum yields of most of the commonly used solutes are greater than 0.85. By a proper choice of solute concentration, the transfer process can be maximized usually near 100 per cent. Thus the most inefficient process of the energy conversion is the ability to produce excited solvent molecules which can transfer energy to the solute molecules.

**SCINTILLATION EFFICIENCY**

A somewhat more practical rating of a scintillator solution is the measure of its scintillation efficiency, $S_z$. There are three main processes that determine the scintillation efficiency: (1) the number of solvent molecules excited (or ionized and recombined); (2) the ability of the excited solvent molecules to transfer the energy to the solute molecules; and (3) the fluorescence quantum yield of the solute molecule. The scintillation efficiency is defined as the ratio of the amount of energy, in the form of photons, released by the solution to the energy of excitation, namely the energy of the ionizing particle, $E_{ex}$.

$$S_z = \frac{h \int \nu n(\nu) \, d\nu}{E_{ex}} = \frac{N_{ph} E_{ph}}{E_{ex}}$$

where $n(\nu)$ is the number of photons of frequency $\nu$, and of distribution from $\nu_1$ to $\nu_2$. $E_{ph}$ is the average energy of photons emitted by the solute and $N_{ph}$ is the number of photons emitted by the solution (on the average) for stopping of a particle of energy $E_{ex}$. Obtaining an absolute scintillation efficiency is very difficult, due primarily to the difficulty of absolute measurement of photon intensity. In a recent article the scintillation efficiency of benzene was reported to be 0.04 for excitation with fast electrons. Using this value and equation for $S_z$ gives

$$0.04 = \frac{N_{ph} E_{ph}}{E_{ex}}$$

If the solute has an average wavelength of emission of 385 nm (about the average for PPO) this would correspond to an energy of 3.2 eV. Substitution of this into the above equation gives

$$N_{ph} = 12.5 E_{ex} (\text{keV})$$

Table 2 lists the number of photons ($N_{ph}$) emitted on the average for electrons of different energy, and the average number of photoelectrons that will be emitted from the photocathode of a photomultiplier tube assuming efficiencies for that wavelength of 15 per cent (for an average DuMont 6292) and 28 per cent (for an average RCA 4501). The last two columns of Table 2 give the expected coincidence counting efficiency as a function of the energy of the electron. High energy electrons are detected with 100 per cent efficiency. The probability that small numbers of photons will coincidentally produce photoelectrons in both photomultiplier tubes is obtained by the relationship

$$\text{prob.} = 1 - 2^{(1-R)} \quad \text{for } R \geq 2$$

where $R$ is the expected number of photoelectrons. Any event giving only enough photons to produce less than 2 photoelectrons, one in each of the coincident photomultiplier tubes, will therefore have a zero probability.

**Solute Concentration.** The scintillation efficiency of a solution is dependent upon the concentration of the solute or solutes (See Chapter 2). Figure 1 shows the two types of concentration dependency usually observed. At low solute concentrations the increase in scintillation efficiency with increasing concentration is primarily due to an increase in transfer efficiency. The chance of a solute molecule diffusing into the range of the energy transfer sphere of an excited solvent molecule increases with increasing solute concentration. At some concentration all (or the maximum possible) of the excited solvent molecules transfer their energy to solute molecules and further increases in concentration usually do not give an increase in solute emission. Further increases in solute concent-
Denotes the probability (Poisson) that an event from that number of photoelectrons will be measured.

Concentration are accompanied by one of two occurrences: a constant scintillation efficiency independent of concentration or a decrease due to the process of self-quenching. For most solutes the concentration giving the maximum scintillation efficiency has to be determined experimentally. Most often it is different for different solutes and may be affected by various solvents. Self-quenching involves the radiationless conversion of the excited solute molecule to the ground state through an interaction with another solute molecule in the ground state. The self-quenching process is greatly dependent upon the solute concentration. As the concentration increases, the probability of interaction of the excited and unexcited solute molecules before the solute emission can occur also increases. This process is thus, diffusion controlled.

**Quenching.** The measured scintillation efficiency can also be affected by the presence of quencher molecules in the solution. Even at very low concentrations (< 1 ppm) some compounds are extremely efficient quenchers. One of the more common quenchers present in a liquid scintillator solution (outside of the sample itself) is oxygen from the air. Not only do quenchers change the scintillation efficiency but in some cases they also

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Table 2.—Expected Average Values of Number of Photons and Photoelectrons as Function of Electron Energy

<table>
<thead>
<tr>
<th>$E_{ex}$ (keV)</th>
<th>$N_{ph}$</th>
<th>Number of Photoelectrons at Photocathode Efficiency of</th>
<th>Efficiency in Coincidence Counting System with Photocathode Efficiency of</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>15%</td>
<td>28%</td>
</tr>
<tr>
<td>1000</td>
<td>12,500</td>
<td>1,875</td>
<td>3,500</td>
</tr>
<tr>
<td>500</td>
<td>6,250</td>
<td>938</td>
<td>1,750</td>
</tr>
<tr>
<td>158</td>
<td>1,980</td>
<td>297</td>
<td>555</td>
</tr>
<tr>
<td>50</td>
<td>625</td>
<td>94</td>
<td>175</td>
</tr>
<tr>
<td>5</td>
<td>63</td>
<td>9</td>
<td>18</td>
</tr>
<tr>
<td>1</td>
<td>13</td>
<td>2 (0.86)°</td>
<td>3.5 (0.97)°</td>
</tr>
</tbody>
</table>

°Denotes the probability (Poisson) that an event from that number of photoelectrons will be measured.

---

Fig. 1.—Relative scintillation or fluorescence yields as the function of solute concentration showing the effect of solutes having self-quenching as opposed to those not having self-quenching.
change the concentration dependency. Figure 2 shows the effect of oxygen, air and nitrogen saturation of toluene solutions of different concentrations of PPO. As the concentration of oxygen increases, the maximum relative scintillation yield is decreased and the concentration at which that maximum is reached is increased.

*Spectral Overlap.* The relationship between the absorption and fluorescence spectra will influence the measured scintillation efficiency. Any overlap of these two spectra will lead to decreases in the observed fluorescence due to self-absorption of the emitted fluorescence. The greater the overlap, the more solute molecules are excited by absorption of their own fluorescence. Once reabsorption occurs, the competitive processes all have a second opportunity to prevent the emission of a photon and there is a resulting decrease in the observed scintillation efficiency.

Figure 3 shows typical absorption and fluorescence spectra of some common scintillator solutes. The amount of overlap is different for each of the solutes. The difference between the average energy of excitation (absorption) and the average energy of fluorescence is called the Stokes loss (or Stokes shift). The magnitude of the Stokes loss is often used as an indication of the amount of self-absorption expected. A large Stokes loss indicates a large difference between the wavelengths of absorption and fluorescence, a small amount of overlap and a small probability of self absorption.

*Influence of the Solvent.* That the measured scintillation efficiency is also a function of the solvent has been discussed extensively by Dr. Laustriat in Chapter 2. The main effect is attributable to differences in the ability of the solvents to transfer the particle energy (quanta) to excited solvent molecules and can be distinguished from the effect of the solvent upon excited solute molecules. Figure 4 shows the variation of the relative scintillation efficiency with different solvents as a function of PPO concentration. The variation is due entirely to the difference in the ability of the solvents to produce excited solvent molecules. Figure 5 shows that the direct excitation of the solute molecules, at least in the case of PPO, shows no solvent effect upon the relative fluorescence yield. There are, however, cases where fluorescence yield and spectra are affected by interactions...
Fig. 3.—Ultraviolet absorption and fluorescence spectra of three commonly used solutes in cyclohexane solutions showing different amounts of self-absorption.
Fig. 4.—Relative scintillation yield of a scintillator solution as a function of the concentration of PPO in three solvents; benzene, toluene and p-xylene. The mole fraction is defined as \( \gamma = \frac{n_2}{(n_1 + n_2)} \) where \( n_1 \) and \( n_2 \) are the number of moles of solvent and solute, respectively.

Fig. 5.—Relative fluorescence yield of a scintillator solution as a function of the concentration of PPO in three solvents; benzene, toluene and p-xylene.

between excited solute molecules and solvent molecules. Some of the ways that the interaction can occur are:

- \( ^1A^* + S \rightarrow A + S + \text{en} \) — quenching
- \( ^1A^* + nS \rightarrow ^1AS^*_n + \text{en} \) — exciplex formation
- \( ^1AS^*_n \rightarrow A + nS + h\nu' \) — exciplex emission

(See Chapter 2 for a discussion of the exciplex complex.) With exciplex formation there is a shift of the emission spectrum to a longer wavelength, but there is no change in the absorption spectrum.

Another type of interaction between the ground states of the solvent and solute molecules may occur:

\[ A + mS \rightarrow AS_m \]

which is then excited

\[ AS_m \xrightarrow{E_{\text{ex}}} ^1AS^* \]

and finally fluoresces

\[ ^1AS^*_m \rightarrow A + mS + h\nu'' \]

This type of interaction produces shifts in both absorption and fluorescence spectra. Both of the above mentioned types of solvent-solute interactions could lead to a lowering of the scintillation efficiency, primarily because the solvated species usually have a longer lifetime and hence a greater probability of radiationless emission (de-excitation).

Influence of Sample. The scintillation efficiency of a counting solution can also be reduced by the addition of the sample either through interaction with the sample itself with the solvent used to incorporate the sample into the counting solution, or with foreign material present in the sample. It is especially important to have the sample in a form compatible with the counting solution.

In some cases the sample has to be dissolved in a second solvent before addition to the counting solution. This can lead to a decrease in the solubility of the solute in the
mixed solvent system. If the solubility is decreased to a value at which the transfer efficiency is less than its maximum value, there will be a decrease in the measured scintillation efficiency.

Temperature. Lowering the temperature of some counting systems can also change the apparent scintillation efficiency by reducing the solubility of the solute to a concentration below that which will give the maximum transfer efficiency. This has happened with counting solutions with p-terphenyl as the solute. In one experiment involving the measurement of $^{233}$U $\alpha$-particles in a toluene solution with p-terphenyl (5 g/l), the p-terphenyl crystallized from the solution upon cooling to about $-5^\circ$ C in a refrigerated scintillation counter. Not only did the concentration decrease below the value required for maximum transfer efficiency, but some of the $^{233}$U (as the di-butyl phosphate complex) was co-precipitated by trapping in the p-terphenyl crystals.

### Relative Scintillation Response

Simplified diagrams of the processes of excitation and emission are shown in Figs. 6 through 8. Figure 6 shows the potential energy diagram as a function of the interatomic distance. The Frank-Condon principle is based on the fact that electron transi-
Fig. 8.—Simplified potential energy surface diagram showing the relationship between the energy of the process and the type of process.

Fig. 9.—Modified Jablonski diagram showing the various processes that can occur upon excitation of an organic molecule.
followed by vibrational cascade to the lowest level. The absorption, fluorescence and phosphorescence spectra are therefore broad bands covering many wavelengths (see Fig. 8).

Most solutes are rated by measuring their relative response in a common solvent to excitation by UV radiation or fast electrons of known energy. The relative fluorescence quantum yields are obtained by measurement of the fluorescence spectra for UV radiation excitation. However, the response to fast electrons, which gives the relative scintillation yield, is the more common rating method (compare Chapter 2).

Fast Electrons. The relative response of a scintillator solution of 7 g/l of PPO and 0.5 g/l of M₂-POPOP (dimethyl-POPOP) in toluene to different energy electrons is given in Figures 10 and 11. For electrons above an energy of 80 KeV, the response-energy relationship is linear:

Relative Scintillation Response =

\[ S.R. = a(E_{ex} + 18) \]  

where \( a \) is the calibration factor depending upon the gain of the electronic system and \( E_{ex} \) is the energy of the electron in keV.

The voltage output of a multiplier phototube is proportional to the number of photoelectrons produced at the photocathode. If the photocathode efficiency is constant, the output can be said to be proportional to the number of photons emitted by the scintillator solution. If a source of monoenergetic electrons is used, the relative number of photons produced by a solution can be obtained by comparison of the relative pulse height distri-

Fig. 10.—Pulse height (number of photons) as the function of the energy of electrons that excite a scintillator solution of PPO (7 g/l) and M₂-POPOP (0.5 g/l) in toluene.

Fig. 11.—Pulse height (number of photons) as a function of the energy of electrons (less 100 KeV) that excite a scintillator solution of PPO (7 g/l) and M₂-POPOP (0.5 g/l) in toluene.

Fig. 12.—Several differential pulse height spectra (relative number of photons) from scintillator solutions of different concentrations of PPO which are excited with the 370 KeV conversion electrons from the decay of \(^{113}\text{mIn}\) dissolved in the solutions.
butions. Figure 12 shows the relative pulse height distributions from solutions of different concentrations of PPO in toluene \(^{15}\) which were excited by the 370 KeV conversion electrons from the decay of \(^{113m}\)In in a source of \(^{113}\)Sn. The number of photons produced by stopping a conversion electron is reduced by decreasing the concentration of PPO. With decreasing concentrations, the peak of the pulse height distribution occurs at progressively lower pulse heights which constitute a measure of the relative scintillation efficiency of the scintillator solutions. The same technique can be used to evaluate the relative scintillation efficiency of various solutes. A major disadvantage in this use of conversion electrons is their short mean path. The sources of conversion electrons must either be dissolved in the scintillator solution or placed so that they will directly bombard the scintillator solution.

**X-rays and Gamma Rays.** Solutions can also be rated by observing their response to excitation by x-rays and gamma rays. With this technique the electrons produced by absorption or scattering of the x-rays and gamma rays are actually measured. Figure 13 shows some typical spectra of photopeaks produced by the total absorption of the energy of some low energy x-rays and gamma rays. \(^{16}\) Again the relative scintillation efficiency can be obtained by comparison of the maxima of the pulse height distributions. Because of the small number of photons produced by x-rays and gamma rays of low energy, the range of variation of scintillation efficiency is limited. High energy gamma rays can be used to determine relative scintillation efficiencies through the Compton scattered electrons produced by the interaction of the gamma rays with liquid. \(^{17}\) The maximum energy that a Compton scattered electron can obtain, \(E_{\text{max}}\), is given by

\[
E_{\text{max}} = \frac{2E_\gamma^2}{2E_\gamma + 0.51} \quad \text{(MeV)}
\]

where \(E_\gamma\) is the energy of the gamma ray (MeV) and 0.51 is the rest mass of an electron. The spectra from a liquid scintillator solution excited by Compton scattered electrons from the 0.662 MeV gamma rays of \(^{137}\)Cs and from the 0.51 and 1.277 MeV gamma rays of \(^{24}\)Na are shown in Fig. 14. The sharp edge of the distribution corresponds to the \(E_{\text{max}}\) value. There are no electrons produced with an energy greater than \(E_{\text{max}}\). For a given gamma ray source, the pulse height at which this Compton edge occurs will vary with the scintillation efficiency of the solution. Figure 15 shows the

---

![Graph](image)

**Fig. 13.**—Differential pulse height distributions (relative number of photons) from liquid scintillator solution excited externally by low energy x-rays and gamma rays. Photopeaks for the 60 KeV gamma rays of \(^{24}\)Am and the 22.5 KeV x-rays of Ag.
Fig. 14.—Differential pulse height distributions (relative number of photons) for a scintillator solution excited by Compton scattered electrons produced by external excitation by high-energy gamma rays of $^{137}$Cs and $^{22}$Na.

Because the relative scintillation efficiency is dependent upon the solute concentration, to obtain the maximum value of the relative scintillation efficiency it is necessary to measure the response of solutions with different solute concentrations. Figures 16 through 20 show some typical plots of relative scintillation yields as a function of solute concentration. To interpret these plots it is necessary to know if a given solute will self-quench or form an excimer which fluoresces.

Self-quenching and Excimer Formation. There are certain structural correlations that are useful in understanding the concentration effects, although these should not be treated as absolute rules. Self-quenching seems to occur in molecules that have unprotected, coplanar chromophores so that there can be a complete mirror image overlap of the chromophores of the excited and unexcited solute molecules. Excimer formation

Fig. 15.—Change in relative pulse height (relative number of photons) distribution for excitation of a scintillator solution with Compton scattered electrons produced by 662 KeV gamma rays of $^{137}$Cs upon addition of different amounts of quencher.
seems to follow the same structural correlations. While these are useful in helping to predict concentration effects on fluorescence, it should be remembered that the structure of the excited state of a molecule may be quite different from that of the molecule in the ground state. Bond lengths and angles may be different, electron densities are sometimes different, and quite often the reactivities of the excited molecules are different, usually more reactive.\textsuperscript{21} Self-quenching and excimer formation seem to proceed by a common pathway:

\[ \text{excimer formation} \]

\[ \text{excimer emission} \]

\[ \text{internal conversion} \]

Fig. 16.—Relative pulse height (RPH) of the Compton edge as function of concentration of 1,1'-binaphthyl and two oxyalkyl derivatives of 1,1'-binaphthyl in toluene solutions.

Fig. 17.—Relative pulse height of the Compton edge as function of concentration of 2-phenyl-5-(4-biphenylyl)-1,3,4-oxadiazole (PBD) and 2-(4-tert-butyl-phenyl)-5-(4-biphenylyl)-1,3,4-oxadiazole (butyl-PBD) in toluene solutions.
again suggesting that both phenomena have the same structural requirements. Not all self-quenching solutes will show excimer emission, however. In some cases, if an excimer is formed, it appears to be completely converted to the ground state by radiationless processes.

Under experimental conditions it is possible to observe an increase in the relative scintillation efficiency at high solute concentrations.\(^9\) In these cases it has been shown that either the wavelength response of the multiplier phototube and the spectrum of the solute are mismatched, with the excimer acting as a wavelength shifter, or that the amount of self-absorption is reduced by the shift in the fluorescence spectrum.

Two techniques of altering the solute mole-
Fig. 20.—Relative pulse height of the Compton edge as function of concentration 2-phenylindole, N-methyl-2-phenylindole and N-n-hexyl-2-phenylindole in toluene solutions.

cule have been demonstrated as ways of eliminating or reducing self-quenching and excimer formation:18-20 (1) destroying the ability of the chromophore of the solute molecule to obtain a coplanar configuration and (2) shielding the chromophore with a large bulky group which prevents the close approach and/or special orientation required (a "bumper" group). Table 3 lists several solutes and gives information about the possibility of self-quenching and excimer formation.

Temperature Changes. Changes in the temperature of solutions containing solutes that can form excimers may lead to changes in the relative scintillation efficiency. This is the result of the temperature dependence of the equilibrium between the excited monomer and excimer molecules:22

\[ A^* + A \rightleftharpoons A_2^* + \text{en} \]

A reduction in the temperature favors the formation of the excimer. An increase in the temperature favors the transformation disproportionation of the excimer into an excited monomer. Figure 21 shows the effect of temperature upon the fluorescence spectrum of a concentrated solution of PPO in toluene. The same type of shift in the fluorescence spectrum is observed with increasing concentrations of PPO at 24° C (Fig. 22). From these two illustrations it can be seen that the emission from the concentrated solution at 100° C is essentially the same as from a very dilute solution and that the fluorescence must all be from the excited monomer.

In time studies, part of the fluorescence from heated concentrated solutions has appeared to be delayed beyond prompt fluorescence.23 This is understandable since the decay time of an excimer is longer than that of a monomer. For PPO the decay times of the monomer and excimer are 1.6 and 7.9 ns (10^-9 s), respectively.24 Because of the longer lifetime of the excimer, there is a greater probability that it will be quenched before it can fluoresce. In aerated concentrated solutions of PPO, the excimer will not be ob-
served because of the preferential quenching of the excimer by oxygen.

**RELATIVE FLUORESCENCE YIELD**

Table 4 lists the relative fluorescence yields of some solutes as a function of concentration and temperature. Compounds without self-quenching show an increase in fluorescence with a decrease in temperature, (e.g., the data for TMQP and 1,1'-BN). Compounds displaying self-quenching and excimer formation show a decrease in fluorescence.

<table>
<thead>
<tr>
<th>Compound</th>
<th>Abbreviation</th>
<th>Self-quenching</th>
<th>Excimer Emission</th>
<th>Comment</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH₃H₂CH₃CH₃CH₃</td>
<td>TMQP</td>
<td>No</td>
<td>No</td>
<td>Co-planarity Destroyed</td>
</tr>
<tr>
<td>R=CH₂CH₂C₆H₅</td>
<td>QP-G16</td>
<td>Very little</td>
<td>No</td>
<td>Bumper Effect</td>
</tr>
<tr>
<td></td>
<td>PPO</td>
<td>Yes</td>
<td>Yes</td>
<td>Co-planar, Unprotected</td>
</tr>
<tr>
<td></td>
<td>PI-N1</td>
<td>Yes</td>
<td>No</td>
<td>Co-planar, Unprotected</td>
</tr>
<tr>
<td></td>
<td>PI-3M-N1</td>
<td>No</td>
<td>No</td>
<td>Co-planarity Destroyed</td>
</tr>
<tr>
<td></td>
<td>PI-3M</td>
<td>Yes</td>
<td>No</td>
<td>Thru Hydrogen Bonding, even through Co-planarity Destroyed</td>
</tr>
<tr>
<td></td>
<td>EPI-N1</td>
<td>Yes</td>
<td>Yes</td>
<td>Co-planar, Rigid</td>
</tr>
<tr>
<td></td>
<td>1,1'-BN</td>
<td>No</td>
<td>No</td>
<td>Not Co-planar</td>
</tr>
</tbody>
</table>

*a* Solubility limited.

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Table 3.—Some Solutes and Their Self-quenching and Excimer Formation Properties Related to Their Structural Properties
**Fig. 21.**—Effect of temperature on fluorescence spectrum of concentrated (204 g/l) solution of PPO in toluene.

![Graph showing the effect of temperature on fluorescence spectrum of PPO in toluene.](image)

**Fig. 22.**—Effect of concentration on fluorescence spectra of toluene solutions of PPO at room temperature.

![Graph showing the effect of concentration on fluorescence spectra of toluene solutions of PPO.](image)

**Table 4.**—Relative Fluorescence Yields of Some Solutes as Function of Temperature and Concentration

<table>
<thead>
<tr>
<th>Compound</th>
<th>Concentration as a Mole Fraction, ( \gamma )</th>
<th>Relative Fluorescence Yield ( \beta ) at</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>100°C</td>
</tr>
<tr>
<td>PPO</td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>1.8 x 10^{-2}</td>
<td>85</td>
</tr>
<tr>
<td>1,1'-BN</td>
<td>9.6 x 10^{-5}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.8 x 10^{-3}</td>
<td></td>
</tr>
<tr>
<td>TMQp</td>
<td>0.24</td>
<td></td>
</tr>
<tr>
<td>i-p-PBD</td>
<td>6.4 x 10^{-4}</td>
<td></td>
</tr>
<tr>
<td></td>
<td>2.5 x 10^{-2}</td>
<td></td>
</tr>
</tbody>
</table>

\( a \) The mole fraction is defined as \( \gamma = n_1/(n_1 + n_2) \), where \( n_1 \) and \( n_2 \) are the number of moles of solvent and solute, respectively.

\( b \) Normalized to a value of 100 for the area of the fluorescence spectrum of a gas-free toluene solution of PPO at a concentration of 3g/l.

\( c \) 2,5-diphenyl-1,3-oxazole.

\( d \) 1,1'-binaphthyl.

\( e \) 2,23,32,43-tetramethyl-p-quaterphenyl.

\( f \) 2-(4-iso-propyl-phenyl)-5-(4-biphenyl)-1,3,4-oxadiazole.
cence with a decrease in temperature at high solute concentrations (e.g., PPO and i-p-PBD). At low concentrations of i-p-PBD, the fluorescence is independent of the temperature.

In selecting a solute, it is desirable to have one with as fast a decay time as possible. Most of the commonly used solutes have decay times between 1 and 2 ns. The faster the decay time, the less probable the chance of quenching before the solute fluoresces. The solute TMQP has a decay time of about 1 ns. With this fast decay time the relative scintillation and fluorescence efficiencies are the same in solutions that are gas-free or saturated with air.

Recently there have been reports of studies of quenching of fluorescence by other competitive processes. Inter- and intramolecular proton transfer have been shown to lead to quenching and shifts in the fluorescence spectra. Hydrogen bonding between an excited solute molecule and one in the ground state has been responsible for quenching the fluorescence of some 2-phenylindole derivatives.

REFERENCES

SECTION II
USES OF THE SCINTILLATION COUNTER

The beginnings of instrumentation for scintillation counting have been described by Birks.\textsuperscript{1} Rapkin in Chapter 4 has reviewed the evolution of liquid scintillation counting from these beginnings, assuming that the reader will turn to other sources—perhaps the best being Birks’ volume—for discussion of the fundamental role of each instrument component. Chapter 4 thus includes the principal improvements in commercial involvements: the bialkali photocathode which has resulted in increased detection efficiency, decreased resolving time and decreased electronic noise in photomultiplier tubes, and more sophisticated system logic. Chapters 5–7 attempt to answer two questions for single isotope, double isotope and low background counting in turn: (1) How can one obtain confidence in his counting data? Expressed differently, how can one accurately assess instrument and counting error, so that differences between sample radioactivity and background, or the radioactivity in another sample, can be assessed? For a complete consideration of instrument performance and accuracy, the reader should turn to Sections IV and V which deal with quenching, quench correction and chemiluminescence. (2) How is the scintillation counter best set up for a particular counting application?

Chapter 8–12 describe less common uses of the scintillation counter. Schram provides an inclusive review of this history, advantages and disadvantages of flow counting, as well as practical information on the technique in Chapter 8.

The usefulness of Cerenkov counting of higher energy $\beta$s in the absence of both scintillator solute and solvent, appreciated only very recently, is emphasized by Parker and Elrick in Chapter 9. Indeed, a recent report\textsuperscript{2} suggests that the Cerenkov radiation of $\beta$ emitters can also be measured on “dry” membrane filters placed in scintillation counting vials, allowing the “liquid” of liquid scintillation counting to be omitted. That the application of this technique will increase is suggested by the exciting discovery of Ross (Chapter 10) that ($^{14}$C), theoretically below the Cerenkov threshold, can be measured as a result of anomalous refractive dispersion in the counting medium.

Chapters 11 and 12 emphasize the suitability of liquid scintillation counters for measuring luminescence rather than radioactivity, with the instrument in a noncoincidence mode. For background on the use-
fulness of chemiluminescence the reader is referred to Chapter 32, a recent monograph on photoluminescence by Parker \(^3\) and the proceedings of a symposium on fluorescence and phosphorescence analysis edited by Hercules.\(^4\)

**REFERENCES**

In the late 1940's, Kallmann at New York University and Reynolds at Princeton independently observed that dissolved activity caused certain organic materials ("scintillators") in solution to fluoresce. That fluorescence could then be measured with a photomultiplier. The first scintillator of consequence was p-terphenyl and the first solvents were aromatic hydrocarbons. Though the field of liquid scintillation counting has been active for 20 years, aromatic hydrocarbons are still preeminent and p-terphenyl is among the better scintillators, though other materials which are more convenient to use have come into greater prominence.

**EARLY DEVELOPMENTS**

Though the basic counting mixtures have undergone relatively little change during the years, the same cannot be said for the instrumentation. The first liquid scintillation counters were little more than an adaptation of the single channel analyzer used for gamma counting (Fig. 1). In these early counters, the sample container was optically coupled to the photomultiplier. The scintillation light was channeled into the photomultiplier using an optical coupling fluid. The output of the photomultiplier was then amplified and counted.

**Fig. 1.**—Single photomultiplier counter. 1952.

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*Mention of firm names or trade products is made for information only and is not to be construed as endorsement by the Massachusetts Institute of Technology, Beckman Instruments Incorporated, or New England Nuclear Corporation.

Edward Rapkin, Ph.D.: Intertechnique Instruments, Inc., Dover, N.J.
coupled to the face of a vertical photomultiplier. A reflector was employed to maximize light collection at the photocathode. The assembly was generally shielded with about 2 inches of lead. Photomultiplier gain could be varied by adjustment of high voltage to a maximum of about 10^6. At the photomultiplier output there was a preamplifier with a gain of perhaps 30 followed by a vacuum tube amplifier (gain 3000), a single channel analyzer, and finally a scaler. Results judged by modern standards were poor. Tritium efficiencies on the order of 20 percent might be obtained, but the "background" was typically in the region of 1000 cpm.

In these early counters the principal contributor to background was electronic noise generated largely in the photomultiplier but also in the preamplifier. Noise arising from the preamplifier and amplifier could be minimized by designs in which the electronic gain was held down, but this necessitated a compensatory increase in photomultiplier gain. Unfortunately, as high voltage in the photomultiplier was increased, so was internal noise generation. Because it was early recognized that some of this effect could be partly overcome by refrigerating the photomultiplier, most early liquid scintillation counters included provision for cooling.

The single photomultiplier counter has never been a commercial success, largely because of inferior performance. In the United States only Baird Atomic attempted to enter the market with such a device, but relatively few units found their way into customers' laboratories. In Europe, however, in the early days, price consideration made single tube counters potentially attractive. Counters produced by Ekco, Panax, and Nuclear Enterprises achieved modest popularity which waned as it became recognized that alternative arrangements provided appreciably better performance.

In the early 1950's, Hiebert and Hayes at Los Alamos devised the first successful coincidence counter. They recognized that the noise component of background could be largely eliminated if two photomultipliers examined the same sample and each gave rise to an output pulse in response to a legitimate decay event. Only "coincidences" were counted. Noncoincident pulses were assumed to arise from noise. In the first of these counters, the coincidence-resolving time was something less than 1 μs. The number of "accidentals" or chance coincidences arising from simultaneous generation of noise in each of the two photomultipliers was on the order of 10 cpm, a substantial reduction from the single tube noise rate of 10,000–20,000 pulse per minute under the chosen operating conditions.

Although the coincidence counter has the disadvantage that light output from a single decay event is divided into halves for each photomultiplier, thereby reducing the signal amplitude. It has the very great advantage of allowing the photomultipliers to be run at higher operating voltages and therefore at higher gains. A significant increase in individual photomultiplier noise may add only a few accidentals. The counting efficiency-to-noise ratio for these early coincidence counters, when compared to their single photomultiplier counterparts, was thereby substantially increased.

As has been the case with so many devices developed in the university and national laboratories, once the early problems were overcome and the basic principles well-understood, private industry pursued further development. This has been especially true of liquid scintillation counting. Continued discussion of the evolution of the modern counter must be centered about developments that took place in the design departments of the various nuclear instrument manufacturers.

**Initial Commercial Activity**

The first commercial coincidence counters were introduced by the Packard Instrument
DEVELOPMENT OF THE MODERN COUNTER

Company, Tracerlab, and the Technical Measurement Corporation. The Packard Tri-Carb system delivered in prototype form in 1953 to Dr. George Leroy and co-workers at the University of Chicago is shown in Fig. 2. The detector included two horizontally opposed photomultipliers (Du-mont Type 6292) housed in a refrigerated compartment. Coincidence-resolving time was 200 ns. Each photomultiplier was powered by its own independent, high-voltage supply which permitted operation at the maximum gain consistent with acceptable noise levels. The sample containers were 60-ml bottles, and silicone fluid was used for optical coupling between photomultiplier and sample.

Though the Packard prototype developed into a commercially successful instrument, the same cannot be said for the early Tracerlab unit which had both electronic problems and a clumsy sample-handling requirement involving manipulation through two rubber gloves permanently fastened to ports in the side of the refrigerator.

The Technical Measurement Corporation unit was a relatively faithful copy of the Los Alamos system concept. Performance was acceptable, and reliability was that of an adequate vacuum tube design. However, the number of units sold was disappointing and continued development effort was not pursued. In the face of competitive refinements, the design became obsolete and TMC activ-
ity in the field of liquid scintillation counting was discontinued as TMC explored other areas of nuclear instrumentation.

Since this first usable Packard counter and the entry of commercial units on the scene, the performance of liquid scintillation counting equipment as judged by counting efficiencies and background has shown marked improvement. Figures 3 and 4, taken from data in sales literature, indicate the quality of performance available over the years from commercial equipment. A brief look at various significant innovations, some of which led to this improved performance, should be instructive in understanding the present state of liquid scintillation counter design.

The early Packard Tri-Carb Spectrometer Model 314 is shown (Fig. 5) with its detector (Fig. 6) and a block diagram (Fig. 7). The system logic follows directly from that of the first prototype, with two important differences: a single high-voltage supply was employed rather than two separate ones and both photomultipliers were operated at the same high voltage. As the block diagram indicates, each of the two photomultipliers performed a different function. Pulse height analysis was performed on the output of one (termed "Analyzer"), while the second ("Monitor") served to verify coincidences. This form of system logic has been termed "unsymmetrical." It will be discussed in some detail in a later section.

The second change from prototype to commercial unit was the addition of a second scaler and an additional discriminator. This arrangement permitted measurement of one isotope in one counting channel and either a portion of that same isotope spectrum or possibly a second isotope in the second counting channel. The instrument was operated manually, timing was mechanical, and the circuitry was completely based on vacuum tubes.

**Automatic Equipment**

The first significant refinement of this design introduced in 1957 was the substitution of a 100 sample automatic sample changer for the manual unit together with

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**Fig. 3.**—Counting efficiency versus energy. Data taken from commercial sources.

**Fig. 4.**—Background for maximum efficiency versus energy. Data taken from commercial sources.
the necessary electronic timer and printout mechanism. (Figs. 8 and 9). The control unit for the sample changer mechanism was of solid state design and represents one of the earliest uses of transistorized circuitry in commercial nuclear instrumentation. Over 400 of these instruments, both manual and automatic, were manufactured from 1954 to 1960. Though by today's standards this equipment is crude, for its time it represented a substantial advance. There is little doubt that the present success of automatic liquid scintillation counting equipment can be traced directly back to these units.

Shortly thereafter, Tracerlab made a second entry into the field with a 40-sample, semi-automatic counter and then a 40-sample automatic unit. In these devices, the photomultipliers were placed at 90° angles rather than the more conventional 180°, the supposition being that light interactions between the photomultipliers would be reduced by preventing the two tubes from facing one another. Although in small measure this is true, the advantage gained in terms of background reduction is inconsequential, especially when good reflectors are employed. A far more interesting aspect of these Tracerlab counters was the use of the EMI Type 9536S photomultiplier, the first routine use of this then-superior device. Counting efficiencies were somewhat better and backgrounds lower than before. Not long thereafter, the EMI photomultiplier be-
came the standard of the industry. The Tracerlab counter was the first to employ transistorized rather than vacuum tube preamplifiers, but it is probable that at that early date (1959) the best vacuum-tube preamplifier designs were less noisy than the best transistorized circuitry.

Despite these innovations, the Tracerlab unit was a commercial failure, no doubt due to lack of recognition of important user requirements. Sample capacity was low: at ten minutes per sample, 40 vials can be examined in 7 hours. The detector was not light-tight and each time the refrigerator lid was opened it was necessary to turn off high voltage, interrupting a count in progress. Inadvertent opening caused erroneous counting results and might have caused irreparable damage to the photomultipliers. At first only a single counting channel was provided. Dual isotope samples were counted by making sequential measurements with different instrument settings. In a later design, a second spectrometer was added to provide simultaneous counting capability in two channels.

In 1960, Packard introduced the 314A series of Tri-Carb spectrometers. These instruments did not differ significantly in system logic from their vacuum tube predecessors, but they were transistorized except for their preamplifiers which retained the original vacuum tube design. The sample handling mechanism remained unchanged as did the general aspect and mode of operation. After initial difficulties were overcome, these first transistorized instruments demonstrated a significantly higher order of reliability than previous or contemporary vacuum tube units.

In 1961, the 314A series was superseded by the 314E Tri-Carb. In this unit, the first

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**Fig. 6.** Packard Model 314 single-sample detector. 1954.

**Fig. 7.** Packard Model 314 block diagram. 1954.
Fig. 8.—Packard Model 314X: the original automatic counter. 1957.

Fig. 9.—Packard Model 314X 100-sample automatic changer. 1957.
totally transistorized liquid scintillation counter, the vacuum tube preamplifier design was replaced by transistorized circuitry, again resulting in increased reliability. An even more significant innovation was related to a revision in system logic.

In previous 2-channel counters, the output of the Analyzer photomultiplier had been directed to a single amplifier whose output in turn was directed to three discriminators. Pulses falling between the lower and mid-level discriminator settings might be routed to one scaler, and pulses falling between the middle and upper levels to a second scaler. Because of limited dynamic range, isotopes that differed substantially in energy could not be satisfactorily counted simultaneously by this arrangement while the poor spectral separation did not permit differentiation of similar energies. For example, in the case of ($^3$H) and ($^{14}$C) in the same sample, the bulk of the ($^{14}$C) spectrum was found above the uppermost working limit of the upper level discriminator. To measure these two isotopes simultaneously, it was necessary to suppress operation of the upper-level discriminator and assume that all counts falling above the mid-level were $^{14}$C. Such an arrangement is inherently unsatisfactory in that it superimposes an integral background on the ($^{14}$C) channel. If a third isotope were present, that isotope could not be differentiated from the ($^{14}$C), nor was it possible to count under "balance point" conditions.

"Balance point" counting was an important aspect of the operation of these early counters that had inherent instabilities in their "front-end" electronics. Photomultipliers, high voltage preamplifiers, and discriminators were all affected by temperature change, often resulting in appreciable changes in overall system gain. By adjusting operating conditions so that an individual spectrum was essentially centered within the counting window established by two discriminator levels while making certain that some of the spectrum lay outside the window, both above and below, the effect of gain change was minimized. If the gain increased, some of the spectrum moved from the counting window above the upper-level and counts were lost, but this tended to be compensated for by a portion of the spectrum lying below the lower level moving into the counting window. Conversely, if the gain diminished, a portion of the spectrum lying in the window might fall below the lower level, but this was presumably compensated for when some of the spectrum originally above the upper level descended into the counting window.

While "balance point" operation had the virtue of providing pseudo-stability, it was necessary that a count loss be accepted. With present-day instrumentation wherein temperature stability is of a significantly higher order than in earlier devices it is no longer particularly advantageous to count at "balance point." In 1961, however, the best performance was obtainable at "balance point," and the Packard 314E series was designed to permit two isotopes to be counted simultaneously under these conditions.

The output from the Analyzer photomultiplier, after suitable preamplification, was divided along two paths, each including its own independent attenuator followed by a fixed gain amplifier, a single channel analyzer with independent upper and lower discriminators and a scaler. A coincidence gate was placed between the output of the single channel analyzer and the scaler (Fig. 10). Again, the second photomultiplier served as a Monitor and was used to verify coincidence.

The advantage of this arrangement was that the overall gain in each of the two channels could be different. With two isotopes of different energies, the isotope of lower energy was counted in one channel with little or no attenuation after increasing high voltage to such a point that the spectrum was counted under "balance point"
conditions between the two discriminator levels. The more energetic isotope was then counted in the second channel, also at "balance point," by attenuating the input signal to that channel only.

In 1961, Nuclear-Chicago entered the liquid scintillation field with a partly transistorized, largely vacuum tube, two-channel 50-sample automatic counter. System logic was comparable to the Packard 314 and 314A series and therefore would not permit "balance point" counting for two isotopes being examined simultaneously. Photomultipliers were the 11-dynode EMI 6097B which provided more gain than could be obtained with 10-stage tubes. Coincidence times were reduced to 60 ns. This first Nuclear-Chicago counter made use of a high-speed parallel printer for data output. Total printout time was on the order of 2 seconds in contrast to the 30 seconds or so required in earlier commercial devices. Though such rapid printing seems superficially attractive, especially nowadays when so many samples are being counted, the parallel printing mechanism has been completely abandoned because of high cost and unacceptable data format.

Another improvement in this early Nuclear-Chicago counter was in the detector assembly mechanism itself. It was the first in which the sample was loaded in light-tight fashion on a single vertically descending elevator, which, when in its upper-most position, made a light-tight seal with the passage through which the sample descended by virtue of felt o-rings. As the elevator descended, a leaf-type shutter closed over the sample making a second light seal before the first was broken. All modern liquid scintillation counters make use of some adaptation of this principle of a double light seal as well as the principle of graded shielding (lead, iron or steel, and copper) also introduced by Nuclear-Chicago in this counter.

Despite many worthwhile features, the original Nuclear-Chicago system was no more successful than the first Tracerlab units. Sample capacity was inadequate and system logic was inferior. Even in 1961, vacuum tube equipment was generally not competitive with fully transistorized instruments.

Channels Ratio

It is to the credit of the Nuclear-Chicago organization that they very quickly realized the shortcomings of this first unit and substituted for it a much superior device. In 1962, Nuclear-Chicago introduced a totally transistorized system, the Model 725, which established for that company an important place in the market and for a time established technological leadership. It was the first instrument to make use of a serpentine sample transport. Sample capacity was 150 (Fig. 11). For the first time, empty sample positions in the transport were automatically
bypassed and selected groups of samples could be examined to the exclusion of others (Group Counting). Three counting channels were provided and recognition was made of the general utility of the Channels Ratio method of performing quench correction. An optional mechanical calculator enabled the user to obtain counts per minute and Channel Ratio values directly in addition to the more usual raw count data.

System logic was a cross between the previous Nuclear-Chicago instrument and the Packard 314E. Two channels were tied together and preceded by a single attenuator. The third channel had its own amplifier and attenuator and could be operated at an overall gain setting different from the other two. This arrangement was quite acceptable for Channel Ratio operation since the ratio must be restricted to a single isotope which should be counted in two channels at the same gain, even if two isotopes are being measured.

In 1962, Packard responded to Nuclear-Chicago's increase in sample capacity by converting the sample changer of the Model 314E from a circular array to a serpentine transport with a 200-vial capacity. The earliest units were unreliable, but subsequent changes overcame the initial difficulties and this transport mechanism, essentially unaltered in concept, continues in successful use today.

A second innovation, incorporated in the 1962 revision of the Packard Model 314E, was the introduction of a polymethacrylate light guide to improve light collection. Previously little thought had been given to optics which consisted merely of parallel aluminum-coated reflecting surfaces. As a result of the work of Horrocks and co-workers at Argonne and Benson at Monsanto Chemical Company, it had been shown that a cylindrical guide, optically coupled to the two photomultipliers and having a hole for the sample perpendicular to the axis of the cylinder with a diameter only slightly larger than the sample vial, gave marked improvement in counting efficiency. By optimizing the design, Packard achieved this augmented efficiency with only a slight increase in background. The modern Packard counter continues to employ a light guide little changed from the earliest unit, although by virtue of improved reflector geometry, other manufacturers are able to achieve results essentially identical to those obtained through use of the light guide.

Though the original design of the Vanguard liquid scintillation counter (Fig. 12) was never completed, the prototype, exhibited and advertised in 1962, introduced

Fig. 11.—Nuclear Chicago 150-sample changer. 1962.
several interesting features that have been incorporated in more recent production instruments. The Vanguard counter had 100-sample capacity in a bench-top configuration. Refrigeration was provided. Digital display was omitted in the interest of economy. A solenoid actuated IBM typewriter with its horizontal data format was used for the first time in place of the adding machine lister heretofore employed by others. The Vanguard counter was also the first to include low count rejection and to provide for automatic repetitive counting of one sample several times before advancing to the next.

**Pulse Summation**

In 1963, the Packard Instrument Company introduced the 3000 Series of Tri-Carb Spectrometers representing a substantial improvement over past instruments. (Fig. 13). Previous designs had been based on "unsymmetrical" system logic in which the output of one photomultiplier was subjected to pulse height analysis while the second provided coincidence information only. In such an arrangement, if light, especially that arising from many low-energy decay events, is not directed equally to the two photomultipliers, there is an apparent broadening of the observed spectrum. Though there is no one universally accepted expression for resolution, one useful measure of performance is the amount of the (\(^{14}\)C) spectrum appearing above all of the (\(^{3}\)H) spectrum in an ideal sample. In an "unsymmetrical" liquid scintillation counter, approximately 40 per cent of all measurable (\(^{14}\)C) is found above all of the (\(^{3}\)H).

In the 3000 Series Tri-Carb, the outputs of the two photomultipliers were summed and examined for coincidence (Fig. 14). By summing phototube outputs, problems resulting from unequal light distribution are essentially eliminated and the system resolution is improved. In such a "symmetrical" arrangement, more than 60 per cent of all (\(^{14}\)C) is found above the (\(^{3}\)H) spectrum. This should be taken as an example of the importance of good system logic. All modern liquid scintillation counters make use of pulse summation. A further advantage of the pulse summation technique is enhancement of the signal-to-noise ratio. Since a noise pulse arising from either photomultiplier will generally not have a coincident counterpart from the other, the pulse amplitude at the output of the summation circuit will be less
than with a legitimate coincidence. In the earlier counters where coincidences were not summed, this difference did not occur. This improvement made it possible to work with amplifiers of lower gain which inherently have lower internal noise generation.

To take maximum advantage of pulse summation, the coincidence time was shortened to 20–30 ns. More photomultiplier gain was obtained by substitution of the 13 dynode EMI Type 9514 B for the previously used 10-stage Type 9536 S. Though the absolute noise rate of each 9514 B was likely to be greater than the 9536 S, the shortened coincidence time resulted in a lowered accidental rate.
The photomultipliers were operated at a fixed high voltage sufficient to permit counting of quenched (1H) or (15Fe) samples at the maximum efficiency of which the tubes were capable. For more energetic isotopes, system gain was diminished through the use of attenuators. By operating the phototubes at constant potential, not only was operation simplified by elimination of the usual high voltage controls, but system stability was enhanced by not traumatizing the tubes with changing conditions. Other manufacturers were quick to accept this improvement, and for some years all commercial devices have followed this lead. The 3000 Series Packard also included some additional refinements to previous concepts. A new detector assembly utilizing a graded shield of copper and lead resulted in improved backgrounds. A third independent counting channel was added, and the precision of attenuation control was improved by cascading three resistor strings (unfortunately each had a separate control) as a substitute for the previously used single 10-turn potentiometer. Though this resulted in improved performance, it added to the complexity of operation by substituting 9 controls where two potentiometers had previously been. The 3000 Series also included an optional mechanical calculator capable of providing counts per minute and Channels Ratio.

A system for background subtraction was incorporated in the 3000 Series. Provided that the sample was counted to preset time, the anticipated background for the counting period was dialed into thumbwheel switches that established a complimentary value in the scalers, at the start of counting. Thus, if counting was to be for ten minutes and the background for a particular channel known to be 25 cpm, the scaler would commence counting at 999750. The first 250 counts then brought the scaler contents to 000000 and were in effect subtracted from the total for the preset time period. If the background was accurately known this technique gave satisfactory results though it had the slight disadvantage of incorrect display, inasmuch as the first count was recorded as 999751 rather than 000001, and a greater disadvantage in that it was only satisfactory for preset time operation and could not be used if counting to preset count.

These instruments included provision for rejection of samples having an activity level less than some chosen figure. This feature, originally incorporated in the short-lived Vanguard liquid scintillation counter, has proved to be useful in that it permits an investigator to scan large numbers of samples but only spend time counting those having sufficient activity to be of interest. Unfortunately, the concept as introduced by Packard was less than satisfactory in that it was not possible to perform both background subtraction and low-count rejection while counting the same sample. Low-count rejection was achieved by examining the scaler contents at the end of the first minute of countings. If they exceeded some preselected level, the sample was counted; if not, the sample was rejected. Because the background subtraction system necessitated resetting the scalers to the compliment of the total anticipated background, it was apparently inconvenient to provide the circuitry that would allow the rejection system to operate under these conditions. In more modern designs, as incorporated in most of today's instruments, this objection has been overcome either by subtracting background during the count or at its conclusion, thereby allowing the one-minute scaler contents to be a reasonably true reflection of the sample count or sample count plus background rate.

The 4000 Series (Fig. 15) Packard TriCarb made its first prototype appearance in 1963, and production units were delivered somewhat later. By that time, the instruments had little that was unusual except the sample handling mechanism, to which, until recently, there was nothing comparable. Three hundred and sixty samples could be
Fig. 15.—Packard series 4000, 320-sample counter. 1963–64.

handled in removable circular trays, each holding 24 counting vials. Programming circuitry allowed selected trays to be examined to the exclusion of the others. In this regard, the equipment satisfactorily met its design objective, which was not so much to provide enlarged sample capacity but to allow individual investigators, sharing the same unit, to be assured of reserved space in the sample changer. In 1967 and 1968, Phillips carried this same concept one step farther with a device having a 420-sample capacity (21 trays of 20 samples each). Through the use of microswitches and trips, a different counting program could be called out for each tray.

In 1963, Nuclear-Chicago introduced a system of background subtraction which, in principle, permitted operation with preset count or preset time. An oscillator generated pulses at the selected background rate which served to block a corresponding number of pulses at the input to the scaler. When count rates were high, this arrangement was satisfactory, but with low count rates and low background rates, errors were substantial and cumulative, since the oscillator operated periodically but the radioactive decay rate was random. When the two rates approximated one another, the likelihood was that random events did not fall between the periodic pulses from the oscillator, while some counts that should have been subtracted were not.

**EXTERNAL STANDARDIZATION, LOGARITHMIC AMPLIFICATION**

In 1964, the ANSitron liquid scintillation counter was introduced (Fig. 16). This unit was the first liquid scintillation counter since the original Packard prototype not to follow directly from its predecessors. Even the ap-
development of the modern counter

appearance of the system was unique: the first liquid scintillation counter in which an integral cabinet design housed both sample transport and mechanics. Though the detector assembly was conventional, closely following the precedent established by Nuclear-Chicago, the remainder of the system was not. Significant background reduction was achieved by means of the quartz-faced EMI 6255 B 13-dynode photomultipliers, the first production use of these superior phototubes. The 200-sample transport was serpentine; samples were moved on adjacent parallel belts running in opposed directions rather than by means of a chain.

Of greater significance was the electronic design and overall system logic. The ANSitron was the first instrument to incorporate the use of Automatic External Standardization for evaluating quenching. At the conclusion of each sample count, a gamma source previously stored in a shielded location was moved adjacent to the sample and short recount was made in a counting channel especially reserved for this purpose. Prior to printout of data from this second count, counting data in this same channel for the sample in the absence of the gamma source were subtracted from the additive count, the remaining data being for the external source alone. By examining a series of quenched samples of known activity, it was possible to provide a calibration curve plotting counting efficiency against external source counts. The efficiency of unknown samples could then be determined through use of the external source and this curve. The value of the automatic external standard method was quickly recognized by all manufacturers and incorporated in one form or another into their equipment. At present, no automatic liquid scintillation counter being manufactured does not include this feature.

Another significant advance incorporated for the first time in the ANSitron was the logarithmic amplifier. Before the ANSitron, only linear amplifiers were used. The necessary dynamic range was achieved through use of multiple attenuators with their attendant complexity. In the ANSitron, the designers made use of symmetrical system logic and pulse summation, but the summation circuit was followed in turn by a logarithmic amplifier and a linear coincidence gate (Fig. 17).

The logarithmic element provided a potential dynamic range of almost four decades although only a bit more than three were used. Thus, if the system was calibrated so that $^{32}\text{P}$ ($E_{\text{max}} = 1710$ keV) covered an energy scale corresponding to 1000 units on a 10-turn helical potentiometer, then $^{14}\text{C}$ ($E_{\text{max}} = 154$ keV) would cover approximately 650 divisions and $^{8}\text{H}$ ($E_{\text{max}} = 18$ keV) approximately 400 divisions. The need for attenuation controls was eliminated and operation thereby simplified. Counting channels could be set by bracketing an individual peak by means of ratemeters. The simplicity of making instrument settings was further
enhanced through the use of "β-set" plug-in modules that carried fixed resistors to establish preselected discriminator levels. Use of the "β-set" assured the investigator of rapid, reproducible and error-free discriminator selection. The substance of this last feature has since been adopted by all manufacturers of liquid scintillation counting equipment. Several producers, notably Beckman, Picker and, most recently, Intertechnique, have adopted the logarithmic amplifier.

The logarithmic amplifier of the ANSitron exhibited some initial difficulties with drift with change in amplifier temperature. This problem was at least partially overcome by installing the amplifier in the controlled constant temperature environment of the refrigerated compartment. Unfortunately, this rather simple solution had a drawback: if the operating temperature was changed, the amplifier performance was altered. In more modern designs, this difficulty has been overcome by the use of integrated circuitry, which combines many elements in a single package, thereby eliminating the effects of a temperature differential between instrument components. In the most recent design from Intertechnique, the logarithmic feedback element is maintained at constant temperature through use of a miniature oven in which temperature is held constant to better than ±1°C. The ANSitron was also the first liquid scintillation counter to incorporate a quartz crystal oscillator that provided a timing standard independent of power line frequency.

A further innovation was "live timing," although that technique had been in use for some years in the field of multichannel analysis. In earlier counters, if a second pulse followed a first too quickly, two distinct types of errors were observed: the second pulse might not have been counted ("coincidence loss") or the second pulse might have been summed in amplitude with the first ("pulse pileup"). In both instances the count was lost, but in the second, the amplitude measurement of the initial pulse was of necessity in error. Investigators counting at moderate rates were not troubled by pileup but were required to make corrections for coincidence loss. One way of overcoming these difficulties was to increase the speed of the electronics. Most manufacturers have followed this approach and have designed faster amplifiers, single channel analyzers, and scalers, but the state of the art, particularly with respect to the single channel analyzer continues to limit performance at high count rates.

"Live timing" overcomes these difficulties. As soon as an input pulse is recognized and processing begun, the linear gate at the output of the amplifier is closed, thereby preventing the next pulse from attacking the system. At the same instant, timing is stopped. The clock is not advanced until the first pulse has been completely processed. When this is achieved, the gate is opened and timing restarted. The total time measured is that in which the system was available for processing coincident pulses, the dead time having been automatically compensated for. Only a few manufacturers of liquid scintillation counter equipment (e.g., Intertechnique) have apparently felt that the added sophistication of the "live timing" system was necessary.

The ANSitron background subtraction system was patterned after the earlier Nuclear-Chicago approach, but in recognition of the problem of attempting to match periodic with random pulses, included provision for storage of up to six periodic pulses until they were matched with random events. Shortly thereafter, Nuclear-Chicago adopted this same approach.

A final point worth noting in the ANSitron was the first attempt at electronic rather than mechanical computation. A simplified method of division permitted the total number of counts accumulated to be divided electronically by fixed preset times. Counts per minute could be obtained at the expense of not being able to operate to preset count.
EXTERNAL STANDARD RATIO

In 1965, Beckman Instruments Inc. introduced the LS 200 Liquid Scintillation Counter (Fig. 18). It was in many ways similar to the ANSitron but carried the previous concept farther, particularly in the realm of computation. A fully electronic calculator was included, permitting counts per minute to be obtained whether the sample had counted to preset time or to preset count. Background subtraction was accomplished by establishing the known background rate on thumbwheel switches and then subtracting after calculation of gross counts per minute. The Teletype page printer with its superior data format was offered for the first time as a standard item. (The same unit had been used previously on specially constructed ANSitron and Packard systems).

Front-end electronics were superficially similar to but very different from those of the ANSitron. The photomultipliers were operated in such a manner that a pseudo-logarithmic response was attained. Their outputs were then linearly summed and subjected to linear amplification followed by pulse height analysis and then coincidence (Fig. 19). While this approach does not give a precise logarithmic energy relationship between isotope spectra, dual isotope separation and overall performance are nevertheless quite good.

The most important aspect of this first Beckman automatic liquid scintillation counter (a concept also included in the Nuclear Chicago Mark I counter introduced at approximately the same time) was the inclusion of the external standard ratio method of quench correction, a significant advance over the gross count method then being used in both the ANSitron and Packard counters. Although the external standard method as originally devised, simplified the determination of counting efficiency, it had some real and potential disadvantages: induced count rate varied with sample volume, with counting vial wall thickness and composition, and with the nonreproducibility of sample or source positioning. (See Chapter 30). The Channels Ratio method of quench correction...
had been based on the observation that the ratio of counts in two channels, each bracketing a different portion of a single isotope spectrum, was an indication of spectral shape. Changes in this ratio indicated spectral shift; hence, quenching. The Nuclear Chicago and Beckman design teams, following the earlier lead of Kaufman and co-workers, applied the same principle to the external standard: the external standard spectrum was examined in two regions and the ratio was computed and related to changes in quenching. Today almost all commercially available liquid scintillation counters enable the user to obtain the external standard ratio rather than, or in addition to, the external standard gross count. Two completely independent counting channels dedicated to external standard ratio determination were added to the original three counting channels. This so-called "3 + 2" system has considerable merit when compared to equipment in which the external standard and sample counting channels are the same and have the same settings, since the optimum sample counting channel may not be best for the external standard. By making fixed external standard channels possible, quenching effects between similar samples not counted under the same conditions may be compared by comparing external standard ratios. This is particularly useful in working with single- and dual-isotope samples that arise from parallel experiments but are best counted with different settings.

The Beckman LS 200 was also the first production instrument in which the RCA photomultipliers now known as Type 4501 were routinely employed. These high-performance unusually noise-free photomultipliers made possible, for the first time, high-performance liquid scintillation counting at room temperature. Most Beckman units delivered to date have been nonrefrigerated. Although room-temperature background levels using the newest photomultipliers can be as low as those obtained under refrigerated conditions, many still favor operating at constant temperatures below ambient.

In 1965, Nuclear-Chicago introduced the Mark I counter, the first liquid scintillation counter to make extensive use of integrated circuitry (Fig. 20). The volume of electronics was substantially reduced and the reliability significantly enhanced. In addition to the previously noted incorporation of external standard ratio computation, the Mark I included an electronic calculator and provision for subtraction of background after gross counts per minute computation. Though the data format arising from the Mark I was perhaps the least satisfactory and most complicated ever to arise from a liquid scintillation counter, the Mark I enjoyed substantial commercial success primarily because of good technical execution and its high order of dependability.

Also introduced in 1965 was the Picker...
Liquimat, a 200-sample automatic counter, again largely patterned on the ANSitron with external standardization and logarithmic amplification. The Liquimat, however, had the unique feature of incorporating facilities for gamma counting. A crystal/photomultiplier assembly was placed in the sample changer mechanism in such a way that the sample elevator passed through a cylindrical hole cut in the crystal perpendicular to the long axis. The elevator mechanism was designed to stop part-way down for gamma counting. At the conclusion of the gamma count, the sample could still be lowered farther to the beta-counting position between opposed photomultipliers or it could be elevated back to the sample transport, allowing the next sample to be examined for gamma activity only. Although in the first units there was evidence of inadequate shielding which resulted in elevated beta backgrounds when there was gamma activity on the transport, this problem was apparently eliminated in later units.

The Packard Model 3375 (Fig. 21) was first offered in 1966. It substituted an electronic calculator for the previous mechanical version. Extensive use was made of integrated circuitry, although the system logic, sample-handling features, and detectors were essentially unaltered from those of previous instruments in the Packard series. A solenoid-activated IBM Selectric Type-writer provided a data format more acceptable than the format from the adding machine lister or mechanical calculator used in the past. Preadjusted counting windows were pushbutton-selectable. Counting performance was improved through use of EMI 9634QR quartz-faced photomultipliers having the “Super S-11” cathode. External standard ratios were determined using the “3 + 2” system introduced by Beckman.

Also in 1966, the Nuclear-Chicago Company offered Unilux I, a 100-sample benchtop liquid scintillation counter, while Beckman offered the LS 100, another 100-sample unit. These counters were notable for their small size, simplicity, and low cost. The Beckman LS 100 and the newer LS 150 were the first automatic counters offered since the Vanguard which had no digital display. Only a single counting channel was provided, but provision was included to permit sequential counting in that channel with up to three different channel settings. Thus 1, 2 or 3 isotope samples could be handled. A trade-off between instrument cost and counting time was achieved. The Unilux I also had a single counting channel and provision for sequential counting of the same sample with different settings. Though useful devices, neither instrument merits extensive discussion as they incorporated no particular advance in terms of features or technology. They were and continue to be stripped-down versions of the more elaborate, large automatic counters. For those who do not require either the sample size or the more intricate features and data presentation of the larger units, these instruments and recently introduced counterparts...
from other manufacturers have proven to be both worthwhile and economical.

Among the inexpensive units, the Nuclear-Chicago Unilux II is an updated version of the Unilux I. While still retaining 100-sample capacity, it is available with refrigeration and 1, 2 or 3 counting channels. The Packard Model 3320 is a low-cost 200-sample refrigerated system. Three counting channels, external standardization, and background subtraction are provided. The Intertechnique Model SL 30, also a 200-sample, 3-channel, controlled-temperature unit, includes provision for two additional channel settings for external standardization similar to those of the "3 + 2" concept. The Picker Model 2211 is a 100-sample instrument having two counting channels and two additional channels for external standardization. In many ways similar to the Beckman LS 100 and the more recent LS 150, the Model 2211 includes neither visual display nor provision for reduced temperature operation.

**Correction for Quenching**

1967 was a year in which both Packard and Beckman announced means to nullify, at least partly, the effects of quenching. The approaches taken were quite different and each is interesting in its own way.

The Packard Model 544 AAA ("Absolute Activity Analyser") was first announced in mid-April of that year. It was originally intended to be an accessory for the Model 3375, but extensive modifications to that unit were required to make it compatible with the Model 544, so that a new basic instrument, Model 3380, was introduced. The Model 3380 was superficially identical to the Model 3375 but included all of the internal modifications required for compatibility with the Model 544. Production units of the Model 544 were delivered in 1968.

The heart of AAA is a system for defocusing the two photomultipliers in response to the observed external standard ratio. Each phototube is surrounded by a defocusing coil. A magnetic field generated by passage of current through the coil is capable of causing the photomultipliers to perform in less efficient fashion than might otherwise be the case. In advance of sample counting, the instrument is calibrated with a series of known quenched standards. Data points representing counting efficiencies versus external standard ratio are determined and the various values are set in banks of switches. When an unknown is counted, the external standard ratio is first determined. If it is not equivalent to the values for one of the standard data points, a first-order correction is made by passage of current through the defocusing coils. The ratio is then redetermined. If this second value does not lie precisely on one of the data points, a smaller second-order correction is made and again the ratio is determined. The process is repeated as many as 15 times, the mean value being reported as about 5. Once the artificially created external standard ratio is identical to the experimentally determined ratio for one of the standard data points, the sample is counted. After counting is completed, the observed cpm value is divided by the now-known counting efficiency and disintegrations per minute are recorded. For dual isotope counting, the operating procedure is identical after having previously established the necessary information for each of two isotopes and the cross-countribution.

The Beckman Models LS 150 and LS 250, direct successors to the earlier LS 100 and LS 200, include provision for altering counting conditions in direct response to quenching effects. The Beckman AQC ("Automatic Quench Calibration") is intended to compensate automatically for varying quench between samples by readjusting system gain before each sample is counted. This is accomplished by examining the external standard ratio in advance of sample counting and then varying gain to bring this
ratio to some previously established value. Gain adjustment is accomplished by varying the degree of amplification. High voltage to the photomultipliers can also be varied, but changing amplification is the better of the two approaches since photomultiplier performance requires considerable time before stabilization after high voltage has been altered.

For single isotope counting, the principle utility of the method is in counting $^{14}C$ and more energetic isotopes that exhibit spectral shift on quenching. The lower level discriminator of a counting channel can be set above zero so that when quenching occurs a portion of the spectrum drops below that discriminator setting. If the system gain is now increased, it may be possible to restore the counting efficiency to approximately that of an unquenched sample by raising that part of the spectrum below the discriminator to the region above where it is counted. The method is of even more potential utility in the counting of two isotopes where, by increasing gain in response to quenching, the cross-contribution of the higher energy isotope in the channel reserved for the less energetic is held relatively constant. The effects of quenching of the more energetic isotope and the reduction of counts in its counting channel tend to be minimized in a manner similar to that described above for single isotope counting. The AQC system does not give the user disintegrations per minute, as does the Packard AAA, but it does give improved counting conditions. The subject of the Beckman AQC is treated more extensively in Chapter 31.

INTERNAL PROGRAMMABLE COMPUTATION

In 1968, Intertechnique announced the Model SL 40 (Fig. 22) which in several respects represented a departure from previous automatic liquid scintillation counting systems. The Model SL 40 is a 200-sample automatic unit with controlled, constant-temperature counting environment. The front-end system logic closely follows that of the ANSitron. Its based on linear summation followed by logarithmic amplification and linear coincidence gate. “Live timing”, a “3 + 2” system for external standardization, and rotary selector switches permitting choice of preadjusted counting channels are included in the instrument.

The principle difference between the SL 40 and earlier instruments is found at the output of the single-channel analyzer. In all previous instruments, scalers with or without display were fed from these outputs. In the SL 40, however, a small programmable core memory computer is used both for storage of counting information and manipulation of data after the count has been completed. Three memories of different sizes (1024, 2048 or 4096 words) are offered. Word length is 12 bits. Memory cycle time is 2 μs and add-time is 6 μs. A read-only memory of 256 4-bit words is used for storage of microinstructions and has a cycle time of 400 ns. Apart from storing counting data and performing normal computation, the
computer memory is capable of storing quench calibration curves, thereby permitting disintegrations per minute to be obtained for single, dual, or triple isotope samples by either the external standard ratio or channels ratio methods of quench corrections.

Programs are also available for handling assay data. For example, a dual isotope sample might be counted and the final printout could be micrograms of aldosterone in a 24-hour urine collection. Similarly, by entering sample weights into the memory, specific activity can be presented. Plotting regression lines and computing the best mathematical expression for such data are also possible. The SL 40 is capable of providing the operator with whatever on-line information he might expect from an off-line computer of comparable size.

At the time of this writing, the most recent liquid scintillation counter to be announced is the Nuclear-Chicago Mark II, a highly modular system with a 300-sample capacity (Fig. 23). The new aspects of the Mark II system are two-fold. A stabilizer is provided to adjust system gain automatically to prevent long-term drift. While the details of the stabilizer have not been completely revealed, it appears that the system has a
plastic scintillator pellet containing alpha activity probably in the form of $^{241}$Am). An alpha emitter is chosen because of its monoenergetic energy spectrum which, even with plastic scintillator, leads to a relatively narrow peak. Several times a day the plastic pellet is automatically placed between the photomultipliers and the count rate in a fixed window and examined. If the rate is not comparable to a preestablished value, indicating that the peak has drifted out of the counting window, the overall system gain is varied to restore the count rate in the window to the standard value. While new to $\beta$-counting, this technique has long been used in gamma pulse height analysis. It is reasonable to expect that it will lead to a high order of reproducibility over a long period, especially if relatively narrow counting windows are employed.

The second interesting aspect of the Mark II is its ability to provide different preadjusted counting conditions for several investigators using the instrument sequentially in unattended fashion. In this, Nuclear-Chicago is following the lead previously established by Phillips which has been mentioned before. A module is provided that allows each of several investigators to choose his own preset time, preset count settings, and any combination of the preadjusted counting windows. Each group of samples is preceded by a coded plug. The code is read at the loading station and preset counts, times, and windows are switched to the desired settings. When the samples of the next investigator, preceded by their coding plug, have advanced to the loading station, the settings are changed again. A wide variety of data-recording devices are offered with the Mark II system.

**Modern Single-sample Counters**

Though liquid scintillation counting began with the single-sample counter, by 1960 almost all the units sold were automatic models or semi-automatic devices designed for subsequent automation. The single-sample counter had practically disappeared from the market. Beckman and Picker never
built such a device, and for some years both Packard and Nuclear-Chicago had apparently discontinued efforts in that direction. This trend is not difficult to understand, since the early units required cumbersome refrigeration systems, the cost and size of which were so great that the system that included them might as well have been automated. With the advent of the bialkali photomultipliers that provide highly acceptable performance at ambient temperature, there has been renewed interest in small, low-cost, single-sample, bench-top, coincidence counters.

The ANSitron BTC was first exhibited in 1966. It was a two-channel device with system logic identical to that of the larger automatic ANSitron counter. External standardization was accomplished by manual insertion of a gamma point source adjacent to the counting chamber. Data were generally recorded manually but provisions were included for a digital lister and for automatic repetitive counting.

In 1968, Intertechnique introduced the Intertechnique Model SL 20 (Fig. 24). Based on system logic very much like that of the ANSitron, the SL 20 is also a two-channel instrument with provision for external standardization and automatic data recording. Counting channels may be adjusted through use of front-panel potentiometers, or fixed settings for common single and dual isotopes may be obtained through use of a single rotary switch. The SL 20 also includes preset windows for external standardization. Design of the sample insertion slide allows the instrument to be used as a continuous flow monitor.

Editor's Note: In late 1969, Beckman also introduced the β-Mate, a single-sample, single-channel counter, which includes provision for quench calibration by external standard-channels ratio.
There was a time in the history of liquid scintillation counting when we knew very well we could have no confidence in the results from our equipment unless we counted everything ten times or more, and unless an investigator was something of a physicist and electronics expert, he was probably not even working in this field. (See Chapter 4.) Today, however, the situation is changed. We pay our money and in due course receive an instrument that is really a wonder to behold. Just looking at the awe-inspiring sight generates a feeling of confidence. One or two hundred samples can be loaded at once, and the results are neatly tabulated by a typewriter and punched on tape for handling by a computer which may even be included in the package. We are tempted to feel that the automatically printed numbers must indeed represent the absolute truth, but actually our confidence must be carefully established and continually reestablished. Neither men nor machines are completely or consistently reliable.

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VARIABILITY

Let us begin with a few generalities. In tracer work, we usually start with some quantity of radioactive material which we will call $Q$. This material is allowed to pass through some process which is being studied. This may be a manufacturing process, a biological process, a water pollution process or almost anything conceivable. Along the way, our quantity $Q$ is divided:

$$Q_0 \rightarrow Q_1 \rightarrow Q_2 \rightarrow Q_3$$

Our aim is to measure these various quantities by liquid scintillation counting. As far as this discussion is concerned, our question is "If I measure one of these quantities many times, to what extent will the various results disagree with each other?" This variability is represented by the statistic called the standard
deviation, often written as the greek letter sigma (σ). If we are concerned with the variability of our quantity \( Q \), we will designate this with a subscript: \( \sigma_Q \).

It is useful to think of this variability as arising from a number of distinct sources. For example,

\[
\sigma_Q = \sigma_{\text{Process}} + \sigma_{\text{Pipette}} + \sigma_{\text{Sepn.}} + \sigma_{\text{Combustion}} + \sigma_{\text{Counting}}
\]

The statisticians\(^1\)\(^2\) tell us, however, that these sources add together not as standard deviations but as their squares as shown:

\[
\sigma_Q^2 = \sigma_{\text{Process}}^2 + \sigma_{\text{Pipette}}^2 + \sigma_{\text{Sepn.}}^2 + \sigma_{\text{Combustion}}^2 + \sigma_{\text{Counting}}^2
\]

A squared standard deviation is called a variance. The individual contributors are called the components of the observed variance \( \sigma_Q \). With the proper experimental design, it is possible to determine each of the variance components, although here we will concentrate on the one that is due to the counting process. It is the easiest to study because we can immediately eliminate all the other components by simply counting the same sample over and over. In this circumstance, our quantity of material \( Q \) will be represented by the disintegration rate \( D \).

**The Theoretical Standard Deviation**

In counting radioactive disintegrations,\(^3\) there is a variability inherent in the decay process. If this is the only source of variation, the standard deviation is equal to the square root of the number of counts:

\[
\sigma_N = \sqrt{N}
\]

However, we are more interested in the count rate

\[
R = \frac{N}{T}
\]

so that in general

\[
\sigma_R = \frac{\sigma_N}{T} = \frac{\sqrt{N}}{T} = \sqrt{\frac{N}{T^2}}
\]

Equation (3) can be written as

\[
\sigma_R = \frac{\sqrt{R}}{\sqrt{T}}
\]

In particular, for the gross counts rate \( G \), Equation (4) is written as

\[
\sigma_G = \frac{\sqrt{G}}{\sqrt{T_G}}
\]

and for the background count rate \( B \)

\[
\sigma_B = \frac{\sqrt{B}}{\sqrt{T_B}}
\]

The net count rate \( S \) is obtained by difference

\[
S = G - B
\]

By a calculation of error propagation, we may obtain the standard deviation of the net sample count rate:

\[
\sigma_S^2 = \sigma_G^2 + \sigma_B^2
\]

or, substituting the right-hand terms of Equations (5) and (6),

\[
\sigma_S^2 = \frac{G}{T_G} + \frac{B}{T_B}
\]

From Equation (7) we may substitute \( S + B \) for \( G \) and rearrange the equation for more convenient consideration:

\[
G = S + B
\]

\[
\sigma_G^2 = \frac{S + B}{T_G} + \frac{B}{T_B}
\]

or

\[
\sigma_S^2 = \frac{S}{T_G} + \frac{B}{T_G} + \frac{B}{T_B}
\]

One more change of units is required because we are interested in the disintegration rate \( D \) rather than the net count rate \( S \). Since

\[
D = \frac{S}{E}
\]
the standard deviation of \(D\) is obtained from
\[
\sigma_D^2 = \frac{\sigma_S^2}{E^2} = \frac{S}{T_0E^2} + \frac{B}{T_0E^2} \left(1 + \frac{T_G}{T_B}\right) \quad (16)
\]

Substituting \(D\) for \(S/E\),
\[
\sigma_D^2 = \frac{D}{T_0E} + \frac{B}{T_0E^2} \left(1 + \frac{T_G}{T_B}\right) \quad (17)
\]

Thus we have in Equation (17) an expression for the standard deviation of the disintegration rate resulting from our expectation that \(a-N = \frac{T_G}{T_B}\).

This equation, or one similar to it, has been suggested by a number of authors\(^4\)\(^5\) as a means of comparing instruments or of arriving at the optimum window setting on a particular instrument. In this form it is a little cumbersome, but it can easily be simplified to fit two common situations. In the author's laboratory, for example, a relatively large amount of time is spent counting the background so that the ratio \(T_G/T_B\) becomes small compared to 1 and can be ignored. If the net count rate of the sample is at least 5 times that of the background, the background term becomes negligible:
\[
\sigma_D^2 = \frac{D}{T_0E} \quad (18)
\]

Conversely, if the background count rate is 5 times the net sample count rate, the first term becomes negligible:
\[
\sigma_D^2 = \frac{B}{T_0E^2} \quad (19)
\]

Note that the values for \(B\) and \(E\) are controlled by the instrument or the instrument settings, while the values of \(D\) and \(T_0\) have nothing to do with the instrument. If we wish to minimize the standard deviation (or variance) and we have a large number of counts relative to background, we will choose an instrument with a maximum value of \(E\) (efficiency). If we plan to work with extremely low count rates, we will choose an instrument with a maximum value of \(E^2/B\).

**Actual Standard Deviation**

There is just one thing wrong. These expressions do not tell the whole story because they are based on the assumption that \(\sigma_N = \sqrt{N}\), when actually \(\sigma_N > \sqrt{N}\). Obviously in evaluating an instrument we wish to know if the actual standard deviation for the number of counts is significantly greater than \(\sqrt{N}\). At the point of actually determining a standard deviation, our motto, as always, will be "Keep Statistics Simple."

Most of us have learned to calculate the standard deviation of a set of \(n\) measurements by the general method\(^6\)\(^7\),
\[
\sigma_x = \sqrt{\frac{\text{Sum} \ (x - \bar{x})^2}{n - 1}} \quad (20)
\]

For small sets of data there is a simpler way\(^6\)\(^9\) that has been found very useful in the author's laboratory:
\[
\sigma_x = (\text{Max} - \text{Min})K \quad (21)
\]

where \(K\) is a factor depending on the number of measurements (\(K = 1/3\) for 10 measurements, \(K = 1/2\) for 4 measurements). This method can be used for groups of 10 or smaller.

Table 1 demonstrates the validity of this short-cut method. Column 1 shows the mean values for 19 different sets of data from the same sample, each set being the result of counting the sample ten different times. Column 2 shows the standard deviations calculated from each set by the general method and Column 3, standard deviations by the short-cut method. Even with the general method, a standard deviation obtained from a small set of data is only a rough estimate of the "true" standard deviation obtained from a very large set of data.

The best value obtainable from the data of Table 1 results from using the general method on the entire set of 190 measurements. This gives a standard deviation of 260. However, in practical work we often wish to check on the standard deviation using ten or even fewer replicates. For this purpose, the short-cut
method can be seen to follow quite closely the results obtained by the general method. For larger groups of data, the short-cut method can be used by simply averaging the standard deviations obtained from several small groups. For the data in Table 1, the average of Column 3 is 280, a value reasonably close to 260.

STABILITY OF THE INSTRUMENT

Now back to the problem of discovering if our scintillation counter is giving us a standard deviation higher than the theoretical $\sqrt{N}$. This is answered on a probability basis by means of a simple test, the F-test.\(^{10}\) Let us look at two examples.

EXAMPLE 1

Mean of 10 replicate counts = 691344
Standard deviation = 1018 d.F. = 9
Theoretical standard deviation = $\sqrt{N}$
\[ \text{d.F.} = \infty \]
F-ratio = $1018^2/691344 = 1.50$
Critical F-ratio at 95 per cent probability = 1.88

EXAMPLE 2

Mean of 10 replicate counts = 209471
Standard deviation = 860 d.F. = 9
Theoretical standard deviation = $\sqrt{N}$
\[ \text{d.F.} = \infty \]
F-ratio = $860^2/209471 = 4.10$
Critical F-ratio at 95 per cent probability = 1.88

The critical F-ratio is obtained from an F-table given in most statistics texts. One chooses a probability level such as 95 per cent and enters the table with the degrees of freedom (d.F.: 1 less than the number of observations; for the theoretical SD, infinity) of the numerator and denominator of the F-ratio. In Example 1, the F-ratio is less than the critical ratio which means that the data do not support the conclusion that the observed standard deviation is greater than the theoretical value. In Example 2, the opposite is true, and we can conclude with 95 per cent probability that there is an additional cause of variability beyond that inherent in the radioactive decay process.

Table 1.—Comparison of Two Methods for Calculating Standard Deviation

<table>
<thead>
<tr>
<th>Mean of 10 Counts</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>(\sqrt{\frac{\sum (X - \bar{X})^2}{n - 1}})</td>
<td>(\frac{\text{Max} - \text{Min}}{3})</td>
</tr>
<tr>
<td>69146</td>
<td>280</td>
</tr>
<tr>
<td>69224</td>
<td>360</td>
</tr>
<tr>
<td>69145</td>
<td>370</td>
</tr>
<tr>
<td>69242</td>
<td>260</td>
</tr>
<tr>
<td>68995</td>
<td>270</td>
</tr>
<tr>
<td>69137</td>
<td>230</td>
</tr>
<tr>
<td>69031</td>
<td>230</td>
</tr>
<tr>
<td>69189</td>
<td>230</td>
</tr>
<tr>
<td>69178</td>
<td>300</td>
</tr>
<tr>
<td>69157</td>
<td>370</td>
</tr>
<tr>
<td>68955</td>
<td>220</td>
</tr>
<tr>
<td>69038</td>
<td>250</td>
</tr>
<tr>
<td>68969</td>
<td>210</td>
</tr>
<tr>
<td>69077</td>
<td>190</td>
</tr>
<tr>
<td>68938</td>
<td>180</td>
</tr>
<tr>
<td>69052</td>
<td>250</td>
</tr>
<tr>
<td>68833</td>
<td>280</td>
</tr>
<tr>
<td>69023</td>
<td>190</td>
</tr>
<tr>
<td>69194</td>
<td>230</td>
</tr>
</tbody>
</table>
Another simple way of examining this question is to use control charts. The results obtained by repeated counts made over a 20-hour period are plotted in Fig. 1. The solid line is drawn through the mean of these values. The two dotted lines are drawn a distance away equal to two times the square root of the mean. Thus if the standard deviation has the theoretical value of the square root of the mean, 95 per cent of the time the values should lie between the dotted lines. These lines are called the upper and lower control limits. In Fig. 1 we see a few points slightly outside as would be expected. Figure 1 then indicates that during that period of time, any variability introduced by the instrument was negligible compared to the variability inherent in the radioactive decay process itself. Therefore Equations (17), (18) and (19), based on the inherent standard deviation, are valid for the particular period in which these data were taken.

Figure 2 shows a similar control chart taken at a later time. Obviously something changed part of the way through the test, although we cannot tell whether it was the power supply, an amplifier, a window setting or something else. One sign of trouble shown by the chart is that there are too many points outside the control limits. Another sign, helpful in detecting trouble, is the occurrence of seven or more points in succession, all lying on one side of the mean. If we had been watching this chart as it developed, we would begin to suspect that trouble was coming, as it did.

Figure 3 is from the same series of counts as Fig. 2 but in an adjacent channel. Here we see that the excursions correspond to those in Fig. 2 but are in the opposite direction. This provides an additional clue to the trouble and
suggests that the power supply was drifting, causing counts to be shifted from one channel to the other. As it turned out, a new power supply cured the problem. In Fig. 3 there is a series of seven points on one side of the mean, giving warning.

The real value of control charts is not after the fact as in these figures but as a continuing record so that trouble can be spotted early and the difficulty repaired before it becomes serious. Otherwise we may find ourselves in a situation in which the experimental results of many days suddenly become suspect.

STATISTICAL DECISION-MAKING

Having established that our instrument is behaving properly, we come to another use of statistics. How can we answer the question, "Is the activity of sample B really different from sample A?" A rough but often useful rule is that the activity of B can be considered different from the activity of A if the measured difference is greater than two times the standard deviation. This assumes that the standard deviation has previously been determined experimentally at the same general level of activity and with the same sources of variability included.

Similarly we may answer the question, "Does my sample activity really differ from zero or is it entirely due to background?"

Here Equation (19) provides the proper measure of the standard deviation if the background count rate has been well determined. Thus the measured activity of the sample can be considered to be above background if it is greater than two times the standard deviation obtained from Equation (19).

The factor of two used above provides a probability level of approximately 95 per cent. For critical decisions, more exact factors are obtained from a t-table in a statistics text. However, simply using a factor of two will help to prevent gross errors of drawing conclusions not justified by the experimental data.

SYMBOLS USED IN THIS CHAPTER

\[ Q = \text{quantity of material} \]
\[ \sigma = \text{standard deviation} \]
\[ N = \text{number of counts} \]
\[ T = \text{time in minutes} \]
\[ R = \text{count rate from an unspecified source} \]
\[ G = \text{gross count rate} \]
\[ T_0 = \text{time spent on gross sample count} \]
\[ T_B = \text{time spent on background count} \]
\[ S = \text{net count rate} \]
\[ D = \text{disintegration rate} \]
\[ E = \text{efficiency} \]
\[ n = \text{number of observations} \]
\[ x = \text{an experimental measurement of any kind} \]
\[ K = \text{factor for converting the range to standard deviation} \]
REFERENCES


6/PRACTICAL ASPECTS OF DOUBLE ISOTOPE COUNTING

Y. KOBAYASHI AND D. V. MAUDSLEY

One of the most useful and most exploited features of a liquid scintillation counter is its ability to determine two isotopes in the same sample simultaneously. For this technique to be successful, the beta spectra of the two isotopes must be sufficiently different to be amenable to separation by pulse height analysis. Fortunately, this is the case for the isotope pairs most commonly used in biomedical research such as (³H) and (¹⁴C), (³H) and (³⁵S), (³H) and (³²P), and (¹⁴C) and (³²P). In instances where the spectra of the two isotopes are very similar, for example with (¹⁴C) and (³⁵S), chemical separation of the isotopes is necessary and they must be counted individually. The counting of doubly labelled samples does not really present any serious difficulties. There are several ways of doing it, some of which may be better than others, depending on the circumstances of a particular experiment.

The data to be presented have been obtained from a variety of liquid scintillation counters, and although all were controlled-temperature units employing linear amplification and pulse summation, most of our comments will also be applicable to instruments employing logarithmic amplification. Since the majority of dual-label work involves the pairing of (³H) with (¹⁴C), these isotopes will be used to illustrate some of the practical considerations of double isotope counting.

CONDITIONS FOR (³H) AND (¹⁴C) COUNTING

Figure 1 shows experimentally determined spectra of (¹⁴C) and (³H) using air-quenched samples in toluene containing 4 g of PPO per liter. They were plotted in such a way that the (¹⁴C) spectrum occupied the full window width of a thousand divisions of the discriminator. Under these conditions the tritium spectrum occupied approximately one-tenth of the discriminator window width.

Consideration of these spectra illustrates two important aspects of double isotope counting. First, the higher energy isotope [in this case, (¹⁴C)] can, if necessary, be counted
without any interference by the lower energy isotope. By setting the lower discriminator at 170 divisions, all the tritium is excluded.

Second, the lower energy isotope [in this case, (\(^3\)H)] cannot be counted without also counting some of the (\(^{14}\)C). The counts of the lower energy isotope must always be corrected for the contribution of the higher energy isotope.

The only real problem in double isotope counting is arriving at the best conditions for counting the lower energy isotope of the mixed pair. Unfortunately, these conditions cannot be categorically listed because there are too many contingencies to cope with. What follows, therefore, is an outline of some of the considerations that must be weighed in arriving at reasonable counting conditions for double isotope analysis.

Let us start with the counting considerations for the higher energy isotope. In a modern counter with pulse summation, more than 50 per cent of the carbon spectrum lies above the tritium spectrum in an air quenched sample. This means that (\(^{14}\)C) can be counted at 50 per cent efficiency without (\(^3\)H). This is known as the isotope exclusion method and its advantages are that (\(^{14}\)C) can be determined very accurately because there is no contamination by (\(^3\)H) and that the calculations are simplified. Further consideration of Fig. 1 shows that there is only a small amount of (\(^3\)H) between 100 and 170 divisions, so that by allowing a small amount of (\(^3\)H) to creep into the (\(^{14}\)C) channel, the (\(^{14}\)C) counting efficiency can be significantly increased (Table 1). With no (\(^3\)H), the (\(^{14}\)C) counting efficiency is about 51 per cent. By including 0.1 per cent of the (\(^3\)H) activity, the (\(^{14}\)C) counting efficiency can be increased by about 15 per cent. With 1.0 per cent (\(^3\)H), the counting efficiency for (\(^{14}\)C) is about 72 per cent.

Now let us consider the counting conditions for the lower energy isotope, (\(^3\)H). Initially we will focus on the ratio of the counting efficiencies of the two isotopes in the lower energy channel and assume that we wish to count tritium at high efficiency with the mini-

### Table 1.—Effect of Increasing (\(^3\)H) Spectrum Spillover into (\(^{14}\)C) Counting Channel on (\(^{14}\)C) Counting Efficiency

<table>
<thead>
<tr>
<th>((^3)H) Counting Efficiency (%)</th>
<th>((^{14})C) Counting Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>50.9</td>
</tr>
<tr>
<td>0.01</td>
<td>57.5</td>
</tr>
<tr>
<td>0.05</td>
<td>62.5</td>
</tr>
<tr>
<td>0.1</td>
<td>65</td>
</tr>
<tr>
<td>0.5</td>
<td>70.3</td>
</tr>
<tr>
<td>1</td>
<td>72.5</td>
</tr>
</tbody>
</table>
USES OF THE SCINTILLATION COUNTER

Fig. 2.—Plot of $(^3\text{H})$ efficiency versus $(^1\text{C})$ efficiency in $(^3\text{H})$ channel for air-quenched samples in toluene-PPO.

Engberg Plots

There is an alternative way a particular counter can be characterized with respect to its double isotope counting ability. This information can be of assistance in choosing the appropriate counting conditions. The procedure is to measure the counting efficiency of $(^3\text{H})$ and $(^1\text{C})$ in a fixed window varying the gain step-wise from 0 to the maximum. The counting efficiencies are then plotted against each other on log-paper. Such a plot is shown in Fig. 3. Since this method was devised by Mr. Harry Engberg of Nuclear Chicago, we will call this the Engberg plot. The upper curve was derived from an air-quenched sample in toluene-PPO. This curve shows that this particular counter has an efficiency of about 60 per cent for $(^1\text{C})$ with 0.1 per cent contribution of $(^3\text{H})$. It can be seen that there is relatively little change in $(^1\text{C})$ counting efficiency when between 0.01 and 1 per cent tritium are included. This portion of the curve represents the bulk of the $(^1\text{C})$ spectrum and only the high energy end of the $(^3\text{H})$ spectrum. The other end of the curve represents the choices available in the $(^3\text{H})$ counting channel. The best ratio between $(^3\text{H})$ and $(^1\text{C})$ can be obtained by drawing a $45^\circ$ line from the base of the curve. Where the curve deviates from the $45^\circ$ line would be the best compromise between the ratio of the counting efficiencies. The actual choice of the final counting condition is dependent on the nature of the sample. The $45^\circ$-line represents a constant ratio be-

Fig. 3.—Engberg plot for isotope pair $(^1\text{C})$ and $(^3\text{H})$. Upper curve is derived from air-quenched samples in toluene-PPO; lower curve from acetone-quenched samples in toluene-PPO.
The Engberg plot gives a more comprehensive picture of the counter characteristics than the other plot presented in Fig. 2 which shows the \(^{14}\text{C}\) efficiency in the \(^{3}\text{H}\) channel as a function of the \(^{3}\text{H}\) efficiency.

The inner curve in Fig. 3 was derived from samples quenched from 72 per cent to 32 per cent \(^{14}\text{C}\) efficiency with acetone as measured by the change in channels ratio. This curve shows that the \(^{14}\text{C}\) counting efficiency can be restored almost completely by manipulating the amplifier gain but that the tritium efficiency can be restored to only a half of its unquenched efficiency, and that the isotope efficiency ratio in the tritium channel has deteriorated. (See Chapter 31.) Even in this condition, \(^{3}\text{H}\) can be counted at 15 per cent efficiency with 5 per cent contribution from \(^{14}\text{C}\). This is better than was possible in the first commercial liquid scintillation counter 15 years ago with an unquenched sample. (See Chapter 4.)

**Conditions for Other Isotope Pairs**

\((^{3}\text{H})\) and \((^{35}\text{S})\)

Figure 4 shows the Engberg plot for the isotope pair, \(^{3}\text{H}\) and \(^{35}\text{S}\). In an air-quenched sample, \(^{35}\text{S}\) can be counted at about 70 per cent efficiency with less than one-tenth per cent \(^{3}\text{H}\) contribution, and \(^{3}\text{H}\) can be counted at 50 per cent efficiency with a \(^{35}\text{S}\) efficiency of 10 per cent.

\((^{3}\text{H})\) and \((^{32}\text{P})\)

Figure 5 is the Engberg plot for the isotope pair, \(^{3}\text{H}\) and \(^{32}\text{P}\). In an air-quenched sample, \(^{32}\text{P}\) can be counted at 90 per cent efficiency with no \(^{3}\text{H}\) contribution, and \(^{3}\text{H}\) can be counted at 60 per cent efficiency with a \(^{32}\text{P}\) efficiency of 2 per cent. The 90 per cent efficiency point for \(^{32}\text{P}\) is not shown simply because it is beyond the range of the graph. This is an easy combination to count.

\((^{14}\text{C})\) and \((^{30}\text{Cl})\)

The Engberg plot for the combination \(^{30}\text{Cl}\) and \(^{14}\text{C}\) is shown in Fig. 6. The plot shows that \(^{30}\text{Cl}\) can be counted with high efficiency with little contribution of \(^{14}\text{C}\) and that \(^{14}\text{C}\) can be counted at 50 per cent efficiency with less than 6 per cent \(^{30}\text{Cl}\). As it turns out, the beta energies of \(^{30}\text{Cl}\) and \(^{131}\text{I}\)
are very similar, the actual values for the two iodine betas being 0.61 and 0.81 Mev. It would be expected that the combination of \(^{14}\text{C}\) and \(^{131}\text{I}\) could be counted effectively in a liquid scintillation counter.

\((^{36}\text{Cl})\) and \((^{32}\text{P})\)

The final Engberg plot is shown in Fig. 7. This is for the combination of \(^{36}\text{Cl}\) and \(^{32}\text{P}\). An interesting point is that the ratio of the beta energies between the two isotopes is 2.4. Ten years ago, it was thought that isotope pairs with beta energy ratios of less than 4 to 5 could not be analyzed effectively with a liquid scintillation counter. With modern counters, however, it can be seen that this is no longer true. In this case, \(^{32}\text{P}\) can be counted at 35 per cent efficiency with only a half a per cent efficiency for \(^{36}\text{Cl}\). The \(^{36}\text{Cl}\) can be counted at 80 per cent efficiency in the presence of 40 per cent of the \(^{32}\text{P}\) in the sample. This is certainly a workable system.

**Simultaneous Equations**

The contribution of each isotope to the total counts observed in the test samples can be calculated with the use of the following simultaneous equations with \(^{14}\text{C}\) and \(^{3}\text{H}\) as examples:

\[
C = (^{14}\text{C}) \text{ dpm in sample (disintegrations per minute)}
\]

\[
H = (^{3}\text{H}) \text{ dpm in sample}
\]

\[
c_1 = (^{14}\text{C}) \text{ efficiency in Channel 1 (100\% = 1.00)}
\]

\[
c_2 = (^{14}\text{C}) \text{ efficiency in Channel 2}
\]

\[
h_1 = (^{3}\text{H}) \text{ efficiency in Channel 1}
\]

\[
h_2 = (^{3}\text{H}) \text{ efficiency in Channel 2}
\]

\[
N_1 = \text{net total observed counts in Channel 1}
\]

\[
N_2 = \text{net total observed counts in Channel 2}
\]

then

\[
N_1 = C(c_1) + H(h_1)
\]

and

\[
N_2 = C(c_2) + H(h_2)
\]
Solving the above equations for \( C \) and \( H \), it will be found that

\[ C = \frac{N_1 - N_2(h_1/h_2)}{c_1 - c_2(h_1/h_2)} \]  

\[ H = \frac{N_2 - N_1(c_2/c_1)}{h_2 - h_1(c_2/c_1)} \]

Under conditions in which all the \(^3\text{H}\) is excluded from the \(^{14}\text{C}\) channel, the equations are simplified as follows:

\[ N_1 = C(c_1) \]  

and

\[ N_2 = C(c_2) + H(h_2) \]

If we now solve these equations for \( C \) and \( H \), it will be found that

\[ C = \frac{N_1}{c_1} \]  

\[ H = \frac{N_2 - Cc_2}{h_2} \]

Equations (3) and (4) are basic equations that can be applied to any liquid scintillation counter being used for double isotope analysis.

**ISOTOPE RATIOS**

*Theoretical Counting Errors*

Let us now consider the problem of isotope ratios; i.e., the relative amounts of each isotope contained in a single sample. In the case of \(^{14}\text{C}\), the error of its determination in double isotope analysis can be independent of the amount of \(^3\text{H}\) simply because all the \(^3\text{H}\) can be excluded from the \(^{14}\text{C}\) analysis channel. The accuracy of the \(^{14}\text{C}\) analysis then becomes a function of the number of counts collected and the accuracy of the counting efficiency determination. The theoretical counting error expressed as the standard deviation for the \(^{14}\text{C}\) determination is the square root of the sum of the squares of the standard deviations of the \(^{14}\text{C}\) count and the background count. (See Chapter 5.)

The total counting error of the \(^3\text{H}\) determination is more involved. The formula for computing the total counting error as the per cent standard deviation of the \(^3\text{H}\) count rate is shown in Fig. 8. This formula is an adaptation by Horton and Tait\(^1\) of Jarrett's formula\(^2\) for the error in the sum of a series of counting determinations. By proper substitution in this formula, it is possible to compute the theoretical counting error as the per cent standard deviation for various isotope ratios and various amounts of total radioactivity.

The total counting error of a sample containing 1000 dpm of \(^3\text{H}\) and 5000 dpm of \(^{14}\text{C}\) counted for 1 minute is about 10 per cent. If this sample were counted for 9 minutes, the counting error decreases by a factor of \(\sqrt{9} = 3\) or 3 per cent. See text for instructions.

---

**Fig. 8**—Nomograph for estimating theoretical counting error of \(^3\text{H}\) determination as per cent standard deviation for various isotope ratios of \(^3\text{H}\) and \(^{14}\text{C}\). Calculations are based on the following conditions: \( H = \) \(^3\text{H}\) count rate in counts per minute in \(^3\text{H}\) channel at 30 per cent; \( b_h = \) background count rate in \(^3\text{H}\) channel = 20 cpm; \( C = \) \(^{14}\text{C}\) count rate in counts per minute in \(^{14}\text{C}\) channel at 65 per cent; \( b_c = \) background count rate in \(^{14}\text{C}\) channel = 10 cpm; \( m = \) ratio of \(^{14}\text{C}\) efficiencies in both channels = 0.1538; \( T = \) counting time = 1 minute. See text for instructions.
Each sample counted 20 minutes (2 \times 10 \text{ mm}).

Counting conditions: (RH) channel: ('H) eff = 18.7\%; (tC) eff = 6.5\%

\(1C\) channel: ('C) eff = 57.8\%; (aH) eff = 0\%

It should be pointed out that when quenching occurs, the ratio of (14C) over (H) tritium also increases.

**Actual Counting Error**

These are theoretical curves based on assumed conditions, but how do they function in practice? The experimental data in Table 2 show the results of counting ten replicates prepared for each ratio listed. The ten values were averaged and the per cent standard deviation for the ten values are shown. Each sample was counted twice for a total of 20 minutes. The (3H) counting conditions chosen were deliberately poor to demonstrate the fact that, even with poor counting conditions, samples containing a moderate amount of radioactivity can be counted with reasonable statistics in a reasonable length of time. Although the average value found for the approximately 1000 dpm (3H) samples was a bit low, the lack of precision may have been due to careless preparation of the replicates. No special precautions were taken in formulating these 60 air-quenched samples. The statistics of this experiment could have been improved by counting longer, especially in the case of the samples which contained only 100 dpm of (14C). It should be noted that it was not possible to determine (3H) in the presence of a hundred times more (14C), but ratios of this order are seldom encountered experimentally. An important conclusion from this experiment is that isotope ratios previously considered impractical for analysis (for example, a 1 to 20 ratio) because of the large inherent counting errors due to the instability of the counting apparatus, can now be counted with an acceptable degree of precision.

**Table 2.** Determination of Standard Deviation Among Group of Ten Replicates Containing Varying Ratios of (14C) and (3H)*

<table>
<thead>
<tr>
<th>Ratio dpm ((\text{H})/(14C))</th>
<th>(3H) dpm</th>
<th>Actual</th>
<th>Found ± % S.D.</th>
<th>(14C) dpm</th>
<th>Actual</th>
<th>Found ± % S.D.</th>
</tr>
</thead>
<tbody>
<tr>
<td>100:1</td>
<td>9891</td>
<td>9886 ± 0.89%</td>
<td>93.98</td>
<td>97.13 ± 4.45%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>10:1</td>
<td>9891</td>
<td>9787 ± 0.74%</td>
<td>1110</td>
<td>1089 ± 1.38%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:1</td>
<td>1043</td>
<td>994 ± 2.25%</td>
<td>1110</td>
<td>1102 ± 0.83%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:10</td>
<td>1043</td>
<td>921 ± 4.91%</td>
<td>10133</td>
<td>9962 ± 0.80%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:20</td>
<td>1285</td>
<td>1147 ± 1.67%</td>
<td>20184</td>
<td>19801 ± 0.73%</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:100</td>
<td>1043</td>
<td>381 ± 118%</td>
<td>104910</td>
<td>107345 ± 0.71%</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Each sample counted 20 minutes \((2 \times 10 \text{ min.})\).

General Comments

Thus far we have discussed as separate items the major factors (the efficiency ratio and the isotope ratio) involved in selecting the best conditions for dual-label work. One of the dangers of sweeping generalizations is the difficulty of establishing which factors will be most limiting in satisfying the usual criteria of accuracy, precision and sensitivity. In some investigations, for example, the isotope ratio may be determined or fixed by other considerations of the experiment quite unrelated to the counting of the final samples. In other instances, the efficiency ratio may be determined or fixed by other considerations of the experiment quite unrelated to the counting of the final samples. In other instances, the efficiency ratio may be limited either by instrument capabilities or by restrictions in sample preparation. The most serious attempt at compromise generalizations for double isotope analysis has been the mathematical treatment by Bush. Her paper is referred to for a more sophisticated approach on this problem. However, the solution to most problems associated with dual-label work is time. (The data presented support the
contention that double isotope counting can be routine, accurate and precise. The main requirement is that sufficient counts be accumulated to meet the statistical requirements for a particular experiment.) Poor efficiencies and low count rates can always be overcome by counting for longer periods. On the other hand, counting time may be severely limited either by instrument availability or, as is more often the case, by the impatience of the investigator.

There have been two important assumptions in this discussion so far: that the instrument is stable over the period of the experiment and that the counting efficiencies of the two isotopes can be accurately determined. The various methods of determining counting efficiency will not be discussed since they are covered in detail elsewhere in this volume. It should also be stressed that the counting characteristics presented in this paper were all determined in homogeneous solutions. With all the hazards and uncertainties associated with efficiency determinations in heterogeneous counting mixtures, it would seem that every effort should be made to avoid them. We have discussed this subject in some detail elsewhere.  

Since the counting of dual-labelled samples is not performed under balance point conditions, small variations in line voltage, amplifier gain, for instance, may cause significant changes in count rate. Assessing instrument stability by counting a standard at balance point is meaningless because the balance point accommodates or conceals changes in instrument stability that could make a mockery of dual-label work. This point was stressed by Okita and co-workers many years ago but is still too often ignored. We recommend the use of the spillover of ($^3$H) into the ($^{14}$C) channel as a routine measure of instrument stability.

**Low Count Rates**

The major practical problem encountered in double isotope analysis is one of low count rates. If the low count rate is due to quenching, a useful endeavor would be to increase the count rate either by reducing quenching through improving the sample preparation or by optimizing the counting solution by manipulating the concentration of the scintillator and possibly the scintillation solvent or by manipulating the amplifier gain. On the other hand, if the low count rate is simply due to low radioactive content, an effort to minimize the background count will be most helpful in reducing the counting time required to accumulate a statistically significant number of counts. For example, we have found that background can be reduced from 10 to 9 cpm in the ($^{14}$C) channel and from 15 to 12 cpm in the ($^3$H) channel by reducing the volume from 10 ml to 5 ml in a low-potassium glass counting vial. Although the reduction in background of one to three counts may seem trivial, it will result in a significant improvement in $(E^2/b)$. Dr. Christopher Longcope of the Worcester Foundation has found that the background for 10 ml of counting solution could be reduced from 15 to 3.2 cpm in the ($^3$H) channel by the use of nylon vials. The ($^3$H) counting efficiency was 25 per cent so the $(E^2/b)$ value was improved from 42 to about 200 for an air-quenched sample. Unfortunately, nylon vials cannot be used with certain compounds because of surface adsorption resulting in $2\pi$ geometry. In such cases, polyethylene vials may be useful in reducing ($^3$H) background. With nylon vials the ($^{14}$C) background was reduced from 10 to 9 cpm.

**History of Double Isotope Counting**

From a biochemical standpoint, the advantage of liquid scintillation counting using two isotopes is that it does away with the requirement for weighing a sample. The first method for using two isotopes was introduced in 1946 by Keston, Udenfriend and Cannan for determining amino acids in biological samples. ($^{131}$I)-pipsylchloride was used as the reagent, and tracer amounts of a ($^{14}$C) amino acid were used as the indicator. The first use of a liquid scintillation counter for a double
isotope analysis of a steroid can be attributed to Avivi, Simpson, Tait and Whitehead.\(^7\) It is of historical interest to note that their liquid scintillation counter was home-made and was used to determine total radioactivity, \(^{3}H\) and \(^{14}C\) in the sample because it could not discriminate \(^{3}H\) from \(^{14}C\). A Geiger counter was used to exclude tritium. The essence of this early report was the suggestion that \(^{14}C\) or \(^{3}H\) acetic anhydride might serve as a more useful labelled reagent than \(^{31}P\)-pipsycloride from many points of view. The application of the acetic anhydride reagent was demonstrated for the steroids cortisone, hydrocortisone and aldosterone (electrocoritin).

The wide attraction of double isotope analysis lies in the very high sensitivity of these radiochemical methods, which is the function of the specific activity of both the labelled reagent and the indicator. The difficulties encountered with the double isotope analyses with a liquid scintillation counter in the early days stemmed mainly from the limitations in instrumentation.\(^3\) The low counting efficiencies for \(^{3}H\), poor isotope discrimination, and poor instrument stability made it a very difficult and delicate procedure. However, these factors are no longer as critical and limiting as they once were.

As a final illustration of the leniency that can now be tolerated in double isotope analysis, the counting conditions used for two recently published methods\(^8^9\) for aldosterone are compared in Table 3. The biochemical aspects of these assays are unimportant except to point out that the stringent requirements for purity of the end product results in the loss of between 70 and 80 per cent of the starting material. The typical count rates in both assays were in the range of 10 net cpm for \(^{14}C\) and 60 net cpm for \(^{3}H\). What should be noted is the low \(E^2/b\) value for \(^{3}H\) for the Tait group compared to Coghlan and Scoggins. Another interesting comparison is the rather high contribution of \(^{14}C\) in the \(^{3}H\) channel tolerated by Coghlan and Scoggins. In spite of these differences, both methods yield equally precise results. It becomes clear that acceptable double isotope analysis with low-level counting can be done with relatively low \(E^2/b\) for \(^{3}H\) or with a relatively large contribution of \(^{14}C\) to the \(^{3}H\) channel. The critical requirement is not as much the counting efficiencies of the isotope as the constancy of counting conditions during the length of the experiment. It should be equally clear that with modern liquid scintillation counters, double isotope counting should be markedly improved because of the higher counting efficiencies, better isotope separation and good instrument stability. For example, the counting time quoted for the aldosterone assay\(^8\) can be reduced from 240 minutes to about 60 minutes to obtain comparable counting statistics.

**Importance of Sample Preparation**

It is apparent that although the modern liquid scintillation counter is a useful and re-

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**Table 3. Comparison of Counting Conditions for Aldosterone Assay**

<table>
<thead>
<tr>
<th></th>
<th>(^{14}C) Channel</th>
<th>(^{3}H)</th>
<th>(^{14}C) Channel</th>
<th>(^{3}H)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Efficiency (%)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A *</td>
<td>42</td>
<td>0.06</td>
<td>7</td>
<td>13</td>
</tr>
<tr>
<td>B †</td>
<td>56</td>
<td>0.015</td>
<td>29</td>
<td>30</td>
</tr>
<tr>
<td>Background (cpm) A</td>
<td>6</td>
<td>6</td>
<td>12</td>
<td>12</td>
</tr>
<tr>
<td>B</td>
<td>8</td>
<td>8</td>
<td>22</td>
<td>22</td>
</tr>
<tr>
<td>(E^2/b)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A</td>
<td>294</td>
<td>14</td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>392</td>
<td>41</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Counting time: A. 240 minutes/sample; B. 200 minutes/sample

* Brodie, Shimizu, Tait and Tait
† Coghlan and Scoggins
liable instrument, in practice its usefulness in double isotope analysis is greatly dependent on sample preparation. The influence of sample preparation has recently been demonstrated by Ronucci and co-workers who investigated the simultaneous determination of (3H) and (35S) in biological samples using the oxygen flask combustion method. Under normal circumstances (35S) can always be counted more easily and accurately than (3H), but these workers found that in practice the accuracy, precision and sensitivity were generally better for (3H) than for (35S). This apparent contradiction stems in part from the reluctance of these workers to vary the isotope ratio, but it is predominantly due, as they point out, to the fact that the errors in trapping (35S) after combustion are greater than for trapping (3H). The generation of legitimate data depends heavily on the proper marriage of optimized instrument settings to good sample preparation.

ACKNOWLEDGMENT

The authors thank Mr. Barton Laney of Nuclear Chicago Corporation for the use of Engberg plots, Figures 4 through 7.

REFERENCES

Even in the early days of liquid scintillation counting, potential for low-level counting was recognized. Because of difficulties associated with low-level counting of tritium by other techniques such as internal gas counting, the first efforts reported were in (3H) counting.1-4

This chapter reviews the efforts of the Southeastern Radiological Health Laboratory in low-level counting by liquid scintillation. We have dealt primarily with (3H) counting but have recognized that low-level counting of other radionuclides may be carried out advantageously by the same technique. Details of the procedures for each radionuclide discussed are in the process of preparation for publication or have been published.5,6 All investigations reported here have been carried out using a Beckman liquid scintillation system except where specifically mentioned.

There are a number of factors affecting the performance of low-level counting systems, and various attempts have been made to correlate these factors in a single formula. The major factors determining the sensitivity of a low-level counting system are background, efficiency, and sample size. Other factors, such as the requirements of time and skill for sample preparation, and the convenience of operation, are important but have relatively little effect on the sensitivity of the system.

BACKGROUND

In older liquid scintillation counters, the thermal electron emission of photomultiplier tubes (PMT) was the major source of back-
The development of bialkali PMT's with "noise" levels of 2000–15000 cpm and the application of nanosecond coincidence and circuitry has reduced the contribution of the electronic system to the background to less than 1 cpm. (See Chapter 4.)

As registered in a modern liquid scintillation counter, background is generally caused by external radiation. Cosmic rays and radiation from radionuclides in shielding and other construction materials are the major components of this external radiation. In areas with high air radon concentration, decay products may plate out on surfaces and contribute to background.

In a background spectrum over the range of approximately 2–2000 keV, there is an undesirable peak close to the tritium spectrum, presumably caused by the bremsstrahlung of the cosmic radiation in the shield. Above approximately 50 keV, the background count/channel in a multichannel analyzer seems to be relatively constant. Efforts are under way in our laboratory to reduce the background for (3H), primarily by changing the geometry of the shielding. It is unfortunate that manufacturers of liquid scintillation counters do not offer instruments with an anticoincidence shield. We estimate that such a shield, which would reduce the background to one-third or less of the original value, would increase the price of the instrument by only 10–15 per cent. This increase, well worth the cost, would substantially improve the sensitivity of liquid scintillation counting and, as it may be noted from the following description, make it competitive with other low-level counting systems for high-energy β- and α-emitters.

While the contribution of external radiation to the background is relatively constant, there are other additional factors responsible for the lack of precision of background observed by many investigators. The variety of reactions that may produce light in the scintillation liquid—phosphorescence, chemiluminescence, and static electricity—are very important.

**Phosphorescence**

In our laboratory a great deal of work has been carried out dealing with phosphorescence and its temperature dependence. Three mixtures were given special attention: a dioxane-water solution with a volume ratio of 80:20 (DW), a p-xylene, Triton N-101 and water emulsion with a volume ratio of 44:16:40 (XTN), and a toluene, Triton X-100, water emulsion with a ratio of 55:25:20 (TTX). The concentration of scintillators was in all cases 7 g diphenyloxazole (PPO) and 1.5 g p-bis(o-methylstyryl)-benzene (bis-MSB) dissolved in one liter of organic solvent. The dioxane contained 120 g naphthalene/1.

The excitation by light of detergent mixtures (XTN and TTX) was 10 times stronger than with dioxane (DW) solution. In all three cases there were three decay components: short (half-life < 0.5 minute), intermediate, and long (half-life 4–6 hours).

The intermediate component had a half-life of 30 minutes for DW, 20–25 minutes for XTN, and 4–6 minutes for TTX. It was found that the emission intensity was reduced by a factor of approximately 2 for DW and 3 for XTN and TTX during the 115-minute interval between 5 and 120 minutes after excitation. The emission intensity of DW was 10 times higher than the other two 5 minutes after excitation. Only the half-life of the intermediate component was subject to reduction by changing the temperature and could be reduced (e.g., by a factor of 2 for DW) by reducing the temperature to less than 16°C. Heating the excited scintillation mixture reduced the phosphorescence substantially, almost to zero, a finding analogous to the thermoluminescence observed in many inorganic scintillators.

Coincidence measurements show that phosphorescence decay is primarily monophotonic. When excitation is intense, random coincidence may occur and pulses in higher energy ranges may be registered. The complexity of phosphorescence suggests the ad-
visability of avoiding it whenever possible. For this reason it has become routine in our laboratory to perform all operations involving scintillation solutions under red light.

**Chemiluminescence**

The production of light as a result of chemical reaction is more complicated than phosphorescence. (See Chapters 32 to 34.) In dioxane-based solutions, impurities, particularly peroxides, cause chemiluminescence. However, high purity dioxane is available and is used along with high-purity scintillators exclusively, in our laboratory. Because of the solubility limitations of DW solution, it is used only for (3H) counting.

In suspension systems, the detergent and carrier can also be selected to minimize chemiluminescence. An advantage of this system is the separation of aqueous and organic phases, and although the surface of the interphase is large and each phase is to some extent soluble in the other, the possibility of a chemical reaction is considerably less than in a true solution. Heating the mixture generally reduces or eliminates chemiluminescence but is inconvenient and not always possible because of chemical reactions that may destroy the scintillators. In some cases, scintillators may be added in concentrated solution to the sample after the latter was heated with a portion of the solvent. In other cases, reducing the temperature may be helpful.9

The selection of any low-level counting system should include the consideration of chemiluminescence. As chemiluminescence may be enhanced by light, exposure of the sample to light should be avoided.

**Static Electricity**

As radioactive sources are extensively used in the industry to eliminate static electricity, it is ironic that in a radioactivity counting system, perturbations from static electricity must be considered. As may be seen from Fig. 1, static electricity should always be considered in the background in (3H) counting when the sample moves an appreciable distance in the sample-changer compartment. To avoid static electricity, it is advisable to arrange samples within the changer compartment so that none travels more than five places immediately before it is counted.

**Efficiency and Sample Size**

The quantum efficiency of PMTs is an essential factor in selecting a low-level counting system for reasons reviewed recently.5 Bialkali PMTs with approximately 30 per cent quantum efficiency are now available. Counting efficiency E is closely related to the degree of quenching and to the sample size, the amount M of the radionuclide (and its carrier) introduced into the scintillation mixture. For water, it has long been recognized that

![Fig. 1.—Spectrum of static electricity in background sample. Indicated energies are approximate and were estimated using (90Sr)-(90Y) and (3H).](image-url)
the product $E \times M$ is the determining factor in low-level tritium counting. This is also valid for other radionuclides if the optimum amount of the sample is available for analysis, as is often the case for $^{14}$C and $^{40}$Ca. For low-level counting of those radionuclides present in a sample at trace levels, such as $^{239}$Pu, conditions may often be found for which the efficiency is close to 100 per cent.

**Correlation of Efficiency, Sample Size, and Background**

We recently defined a $Y$-value for the comparison of low-level counting systems. This value, (in picocuries) the minimum limit of detection at a 1-σ confidence level and a one-minute counting time, correlates background $B$ (cpm), efficiency $E$ (cpm/dpm), and the amount of sample in the scintillation mixture $M$ (g):

$$Y(\text{pCi/g}) = \frac{\sqrt{B}}{2.22 \times E \times M} \quad (1)$$

If the optimum amount of carrier is not available, Equation (1) is replaced by

$$Y(\text{pCi/sample}) = \frac{\sqrt{B}}{2.22 \times B} \quad (2)$$

Due to the efficiency dependence of background, 100 per cent efficiency does not necessarily represent the optimum $Y$-value. Experiments with many radionuclides in our laboratory have shown that optimum $Y$-values are usually at efficiencies between 80–90 per cent for radionuclides with a maximum $\beta$-energy of over 200 keV.

$^{3}$H Counting

Low-level counting of $^{3}$H has undergone vast improvements in recent years. There have been three general approaches for incorporating a large amount of water in the scintillation mixture: solution in dioxane-based mixtures, conversion to a compound such as benzene, and suspension in aromatic solvents. The lowest $Y$-value obtainable with a dioxane-based mixture is 1.1 pCi/g H$_2$O using the solution previously abbreviated as DW. In suspension systems the best $Y$-value for Triton X-100 seems to be 1.4, as reported by Williams. The application of a modern liquid scintillation counter with bialkali PMTs could reduce this value to 1.0 pCi/g H$_2$O. The lowest $Y$-value for a Triton N-101 suspension is approximately 0.6 pCi/g H$_2$O with the composition abbreviated previously as XTN.

Several authors report instabilities associated with suspension counting. Refrigerated liquid scintillation counters have been used in all cases where these instabilities are mentioned. The suspension of water in an aromatic solvent is based on the surface tension of the produced water bubble in the organic liquid. Due to temperature dependence of surface tension, a large temperature reduction may change the equilibrium established between the two phases at room temperature and could be the cause for reported instabilities of the suspension system. (See Chapter 19 for temperature optima of suspensions.) Two corrective techniques have been proposed. The first consists of repeated shaking of the mixture as it is cooled. During this process, the distorted equilibrium is redistributed at intermediate temperatures until the final temperature is reached. The second technique consists of heating the counting vial for approximately 30 minutes at approximately 40°C. The actual effect of this treatment is not entirely clear, but apparently a stable and irreversible condition can be established which can tolerate a reduction of 20°C without noticeable change in the equilibrium of the system. The suspension system developed in our laboratory can be used between 17 and 25°C. It is stable and reproducible within this temperature range, so that neither treatment is necessary for reproducible results.

Low-level $^{3}$H counting in urine has been made possible by the advances in suspension counting. The major source of uncertainty is the presence of other radionuclides in raw urine, particularly $^{14}$C and natural $^{40}$K. 
Activities of 10 nCi/1 or more are easily detectable with a Triton N-101:p-xylene (7-g PPO and 1.5-g bis-MSB/l) mixture in a volume ratio of 1:2.5. Eight ml of urine and 17 ml of the above mixture result in a Y-value of 1.4 pCi/ml (E = 0.15, B = 12).

The most sensitive technique for low-level counting of (3H) consists of a synthesis of benzene through the reaction of calcium carbide and water followed by a trimerization of the resulting acetylene to benzene. Due to an isotope effect, the specific activity of (3H) in benzene is only 70-80 per cent of that in the water, and the process is time consuming and elaborate. The Y-value of this method, if 25 ml of benzene were synthesized, would be 0.3 pCi/g H2O.

(14C) Counting

Low-level counting of (14C) by liquid scintillation is considerably more complicated than that of (3H). Inasmuch as the conversion of the sample to CO2 is usually the start of the (14C) procedure, the introduction of a sufficient amount of CO2 into the scintillation liquid is necessary. Many chemical reactions have been proposed for converting CO2 to a suitable compound. The only reaction with wide acceptance is the conversion of CO2 to a carbide and the trimerization of the latter to benzene.15 The Y-value of this process, if 25 ml benzene were prepared, would be 0.12 pCi/gC (E = 0.85, B = 20, and M = 20 g). This excellent sensitivity depends however, on very elaborate equipment and techniques. The preparation of 25 ml of benzene would require approximately a week, and a rather large sample is needed. For these reasons, benzene synthesis is not feasible for the routine monitoring of (14C) in environmental samples.

Another technique has recently been developed in our laboratory. The essential features of our procedure are as follows: the sample is converted to CO2 and is reacted with lithium hydroxide to produce lithium carbonate. Due to large differences in the solubility of LiOH and Li2CO3 in water-alcohol mixtures, the recovery of Li2CO3 is large and can be made quantitative by the evaporation of the solvent. The critical portion of this procedure is the removal of entrapped solvent from the Li2CO3 crystals by drying in a desiccator overnight. A counting vial is then filled with 12.5 g of the Li2CO3 and 15 ml of an aromatic solvent scintillator. This mixture has been counted with 55 per cent efficiency and a background of 20 cpm, resulting in a Y-value of 1.8. The Y-value of this method is considerably higher than that of the benzene synthesis, but it is sensitive enough for monitoring environmental (14C) and for (14C) dating in the large-volume liquid scintillation counter described later in this chapter. An additional method, oxygen combustion, is described in Chapter 23.

(45Ca) Counting

The introduction of an inorganic ion into a scintillation mixture may be accomplished by several techniques. (See Chapter 18.) A number of organic compounds have been used to extract ions from aqueous solutions and introduce the organic phase then being introduced into the scintillation liquid. The maximum amount of sample that may be introduced into the scintillation liquid by this technique is usually quite limited. A second technique consists of suspending an insoluble salt in an aromatic solvent. The (14C) procedure described above is an example of this technique. If there is not enough sample to fill the entire counting vial, a technique such as the one described by Bollinger et al16 may be applied. A third approach may be employed if the scintillation mixture contains a large amount of water: a water soluble salt may be used to incorporate adequate amounts of the sample into the scintillation mixture. Depending upon the available sample size, the latter two techniques may both be used for calcium. Calcium fluoride is known to be an excellent light conductor. It is insoluble in water and moderately concentrated acids and bases and
is easy to prepare. Up to 30 g of CaF₂ may be added to a conventional counting vial resulting in a Y-value of 0.4 pCi/gCa (E = 0.30, B = 20 cpm).

For determination of (⁴⁵Ca) in samples where less than 15 g calcium are available, an aqueous solution of calcium chloride may be used. P-xylene (containing 7 g PPO and 1.5 g bis-MSB/1) is mixed with Triton N-101 in a volume ratio of 2.5:1. Fifteen ml of this solution can incorporate 10 ml of water containing a large amount of inorganic ions. A variation of the added amount of Ca between 0 and 800 mg changes the efficiency from 100 per cent to 85 per cent, yielding a Y-value of 3 pCi/g Ca. This technique is well suited for radiobioassay of calcium in urine and feces.

These values compare favorably with those reported by Hardcastle et al.¹⁷ of 20 pCi/g Ca (E = 0.85, M = 150 mg, and B = 30 cpm). The best Y-value with their procedure, if the volume of the sample were increased from 15 to 25 ml and if a modern liquid scintillation counter were used, would be approximately 10 pCi/g Ca.

(⁵⁵Fe) Counting

Liquid scintillation is particularly suitable for low-level counting of this radionuclide. The major problem in counting iron is the conversion of the ferric-ion to colorless compounds. Cosolito et al.¹⁸ obtained a Y-value of 0.33 pCi/mg Fe (B = 6 cpm, M = 20 mg and E = 0.17) by extracting iron with Di-(2-ethylhexyl)-phosphoric acid (EHPA) and the addition of 5 ml of EHPA to 15 ml of a toluene-based scintillation liquid containing 50 g/l of naphthalene. The Y-value of this technique in a modern liquid scintillation counter would be approximately 0.2 pCi/mg Fe.

Iron fluoride is colorless and has considerable solubility in water. If iron hydroxide is dissolved in a limited amount of HF, the resulting solution may be introduced in a suspension as described for (⁴⁵Ca). The only critical point in this procedure is the need for careful control of the amount of HF. The counting efficiency is changed only slightly by the addition of 0-250 mg of iron. The Y-value of this system is 55 pCi/g Fe (E = 0.1, M = 0.25 g and B = 9 cpm). This technique is well suited for low-level counting of iron in environmental samples.

(⁸⁵Kr) Counting

Low-level counting of krypton by liquid scintillation is based on the solubility of krypton in aromatic solvents. Curtis et al.¹⁹ report a simple liquid scintillation technique which, however, suffers from lack of reproducibility due to partial evaporation of krypton from the liquid phase. Sax et al.²⁰ report a plastic scintillation technique with a Y-value of 2 pCi/ml Kr (B = 25, M = 1.2 ml, and E = 0.95). A newly developed procedure in our laboratory for determination of (⁸⁵Kr) is accurate, sensitive and reproducible.

An evacuated glass vial (25 ml) with a luer fitting is connected to a krypton container and filled with krypton to a pressure of approximately 500-600 mm mercury. The vial, which is closed with a luer stopcock, is removed and connected to a 50-ml syringe filled with an aromatic-based scintillator. If this solution is deaerated, krypton is easily soluble in the aromatic solvent and the vial can be filled to the top with liquid within a few minutes. The stopcock is then removed and the vial sealed with a luer stopper. No krypton loss has been observed if the vial is left open for several minutes. Removal of the stopcock does not endanger the results even if it is carried out slowly. The counting efficiency of a sealed vial does not change over several months. The Y-value of this system is 0.14 pCi/ml Kr (E = 0.9, B = 22, and M = 17-20 ml).

(⁸⁷Rb) Counting: Determination of Natural Rubidium

Although for determining natural rubidium there are more sensitive techniques such as atomic absorption spectroscopy, the ease of operation of liquid scintillation offers some
advantages for determination of rubidium in environmental samples. Rubidium is easily separated by ion exchangers of the metal hexacyanoferrate-II type. The limitations of determining rubidium are given by the long half-life \( (4.7 \times 10^4) \) which results in a very low rate of isotopic decay and the abundance of \( ^{87}\text{Rb} \), 27.8%, resulting in only 29 dpm/mg of natural rubidium. The efficiency of rubidium in a mixture described for \(^{43}\text{Ca}\) is close to 100 per cent, but the optimum efficiency is 90 per cent for a background of 16 dpm. The \( Y \)-value is consequently 2 pCi/sample.

\((^{147}\text{Pm})\) Counting

The abundance of this radionuclide in the fission process and, consequently, its relatively low price, have led to a number of new applications for promethium; e.g., for production of self-luminous compounds. Determination of the radionuclide in environmental samples and radiobioassay of dial painters are two areas where liquid scintillation counting may be advantageously used.

Praseodymium (10 mg) is used in our laboratory as carrier for determination of radiochemical recovery of promethium. After anion exchange separation, the two possible interfering elements (Ce and Eu) are separated by their differences in valency from promethium if their presence is suspected. The radiochemical recovery is determined by weighing praseodymium oxalate that is subsequently dissolved in an EDTA solution. The final scintillation mixture is identical to the one described for \(^{143}\text{Ca}\). With an efficiency of 85 per cent and a background of 12 cpm, the \( Y \)-value corresponds to 1.8 pCi/sample.

Counting \(^{90}\text{Sr}, \ (^{90}\text{Sr}) \) and \(^{90}\text{Y}\)

Low-level counting of these radionuclides is similar to that described for \(^{147}\text{Pm}\). Inasmuch as strontium oxalate is insoluble in EDTA, the carbonate that can be dissolved in a few drops of hydrochloric acid is used instead. The \( Y \)-value for these radionuclides is approximately 2 pCi/sample.

\(^{90}\text{Sr}\) is usually accompanied by \(^{90}\text{Sr}\). In a conventional liquid scintillation counter, 10–20 per cent of \(^{89}\text{Sr}\) counts are registered in the \(^{89}\text{Sr}\) channel if the latter is set for an efficiency of 80 per cent. This is inadequate for environmental \((^{89}\text{Sr})-(^{89}\text{Sr})\) analysis. However, it permits an estimation of \(^{90}\text{Sr}\) level in the presence of \(^{90}\text{Sr}\) while a quantitative assessment is easily made by a recount of the sample after two weeks. The ingrowth of \(^{90}\text{Y}\) corresponds to the \(^{90}\text{Sr}\) level of the sample.

\((^{18}\text{Cl})\) Counting

The only sensitive low-level counting technique previously reported for chlorine is that of Ronzani and Tamers. They converted chlorine to \(\text{SiCl}_4\) which can be added up to 55 per cent by volume to a scintillation liquid with a resulting \( Y \)-value of 0.4 pCi/g Cl. This value may be reduced to 0.25 pCi/g Cl by optimization of the procedure and application of a modern liquid scintillation counter. Although their technique is sensitive, the labor requirements are high, and we have sought other alternatives.

Using a technique similar to that described for \(^{14}\text{C}\), the ideal chlorine salt for introduction into a liquid, is lithium chloride. The preparation of \(\text{LiCl}\) presented severe difficulties, and sodium chloride was consequently used. The difference in solubility of \(\text{NaOH}\) and \(\text{NaCl}\) in alcohol made the preparation rapid and easy. Eleven grams of sodium chloride may be introduced in a vial and counted with 95 per cent efficiency. The \( Y \)-value under optimum conditions is 0.43 pCi/g Cl \((E = 0.85, M = 6.6 \text{ g}, \text{ and } B = 20 \text{ cpm})\).

Counting Plutonium Isotopes

Numerous low-level counting techniques involving liquid scintillation have been proposed for plutonium. The sensitivity of these is inferior to other techniques such as solid state counting, primarily because of the rela-
tively high background levels encountered in liquid scintillation, but the ease of operation, particularly in sample preparation, has made liquid scintillation popular for plutonium counting. The efficiencies for the four major isotopes: \( ^{239}\text{Pu} \), \( ^{236}\text{Pu} \), \( ^{238}\text{Pu} \) and \( ^{241}\text{Pu} \), are essentially close to 100 per cent as described, for example, by Lindenbaum et al.\(^{22}\)

We have obtained similar results using a suspension similar to that described for \( ^{45}\text{Ca} \). A large amount of inorganic solids can be tolerated, limited by their solubility. Our suspension system is less subject to quenching than the system described by Lindenbaum. The \( Y \)-value of either system is 2.4 \( \text{pCi/sample} \) (\( E = 0.95 \) and \( B = 25 \text{ cpm} \)).

**OTHER RADIONUCLIDES**

Many other \( \alpha \)- and \( \beta \)-emitters have been counted successfully by liquid scintillation in our laboratory and elsewhere. The principle of the techniques are essentially as described for those radionuclides discussed in this paper. For radionuclides with a maximum \( \beta \)-energy emission of 200 keV or more, counting efficiency is usually 100 per cent and the optimum \( Y \)-value depends on the proper correlation of efficiency and background. The suspension described for \( ^{45}\text{Ca} \) is applicable to the low-level counting of the majority of radionuclides. Gaseous radionuclides may be counted in a system similar to that described for \( ^{85}\text{Kr} \).

**ALPHA SPECTROSCOPY BY LIQUID SCINTILLATION**

Horrocks has proposed an ingenious technique for alpha spectroscopy of \( \alpha \)-emitting radionuclides.\(^{23,24}\) The carrier-free nuclide is introduced in 250 \( \mu \text{l} \) of a scintillation liquid and counted with one PMT in association with a multichannel analyzer. Although the resolution of this technique is inferior to that of solid state spectroscopy, for example, it is expected to find wide acceptance after proper modifications are made for routine laboratory use.

**A LARGE-VOLUME LIQUID SCINTILLATION COUNTER**

The maximum sample size in a conventional liquid scintillation counter is determined by the capacity (25 ml) of the counting vial. Several large-volume liquid scintillation counters (LVLSC) have been described.\(^{4,25}\) The optimum conditions of an LVLSC are also governed by the \( Y \)-value. Our LVLSC consists of a cylindrical Teflon tube with an internal diameter of 5 cm and two Teflon windows. To determine the optimum volume of an LVLSC, tubes of varying lengths are cut and filled with the scintillation mixture. That for \( ^{3}\text{H} \) is 250–300 ml, with a \( Y \)-value of 0.25 if the Triton N-101 suspension is used. Recently we have constructed an anticoincidence shield which preliminary experiments show can substantially reduce background. The preliminary \( ^{3}\text{H} \) \( Y \)-value of our LVLSC is 0.15 \( \text{pCi/g H}_{2}\text{O} \). This system may be applied to other radionuclides such as \( ^{14}\text{C} \) and \( ^{35}\text{Cl} \). Detailed results of these investigations will be published in the near future.

**OUTLOOK**

From the foregoing description it is clear that liquid scintillation is an effective technique for low-level counting of radionuclides. Recent developments have increased the detection capability of this technique considerably. The major drawback of liquid scintillation at present is the contribution of external radiation to the background. It is hoped that instrument manufacturers will attempt to offer anticoincidence shield as an option in the near future. This option would enable those involved in environmental surveillance to use this useful technique for determination of radionuclides such as \( ^{90}\text{Sr} \).

The development of more sophisticated scintillation mixtures will certainly place more strict requirements on temperature control of liquid scintillation instruments. Temperature dependence of phosphorescence and chemiluminescence is an additional reason for...
desirability of temperature control. We feel that it is already desirable to be able to operate our liquid scintillation counters between 10–25°C with an accuracy of ±1°C.

REFERENCES

The development of equipment for continuous scintillation counting may be ascribed in large part to the prior introduction of automated systems for the chromatographic separation of various compounds, particularly amino acids. While methods were worked out which replaced the discrete analysis of chromatographic fractions with a continuous colorimetric analysis (amino acids), with ultraviolet spectrophotometry (nucleotides), or with other techniques, the need for methods that would permit the simultaneous monitoring of radioactivity also became apparent.

Our involvement in the development of methods for the continuous counting of aqueous solution arose around 1955 from our interest in the metabolism of sulfur amino acids. The ($^{35}$S)-labeled substances encountered in these studies were separated by ion-exchange chromatography. The radioactivity in the effluent was measured with a Geiger counter. The rather large amounts of salt left after evaporation of the chromatographic fractions however, led to excessive self-absorption. Heterogeneous samples often had to be converted to BaSO$_4$, and then plancheted to obtain accurate and comparable results.

While it proved quite easy to conceive of methods for the continuous recording of hard $\beta$-emitters such as ($^{32}$P) or $\gamma$-emitters such as ($^{131}$I) in flowing aqueous solutions, the $\beta$-particles from ($^{35}$S), ($^{14}$C) and ($^3$H), which are most frequently used for labeling organic and biological substances, offered no simple solution. The introduction of liquid scintillators did not at first solve the problem because of their poor capacity to incorporate water. At the time they were introduced, no satisfactory recipes had yet been worked out to circumvent this difficulty, and most chromatographic effluents contain salts, acids or bases that lower sample solubility or increase quenching in aqueous scintillators.

To solve the problem of self-absorption...
by the solvent, a way had to be found of spreading the solution into a thin film so that it was in close contact with the detector. This was first achieved by Bangham, using classical equipment consisting of a proportional counter with a large mica window in contact with the aqueous stream. The efficiency was low, however, and amounted to 1% for (14C). In the meantime, plastic scintillators had become available and were chemically inert to most aqueous solutions and possessed greater mechanical strength than large organic crystals. They offered the opportunity of building more sophisticated cells with the liquid stream in direct contact with the fluor, thus avoiding any absorption of radioactivity by a window or membrane. Our first cell was constructed from this material and allowed us to increase the efficiency for (14C) and (35S) to 6%.

The advantages of such a method for the continuous recording of the radioactivity of chromatographic effluents appeared numerous: (1) No quenching is likely to occur. (2) Since the geometry and the self-absorption remain constant (small changes in the density of the effluent will not affect the self-absorption significantly), continuous counting is performed with constant efficiency. (3) Most important, in the study of intermediary metabolism with labeled molecules, it offers an easy way to detect unknown metabolites because no specific reaction or property is necessary to show their presence. For example, the chromatographic study of taurine metabolites in the rat rapidly led us to the discovery of the conversion of taurine to isethionic acid (OH-CH2-CH2-SO3H), which could be identified by isotopic dilution but was otherwise very difficult to detect by classical means. (4) The method is non-destructive, i.e., counting does not affect subsequent analysis or recovery of the sample. Results similar to ours were obtained by Funt and Hetherington in 1959 using a cell of simpler construction which consist of small bore plastic scintillator tubing.

Data on the use of suspended fluorors for the counting of aqueous solutions were published in 1958 by Steinberg. In his first experiments he used a plastic scintillator in the form of thin filaments but soon replaced them with anthracene crystals offering the advantage of a much higher efficiency. For the first time, counting of (3H) appeared to be practical. Steinberg's findings led us to reconsider our concepts concerning the construction of continuous counting cells and to adopt the suspension counting method. Anthracene-packed cells were developed by us and others and are still used for many purposes, especially for the monitoring of amino acids.

Looking back at the evolution of flow-cells, it may be concluded that the lack of miscibility of aqueous solutions in organic scintillators was ultimately turned into an advantage. Flow-cell design has been improved and simplified, and most flow-cells used today are shaped like ordinary counting vials so that they can be easily inserted into conventional liquid scintillation counters.

At the time of the aforementioned investigations, there were efforts to record radioactivity in aqueous effluents continuously by homogeneous counting methods. Until recently the results were rather discouraging. Although satisfactory efficiencies could be obtained, high scintillator-to-effluent ratios had to be used which rendered the method prohibitive from an economic point of view. The introduction of emulsion counting and of dioxane-based scintillators has modified this situation somewhat, and continuous homogeneous systems may now be envisaged in cases where anthracene cannot be used.

The use of suspended anthracene has been thoroughly discussed by Piez. More general accounts of flow monitoring have been published by Rapkin. The properties of organic scintillators have been previously described by the present author and have
FLOW-MONITORING OF AQUEOUS SOLUTIONS

been thoroughly discussed in a recent book edited by Horrocks.12

FLOW-CELL DESIGN

General Characteristics

Flow-cells designed for the continuous counting of weak β-emitters should satisfy a series of conditions: (1) Their geometry must provide the highest possible surface-to-volume ratio between the fluor and the solution, while their shape and overall dimensions must allow them to be inserted between two photomultipliers or, when conventional scintillation counters are used, to take the place of a standard counting vial. (2) Mixing the solution inside the cell and the connecting lines must be avoided so as not to reduce the resolution between successive portions of the fluid. This can be achieved by using small cross-sections for the fluid lines and the counting cell. (3) The "effective" volume of the cell should be large enough to accumulate a sufficient number of counts during the passage of a radioactive portion of the liquid. To avoid loss of resolution, the volume should remain compatible with the rate of change in radioactivity. In chromatographic experiments, for instance, the cell volume will usually be of the same order as the fractions collected for the analysis of the effluent. (4) The cell must be leak-free and resist the back-pressure of the system in which it is incorporated. Its flow-resistance should not add too greatly to the overall back-pressure.

The various cells designed until now will be described in the next paragraphs and belong to three types:

(1) "External" counting cells. In this type of cell, the first to be described in the literature, the aqueous fluid is circulated through a cell of plastic scintillator.

(2) Cells packed with suspended fluor particles or crystals. The anthracene cell belongs to this type and has been the most popular system during the past years, although other cell-types have proven more adequate in specific cases and may still gain in acceptability.

(3) Homogeneous counting systems. In such systems, the fluid is continually mixed with a liquid scintillator before passing through the cell. Until now, this type of cell has provided by far the highest efficiencies for ($^3$H), but it implies that the flowing solution be miscible with the liquid scintillator. One is consequently faced with exactly the same problems as in the discrete counting of aqueous samples by mean of liquid scintillators.

Plastic Scintillator Cells of the External Type

When we constructed our first flow cell from plastic scintillator,13,14 it occurred to us that this material was rather brittle and could not easily be machined. The cell body was therefore made from a grooved block of nonscintillating Perspex sandwiched between two thin sheets of plastic scintillator. The thickness of these sheets (0.3 mm) was selected so as to absorb completely the radiation of ($^4$C) and ($^3$S) without unduly increasing the background. The observed efficiency was about 6 per cent for ($^4$C) with a background of about 150 cpm for an unshielded cell. Although much higher efficiencies were obtained afterwards, this first result led us to important conclusions as to the applicability and advantages of the method. Besides the possibility of counting aqueous fluids without any alteration and with an acceptable efficiency, no retention of radioactivity or significant loss of resolution were observed. Efficiency was further unaffected by the presence of salts, acids or bases in the buffers used for the chromatography.

Soon after the publication of this cell design, it became possible to build similar cells in a much simpler way, thanks to the availability of small-bore plastic scintillator tub-
ing. Cells made of such tubing wound into a flat coil, described by Kimbel and Willenbrink \(^{15}\) and by Funt and Hetherington,\(^3\) were soon made commercially available.

Selection of the cross-section and of the wall thickness resulted from a compromise between several factors. Self-absorption by the liquid decreases when the lumen is reduced and a higher intrinsic efficiency is therefore attained. For small cross-sections, however, the count rate actually observed per unit length of coil will decrease. Although this can be partially compensated for by using a longer length of tubing, one is nevertheless limited first by the fact that the wall thickness may not be reduced beyond the maximum range of the \(\beta\)-particles and second, because the multilayer spiral that would result from a longer length of tubing is disadvantageous from the point of view of light transmission. The back-pressure of such cells may also become excessive. The inner diameters of the tubings used by Kimbel and Willenbrink \(^{15}\) and by Funt and Hetherington \(^3\) were 0.7 and 0.6 mm, the wall thicknesses 0.4 and 0.45 mm. The lengths of tubing used were 80 and 100 cm, giving internal volumes of 0.3 and 0.27 ml, respectively. The efficiencies observed by Funt and Hetherington were 76.4 per cent for \((^{32}\text{P})\), 51.4 per cent for \((^{22}\text{Na})\), and 5.7 per cent for \((^{14}\text{C})\). A similar cell has been used for measuring \((^{45}\text{Ca})\) by Pickering et al.\(^{16}\) who observed an efficiency of between 7.15 and 10.9 per cent for this isotope.

**Continuous Counting with Suspended Fluors**

The potentialities offered by suspended fluor for the counting of aqueous solutions were first emphasized by Steinberg.\(^4\) By dispersing a finely divided fluor in a solution, a situation is approached resembling that in which the sample is dissolved in a liquid scintillator. An intimate contact is achieved between the sample and the fluor, and many problems related to insolubility of the sample in organic solvents or to chemical quenching are eliminated. For a number of practical rather than theoretical reasons, the method has found little acceptance for the counting of discrete samples and has not been further developed. It has proved very suitable for the construction of continuous flow cells with much higher efficiencies than can be attained with cells of the external type.

Several types of fluor have been tested by Steinberg \(^{14-16}\) for the discrete counting of aqueous samples. In his first series of experiments, filaments of Pilot-B plastic scintillator, 0.043 cm in diameter, were packed in a bundle inside a glass tube fitting into a conventional scintillation counter. The sample was introduced into the void space of the bundle by capillary action. In a simpler version of the same technique, the filaments were cut into short fragments and suspended in the sample. With the bundle technique, efficiencies up to 35 per cent were observed for \((^{14}\text{C})\), and for the first time promising results were also obtained for \((^{2}\text{H})\) which could be counted with an efficiency of 0.7 per cent. When loosely suspended in the radioactive solution the filament fragments proved far less efficient [11.5% for \((^{14}\text{C})\)].

In later experiments, several crystalline organic scintillators were tested: anthracene, PPO, POPOP, t-stilbene, and diphenylstilbene. Among these, anthracene was found the most suitable and the following efficiencies were reported for several isotopes when 1 g of blue-violet fluorescence grade anthracene (Eastman Kodak) was suspended in 3 ml of solution: \((^{2}\text{H})\), 0.5 per cent; \((^{14}\text{C})\), 16–20 per cent (according to batch); \((^{45}\text{Ca})\), 49 per cent and \((^{32}\text{P})\), 78 per cent. In the presence of an excess of anthracene, efficiencies up to 54 per cent were obtained for \((^{14}\text{C})\). Among the \((^{14}\text{C})\)-labeled substances tested by Steinberg were amino-acids, peptides, proteins and glucose.
His results were further checked in the presence of salts, diluted acids and alkali. To counteract anthracene's tendency to float and to achieve a good contact between the scintillator and the solution, some detergent had to be added to the suspension. (The same technique is used today for the packing of flow cells.)

The advantages of suspended anthracene for the continuous counting of aqueous solutions were exploited by Schram and Lombaert \(^1\), \(^1\) and by Rapkin and Packard. \(^2\)

The first cell described by Schram and Lombaert \(^1\) consisted of a Teflon block in which a zig-zag path had been grooved. This groove was covered with a sheet of Perspex and faced two photomultipliers placed at a right angle, with a Perspex prism serving as light-guide. The cell was filled with small anthracene crystals (Fluka, Switzerland) which had been calibrated by sieving. Its free space was about 1 ml and efficiencies up to 40 per cent were recorded for (\(^{14}\)C) when the cell was used in connection with an amino-acid analyzer.

The first cell developed by Rapkin and Packard \(^2\) consisted of a cylindrical polyethylene coil filled with crystalline anthracene and was positioned between opposite photomultipliers. Practical difficulties were encountered in obtaining uniform packing, and the coil-shaped cell was replaced by a simpler design consisting of a straight glass tube (7 mm in diameter and 50 mm long). In the instrument described by Rapkin and Gibbs \(^3\) and shown in Fig. 1, this tube was inserted in a hole drilled vertically in a cylindrical polymethacrylate block, placed between the photomultipliers and acted as a light-guide. This design proved quite adequate and did not impair the resolution of peaks when used with commercial amino-acid analyzers. Its efficiency was above 50 per cent for (\(^{14}\)C) and 1–2 per cent for (\(^{3}\)H).

At the same time, Schram and Lombaert \(^1\) further developed their own cell design. Their Teflon anthracene-cell was soon replaced by a flat coil consisting of a 60-cm length of polyethylene tubing 2.3 mm in diameter. To ensure proper packing, it was filled while unwound and coiled afterwards into a flat spiral fitting in a thin Lucite.

![Fig. 1.—Anthracene flow cell developed by Rapkin and Gibbs (courtesy of the authors).](image-url)
holder filled with silicon oil for optical coupling. (See Fig. 2.) This holder could easily be sandwiched between the windows of two opposite photomultipliers mounted with springs to ensure proper contact.

The mesh size of the anthracene used to fill the coil was carefully selected by means of standard sieves. A compromise had to be made between efficiency and flow resistance, since both increase with decreasing particle size. The counting efficiencies obtained were 55 per cent for (14C) and 2 per cent for (3H) with 150-μm anthracene crystals. The background amounted to about 60 cpm without refrigeration.

The diameter of the tubing was kept rather small to minimize loss of resolution and improve light transmission through the anthracene bed. Although the anthracene bed looks opaque, its efficiency is only slightly altered when its thickness is increased. Comparative measurements performed with short column segments have shown that when the diameter of the anthracene column is increased from 4 to 8 mm the efficiency for (3H) decreases only by about 7 percent.

Count rates observed for (3H) with various anthracene columns were as follows:

<table>
<thead>
<tr>
<th>Diameter</th>
<th>Dry Weight of Anthracene</th>
<th>cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 mm</td>
<td>25 mg</td>
<td>4545</td>
</tr>
<tr>
<td>4 mm</td>
<td>75 mg</td>
<td>13022</td>
</tr>
<tr>
<td>8 mm</td>
<td>100 mg</td>
<td>16878</td>
</tr>
<tr>
<td>8 mm</td>
<td>300 mg</td>
<td>48602</td>
</tr>
</tbody>
</table>

The absence of distortion of peaks obtained by chromatography was checked by comparing the curves for identical samples introduced in succession on the same ion-exchange column which contained two compounds chosen for their close appearance in the eluate: e.g., taurine and urea.

The cylindrical cell described by Rapkin and Gibbs was subsequently improved by replacing the glass tube, which was responsible for elevated backgrounds, with a Lucite or a translucent Kel-F detector cell. Still lower backgrounds were obtained by Piez, using a U-shaped quartz-cell (Fig. 3) with a larger arm containing the anthracene. Symmetrical U-shaped cells with larger volume and made of Lucite or Kel-F were used by Elwyn in cases where the level of radioactivity was marginal. Increasing the volume of the cell gave better statistics, but partial overlapping of the peaks reduced the resolution attained in the chromatographic step. The use of larger cell volumes was responsible for higher backgrounds that could, however, be reduced by improved shielding.

**Continuous Counting with Liquid Scintillators**

The use of liquid scintillators for the continuous counting of aqueous solutions has long met with little success for several reasons: (1) Aqueous column effluents are poorly soluble in liquid scintillators and their salt content is often a source of trouble. High scintillator-to-effluent ratios must therefore be used, rendering cost of the method prohibitive. As far as the scintillator is concerned, anthracene cells can be run at practically no cost. (2) The efficiency of liquid scintillators varies with their water content and with the composition of the aqueous fluid. (3) The method is destructive.
Except for the last problem, the situation has improved in recent years and the objections to the use of liquid scintillators are not quite as valid as they used to be.

Liquid scintillators offer two definite advantages over other methods: (1) In the case of (3H) they have a much higher efficiency than suspended scintillators. This aspect is of paramount importance because the use of increased flow rates in present-day automatic amino-acid analyzers and similar systems has resulted in a shorter dwell-time of the fluid in the counting cell, further lowering a counting efficiency that was already marginal. (2) Contamination of the cell is less likely to occur. Substances that tend to become adsorbed on anthracene will in many cases be counted without difficulty using the homogeneous counting method. Fluids containing organic solvents that tend to dissolve anthracene and other organic scintillators may also be counted by this method, if they do not cause severe quenching.

A comparative study of the advantages of suspension and homogeneous counting for the flow monitoring of aqueous solutions was presented several years ago by Scharpenseel and Menke using a liquid scintillator that was a mixture of toluene and ethanol (9:6 v/v) containing PPO and POPOP. This mixture was added to the chromatographic effluent by means of a metering pump in the ratio 30:1 and circulated through a coil of potassium-free glass placed between two photomultipliers. Although the ratio of scintillator to aqueous solution was high, salt precipitation still caused occasional blocking of the lines. Scharpenseel and Menke insisted on the importance of efficient mixing, achieved by means of a vibrating rod inserted in the fluid line and actuated by a magnetic stirrer. The efficiency attained for (3H) was 6 per cent, obviously far better than could be attained with suspended scintillators.

An important contribution in the field of continuous homogeneous counting has recently been published by Hunt who gives an interesting account of the possibilities offered by this method. He describes the use of a liquid flow cell in connection with a flow spectrophotometer and a multipoint recorder for the continuous counting of (3H) and (14C) in enzymatic digests of RNA, although his system has more universal applicability. The coils he used were made from polyethylene tubing (0.085 or 0.070 inches in diameter) by wrapping the tubing around a test tube, heating in boiling water until soft, and cooling rapidly. These coils are contained in
USES OF THE SCINTILLATION COUNTER

an outer casing made by cutting off the top of a polyethylene counting vial to fit into conventional scintillation counters. (See Fig. 4.) Another cell consisting of two concentric helices of Teflon tubing embedded in clear plastic was not further investigated because of its poorer efficiency for \(^{3} \text{H}\). Before counting, the chromatographic effluent was mixed with a dioxane-based scintillator mixture by pumping part of the effluent, split at a T junction, at 3 ml/hr into one arm of a mixing chamber containing a glass-covered magnetic stirring bar. The scintillator solution was pumped into the chamber through a second arm at 27 ml/hr giving a 10 percent aqueous content in the scintillator.

The different scintillators Hunt assayed under these conditions for their ability to count \(^{3} \text{H}\) and \(^{14} \text{C}\) in the presence of varying amounts of salt were: (a) A dioxane solution of 100 g naphthalene, 10 g PPO and 0.25 g POPOP per liter. (b) The same dioxane solution with 10 percent by volume of a mixture of butan-1-ol (100 parts), 2,2' -diethylhexylamine (11.7 parts) and acetic acid (2 parts). (c) Bray’s solution: a dioxane solution of 60 g naphthalene, 4 g PPO, 0.2 g dimethyl POPOP, 100 ml methanol and 20 ml ethylene glycol per liter. (d) Bray’s solution with 2 percent by volume of 2,2' -diethylhexylamine (11.7 parts) and acetic acid (2 parts).

Table 1 summarizes some of Hunt’s findings: the efficiency of his flow cell compared with the results obtained when 10 ml of the same solutions were counted in polyethylene vials. The choice of mixtures proposed depended on the salt concentration used. Despite its lower efficiency, mixture d was generally preferred over c because of a smaller drop in efficiency over the range 0–0.6 m (20 percent instead of 40 percent). Hunt concluded that, even at the lowest \(^{3} \text{H}\) efficiency (4 percent) the figure of merit (efficiency $\times$ effective cell volume) still compared favorably with that of an

<table>
<thead>
<tr>
<th>Scintillator Solution</th>
<th>Isotope</th>
<th>Efficiency (%)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Polyethylene Coil</td>
</tr>
<tr>
<td>a</td>
<td>(^{14} \text{C})</td>
<td>69.6</td>
</tr>
<tr>
<td>b</td>
<td>(^{3} \text{H})</td>
<td>13.6</td>
</tr>
<tr>
<td>c</td>
<td>(^{14} \text{C})</td>
<td>69.2</td>
</tr>
<tr>
<td>d</td>
<td>(^{3} \text{H})</td>
<td>9.4</td>
</tr>
<tr>
<td></td>
<td>(^{14} \text{C})</td>
<td>68.6</td>
</tr>
<tr>
<td></td>
<td>(^{3} \text{H})</td>
<td>10.1</td>
</tr>
<tr>
<td></td>
<td>(^{14} \text{C})</td>
<td>65.4</td>
</tr>
<tr>
<td></td>
<td>(^{3} \text{H})</td>
<td>6.5</td>
</tr>
</tbody>
</table>
anthracene cell, although ($^{14}$C) efficiency was lower. The 1:10 effluent-to-scintillator ratio is still acceptable from an economic point of view, and dioxane apparently does not permeate polyethylene walls as toluene may.

No thorough study has yet been published concerning the advantages of emulsion counting for the continuous monitoring of aqueous solutions. Since the efficiency of liquid scintillators containing a detergent as Triton-X 100 is by far less sensitive to quenching than true solutions, higher efficiencies and better resolution of ($^3$H) and ($^{14}$C) may be anticipated from such a system.

PRACTICAL ASPECTS

Early flow cells were sometimes characterized by rather high backgrounds caused by the absence of an adequate shielding; the use of large light-guides that acted as a source of Čerenkov radiation or were responsible for optical interaction between the photomultipliers; the use of glass as construction material; and the absence of cooling, resulting in higher noise from early photomultipliers. Most of these inconveniences have been overcome in present-day instruments.

Self-Absorption in Two-phase Systems

Authors generally agree about the absence of chemical quenching when passing colorless solutions through plastic-scintillator or anthracene-packed flow cells. Counting efficiencies remain constant for each isotope over a wide range of activities and for a wide choice of buffer compositions, although a significant change in the density of the fluid may affect the self-absorption and should be taken into account. Conventional standardization methods are inadequate for checking for this effect, but they are not necessary in most cases. When available, an external standard can be used to check for variations in instrument performance and to monitor the counting efficiency of colored solutions. The absence of chemical quenching makes two-phase systems very attractive and has been one of the reasons for their success. (See Chapter 19.)

Quenching in Homogeneous Systems

When monitoring an aqueous stream by means of a liquid scintillator, the same rules will apply as when counting discrete samples. External standardization or channel ratio will be useful for determining efficiency ad for calculating the necessary corrections. It is fortunate that the composition of many chromatographic effluents remains relatively constant and that the amount of material present in the peaks is small. The amount of quenching and the efficiency will thus remain constant in many cases.

Effective Volume and Counting Efficiency of Flow Cells

The true counting efficiency of a flow cell is equal to the observed count rate divided by the amount of radioactivity present in the cell. The latter figure can only be determined if the effective volume of the cell is known, and several methods have been used for this purpose. For suspended scintillators, Schram and Lombaert have used an acid solution of known molarity which is circulated through the cell until it reaches the outlet. Inlets and outlets are then carefully taken off, filled with distilled water and reinstalled. More water is passed through the cell while collecting the effluent until it is neutral. Titration of the acid effluent with alkali gives the effective volume of the cell. After this figure has been determined, the counting efficiency can be established by circulating a solution of known specific radioactivity through the detector.

One should be aware that the efficiency of a flow cell is only a partial measure of its performance and that the sensitivity attained
will depend on its volume and on the flow rate of the liquid.

Contamination of Flow Cells by Adsorbed Radioactivity

Adsorption of various substances on solid fluors has been reported by several authors and may limit the usefulness of a particular kind of flow cell.

Adsorption of \((^{32P})\text{-phosphate}\) ions on the walls of a plastic scintillator cell was observed Funt and Hetherington.\(^5\) The absorbed radioactivity could be removed by rinsing with a versene solution. Using a similar device, Sjöberg and Agren\(^22\) obtained satisfactory results when recording the radioactivity of acid-soluble \((^{32P})\)-labeled nucleotides eluted with formic acid or formic acid-ammonium formate buffers.

Crystalline anthracene has been widely used for monitoring labeled amino acids after their separation by chromatography on ion-exchange columns. No retention of these amino acids or their metabolic products by the suspended scintillator was reported, even in the case of the aromatic amino acids phenylalanine and tyrosine.\(^15\) Untreated amino acid solutions may contaminate anthracene cells because of the presence of impurities that are otherwise retained by the ion-exchange column.\(^9\) Elwyn has also observed contamination from standard amino acid samples, normally not retained, that had been left at room temperature for some time.\(^21\) Microorganisms were assumed to be responsible for this effect.

Substances likely to cause accidental contamination of the counting cell may usually be removed from the stream by inserting a piece of tubing filled with anthracene ahead of the counting cell. The anthracene can easily be replaced and will act as a filter for tracer contaminants that would otherwise spoil the detector and for suspended particles that might eventually clog the system.

Although anthracene has proved quite adequate for the monitoring of amino acids, difficulties have been encountered with other substances such as polysaccharides, proteins, nucleic acids and phosphate. In experiments with \((^{14C})\)-labeled proteins, Schram and Lombaert\(^18\) were able to avoid adsorption by mixing the fluid stream with 0.1 NaOH before it was circulated through the cell. Adsorption of nucleotides was reported by Hunt\(^22\) and motivated him to another kind of flow cell (See above). Adsorption of \((^{32P})\)-phosphate has been observed by Martin\(^24\).

Operation of Anthracene Flow Cells

For practical reasons, users of anthracene counting cells should be able to pack their cells themselves. To avoid air being trapped between the suspended fluor particles, the anthracene bed must remain immersed and cells thus cannot easily be shipped ready to use. Clogging or contamination from use may also require repacking of the cell by the user.

Filling the counting cell is most conveniently achieved by means of an anthracene suspension in water. Detergent should be added to facilitate wetting the suspended particles which otherwise tend to float. Commercial anthracene batches are heterogeneous as far as the dimensions of the crystals are concerned, and fines must be discarded by decantation before use. The suspension is poured into the cell by shaking to ensure proper packing and to remove possible air bubbles. Quartz wool may conveniently be used for the plugs at the bottom and top of the fluor bed. When properly packed and handled with care, an anthracene cell should last indefinitely and has been reported to show no change of efficiency for more than 6 months.\(^21\) The flow resistance of the cell should also remain low.

Anthracene has been used mostly for the monitoring of aqueous solutions in which it is perfectly insoluble. Some chromatographic separations may require the addition of organic solvents in which anthracene might be more or less soluble. To check for this, a
few anthracene crystals should be suspended in the solvent mixture and put on a magnetic stirrer for some time. Solubilization of the anthracene may easily be shown by measuring the optical density of the supernatant solution at 376 nm. We have observed in this manner that no significant solubilization occurred in 50 percent (v/v) solutions of formic or acetic acid. Ethyl alcohol and formamide will slightly dissolve anthracene.

Miscellaneous

Flow cells should be designed to resist the pressure of the system in which they are incorporated. Leakage of the cell or its connectors could impair the results and would be disastrous if the liquid happened to reach the electronic part of the detector. In addition to being leak-free, flow cells should be light-tight. The tubing used for inlets and outlets may easily act as a light-pipe and care should therefore be taken to use sufficiently long connecting lines made of opaque material such as black Teflon.

Chemiluminescence has been reported in several cases where suspended and liquid scintillators were used. (See Chapter 19.) Whenever this is suspected, a nonradioactive sample should be run through the cell. Chemiluminescence of dioxane-based scintillator solutions was observed by Hunt, but they could be eliminated by cooling the flow cell and detector below 10°C. (See Chapters 32–34.)

RESULTS

Data Recording

During their early development, flow detectors were used in connection with a ratemeter and strip-chart recorder. The principal advantage of such a system is that the separations can be easily visualized. On the other hand, the use of logarithmic instead of linear ratemeters is advantageous because small peaks can more easily be detected while large ones are still correctly recorded. Calculation of the results from analog recorders may be tedious and difficulties may arise when the peaks are not symmetrical or when jittering of the pen does not allow curve estimation with sufficient precision. Increasing the time constant of the ratemeter gives smoother curves but precision is reduced at the same time.

Simplicity of calculations prompted investigators such as Piez to use digital systems which usually proved more expensive but which become convenient with the availability of cells that fitted in conventional liquid scintillation counters equipped with printers and timers. Digital recording may be achieved by setting the counter for repetitive counting over identical time intervals. The results are printed in succession at the conclusion of each counting period and may afterwards be plotted on a graph. The radioactivity of a given peak is obtained by summation. The time needed for printing and resetting of the scaler should be as short as possible in order not to lose an excessive number of counts between successive counting intervals. The counter should include a high-speed printer or a system allowing counting to go on while printing proceeds. Digital counting offers definite advantages over analog counting for accurate calculation of results and statistical error.

In many cases radioactivity curves will have to be compared with curves obtained by other methods such as colorimetric analysis for amino acids or spectrophotometry for nucleotides. Since the same portion of liquid is registered at different moments by the two systems, it is necessary to know exactly how much one curve lags behind the other. In some cases a small shift may be observed because of an isotope effect [for instance, in the ion-exchange chromatography of (14C)-labeled amino acids]. Piez observed that (13C) specific radioactivity increased from the leading to trailing sides of the amino-acid concentration peak. The effect was maximum for phenylalanine which showed a
volume displacement of 2.5 ml (See Chapter 14.)

**Precision and Sensitivity**

The precision of the results obtained with flow cells depends mostly on two factors: the flow-rate, and the statistical error, which is itself dependent on the signal-to-noise ratio and on the number of counts available. Constant flow rate is important in that the dwell time of each portion of liquid in the counting cell should be the same. This can easily be attained by using precision metering pumps. The statistical error is less susceptible to improvement in continuous measurements. When performing discrete measurements, the statistical error can be improved very simply by lengthening the counting interval. In continuous measurements one is forced to consider manipulating other factors discussed above.

*Flow-rate* is usually fixed by the kind of experiment performed. In chromatographic separations, lowering the flow rate would mean longer experiments and would make the method less attractive.

*Effective cell-volume* should be as large as possible to increase the number of counts recorded, but at the same time it should remain compatible with the resolving power of the separation method involved in the experiment. For chromatographic separations, the cell volume will in general be the same as that of the fractions found adequate for the chemical or physical-chemical analysis of the effluent.

*Duration of counting periods* must remain within reasonable limits so as not to impair the resolution obtained in the preceding steps. The optimal time of measurement will correspond to the passage of one cell volume if the latter has been correctly chosen.

It will be noted that flow rate, cell volume and time of measurement are interrelated and that it is rather difficult to change these parameters. To increase statistical precision in marginal cases where very low levels of radioactivity must be detected, some authors have sacrificed some resolution to precision by increasing cell volume.

The true efficiency (the number of counts observed per the amount of radioactivity present in the flow cell) is a rather arbitrary figure and does not always give a true idea of cell performance. A more realistic parameter is the *figure of merit* which is equal to the product of cell efficiency and volume.

One can also consider a dynamic parameter the *apparent efficiency*. Depending on the flow rate, a different number of counts will be collected for the same peak while the true efficiency of the cell remains constant.

The accuracy of continuous measurements will ultimately depend on the total number of counts collected and on the *signal-to-noise ratio*. When considering the case of chromatographic peaks, the accuracy will therefore decrease with the peak width. If the shape of these peaks is assumed to be Gaussian, their maximum height should always be about twice as large as their mean height. If 99 percent of the radioactivity of a peak is contained in five successive fractions of same volume, 39 percent of the radioactivity should be found in the middle fraction although the average radioactivity of each fraction is only 20 percent. For instance, if the background is 25 cpm, a peak containing 60 cpm will be twice the background at its maximum. Small peaks may show up, although the overall accuracy may be rather poor.

**Example**

With all the above factors in mind, it is possible to calculate the theoretical number of counts which must be present in a peak to achieve a fixed accuracy of measurement.

Experimental conditions:
- Effective volume of flow cell: 1 ml
- Flow rate: 30 ml/hr
- Time of measurement: 2 min
- Width of peak: 6 ml

*Uses of the Scintillation Counter*
Background: 25 counts/min
Apparent efficiency: 50 percent (in this case it is equal to the true efficiency)

Number of counts necessary to attain 1 percent accuracy:
At 68 percent confidence level, $2.1 \times 10^4$ dpm
At 95 percent confidence level, $8.1 \times 10^4$ dpm

Number of counts necessary to attain 5 percent accuracy:
At 68 percent confidence level, $1.5 \times 10^3$ dpm
At 95 percent confidence level, $4.1 \times 10^3$ dpm

It is thus apparent that amounts of radioactivity which are orders of magnitude above background levels must be available if any sort of precision is to be obtained.

Resolution of Double-labeled Samples

With monitoring fluids containing two isotopes, the record for the less energetic isotope will always be cross-contaminated by the more energetic isotope so that the analog curves or digital records cannot be interpreted at once. Correction for this cross-contamination may require much computation, and efforts have been made towards automatic compensation. An analog system for this purpose has been described by Lowe. Automatic compensation for cross-contamination has similarly been incorporated in commercial equipment. In such systems spectral overlap is eliminated by automatically subtracting a fixed percentage of the counts appearing in the channel assigned to the more energetic isotope from the counts in the channel assigned to the less energetic one (see also Chapter 31). The distribution of the more energetic isotope in both channels must therefore be established in advance, and the efficiency of the cell must remain constant for the duration of the experiment. (See Chapter 31.) It should be remembered that the energy resolution of dual-labeled samples is far less satisfactory with anthracene flow cells than with liquid scintillators.

PRACTICAL APPLICATIONS OF FLOW MONITORING

Up to now many applications of flow-monitoring have been concerned with the counting of amino acids and their derivatives by means of anthracene-packed cells. The method has, however, much wider capabilities, as indicated below. Because of their low efficiency, plastic scintillators have been replaced by anthracene or liquid scintillators for most practical applications.

Continuous Counting of Aqueous Solutions with Anthracene

Anthracene has been found particularly suitable for counting amino acids. Flow cells packed with this fluor have been widely used in connection with automatic amino acid analyzers. When the radioactivity is recorded with a rate meter, extra instrumentation can be spared by using the third channel on the recorder of the amino acid analyzer, a channel normally used to record the absorbancy of the lower sensitivity cell at 570 nm. In cases where the response of the higher sensitivity cell is off-scale because of the presence of high amino acid concentrations, one should resort to the absorbancy at 440 nm which is generally lower and can readily be related to the absorbancy at 570 nm. So that scintillation photons are not absorbed by the color developed in the presence of ninhydrin, counting must be performed before the addition of the reagent. The radioactivity trace will therefore precede the ninhydrin trace by about 18–20 minutes.

Anthracene flow cells have been used in our laboratory for other purposes as well. The following examples illustrate some of these applications.
Metabolism of sulfur amino acids. Our first experiments involving the flow monitoring of weak $\beta$-emitters were concerned with the metabolism of the sulfur amino acids and some of their derivatives. Column radiochromatography was used to trace the metabolites of taurine and related compounds. One of these metabolites was identified as being isethionic acid.

Biosynthesis of histones. In the course of more general studies on protein biosynthesis, the preferential synthesis of histones was followed by measuring the rates of incorporation of labeled arginine and lysine as compared to those of other amino acids. These rates were determined by radiochromatography after hydrolysis of the proteins synthesized.

Activation of amino acids in the course of protein biosynthesis. The specificity of activating enzymes and transfer nucleic acids has been checked by the radiochromatographic assay of the amino acids selectively retained from a mixture of labeled amino acids.

Tritiation of proteins. Automatic ion-exchange chromatography combined with flow monitoring has been used to study the extent by which the several amino acids of a protein are tritiated either by the Wilzbach method or by catalytic exchange.

Radiochemical purity of amino acids. In the course of systematic checks performed in the past years on commercial ($^{14}$C)-labeled amino acid preparations, some batches of isoleucine were found to contain appreciable amounts of allo-isoleucine. Similar results were also obtained by other authors.$^5$ Radiochromatography offers an easy way to check for such foreign substances and should often be applied more systematically.

Homogeneous Counting of Aqueous Solutions

Notwithstanding its advantages for a wide array of applications, anthracene has often shown limitations when compounds other than amino acids had to be counted, particularly in respect to nucleotides. ($^{32}$P)-labeled nucleotides have been counted using plastic scintillator tubing,$^{23}$ but the efficiency of such a cell for ($^{14}$C) is poor and for ($^{3}$H) is negligible. Homogeneous counting seems to be the method of choice. A comprehensive account of such a method has been published by Hunt.$^{22}$

Although emulsion by means of detergents has apparently been used only for discrete counting (see Chapter 19), it is likely to find more widespread application. In our laboratory, for instance, we have proved its applicability to the continuous counting of concentrated sucrose solutions from density gradients.

In some cases, continuous liquid scintillation counting could also offer advantages for the counting of radioactivity contained in organic solvents which will dissolve anthracene or plastic scintillators. The possibilities are limited by the quenching properties of many such solvents.

Monitoring gas flow. We have not mentioned the use of flow cells for counting volatile substances. Although this does not fall within the scope of the present chapter, we would like to stress the importance of such techniques for the measurement of radioactivity in the effluents of gas chromatography columns. During the past years, several approaches have been described in the literature.$^{27}$ These include counting in the gaseous phase by means of plastic scintillator tubing or anthracene-packed flow cells, sometimes after chemical conversion of the labeled substances into hydrogen and carbon dioxide; fractionation of the effluent by absorption on successive cartridges of silicone-coated anthracene; and absorption by liquid scintillators.

Cerenkov counting. Although this method does not apply to the low-energy $\beta$-particles of ($^{14}$C), ($^{35}$S) and ($^{3}$H) in aqueous solutions, more energetic isotopes such as ($^{32}$P) may be counted without the use of any scintillator, thanks to the production of Cerenkov radia-
tion which can be detected by the photomultipliers of a liquid scintillator counter. The efficiency found by Clausen was 19 percent and was not affected by the presence of HNO₃, H₂SO₄, KOH or NaOH in rather high concentrations. NaCl, KCl, HCl, and ethanol seemed to increase the efficiency slightly (see Chapters 9 and 10).

REFERENCES

CERENKOV COUNTING AS A MEANS OF ASSAYING \(\beta\)-EMITTING RADIONUCLIDES

R. P. PARKER and R. H. ELRICK

An important recent advance in methods of radioassaying solutions of hard \(\beta\)-emitters (\(E_{\text{max}} > 0.5\) MeV) is the use of liquid scintillation counting equipment to detect the Cerenkov light emitted by the \(\beta\)-particles as they travel through the solution. Reasonable detection efficiencies can be achieved, and, since no scintillator need be added and chemical quenching is absent, the sample may be dissolved in a wide variety of solvent systems ranging from organic solvents to strongly acidic oxidizing agents such as perchloric acid. Sample preparation is simple and the sample is readily recoverable for further chemical processing.

The faint, bluish-white light (now termed "Cerenkov light") that is emitted from solutions of strongly radioactive sources was first observed in 1910 by Madame Curie,\(^1\) but many years elapsed before Mallet\(^2-4\) made a deliberate study of the phenomenon. His work preceded that of Cerenkov who did not begin his comprehensive series of experiments until 1934. His results were in good agreement with the theoretical explanation subsequently provided by his colleagues Frank and Tamm.\(^5,6\)

The invention of the photomultiplier enabled the phenomenon to be used as a method of radioassay. Some of the first work was carried out in 1950 by Belcher\(^7\) who observed in aqueous solutions of radionuclides a feeble luminescence attributable either to excitation of the substrate or to Cerenkov emission. Belcher made an experimental and theoretical study of the problem and showed that the intensities of the observed effects of a range of high-energy \(\beta\)- and \(\beta\)-\(\gamma\) emitting nuclides were very largely attributable to the Cerenkov effect. With his apparatus, which included one of the early types of photomultiplier, a counting efficiency of 11 per cent was obtained for \(^{42}\text{K}\) (\(\beta_{\text{max}} 3.6\) MeV) and 4 per cent for \(^{32}\text{P}\) (\(\beta_{\text{max}} 1.7\) MeV).

Although Anderson and Belcher\(^8\) utilized this effect in constructing a standard light source, over the next ten years it was used

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very little except for a few specialized applications using custom-built apparatus. Only recently has it been used as a routine method of assaying $\beta$-emitters, due largely to the realization that the Cerenkov emission from small volumes of radioactive solutions can be satisfactorily detected by commercial liquid scintillation counters.

The present paper reviews those features of the Cerenkov technique directly applicable to the problems of radioassay and discusses the usefulness, limitations and practical realization of this method. Unless otherwise stated, all the results quoted have been obtained using commercial automatic liquid scintillation counters fitted with two photomultipliers arranged at $180^\circ$ to each other and connected by a fast coincidence circuit.

### Nature of the Cerenkov Effect

Comprehensive reviews are given by Marshall and Jelley, but a brief description is given here in order to understand the technique.

When a charged particle passes through a dielectric medium, local electronic polarization will be produced along its path. Immediately after its passage, these polarized molecules return to their quiescent state with the emission of electromagnetic radiation. When it passes into the medium, the velocity of the charged particle, $v$, is unaltered except for subsequent ionization and radiation losses. The electromagnetic radiation emitted will be propagated with a phase velocity of $c/n$, where $n$ is the refractive index of the medium. When $v < c/n$ (i.e., $\beta n < 1$), the emitted electromagnetic radiation from individual molecules will interfere destructively. If the velocity of the particle is greater than that of light in the medium ($\beta n > 1$), there will be a certain direction in which the wavelets interfere constructively. The resulting pulse of light is known as Cerenkov radiation and is analogous to the shockwave caused by a supersonic aircraft.

\[ v = \beta c, \text{ where } c \text{ is the velocity of light and } \beta \text{ the dimensionless velocity ratio.} \]

The radiation is emitted in a conical shape of half-angle $\phi$, where

\[ \cos \phi = 1/\beta n \]

and it can be seen that there is a threshold energy $E_{\text{min}}$ given by

\[ \beta_{\text{min}} = 1/n \]

In Fig. 1 the threshold energy for electrons is plotted against refractive index; for water it is equivalent to an energy of 0.263 MeV. The technique is not suitable for the measurement of low-energy $\beta$-emitters, although the range can be extended by using materials of high refractive index. [See Chapter 10 concerning ($^{14}$C).] The highly directional nature of the light (Fig. 2) affects the light collection efficiency of a multiple photomultiplier system in the usual fixed geometrical arrangement.

The theoretical treatment of Frank and Tamm gives the number of photons, $N$, emitted within the spectral range $\lambda_1$ to $\lambda_2$ by a $\beta$-particle of energy $E$ in a distance $dx$ as

\[ N = 2\pi \alpha \left( \frac{1}{\lambda_2} - \frac{1}{\lambda_1} \right) \cdot \left( 1 - \frac{1}{\beta^2 n^2} \right) dx \]

where $\alpha$ is the fine structure constant $1/137$. It is assumed that the medium is nondispersive and that the energy (and therefore $\beta$) is constant over the distance $dx$. Thus there is a continuous spectrum of radiation emitted, with a greater number of photons produced at short wavelengths, the spectral limits being defined by the relation $\beta n > 1$. The energy is concentrated mainly in the ultraviolet. It extends into the visible but becomes negligible in the infrared.

Cerenkov radiation is quite distinct from both fluorescence and bremsstrahlung, and the duration of the light flash is very short (< 1 ns).

### Calculation of Photon and Photo-Electron Yield

To evaluate the number of photons of Cerenkov light emitted by a particle, we re-
quire its velocity as a function of the distance transversed in the medium. This is given by Berger and Seltzer\(^{17}\) who for various media tabulate range and rate of energy loss in terms of their ability to stop \(\beta\)-particles of different initial energies. Thus the distance traveled can be expressed in terms of the energy of the particle.

If \(N_E\) is the number of photons emitted for a \(\beta\)-particle of energy \(E\) entering a medium of refractive index \(n\), Equation (3) can be expressed as

\[
N_E = C \int_{E_{min}}^E \left(1 - \frac{1}{\beta^2 n^2}\right) dx \quad (4)
\]

which has been evaluated numerically for various values of \(E\). The medium (water) is assumed to be nondispersive and the constant

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**Fig. 1.** Electron threshold energy for Cerenkov emission as function of refractive index of medium. Refractive indices are for 589 nm light at 20° C.

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**Fig. 2.** Half-angle of cone of emission of Cerenkov light in water as function of electron energy.
C = 2πα (1/λ₂ - 1/λ₁), where λ₁ and λ₂ are taken as 300 nm and 700 nm, respectively, to cover the photomultiplier spectral response range. The medium has been assumed infinite, so that no account has been taken of the effects of the escape of β-particles from the counting vial. Belcher⁷ has shown these corrections to be small.

After appropriate normalization, the distribution of the number of Cerenkov photons emitted per disintegration is obtained by weighting the values of \( N_R \) by the β-spectrum of the radionuclide. Some representative results are given in Table 1. For example, \(^{36}\text{Cl}\) emits about 40 photons per disintegration for the most energetic β-particles, the mean value being 6. Forty per cent of the \(^{36}\text{Cl}\) disintegrations have energies of emission below the Cerenkov threshold and cannot be detected. Figure 3 shows the β-spectrum of \(^{36}\text{Cl}\) and the associated probability distribution of the Cerenkov photons.

To estimate the number of photoelectrons emitted, account must be taken of the spectral response of the photocathode used. (See Chapter 4.) The average number of photoelectrons as a function of the initial energy of the β-particle is shown in Fig. 4 for various types of photocathodes. The spectral responses have been taken from the manufacturer’s literature and additional allowances made for the optical absorption of soda glass vials and windows. The values can be taken only as an indication of the performance of a particular photomultiplier, but the advantage of high-quantum-efficiency photocathodes is obvious.

It has been assumed that all the Cerenkov light will reach the photocathode. In practice, the directional nature of the light and the need to share it between two photomultipliers in coincidence mean that only a few photoelectrons can be detected, and the whole process will be subject to statistical fluctuations.¹⁸ The problem is similar to that existing in the measurement of \(^{3}\text{H}\), and for this reason liquid scintillation counting equipment is suitable.

**Table 1.—Calculated Numbers of Cerenkov Photons Emitted on Average in Wavelength Band 300 to 700 nm per Disintegration of β-emitter in Water**

<table>
<thead>
<tr>
<th>Radionuclide</th>
<th>( E_{\text{max}} ) (MeV)</th>
<th>Mean</th>
<th>Maximum Energy</th>
<th>Proportion of β-spectrum Above Cerenkov Threshold (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(^{36}\text{Cl})</td>
<td>0.71</td>
<td>7</td>
<td>40</td>
<td>60</td>
</tr>
<tr>
<td>(^{38}\text{Ti})</td>
<td>0.77</td>
<td>5</td>
<td>47</td>
<td>53</td>
</tr>
<tr>
<td>(^{38}\text{Na})</td>
<td>1.39</td>
<td>30</td>
<td>160</td>
<td>84</td>
</tr>
<tr>
<td>(^{32}\text{P})</td>
<td>1.71</td>
<td>40</td>
<td>210</td>
<td>90</td>
</tr>
</tbody>
</table>

* Values given are mean numbers and maximum obtainable for the most energetic β-particles.
ers, background count rates of 25-40 cpm for glass vials containing 10-20 ml of water have been reported. This is about half the background obtained using a liquid scintillator at the same instrument settings. The causes of the background are thermal and electronic noise, Cerenkov emission in the photomultipliers, counting vial and sample due to environmental $\beta$- or $\gamma$-radiation, and cosmic rays. (See Chapter 7.) We have shown experimentally that using low-noise photomultipliers and coincidence techniques reduces the noise component and Cerenkov effects in the photomultiplier to a few counts per minute.

Haberer and Clausen both observe that the background count rate is increased by only about 10 per cent when 20 cc of water is added to an empty vial, pointing to the vial itself as the main contributor to the background. Läuchli has used vials of different compositions, obtaining backgrounds of 25-30 cpm with glass, 9-15 cpm with plastic (polyethylene and polystyrene) and 5-7 cpm with nylon. We have observed much larger variations for glass vials, depending greatly on the type of glass. Since the efficiency of Cerenkov production in water and plastic is not appreciably different, the natural radioactivity in the vial, especially $^{40}$K, is likely to be the principal component of the background.

Fluctuations in background count rate may also be due to the presence of high energy $\gamma$-emitting samples nearby. Long-term phosphorescence may occur in both the vial and the solvent. (See Chapter 7.)

**DETECTION EFFICIENCY**

The theoretical considerations outlined above show that the detection efficiency will greatly depend on the $\beta$-energy of the nuclide, the characteristics of the photomultiplier, the geometrical and optical arrangement of the counting vial and the refractive index and

**Fig. 4.—Number of photoelectrons emitted from various types of photocathode as function of initial energy of electron in water. Types correspond to those used for EMI photomultipliers.**
density of the solvent. These factors will be considered individually and in relation to commercial liquid scintillation counters, but in practice they are inseparable.

**Effect of Variations in Counting Volume**

Maximum counting efficiency corresponds to sample volumes between 5 and 12 ml, as shown by various workers\(^{15,20}\) and illustrated in Fig. 5 by our own work using Tracerlab LSC-30 equipment. The optimum volume obtained from considerations of efficiency is relatively independent of \(\beta\)-energy, but the variation in response with volume can be very dependent on the radionuclide considered.

In many cases the size of sample used is limited only by the vial size. Since the background is almost independent of sample volume, the greatest sensitivity for a given vial size and specific activity is achieved by using the maximum volume possible.

**Effect of \(\beta\)-energy and Photomultiplier Characteristics**

We have measured standards of a number of \(\beta\)-emitting nuclides by Cerenkov counting in both a Tracerlab LSC-30 fitted with S11 response photomultipliers and a Beckman Series LS-200 equipped with the high quantum efficiency RCA 4501-V3 photomultiplier tubes. The detection efficiencies obtained for 10 cc volumes are given in Table 2. They depend on the instrument settings which were adjusted to give approximately maximum counting efficiencies, or "goodness," as defined by Greenfield and Koontz.\(^{22}\)

Clearly the use of high quantum efficiency photocathodes results in increased detection

![Fig. 5.—Variation of Cerenkov counting efficiency of \((^{203}\mathrm{TI})\) with volume for Tracerlab LSC-30 liquid scintillation counter.](image)

### Table 2.—Experimentally Determined Cerenkov Counting Efficiencies

<table>
<thead>
<tr>
<th>Nuclide</th>
<th>(E_{\text{max}}) (MeV)</th>
<th>(%) of Disintegration</th>
<th>S11 Photocathode</th>
<th>RCA 4501-V3 Photocathode</th>
</tr>
</thead>
<tbody>
<tr>
<td>((^{203}\mathrm{TI}))</td>
<td>0.77 (98%)</td>
<td></td>
<td>1.3</td>
<td>2.6</td>
</tr>
<tr>
<td>((^{133}\mathrm{Cs}))</td>
<td>0.51 (92%), 1.17 (8%)</td>
<td></td>
<td>2.1</td>
<td>4.7</td>
</tr>
<tr>
<td>((^{24}\mathrm{Cl}))</td>
<td>0.71 (98.3%)</td>
<td></td>
<td>2.3</td>
<td>4.7</td>
</tr>
<tr>
<td>((^{198}\mathrm{Au}))</td>
<td>0.96 (99%)</td>
<td></td>
<td>5.4</td>
<td>5.3</td>
</tr>
<tr>
<td>((^{47}\mathrm{Ca}))</td>
<td>0.66 (83%), 1.94 (17%)</td>
<td></td>
<td>7.5</td>
<td>14.8</td>
</tr>
<tr>
<td>((^{40}\mathrm{K}))</td>
<td>1.32 (89%)</td>
<td></td>
<td>14</td>
<td>31</td>
</tr>
<tr>
<td>((^{85}\mathrm{Na}))</td>
<td>1.39 (100%)</td>
<td></td>
<td>18</td>
<td>40</td>
</tr>
<tr>
<td>((^{88}\mathrm{Rb}))</td>
<td>0.68 (8.5%), 1.77 (91.5%)</td>
<td></td>
<td>23</td>
<td>46</td>
</tr>
<tr>
<td>((^{31}\mathrm{P}))</td>
<td>1.71 (100%)</td>
<td></td>
<td>25</td>
<td>50</td>
</tr>
<tr>
<td>((^{144}\mathrm{Ce}-^{144}\mathrm{Pr}))</td>
<td>2.98 (97.7%)</td>
<td></td>
<td>54</td>
<td>75</td>
</tr>
<tr>
<td>((^{42}\mathrm{K}))</td>
<td>2.0 (19%), 3.6 (82%)</td>
<td></td>
<td>60</td>
<td>75</td>
</tr>
<tr>
<td>((^{106}\mathrm{Ru}-^{106}\mathrm{Rh}))</td>
<td>2.0 (3%)</td>
<td>2.4 (12%)</td>
<td>62</td>
<td>85</td>
</tr>
<tr>
<td></td>
<td>3.1 (12%), 3.6 (70%)</td>
<td></td>
<td></td>
<td>75.3</td>
</tr>
</tbody>
</table>
efficiencies, but the efficiency will also depend on the geometry and optical arrangement of the counting chamber and on the discriminator level of the coincidence and summation circuits. It is difficult to separate the effect of altering the type of photocathode from the other instrument variables.

For any β-emitting nuclide, the detection efficiency may be predicted from Fig. 6 in which it is plotted as a function of maximum β-energy. The values are only approximate because of differences in the shape of β-spectra, difficulties in calculation for heteroenergetic β-emitters, and the calculation of the direct effects of any γ-emissions.

Effect of Optical Arrangement

Because of the directional nature of Cerenkov light and the relatively small number of photons involved, many events are not detected by diametrically opposed photomultipliers and are lost. Higher efficiencies result if the coincidence condition is removed, but the effects of noise and chemiluminescence can make this disadvantageous. Care must be taken in using such a direct summation technique since anomalous results have been observed due partly to multiple pulsing in the photomultiplier.22

A number of workers have reported improved detection efficiencies with plastic vials.12,14,19 The probable explanation of the improved efficiency is better optical transmission, particularly in the ultraviolet, coupled with some removal of the severe directional dependence of the Cerenkov light through light dispersion by the plastic. Some types of plastic give greatly enhanced efficiencies that cannot be explained solely on this basis. Elrick and Parker14 detected (44Ca) ($\beta_{\text{max}}$ 0.25 MeV) and (14C)($\beta_{\text{max}}$ 0.155 MeV) with low efficiency in aqueous solutions with polystyrene vials. Both these nuclides have maximum β-energies below the Cerenkov threshold in water. One explanation is a weak scintillator action in polystyrene and the reduced Cerenkov threshold in polystyrene (Fig. 1).

Effect of Wavelength Shifters

A large proportion of the Cerenkov radiation is emitted in the ultraviolet and is ab-
sorbed by the glass counting vial and photomultiplier envelope. The detection efficiency is increased by absorbing some of the ultraviolet light and reemitting the energy in the visible region, using a suitable wavelength shifter. Elrick and Parker used the water-soluble sodium potassium salt of 2-naphthylamine-6, 8-disulphonic acid at a concentration of 100 mg/l of sample and obtained double the efficiency for maximum $\beta$-energies up to about 2 MeV (Table 2). A contributing factor to this enhancement is that the directionally emitted ultraviolet energy is reemitted isotropically.

Fluorescent indicators such as $\beta$-methyl umbelliferone can be used in a similar manner, but their effect depends upon pH. Läuchli reports on the use of 7-amino-1, 3-naphthalene-disulphonic acid (ANDA), the optimum concentration being 5 mM for $\beta$-energies less than 1 MeV, and 2.5 mM for higher $\beta$-energies. The addition of the wavelength shifter causes an upward shift of the pulse height spectrum, resulting in improved detection efficiency, particularly with samples containing tissue.

Clearly a useful improvement in efficiency is obtained with wavelength shifters and is most marked for the lower-energy $\beta$-emitters. The disadvantages are more complicated sample preparation and recovery.

**Effect of Variations in Refractive Index**

As shown by Equation (3), the photon yield in Cerenkov emission increases with the refractive index of the medium in which the particle travels. Using ($^{40}$K) and ($^{144}$Ce $\rightarrow ^{144}$Pr), Haberer studied the variation of count rate for sugar solutions over the relatively large range in refractive index of 1.33 to 1.44 and found an overall increase of about 7 per cent (less than 1 per cent for a change in refractive index of 0.01). This has been confirmed by other workers who note that any effect will be more apparent with $\beta$-emitters near the threshold, such as ($^{36}$Cl) and ($^{20}$Tl). With these nuclides a 10 per cent increase in efficiency results from an increase of 0.01 in refractive index. (See the discussion by Ross of anomalous refractive dispersion in Chapter 10.)

**Effect of Variations in Density**

Since the stopping power of a medium increases with density, a decrease in density should result in a longer path and hence in a larger pulse and improved detection efficiency. Again the effect will be most pronounced for low-energy $\beta$-emitters. It has been shown that a decrease of 7 per cent in a specific gravity results in a 10 per cent increase in detection efficiency for ($^{20}$Tl). For ($^{3}$P) ($\beta_{\text{max}}$ 1.76 MeV), the increase is less than 1 per cent.

When using organic solvents, enhanced efficiencies may be obtained because of weak scintillation effects. These may be seriously affected by the presence of impurities and are often not reproducible between samples.

**PULSE HEIGHT ANALYSIS**

Since the Cerenkov photon yield is a function of the energy of the particle, the resulting pulse height spectral distribution will depend on the $\beta$-spectrum of the nuclide. Figure 7 shows the pulse height spectra of ($^{1}$Cl), ($^{32}$P) and ($^{42}$K) obtained using photomultipliers with bialkali photocathodes. Separation between $\beta$-emitters of widely different energies is possible, but any loss of photons due to inferior light collection or self-absorption of the light by the solution will degrade the quality of the spectra obtained.

**ABSENCE OF CHEMICAL QUENCHING**

With liquid scintillators, interactions between the sample and the solvent or solute components of the scintillator system may interfere with the efficiency of energy transfer or fluorescence. This is termed “chemical quenching.” Since the Cerenkov effect is not a type of fluorescence due to excited isolated molecules, virtually no chemical
Pulse amplitude (volts)

Pulse rate per channel (arbitrary units)

**Pulse Amplitude (Volts)**

Quenching is to be expected\(^{28}\) as has been shown experimentally.\(^{12,14}\)

It therefore follows that samples may be dissolved in any suitable solvent, including those known to exhibit chemical quenching with liquid scintillators, such as water and perchloric acid. Chemiluminescence can occur, and time should be allowed for this to decay to acceptable levels. Normally a twin photomultiplier arrangement connected in a coincidence circuit is required.

**Color Quenching**

This term refers to the drop in count rate due to the attenuation of the Cerenkov light by any optical absorption bands present in the solution. Using the techniques outlined above, the relative number of photoelectrons emitted, on the average, from photocathodes of various types have been calculated and are listed in Table 3 as a function of the wavelength of Cerenkov light. In each case the total number of photoelectrons has been normalized to 100. Even after allowance for the reduced transmission of soda glass at short wavelengths, the wavelength band between 300 and 400 nm is the most important, although that between 400 and 500 nm is also a significant contributor. Optical absorption in the visible or near-ultraviolet will therefore affect the efficiency of Cerenkov detection, particularly at short wavelengths. Since many organic samples possess absorption bands in these regions of the spectrum, color quenching is often appreciable and the following methods for assessing its effect have been considered.\(^{14}\)

**Decoloration of Sample**

This is a straightforward method if a practicable chemical procedure exists that does not introduce excessive chemiluminescence. Rapkin\(^{26}\) and Mahin and Lofberg\(^{27}\) have reviewed suitable techniques for organic compounds.

**Internal Standardization**

The degree of quenching may be determined by adding a small, accurately known amount of the radionuclide and recounting.

### Table 3.—Relative Numbers of Photoelectrons Emitted in Different Wavelength Bands for Photocathodes Irradiated by Cerenkov Light

<table>
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<tr>
<th>Wavelength Band (nm)</th>
<th>Type of Photocathode</th>
<th>300-400</th>
<th>400-500</th>
<th>500-600</th>
<th>600-700</th>
<th>700-800</th>
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<tr>
<td>300-400</td>
<td>S 11</td>
<td>51</td>
<td>38</td>
<td>10.5</td>
<td>0.5</td>
<td>0</td>
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<tr>
<td>400-500</td>
<td>Super S 11</td>
<td>49</td>
<td>38</td>
<td>12</td>
<td>1.0</td>
<td>0</td>
</tr>
<tr>
<td>500-600</td>
<td>S 20</td>
<td>51</td>
<td>35.6</td>
<td>11.3</td>
<td>2.0</td>
<td>0.1</td>
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<tr>
<td>600-700</td>
<td>RCA 8575</td>
<td>57.4</td>
<td>35.9</td>
<td>6.5</td>
<td>0.2</td>
<td>0</td>
</tr>
<tr>
<td>700-800</td>
<td>Bialkali (K-Cs)</td>
<td>53.4</td>
<td>37.5</td>
<td>8.8</td>
<td>0.3</td>
<td>0</td>
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</tbody>
</table>

* Total number of photoelectrons is normalized to 100 in each case.

**Fig. 7.—Pulse height spectra obtained with bialkali tubes for \(^{36}\)Cl \(\beta_{\text{max}} 0.71\) MeV, \(^{32}\)P \(\beta_{\text{max}} 1.71\) MeV and \(^{42}\)K \(\beta_{\text{max}} 3.6\) MeV.**
Francois notes that over a few days certain types of sample, such as urine, undergo optical changes. The calibration procedure should therefore be carried out soon after the initial measurement.

**External Standardization**

Changes in the pulse-height spectrum induced by an external $\gamma$-source are correlated with the degree of quenching. When applied to Cerenkov counting, the $\gamma$-energy must be high enough to liberate enough Compton electrons of energy in excess of the Cerenkov threshold. ($^{137}\text{Cs}$) is therefore unsuitable as an external standard and ($^{226}\text{Ra}$) has been used. The resulting pulse-height spectrum is shown in Fig. 8. A significant proportion of the Cerenkov emission is in the vial, and Elrick and Parker have shown that variations between vials can cause significant errors.

A typical quench correction curve is illustrated in Fig. 9. Over most of the range, variations using different colored pigments are small, but there is a divergence at high dye concentrations. The external standardization technique is practicable although somewhat inaccurate. It is more sensitive for the higher energy $\beta$-emitters since the slope of the calibration curve (Fig. 9) is less.

**Channels Ratio**

The shift in the pulse-height spectrum due to color attenuation is measured by the change in the ratio of the count rates for two
different discriminator levels. Figure 10 shows the correction curves that we have obtained using both S-11-type photocathodes and RCA 4501-V3 photomultipliers. The method is more sensitive for the higher quantum efficiency photocathodes, and variations between colors are small except at high dye concentrations.

The channels ratio technique may be employed in conjunction with an external standard, thus avoiding inaccurate results at low count rates and reducing the effects of variations in sample volume. (See Chapter 29.)

Spectrophotometric Methods

The magnitude of color quenching can be estimated by determining the optical transmission of the sample at various wavelengths. While generally applicable, this technique is laborious and is convenient only for groups of samples where an estimation of optical transmission at only one wavelength is sufficient.

Applications

Cerenkov counting has been applied in radioassay in two main ways: high-sensitivity counting of large sample volumes and sample counting using commercially available liquid scintillation counters.

High-sensitivity Counting

While the detection efficiency obtained in Cerenkov counting is less than that obtained by some other techniques (notably liquid scintillation counting), large volumes can be counted, the useful size being limited only by mechanical considerations and self-absorption of the Cerenkov light. Thus the Cerenkov technique can be the preferred method for determining high-energy β-emitters at low specific activities in large volumes of solution and is particularly adaptable to flow monitoring. (See Chapter 8.)

Rippon9 has considered the problem of monitoring hard β-emitting fission products from water-cooled reactors. Due to the low light-collection efficiency of his apparatus, only poor pulse height discrimination was achieved between fission products and other reactor coolant activity, although adequate sensitivity was achieved for the higher fission product-to-background ratio obtained in samples from individual fuel channels.
The determination of $^{90}$Sr in radioactive waste water prior to disposal has been discussed by Yamada. The $^{90}$Y daughter ($\beta_{\text{max}} 2.25$ MeV) is detected by Cerenkov emission in an 8 l sample viewed by two photomultipliers in coincidence. Yamada says that concentrations of $^{90}$Y down to $8 \times 10^{-14}$Ci/cc can be readily detected in a counting time of 5 minutes. (See Chapter 7.)

**Sample Counting**

The increasingly widespread use of Cerenkov counting for samples containing $\beta$-emitters of energies greater than 0.5–1 MeV stems from the virtues of minimal sample preparation, the absence of chemical quenching and the fact that chemical processing after counting is simplified by the absence of a scintillator.

$^{90}$Sr in human urine following radioactive accidents can be rapidly determined using the Cerenkov technique as described by Narrog. The urine is decolorized using sodium salts. [Potassium salts contribute to the background through the presence of $^{40}$K.] With a typical liquid scintillation counter ~ 1 pCi/cc can be measured in a 10-minute counting period.

Francois has used the method for the routine determination of exchangeable potassium in human urine, using $^{42}$K as tracer. The detection efficiency is 62 per cent, falling to 48 per cent for urine unless decolorization is achieved, preferably by mineralization. The advantage is the ability to measure $^{42}$K in large volumes without a contribution from any $^{9}$H or $^{14}$C which may also be present.

Another biochemical application is that of Braunsberg and Guyver. Steroid hormone uptake in tissue slices is studied in vivo using high energy $\beta$-emitters such as $^{24}$Na, which are determined by Cerenkov counting. The technique is particularly useful for samples in buffer solutions or samples insoluble in organic solvents. The authors point out that it can be used for the automatic measurement of chromatographic column effluents.

In studies on ion transport in plants, Läuchli has used Cerenkov counting to determine the $^{86}$Rb tracer. A wavelength shifter is used to improve the detection efficiency and partly compensate for loss in efficiency due to the presence of plant tissue. These last two applications indicate that the sample need not be totally in solution. Mr. A. Davies (personal communication) routinely uses the Cerenkov mode of counting for assaying $^{32}$P-labeled compounds of low molecular weight in bacteria. The use of a wavelength shifter more than doubles the detection efficiency obtained.

**SUMMARY**

Using liquid scintillation counting equipment, Cerenkov counting is both a practicable and useful technique for assaying $\beta$-emitters with a maximum energy above about 1 MeV. The requirements imposed on sample preparation are far less rigorous than in liquid scintillation counting, a great asset when large numbers of samples are to be measured or the sample is needed for subsequent additional processing. Allowance must be made for any attenuation of the Cerenkov light by optical absorption bands in the sample. An external standard of sufficient $\gamma$-energy, such as $^{226}$Ra, provides a suitable method of estimating the correction required.

The recent introduction of high quantum efficiency photomultipliers has brought about a marked improvement in the detection efficiencies of the lower energy $\beta$-emitters over those obtained a few years ago. The addition of a wavelength shifter improves the efficiency still more and is beginning to be a routine procedure for some purposes.

Compared with liquid scintillation techniques, the increased sample volume and lower background obtained with the Cerenkov method can often mean that the latter has a higher factor of merit, particularly for $\beta$-emitters in excess of 2 MeV where the Cerenkov detection efficiency is high. It is likely that Cerenkov counting will become increasingly important as a method of high-sensitivity $\beta$-detection.
A major improvement in detection efficiency depends on the development of counters specifically designed for Cerenkov detection. Coincidence circuitry should be retained to limit noise and the effects of chemiluminescence. A multiple photomultiplier arrangement can be envisaged that will minimize the effect of the directional nature of the Cerenkov light, particularly at low energies where an improvement in efficiency would be most rewarding. Such detectors can be justified for special purposes, but then the great advantage of being able to use commercially available automatic liquid scintillation counting equipment would be lost.

ACKNOWLEDGMENTS

The authors wish to acknowledge the help of Dr. C. G. Raison and of Nuclear Enterprises (GB) Limited for the use of counting facilities, and Professor J. W. Boag, Director of the Physics Department, for his interest and advice.

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10/CERENKOV RADIATION: PHOTON YIELD APPLICATION TO (¹⁴C) ASSAY

H. H. Ross

For several years, the Analytical Chemistry Division at Oak Ridge National Laboratory has been investigating the design of radiation-stimulated light sources.¹ ² Sources that use radioisotope decay energy for activation of emission have many potential applications in analytical instrumentation. They are stable, long-lived, require no external source of power, and often they can be made to operate in a pulsing mode that may be important when digital logic detection systems are used.

The major portion of our work has been concerned with energy conversion via an organic or inorganic fluor. These materials are usually combined physically with the isotope of interest. The solid mixture or solution is sealed into a transparent cell for use. One disadvantage of this sort of arrangement is that the fluorescent emission is usually restricted to a very narrow wavelength band (often only 100–150 nm wide and occasionally much narrower). For optical measurements where a wide spectral range is desired, several different light sources would have to be used. The use of multiple light sources in a practical analytical instrument would be undesirable both from mechanical and optical standpoints.

We were already aware that Cerenkov radiation is broadband in nature, theoretically extending from the microwave to the x-ray region,³ and the idea of using β-induced Cerenkov radiation suggested itself as a means of designing a broadband light source. A preliminary literature survey was conducted to determine how to calculate the photon yield that we might expect from an aqueous system containing a β-emitting isotope, since this is an important parameter of a practical lighting device. To our surprise, we could not locate any calculations of Cerenkov photon yield as a function of spectral distribution or β-energy, so we decided to make these calculations for the specific case of electrons in water up to an
energy of 4 MeV. (See a similar approach by Parker and Elrick in Chapter 9.)

**PHOTON YIELD CALCULATION**

The threshold condition for the formation of Cerenkov radiation is

\[
\beta n = 1
\]

where \(\beta\) = particle relative phase velocity, velocity of particle \(v\)/speed of light \(c\), and \(n\) = refractive index of the transparent medium. For relativistic electrons, \(\beta\) is related to electron energy \(E\) by

\[
\beta = \left[ 1 - \left( \frac{E\text{(MeV)}}{511} + 1 \right) \right]^{1/2}
\]

In water, where \(n = 1.332\), \(\beta\) must exceed 0.7508 for the generation of Cerenkov radiation by electrons. Substituting \(\beta = 0.7508\) in Equation (2) and solving for \(E\) gives 263 KeV as the lower energy threshold. The Cerenkov energy threshold for electrons as a function of refractive index is illustrated in Fig. 1; the positions of several solvent systems are indicated.

From the theory of Frank and Tamm, \(^4\) the number of photons per unit path length of an electron over a selected spectral region is

\[
\frac{dN}{dx} = 2\pi \alpha \left( \frac{1}{\lambda_2} - \frac{1}{\lambda_1} \right) \left( 1 - \frac{1}{\beta^2 n^2} \right)
\]

photons/cm

where \(\alpha\) = fine structure constant \(= e^2/\hbar c = 1/137\), \(\lambda_1\) = upper limit of selected wavelength region (cm.), and \(\lambda_2\) = lower limit of selected wavelength region (cm.). For example, over the wavelength region 400 to 600 nm,

\[
\frac{dN}{dx} = 380 \left( 1 - \frac{1}{\beta^2 n^2} \right) \text{photons/cm}
\]

To determine the total photon yield from a given Cerenkov event, Equation (4) would normally be integrated over the limits of \(x\), the particle path length. For the case of electrons in water, this simple solution cannot be used, however, since \(x\) is a function of the original electron energy. \(\beta\) also becomes a variable since energy degradation of the electron occurs during its passage through the liquid, and the low energy Cerenkov cutoff at 263 KeV must be considered.

Our calculation of photon yield in water incorporates these factors for the specific case of electrons up to 4 MeV. The variables \(x\) and \(\beta\) were established in terms of electron energy \(E\) and Equation (4) was transposed to the \(dN/dE\) form.

Empirical range-energy relationships for electrons in water were generated based on the techniques of Glendenin. \(^5\) These slightly modified forms are in good agreement with experimental data:

\[
R = \left[ (-5.08 \times 10^{-10}E) + (3.72 \times 10^{-5}) \right] E^{1.54}
\]

\[
R = (4.82 \times 10^{-4}E) - 0.098
\]

where \(R\) = range of electron in water (cm) and \(E\) = energy of electron (KeV). Equation (6) is used for electron energies up to 1 MeV and Equation (7) between 1 and 4 MeV. Although the actual experimental values for the ranges of electrons could have been used in the calculation to be described, the above equations provided a convenient entry into the computer program and were also used in the energy calculations.

Electron energy degradation as a function of distance traveled (\(dE/dx\)) can be evaluated
Table 1.—Cerenkov Photon Yields of Electrons in Water for Selected Spectral Regions

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from theoretical equations, but their use is somewhat cumbersome, and a significant deviation between the theoretical and experimental result is observed at low electron energies. Therefore, a linear regression technique was applied to Equations (6) and (7) for the \( dE/dx \) calculation. The results obtained in this way were in good agreement with the experimental values and had the advantage that they could be easily generated in the computer program.

Because a simple analytical solution for \( dN/dE \) using Equations (4, 6, and 7) did not appear to be likely, a numerical integration procedure was developed using both Simpson and trapezoidal methods for comparison. In addition to the range and energy degradation factors, the low energy cutoff at 263 KeV was included in the calculation as the lower integration limit. Table 1 shows the results of the photon yield integration. For an electron having an original energy \( E \), the total number of photons produced in each of seven spectral ranges is indicated. Photon yields over wider spectral segments are obtained by simply adding the results of each individual segment. For example, a 1 MeV electron (\( \beta \)) creates 23.4 photons in the 400–600 nm spectral range before degrading to the 263 KeV threshold level. We admit that additional refinements could have been included in the calculation, but we did not feel that the calculated response would be changed significantly.

**Cerenkov Radiation from \( ^{14}C \)**

One of the most important applications of the liquid scintillation counter is the measurement of \( ^{14}C \), but the low-energy Cerenkov cutoff at 263 KeV precludes the detection of this isotope in completely aqueous systems. Earlier we noted that the threshold energy is solely a function of solvent refractive index. It appeared that if we could locate a highly refractive solvent greater than 1.559 (from
Equations 1 and 2), we might just be able to detect ($^{14}$C) in a Cerenkov system. A quick check of the refractive index tables revealed a number of solvents greater than 1.559. Because of its availability, we selected α-bromonaphthalene ($n = 1.6582$) for our preliminary study.

On the basis of the index of refraction of α-bromonaphthalene and the β-distribution of ($^{14}$C), we calculated that the maximum theoretical efficiency that we could observe would be 1.3 per cent. We also did a photon yield calculation for $n = 1.658$, and on the basis of this result estimated that our actual experimental detection efficiency would be closer to 0.1 per cent. We were not impressed with the practical application of the technique, and more for academic interest than anything else, we measured the ($^{14}$C) detection efficiency in α-bromonaphthalene and found the observed response to be 12.3 per cent.

We immediately suspected our scintillation counter, but a thorough electrical check indicated no malfunction. We also considered fluorescence of the solvent. When we checked for fluorescence using xenon excitation, however, none was observed. (This represents a factor of $1.2 \times 10^3$ less fluorescence than a toluene solution of dimethyl POPOP under similar excitation conditions.) Chemiluminescence was also ruled out as the cause. This sample and others were counted for several days without any change in the observed efficiency. We were completely at a loss to explain our observed results.

A careful consideration of the problem indicated that the index of refraction of the solvent must be much greater than we originally thought. It then became apparent that our system was operating in the region of anomalous refractive dispersion.

The refractive index of a liquid can be expressed as

$$n_\nu = 1 + \frac{a}{\nu_0^2 - \nu^2}$$

(8)

where $n_\nu$ = refractive index at $\nu$, $\nu$ = light frequency, $\nu_0$ = characteristic oscillator frequency, and $a$ = constant.

When $\nu$ is small with respect to $\nu_0$, small changes in $\nu$ only slightly affect $n$. However, as $\nu$ approaches $\nu_0$, $n$ becomes very large. A characteristic oscillator frequency of a solvent is reflected in the molecular and electronic absorption bands of the molecule. Those affecting the index of refraction are most often found in the ultraviolet and x-ray regions but may occasionally be found in the visible. In these narrow absorption regions where anomalous dispersion exists, Cerenkov radiation may be observed if the energy threshold condition can be satisfied.

We feel that this effect explains our results with α-bromonaphthalene. In keeping with our interpretation, α-bromonaphthalene exhibits an intense absorption edge at 300 nm, although we have not been able to measure directly the index of refraction in this wavelength region. Since our original experiments, we have found other solvents that show good detection sensitivity for ($^{14}$C). We hope to be able to report on these in the near future.

From these results, it appears that Cerenkov counting techniques may be even more important to isotope assay than originally anticipated. The biological interest in ($^{14}$C) alone warrants a complete investigation of the method.

REFERENCES

CHEMILUMINESCENCE AS AN ANALYTICAL TOOL

D. A. KALBHEN

Since the development of highly sensitive photodetectors and photomultipliers about 20 years ago, chemiluminescence phenomena in organic chemistry have been extensively studied. They have been found to occur in a great number of organic and bioorganic reactions. Although the reaction mechanism of many light-emitting chemical processes is not yet completely understood, the various factors that may determine the quantity and efficiency of the light emission have been investigated and are relatively well known.¹ (See Chapter 32.)

QUANTITATIVE MICROCHEMICAL ASSAY

The intensity of the luminescence reaction, for example, which occurs in a mixture of luminol and hydrogen peroxide, is known to be dependent on the concentration of metal salts that catalyze the reaction. Based on this and other chemical reactions, many procedures have been developed which can be used for microassays of various compounds. Some substances which may be determined by a chemiluminescence method are listed in Table 1. Many other organic and inorganic compounds such as organic peroxides, glucose, vitamin C, nitroaniline, aniline, resorcinol, pyrogallol, methyl-, ethyl- and propylalcohol may be determined by chemiluminescence using the luminol reaction.¹⁹

Under optimal conditions, the sensitivity of this new analytical method can exceed even activation analysis, which may suggest the importance of chemiluminescence methods for future research. Although with their highly sensitive photomultipliers and their excellent counting electronics liquid scintillation counters are most useful measuring devices for these methods, the applicability of these instruments has so far been quite underestimated and unrecognized.

BIOLUMINESCENCE ASSAY

Following the suggestion of Tal et al.,¹⁴ in our laboratory we have elaborated a routine method for the quantitative microde-
termination of adenosine triphosphate (ATP) using the bioluminescence reaction with firefly enzyme and a liquid scintillation counter as a photodetector. With this instrument, the sensitivity of ATP assay is greatly increased and the costs for enzyme are markedly reduced. (In the following chapter, Schram discusses an essentially similar procedure for ATP determination developed in his laboratory.) In recent years we have done several thousand ATP determinations with a liquid scintillation counter. I am quite sure that other analytical methods based on chemi- or bioluminescence reactions can easily be performed with this instrument. I hope that these enlarged possibilities of application will be included in the design of future liquid scintillation counters and in the experimental methodology of investigators who use counters.

REFERENCES

A number of papers \textsuperscript{1-7} have recently drawn attention to the possibilities offered by scintillation counters for the measurement of the bioluminescence produced by firefly extracts in the presence of adenosine triphosphate (ATP). The availability of scintillation counters in many biochemical laboratories and the universal importance of ATP in life processes have prompted us to investigate further this very promising method. We have particularly considered two aspects: the control over the bioluminescent reaction rate, and the counting methodology.

Comprehensive accounts on the various aspects of bioluminescence have been published by Seliger and McElroy,\textsuperscript{8} Hastings,\textsuperscript{9,10} and Strehler.\textsuperscript{11} Although scintillation counters are probably suitable for the measurement of several kinds of luminescent reactions, we have concentrated our attention on the firefly luciferin-luciferase system. The following substances are involved in this system: dehydroluciferin ($L_2$), adenosine triphosphate (ATP), the enzyme luciferase $E$ and oxygen. According to our present knowledge, the reaction proceeds in several steps which may be summarized as follows:\textsuperscript{10}

$$
\text{Mg}^{++} \\
LH_2 + ATP + E \xrightarrow{} E-LH_2-AMP + pp \\
E-LH_2-AMP + O_2 \rightarrow E-L-O^-* + \text{product} \\
E-L-O^-* \rightarrow E-L-O^- + h_\nu
$$

According to this scheme, the oxidation of the activated substrate $E$-AMP-$L_2$ by molecular oxygen yields an energy-rich intermediate $E$-$L$-$O^-$* which then liberates photons upon deactivation with a quantum yield of one. It has been further observed that the luminescent reaction is inhibited by the end product of the reaction. Pyrophosphate liberates the enzyme from this inhibitory complex but at the same time counteracts the activation step.

**CONTROL OF REACTION RATE**

The time course of the luminescence shows a rapid rise followed first by a rapid decay.
USES OF THE SCINTILLATION COUNTER

Fig. 1. Luminescence decay curves at different enzyme dilutions.

and then by a slow decay lasting for hours. The proportionality between ATP concentration and luminescence, observed at the maximum of the curve, is the basis of various methods for the essay of ATP. Until now, the rapid decay of the luminescence has been a drawback for its measurement with scintillation counters. Because of the time spent in introducing the vial in the counter, it is not possible to record the starting portion of the curve, and since the luminescence must be measured over short time intervals, the time elapsed between mixing the sample and counting is very critical.

It occurred to us that these practical drawbacks might be circumvented if the reaction were slowed down by dilution of the enzyme preparation (Fig. 1). Under these conditions the initial maximum is less pronounced and the luminescence decays exponentially over a relatively prolonged period of time. Along the exponential portion of the curve, the light intensity happens to be proportional to the ATP concentration in contradistinction to the distal portion of the curve where the linear relationship is no longer observed. The decay curves obtained at various ATP concentration are parallel over the interval used for counting (Fig. 2) and a linear relationship is observed between luminescence and the ATP concentration (Fig. 3).

Since the luminescence yield of the luciferin-luciferase system is very sensitive to the presence of foreign substances and is dependent on the enzyme batch and age, standard curves should always be prepared for each series of experiments. Samples should preferably be assayed in duplicate, one of them containing an internal ATP standard. The reduction in luminescence efficiency from dilution of enzyme is compensated for by the fact that counting can be performed over longer periods and under less critical conditions.

COUNTING METHODOLOGY

Most instruments used for the routine measurement of bioluminescence have been
of the analog type and could be used in connection with a strip-chart recorder to facilitate the observation of the luminescence time course, while scintillation counters act as quantum counters and give a digital response. Single photons can be detected and very high sensitivities attained. Some specific features of scintillation counters are considered here in connection with their use for bioluminescence measurements.

**Single Channel and Coincidence Counting**

Scintillation counters are equipped with a coincidence circuit that is actually not useful in the present situation as light emitted by chemiluminescent or bioluminescent systems consists of isolated photons and not, as in the case of the scintillation counting of radioactive nuclides, the nearly instantaneous emission of bursts of photons. Coincident photons will only occur by chance and their number \( N_c \) will be proportional to the square of the luminescence rate according to the following equation:

\[
N_c = 2 \times \tau \times N_1 \times N_2 \tag{1}
\]

(\( N_1 \) and \( N_2 \) = number of pulses delivered by each of the photomultipliers which are proportional to the ATP concentration; \( \tau \) = resolving time of the counter). If \( N_1 = N_2 \),

\[
N_c = 2 \times \tau \times N^2 \tag{2}
\]

The higher resolving times of coincidence scintillation counters reduces their efficiency for luminescence measurements and they should therefore be operated as single channel counters.

**Gain, High Voltage and Discriminator Settings**

The pulses originating from luminescence photons cannot be differentiated from background pulses. On the other hand, increasing the gain or the high voltage may increase both the background and the efficiency, resulting in a greater figure of merit \( (E^2/B) \). We have also become aware of an artifact that might become a source of serious trouble if not taken care of. We observed that when the voltage was increased, the relationship between luminescence and ATP concentration changed gradually from linear to exponential and the decay curve became steeper. In subsequent experiments, a single vial was counted simultaneously in two adjacent channels with the rather surprising result that the decay rate in the higher energy channel was more rapid than in the lower-energy channel. It was assumed that the higher energy pulses were originating from coincident photons. In analog systems where the output current of the photomultipliers is measured instead of individual pulses, coincidence pulses will give rise to higher currents that will hence remain proportional to the luminescence. In digital systems, however, coincident photons will give rise only to a single pulse, albeit of higher energy. Because the number of pulses is proportional to the square of the number of incident photons, the linear relationship between luminescence and ATP concentration is no longer maintained and the decay curve becomes steeper.

The equation for the decay curve of coincident photons is

\[
N_c = N_o^2 \times e^{-2\lambda t} \tag{3}
\]

as compared with

\[
N = N_o \times e^{-\lambda t} \tag{4}
\]

for single photons. The presence of coincidence pulses can easily be checked for by counting in two energy windows and looking for the parallelism of the decay curves. For maximum sensitivity the second window should cover about 5 per cent of the total spectrum on the high energy side. We have verified that increasing the gain (amplifying the pulses after they have been delivered by the photomultipliers) did not alter the proportionality between luminescence and ATP concentration (Fig. 4). Coincident pulses can only be avoided either by decreasing the high voltage or by reducing the ATP concentration. In some scintillation counters the voltage applied to the first dynode is fixed. In this
deplete the endogenous ATP which would otherwise contribute an excessive background. Prior to use, this solution is brought to 20° C. For each assay, 10 μl of sample solution is added to 100 μl of the enzyme preparation. Counting is performed over one or more minutes, starting exactly ten minutes after the mixing of reactants. A standard curve is established in the same way using from 0.1 to 10 pM (10^-12 M) of ATP per counting vial.

The sensitivity of the method is largely dependent on the residual luminescence of the enzyme preparation. Using a model 2002 Packard scintillation counter set at the minimum voltage, the following average figures (expressed in counts per minute) were obtained:

<table>
<thead>
<tr>
<th></th>
<th>GAIN</th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Photomultiplier background</td>
<td>521</td>
<td>1023</td>
<td>1395</td>
</tr>
<tr>
<td>Residual luminescence</td>
<td>2.7 × 10^3</td>
<td>6.7 × 10^3</td>
<td>10.4 × 10^4</td>
</tr>
<tr>
<td>ATP (1 pM)</td>
<td>31 × 10^2</td>
<td>65 × 10^2</td>
<td>88 × 10^4</td>
</tr>
</tbody>
</table>

**APPLICATIONS**

The following standard procedure has been tentatively adopted for the assay of ATP:

Dissolve the content of one vial of firefly extract (Sigma FLE-50) in 5 ml distilled water, which gives a final concentration of 0.05 M K₂HSO₄ and 0.02 M MgSO₄, pH 7.4. Store this concentrated solution in a freezer. The working solution is prepared by diluting the concentrated solution ten-fold with the same buffer and leaving it overnight at 4° C to deplete the endogenous ATP which would otherwise contribute an excessive background. Prior to use, this solution is brought to 20° C. For each assay, 10 μl of sample solution is added to 100 μl of the enzyme preparation. Counting is performed over one or more minutes, starting exactly ten minutes after the mixing of reactants. A standard curve is established in the same way using from 0.1 to 10 pM (10^-12 M) of ATP per counting vial.
The standard deviation of the method is less than 2 per cent.

Until now, firefly extracts have been used chiefly for the determination of ATP for which they are highly specific, but they can be adapted to assay related substances such as AMP and ADP, after their enzymatic conversion to ATP, and to study a great number of systems in which ATP interacts. In our laboratory, assays were performed in connection with the kinetics of amino acid activation and with the mechanisms of photophosphorylation. It should be noted that the high sensitivity of the method permits the dilution of many systems to such an extent that enzymatic reactions will be essentially halted and most interfering substances will be present in harmless concentrations.

Some of the observations reported in this chapter may be applied to the measurement of other bioluminescent or chemiluminescent systems. (See Chapter 11.)

ACKNOWLEDGMENT

The author gratefully acknowledges the technical assistance of Michele Hayet.

REFERENCES

**Section III**

**THE LABELED SAMPLE**

**A. Special Problems with Radioisotopically Labeled Samples**

In measuring the radioactivity of a sample, the influence of the previous history of the labeled compound of interest is often neglected. In Chapter 13, Geller and Silberman review several examples of radiochemical instability and specific precautions against them, while referring the reader to several previous catalogues of optimal storage conditions, with caution.

Much scintillation counting involves samples that have been subjected to chemical separation or purification and rests on the assumption that the physiochemical behavior of a substance and its isotopically labeled tracer are identical. Klein's comprehensive review of isotope effects in Chapter 14 serves as an important reminder that one has to make sure that this assumption of tracer identity is valid, and provides criteria for determining whether it is valid. Litt and Carter in Chapter 15 emphasize another generally neglected source of error in counting, the adsorption of labeled samples to scintillation vial walls. In presenting several examples, the authors present methods of detecting the phenomenon and protecting against it.

**B. Lipophilic, Hydrophobic Samples**

Chapter 16, by Thomas and Dutton, provides an example of procedures for collecting and counting labeled samples which are soluble in toluene, the most commonly used scintillator solvent (See Chapter 2). Lipids and sterols, and many organic chemicals which are only indirectly of biological origin, fall into this category. The interested reader should also refer to Chapter 24, a description of preparative gas-liquid radiochromatography by Gordon and Muhs; and to Snyder's Chapter 25 on the radioassay of thin-layer chromatograms. The lucky reader who wishes to count lipophilic samples, but does not need to subject them to chromatographic fractionation, need go no further than Chapters 2 and 3 for information on optimal scintillator mixtures.

**C. Aqueous Samples**

In Chapters 17 and 18 Bray and Mueller evaluate scintillator mixtures for counting low-energy $\beta$s in samples which are not miscible with toluene. For the most part such samples are aqueous; methods of counting them in dioxane, in "solubilizers" which can in turn be dissolved in toluene, and in suspension are reviewed and compared. Mueller examines the particular conditions imposed by samples which are, or which contain, significant quantities of inorganic salts. Aqueous samples may be also counted in heterogeneous systems, where they are not in solution (Chapters 9 and 20), or on solid supports such as...
membranes or filter paper (Chapter 21). They also may be subjected to combustion which renders them miscible with aromatic solvents (Chapters 22 and 23).

D. Solid Samples and Heterogeneous Counting Systems

If a sample is not in solution and is not dissolved by a common scintillator solvent-primary solute mixture, it can be counted in suspension or emulsion, or precipitated onto a solid support. Greene reviews the potential problems in suspension counting in Chapter 19 and emphasizes the desirability of selecting an optimal system for a specific type of sample. Furlong demonstrates in Chapter 20 that the measurement of $^3$H radioactivity in samples on solid supports is not as straightforward as it first seems. Inefficiency in counting due to self-absorption, which cannot be monitored by standard quench-correction procedures (Chapter 29) can also be significant with more energetic $\beta$s such as $^{14}$C.*

E. Solid Samples and Homogeneous Counting Systems

There are two fundamental approaches to the problem of how one reduces a solid or semisolid material to a form which can be dissolved in a scintillator solvent.

The first involves the use of hydrolytic agents. Pollay and Stevens review the use of several commercial “solubilizers” with tissue samples in Chapter 21. These have also been examined for aqueous samples in Chapters 17 and 18.

The second involves combustion of the samples. The “wet combustion” technique discussed by Mahin and Lofberg in Chapter 22 is an extension of the use of hydrolytic conditions. Oxygen flask combustion, thoroughly reviewed by Davidson, Oliverio, and Peterson in Chapter 23 involves the reduction of organic samples to H$_2$O for $^3$H counting and CO$_2$ for $^{14}$C counting. The chapter includes detailed instructions on how to set up a manual oxygen flask combustion procedure, as well as new information on automatic combustion trains for $^3$H and $^{14}$C counting.

F. Counting Samples Obtained by Special Preparative Techniques

For both toluene-soluble and aqueous samples, there are special techniques for fractionation and purification which generate particular sets of problems for investigators who wish to determine the radioactivity of separated compounds. Chapters 24 to 27, respectively, involve applications of the sample handling techniques discussed in the preceding chapters of this section to: preparative gas-liquid chromatography (GLC), thin-layer chromatography (TLC), cesium salt density gradients, and acrylamide gels. Each of these procedures is in widespread use, and each presents different problems for radioassay.

* Bransome, E. D., Jr., and Grower, M. F., submitted for publication.
Prior to 1953, it was believed that the low average energy of the $\beta$-emission of $^{14}$C and $^3$H would result in little molecular damage to a radiochemical. The dynamic increase in the synthesis and use of high specific-activity radiochemicals following this period made it apparent that these compounds were indeed subject to marked changes because of self-radiolysis. These marked changes must be considered in the counting of any radiochemical that has been stored for several months or longer.

Radiochemical decomposition of $^3$H-labeled compounds may result in the loss of $^3$H during storage. For example, disproportionation of glucose-3-(3H) with subsequent oxidation of the glyceraldehyde-(3H) results in the removal of (3H) from the organic compounds in solution. The destruction of the molecular structure of orotic-5-(3H) by radiolysis gives rise to "labile" (3H) in the form of tritiated water and unlabeled chemical impurities. $^{14}$C-labeled compounds can also give rise to a loss of activity resulting from decomposition during storage; for example, decarboxylation of carboxyl labeled fatty acids and the decomposition of glucose-1-($^{14}$C).

Some decomposition products of labeled compounds result in colored impurities that cause considerable quenching, while other breakdown products may be selectively absorbed onto the storage container wall. Precipitation and crystallization can also occur, effectively decreasing the activity available for counting. An example is provided by the compound epinephrine: its primary decomposition products are colored adrenochromes, and its secondary products are insoluble in the original storage solution. Clearly decomposition is a subject for consideration in counting. Some recent findings related to minimizing radiochemical decomposition are the principal subjects to be considered in this chapter.

There have been a number of publications dealing with the theoretical aspects of radio-decomposition and the more practical aspects of preventing and decreasing this decomposition. From the results of observations during the late fifties and early sixties, some basic tenets were developed for the storage of radiochemicals. At various intervals during...
this period, papers appeared with long tables of radiochemicals which had been stored at various temperatures and usually arbitrary time periods. These tables made it possible for the user of radiochemicals to ascertain what might be expected with his own radiochemicals stored under similar circumstances.

The problem encountered was that one almost never duplicated the decomposition rates reported by other investigators, and often one could not even duplicate his own decomposition rates comparing different preparations of the same compound. Even worse, samples of the same lot of the radiochemical often displayed different stability rates when stored by different investigators under slightly different conditions. In seeking answers to the problem of variability, a number of laboratories have recently reexamined existing practices for coping with radiodecomposition.

**FREEZING**

The misconception that all labeled compounds should be stored in the freezer is slowly being dispelled. Thought must be given to the freezing of the solvent with subsequent zone localization of the solute since freezing frequently results in considerable decomposition. The reason may be the effective removal of the protecting solvent in the freezing process and/or an increase rate of reaction of reactants in frozen solutions. The current practice in our laboratories, barring specific information to the contrary, is to store all labeled compounds in a solution as cold as possible but above the freezing point of the solution. See Table 1 for a comparison of decomposition rates of a steroid stored at various temperatures, the lowest of which resulted in the freezing of the solvent.

**IMPURITIES**

It has been demonstrated that chemical impurities have a deleterious effect on the stability of labeled compounds. Even the best of storage conditions can be of little avail if prior care is not taken in their removal. The compound, Esterone-6,7-(H) (40 c/mM) had a variable decomposition rate of 2 per cent in six months to 50 per cent in six weeks. It was noted that a particular partition column used for final purification resulted in a highly stable product (decomposition less than 1% /17 months) while other columns did not, in spite of the fact that in each case the material after purification was radiochemically pure to paper chromatography and reverse isotopic dilution analysis.

The reason for the then curious observation is apparent from the next example. Cortisone-4-(14C) frequently exhibited a decomposition rate of 20 per cent/month. Removal of its radioactive decomposition products on a Bush B5 chromatogram did not improve its stability. When a completely different type of partition chromatography was used, a small amount of colored, nonradioactive oil was isolated in the early chromatographic fractions. The removal of this oil gave a product which was now very stable. The addition of these nonradioactive tars to the now-stable steroid again reproduced its former decomposition rate. Thus the addition or removal of the unlabeled tars resulting from solvents and/or synthesis were the predominant factor responsible for the unusual decomposition. Our current practice is to change purification systems whenever a labeled compound (steroid or otherwise) shows undue instability. Admittedly, the process is strictly qualitative in approach due to the obvious difficulties in detecting un-

<table>
<thead>
<tr>
<th>Temperature</th>
<th>2 months</th>
<th>18 months</th>
<th>20 months</th>
</tr>
</thead>
<tbody>
<tr>
<td>Room</td>
<td>2%</td>
<td>50%</td>
<td>75%</td>
</tr>
<tr>
<td>0° to -5° C</td>
<td>1%</td>
<td>8%</td>
<td>15%</td>
</tr>
<tr>
<td>0° to -5° C</td>
<td>5%</td>
<td>90%</td>
<td>100%</td>
</tr>
</tbody>
</table>

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labeled impurities. The presence of unlabeled impurities in a labeled compound can sometimes be more readily discerned by undue decomposition.

In a recent publication, Frankel and Nalbandov have demonstrated that the evaporation of steroids in commercial solvents results in rates of decomposition dependent on the purity of the solvent. Crosby and Aharonson have studied the fluorescence of tars present in commercial solvents and noted that their removal could be effected by passage through a charcoal column followed by distillation. Our own efforts were to find out if these fluorescent tars were causing the decomposition described by Frankel and Nalbandov and to determine whether some of the more involved solvent purifications employed by Frankel and Nalbandov were necessary or whether the simple method of Crosby and Aharonson would suffice.

In Table 2, the per cent decomposition of estrone-6,7-(3H) after storage with a variety of common solvents purified by distillation and charcoal is given. In each case, a 500-ml sample of solvent was evaporated to dryness in vacuo at 50°C and the residue dissolved in 1 ml of distilled solvent. This residue-containing solvent was used as the storage solvent for the steroid. The per cent decomposition was determined by paper chromatography and quantitated with 1 per cent standards. As seen in Table 2, the solvent tars causing the greatest damage came from commercial methanol. Purification by means of a charcoal column improved the stability rate except in the case of methylene chloride. Distillation was the most favorable in all instances. Distillation followed by passage over a charcoal column was little better than charcoal alone. This suggests that an impurity was picked up in the solvent after the distilled solvent passed through the column. The above results indicate that a simple glass distillation is both necessary and sufficient for treating solvents used for the synthesis and storage of labeled compounds.

In one attempt to investigate what was causing the decomposition in these nondistillable solvent tars, we examined them for heavy metal ions. Tables 3 and 4 describe the presence and the effect of heavy metal ions in methanol on estrone-6,7-(3H) during a brief period of storage.

Table 3.—Analysis of Nondistillable Tars from 100 ml of Commercial Methanol*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Fe</th>
<th>Cu</th>
</tr>
</thead>
<tbody>
<tr>
<td>Untreated Tars</td>
<td>130 µg.% (or 1.3 µg./sample)</td>
<td>70 µg.% (or 0.7 µg./sample)</td>
</tr>
<tr>
<td>Versene-washed Tars</td>
<td>0% (sensitivity 30 µg.%)</td>
<td>0% (sensitivity 30 µg.%)</td>
</tr>
</tbody>
</table>

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period of storage. Table 4 shows that the methanol tars containing both iron and copper ions caused the steroid to decompose rapidly, while tars without the cations caused less damage.

Even under the most favorable storage conditions and with the absence of destructive impurities, a radiochemical will decompose at one rate or another. The question occurs as to how best to treat the contaminated materials when further storage is required. In Fig. 1, curves are given for the per cent decomposition versus the storage time of estrone-6,7-(3H) stored under a variety of conditions. Four points exist for each curve: zero, 2, 18, and 20 months. Although only a few points are available, it can be seen that all curves are essentially similar. None is linear, in spite of the fact that decomposition was fostered by the alteration of temperature or by the use of unfavorable solvents. The initial decomposition is almost linear, but then the curves rise sharply, suggesting an autocatalytic process. These curves suggest that the best approach toward a contaminated, labeled compound in storage is to purify it before or just after several percent of impurities have formed. To delay several months may result in a considerable loss of expensive material, especially if that delay allows the slopes of the decomposition curves to rise rapidly. It is reasonable to expect that, in addition to a loss of sample, the counting-related problems discussed above will only increase.

Fig. 1.—Decomposition of estrone-6,7 (3H), 42 Ci/mM during storage. (A) — 5° C in benzene; (B) + 5° C in benzene:ethanol (1:1); (C) room temperature in benzene; (D) + 5° C in benzene:ethanol (4:1); (E) + 5° C in benzene.

Table 4.—Estrone-6,7-(3H), 42 c/mMole, 1 mCi/ml Benzene:Ethanol (9:1) 5° C Stored Five Weeks in Presence of Methanol Tars Before and After Washing with 0.2% Versene Solution*

<table>
<thead>
<tr>
<th>Sample</th>
<th>Treatment</th>
<th>% Decomposition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Estrone-6,7-H^3</td>
<td>No tars</td>
<td>0%</td>
</tr>
<tr>
<td>Estrone-6,7-H^3</td>
<td>Untreated tars</td>
<td>6.5%</td>
</tr>
<tr>
<td>Estrone-6,7-H^3</td>
<td>Washed tars</td>
<td>1.5%</td>
</tr>
</tbody>
</table>

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REFERENCES


The use of radioactive tracers in biological and medical measurements assumes that the substitution of one isotopic atom for another of the same chemical species has no effect on the measurement made. This assumption hinges on two stipulations: (1) that the isotopic substitution involve only a small mass change in the atom replaced and (2) that the substitution be made in a nonreactive region of the molecule. By a curious coincidence, the three major radioactive tracers used in biological and medical studies, \(^{32}\text{P}\), \(^{14}\text{C}\), and \(^{3}\text{H}\), were introduced in order of increasing mass effect and each required the development of more sensitive equipment for its detection. This sensitivity and the capability of determining two isotopes by liquid scintillation counting, as well as the instrumental capacity for large numbers of samples, then made it possible to detect very small contributions to molecular reactivity. The resultant of these trends has been an increasing awareness of the limitations of the original assumption, of the prevalence of isotope effects, and of the appropriate measures to be taken to counter them.

Within the scope of this volume, it is appropriate to deal with five aspects of isotope effects: their origin and magnitude, effects in biological processes prior to sampling, effects during purification of samples for counting, the differentiation of isotope effects from radiochemical impurities, and countermeasures in separation and computation procedures. Details of these topics may also be found in the Symposium on Isotope Mass Effects\(^1\) and in reviews by Klein\(^2\) and by Simon and Palm.\(^3\)

**ORIGIN AND MAGNITUDE OF ISOTOPE EFFECTS**

Traditionally, isotope effects are regarded as manifesting themselves in the transition state of a reaction in which a chemical bond is broken. In kinetic terms, the specific rate
constant, which is the ratio of the normal reactant rate to that of the isotopically substituted reactant, is made up of three terms:

\[
k_1/k_2 = (\text{MMI}) \times (\text{EXC}) \times (\text{ZPE}),
\]

where the mass moments of inertia (MMI) are the ratio of translational and rotational partition functions for light and heavy isotopic species, EXC is the ratio of vibrational excitation factors for light and heavy species in all modes of vibration, and ZPE is the ratio of all vibrational zero point energies. The difficulty in evaluating \( k_1/k_2 \) directly for molecules of biological interest, lies in the requirement that the normal vibration frequencies for the reactants and the transition state be known. To construct these from the fundamental frequencies by direct computation requires an inordinate amount of calculation: the number of terms to be evaluated grows as \( 3N - 6 \), where \( N \) is the number of atoms in the molecule. Wolfsberg and Stern have designed computer programs capable of handling up to 30 atoms, but experimental verification of their values is best carried out on gaseous reactants. Bigeleisen has estimated the maximum ratio of specific rate constants for a series of isotopic pairs. Those most commonly used in analytical or biological tracer studies are listed in Table 1. Examination of these values shows, for example, that (\(^{31}\text{P})\) can be expected to react at a rate 2 per cent faster than (\(^{32}\text{P})\), (\(^{12}\text{C})\) some 50 per cent faster than (\(^{14}\text{C})\), and protium sixty-fold faster than (\(^{2}\text{H})\). These values are for reactions involving a primary bond to the isotope itself. The effect of substitution in a secondary linkage upon the reaction will be less.

It is a common misconception that changes in the reactivity of a molecule are due solely to the incremental mass change; by this reasoning, the per cent mass difference caused by tritium substitution in a molecule of molecular weight 400 should be imperceptible and isotope effects should be absent. Comparison of a (\(^{3}\text{H})\)- and a (\(^{1}\text{H})\)-labeled compound would involve no mass difference, and it would be unreasonable to expect any differences in molecular behavior to be present. Experiences with isobaric fractionation, such as those involving (\(^{14}\text{N})\text{H}_2\text{D} \) and (\(^{15}\text{N})\text{H}_2\) have shown, however, that appreciable differences in vapor pressure can be demonstrated in the absence of mass differences. The nuclear properties of the isotopic atom are not averaged over the entire molecule but are localized and preserved in a given region. Wolfsberg and Stern have shown that when all bonds in which the isotopic atom participates have been included in their calculations, the remainder of the molecule contributes very little to the isotope effect.

### Biological Consequences of Isotope Effects

Biological systems are known to be capable of discriminating between isotopes of hydrogen, carbon, nitrogen and sulfur, and this ability has been used to determine the rate-limiting step in enzymatic reactions. (See, for example, Simon and Palm.) Although a variety of labeled compounds have been used to study this capability, the majority of investigators (and the totality of radiochemical suppliers) are more interested in avoiding such effects in the materials used. Until the advent of dual isotope measurements by liquid scintillation counting, the demonstration of the presence or absence of an isotopic

<table>
<thead>
<tr>
<th>Isotope Pair</th>
<th>Ratio</th>
</tr>
</thead>
<tbody>
<tr>
<td>((^{1}\text{H})/(^{1}\text{H}))</td>
<td>60</td>
</tr>
<tr>
<td>((^{1}\text{H})/(^{2}\text{H}))</td>
<td>18</td>
</tr>
<tr>
<td>((^{12}\text{C})/(^{14}\text{C}))</td>
<td>1.5</td>
</tr>
<tr>
<td>((^{12}\text{C})/(^{13}\text{C}))</td>
<td>1.25</td>
</tr>
<tr>
<td>((^{15}\text{N})/(^{14}\text{N}))</td>
<td>1.14</td>
</tr>
<tr>
<td>((^{18}\text{O})/(^{16}\text{O}))</td>
<td>1.19</td>
</tr>
<tr>
<td>((^{3}\text{H})/(^{2}\text{H}))</td>
<td>1.02</td>
</tr>
<tr>
<td>((^{32}\text{S})/(^{35}\text{S}))</td>
<td>1.05</td>
</tr>
</tbody>
</table>

Table 1.—Maximum Ratios of Specific Rate Constants for Various Isotopes
effect was an onerous task, requiring extremely accurate chemical measurements in the whole animal, or the use of an in vitro system. Once it became possible to compare the rate of metabolism of a (3H)-labeled compound to that of its (14C) counterpart, this difficulty was eliminated, and the general absence of unanticipated isotope effects in metabolic processes could be established. Osinski has underscored the utility of steroids labeled in the 1,2 position with (3H) by demonstrating that (3H)- and (14C)-cortisol were converted to 3a, 11β, 17, 21 tetrahydroxypregnane-20-one at the same rate. MacDonald and co-workers showed that 7α(3H) and 4-(14C)-3β hydroxyandrosterone-17-one appeared in urine with the same isotope ratio as the administered material. Kowarski et al. recovered 1,2-(3H) aldosterone of undiluted specific activity from the urine of adrenalectomized patients, while Gold and Crigler were unable to find any isotope effect of tritium in 1,2-(3H) cortisol or its metabolic products, when compared to 4-(14C) cortisol. Specific isotope effects have been sought, and found absent, in cholesterol, delta cholesterol, delta amino levulinic acid, bilirubin and mesobilirubinogen, bile acids, and phenobarbital. The sole report of an isotopic effect involving (3H) in a non-reactive position is that of Baugnet-Mahieu, Goutier and Semal who found isotopic discrimination between (3H)- and (14C)-labeled thymidine during phosphorylation in vitro.

ISOTOPE EFFECTS IN SAMPLE PURIFICATION FOR LIQUID SCINTILLATION COUNTING

In sharp contrast to the absence of effects in biological systems, there is overwhelming evidence of fractionation effects during the analytical separation of labeled compounds. Enrichment of one isotope, or separation of an (3H)-labeled compound from its (14C)-labeled counterpart has been found in amino acids, carbohydrates, sterols, bile acids, fatty acids, purines, organic acids, and formaldehyde. By far the largest group, numerically, are steroids. Most of these reports are concerned with fractionation effects in aldosterone. Selected examples of compounds undergoing fractionation are shown in Figs. 1 through 7. They illustrate that analytical isotope effects are to be expected in partition, adsorption and ion exchange processes; in columns, thin layer chromatograms, counter current distributions and distillation; in any procedure used to separate two compounds, that has any degree of selectivity, one may expect to find fractionation of isotopically labeled compounds. This results in rising or falling specific activities or isotope ratios across a chromatographic peak, it eliminates the criterion of constant specific activity as an indicator of purity in chromatograms or crystallization, and it makes difficult the estimation of the true proportions of isotopes in an isolated sample.

DIFFERENTIATION OF ISOTOPE EFFECTS FROM RADIOCHEMICAL IMPURITIES

Perhaps the most important task facing the investigator who encounters isotope fractionation in his analytical separation is to establish that it is fractionation and not a radiochemical impurity that he sees. He may avail himself of several simple physical procedures in such an instance; for example, peak sectioning and rechromatography of the isolated portion. Brown et al. took the center portion of their (3H)-methoxyacetic acid peak and diluted it with unlabeled carrier. Repeated sectioning and rechromatography established that the displacement of (3H) from mass was an isotopic effect. Gottschling and Freeze took samples from their ion exchange column at the leading and trailing edge of the peak and chromatographed these samples on paper in a solvent system possessing a different selectivity from the column. Identical spots of different specific activity confirmed the fractionation of (3H)-labeled aminopurine. Gold and Crigler sectioned their
column peak fractions into two halves and showed that the slope of the isotope ratio persisted in the rechromatographed portion. Still another approach was used by Laragh, Sealey and Klein, who developed a paper chromatogram of doubly labeled aldosterone diacetate, sectioned the radioactive spot and applied each portion to a separate partition.

Fig. 1.—Fractionation of 1,2-(3H) aldosterone from 4-(14C)-aldosterone on celite partition column. Cross-hatched area: (14C); outline: (3H). Dots indicate isotope ratio (right-hand ordinate). (Reproduced by permission of J. Chromatogr. 51)

Fig. 2.—Adsorption chromatography of 2,4-(3H)-labeled bile acids with 24-(14C) bile acids. Left: lithocholic acid on alumina column; center: chenodeoxycholic; right: cholic acid on silica gel thin-layer plates. (Reproduced by permission of J. Lipid Res. 21)
Fig. 3.—Ion-exchange chromatographic separation of 5-(3H)-proline (cross-hatched area) from 1-(14C)-proline. Column dimensions: 155 × 0.9 cm; temperature: 50°; buffer: sodium citrate pH 3.28.

column (Fig. 8). Within the accuracy of the measurement, the degree of isotope fractionation was identical in all of the segments.48

The possibility of a radiochemical contaminant escaping such procedures is quite small, but it has been our concern for several years to extend the concepts of radiochemical purity and identity to include such unlikely events.54,58 Using a variety of synthetic chromatographic models, constructed by computer,3,37,58 we have systematically identified the opportunities for, and eliminated the likelihood of, a small contaminating component simulating a fractionation effect.48 By appropriate computation procedures, it is also possible to establish, that a given isotopic displacement will reflect the presence of isomeric forms within a single labeled species.58

Based on these experiences, it is possible to provide several guidelines that will enable the investigator to make reliable decisions concerning the identity and purity of his compounds.57 The first of these is that the specific activity or isotope ratio across the peak should exhibit a straight line when plotted on semi-logarithmic paper against fraction number. It is often worth calculating the least squares fit of these points to determine whether or not a systematic, perceptible curvature is present, or whether there is a departure from the line at either end of the peak. If a departure is evident, more sophisticated analyses can be used to determine the nature and magnitude of contamination.56,58

The second guideline is that the cumulative percent of material in each isotopic peak can be transformed (from a table of values or by computer calculation) to the corresponding area under a unit normal curve. When this is done, these values form a linear relationship to fraction number, and cross the x-axis at the midpoint of the peak, M. The displacement can be calculated from the difference in the

Fig. 4.—Adsorption chromatography of 1-(14C)- and 2-(3H)-labeled acetates of cholesterol on silica gel. Cross-hatched area: (3H); outline: (14C).
two midpoints and is usually normalized as $\Delta M$ per cent, by multiplying the value by $100/M_1$, where $M_1$ is the reference peak. The slope of the relationship between unit normal and fraction number is the reciprocal of the peak dispersion $\sigma$. Again, the use of a least squares analysis of the points is quite informative and yields highly accurate estimates of $M$ and $\sigma$ from the intercept and slope, as well as their errors. With these parameters in hand, the classifications of chromatographic peaks shown in Table 2 can be made. An example of such distinctions in practice is shown in Fig. 9, data taken from Hofmann, Szczepanik and Klein. The purity of ($^3$H)-labeled bile acids was compared to that of commercially available ($^{14}$C) forms of the same compound, and the criterion employed was the ratio of dispersions. To our pleasure, lithocholic and cholic acids prepared by enolic exchange of their keto derivatives proved to be of identical purity to the ($^{14}$C) varieties, but to our surprise, the chenodeoxycholic acid standard proved to be less pure than the product we

![Graph showing isotope fractionation of (3H)-labeled methyl linolate on AgNO$_3$-silica gel columns. (A) 150 $\times$ 12 mm; (B) 230 $\times$ 12 mm. Circles: mass; diamonds: tritium; triangles: specific activity. (Reproduced by permission of J. Chromatogr. 39)](image)

### Table 2—Diagnostic Features in Peak Analysis

<table>
<thead>
<tr>
<th>Quantity</th>
<th>Magnitude</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>$\Delta M_{%}$</td>
<td>$&gt; 10%$</td>
<td>Chemically different species</td>
</tr>
<tr>
<td>$\Delta M_{%}$</td>
<td>$\leq 1%$</td>
<td>Isotope fractionation</td>
</tr>
<tr>
<td>$\sigma_2/\sigma_1$</td>
<td>1.03</td>
<td>Second species is pure, even though displaced</td>
</tr>
<tr>
<td>$\sigma_2/\sigma_1$</td>
<td>1.03-1.10</td>
<td>Multiplet form of second species</td>
</tr>
<tr>
<td>$\sigma_2/\sigma_1$</td>
<td>$&gt; 1.20$</td>
<td>Second species almost certainly contaminated</td>
</tr>
</tbody>
</table>
had prepared. Inspection of the chromatogram in the center of Fig. 4 will confirm that there is a portion of the radiocarbon peak at the left that does not appear to be part of a true gaussian distribution.

**COUNTERMEASURES TO ISOTOPE FRACTIONATION EFFECTS**

**Separation Procedures and Isotope Location**

When it becomes obtrusive in analytical measurements, it is often possible to influence the extent of isotope fractionation by selecting an alternatively labeled form of the molecule. Usually, the greater the proximity of the isotopic atom to the major functional group, the greater the isotope effect observed. For example, Klein and Szczepanik mapped the effect of position on amino acid fractionation effects with the results shown in Fig. 10, illustrating that the contribution diminished with distance from the carboxyl group. The position effects showed a strong positive effect in position 3 and increasing negative displacement with greater distance from the head group of the amino acid (Fig. 11). These effects were attributed to interactions of the with the amino group and with the ion exchange resin, respectively. Hofmann, Szczepanik and Klein compared labeled lithocholic, chenodeoxycholic and cholic acids with their labeled counterparts and found isotopic

![Fig. 6. Countercurrent distribution of 1-(14C)-xylose. Solid circles; radioactivity; open circles: mass. Lower graph: specific activity. (Reproduced by permission of J. Chromatogr.)](image)

![Fig. 7. Distillation of aqueous solutions of (3H) formaldehyde under various conditions: (I) Distillation through normal Claisen apparatus at 80 mm pressure; initial concentration 0.32 M. (II) Distillation through 10-cm Vigreux column at 80 mm pressure; initial concentration 0.32 M. (III) Distillation through 20-cm column at 80 mm pressure; initial concentration 0.34 M. (IV) As in II, at normal pressure. (V) Distillation through vacuum-jacketed column (25–30 Plates) at atmospheric pressure. (Reproduced by permission of Z. Naturforsch.)](image)
displacements of 0.91, 0.41 and 0.02 per cent, respectively, indicating that as the total interaction of the molecule with the adsorbent increases from mono hydroxy to tri hydroxy acid, the contribution of the isotopic atom diminishes or vanishes completely. Where possible, it is particularly desirable to avoid the use of (3H) in positions immediately adjacent to a double bond. Several groups have prepared (3H)-labeled products of this kind; e.g., Sgoutas and Kummerow have prepared (3H)-labeled linoleic acid, and Paliokis and Schroepfer have synthesized 6β(3H)-Δ7 cholesterol. Employing silver nitrate columns, both groups have encountered unusually large fractionation effects. In the latter report, one of the metabolites moved with such an altered mobility as to be completely separated from its averred (14C) counterpart.

Separation Procedures and Solvent Composition

It also appears possible to influence the degree of isotope fractionation by modifying parameters of the separation process such as...
Fig. 9.—Relative purity of 2,4-(3H) versus 24-(14C) lithocholic, chenodeoxycholic and cholic acids. (Reproduced by permission of J. Lipid Res.21)

Fig. 10.—Relationship between isotope position and displacement of (14C)-labeled from unlabeled amino acid. (Reproduced by permission of Anal. Chem.28)

Fig. 11.—Relationship between isotope position and displacement of (3H)-labeled from unlabeled amino acid. (Reproduced by permission of Anal. Chem.28)
solvent composition. Laragh, Sealey and Klein originally reported a difference in mobility between (3H)- and (14C)-labeled aldosterone of 0.65 per cent, while Cejka and Venneman found the displacement to be 1.85 per cent. This was shown to be a function of the stationary phase composition of the columns by Cejka and co-workers. They were able to alter the fractionation of 1,2-(3H) aldosterone from 4-(14C) aldosterone over a three fold range (Fig. 12) and of 1,2-(3H) cortisone from 4-(14C) aldosterone over a ten-fold range by altering the methanol composition of a celite column. The fractionation effect was far larger on short columns with low plate numbers than on long columns (Fig. 13), indicating the participation of factors other than basic differences in partition coefficients. A similar effect has been noted by Klein and Szczepanik for (3H)- and (14C)-labeled proline on ion exchange columns: under conditions of constant column length but changing peak dispersion, the separation between radioactive forms was inversely related to $M/\sigma$. Data illustrating this finding are shown in Fig. 14.

Computation Procedures in Isotope Dilution Assays

Whenever isotope fractionation has been encountered in an isotope dilution assay, the experimenter has had to deal with it in an empirical manner. Ulick and coworkers, for example, took the isotope ratio in the center of the peak as representative of the true ratio. Gold and Crigler recommended taking the entire peak, or at least the central portion, while Benraad and coworkers surmised that symmetrical cutting was essential. Laragh, Sealey and Klein began from the equation governing the isotope ratio:

$$\frac{3H}{1C} = \phi = \frac{M_{10}}{M_{1}} \exp \left[ \frac{-(M_{2} - M_{1})(M - X)}{\sigma_{1}^{2}} + \frac{\sigma_{1}^{2} - \sigma_{2}^{2}(M - X)}{2\sigma_{1}^{2}\sigma_{2}^{2}} \right]$$

where $n$ is the number of counts, $M$ is the midpoint of the peak, and $\sigma$ its dispersion for isotopes 1 and 2, $x$ is the volume eluted or distance migrated, $\bar{M}$ is the midpoint of the mixed peak: $(M_{1} + M_{2})/2$. They pointed out that simply selecting the isotope ratio at the peak maximum is an erroneous procedure.

![Fig. 12. Relationship between stationary phase composition and isotope fractionation of 1,2-(3H) aldosterone from 4-(14C) aldosterone. (Reproduced by permission of J. Chromatogr.)](image1)

![Fig. 13. Relationship between plates generated by column and isotope fractionation of 1,2-(3H) aldosterone from 4-(14C) aldosterone. (Reproduced by permission of J. Chromatogr.)](image2)
where SE(o-3) and SE(o2) are obtained by probit analysis. The true mathematical measure of isotope dilution is simply the ratio of the total area under one peak to the total area under the other peak, and its reliability is a function of the error estimates of the constituent dispersions $\sigma_1$ and $\sigma_2$.

Because: (1) it requires that $\bar{M}$ be known and $\phi$ will be in error to the extent that $\bar{M}$ does not equal the $x$ value selected; (2) it assumes that no correction for any differences in dispersion is required; (3) it assumes no scatter in the values for the isotope ratio, i.e., that they all fall on a straight line with no error; and (4) it does not provide an estimate of the error function for $\phi$ and, hence, for the error in the dilution factor itself.

In reality, the exponential function in Equation (2), evaluated at the mixed midpoint where $\bar{M} = x$, reduces to 1, leaving

$$\phi = \frac{M_2 \sigma_1}{M_1 \sigma_2}$$

and from which the associated error function can be obtained from

$$SE\phi = \phi \left[ \frac{SE(\sigma_1)}{\sigma_1} \right]^2 + \left[ \frac{SE(\sigma_2)}{\sigma_2} \right]^2$$

where $SE(\sigma_1)$ and $SE(\sigma_2)$ are obtained by probit analysis. The true mathematical measure of isotope dilution is simply the ratio of the total area under one peak to the total area under the other peak, and its reliability is a function of the error estimates of the constituent dispersions $\sigma_1$ and $\sigma_2$.

It is generally recognized that the existence of isotope fractionation can present a hazard to isotope dilution measurements, not merely in the estimation of the final ratio, but also by favoring the enrichment or depletion of one isotope during the purification steps preceding the measurement, and that removal of a symmetrical peak eliminates much of this hazard. The possibility of artifacts is also reduced when the ratio of the two isotopes is kept close to unity: the slope of the ratio across the peak is reduced and counting statistics are similar for the two isotopes.

Laragh et al. also studied the strategy of truncation in some detail. Keeping in mind the desirability of maintaining the isotope ratio, while removing the contribution of possible contaminants, they computed the contribution to total peak area of a symmetrical region about the maximum as a function of $y' / y_{\text{max}}$, where $y_{\text{max}}$ is the counts at the maximum and $y'$ is the counts at a distance $x$ on either side. These values are shown in Fig. 15 and have the following utility: Suppose one takes the fractions on either side of the maximum at which the ratio activity has fallen to 20 per cent of the maximum and asks, what
### Table 3.—Determination of Isotope Ratio and its Error from Recoveries and Dispersions

<table>
<thead>
<tr>
<th>Run</th>
<th>Number of Samples</th>
<th>Total (HI)</th>
<th>Total (14C)</th>
<th>$\sigma$ (HI)</th>
<th>$\sigma$ (14C)</th>
<th>(HI/14C)</th>
<th>Error (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>311A</td>
<td>6</td>
<td>5266</td>
<td>417</td>
<td>7.98 ± 0.20</td>
<td>9.14 ± 0.42</td>
<td>12.62 ± 0.66</td>
<td>5.2</td>
</tr>
<tr>
<td>311B</td>
<td>8</td>
<td>8566</td>
<td>840</td>
<td>4.21 ± 0.39</td>
<td>4.45 ± 0.44</td>
<td>10.20 ± 1.33</td>
<td>13.0</td>
</tr>
<tr>
<td>311C</td>
<td>9</td>
<td>9251</td>
<td>1263</td>
<td>4.31 ± 0.24</td>
<td>4.25 ± 0.10</td>
<td>7.33 ± 0.44</td>
<td>6.0</td>
</tr>
<tr>
<td>311D</td>
<td>6</td>
<td>6211</td>
<td>1038</td>
<td>3.80 ± 0.17</td>
<td>3.89 ± 0.19</td>
<td>5.98 ± 0.40</td>
<td>6.7</td>
</tr>
<tr>
<td>Average:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>7.7</td>
</tr>
<tr>
<td>321</td>
<td>22</td>
<td>93960</td>
<td>93879</td>
<td>5.48 ± 0.13</td>
<td>5.50 ± 0.14</td>
<td>1.001 ± 0.035</td>
<td>3.5</td>
</tr>
<tr>
<td>322</td>
<td>21</td>
<td>77045</td>
<td>50470</td>
<td>5.22 ± 0.08</td>
<td>5.31 ± 0.08</td>
<td>1.526 ± 0.003</td>
<td>2.1</td>
</tr>
<tr>
<td>323</td>
<td>29</td>
<td>67005</td>
<td>64371</td>
<td>8.04 ± 0.10</td>
<td>8.11 ± 0.11</td>
<td>1.041 ± 0.019</td>
<td>1.8</td>
</tr>
<tr>
<td>324</td>
<td>17</td>
<td>29572</td>
<td>28312</td>
<td>4.75 ± 0.02</td>
<td>4.73 ± 0.03</td>
<td>1.044 ± 0.008</td>
<td>0.8</td>
</tr>
<tr>
<td>325</td>
<td>22</td>
<td>49470</td>
<td>45163</td>
<td>8.01 ± 0.12</td>
<td>7.98 ± 0.13</td>
<td>1.095 ± 0.024</td>
<td>2.2</td>
</tr>
<tr>
<td>326</td>
<td>14</td>
<td>24961</td>
<td>22574</td>
<td>3.97 ± 0.02</td>
<td>3.91 ± 0.03</td>
<td>1.106 ± 0.010</td>
<td>0.92</td>
</tr>
<tr>
<td>Average:</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.9</td>
</tr>
</tbody>
</table>

It should be emphasized that the introduction of these computations by Laragh et al. did not show previous values to be outside the standard errors of the correct estimates. Bearing in mind the range of biological variation normally encountered in such assays, it is evident that isotope fractionation did not give rise to misleading values. Nevertheless, when further extensions in precision and accuracy are required, application of these procedures can enhance the value of isotope dilution techniques.

### Summary

Isotope effects are widely prevalent in radiochemical measurements, especially those involving (HI) and (14C). The overwhelming majority arise during analytical procedures designed to isolate and purify the labeled molecules of interest. They are to be expected even in low resolution processes, and they give rise to changing specific activities or isotope ratios where constancy is anticipated. Although these effects are seldom so obtrusive as to prevent the establishment of the purity and identity of a compound, they may require extra precautions in isotope dilution assays, where safeguards against accidental enrichment or depletion should be observed.
REFERENCES

15 SAMPLE ADSORPTION PROBLEMS IN LIQUID SCINTILLATION COUNTING

G. J. LITT and H. CARTER

In the handling and analysis of high specific-activity radionuclides, it has long been recognized \(^1\), \(^2\) that adsorption to the walls of common containers may be of great significance. Although there have been periodic references to this problem with labeled biochemicals, there does not appear to be a widespread appreciation for the possible magnitude of the effect. This has become of much greater importance in recent years because of the demand for tracers of ever-increasing specific activity (therefore lower mass), in many cases approaching the theoretical maximum. With recent advances in automation, liquid scintillation counting has evolved into a procedure of deceptive operational simplicity, and often the significance of such effects as wall adsorption may be overlooked.

The general phenomenon of surface adsorption is a very well-known problem in microanalysis. In the field of gas chromatography, for example, a great deal of effort is expended in either deactivating the column support (or walls) or in searching for less adsorptive surfaces. \(^3\) Steroids are probably the best known class of compounds where this is often of critical importance. \(^4\) The recent surge in popularity of adsorption thin layer chromatography has illustrated the often irreversible binding of a wide variety of materials to an activated surface. Some of the difficulties in recovery of substances by scraping and eluting the adsorption medium can even be attributed to adsorption to the glass plate itself, a phenomenon observed in our laboratories with labeled steroids. The strong adsorption of proteins to glass has been noted, \(^5\) and, the use of denaturants such as urea or of enzymes is often necessary to clear the surface. Even gases such as carbon dioxide have been shown to bind to the walls of various sampling containers. \(^6\) As a final example, the addition of small quantities of relatively polar compounds such as isoamyl alcohol, as site saturators, has been shown to be necessary in the extraction and handling of many trace metabolites. \(^7\)

\(^1\) GERALD J. LITT, Ph.D.: Analytical Department; HENRY CARTER, B.Sc.: Assay Laboratory, New England Nuclear Corporation, Boston, Mass.
In the general field of liquid scintillation analysis, Hayes has reported on counting anomalies with benzoic acid-(\(^{14}\)C) in a toluene system. Petroff et al., suggested the use of silanized glass vials for preventing adsorption of some inorganic compounds, and in a later review Turner considered the problem of adsorption in the counting vial. He noted the effect with such diverse materials as fatty acids, a number of polymers, and several inorganic ions. The addition of unlabeled carrier, the treatment of the glass vial with dimethylchlorosilane, or the use of plastic vials were suggested as procedures to minimize the effect. Kandel and Gornall have reported on the glass adsorption of \(^{(14)}\)-labeled aldosterone and have shown the effectiveness of both added carrier and the use of a competitive "site saturator," in this case, hydrocortisone.

Blanchard and Takahashi have utilized the strong adsorptive properties of submicron silica to minimize binding to the glass walls, in effect preserving \(4\pi\) counting geometry although one would anticipate some energy adsorption problems in applying this procedure to weak \(\beta\)-emitters such as \(\alpha\). These workers also reported on the wall adsorption of such materials as sodium sulfite \((^{35}\)S) and several polyacrylamides labeled with \((^{14}\)C).

Over the years, experience in our own laboratories has indicated that widely variable counting rates shown by a number of compounds can often be attributed to adsorption by the counting vial, the dilution flask, or the transfer pipette. In most cases, the vial seemed of greatest importance since the addition of small amounts of unlabeled carrier seemed to stabilize the count rates and lead to an "acceptable" radioassay. The studies reported in this chapter were designed to evaluate the influence of carrier concentration and the effect of the counting medium on the reliability of counting rates for a variety of substrates. We also present the results of preliminary investigations of methods for eliminating the surface adsorption phenomenon.

**Experiments**

In the initial screening, a number of important labeled biochemicals were surveyed for counting anomalies that could be related to adsorptive effects. This group included two basic amino acids, \(\mathrm{L-arginine-}\left(\text{H}\right)\) (G) and \(\mathrm{L-lysine-}\left(\mathrm{H}\right)\) (G), two steroids, testosterone-1,2-\((\text{H})\) and desoxycorticosterone-1,2-\((\text{H})\) (N), thymidine-methyl-\((\text{H})\), \(\mathrm{DL-norepinephrine-7-}\left(\text{H}\right)\) (N) and bradykinin-2-[\(\mathrm{L-proline-}\left(\text{C}\right)(\mathrm{U})\)] triacetate. Of this group, only the basic amino acids and the polypeptide bradykinin, showed strong adsorption. There were indications of counting variability with testosterone, but careful checking showed this to be within the normal range of error. The bulk of the investigation was limited to the \((\text{H})\)-labeled amino acids (specific activities of 1.32 Ci/mM for the arginine and 3.96 Ci/mM for the lysine) and the bradykinin-\((\text{C})\) (specific activity 35 mCi/mM. Angiotensin II-[1-aspartic-5-isoleucine-\((\text{C})\) (U)], specific activity 236 mCi/mM, was used as a final example.

Packard Tri-Carb liquid scintillation spectrometers (Models 3310 and 3314) were utilized with freezer temperatures of 3-6° C and operated at the balance point for the isotope being counted. Where the data were corrected for efficiency, internal standardization was used for \((\text{H})\)-labeled compounds and a modified channels ratio procedure for the \((\text{C})\) materials. Based on their utility for the type of sample being counted, three basic scintillation cocktails were chosen from the standard group utilized in our laboratory: (1) the dioxane system, based on Eastman White Label Dioxane and containing 12 g of PPO, 0.5 of POPOP, 125 ml of dimethoxy ethanol, 125 ml of anisole, and 133 ml of water per liter; (2) 30 percent methanol-toluene containing 40 ml of Liquifluor and 300 ml of methanol per liter of final mix; and (3) the primene system based on tolu-
Effect of Carrier Concentration and Counting System

A series of volumetric flasks containing increasing amounts of unlabeled carrier and a constant amount of radioactive compound were prepared. For both arginine and lysine, the solvent employed was 0.01 N hydrochloric acid. Aliquots were then removed from each flask and carefully transferred to the appropriate counting vial and the pipette rinsed several times with the scintillation cocktail. The vials were then cooled, dark-adapted in the instrument freezer for at least one hour, and counted.

This initial survey led to two major conclusions: (1) the stability of the counting rate strongly depended on the carrier concentration and (2) there was a pronounced increase in counts as the carrier was increased. As might be expected, both effects were very dependent on the particular counting cocktail used. Figure 1 shows the change in counting rates of arginine-(3H) with increasing quantities of carrier as a function of time in the 30 percent methanol-toluene system. It can be seen that the counting rate does not become stable until a carrier concentration of about 500 μg/ml is added. (Fifty-microliter aliquots of the original dilution were counted so that the carrier represented about 25 μg added per counting sample.) The same effect was seen when a dioxane system was used (Fig. 2), although it was much less pronounced. Counting in the Primene system resulted in a similar curve, and virtually identical results were obtained in all of the systems when lysine was studied.

Fig. 1.—Effect of carrier concentration on counting stability and rate for L-arginine (3H) (G) counted in 30 per cent methanol-toluene system. Fifty-microliter aliquots of diluted solutions were counted.

---

ARGinine
30% Methanol

<table>
<thead>
<tr>
<th>Carrier Concentration (μg/ml)</th>
<th>CPM</th>
</tr>
</thead>
<tbody>
<tr>
<td>No Carrier</td>
<td></td>
</tr>
<tr>
<td>10</td>
<td></td>
</tr>
<tr>
<td>25</td>
<td></td>
</tr>
<tr>
<td>50</td>
<td></td>
</tr>
<tr>
<td>200</td>
<td></td>
</tr>
<tr>
<td>500</td>
<td></td>
</tr>
</tbody>
</table>

---

**THE LABELED SAMPLE**

ard glass micropipettes were employed with no correction being applied for possible adsorption to the inner walls of the pipette as evidence showed that the counting container was the principal contributor.

---

* New England Nuclear Corp., Boston, Mass.; contained 4 g of PPO and 0.05 g of POPOP per 42 ml of toluene.
† Rohm and Haas, Philadelphia, Pa.
It is interesting to note that although in both Fig. 1 and 2 the count rate appears to be stable at a carrier concentration of 500 µg/ml (in the original dilution) for both the 30 percent methanol and Primene system in Fig. 3, neither curve attained a plateau at this concentration. The present study did not include the use of still greater amounts of added carrier.

While carrier sensitivity to the counting of the basic amino acids arginine and lysine is demonstrable in all three counting cocktails, we have not observed this with steroids, thymidine, or norepinephrine with which the counts are stable over time and are unaffected by added carrier. We did not evaluate bradykinin and angiotensin for this effect.

![Graph](image_url)

**Fig. 2.**—As in Fig. 1 counting in dioxane system.

In Figs. 1 and 2 it is apparent that, in addition to the effect on counting stability, the absolute counting rate was significantly altered by the addition of carrier. In Fig. 1, even at so-called zero time (this actually represented approximately 1 hour standing in the instrument freezer before counting), there was a change from about 25,000 cpm with no carrier to about 37,000 cpm when the maximum amount was added. The relative importance of the counting system utilized is summarized for arginine in Fig. 3. It can be seen that while the results obtained in the dioxane system were relatively insensitive to carrier concentration, counts in the 30 percent methanol-toluene cocktail were quite dependent on it, and even large differences obtained with the Primene system.

![Graph](image_url)

**Fig. 3.**—Effect of carrier concentration on absolute counting rate of L-arginine(³H) (G), using first recorded count after sample cooling in all three counting systems studied.
Effect of Counting Vial

Since there was a strong adsorption of the basic amino acids arginine and lysine to the walls of the counting vial, dilution flask, or transfer pipette we investigated the effect of modifying the walls of the respective containers. The procedures used were basically the same as those in the carrier concentration study. The principal difference was that counting was done in silanized glass and plastic vials in addition to the standard untreated glass vials and that all counts were corrected for quenching. To demonstrate the differences between the various containers, we chose the Primene system since it displayed the widest differences in count rate with both arginine (Fig. 3) and lysine.

Figure 4 shows the effect of the vial type on arginine-(3H) dpm as a function of carrier concentration. Virtually identical results were obtained with lysine (Fig. 5). Several important points can be made from consideration of these data: (1) Although at about 500 µg/ml of carrier the absolute counting rate in an untreated vial is within about 5 percent of that in either a silanized or plastic vial, the curves in both figures are relatively steep; a small variation in the amount of carrier added could have a significant effect on the result. (2) The evidence for adsorption is particularly good since the counts in the silanized container reached a plateau in both Fig. 4 and 5 at about 200 µg/ml of carrier, a point at which the count rate in the untreated container was rising sharply. This was even more apparent with the plastic container. (3) Although silanizing by our method appeared to modify many of the active adsorptive sites, some still remained as evidenced by the very sharp initial slope compared to that of the plastic vial. (4) The effect cannot be completely
ascribed to interaction with sites peculiar to the structure of glass as there is still a curvature for the plastic vial below 100 µg/ml of carrier. Dr. Jack Davidson's suggestion that this curvature was due to adsorption within the transfer pipette has been borne out, but we have found that the effect varies significantly from pipette to pipette.

There was very little apparent effect on the observed count rates when the dilution flasks were also silanized. Figure 6 combines the data for both the treated and untreated flasks. It can be seen that most of the points are identical at a given carrier concentration or fall within a reasonable error. The only point with a significant deviation was for the silanized vial at 50 µg/ml of carrier.

Magnitude of Effect

In the preparation of some labeled compounds, cold carrier material is either not obtainable or quite valuable. Although readily available today, the polypeptides, bradykinin and angiotensin II when the labeled molecules were synthesized, were quite dear. The liquid scintillation counting done at New England Nuclear was, of necessity, without added carrier. A recent reinvestigation of the counting rates of the (14C)-labeled polypeptides is described below.

At nominal specific activities of 35 mc/mM for the bradykinin-2-[L-proline-14C] (U) triacetate and 236 mCi/mM for the angiotensin II-[1-aspartic-5-isoleucine-14C] (U) the samples were counted in the dioxane and 30 percent methanol-toluene systems used in the previous experiments. Samples were counted in duplicate in untreated glass, silanized glass, and plastic vials. Correction was applied with a modified channels ratio procedure. The values obtained are shown in Table 1. In the dioxane system, a very significant percentage of the dpm were simply not seen if an untreated glass counting vial was used, particularly with bradykinin. Angiotensin II appears to have exhibited much less adsorption but the importance of the proper choice of counting system is apparent from the table. While the absolute efficiency of bradykinin was not more than 5 percent higher in the plastic vials, almost 27 percent more dpm were found with the dioxane system and 65 percent more dpm were recovered in the 30 percent methanol-toluene cocktail.

**DISCUSSION**

Although the magnitude of surface adsorption is quite different for various classes of compounds and is quite dependent on the specific scintillation cocktail used, the significance of the data reported here lies in this very nonpredictability and in the fact that the standard methods of correction cannot compensate for the effect. This is shown quite graphically in the example with labeled bradykinin. Over the years, it has been our experience that erratic counting can very frequently be ascribed to adsorption, and the addition of a pinch of carrier has often been
observed to help. From the data with arginine, lysine and bradykinin, it is clear that mere attainment of a reproducible counting rate gives no assurance that this accurately reflects the amount of radioactivity present. There are dangers inherent in recommending the addition of large amounts of carrier to overcome adsorption effects, and two of these, the possible quenching caused by interference with the energy transfer process and the approach to the limit of solubility, are particularly important. While the former can be corrected for, the latter may cause erratic results.

A preliminary counting in both glass and plastic containers is advisable and may indeed be mandatory when carrier is not available. The method of Blanchard and Takahashi 12 previously discussed may present an alternate approach to preventing wall absorption, at least for (14C)-labeled compounds. In our laboratories, we have also experimented with the possible applicability of displacement or competitive absorption, discussed for the counting of aldosterone by Kandel and Gornall 11 who used hydrocortisone to saturate adsorptive sites, this method can sometimes be useful when no carrier is available. We have found, however, that it is difficult to predict the effectiveness of the competitor in saturating the sites. For example, although arginine is somewhat effective in blacking the sites for bradykinin, silanizing the container is more effective, but neither of these procedures is as effective as counting in a plastic vial.

The importance of the counting system used should not be underemphasized. The relative competition of components of the solvent can make very great differences. In the present study, the dioxane system employed contained a fair quantity of water. Adsorptive effects were much lower than in the other cocktails, the water presumably saturating the sites. The contribution of the solvent system should be determined for each individual case.

It is clear that much remains to be done regarding the phenomenon of adsorption. We anticipate that the effect would have been significantly reduced if samples were counted at ambient temperature, but no definitive data are available showing this. The utility of other types of glass treatment and the use of vials of other material such as nylon have not been investigated. There are indications that new procedures for treating glass may be much more effective in removing active sites than silanizing 14 and that new types of glass may exhibit less adsorptive effects.15

### Table 1

<table>
<thead>
<tr>
<th>Counting System</th>
<th>dpm</th>
<th>dpm</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Dioxane</td>
<td>30% Methanol-toluene</td>
</tr>
<tr>
<td>Bradykinin-2-[(L-Proline-C14 (U)] Triacetate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. Glass Vial</td>
<td>26,609</td>
<td>20,051</td>
</tr>
<tr>
<td>II. Silanized Vial</td>
<td>25,264</td>
<td>20,398</td>
</tr>
<tr>
<td>III. Plastic Vial</td>
<td>28,752</td>
<td>24,713</td>
</tr>
<tr>
<td></td>
<td>32,821</td>
<td>34,122</td>
</tr>
<tr>
<td>Angiotensin II [1-aspartic-5-isoleucine-C14 (U)]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>I. Glass Vial</td>
<td>25,631</td>
<td>22,773</td>
</tr>
<tr>
<td>II. Silanized Vial</td>
<td>25,557</td>
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<tr>
<td>III. Plastic Vial</td>
<td>25,441</td>
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<td>25,219</td>
<td>26,691</td>
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<td>26,507</td>
<td>27,991</td>
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<td>26,523</td>
<td>27,443</td>
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</table>
We feel that the results reported here are of sufficient magnitude to emphasize the problem, although admittedly restricted to a few compound classes. Combined with our past experiences, this information has prompted us to consider the institution of a screening program to uncover cases where significant adsorptive effects occur and to develop procedures for minimizing the effect when observed.

Note: After writing this chapter, the authors learned of a publication of Davidson and Oliverio who demonstrated similar effects and showed that added carrier stabilized the counting rate of methylglyoxalbis [guanylhydrazone (14C)] and that a plastic vial was effective. Davidson and Oliverio discussed some of the implications of the adsorption phenomena in liquid scintillation counting.

REFERENCES

5. Rothstein, F.: Personal communication.
16/PREPARATION AND COUNTING OF LIPOPHILIC SAMPLES

P. J. THOMAS and H. J. DUTTON

A chemist generally classifies organic compounds as being more soluble in ether than in water. The budding biochemist quickly discovers that the majority of compounds of physiological interest unfortunately do not fall into this category. (See Chapters 17–23 and 25–27.) Lipids are the exception. By definition, lipids are naturally occurring substances that are more soluble in ether than in water.

The physical properties of this class of compounds tend to make life easier for the lipid chemist. This is particularly evident when he comes to do liquid scintillation counting. Nearly all lipid samples form homogeneous solutions in the most common scintillator solution: toluene containing 4–5 g/l of 2, 5-diphenyloxazole (PPO). The selection of solvents for scintillation counting has been discussed by Laustriat in Chapter 2. We shall only say that toluene remains the solvent of choice for lipophilic samples, as it has been since the early work of Hayes et al.¹ and Davidson et al.²

SAMPLE PURIFICATION

In performing a metabolic experiment using radioactive tracers, the lipid chemist is faced with many of the problems common in other biochemical studies. The task is to demonstrate that particular atoms of a metabolic precursor are incorporated, more or less specifically, into a chemically distinct intermediate or product molecule. To do this, the investigator must establish the chemical nature of the labeled products, generally by separating them into classes and, perhaps ultimately, into pure components. If sufficient separation cannot be achieved by solvent extraction, the modern chemist usually turns to chromatography. It may be

¹Mention of firm names or trade products is made for information only and is not to be construed as endorsement by the United States Department of Agriculture.

Paul J. Thomas, Ph.D.; Herbert J. Dutton, Ph.D.: Chemical and Physical Properties Investigations, Oilseed Crops Laboratory, United States Department of Agriculture, Agricultural Research Service, Northern Utilization Research and Development Division, Peoria, Ill. Present address of Dr. Thomas: Gastroenterology Unit, Mayo Foundation, Rochester, Minn.
useful, then, to consider some of the general techniques of liquid scintillation counting used to assay radiotracer in lipophilic compounds separated by chromatography.

Chromatographic separations may be classified by method as gas-liquid or gas-solid, liquid column, thin-layer, and paper; and they may be classified by intent as preparative or analytical. These two categories are not entirely independent. Until quite recently, liquid columns have been most often used for preparative separations, owing to the lack of sensitive universal detectors. Gas chromatography has been used mostly for analysis, with the sample being consumed or simply lost after detection. This has largely been due to the inconvenience of scaling up gas columns for large samples and to the difficulty experienced in recovering the sample from the effluent vapor. (See Chapter 24.)

All these considerations influence the selection of a method for measuring radioactivity in the separated components. It would generally be more convenient to monitor the flowing stream continuously during gas or liquid column chromatography. Monitoring can be done with continuous flow cells in scintillation counters \(^5\) (See Chapter 8), but the sample can be observed in the counter for only a short time. A relatively high concentration of radioisotopes is required in the stream to produce a significant signal. If the sample is collected in serial fractions, these can be assayed for radioactivity at leisure, and with samples of low activity the time of counting can be increased to give meaningful statistics. Considering the ease with which radioactive isotopes can be measured in automatic liquid scintillation counters, this latter approach does not involve serious inconvenience.

**Analytical Columns**

In analytical separations or where the utmost sensitivity is desired, the entire sample can be collected directly in scintillation vials placed in an automatic fraction collector. Such a system has been described by Dutton.\(^6\) A toluene-base scintillation solution is used to scrub the effluent vapor of a gas chromatographic column and is then automatically collected in vials for subsequent counting. No splitting of the stream is necessary when a nondestructive (e.g., thermal conductivity or argon-ionization) detector is used. When a flame ionization detector is used, a stream flow-splitter is required. Similar fractionation may be achieved with the apparatus described by Karmen et al.,\(^4\) in which the sample is trapped by passing the hot effluent gas over silicone-coated crystals of anthracene or \(p\)-terphenyl packed in small glass cartridges. When the cartridges are placed in toluene-filled scintillation vials, the sample and terphenyl dissolve and the terphenyl serves as a scintillator for subsequent counting.

Fractions from an analytical liquid column can also be collected directly in scintillation vials, but the probability of chemical quenching must be considered, particularly if tritium is to be counted. Commonly used solvents for liquid chromatography, such as chloroform and acetone, are strong quenching agents. At a concentration of 3 percent of the final volume, chloroform can reduce \(^3\H\) counting efficiency by 50 percent. The concentration of these chromatographic solvents should be kept to a minimum. Quenching is discussed in detail in Chapters 28–32. If the isotope concentration in the effluent is low, larger volumes of solvent should be removed by evaporation before adding the scintillation “cocktail.” Removal of solvent can usually be conveniently carried out by directing a stream of warm air over the vials in the fraction collector already filled with the column effluent. The solvent-free vials, still in the fraction collector, can then be filled automatically with scintillator solution, using the pump and valve arrangement described by
This minimizes the possibility of getting the vials out of sequence during the extra handling that would otherwise be required in drying and refilling them. This procedure works best, obviously, when a uniform rate of flow is maintained through the chromatographic column. Fractions can then be collected on a constant time basis, and the scintillator solution can be pumped at a constant rate to leave about 10 ml in each vial. The vials are finally capped and numbered before removing them from the fraction collector.

Preparative Columns

In the more common applications of liquid column chromatography and in some applications of gas chromatography, only a small part of the effluent need be assayed for radioactivity; the remainder is preserved for other experiments. The simplest procedure is to pipet a portion of each collected fraction into a scintillation vial and add the scintillator solution. This is not much work with the newer semiautomatic pipettors and dilutors, but it could be completely automated by splitting the effluent stream so that a constant small portion is collected in scintillation vials. In practice, it is difficult to make a stream-splitter sufficiently quantitative for reliable sampling at low flow rates, although some good designs have been published. In principle, it should be possible to sample the effluent on a time basis, through a pneumatic or solenoid-actuated three-way valve to divert the stream to scintillation vials. In practice, it is difficult to make a stream-splitter sufficiently quantitative for reliable sampling at low flow rates, although some good designs have been published. In principle, it should be possible to sample the effluent on a time basis, through a pneumatic or solenoid-actuated three-way valve to divert the stream to scintillation vials. In practice, it is difficult to make a stream-splitter sufficiently quantitative for reliable sampling at low flow rates, although some good designs have been published. In principle, it should be possible to sample the effluent on a time basis, through a pneumatic or solenoid-actuated three-way valve to divert the stream to scintillation vials. In practice, it is difficult to make a stream-splitter sufficiently quantitative for reliable sampling at low flow rates, although some good designs have been published.

Fraction Spacing

When the assay of radioactivity in column effluents is discontinuous, as in the examples discussed, it is important that attention be given to fraction spacing. With preparative-scale liquid columns, fraction size is often dictated by the largest size containers that can be accommodated by the fraction collector. Several hours may be required for complete elution, and the fractions collected may number more than 200. Here it would be unnecessary to assay each fraction. With the higher elution rate, higher resolution of vapor-phase columns, and some improved liquid columns, fractions should be closely spaced to preserve the degree of resolution attained in the original separation. Close spacing of the fractions, also decreases sensitivity. This becomes the limiting factor with samples of low specific activity, particularly in gas chromatography, where the size of the sample which can be applied is severely limited.

Many experiments have been reported in which sensitivity was maximized by collecting each peak in a single fraction, but this method yields only qualitative results with poor resolution. A minor compound of high specific activity could be mistakenly identified if it overlapped a major component of low specific activity. If fractions are not collected over equal time periods, the contribution of background due to slow bleed of degradation products will be unequal. For any reliable quantitative results, chromatographic fractions must be spaced uniformly and closely.

Increasing Resolution and Sensitivity by Averaging

Both the resolution and the sensitivity of radiochromatographic analysis can be improved by repeating the analysis and averaging the results. We have recently described a digital computer program that will combine and average the results of two to four repeated analyses on identical samples and present the results graphically on a Cal-Comp x-y digital plotter. The procedure may be applied in principle to any separation process in which serial fractions are analyzed, but the greatest benefit will be
noted in techniques where the amount of sample separated in a single run is limited, as in gas or thin-layer chromatography. It is also necessary that the separation be readily reproducible. So far we have applied the calculation method only to samples separated by gas chromatography, but it appears also to be well suited to the zonal scanning of thin-layer chromatograms described by Snyder.9 (See Chapter 25.) Here sensitivity is improved because the final result is based on the analysis of a larger sample. Resolution is also improved because, in general, the composition of respective fractions is not identical from one run to the next. This has an effect similar to that of spacing fractions closer together. Because of the discrepancy in spacing, of course, the counts in corresponding fractions cannot simply be averaged for the successive runs; this would reduce the resolution. Instead, the count for each fraction is assigned a retention value relative to some reference standard, such as methyl stearate. A scale of relative retention values from 0 to 2.5 times that of methyl stearate is divided into 1250 increments, each represented by 1/100 of an inch on the plotter. A count is calculated at each increment for each of the separate runs by straight-line interpolation between the data points on either side. The calculated values from the individual runs are then added and divided by the maximum to give a "per cent of full-scale" figure recorded by the plotter at each increment. When these points are connected, the resulting graph of radioactivity versus relative retention time approximates a smooth curve and is, in essence, a radiochromatogram. When the chromatographic mobility of a labeled product is to be compared with the mobility of a standard, a second variable is generally measured to locate the standard. This could be a different isotopic label \[(^{14}C)\] versus

![Graph](image)

**Fig. 1.**—Profiles of radioactivity (dotted line) and thermal conductivity (solid line) in effluent stream of gas-liquid chromatograph, averaged from three repeated analyses. Sample consisted of methyl esters prepared from seed lipids of soybean plant exposed to \((^{14}C)\)-carbon dioxide. Average specific activity of methyl esters was 0.6 nCi/mg.
or simply the distribution of the sample by weight, when the gross composition by weight is known. In either case, it is convenient to plot the function corresponding to the standard on the same graph with the radioactivity profile. This can easily be done with our computer program, provided the data are available in digital form. The output of gas-chromatography detector, for example, would first have to be put through an analog-to-digital converter.

Figure 1 compares data from thermal conductivity analysis and radioactivity analysis by scintillation counting of \(^{14}C\)-labeled soybean methyl esters. The sample was isolated from soybeans 12 hours after briefly exposing a plant to \(^{14}C\) carbon dioxide. The average specific activity of the methyl esters was 0.6 nCi/mg.

The profile shown in Fig. 1 is the calculated average of three repeated analyses of 1-mg samples. Data from the thermal conductivity detector were recorded at 8-second intervals on paper tape by an Infotronics CRS30E digital readout system (Fig. 2). The fraction collector holding the scintillation vials was triggered on every fifth interval of the digitizer, by connecting it through a stepping relay. This provided a precise synchronization of the thermal conductivity and radioactivity data.

As each component is eluted from the column, a lag of 15 to 20 seconds is generally observed between the maximum thermal conductivity recorded and the maximum concentration of radioactivity collected in the vials. This sort of delay can be compensated for empirically in the plotting program, but it leads to a degree of uncertainty in correlating peaks, particularly when the labeled compounds are not observed during the thermal conductivity analysis. A difference of 3 percent or less between peak positions may therefore not be significant, but when greater precision is required, the double isotope technique is preferred.

Some loss in resolution of the radioactivity profile may be noted in Fig. 1. The peaks are broader and the valleys between them are higher than in the thermal conductivity analysis. This is an inevitable result of collecting serial fractions, and the degree of peak broadening is related to fraction spacing, as discussed above.

Fig. 2.—Gas chromatograph and fraction collector equipped for automatic condensation and serial collection of effluent in a liquid scintillation counting mixture. Recorder and digital readout system for thermal conductivity detector are at right.
Double-isotope analysis by scintillation counting is straightforward with samples that form homogenous solutions in toluene. Counting efficiency in the presence of variable amounts of quenching agent is easily determined using the external standardization or channels-ratio methods described in Chapters 6 and 29.

Figure 3 shows the graph plotted from simultaneous gas chromatographic analysis of methyl esters containing \(^{14}\text{C}\) and \(^{3}\text{H}\). The \(^{14}\text{C}\)-labeled sample was isolated from a methionine-dependent strain of *Agrobacterium tumefaciens* grown in a liquid medium containing \(^{14}\text{C}\)-methyl-methionine. \(^{3}\text{H}\)-labeled methyl esters of the normal 16-, 17-, 18-, and 19-carbon fatty acids were prepared by the method of Mounts and Dutton and mixed with the \(^{14}\text{C}\) sample before injection into the gas chromatograph. The \(^{3}\text{H}\)-labeled standards served as markers to aid in the identification of the \(^{14}\text{C}\)-labeled components. Since both isotopes were measured on the same collected fractions, relative mobility could be determined with high precision. In gas-chromatographic separations, differences in retention time of about 1 percent between the \(^{14}\text{C}\) and \(^{3}\text{H}\)-labeled compounds can be observed and considered significant. Smaller separations (0.3%) due to isotopic fractionation may be deduced from \(^{3}\text{H}/^{14}\text{C}\) ratios across the peak, as described by Klein et al. (See Chapter 14.)

In contrast, double isotope analysis with proportional counters or ionization chambers would leave many uncertainties about the relative amounts and mobilities of the two isotopically labeled compounds, since stream splitting, followed by separate combustion and reaction trains for each isotope, would be required. Monitoring chromatographic separations by liquid scintillation counting of serial fractions offers the distinct benefits of simplicity, sensitivity, and precision for dual-label analysis.

**REFERENCES**

17/DETERMINATION OF RADIOACTIVITY IN AQUEOUS SAMPLES

G. A. Bray

The use of liquid scintillation counters to assay radioactivity from β-emitting isotopes has become widespread. Along with the increased availability of this technique has come a search for better methods of preparing samples. The ensuing paragraphs focus on the problem of preparing aqueous samples containing (3H) or (14C) for assay in a liquid scintillation counter. A variety of methods have been proposed for this purpose and they will be reviewed; the use of dioxane-based solutions will be discussed in detail; and toluene- and dioxane-based liquid scintillation solutions will be compared as methods for counting radioactivity in serum and urine.

Ideally, a liquid scintillation solution should have the following properties; (1) The reagents should be readily available. (2) They should be relatively inexpensive. (3) Once prepared, the solution should be stable for a reasonable period of time. (4) The scintillation solution should have a high efficiency for the conversion of energy from radioactive particles into light. (5) The liquid scintillator should be miscible with a wide variety of experimental samples. Many of these qualities are to be found in solutions utilizing toluene as the principal solvent and containing PPO (2,5-diphenyloxazole) as the primary scintillator and a wavelength shifter such as POPOP (1,4-bis-[2(5-phenyloxazolyl)]-benzene). (See Chapter 3.)

The major drawback to toluene-based scintillators is their low miscibility with water. I first became aware of this limitation ten years ago while working as a renal physiologist at the National Institutes of Health. Determination of radioactivity in plasma and urine was difficult with any of the systems available prior to 1958. Since that time a number of methods have become available for assaying radioactivity in aqueous samples. These have been summarized in Table 1.

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Anthracene

One approach was to find scintillators that would dissolve in water and convert the radioactivity into photons. The first important example of this group was developed by Steinberg. In his method, 1 g of anthracene is accurately weighed into a counting vial to which 3 ml of the aqueous solution are added. These two phases are brought into contact by adding a wetting agent such as triton X-100. The wetting agent, anthracene and sample are thoroughly mixed before being put into the liquid scintillation counter. Efficiency is approximately 20 percent for (14C) but only one percent for (3H). The low efficiency for (3H) and the difficulty of preparing 1-g samples of anthracene for large numbers of individual vials are its principal drawbacks.

Toluene and Organic Solvents

The addition of organic solvents to toluene-based scintillation solutions to increase their miscibility with water has provided a second general method for assaying radioactivity in aqueous samples. When ethanol is added to a toluene scintillator, it is possible to incorporate small amounts of water and yet retain a homogeneous system. There are severe limitations in the volume of water that can be incorporated, and efficiency is relatively low due to the quenching produced by ethanol.

The solubility of water in toluene can also be increased by the addition of various "solubilizers." Hydroxide of hyamine, the first to be used is a quarternary ammonium base \([p-(diisobutyl-cresoxy-ethoxyethyl)]\) dimethyl-benzylammonium hydroxide which is soluble in methanol. Passman, Radin and Cooper have used it to trap carbon dioxide before adding it to a toluene-based liquid scintillator, but it has also been used for the digestion of tissues prior to assaying their radioactivity. The principal disadvantage of hyamine is the quenching it produces. This difficulty has led to the search for other solubilizers that quench less. Phenethylamine and ethanolamine have largely replaced hyamine as an agent for trapping CO₂. Two other solubilizers have recently become
available, one from Nuclear-Chicago Corporation called NCS and the other from Beckman Instruments Inc, called Bio-Solv. This latter product consists of three separate solutions used under different experimental conditions. The three Bio-Solv solubilizers will be compared with NCS and with hyamine in experiments reported below.

Combustion

A third general approach to the problem of counting radioactivity in aqueous samples has been to convert the radioactivity into CO₂ and water.¹⁰ The carbon dioxide and water can be trapped and counted separately. This method is discussed in detail in Chapter 23. It has been found particularly useful in gas chromatography where the effluent from the chromatographic column can be trapped and converted to CO₂ and water and counted with 4π geometry.

Suspensions

The formation of a suspension provides a fourth general way of preparing aqueous samples for assay with the aid of a liquid scintillation counter.¹¹⁻¹⁴ These methods are most useful for fine particles and are dealt with in greater detail in Chapter 19.

Dioxane Scintillators

The dioxane-based scintillators represent a fifth approach to the problem of providing homogeneous systems for counting water-soluble isotopes in a liquid scintillation counter, and this method will be dealt with in more detail below.

Development of a Dioxane-based Liquid Scintillator

It was recognized early that a mixture of dioxane, naphthalene, and a primary scintillator such as diphenyloxazole converted radioactivity into photons.¹⁵ Little use was made of this observation, however, because early liquid scintillation counters housed their photomultiplier tubes in a deep freeze at temperatures below the freezing point of dioxane. (See Chapter 4.) With improvement in the characteristics of the photomultipliers, it is no longer necessary to use low temperatures, and thus dioxane-based systems have become more widely used.¹⁶⁻²⁴ Their principal advantage is the quantity of water that can be counted in a homogeneous solution. Their disadvantage is the slight reduction in peak pulse height relative to toluene.²⁵ My attention was focused on the need for a better liquid scintillator to count isotopes dissolved in water by the difficulty in assaying tritiated acetazolamide, a water-soluble drug which alters renal function. Steinberg had just developed a system for assaying the radioactivity in aqueous solutions utilizing anthracene.² We tried this method but found that the efficiency for (³H) was too low for our experiments. Next we tried a dioxane-based system to which naphthalene was added, and the efficiency of this mixture was much better, but dioxane freezes at 11.7° C and the sample-changer in the liquid scintillation counter was set at −5° C. Thus, all of our samples froze with unpredictable variation in counting. The next step suggested by Steinberg was to add antifreeze to the dioxane. Our original choice was ethylene glycol, a widely used, permanent antifreeze which solved our problem. Our system could hold up to 30 percent water with an efficiency above 10 percent for (³H).

The composition of the liquid scintillator which was subsequently published,¹⁸ differed from the original preparation because we found that the substitution of methanol for most of the ethylene glycol provided higher efficiency and a greater versatility in the samples which could be prepared as homogeneous solutions. The reasons for the selection of specific solvents can be seen in Figs. 1 and 2 and Tables 2 and 3. Figure 1 shows the effect of increasing concentrations
Fig. 1.—Effect of naphthalene on count rate for \((^{14}C)\) and \((^3H)\). PPO concentration was 0.4 per cent and POPOP 0.02 per cent. Radioactivity was present in 0.1 ml of water. Samples were counted in Nuclear-Chicago Mark I with maximum gain and wide integral window. Ordinate is percentage of counts reached when naphthalene was 20 per cent.

Table 2.—Solubility of Acidic, Basic and Salt Solutions in Mixtures of Dioxane and Methanol

<table>
<thead>
<tr>
<th>Dioxane (ml)</th>
<th>Methanol (ml)</th>
<th>Miscibility After Adding One Milliliter</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>1 N HCl</td>
</tr>
<tr>
<td>9</td>
<td>1</td>
<td>clear</td>
</tr>
<tr>
<td>9</td>
<td>2</td>
<td>clear</td>
</tr>
<tr>
<td>9</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>9</td>
<td>4</td>
<td>—</td>
</tr>
</tbody>
</table>

of naphthalene on the count rate of radioactivity using both glucose-\((^{14}C)\) and tritiated water. The data on the ordinate are expressed as per cent of the maximal count obtained when 20 percent naphthalene was present. As the naphthalene concentration was raised from 1 to 6 percent there was a substantial increase in the count rate for both \((^3H)\) and \((^{14}C)\). When the concentration of naphthalene was increased above 6 percent, however, the increase in the efficiency was small since the count rates for \((^{14}C)\) and \((^3H)\) were both at or above 90 percent of the maximal rate.

Table 3.—Solubility of Basic Solutions in Mixtures of Dioxane, Methanol and Ethylene Glycol

<table>
<thead>
<tr>
<th>Experiment 1</th>
<th>Dioxane (ml)</th>
<th>Methanol (ml)</th>
<th>Ethylene Glycol (ml)</th>
<th>Miscibility After Adding One Milliliter of 1 N NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.5</td>
<td>1</td>
<td>clear</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1.0</td>
<td>1</td>
<td>clear</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1.5</td>
<td>1.5</td>
<td>clear</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Experiment 2</th>
<th>Dioxane (ml)</th>
<th>Methanol (ml)</th>
<th>Ethylene Glycol (ml)</th>
<th>Miscibility After Adding One Milliliter of 0.5 N NaOH</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>1.0</td>
<td>0</td>
<td>cloudy</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.9</td>
<td>0.1</td>
<td>clear</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.8</td>
<td>0.2</td>
<td>clear</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.6</td>
<td>0.2</td>
<td>cloudy</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>0.4</td>
<td>0.2</td>
<td>cloudy</td>
<td></td>
</tr>
<tr>
<td>9</td>
<td>—</td>
<td>0.2</td>
<td>cloudy</td>
<td></td>
</tr>
</tbody>
</table>
Table 4.—Composition of Liquid Scintillator (Bray's Solution)

<table>
<thead>
<tr>
<th>Component</th>
<th>Quantity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Naphthalene</td>
<td>60 g</td>
</tr>
<tr>
<td>PPO</td>
<td>4 g</td>
</tr>
<tr>
<td>POPOP</td>
<td>0.2 g</td>
</tr>
<tr>
<td>Methanol</td>
<td>100 ml</td>
</tr>
<tr>
<td>Ethylene glycol</td>
<td>20 ml</td>
</tr>
<tr>
<td>Dioxane to make 1 liter</td>
<td></td>
</tr>
</tbody>
</table>

Similar data have been presented by others. 17,18 Counter-balancing the rise in efficiency with added naphthalene was the fact that the quantity of water which could be dissolved was substantially reduced. For this reason a concentration of 6 percent naphthalene was selected as a reasonable compromise between maximal solubility for water and maximal efficiency. Other systems using naphthalene and dioxane have been published with concentrations of naphthalene up to 15 percent. As can be seen in Fig. 1, the count rate will be slightly higher in these solutions, but for our purposes the increased solubility of water more than justified the small loss in counting efficiency with the lower concentration of naphthalene.

The effect of diphenyloxazole on the count rates for (3H) and (14C) is shown in Fig. 2. As the concentration of diphenyloxazole was increased from 0.025 percent to 0.2 percent, there was a striking rise in the relative count rate for both isotopes. Between 0.2 and 0.4 percent there was a further small increase but little increase beyond 0.4 percent. The system reported in Table 4 contains 0.4 percent.

The effect of methanol on the solubility of acidic, basic and salt solutions is shown in Table 2. In these experiments 9 ml of dioxane were added to each vial and volumes of methanol varying between 1 and 4 ml were added to separate vials. Solutions of 1 N NaOH were the least soluble. Not until 4 ml of methanol were added to 9 ml of dioxane was a clear solution obtained with 1 N NaOH. The solubility for solutions containing NaOH was improved by adding small amounts of ethylene glycol in place of some of the methanol. This is shown in Table 3. The addition of 1 ml of ethylene glycol and 0.5 ml of methanol to 9 ml of dioxane produced a clear solution with 1 ml of 1 N NaOH. When various mixtures of ethylene glycol and methanol were used, as shown in experiment 2, a clear solution was obtained with 0.9 and 0.8 ml of methanol and 0.1 or 0.2 ml of ethylene glycol. On the basis of these experiments the liquid scintillator we published contained 10 percent methanol and 2 percent ethylene glycol (Table 4). 18

I was neither the first 15 nor the last to report systems in which dioxane and naphthalene were the principal ingredients in a system designed for counting radioactivity contained in aqueous solutions. These systems have contained between 5 and 15 percent naphthalene and the effects of these variations in naphthalene concentrations on count rate have been shown in Fig. 1. The concentration of diphenyloxazole has also varied from 0.4 to 1 percent and the effects of such variations can be found in Fig. 2. Variation within these ranges would have relatively small effects on count rate.

Several solvents have been tried in order to prevent dioxane from freezing. Ethylene glycol, 20 methanol 15 and ethanol 26 have all been used as have anisole and dimethoxythethane 27 and the monomethyl 25 and monoethyl 16 ethers of ethylene glycol. If the liquid scintillation counter is used at ambient temperature, these added solvents can be omitted, but the solubility for aqueous solutions is then often reduced.

To gain more insight into the effect of adding solvents to a dioxane scintillator, three such solutions 15,27,28 have been compared with three toluene-based systems 29.
For this experiment, absolute standards of toluene-\(^{14}\text{C}\) and toluene-\(^{2}\text{H}\) obtained from New England Nuclear Corp. were added to duplicate samples of each of the six solutions listed in Table 5. Solutions 1, 2 and 6 had toluene as the principal solvent and the other three dioxane. The efficiency for \(^{14}\text{C}\) was nearly identical in all systems, although two of the toluene-based systems (1 and 2) were more efficient in counting \(^{2}\text{H}\). The amount of water that could be maintained in a single phase was greatest in solutions 3 and 5. System 4 had a slightly lower capacity and system 6 held much less water. From these data, it would appear that scintillation solution 3 or 5 had the advantage of dissolving more water.

### Application of Liquid Scintillation Counting to Problems in Biology

Many papers in the scientific literature deal with the problem of assaying the radioactivity in water-soluble components utilizing techniques of liquid scintillation, but few provide comparative data on the effectiveness of various methods.\(^6\)\(^-\)\(^7\) To provide such information, a dioxane-based system\(^9\) was compared with a toluene system in which the aqueous samples were dissolved with the aid of solubilizers. These solubilizers provided a new and potentially better method for the assay of radioactivity in aqueous samples. The solubilizers used in these experiments were hyamine, NCS and several Bio-Solv solubilizers. NCS, made by Nuclear-Chicago, is an organic base soluble in toluene or dioxane that can be used to trap carbon dioxide and to digest protein. The Bio-Solv solubilizers, introduced by Beckman, consist of three organic mixtures, each of which has a specific use. BBS-1 and BBS-3 are general-purpose solubilizers, while BBS-2 is a solubilizer for alkaline materials. Hydroxide of hyamine, the third solubilizer, has been in use for a number of years.

### Assay of Radioactivity in Serum

Two experiments were performed: one to assay \(^{14}\text{C}\) and the other to measure \(^{2}\text{H}\). For the first experiment, 2 \(\mu\text{Ci}\) of pyruvate-3-\(^{14}\text{C}\) was mixed with 10 ml of human serum and 0.2 ml added to counting vials in duplicate. Half-milliliter aliquots of NCS, Hyamine, Bio-Solv BBS-1 and Bio-Solv BBS-3 were added to each of four vials. The serum and solubilizer were thoroughly
mixed. A toluene scintillator was added to two vials from each group and the dioxane-based scintillator was added to the other two. The results are presented in Table 6. All vials had been adapted to the dark in the cold of the liquid scintillation counter for at least ten hours prior to counting. Only the dioxane-hyamine and toluene-BBS-3 systems were homogeneous; all others had precipitates or varying degrees of cloudiness. Although two of the samples (1 and 8) had substantially lower counts, there was little choice among the efficiencies of the other seven. The simplest to prepare, however, was the one in which serum was added directly to the dioxane scintillator. Although the proteins precipitated in this system, it seemed to have little effect on the efficiency with which the radioactivity was assayed. Larger volumes of serum can also be added directly to the dioxane scintillator.

For the second experiment, serum was enriched with glucose-1-(3H) (Table 7). An aliquot of 0.2 ml of the radioactive serum was added to vials containing a dioxane scintillator or a toluene scintillator containing 5 percent Bio-Solv BBS-3. The count rate was substantially higher in the toluene-Bio-Solv mixture. Thus, when (3H) is the radioactive isotope, there is a definite advantage to the use of Bio-Solv BBS-3 which is not present when the isotope is (14C). Note that under the experimental conditions above, the mixture was hazy. With the addition of more solubilizer (and dilution of ionic strength), more clarity could be expected.

Many techniques have been described for counting radioactivity in serum, but few are as simple as the one described above (Table 6). Thus, with (14C) and, presumably, (35S) in a chemical form that is soluble in methanol or water, the radioactivity again could probably best be assayed by adding the serum directly to a dioxane-based scintillator. To avoid small changes in pH, the scintillator might be buffered as suggested recently by Das and Toennies. However, when the isotope is (3H), particularly if low counts are expected, Bio-Solv BBS-3 in a toluene scintillator has quantitative advantages over dioxane.
**Table 8.—Assay of Radioactive Palmitate-1-\(^{14}\text{C}\) in Incubation Medium***

<table>
<thead>
<tr>
<th>Scintillation Solution</th>
<th>Additive (0.5 ml)</th>
<th>Description</th>
<th>cpm $\times 10^8$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene †</td>
<td>Hyamine</td>
<td>Cloudy; 2 phases</td>
<td>138.6</td>
</tr>
<tr>
<td>Dioxane ‡</td>
<td>Hyamine</td>
<td>Clear</td>
<td>143.6</td>
</tr>
<tr>
<td>Toluene</td>
<td>NCS</td>
<td>Clear</td>
<td>168.3</td>
</tr>
<tr>
<td>Dioxane</td>
<td>NCS</td>
<td>Opaque</td>
<td>152.2</td>
</tr>
<tr>
<td>Toluene</td>
<td>Bio-Solv (BBS-1)§</td>
<td>Yellow; thick</td>
<td>162.8</td>
</tr>
<tr>
<td>Dioxane</td>
<td>Bio-Solv (BBS-1)</td>
<td>Gel; 2 phases</td>
<td>146.0</td>
</tr>
<tr>
<td>Toluene</td>
<td>—</td>
<td>Clear; water drops</td>
<td>35.6</td>
</tr>
<tr>
<td>Dioxane</td>
<td>—</td>
<td>White precipitate</td>
<td>159.5</td>
</tr>
</tbody>
</table>

* Radioactivity was contained in 0.1 ml of Krebs-Ringer bicarbonate buffer with 4% bovine albumin and palmitate-1-\(^{14}\text{C}\)OOH, 0.1 $\mu$Ci, was added to each vial.
† Toluene solution: Solution 1, Table 5.
‡ Dioxane solution: Solution 3, Table 5.
§ BBS-1 solubilizer has less ability to solubilize samples of high ionic strength than BBS-3.

**Assay of Radioactive Palmitate in Buffered Albumin Solution**

An incubation mixture of Krebs-Ringer bicarbonate buffer with albumin as an acceptor for free fatty acids is used for in vitro studies with adipose tissue. To compare methods for measuring radioactivity in such a medium, samples were prepared and enriched with 0.1 $\mu$Ci of radioactive palmitic acid per ml. Aliquots of 0.1 ml were added in duplicate to 16 counting vials. A half-milliliter aliquot of Hyamine, NCS or Bio-Solv BBS-1 was added to each of four vials, and a toluene or dioxane scintillation mixture was added to a pair of vials with each solubilizer. Table 8 summarizes this experiment. Toluene alone did not extract the palmitic acid; the counts were less than 25 percent of those in the other samples. The samples dissolved in NCS were only 3.4 percent higher than those in Bio-Solv BBS-1 and only 5.2 percent higher than those in the sample added directly to the dioxane-based scintillator. Thus, the protein precipitate formed by adding albumin to the dioxane-based system did not lower the count rate appreciably. As with serum, the simplest approach to counting such an incubation medium was to add it directly to the dioxane-based liquid scintillator.

**Assay of Tritiated Water in Urine**

Pooled human urine was enriched with tritiated water and samples were prepared for counting as described in Table 9. In this experiment the two general-purpose Bio-Solv preparations BBS-1 and BBS-3 were compared. The addition of BBS-3 produced a clear solution but BBS-1 did not. (As noted in Table 8, it is more sensitive to high ionic strength.)

**Table 9.—Assay of Tritiated Water in Urine***

<table>
<thead>
<tr>
<th>Scintillation Solution</th>
<th>Volume Urine</th>
<th>Additive</th>
<th>Description</th>
<th>cpm $\times 10^4$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene †</td>
<td>1.0</td>
<td>Bio-Solv (BBS-1, 2.0 ml)</td>
<td>Cloudy</td>
<td>87.3</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>(BBS-3, 2.0 ml)</td>
<td>Clear</td>
<td>197.0</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>NCS (1.5 ml)</td>
<td>Clear</td>
<td>39.9</td>
</tr>
<tr>
<td>Dioxane ‡</td>
<td>1.0</td>
<td>—</td>
<td>Hazy</td>
<td>98.6</td>
</tr>
<tr>
<td></td>
<td>2.0</td>
<td>—</td>
<td></td>
<td>101.8</td>
</tr>
<tr>
<td></td>
<td>3.0</td>
<td>—</td>
<td></td>
<td>88.7</td>
</tr>
<tr>
<td></td>
<td>1.0</td>
<td>Decolorized §</td>
<td>Clear</td>
<td>116.3</td>
</tr>
</tbody>
</table>

* Radioactivity was contained in pool of urine to which tritiated water had been added.
† Toluene scintillator: Solution 1, Table 5.
‡ Dioxane scintillator: Solution 3, Table 5.
§ Decolorizing done by adding activated charcoal to aliquot of urine and then filtering it.
strength.) Urine samples of 1, 2 and 3 ml were added directly to the dioxane-based scintillator, and an aliquot of urine was decolorized by adding activated charcoal and filtering prior to addition of the liquid scintillator. The count rate of urine containing BBS-3 as a solubilizer was nearly twice as great as that obtained with any of the other preparations, and it would appear that the use of this solubilizer can substantially increase the detection of (3H) in urine. This was not the case, however, when (14C) was assayed in urine (Table 10). For this experiment, radioactivity in the form of glucose-6-(14C) was added to the same pool of urine used for the (3H) experiments in Table 10. One milliliter of urine and 2 ml of Bio-Solv BBS-3 were mixed prior to adding the toluene scintillator, and these results were compared with data with the dioxane-based scintillator. In this experiment, there was no significant advantage to the BBS-3 solubilizer. In both experiments the samples had been cold and dark-adapted prior to counting.

It would appear that for urine the use of Bio-Solv BBS-3 has substantial advantages in assaying for (3H) but that these advantages are no longer present when the radioactivity is in the form of (14C). From these experiments, NCS solubilizer appears to have little usefulness for counting urine.

**Assay of (14C) Inulin and (14C) Urea from Micropuncture Samples**

Drs. Cortell, Gennari and Schwartz of the Renal Service at the New England Medical Center Hospitals observed that repeated counts of samples containing (14C) inulin showed a progressive drop in count rate amounting to just over 20 percent of the original count. Solutions prepared in the same way using (14C) in the form of urea showed no such decline in count rate. It thus appeared that with the passage of time, small amounts of radioactivity in the inulin solutions were escaping assay by the scintillation process. Inulin is a polymer of fructose with a molecular weight of approximately 5000. Glycogen and other large carbohydrate polymers are known to be insoluble in any of the currently available liquid scintillators, but the problem with inulin had not been noted previously. One explanation for the decline in count rate of inulin might be that it was precipitating from dioxane with the passage of time. It seemed of interest to test the possibility that Bio-Solv solubilizers might be useful in overcoming this problem, and solutions of (14C) labeled inulin and urea were prepared in 0.5 ml of water. The dioxane scintillator was added to one pair of vials with each isotope, and 1 ml of Bio-Solv BBS-1 or BBS-3 was added in duplicate to the other samples before adding the toluene scintillator. Ten-minutes counts were obtained repetitively on all samples (Table 11). The count rate for inulin in the dioxane-based system declined with time, while the count rate for urea did not. Bio-Solv BBS-1 did not prevent the decline with time and was less efficient than dioxane. Bio-Solv BBS-3, however, prevented the decline and is the preferred method for counting inulin-(14C).

---

<table>
<thead>
<tr>
<th>Scintillation Solution</th>
<th>Volume Urine</th>
<th>Additive</th>
<th>Description</th>
<th>cpm x 10^4</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toluene †</td>
<td>1.0</td>
<td>Bio-Solv (BBS-3, 2.0 ml)</td>
<td>Clear</td>
<td>373.5</td>
</tr>
<tr>
<td>Dioxane ‡</td>
<td>1.0</td>
<td>Bio-Solv BBS-3</td>
<td>Clear</td>
<td>348.4</td>
</tr>
</tbody>
</table>

* Radioactivity was contained in pool of urine to which radioactive glucose-6-(14C) was added.
† Toluene scintillator: Solution 1, Table 5.
‡ Dioxane scintillator: Table 4.
Table 11.—Comparison of Two Methods for Counting Radioactive Inulin Obtained from Micropuncture Studies

<table>
<thead>
<tr>
<th>Sample *</th>
<th>Scintillation Solution</th>
<th>Additive</th>
<th>Repetitive Counts* (cps × 10⁹)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Inulin-(¹⁴C)OOH</td>
<td>Toluene †</td>
<td>Bio-Solv (BBS-1)</td>
<td>71.8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bio-Solv (BBS-3)</td>
<td>80.6</td>
</tr>
<tr>
<td></td>
<td>Dioxane §</td>
<td>—</td>
<td>78.9</td>
</tr>
<tr>
<td>Urea-(¹⁴C)</td>
<td>Toluene</td>
<td>Bio-Solv (BBS-1)</td>
<td>14.7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Bio-Solv (BBS-3)</td>
<td>17.8</td>
</tr>
<tr>
<td></td>
<td>Dioxane</td>
<td>—</td>
<td>17.2</td>
</tr>
</tbody>
</table>

* Sample prepared by adding 0.01 ml of radioactive solution to 0.5 ml of water in counting vial in duplicate. † Each vial was counted for 10 minutes. Samples were recycled every 130 minutes with the 1st, 3rd, 5th and 7th counts reported. § Toluene scintillator: Solution 1, Table 5. § Dioxane: Solution 3, Table 5.

**Buffered Dioxane Scintillator**

The versatility of dioxane-based scintillators as solvents for aqueous samples has been illustrated in several experiments. It should be pointed out that dioxane is miscible with organic solvents such as benzene, xylene, heptane, and other organic samples. The potential usefulness of these scintillators has recently been extended in experiments by Das and Toennies 23 who were concerned with assaying bacterial proteins and culture media. They found that the addition of a buffered aqueous phase to a dioxane-based scintillator 28 increased the capacity for proteins digested in alkali and served to minimize quenching due to variations in pH. This adaptation of dioxane-based scintillators may well find other applications to biological problems.

**SUMMARY**

Some of the methods available to assay radioactivity in aqueous samples have been reviewed and the use of dioxane-based scintillators has been discussed in detail. The efficiency for counting (¹⁴C) was similar in several systems, but the efficiency for (³H) was higher in toluene-based scintillators. The merits of various concentrations of naphthalene were discussed, and it was shown that higher quantities of naphthalene reduced the solubility for water.

Several methods for counting urine and serum were compared. The simplest method for counting radioactivity in serum was to add the sample directly to a dioxane scintillator. The protein precipitate did not produce significant quenching. When (³H) was being assayed, higher count rates were achieved by adding Bio-Solv BBS-3, an organic solubilizer. The simplest method for counting urine was again to add it directly to the dioxane scintillator. BBS-3 also increased the efficiency for assaying (³H) in urine but did not make a significant difference with (¹⁴C) efficiency for carbon. Neither BBS-1 nor NCS was useful in counting serum or urine.

Studies with inulin-(¹⁴C) have shown that there was a loss of radioactivity with time when added to a dioxane scintillator. This problem was solved by adding Bio-Solv BBS-3.

The potential usefulness of a buffered dioxane scintillator was discussed.

**REFERENCES**

INVESTIGATION OF HOMOGENEOUS COUNTING SYSTEMS FOR AQUEOUS INORGANIC SALTS AND ACIDS

E. B. Mueller

One of the earliest applications of liquid scintillation counting was in the detection of $\beta$-emitting radionuclides in the form of common inorganic ions. With the exception of the carbonate ion, however, the number of inorganic samples being counted has been so eclipsed by the number of species derived from organic systems that relatively little attention has been given to the solubilization of inorganic acids, bases and salts. Their solubilities in liquid scintillators are important for two reasons: because it is often convenient to isolate radioactive carbonates, sulfates, phosphates and chlorides as the end-products of tracer experiments; and because the corresponding unlabeled ions are frequently found in aqueous solutions containing labeled organic solutes which are to be counted. In preparing this latter type of sample, a common procedure is the addition to a scintillator which will dissolve the water and which contains some alcohol or ether in which the organic species is expected to be reasonably soluble. If a precipitate forms in the final sample, it is sometimes assumed that only the salt has come out of solution, and that this will not interfere with the counting. The propriety of this assumption deserves some consideration. Another assumption that appears to have been fairly common is that if the sample is small enough, either in aqueous volume or in mass of the electrolyte, it will be adequately dissolved by a scintillator that will hold the same volume of pure water. Several years ago, in trying to count some of the anions mentioned above in the scintillator systems most often used then for counting small aqueous samples, we found evidence that significant fractions of the samples were not in solution, although there was no visible phase separation. We
then undertook a dual investigation, hoping both to find reliable methods of recognizing unacceptable samples, and to determine which scintillator solutions were most suitable for counting the common aqueous anions of \((^{14}\text{C})\), \((^{35}\text{S})\), \((^{36}\text{C})\) and \((^{32}\text{P})\).

Criteria of an Acceptable Sample

It seems particularly appropriate in this book to discuss the first of these objectives in some detail, since the question of what constitutes an acceptable counting sample is a general focal point of interest. The criteria of acceptability we have adopted are the following sample properties: stability over a counting period of several days; reproducibility in replicate samples of the same composition; susceptibility to a reasonable method of determining counting efficiency, and preferably to one of the automatic methods (by sample channels ratio or external standardization); compatibility with a useful range of sample sizes and concentrations at ambient or reduced temperatures; and reasonable counting efficiency. Application of these criteria originally led us to limit our consideration to single phase systems (to true solutions). The gel systems seemed to suffer from several disadvantages: when made viscous enough to support significant amounts of water without separating, the physical distribution of the gel in the vial was difficult to reproduce, causing variable counting efficiency; the Triton-X-100 system was very sensitive to electrolyte concentration, pH, and temperature, even at constant aqueous concentration, exhibiting a complicated series of phase changes; even more serious was the question of whether accurate efficiency determinations could be made by means of a quench correction calibration curve, or, indeed, could be made at all. Some of the problems of the Triton system have been explored quite thoroughly in the literature.\(^1-5\) More recent experience with Triton X-100 has indicated that, although its phase diagrams are complicated, and not necessarily reproducible from batch to batch, there are useful ranges of composition in which the efficiency can be determined accurately by internal standardization with a compound sufficiently similar in solubility to the sample being measured. For the higher-energy \(\beta\)-emitters particularly, the gel systems may give the highest figures of merit and quite satisfactory results in other respects as long as the optical characteristics of the samples are reproducible. The importance of the last point will be considered further in connection with some of the “solution” systems. For further discussion of gels, see Chapter 19.

**Experimental**

The six scintillator compositions we have now studied in most detail are a toluene-ethanol solution; the mixture of \(p\)-dioxane, methanol, ethylene glycol and naphthalene developed by Bray;\(^6\) a solution of Hyamine hydroxide (1 N in methanol, Nuclear-Chicago Corporation) in toluene or dioxane; NCS (Nuclear-Chicago Corporation); and the recently-introduced Bio-Solv BBS-1 and Bio-Solv BBS-3 (Beckman Instruments, Inc.) in toluene solution. In all cases the fluors used were 2,5-diphenyloxazole (PPO) and 1,4-di-2-(5-phenyloxazoly) benzene (POPOP). The dioxane solvent was scintillation grade, and other solvents were reagent or the best available grade. Borosilicate glass vials were used, except in a few experiments with polyethylene vials.

The aqueous acid or the sodium salt of each of the following radioactive anions was diluted to an appropriate specific activity, and an aliquot was added to a sample of the same species mixed with scintillator: carbonate-(\(^{14}\text{C})\), sulfate-(\(^{35}\text{S})\), chloride-(\(^{36}\text{Cl})\), and phosphate-(\(^{32}\text{P})\). When it was desirable to determine absolute counting efficiencies, standardized solutions were obtained from Nuclear-Chicago Corporation, or, in the case of \((^{14}\text{C})\) and \((^{36}\text{Cl})\), the solutions were sometimes standardized by comparison with liquid
scintillation samples prepared with known amounts of toluene-(\(^{14}\)C) or chloroform-(\(^{26}\)Cl), respectively, using channels ratio curves. Counting was carried out at the indicated temperatures in various Nuclear-Chicago counters, principally in the Mark I model equipped with a (\(^{133}\)Ba) external standard.

Four kinds of tests were applied to ascertain whether the radioactive material was completely dissolved in the scintillator; counting rates and sample channels ratios were followed for several days to detect changes due to slow removal of radioactive material from solution by precipitation, adsorption, or volatilization; samples were examined visually by transmitted and by scattered light, swirling the liquid gently to reveal any loose precipitates, and inverting the vials to reveal any transparent precipitates adhering to the glass; samples were counted in three channels chosen to give suitable sample channels ratios and external standard ratios, so that the two ratios could be compared with each other and with efficiency; and selected samples were transferred by pipet to clean vials for recounting, while the original vials were rinsed and refilled with clean scintillator and recounted. Some of these techniques have been described in more detail elsewhere.7

Effects of Sample State on the Pulse Height Spectrum

Since no quantitative measurement of sample radioactivity can be made without a knowledge of the counting efficiency, it is imperative that the sample fulfill the conditions required by at least one of the methods for determining efficiency. (See Chapter 29.) In the case of ratio methods, the shape of the pulse height spectrum must vary in a predictable way with quenching. Other unpredictable changes in the spectrum, such as those caused by precipitation or adsorption of the radioactive material, cannot be allowed. Because of the possibility that some radioactive material may be occluded or coprecipitated if a second phase forms in a sample solution, any accidental phase separation should be treated with great suspicion and should be avoided if possible. Even if convincing evidence could be obtained that no radioactive material had entered the second phase, the possibility of error in the efficiency determination due to spurious optical effects introduced by a second liquid or solid phase would remain. Such effects can result in an erratic value of either the sample channels ratio or the external standard ratio, that is, the efficiency indicated by one or both ratios may be incorrect. Such samples are sometimes identifiably abnormal on double-ratio plots. For example, consider the following data for a series of samples containing aqueous carbonate in a toluene-based scintillator solution but labeled with (\(^{14}\)C)-toluene. They were counted at three different temperatures. Each time the temperature was reduced, an additional number of samples exhibited the formation of a second phase, either a precipitate or an aqueous liquid, neither of which might be supposed to contain toluene. The effects of phase separation on the pulse height spectra, however, were such that of the 53 samples observed to contain two phases at the time of counting, 32 could be identified as abnormal by double-ratio plots and 24 by plots of efficiency-versus-sample channels ratio. This might suggest that the external standard ratio quench correction is less likely to be a reliable method of determining efficiency than the sample channels ratio for a sample containing an unlabeled second phase, but it really indicates that neither can be depended on when there is phase separation. The effects of spectral distortions produced by haziness, opalescence, and scattering of light by fine suspended precipitates are also erratic and
are likely to lead to small errors (of the order of 1%) in the determination of disintegration rate.

As might be expected, the influence of such optical effects on counting efficiency decreases with increasing $\beta$-energy. Some typical results for $^{36}$Cl are illustrated by the data for a set of samples containing various volumes of aqueous sodium chloride solution (0.01-1.0M) in ethanol and toluene (2:3 v/v), as shown in Fig. 1. Of eight "standard" samples labeled with chloroform-$^{36}$Cl, five were optically clear, two separated into two liquid phases, and one was opalescent and on the verge of phase separation. These last three are clearly distinguishable on the double ratio plot in Fig. 1, but all eight were nevertheless counted at the same efficiency, ± 0.5 percent. Because of the very small range of aqueous volumes that dissolved in this solvent system at 0°C, the accessible region of quenching was short for the set of NaCl samples, and the slopes of the curves were deduced from data on samples in other solvents counted simultaneously. (The fact that the chloroform and the sodium chloride samples had nearly the same activity was fortuitous, but it allowed the counting rates to be plotted in close proximity and with less confusion of points than would have resulted from conversion of these values to efficiencies. Slightly greater accuracy in measuring the larger volume of the chloroform solution is reflected in the smaller spread in counting rates for standards than for the "good" chloride-$^{36}$Cl samples.) Of the 20 chloride ion samples, twelve were clear and one opalescent; these appear on or very close to the double ratio curve for the ($^{36}$Cl)- chloroform standards. The remaining seven contained a second liquid phase or a precipitate on the bottom of the vial; these account for the wide deviations from the double ratio curve in Fig. 1 with sample channel ratios displaced in the direction of greater quenching, as expected. Only three of these were actually counted with lower efficiencies than the "good" samples despite the aberrant ratio values.

**VISUAL INSPECTION**

A careful visual test is the first and most important test to be applied. When a sample is not a clear homogeneous liquid, further evidence should be sought as to whether the radioactive material is being counted with the efficiency expected from its measured ratio or expected from counting an internal standard completely dissolved in the scintillating phase. When it is necessary to use an opalescent or gelatinous sample system, good
precision can probably be obtained only by keeping sample composition within a range in which only small changes can occur in the optical properties or in the possible distribution of the sample material between phases.

Double ratio plots. In identifying samples in which some radioactive material has been removed from solution through precipitation or wall adsorption, double ratio plots (as in Fig. 1) have proved very reliable for \( ^{14}C \) and \( ^{32}S \). Such samples have lower counting efficiencies than standard (dissolved) samples with identical sample channels ratios or external standard ratios. The reliability of the double ratio test and its correlation with changes in efficiency decrease with increasing \( \beta \)-energy; e.g., for \( ^{35}Cl \) and \( ^{32}P \). Opalescence, slight cloudiness, and the whitish wall deposits often seen in Biosolv samples may sometimes cause aberrations.

We have concluded that a double ratio plot is definitely worthwhile whenever a sample composition has not been previously tested. This precaution should be equally valid when the sample contains radioactive organic compounds as long as suitable standard samples are available to establish a double ratio curve. For a sample that does not meet the double ratio test, an excellent chance exists that its activity, as determined by either external standard or sample channels ratio or by internal standardization, will be in error.

Use of Polyethylene Vials

Plastic vials were of interest in this study for two reasons: it was likely that they would show less tendency than glass to adsorb anions from scintillator solutions; and it seemed possible that, by introducing a light-scattering wall, the ratios and efficiencies of samples with internal light scattering would no longer appear anomalous. Only a few experiments were performed with polyethylene vials because of their instability over long counting periods; there was a continuous drifting of the sample or external standard channels ratios. The results of transferring counted samples to clean vials indicated that the plastic walls were freer of adsorbed layers or adhering precipitates than was glass. These two forms of insoluble material are the ones most likely to produce measurable shifts in the pulse height spectra of \( ^{32}P \)-phosphates. When a phosphate precipitate did form in a plastic vial, it was less likely to be detected by a double ratio test than the same precipitate in a glass vial, apparently because the counting geometry factor is better for a loose precipitate than an adherent one. For \( ^{14}C \) and \( ^{32}S \), it appeared that pulse height spectra of clear and cloudy samples were slightly better resolved in polyethylene than in glass, but the difference from glass was quite small.

Comparison of Solvent Systems

To dissolve the anionic species under consideration in most of the organic solvent systems we have examined, some minimum amount of water had to be present. The more concentrated the aqueous solution of the salt or acid, the larger the minimum volume required to achieve solution. Below this minimum volume, the electrolytes precipitated. Thus, the range of acceptable sample volumes decreases as the aqueous electrolyte concentration increases. In general, if one does not know the solubility of an inorganic species in a particular solvent system, he should use an aqueous volume near the maximum that can be maintained in a single phase. He will then have the best chance of keeping the radioactive electrolyte in solution although there may be some loss in counting efficiency.

Ethanol and Hyamine Hydroxide Solvents. Because of their relatively high quenching, ethanol and Hyamine hydroxide (1 N in methanol) are not practically used at concentrations much greater than 40 percent by volume in toluene. At this concentration, counting efficiency is low, and the capacity
to dissolve water is small (Table 1). Since their compatibility with salts and acids is even smaller, it is fortunate that better solvent systems are now known. The 40 percent ethanol solvent was found to dissolve HCl and NaCl (up to 0.1 N in water) without precipitation, up to the volume at which two liquid phases separate. This was approximately 4.5 percent by volume at 0°C and 5 percent at 14°C. Chloride ion concentrations of 0.5 M or greater were not soluble. No significant amounts of the other salts or acids were soluble.

Forty per cent Hyamine hydroxide in toluene failed to dissolve any of the anions at concentrations above 0.01 N, with the exception of chloride ion, which, as HCl, was soluble to 1.0 N in the ratios of 0.02 to 0.04 ml acid per milliliter 40 percent Hyamine solution. Substitution of dioxane for toluene as a primary solvent in a few experiments did not increase the solubilities of salts and acids in Hyamine hydroxide.

**Bray’s Solution.** (See Chapter 17.) Bray’s solution was found to be a good solvent for the acids studied but a poor solvent for the salts, with the exception of sodium chloride. The upper limit of solubility for most solutions was somewhat less than that found for water at room temperature (0.50 ml H₂O/ml Bray’s solution), decreasing with increasing electrolyte concentration. At 0.1 N, sodium sulfate was not soluble, and at 0.01 N, it was soluble only in the range between 0.34 to 0.42 ml/ml. Sodium carbonate at 0.02 N was soluble only from 0.26 to 0.38 ml/ml. It should be noted that CO₂ can be lost by volatilization from Na₂CO₃ or NaHCO₃ samples in Bray’s solution, either at the time of preparation of the sample or from any vial not tightly capped. Sodium chloride was soluble in the presence of much less water, with minimal acceptable volumes of about 0.01 ml/ml at 0.1 M, and less than 0.004 ml/ml at 0.04 M.

No evidence was found of precipitation or adsorption from acid solutions at volumes greater than 0.02 ml/ml. This corresponds to a sample volume of 100 µl in 5.0 ml, certainly a reasonable minimum to maintain in practice.

**Bio-Solvs.** Minimum values in the soluble volume were not observed for most of the electrolyte solutions when added to Bio-Solv in toluene. This is probably due to the water already present in Bio-Solv. Volumes at which two liquid phases would separate (maximal volumes) were not measured for most of the solutions, since it was considered that the opalescence at much smaller volumes was already too severe for good liquid scintillation counting. At the 1:5 ratio of

<table>
<thead>
<tr>
<th>Solvent System</th>
<th>Ml H₂O/ml Solvent at End Point</th>
<th>Type of End Point</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0°</td>
<td>12-15°</td>
</tr>
<tr>
<td>Bray’s solution</td>
<td>.34</td>
<td>.50</td>
</tr>
<tr>
<td>Bio-Solv (BBS-1)/toluene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:5 v/v</td>
<td>.106</td>
<td>.114</td>
</tr>
<tr>
<td></td>
<td>.075</td>
<td></td>
</tr>
<tr>
<td></td>
<td>.25</td>
<td>.25</td>
</tr>
<tr>
<td></td>
<td>.185</td>
<td></td>
</tr>
<tr>
<td>Bio-Solv (BBS-3)/toluene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>1:5 v/v</td>
<td>.134</td>
<td>.155</td>
</tr>
<tr>
<td></td>
<td>.106</td>
<td></td>
</tr>
<tr>
<td>NCS/toluene</td>
<td></td>
<td></td>
</tr>
<tr>
<td>.6 N</td>
<td>.25</td>
<td>.28</td>
</tr>
<tr>
<td>.28 N</td>
<td>.23</td>
<td></td>
</tr>
<tr>
<td>.14 N</td>
<td>.133</td>
<td>.15</td>
</tr>
<tr>
<td></td>
<td>.126</td>
<td></td>
</tr>
<tr>
<td>Hyamine hydroxide/toluene 2:3 v/v</td>
<td>.054</td>
<td>.067</td>
</tr>
<tr>
<td>Ethanol/toluene 2:3 v/v</td>
<td>.049</td>
<td>.074</td>
</tr>
<tr>
<td></td>
<td></td>
<td>.067</td>
</tr>
</tbody>
</table>
Bio-Solv to toluene recommended by the manufacturers, opalescence was observable at volumes considerably smaller than the 0.085 ml/ml suggested sample size. However, we observed no sharp change in sample appearance as aqueous volume was increased, and larger sample volumes were counted satisfactorily. When 2:5 solutions of Bio-Solv in toluene were allowed to stand, a heavy precipitate formed in Bio-Solv BBS-3, and this solution was not used. A smaller amount of precipitate in the Bio-Solv BBS-1 was removed by filtration before use.

Changes that occurred in the Bio-Solv system as temperature was reduced were different from those observed in other systems. At ambient temperatures, many samples were found to have deposits on the walls of the vials, above the liquid levels; these took the form of white streaks which appeared nearly opaque aggregates of small particles or droplets or translucent films. Since no evidence was found that any of the radioactive anionic species was not in solution in these samples, it may be that these deposits consisted of organic components of the Bio-Solv. These deposits tended to disappear as temperature was reduced, and at 0° they were not observed. Opalescence increased as temperature was reduced, but coalescence into two distinct liquid phases never occurred in any sample which was considered "good" at room temperature.

NCS. The solubility of aqueous electrolyte solutions in NCS decreased with temperature. It was significantly lower at 0° C, for instance, than at 10 or 15°. (We have found the latter region convenient for work in a counter with temperature control.) Solubility was limited at the low end of the aqueous volume range, as for most other solvent systems, by precipitation or adsorption of the anionic species; the minimum volume required increased with NCS concentration. As large volumes of water or dilute solutions were added, a gradual transition to a viscous gel was observed in concentrated (0.6 N) NCS. The properties of such gels as counting media have not yet been investigated. At lower NCS concentrations in toluene, the upper solubility limit was marked by a narrow region of opalescence followed by phase separation which yielded a cloudy mixture. With salt solutions, precipitation often preceded liquid phase separation. Precipitates were also seen in many acid solutions at 0°.

The solubility in NCS of salt solutions up to 0.5 N and of acids up to 1.0 N exceeded the solubility of water; 1.0 N acids were approximately twice as soluble as water, while 2.0 N acids had nearly the same solubility as water.

**Counting Efficiency and Figure of Merit.**

A commonly chosen figure of merit is the product of the counting efficiency and the sample size. We shall consider the figures of merit of four systems (Bray's solution, NCS, and the Bio-Solv) for the four anionic species at various sample volumes.

For small aqueous volumes (up to 0.3 ml in a total sample volume of 5.0 ml, for instance), the efficiencies of the four systems are nearly the same, if the appropriate Bio-Solv and NCS concentrations are used. For the higher-energy radionuclides, (36Cl) and (32P), counting efficiencies can be made high enough, even in samples containing large volumes of water, to be considered nearly equal. The figure of merit for these isotopes is nearly independent of the degree of quenching. When large aqueous volumes are to be counted, the maximum soluble volume in a given system is the figure of interest. Only for the lower energy isotopes at large volumes is the product of efficiency and volume important.

For counting very small sample volumes (less than 200 μl) containing the weaker β-emitters, when it might be considered undesirable to dilute the sample and thereby reduce efficiency, the system of choice might be Bio-Solv BBS-3, since the danger of adsorption or precipitation would be mini-
mized. Very large volumes of acid (2 ml or more) could be counted either in Bray's solution or in NCS; salts would best be counted in NCS. At intermediate volumes the situation is more complex, depending on the particular behavior of the species of interest in each solvent. For instance, sodium carbonate would best be counted in NCS or BioSolv BBS-3, because volatilization has been observed from the other two solvents. The maximal figures of merit for these two systems are quite comparable for this salt.

ACKNOWLEDGMENT

The able assistance of Michael Gezing in preparing and counting the samples is gratefully acknowledged.

REFERENCES

One of the major technical difficulties in the use of liquid scintillation counting in biochemical research is the incompatibility between polar compounds and solvents having adequate energy transfer properties to allow efficient detection of radiation. (See Chapter 2.) One approach to the problem of incompatibility is the use of heterogeneous systems in which the labeled polar material is in one phase and the scintillation solution is in the other. Another approach, to deposit the labeled material on an inert support such as filter paper prior to counting, is covered in Chapter 20.

Heterogeneous counting procedures include suspension of insoluble radioactive material in scintillator solutions or gels, the addition of an insoluble fluor to aqueous solutions of isotopes, and, more recently, the use of surfactants to obtain stable dispersions of scintillator and sample.

SUSPENSION COUNTING

The major techniques in use before 1964 have been reviewed in two excellent books, and will be covered only briefly here.

Hayes et al. resorted to the simple procedure of dispersing finely divided solids in scintillator and correcting for the loss in efficiency as the radioactive material settled. Gelation of suspensions to prevent settling has been accomplished by the addition of aluminum stearate to the sample followed by heating or by the preparation of thixotropic scintillator gels with Thixcin or colloidal silica (Cab-O-Sil). Such gels are especially useful for applications involving the counting of relatively large quantities of a solid labeled with an isotope emitting a \( \beta \)-particle at least as energetic as that of \( { }^{14} \text{C} \). Colloidal silica is the most popular of the gelling agents. Addition of dry silica to each counting vial is bothersome, and the thicker premixed gels are difficult to dispense and to mix with the sample. Recently a new procedure for the formation of a gelling agent in the sample vials has been described by Bollinger et al. This method involves incorporation of a branched chain aliphatic primary amine into the scintillation solution.
After mixing scintillator with sample, a small amount of toluene diisocyanate is added and the vial is shaken. The isocyanate reacts with the primary amine to form a substituted toluene diurea which forms a transparent gel with toluene. Gel formation occurs within 30 seconds and the gel has sufficient rigidity to support glass beads.

An opposite approach was taken by Steinberg who added solid fluors (anthracene, plastic scintillators) to aqueous solutions. This procedure has found great application in the preparation of flow cells where the anthracene crystals are tightly packed and the interstitial water layers are thin, but it is not sensitive or convenient enough for routine sample counting. (See Chapter 8.)

With sufficient care, most of these methods give good results when counting the more energetic $\beta$-emitters. When ($^3$H) is counted, self-absorption difficulties lead to unreproducible results except when experiments are done with the most stringent care. The energy of the mean ($^3$H) beta is 9 keV which corresponds to a range of 0.5 $\mu$m in media with a specific density of 1. Since most of the particles and liquid films or droplets obtained by use of these suspension procedures are many micra thick, only those $\beta$-particles emitted near the interface of the radioactive material and fluor will cause light emission. For a tritiated material of a given specific activity, the count rate would be a function of its surface area.

**EMULSIONS WITH TRITON $^*$ SURFACTANTS**

With these limitations of suspension counting in mind, Patterson and I sought an emulsifier capable of producing a stable dispersion of aqueous sample and scintillation solution which would be of sufficiently small dimensions to allow an average ($^3$H) $\beta$-particle to span several water layers but which would still retain the desirable properties of suspension counting. After evaluating several emulsifiers that proved to be strongly luminescent, strongly quenching or which produced inadequate dispersion, we found that Triton X-100 had some of the desired properties. Triton X-100 had been used previously to solubilize small amounts of tissue in scintillation solution for counting with reasonable efficiency but had not been tested with high concentrations of water. Low concentrations of a similar surfactant (0.5%) had been used in conjunction with colloidal silica to prepare dispersions containing 50 per cent water, but the ($^3$H) efficiency was very poor (0.3%).

Considering the complexity of emulsion technology, our first approach was rather naive, but we were able to find several acceptable mixtures.

**Effects of Various Solutions.**

The counting properties of two of these are given in Tables 1 and 2. Since our first report, several workers have investigated the properties of such systems in considerably greater detail. Their studies have shown that large volumes of aqueous solutions can be counted with high efficiency by use of these systems but that if optimum performance (the highest value of water content times counting efficiency) is required, it is usually necessary to adjust the composition of the mixture to suit the composition of the aqueous phase. If less critical performance is required, it is possible to use a single scintillation mixture for counting several dissimilar aqueous solutions. One of the purposes of this article is to emphasize the parameters affecting the counting properties of the emulsions so that workers can make the changes necessary to optimize counting performance of their particular systems.

**Luminescence of Triton Surfactants.** We reported in 1965 that Triton X-100 contained a luminescent impurity which could be removed by treatment with silica gel.

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*$^*$ Trademark, Rohm and Haas, Inc.
Table 1.—Counting Efficiency of tT 21 Emulsions

<table>
<thead>
<tr>
<th>Aqueous Phase</th>
<th>Added Activity dpm</th>
<th>Observed Activity cpm</th>
<th>Efficiency %</th>
<th>Figure of Merit</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>1.4 × 10⁴ (H³)</td>
<td>1.34 × 10⁴</td>
<td>10</td>
<td>230</td>
</tr>
<tr>
<td>0.1 N HCl</td>
<td>1.4 × 10⁴ (H³)</td>
<td>1.24 × 10⁴</td>
<td>9</td>
<td>207</td>
</tr>
<tr>
<td>1.0 N HCl</td>
<td>1.4 × 10⁴ (H³)</td>
<td>0.95 × 10⁴</td>
<td>7</td>
<td>161</td>
</tr>
<tr>
<td>0.1 N NaOH</td>
<td>1.4 × 10⁴ (H³)</td>
<td>1.25 × 10⁴</td>
<td>9</td>
<td>207</td>
</tr>
<tr>
<td>1.0 N NaOH</td>
<td>1.4 × 10⁴ (H³)</td>
<td>1.08 × 10⁴</td>
<td>8</td>
<td>184</td>
</tr>
<tr>
<td>0.1 M, pH 6.8, K phosphate</td>
<td>1.4 × 10⁴ (H³)</td>
<td>1.36 × 10⁴</td>
<td>10</td>
<td>230</td>
</tr>
<tr>
<td>0.2 M, pH 6.8, K phosphate</td>
<td>1.4 × 10⁴ (H³)</td>
<td>1.39 × 10⁴</td>
<td>10</td>
<td>230</td>
</tr>
<tr>
<td>0.4 M, pH 6.8, K phosphate</td>
<td>1.4 × 10⁴ (H³)</td>
<td>0.96 × 10⁴</td>
<td>7</td>
<td>161</td>
</tr>
<tr>
<td>H₂O</td>
<td>None</td>
<td>52</td>
<td></td>
<td>1</td>
</tr>
<tr>
<td>H₂O</td>
<td>2035 (4C)</td>
<td>1377</td>
<td>68</td>
<td>1564</td>
</tr>
<tr>
<td>0.1 N HCl</td>
<td>2035 (4C)</td>
<td>1307</td>
<td>59</td>
<td>1357</td>
</tr>
<tr>
<td>1.0 N HCl</td>
<td>2035 (4C)</td>
<td>1252</td>
<td>62</td>
<td>1426</td>
</tr>
<tr>
<td>0.1 N NaOH</td>
<td>2035 (4C)</td>
<td>1317</td>
<td>65</td>
<td>1495</td>
</tr>
<tr>
<td>1.0 N NaOH</td>
<td>2035 (4C)</td>
<td>1194</td>
<td>59</td>
<td>1357</td>
</tr>
<tr>
<td>0.1 M, pH 6.8, K phosphate</td>
<td>2035 (4C)</td>
<td>1249</td>
<td>61</td>
<td>1403</td>
</tr>
<tr>
<td>0.2 M, pH 6.8, K phosphate</td>
<td>2035 (4C)</td>
<td>1263</td>
<td>62</td>
<td>1426</td>
</tr>
<tr>
<td>0.4 M, pH 6.8, K phosphate</td>
<td>2035 (4C)</td>
<td>1055</td>
<td>52</td>
<td>1196</td>
</tr>
<tr>
<td>H₂O</td>
<td>None</td>
<td>66</td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

* Mixture contained 5 ml of aqueous phase and 17 ml of tT 21 (toluene: Triton 2:1). Labeled compounds were tritiated water or (14C) benzoic acid.

† Figure of merit (% aqueous phase × % counting efficiency.)

Benson later reported that charcoal treatment was required to remove the last traces of luminescent impurity from Triton surfactants. We have not detected this impurity in any batches of Triton obtained recently, and the silica gel treatment has not been required, but all samples of Triton surfactants tested have another impurity which emits light when toluene solutions of the emulsifiers are mixed with aqueous alkali. This base-induced chemiluminescence is initially very intense (≈ 2 × 10² cpm on a channel set up for quenched tritium counting) but decays to background levels within a few hours (the decay of light emission can be resolved into three first-order processes with half lives of 0.5, 2, and 10 minutes). This impurity can be removed by treatment with moist Dowex-1 hydroxide, but the alkali-inducible luminescence returns on storage of several months. The phenomenon of chemiluminescence induced by alkali in the presence of oxygen is discussed elsewhere in this volume. If proper precautions such as acidification or storage of alkaline samples before counting are taken, artifactual results can be avoided and the luminescence will not cause serious difficulty.

Table 2.—Counting Efficiency of tT 76 Emulsions

<table>
<thead>
<tr>
<th>Aqueous Phase</th>
<th>Added Activity dpm</th>
<th>Observed Activity cpm</th>
<th>Efficiency %</th>
<th>Figure of Merit</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₂O</td>
<td>1.41 × 10⁴ (H³)</td>
<td>6939</td>
<td>5</td>
<td>215</td>
</tr>
<tr>
<td>H₂O</td>
<td>2035 (4C)</td>
<td>1119</td>
<td>55</td>
<td>2365</td>
</tr>
<tr>
<td>0.1 N HCl</td>
<td>2035 (4C)</td>
<td>1065</td>
<td>52</td>
<td>2236</td>
</tr>
<tr>
<td>1.0 N HCl</td>
<td>2035 (4C)</td>
<td>876</td>
<td>43</td>
<td>1849</td>
</tr>
<tr>
<td>0.1 N NaOH</td>
<td>2035 (4C)</td>
<td>1112</td>
<td>55</td>
<td>2365</td>
</tr>
<tr>
<td>1.0 N NaOH</td>
<td>2035 (4C)</td>
<td>1127</td>
<td>55</td>
<td>2356</td>
</tr>
<tr>
<td>0.1 M, pH 6.8, K phosphate</td>
<td>2035 (4C)</td>
<td>1157</td>
<td>57</td>
<td>2451</td>
</tr>
<tr>
<td>0.2 M, pH 6.8, K phosphate</td>
<td>2035 (4C)</td>
<td>1164</td>
<td>57</td>
<td>2451</td>
</tr>
<tr>
<td>0.4 M, pH 6.8, K phosphate</td>
<td>2035 (4C)</td>
<td>1124</td>
<td>56</td>
<td>2408</td>
</tr>
<tr>
<td>H₂O</td>
<td>None</td>
<td>62</td>
<td></td>
<td>1</td>
</tr>
</tbody>
</table>

* Counting mixture contained 10 ml of aqueous phase and 13 ml of tT 76 (toluene: Triton 7:6). Labeled compounds used were tritiated water or (14C) benzoic acid.
phase transitions for a series of Triton surfactants that differ in the length of the polyethylene glycol chain. In systems containing 20 per cent water and an 80 per cent solution of one part of Triton in two parts of toluene, similar phase transitions occur with the various members of the series as a function of temperature, but the temperature at which a given transition occurs depends on the particular Triton surfactant. A typical mixture might be milky and mobile at high temperature. If it was left undisturbed at this temperature the phases would separate. As the mixture was cooled, a single, clear, mobile phase might form which would persist for a few degrees and then become cloudy and viscous. On further cooling, the mixture would become clearer and more viscous until the freezing temperature was reached and the emulsion broke. For example, clear, mobile mixtures are formed between 46 and 51°C with Triton X-102, between 31 and 39°C with Triton X-100, between 15 and 23°C with Triton X-114, and between -2 and 3°C with a 1:1 mixture of Triton X-45 and Triton X-114. As shown in Tables 3 and 4, either the clear mobile solution or the viscous translucent emulsion is suitable for counting. Since mixtures of two Triton surfactants have transition temperatures intermediate between those of the two components, it is possible to obtain the desired physical state over a wide temperature range.

A further transition that has been observed in some systems at temperatures below that of the translucent emulsion but above freezing is the formation of an opaque, milky gel. A transition such as this would be expected to occur at low temperature with Triton X-102 but may not have been seen for kinetic reasons, since phase transitions are very slow in systems with high viscosity.

The temperature-dependent phase changes are an expression of the effect of temperature on the solubility of the Triton surfactants in organic solvents and water. Nonionic emulsifiers of this type are soluble in water primarily because of the hydrogen bond formation between water and the oxygen atoms of the polyether chain. Since these bonds can be disrupted at high temperatures, raising the temperature decreases the water solubility of the surfactant, while increasing solubility in organic solvents. The relative hydrophilicity and lipophilicity of these surfactants is a function of temperature as well as the size of the polar and nonpolar parts of the molecule. Addition of salt to the aqueous

Table 3.—Counting Efficiency of Tritium in Triton-Toluene-Water Mixtures at Low Temperature*  

<table>
<thead>
<tr>
<th>Triton</th>
<th>Physical State of Mixture</th>
<th>Counting Rate (cpm)</th>
<th>Counting Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-100</td>
<td>Viscous, translucent emulsion</td>
<td>14,151</td>
<td>19.5</td>
</tr>
<tr>
<td>X-102</td>
<td>Viscous, translucent emulsion</td>
<td>14,082</td>
<td>19.4</td>
</tr>
<tr>
<td>X-45:X-114 (1:1)</td>
<td>Clear, mobile solution</td>
<td>12,771</td>
<td>17.6</td>
</tr>
</tbody>
</table>

* Each vial contained 17 ml of mixture of one volume of Triton to two volumes of scintillation solution and 5 ml of water containing $7.25 \times 10^4$ dpm of tritiated water. Samples were counted at 0°C with Packard Tri-Carb Liquid Scintillation Spectrometer Model 3375; gain, 80 percent; lower discriminator, 50; upper discriminator, 1000.

Table 4.—Counting Efficiency of Tritium in Triton-Toluene-Water Mixtures at Ambient Temperature*  

<table>
<thead>
<tr>
<th>Triton</th>
<th>Physical State of Mixture</th>
<th>Counting Rate (cpm)</th>
<th>Counting Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>X-114</td>
<td>Clear, mobile solution</td>
<td>7670</td>
<td>10</td>
</tr>
<tr>
<td>X-114:X-100 (2:1)</td>
<td>Clear, mobile solution</td>
<td>7133</td>
<td>9.4</td>
</tr>
<tr>
<td>X-102</td>
<td>Viscous, translucent emulsion</td>
<td>7934</td>
<td>10.4</td>
</tr>
</tbody>
</table>

* Each vial contained 5 ml of water with $7.6 \times 10^4$ dpm of tritiated water and 17 ml of mixture of two parts scintillation solution to 1 part Triton. Samples were counted at approximately 22°C with Packard Tri-Carb Model 3375 Liquid Scintillation Spectrometer; gain, 65 percent; lower discriminator, 35; upper discriminator, 1000. Mixture containing Triton X-114 was clear between 17 and 21°C and mixture containing both Triton X-100 and Triton X-114 was clear between 22 and 28°C.
phase tends to mimic temperature increase, and addition of acid, which associates more strongly with the polyether than water does, tends to mimic temperature decrease.

*Composition and Counting Properties of Triton-Toluene Emulsions.* Due to recent efforts of several workers,\textsuperscript{15-18} considerable information is available about the physical and counting properties of a large number of mixtures of toluene, Triton X-100 and various aqueous solutions. Van Der Laarse\textsuperscript{15} constructed phase diagrams for toluene Triton X-100 and water or 10 per cent NaCl. He reported that the appearance of the mixtures ranged from clear through opalescent to opaque white, but that very few separated on storage at 3° C and that most could be used for counting. Although the use of 10 per cent NaCl in place of water increases the area of the phase diagram, giving opaque white or separating emulsions, mixtures with acceptable counting properties were found. The optimal mixture had a higher merit number (per cent aqueous solution content times counting efficiency) for (\textsuperscript{3}H) counting than did the optimal mixture for water. Working at 3° C using toluene containing 4 g/l of PPO and 0.3 g/l POPOP and minimizing the oxygen content by gassing the aqueous and toluene Triton X-100 solutions, Van Der Laarse found that a mixture of 55 per cent toluene scintillant, 25 per cent Triton X-100 and 20 per cent water was optimal for tritiated water counting (merit number 420) and that a mixture of 38 per cent toluene scintillant, 22 per cent Triton X-100 and 40 per cent aqueous phase was optimal for counting 10 per cent NaCl solutions (merit number 549).

Using a slightly different method to find the optimal counting mixture for tritiated water, Williams\textsuperscript{18} prepared a series of solutions of Triton X-100 in toluene over the concentration range of 15 per cent to 60 per cent Triton. He then determined the counting properties of mixtures of these solutions with various amounts of tritiated water. His results show that toluene Triton X-100 mixtures containing between 30 per cent and 40 per cent Triton X-100 are the best for counting tritiated water at 4° C. The compositions of his four most efficient mixtures are given in Table 5. It should be pointed out that the counting properties of the first three mixtures (those containing toluene and Triton in the proportions of 7:3, 2:1 and 65:35) are very sensitive to changes in water content and that efficiencies decrease very sharply on either side of the optimum. The other mixture (3 parts toluene: 2 parts of Triton) is not nearly as susceptible to slight changes in water content. In spite of its lower efficiency it might be preferable for routine use, since slight compositional variations would not lead to marked changes in counting properties. Williams has reported that results obtained by use of these mixtures are quite reproducible.

Suggesting the term "colloid counting" in place of "emulsion counting," Fox\textsuperscript{16} has carried out the extremely laborious job of constructing phase diagrams with counting merit contours at room temperature (21° C for phase diagrams, 25° C for counting properties) for mixtures of toluene, Triton X-100 and various aqueous solutions commonly encountered in biochemical research. The compositions of his optimal counting

<table>
<thead>
<tr>
<th>Table 5.—Results for Scintillator Mixtures Recommended by Williams\textsuperscript{18}</th>
</tr>
</thead>
<tbody>
<tr>
<td>Scintillator Composition</td>
</tr>
<tr>
<td>% vol. Triton</td>
</tr>
<tr>
<td>30.0</td>
</tr>
<tr>
<td>33.3</td>
</tr>
<tr>
<td>35.0</td>
</tr>
<tr>
<td>40.0</td>
</tr>
</tbody>
</table>
mixtures are given in Table 6. As expected from the effects of aqueous solutes on the interaction of water with the polyethylene glycol chain of the surfactant, the compositions of these mixtures vary widely as the character of the aqueous system is changed. In selecting these optimal mixtures, special attention has been paid to the stability of each system, and in some cases small sacrifices in counting efficiency have been made to obtain increased stability.

In contrast to the preceding work which was directed toward finding conditions for incorporation of the greatest volume of aqueous solution while maintaining high (3H)-counting efficiency, Turner \(^1\) has concentrated on the inertness of toluene-Triton mixtures toward a variety of aqueous solutions, some of which have adverse effects on other scintillators. For routine use he recommended that the concentration of aqueous phase be kept below 10 per cent and that the mixture be used primarily for the counting (14C) or other isotopes with equally or more energetic \(\beta\)-emissions. Under these circumstances the mixtures are clear fluids and, as pointed out by Benson,\(^2\) the samples are solubilized rather than emulsified, although there appears to be no practical necessity to distinguish between solubilization and emulsification. Table 7 compares the counting efficiency of Triton scintillant and Bray’s scintillant (see Chapter 17) for a series of disparate aqueous solutions. Of particular interest is the failure of 2-N nitric acid to affect the counting properties. Since in Bray’s solution, nitric acid seems to destroy the fluor, its lack of effect in the Triton system indicates that the aqueous solution is effectively cloistered by the surfactant and does not have access to the scintillators. Turner also points out that in the region of 1.4 to 2 g of aqueous solution per 10 ml of scintillant (1 part Triton X-100: 2 parts of toluene) phase separation occurs, giving a cloudy lower phase and a clear upper phase. Glucose-(14C) distributed mainly into the lower phase. The two phases probably consisted of mixtures of the solubilized aqueous sample seen at lower water concentrations and the emulsion seen at higher concentrations. This unstable region of aqueous solution: scintillant corresponds to the transition from solubilization to emulsification discussed by Benson\(^3\) and the discontinuity reported by Williams.\(^4\) Such instability can be avoided by either increasing or decreasing the sample size.

### Table 6.—Optimal Composition of Phosphor Mixtures Recommended by Fox\(^6\)

<table>
<thead>
<tr>
<th>Aqueous Solution</th>
<th>Basic Phosphor Mix</th>
<th>Optimal Vol.</th>
<th>Counting Efficiency ((^{14})C)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Water</td>
<td>1:1</td>
<td>8</td>
<td>6.0</td>
</tr>
<tr>
<td>2 (M) NaCl</td>
<td>3:7</td>
<td>8</td>
<td>7.0</td>
</tr>
<tr>
<td>8 (M) Urea</td>
<td>1:1</td>
<td>8</td>
<td>6.0</td>
</tr>
<tr>
<td>5% TCA</td>
<td>13:7</td>
<td>10</td>
<td>8.5</td>
</tr>
<tr>
<td>5% HClO(_4)</td>
<td>3:1</td>
<td>3</td>
<td>6.0</td>
</tr>
<tr>
<td>5% Sucrose</td>
<td>2:3</td>
<td>6</td>
<td>5.0</td>
</tr>
</tbody>
</table>

Data were obtained 1-12 hours after mixing. Counting efficiency for each isotope is expressed as absolute percentage efficiency and Merit Value in parentheses. Values are based on optimal PPO concentration quoted. Concentration of POPOP was 2.5 per cent by weight of that of PPO.

Below each value is per cent change in initial counts over 10 hours following mixing of aqueous solution with phosphor (− = decrease, + = increase).

Mixtures were clear or very slightly opalescent; 8 M urea and 5 per cent sucrose produced viscous gels; water and 2 \(M\) NaCl were viscous but fluid; 5 per cent TCA and 5 per cent HClO\(_4\) were not viscous.
Table 7.—Comparison of Triton Scintillant and Bray's Scintillant Under Various Conditions (Turner7)

<table>
<thead>
<tr>
<th>Aqueous Solution Added per 10 ml Scintillant</th>
<th>Triton</th>
<th>Appearance of Sample*</th>
<th>Bray's</th>
<th>Counting Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>Clear</td>
<td>Clear</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Water</td>
<td>Clear</td>
<td>Clear</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2N HCl</td>
<td>Clear</td>
<td>Crystals †</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2N HCl</td>
<td>Clear</td>
<td>Green †, crystals †</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2N H₂SO₄</td>
<td>Clear</td>
<td>Green †</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2N H₂SO₄</td>
<td>Clear</td>
<td>Pale green †, crystals †</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2N HNO₃</td>
<td>Clear</td>
<td>Nearly colorless †</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.2 N NaOH</td>
<td>Clear</td>
<td>Precipitate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.02 N Na₂CO₃</td>
<td>Clear</td>
<td>Precipitate</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.05 M phosphate</td>
<td>Clear</td>
<td>Precipitate</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

A NEW TRITON-XYLENE EMULSION

The studies summarized so far have all been concerned with adjusting the composition of counting mixtures to accommodate different aqueous phases. It is also possible to make adjustments to allow changes in the organic phase.

Recently in my laboratory we have evaluated the counting properties of a xylene-naphthalene-Triton X-114 system. Although our data are not extensive enough to define the system properly, they are included to exemplify the extension of the emulsion technique to other solvent systems, which for some applications may have advantages over the toluene-Triton X-100 systems. The Packard Instrument Company produces a prepared scintillant of unknown composition under the name “Insta-Gel” which has similar (in some cases superior) properties. While the system described here works best near 0°C, “Insta-Gel” is reported to show optimal behavior at 12°C. Aside from the fluors obtained from the Packard Instrument Company, chemicals were reagent grade and not specially purified for scintillation counting. Triton X-114, which is recommended for preparation of xylene-water emulsions in technical literature from Rohm and Haas, was used as received. All the Triton surfactants and the Packard Instrument Company “Insta-Gel” showed alkali-inducible luminescence which decayed to acceptable levels after a few hours of storage. A single scintillation mixture, consisting of one part Triton X-114 and two parts solution containing 100 g of naphthalene, 10 g PPO, and 0.8 g of POPOP per liter in xylene, was used in these experiments. All counting was done between 0° and 1° C using a Packard Tri-Carb Model 3375 Liquid Scintillation Spectrometer. The physical appearance of mixtures of the scintillant and water after 24 hours of storage on ice is given in Table 8. The be-

Table 8.—Physical Properties of Mixtures of Water and Xylene-Naphthalene-Triton X-114 Solution at 0°C

<table>
<thead>
<tr>
<th>Water Content (%)</th>
<th>Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>5 and 10</td>
<td>Clear, mobile</td>
</tr>
<tr>
<td>15</td>
<td>Separated (upper phase, clear; lower phase, hazy)</td>
</tr>
<tr>
<td>20–55</td>
<td>Hazy-to-translucent, viscous solution or gel with greatest viscosity in middle of range</td>
</tr>
<tr>
<td>60–95</td>
<td>Separated semi-opaque to opaque</td>
</tr>
</tbody>
</table>

* At 4°C.
† Crystals were probably naphthalene and redissolved when samples were brought to room temperature.
‡ Bray's scintillant mixed with aqueous acid samples did not show characteristic purple color due to POPOP.
§ Sample quenched so much that channels ratio method of determining counting efficiency could not be used.
N-hexadecane-1-(14C) was used as internal standard in this case.
behavior of these mixtures is similar to that reported for Triton X-100 and toluene, in that water contents less than 10 per cent are solubilized. The mixture containing 15 per cent water separates into two phases. Emulsions ranging from turbid to translucent and from viscous to gelled are obtained with higher water content until another unstable range which produces emulsions of variable opacity is reached. The major difference between the two systems is that the Triton X-114-xylene-naphthalene system is compatible with higher water concentrations than the Triton X-100 toluene system. On several days' storage, emulsions containing 45 to 55 per cent water show some separation. In preliminary experiments with xylene in the absence of naphthalene, it was observed that the 50 per cent water mixture was quite clear and that a stable, hazy fluid dispersion could be obtained with 75 per cent water.

It was found that use of mixtures of Triton X-100 and Triton X-114 in place of Triton X-114 allowed the preparation of clear emulsions containing 50 per cent water at temperatures compatible with the characteristics of the Triton X-100 content. With Triton X-114 the emulsion with 50 per cent water is translucent at 0°C and becomes more hazy as the temperature is raised. With a Triton X-100 to Triton X-114 ratio of 1:9, the emulsion is clear at 0°C and becomes hazy at 5°C. With a 2:8 ratio, the emulsion containing 50 per cent water forms an opaque gel at 0°C which becomes clear at 3°C and hazy at 8°C. With a ratio of 3:7, the emulsion clears at 6°C and becomes hazy at 11°C. When the counting properties of the hazy translucent Triton X-114 emulsion were compared with those of the clear 1:9 Triton X-100-Triton X-114 mixture, no significant differences were found. We have not attempted to obtain a stable emulsion with 75 per cent water, but one might be achieved if the appropriate mixtures of surfactants were used.

**Phase Transitions**

Table 9 shows the relationship between counting rate and volume of tritiated water added. It can readily be seen that nothing is to be gained by use of more than 40 per cent water since further addition of labeled water does not cause an increase in the counting rate. It should also be noticed that for the indicated time periods, the samples with less than 10 per cent or more than 40 per cent water have relatively stable counting rates while the activity of those with intermediate water content slowly increases. This increase in counting rate is probably due to a slow phase transition which appears to reach completion within 24 hours. The samples with 35 per cent water or less showed no change when counted several days later. After standing in the counter several days, the vials with

<table>
<thead>
<tr>
<th>% H2O</th>
<th>Volume H2O</th>
<th>Volume Scintillation Mixture</th>
<th>Counting Rate (cpm) After Storage at 0°C for Specified Times</th>
<th>Spectrometer settings: gain, 80 per cent; lower discriminator, 50; upper discriminator, 1000 (Packard 3375 Liquid Scintillation Spectrometer). Counting efficiency for (3H) in vial containing 40 per cent water was 19.2 per cent.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5</td>
<td>1</td>
<td>19</td>
<td>1033</td>
<td>1024</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>18</td>
<td>1943</td>
<td>1966</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>16</td>
<td>3594</td>
<td>3887</td>
</tr>
<tr>
<td>20</td>
<td>4</td>
<td>15</td>
<td>3970</td>
<td>4212</td>
</tr>
<tr>
<td>25</td>
<td>5</td>
<td>14</td>
<td>4722</td>
<td>4944</td>
</tr>
<tr>
<td>30</td>
<td>6</td>
<td>13</td>
<td>5620</td>
<td>5680</td>
</tr>
<tr>
<td>35</td>
<td>7</td>
<td>12</td>
<td>5873</td>
<td>5921</td>
</tr>
<tr>
<td>40</td>
<td>8</td>
<td>11</td>
<td>5910</td>
<td>5921</td>
</tr>
<tr>
<td>45</td>
<td>9</td>
<td>10</td>
<td>5649</td>
<td>5709</td>
</tr>
<tr>
<td>50</td>
<td>10</td>
<td>9</td>
<td>5649</td>
<td>5709</td>
</tr>
<tr>
<td>55</td>
<td>11</td>
<td>8</td>
<td>5709</td>
<td>5700</td>
</tr>
<tr>
<td>60</td>
<td>12</td>
<td>7</td>
<td>5846</td>
<td>5844</td>
</tr>
<tr>
<td>65</td>
<td>13</td>
<td>6</td>
<td>5875</td>
<td>5875</td>
</tr>
<tr>
<td>70</td>
<td>14</td>
<td>5</td>
<td>5921</td>
<td>5921</td>
</tr>
</tbody>
</table>

Table 9—Effect of H2O Content on (3H) Counting Rate in Xylene-Naphthalene-Triton X-114 System
the higher water contents showed about a 10 per cent drop in counting efficiency probably attributable to partial separation. The counting rate was restored by shaking the vials. Although our results have been somewhat variable, perhaps because of differences in reagents used to prepare different batches, \(^{(3H)}\)-counting efficiencies of 27.5 per cent have been obtained in samples with 20 per cent water and 16.5 per cent in emulsions containing 50 per cent water.

Insta-Gel emulsions containing 20 per cent water have acceptable physical properties near 0°C and routinely give \(^{(3H)}\)-counting efficiencies of 30 per cent. Increasing the concentration of water to 45 per cent gives a rigidly gelled sample clear in the 10°C to 15°C region but which is opaque near 0°C. The transition from clear to opaque is rather slow, enabling samples to be counted at lower than optimal temperatures before the phase change has occurred. Under these conditions a \(^{(3H)}\)-counting efficiency of 18.5 per cent has been obtained before the transition but falls to 8.5 per cent after the sample becomes opaque. Insta-Gel is purported to show optimal behavior at 12°C, but we have not tested it at this temperature and cannot fully evaluate its performance relative to the Triton X-114-xylene-naphthalene system.

Effects of Aqueous Solutions

The influence of aqueous solution composition on the behavior of 20 per cent and 50 per cent emulsions is shown in Table 10. The ability of the Triton-xylene-naphthalene system to accommodate concentrated solutions of salt, acid or alkali appears to be more limited than a 2:1 toluene Triton X-100 system, but a reasonably broad range of such solutions still have acceptable counting properties. The samples with 0.5 M \((NH_4)_2SO_4\) have fairly good counting rates even though a vial with 50 per cent of the aqueous solution has a rather large milky lower phase which appears to contain a finely dispersed precipitate. The counting of such a two-phase system would probably be less acceptable if the \(^{(3H)}\) were in another compound such as protein instead of water. Samples containing 50 per cent plasma and 50 per cent scintillation mixture form a semi-

<table>
<thead>
<tr>
<th>Aqueous Solution</th>
<th>Counting Rate Relative to Water (%)</th>
<th>Appearance</th>
</tr>
</thead>
<tbody>
<tr>
<td>20% aqueous phase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.5 N HCl</td>
<td>75.8</td>
<td>hazy gel</td>
</tr>
<tr>
<td>1 N HCl</td>
<td>73.1</td>
<td>hazy gel</td>
</tr>
<tr>
<td>2 N HCl</td>
<td>71.0</td>
<td>hazy gel</td>
</tr>
<tr>
<td>3 N HCl</td>
<td>57.2</td>
<td>hazy gel, yellowish</td>
</tr>
<tr>
<td>0.5 M ((NH_4)_2SO_4)</td>
<td>89.7</td>
<td>clear, mobile (trace of precipitate)</td>
</tr>
<tr>
<td>1 M ((NH_4)_2SO_4)</td>
<td>53.9</td>
<td>separated (precipitated?)</td>
</tr>
<tr>
<td>1 N NaOH</td>
<td>76.9</td>
<td>clear mobile</td>
</tr>
<tr>
<td>2 N NaOH</td>
<td>42.1</td>
<td>separated</td>
</tr>
<tr>
<td>Plasma</td>
<td>58.1</td>
<td>cloudy gel</td>
</tr>
<tr>
<td>Hemolyzed plasma</td>
<td>47.9</td>
<td>cloudy gel</td>
</tr>
<tr>
<td>(0.3% hemoglobin)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

50% aqueous phase

| 0.5 N HCl        | 79.4                              | slightly hazy, viscous |
| 1 N HCl          | 70.5                              | clear, viscous         |
| 2 N HCl          | 59.1                              | hazy, viscous          |
| 3 N HCl          | 20.7                              | separated              |
| 0.5 M \((NH_4)_2SO_4\) | 79.1 | separated (precipitate in lower phase) |
| 1 M \((NH_4)_2SO_4\) | 33.5 | separated (precipitate in lower phase) |
| 0.5 N NaOH       | 83.5                              | hazy, viscous          |
| 1 N NaOH         | 77.0                              | separated              |
| 2 N NaOH         | 33.2                              | separated              |

Spectrometer settings: gain, 80 per cent; lower discriminator, 50; upper discriminator, 1000 (Packard 3375).
Table 11.—Relative Counting Efficiencies of Tritiated Toluene and Tritiated Water in Xylene-Naphthalene-Triton X-114 Water Emulsions

<table>
<thead>
<tr>
<th>Water Content of Emulsion</th>
<th>Toluene-(¹H) Counting Efficiency</th>
<th>H₂O-(¹H) Counting Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>25%</td>
<td>22.4%</td>
<td>21.2%</td>
</tr>
<tr>
<td>50%</td>
<td>16.5%</td>
<td>16.2%</td>
</tr>
</tbody>
</table>

Spectrometer settings: gain, 80 per cent; lower discriminator, 50; upper discriminator, 1000 (Packard 3375).

opaque, viscous emulsion in which (¹H)-counting efficiency approaches zero. (See Chapter 17). A more thorough investigation of the effects of composition on the counting properties of any of the above systems in the manner used by Van Der Laarse, Williams or Fox would undoubtedly reveal mixtures with better counting efficiencies than those reported here, but even without elucidation of the optimal mixture, the emulsions containing the larger quantities of water have significantly higher figures of merit (~ 800) for tritiated water than have been reported for any toluene-Triton X-100 mixture. The counting efficiency of (¹C)-toluene has been found to be 83 per cent in an emulsion containing 20 per cent water and 79 per cent in one containing 50 per cent water.

Self-absorption of (¹H) βs. The problem of self-absorption of tritium β-particles has been evaluated in emulsions containing 20 per cent and 50 per cent water. As shown in Table 11, tritiated toluene and tritiated water are counted with about the same efficiency, indicating that in these systems the range of the (¹H) βs is long compared to the size of the layers of the two phases. Van Der Laarse has reported that relative counting efficiencies of tritiated toluene and water are often quite different in some of the mixtures. The mixtures giving the greatest differences were opaque-white in appearance, indicating the presence of large droplets. In clear or opalescent systems, the counting efficiencies of the two compounds were much closer; in some cases, the counting efficiencies for water were higher than those for toluene. Because it is difficult to imagine a physical basis for tritiated toluene (which is in the same phase as the fluors), being counted with a lower efficiency than tritiated water, (which is in the aqueous phase), it is probable that small differences are due to errors in the measurement of counting efficiencies.

The structure of hazy emulsions is probably very complex (see Winsor for a review of the behavior of amphiphilic compounds), but since their counting efficiencies are as good as the clear emulsions, the dispersions of the two phases do not seem to be significantly different regarding the range of (¹H) βs with sufficient energy to be detected. Studies of the solubilization of ethyl benzene in water, using polyethylenoxide nonylphenyl under carefully controlled conditions where the mixtures have regular structures, have shown the dimensions of the dispersed organic phase to be under 100 Å, a distance which is quite small when compared to the range of an average (¹H) β-particle.

Quenching. Because effect of water content on photon yield in these emulsion systems has not been studied, it is not possible to evaluate quenching by the aqueous phase, but since the tritium counting efficiency in mixtures with dilute HCl is always lower than in comparable mixtures with water, it is probable that such quenching occurs and that one of the basic goals of emulsion counting has not been achieved. In view of the indication that the components of the organic phase are isolated from those of the aqueous phase, and since quenching by water and polar compounds supposedly involves collision between an excited aromatic molecule and a polar molecule, such quenching was not expected. (See Chapter 1–3 and
28.) Measurement of the absorption spectrum of Triton X-114 in ethanol revealed absorption maxima at about 278 mμ and 284 mμ. Since these absorption bands overlap the emission bands of xylene and toluene, excitation energy could be transferred from the solvents to Triton but not back again. Such a unidirectional energy transfer would trap a portion of the excitation energy on the surface of the organic phase where it is accessible to dissipation by interaction with the planar molecules. Naphthalene may increase counting efficiency by reducing the extent of trapping at the phase interface. If such a mechanism is important, quenching could be reduced by two changes in procedure: (1) using a different solvent such as 2-ethyl-naphthalene 21 with a fluorescence emission peak at appreciably longer wavelengths than the absorption peak of the Triton surfactants and (2) using a surfactant in which the hydrophobic group is aliphatic rather than aromatic, since aliphatic groups would be incapable of accepting energy from excited aromatic molecules. The use of aliphatic emulsifiers would also be expected to minimize chemiluminescence, although such agents may be much less effective at producing the desired emulsions. With these considerations in mind it is reasonable to expect that further investigation of systems for emulsion counting will produce significant improvements in the procedure.

CONCLUSION

As may be judged by the experience of various investigators, many of the problems in the liquid scintillation counting of aqueous solutions can be solved by use of emulsions, but unless appropriate precautions are taken, variable or artifactual results may be obtained. The major problems result from unstable emulsions and from droplet dimensions which are large compared to the range of the β-particle to be measured. These problems can be evaluated in any experimental system by checking the reproducibility of sample counting and by measuring the relative counting efficiencies of tritiated water and tritiated toluene. If the counts are not sufficiently reproducible or if there are significant differences in the counting efficiencies of tritium in the aqueous and organic phases, it is usually possible to overcome these difficulties by alteration of the counting mixture. In many cases, selection of a procedure for counting aqueous or polar samples is a matter of personal preference, but when difficulties with these techniques arise, it should be possible to design an emulsion system suitable for counting almost any type of sample.

REFERENCES


The counting of organisms, organelles, or precipitates labeled with radioactive isotopes after entrapment on various types of filter media has been a standard research technique for a number of years. One common way to locate radioactive materials on paper chromatograms is to cut out and count selected spots. Solid supporting media for counting have also been used in techniques where numbered discs of filter paper or other porous media serve as a carrier for substances which can be precipitated, washed, and dried directly on each disc, thus saving a considerable amount of work in cleaning up the individual precipitates.

In all of these cases, liquid scintillation counting is often employed. Using filter paper as the solid support, (3H), (14C) and (35S) cannot be satisfactorily counted in an ionization chamber, but they can be simply and reliably counted by liquid scintillation methods. On membranes of cellulose acetate, nitrocellulose or other surface filtration media, the isotopes above have been counted successfully in windowless, gas-flow ionization chambers and by liquid scintillation.

The use of liquid scintillation counting of samples on solid supports is widespread, although the special features of this counting technique do not always seem to be recognized. These features are interrelated but can show up in the following ways: (1) the efficiency of counting on a solid support may be less than that for counting in free solution, especially for isotopes emitting particles of low energy; (2) quenching depends primarily on the physical form of the material being counted and also on the supporting medium; (3) the β energy spectrum is shifted downward by the solid support; (4) external standardization provides little or no information on the relative counting efficiency from sample to sample;

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and (5) the channels ratio method for quench correction can be used to obtain the absolute concentrations of isotope but the ratios applying to quenching in free solution are not necessarily applicable to samples on solid media.

**ANOMALOUS COUNTING WITH TRITIUM**

These considerations are all accentuated in the case of tritium counting because of its very low $\beta$-particle energy. The features listed above will be illustrated with this isotope. A specific example of the type of complication that can arise in the liquid scintillation counting of tritiated substances on filter paper discs has been discussed in an earlier paper.\(^8\) The incorporation of tritiated thymidine triphosphate into DNA catalyzed by DNA polymerase was being assayed. At various time intervals samples were placed on paper discs, washed in ice-cold 5 per cent trichloroacetic acid, washed in ethanol and dried. A sample was dried directly on a disc without washing to obtain the total radioactivity of substrate. The radioactivity on these discs was determined by liquid scintillation and the ratio of incorporated counts to total counts was used to calculate the amount of substrate incorporated. Similar procedures are commonly used in biomedical research, and their validity depends, of course, on the assumption that substrate and product will count with the same efficiency. This assumption did not prove to be true for ($^3$H)-TTP as substrate and ($^3$H)-DNA as product when the were counted on filter paper in a toluene-based scintillation medium. The discrepancy appeared initially when the results of such experiments were compared with identical assays using a ($^{32}$P)-labeled substrate. A rate only one-sixth of the incorporation rate found with ($^3$H) substrate was found.

The explanation for these differences was that the triphosphate molecules were small enough to penetrate the paper fibrils, whereas DNA molecules were not able to do so. When the dried discs were placed in scintillation solution, $\beta$-particles from the ($^3$H)-DNA were much closer to phosphor molecules, but the radiation from ($^3$H) triphosphate was absorbed by the surrounding cellulose structure before it could reach the solution of scintillator. The more energetic ($^{32}$P) $\beta$s were virtually unaffected by the dimensions of the paper fibrils. The aliquot of assay medium placed on a disc and dried without washing contained mostly the ($^3$H) triphosphate which thus was counted with one-sixth the efficiency of the ($^3$H)-DNA on the acid-precipitated and washed discs. Since the ($^3$H) radioactivity of the unwashed disc was used to represent the total counts, division by this value increased the apparent specific activity of the ($^3$H)-DNA product.

**Test of Local Quenching Hypothesis**

If the explanation involving differential local quenching in paper fibers is valid, one would predict that ($^3$H)-DNA molecules of different lengths would count with different efficiencies. The results of a simple test confirming this prediction are given in Table 1. ($^3$H)-DNA was subjected to hydrolysis by pancreatic DNAse. At various time intervals, aliquots were placed on filter paper discs. One set of these discs was dried directly without washing. To ascertain the extent of hydrolysis, another set was washed in ice-cold 5 per cent TCA three times then washed in 95 per cent ethanol and dried. All discs were counted in liquid scintillation solution. As an additional internal control, ($^{14}$C)-DNA was also present, dual counting was performed, and the data were processed to yield data on the separate isotopes. It can be seen in Table 1 that a progressive lowering of the tritium counts occurs with the unwashed discs, amounting finally (in this experiment) to 44 per cent of the
In a volume of 1 ml, the reaction mixtures contained potassium phosphate buffer, pH 7.0 (0.1 M), MgCl₂ (0.02 M), calf thymus DNA (0.5 mg), (H)-DNA (24 μg with 20,000 cpm/μg at 14% efficiency), (C)-DNA (2.3 μg with 2,000 cpm/μg at 78% efficiency) and pancreatic DNAase (25 μg).

At the time intervals indicated, duplicate 25-μl aliquots of reaction mixture were placed on 2.1-cm discs of Whatman p541 filter paper. The discs of one set were dried under a heat lamp with no further treatment while the other set was placed in an ice-cold solution of 5 per cent (w/v) TCA for at least 10 minutes. The discs were then washed twice in 5 per cent TCA, twice in 95 per cent ethanol, and dried under a heat lamp.

The discs were placed in 5 ml of scintillation solution containing 4 per cent (w/v) PPO and 0.1 per cent (w/v) POPOP in toluene and counted on a Packard Series 314EX scintillation spectrometer with channels set to differentiate (C) and (H).

original value when 28 per cent of the DNA remains acid insoluble.

"Self-absorption" of Tritium

The higher value (122%) for (H)-DNA on the washed sample represents an enhancement of counting for the DNA when the salts of the incubation medium were washed away in the acid and were not dried down with the DNA. Since all percentages in Table 1 were calculated from the zero-minute unwashed sample, this may explain the differences between the per cent hydrolyzed DNA indicated by the two isotopes. The (C) counts on the unwashed samples were all about the same, indicating that this local self-absorption quenching was not a factor for (C) counting in this experiment. The filter paper discs in this experiment were composed of a relatively hard, compressed fiber, and it is not known whether a more bibulous paper might have presented a local environment which could have affected β-emitters of somewhat higher energy than (H) such as (S) or (C).

Tritium Counting on Various media

The great variety and low cost of tritiated substances promoted an examination of the counting of tritiated compounds of different sizes on other common support media to see if the variability of counting on filter paper could be avoided. Hydrolyzed and native (H)-DNA were chosen for this comparison, and samples of each containing identical amounts of isotope were dried onto different support media and counted. Results are listed in Table 2 as the percentages of the radioactivity in the degraded sample [a 95% hydrolyzed (H)-DNA which consists largely of tri- to octa-oligonucleotides] relative to radioactivity in the undegraded (H)-DNA counted under the same conditions.

From the results of Table 2, it can be seen that counting is slightly poorer on 3 MM paper (which is more absorbent than the 541 paper) and that anomalous counting of tritium shows up again on ion exchange paper. These papers are often used for quantitative chromatography. Precaution must be taken in the interpretation of data obtained on compounds of different sizes when counting is done directly on samples cut from chromatographic sheets. By contrast, the counts obtained for degraded (H)-DNA on cellulose acetate or fiber glass discs are slightly elevated compared to (H)-DNA on these media. These results lend additional credence to the hypothesis of fiber penetration in the case of paper discs. The cellulose acetate filter consists of a nonporous matrix of plastic punctured with holes of uniform diameter. The glass fiber disc is a compressed mat of very tiny glass threads. In neither of these
media are there fibrils into which small molecules could be absorbed; hence, local quenching conditions would be the same for molecules of all sizes. Substances forming bulky precipitates may entrap part of the material mechanically, and this would be expected to cause local self-absorption. This effect may account for the slight elevation of (3H)-oligonucleotide efficiency over that of (3H)-DNA since the latter may have a greater tendency to clump. It seemed reasonable that a scintillation solution in which a relatively large amount of aqueous sample could be dissolved might overcome the local self-absorption effect completely by infiltrating the bound water of the paper fibers and increasing the availability of phosphor molecules within the paper matrix. It can be seen, however, that the relative efficiency of counting in a dioxane-based solvent was found to be not much higher than that in toluene. The absolute counts for identical samples were actually 7 per cent lower in dioxane solution. When the aqueous samples were pipetted directly into the two scintillation solutions, the (3H)-oligonucleotides counted very well in dioxane in which they were soluble to a large extent but counted poorly in toluene in which they were insoluble, compared in each case to (3H)-DNA which is sparingly soluble in dioxane and precipitates in toluene. An incidental observation made while counting samples in dioxane is of interest here. Successive counts made on a (3H)-succinic acid standard sample on a disc placed in dioxane scintillation solution showed a gradual increase over a 72-hour period. This suggests that substances soluble in the dioxane solution may slowly dissolve from a disc. From the results on Tables 1 and 2, at a given time during this process, the efficiency of counting such small molecules could vary anywhere from 44 to 150% of that for large molecules.

Alteration of Energy Spectrum

A confirmation of the fact of local quenching on paper discs can be found in an analysis of the tritium β energy spectrum for small and large molecules on paper discs. The results of such a comparison are given in Table 3 where the percentage of total counts in each of six counting “windows” is given for (3H)-oligonucleotides and for (3H)-DNA on filter paper discs. It is apparent that the emission spectrum has been shifted downward in the case of oligonucleotides. Total counts for this sample were actually only a third of those for (3H)-DNA.

<table>
<thead>
<tr>
<th>Support Medium</th>
<th>Counting Solution</th>
<th>Per cent cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>541 filter paper</td>
<td>Toluene</td>
<td>34</td>
</tr>
<tr>
<td>Cellulose ester</td>
<td>Toluene</td>
<td>115</td>
</tr>
<tr>
<td>Glass fiber</td>
<td>Toluene</td>
<td>28</td>
</tr>
<tr>
<td>3 MM filter paper</td>
<td>Toluene</td>
<td>38</td>
</tr>
<tr>
<td>Ion exchange paper</td>
<td>Toluene</td>
<td>20</td>
</tr>
<tr>
<td>None</td>
<td>Dioxane</td>
<td>155</td>
</tr>
<tr>
<td>None</td>
<td>Dioxane</td>
<td>44</td>
</tr>
</tbody>
</table>

Table 2.—Comparison of Counting on Various Media
COUNTING OF SAMPLES ON SOLID SUPPORTS

Table 3.—Tritium Energy Spectrum for Samples on Discs

<table>
<thead>
<tr>
<th>Potentiometer Settings</th>
<th>Native (3H)-DNA (%)</th>
<th>Degraded (3H)-DNA (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>100–∞</td>
<td>100</td>
<td>100</td>
</tr>
<tr>
<td>100–200</td>
<td>24</td>
<td>34</td>
</tr>
<tr>
<td>200–300</td>
<td>23</td>
<td>26</td>
</tr>
<tr>
<td>300–400</td>
<td>20</td>
<td>17</td>
</tr>
<tr>
<td>400–500</td>
<td>11</td>
<td>15</td>
</tr>
<tr>
<td>500–600</td>
<td>9</td>
<td>3</td>
</tr>
<tr>
<td>600–700</td>
<td>5</td>
<td>1</td>
</tr>
</tbody>
</table>

Samples of equivalent solutions of approximately 1000 cpm of either native (3H)-DNA or of a 95 per cent hydrolyzed (3H)-DNA were pipetted onto Whatman 541 filter paper discs, dried, and placed in dioxane scintillation solution (described in Table 2). Using the standard counter settings, which resulted in approximately 24 per cent peak efficiency for (3H) in solution, the counting window was adjusted to successively higher increments and the counting rate recorded. The per cent of the counts appearing in each window was calculated for both samples.

**Correction for Variations in Efficiency**

Two methods in common use for determining counting efficiencies for individual samples are external standardization and channels ratio. (See Chapter 29.) The results of applying these procedures to the counting of samples on solid supports are given in Tables 4 and 5. One would not expect that the efficiency of counting an external standard would reflect in any way the local quenching conditions that exist on a solid support contained in the scintillation vial, but common sense and logic may not always characterize the eager investigator trying out his new liquid scintillation spectrometer with all of its fascinating capabilities. He will be dismayed to find a monotonous consistency of external standardization data in the light of evidence for widely varying efficiencies for his samples. From

Table 4.—Comparison of Counting on Discs with Counting in Solution

<table>
<thead>
<tr>
<th>Compound</th>
<th>Condition</th>
<th>Actual % Efficiency</th>
<th>Channels Ratio</th>
<th>Predicted % Efficiency</th>
<th>External Standard (cpm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>(3H)-DNA</td>
<td>Solution</td>
<td>14.8</td>
<td>0.821</td>
<td>30.5</td>
<td>119,000</td>
</tr>
<tr>
<td>(3H)-DNA</td>
<td>Disc</td>
<td>16.8</td>
<td>0.822</td>
<td>30.0</td>
<td>126,000</td>
</tr>
<tr>
<td>Degraded (3H)-DNA</td>
<td>Solution</td>
<td>26.2</td>
<td>0.831</td>
<td>29.2</td>
<td>126,700</td>
</tr>
<tr>
<td>Degraded (3H)-DNA</td>
<td>Disc</td>
<td>9.1</td>
<td>0.814</td>
<td>31.3</td>
<td>116,500</td>
</tr>
</tbody>
</table>

Samples of 25 μl of native (3H)-DNA or of a 70 per cent hydrolyzed (3H)-DNA in solution (as in Table 2) were either pipetted directly into 5 ml of dioxane scintillation solution (described in Table 2) or placed on 2.1-cm discs of Whatman 541 filter paper dried and immersed in 5 ml of this scintillation solution. A Nuclear Chicago Mark I counter was adjusted to count a standard sealed sample of (3H)-toluene in toluene scintillator solution at maximum efficiency in one channel (40.1%). A second channel was set to exclude the lower third of the (3H) energy spectrum and a third channel was used to monitor the external standard. A commercial set of ethanol-quenched standards was used to determine the efficiency predicted by the channels ratio.

Table 5.—Comparison of Counting on Filter Paper versus Cellulose Ester Filters

<table>
<thead>
<tr>
<th>Compound</th>
<th>Support</th>
<th>Per cent cpm</th>
<th>Channels Ratio</th>
<th>Predicted % Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>(3H)-DNA</td>
<td>Filter paper</td>
<td>100</td>
<td>.788</td>
<td>34</td>
</tr>
<tr>
<td>(3H)-DNA</td>
<td>Cellulose esters</td>
<td>115</td>
<td>.840</td>
<td>28</td>
</tr>
<tr>
<td>(3H)-TTP</td>
<td>Filter paper</td>
<td>100</td>
<td>.775</td>
<td>35</td>
</tr>
<tr>
<td>(3H)-TTP</td>
<td>Cellulose esters</td>
<td>550</td>
<td>.829</td>
<td>29</td>
</tr>
</tbody>
</table>

Aliquots of 25 μl of either native (3H)-DNA (as in Table 2) or (3H)-TTP (containing approximately 20,000 cpm) were placed on 2.1-cm discs of Whatman 541 filter paper or on 2.5-cm Millipore HA cellulose ester filter discs, dried, and placed in 5 ml of toluene scintillation solution. Counting followed the procedure described in Table 4.
the data on the energy spectrum, we might more reasonably expect the ratio of counts in fixed channels to reflect the efficiency of counting on the solid support, but when the channels are selected and the efficiency curve drawn from the usual set of quenched standards [e.g., (\(^3\)H)-toluene in solution with various amounts of ethanol] furnished by the instrument manufacturer, it can be seen that there is little correlation between the efficiency predicted by the channels ratio and the actual counts of the sample. The results in Table 5 show that there is a definite variation in the channels ratio when comparing counting on filter paper with that on cellulose acetate filters, but these variations are the same for either large or small molecules. Since the transition from outside-the-fiber to inside-the-fiber efficiencies involves five-fold differences, it is not surprising that we have found it difficult to determine channel settings that permit an accurate prediction of efficiencies on filter paper.

**RECOMMENDATIONS FOR COUNTING ON SOLID SUPPORTS**

In the light of these observations, the following recommendations for using a solid support in liquid scintillation counting can be made: (1) The efficiency of counting substrates and products should be determined under conditions identical to those used in the assay technique. (2) Wherever possible, an internal standard should be included to check sample-to-sample variability. (3) The effect ("self-absorption") of buffer salts, chromatographic elution solvents, etc., on the counting of samples that are dried directly should be measured. (4) If channels-ratio quench corrections are necessary to determine absolute counting rates, they should be determined using a progression of known amounts of each of the labeled substances on the solid supports utilized. (5) Whenever possible, surface filtration media should be used to avoid the difficult-to-predict local quenching conditions of filter paper.

**ACKNOWLEDGMENT**

The author wishes to express his appreciation to Mr. D. P. Willis for technical assistance in portions of this research.

**REFERENCES**

The efficient measurement of radioactivity in biological materials by liquid scintillation spectrometry requires close contact between the sample and the counting solution. Unfortunately, many past attempts at soft tissue solubilization have led to unsatisfactory levels of counting efficiency due either to incomplete solution of the sample in the scintillant or to quenching induced by the treatment procedure. Since acid hydrolysis and combustion techniques are reviewed in Chapters 22 and 23, the major emphasis of this chapter will be on the more desirable and popular methods utilizing strong bases and detergents.

Criteria for Evaluating Solubilization Methods

The major considerations in evaluating the methods utilized for producing homogeneous counting solutions from biological materials are: (1) ease of sample preparation, (2) maximum or optimum tissue weight or volume handled by the solubilization method, (3) maximum efficiency as related to tissue weight and expense, (4) chemiluminescence, and (5) variety of tissues which can be treated by the method. A summary of some of these criteria as applied to various solubilizing agents is presented in Table 1. These criteria are also applicable to nonbiological samples, although they usually assume greater importance in biological experiments where low specific activity, limited sample size, and the use of weak $\beta$-emitting isotopes are commonly encountered.

To establish the validity of a quench curve (efficiency versus external standard ratio or channel ratio) based on the usual toluene standards, it is necessary to compare a similar curve based on quench standards utilizing the solubilizing agents and tissues chosen by the investigator. In this way recalibration with the sealed toluene quench
standards can be used with some confidence. It is also necessary for the investigator, after selecting a solubilization method, to determine the background, stability, and self-absorption of each tissue dissolved in the solvent flour system.

TECHNIQUES AND AGENTS USED FOR TISSUE SOLUBILIZATION

* Methanolic Potassium Hydroxide

In the past, digestion of tissue with strong inorganic bases has enjoyed wide popularity, alone or in combination with other solubilizing agents. When used alone, this method provides rapid digestion of a variety of animal tissues. In most cases, the alkaline digest is brought into solution with toluene by the use of a secondary solvent such as ethylene glycol monobutyl ether. This rather inexpensive method suffers from a limitation on sample size and from unacceptably low levels of counting efficiency, particularly for (3H).

* Formamide

Formamide has been used successfully in digesting such diverse samples as bacterial cell wall and animal epidermis. Although this agent is a stronger dissolver of tissue than many of the solubilizing agents, it is also a very strong scintillation quencher. For certain tissues such as cuticle, it may be useful for isotopes of greater energy than those usually found in most biological experiments.

* Hyamine-10-X

This high molecular weight quaternary amine from Rohm and Haas, Inc., has been employed in the free hydroxide and chloride forms. An excellent review of the versatility of the hydroxide of Hyamine-10-X has been presented by Rapkin. The solubility of both the hydroxide and chloride form has been enhanced by alcoholic solutions with some reduction in counting efficiency. The production of the free base is formidable and commercial preparations are not inexpensive. Its popularity is justified by the high figure of merit (weight of tissue X efficiency) for both (14C) and (3H) in a wide variety of biological materials and by the rather simple procedure used for sample preparation. Expense, length of solubilization procedure, and significant chemiluminescence are distinct disadvantages. The last can be dealt with by dark adaptation, acidification of samples, or limitation of sample size. (See Chapters 33 and 34.)

The chloride form of Hyamine-10-X shares most of the advantages and disadvantages of the free base. The available experience indicates that although smaller sample sizes are best used with this form of hyamine, the efficiencies for low energy isotopes (1.5 Molar solution) are greater than that reported for the free base.

* NCS

This relatively new solubilizing agent from Nuclear-Chicago Corp., is a mixture of toluene and soluble quaternary ammonium bases. Recently this reagent was rather extensively studied and compared with Hyamine solutions. Solubilization of solid tissue samples is simple but requires several days to effect complete solution of most samples. Maximum efficiency and short preparation time can be realized by using tissue homogenates (1:4). The advantages associated with the use of homogenates is somewhat offset by the smaller sample size that can be incorporated into the counting vial and the inaccuracy associated with pipetting of tissue suspensions. In the case of biological fluids, the period of digestion is rapid and the method quite simple. In our experience, excellent counting efficiencies have been attained without bleaching the samples and the chemiluminescence usually associated with alkaline digests is minimal and in most instances does
not require acidification or prolonged periods of dark adaption. The efficiencies for \(^{14}\text{C}\) and \(^{3}\text{H}\) as listed in Table 1 are those found at maximum sample size. At a sample size of 100 mg., an efficiency of 90 per cent for \(^{14}\text{C}\) and 35 per cent for \(^{3}\text{H}\) should be expected on most counting instruments. To benefit from the high figure of merit obtained with this base, large quantities of the solubilizer are needed. As shown in Table 1, the cost involved in the use of this agent is significant and for large samples almost prohibitive.

### Table 1.—Methods for Solubilizing Biological Material

<table>
<thead>
<tr>
<th>Solubilizing Agents</th>
<th>Scintillation Solvents</th>
<th>Optimum Tissue Weight or Volume *</th>
<th>Sample Preparation</th>
<th>Efficiency *</th>
<th>Approximate cost per 100 mg. Tissue †</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nitric acid (^{14})</td>
<td>PPO, POPOP Naphthalene, Dioxane, Ethylene glycol</td>
<td>&lt; 75 mg.</td>
<td>Rapid</td>
<td>38% (^{14}\text{C}) 4% (^{3}\text{H})</td>
<td>Negligible</td>
</tr>
<tr>
<td>Formamide (^{9,10})</td>
<td>Toluene-ethanol PPO or Toluene PPO POPOP</td>
<td>&lt; 50 mg.</td>
<td>Rapid</td>
<td>50–60% (^{14}\text{C}) 3% (^{3}\text{H})</td>
<td>Negligible</td>
</tr>
<tr>
<td>2 N Methanolic potassium hydroxide (^{10,11})</td>
<td>PPO, POPOP, Ethylene glycol monobutyl ether, Toluene</td>
<td>&lt; 100 mg. ‡</td>
<td>Rapid</td>
<td>60% (^{14}\text{C}) 8% (^{3}\text{H})</td>
<td>Negligible</td>
</tr>
<tr>
<td>1.5 M Methanolic Hyamine-10-X Chloride and Triton-X-100 (^3)</td>
<td>Toluene, PPO POPOP</td>
<td>100 mg.</td>
<td>Slow</td>
<td>90% (^{14}\text{C}) 18.2% (^{3}\text{H})</td>
<td>$0.05</td>
</tr>
<tr>
<td>1.0 M Methanolic Hyamine-10-X Chloride Pretreatment with N-KOH (^1)</td>
<td>NE213 scintillator based on Xylene and Naphthalene</td>
<td>&lt; 50 mg.</td>
<td>Moderately slow</td>
<td>60–90% (^{14}\text{C})</td>
<td>Negligible</td>
</tr>
<tr>
<td>1.0 M Methanolic Hyamine-10-X Hydroxide (^14,15)</td>
<td>Toluene, Butoxy-ethanol and PPO §</td>
<td>100–1000 mg. up to 1 ml. plasma</td>
<td>Slow</td>
<td>50% (^{14}\text{C}) 20% (^{3}\text{H})</td>
<td>$0.11</td>
</tr>
<tr>
<td>NCS Reagent (quaternary ammonium bases) (^6)</td>
<td>PPO, POPOP Toluene</td>
<td>&gt; 1000 mg.</td>
<td>Slow for whole tissues but moderate for homogenates</td>
<td>50% (^{14}\text{C}) 20% (^{3}\text{H})</td>
<td>$0.42 (solid tissues) $0.11 (plasma)</td>
</tr>
<tr>
<td>1 N NaOH and Bio-Solv BBS-2 (^5,11)</td>
<td>PPO, POPOP Toluene</td>
<td>Up to 150 mg.</td>
<td>Rapid</td>
<td>90% (^{14}\text{C}) 35% (^{3}\text{H})</td>
<td>$0.08</td>
</tr>
<tr>
<td>Bio-Solv BBS-3 (^5,11)</td>
<td>PPO, POPOP Toluene</td>
<td>Up to 1 ml.</td>
<td>Rapid</td>
<td>85% (^{14}\text{C}) 46% (^{3}\text{H})</td>
<td>$0.03</td>
</tr>
</tbody>
</table>

* Varies somewhat with type of tissue.
† Exclusive of flour and primary solvent.
‡ Although large samples (up to 500 mg.) have been incorporated in KOH-diatol mixture, our experience indicates significant self-absorption above 150 mg.
§ Other solvent systems used with less efficiency.
‖ Considerably higher efficiency at lower tissue weights.

**Bio-Solv Solubilizers**

This new group of solubilizing agents from Beckman Corp., represents salt insensitive BBS-3 and alkaline-resistant BBS-2 detergent systems. These systems were initially marketed by Sentol Associates, Inc., as Colosolve and apparently are indistinguishable in composition from the Bio-Solv.\(^4\)

The solubilization of most soft animal tissues by Bio-Solv BBS-2 requires pretreatment of the tissue with 1 N sodium hydroxide.\(^5,11\) Approximately 100 mg. of most solid
tissues can be digested to an almost clear solution in this strong base within one hour at temperatures up to 60°. There is some recent evidence that at 130° C more rapid digests of greater clarity can be obtained. This latter method requires the use of a sand bath and 1 ml of 2 N NaOH for tissue weights up to 100 mg. The alkaline digest is then neutralized with the BBS-2 solution as shown in Fig. 1. It is necessary to determine the quantity of solubilizer needed to reach a pH of 7 for each batch of Bio-Solv (BBS-2) since some variation has been noted between different batch numbers. The type or quantity of tissue to be digested is unimportant in determining this relationship between BBS-2 and 1 N sodium hydroxide. It is a simple matter to determine for each batch of solubilizer the quantity of BBS-2 needed to neutralize the amount of 1 N sodium hydroxide to be used for any group of samples. The end point can be determined with litmus paper or chemical indicators (phenol red) and the solubilizer can be premixed with the appropriate amount of toluene scintillator solution, thus expediting final sample preparation. It is usually found that 1 ml of alkaline digest requires 1.6 ml of BBS-2. Up to 12 ml of the scintillation solution can be tolerated. Excessive amounts of the scintillant (Fig. 1) will produce some phase separation which can lead to serious self-absorption. In practice, this possibility is remote. Chemiluminescence has not been a problem nor has it been necessary to bleach highly colored tissues (such as the liver) to realize excellent counting efficiencies. The cost per 100 mg. of tissue is acceptable although sample size is limited to a maximum of 150 mg. for solid tissue.

Plasma and urine samples are handled rapidly without prior digestion. The quantity of fluid by weight greatly exceeds that of solid tissues and excellent efficiencies are seen for both (14C) and (3H). The BBS-3 useful for biological fluids may be premixed with the scintillation solvent. It may be nec-

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**Fig. 1—Appearance of counting solutions utilizing Bio-Solv BBS-2 solubilizer.** Vial A contains 1 ml of alkaline tissue digest (pH 13). Vial B with an insufficient amount of BBS-2 (pH 9) appears turbid. Vial C contains a clear solution obtained when alkaline digest is properly neutralized by solubilizing agent (pH 7). Addition of toluene scintillation solution does not alter pH and provides a clear counting solution (Vial D). Vial E represents the appearance of solution when excessive quantities of toluene-fluor (> 12 ml) are used or when the neutralization of digest is incomplete.
necessary to add two to three drops of 4 percent stannous chloride in 0.1 N hydrochloric acid to the plasma and BBS-3 mixture to produce a clear solution. It is also suggested that the solution be added to the solubilizer after the urine samples are dissolved in it.

SUMMARY

The final selection of a solubilizing agent must necessarily depend on the experimental conditions and the needs of the investigator. In the case of extremely low specific activity and unlimited tissue availability, the high figure of merit acquired with the use of Hyamine-10-X hydroxide and NCS must be considered a compelling argument for their use. The greater relative and absolute sensitivity offered by NCS is partially offset by its cost. When the sample size is limited, particularly with low sample specific activity, the Bio-Solv solubilizers are superior to any presently available agents. The solubilization of biological fluids is easily accomplished both with the Bio-Solv and with NCS, although the former compounds are considerably less expensive.

REFERENCES

DETERMINATION OF SEVERAL ISOTOPES IN TISSUE BY WET OXIDATION

D. T. MAHIN and R. T. LOFBERG

As the other chapters in this section show, the investigator has an increasing number of methods available to prepare samples for liquid scintillation counting. In general, there is a separate method for nearly every application; e.g., the radioactivity in plasma and urine may be measured by one method, in biological tissues by a second, and in chromatographic or gel electrophoretic preparations by others. Absolute counting efficiencies vary among methods and often among samples using any one given method. This requires the preparation of several sets of quench correction curves and may lead to confusion.

For samples of a particular type and size measured in homogeneous solution, there is an optimum combination of phosphor solvent system, solubilization or decolorization method, and spectrometer adjustment to compensate for quenching. If measurement of a large number of similar samples is contemplated, it might be economical for an investigator to establish this optimum prior to his experiment. On the other hand, an investigator might be willing to accept a less than optimum system if the method were simple and the reagents inexpensive, if it could be applied to a wide variety of sample types and isotopes, if samples were stable and free of luminescence, and if quenching among samples were reasonably uniform. These features characterize the wet combustion technique to a remarkable degree.

This discussion will be divided into two parts: First, wet oxidation in general will be discussed and a particular method will be described. Second, completion of the sample preparation by addition of phosphor-solvent solution will be discussed. A specific method will be described which we have found appropriate for use in many different situations.

The citation of commercial names of materials and equipment used in this report does not constitute recommendation or approval of their use by the Department of Defense or the United States Government.

DORSEY T. MAHIN, M.D.; ROBERT T. LOFBERG, Ph.D.: Radiation and Radiochemistry Section, Division of Nuclear Medicine, Department of the Army, Walter Reed Institute of Research, Walter Reed Army Medical Center, Washington, D.C.
Biological fluids and tissues are composed mostly of water and constituents that are most easily solubilized in water, and many isolates or extracts of biological interest are also most readily soluble in water. In many experimental situations, the investigator is confronted with the need to achieve a homogeneous single phase between aqueous solution and phosphor solvent. He may encounter different sorts of material: (1) aqueous solutions of small molecules or ions, which may be either noncolored or may have sufficient color to cause significant and often variable color quenching; (2) aqueous solutions, colored or noncolored, containing large molecules; (3) tissues composed mostly of water having firm structure and containing large molecules and variable concentrations of pigments; and (4) dry solids, colored or noncolored, such as segments of paper chromatograms or filtrates collected on filter discs.

Unless color quenching is uniform among samples, quench correction of counting rate of individual samples is necessary, or samples may be decolorized during preparation by addition of $\text{H}_2\text{O}_2$. Significant delay before counting may be necessary to permit decay of luminescence which is particularly troublesome in the measurement of $^3\text{H}$ and may have decay half-times of hours to days. Chemiluminescence can be eliminated by acidification (1,2) or, if due to $\text{H}_2\text{O}_3$, by addition of catalase, but these constitute additional steps in preparing samples by alkaline digestion. (See Chapters 33 and 34.)

Considering the above, it seems most reasonable that samples be digested initially by an oxidizing acid which solubilizes, acidifies, and eliminates or minimizes color quenching. During digestion, decolorization by the addition of $\text{H}_2\text{O}_2$ eliminates or minimizes color quenching.

**SAMPLE DIGESTION**

Many procedures for the preparation of biological samples for scintillation counting have been reported in the literature. The dry combustion technique for $^{14}\text{C}$ or $^3\text{H}$ is too complex and time consuming to be easily applied to measurement of a large number of samples, although recent progress has been made in development of automated equipment to perform this process. (See Chapter 23). Two general wet methods have been suggested. The first is solubilization in basic solutions such as inorganic, alkali hydroxides or quaternary organic bases, but this method has several disadvantages. If more than a few hundred micrograms of solubilized protein or other large molecules are contained in each sample, significant chemiluminescence may occur. Since alkaline digestion does not decolorize pigments such as heme, color quenching also results. The second method involves wet oxidation, exemplified by the method of Jeffay, et al. (1,2) for $^{35}\text{S}$ and by the present method.

The Jeffay method, which is applicable to a variety of samples and isotopes, allows the preparation of many samples at one time, but several steps are necessary and there is a modest explosion hazard requiring use of a fume hood. The perchloric acid is volatilized while the sample is completely oxidized to inorganic constituents, and volatile isotopic compounds, as might be formed with $^3\text{H}$ or $^{14}\text{C}$, are lost.

A few years ago Cameron reported a wet oxidation technique for iron colorimetry in blood. This method can be utilized for liquid scintillation sample preparation in many circumstances and during the past several years has been used to prepare many thousands of samples easily, safely and economically. Heme and other derived pigments in biological samples are usually quite resistant to solubilization in a colorless form, but digestion of samples in 70 per cent perchloric acid and 30 per cent hydrogen peroxide at 60–80°C for a short period provides sufficient oxidation to solubilize and decolorize.
Loss of \((^{14}C)\)

Oxidation conditions are mild enough that organic material is not completely oxidized to carbon dioxide and water. Only the most easily oxidized groups such as the double bonds in resonating colored structures such as porphyrins, aromatic methyl groups, and terminal carbons in carbohydrates are attacked. Polymeric biological molecules are hydrolyzed into smaller oxidized subunits which are normally colorless and soluble but not volatile.

These chemical considerations are particularly relevant to the measurement of \((^{14}C)\). Oxidation of \((^{3}H)\) to water or oxidation of metallic ions is of less concern because significant loss through volatilization from the sample is not likely to occur. Table 1 shows the loss of radioactivity when uniformly labeled \((^{14}C)\)-tagged glucose was incubated with this oxidizing solution. The modest loss of activity after two hours incubation was compatible with predominant oxidation of the tagged terminal carbons that presumably comprised approximately one-sixth of the total generally labeled glucose carbons. It should be stressed that this loss of volatile radioactivity is of concern only when \((^{14}C)\)-tagged materials are being measured. In many compounds \((^{14}C)\) is stable to wet oxidation conditions after prolonged incubation. For example, no loss of activity was observed when thymidine-2-\((^{14}C)\) and thymidine-6-\((^{3}H)\) (nominal) were incubated for five hours.

Individual compounds should of course be tested prior to an experiment. This can be done by incubating an aliquot of labeled compound with perchloric acid and \(H_2O_2\) and comparing counting rate to that observed when a similar aliquot is added, just before counting, to a counting vial containing similar volumes of perchloric acid, \(H_2O_2\) and phosphor-solvent solution. Adding radioactivity just prior to measurement minimizes the likelihood of losing significant radioactivity through oxidation to volatile compounds.

Wet oxidation can be used for assay of \((^{40}Ca), \,(^{55}Fe), \,(^{57}Co), \,(^{32}P), \text{ or } (^{35}S)\) without hazard of losing volatile radioactivity.

During several years of experience with this method, two chemical curiosities have been observed. \((^{14}C)\) in ring-tagged chloroquine (a usually stable chemical configuration) was completely oxidized to \((^{14}C)O_2\) by wet combustion when blood or tissue samples containing chloroquine were digested but not when chloroquine alone was digested. It proved to be due to the presence of ferric ion that catalyzed oxidation of chloroquine by \(H_2O_2\). \((^{14}C)\) loss could be almost entirely eliminated by complexing ferric ion with phosphate after adding a small amount of concentrated \(H_3PO_4\) during digestion.

The second curiosity was observed during digestion of samples containing \((^{32}P)O_4\) ion. During incubation, metal foil inside the scintillation vial cap often becomes oxidized and a small but usually harmless precipitate may drop into the scintillation solution. If \((^{32}P)O_4\) ion is present, however, it complexes with the precipitate and is removed from solution. The indications of this phenomenon were loss of count rate and evidence of self-absorption quenching for \((^{32}P)\) alone when it was measured in the presence of other

<table>
<thead>
<tr>
<th>Glucose Concentration*</th>
<th>1 Hour</th>
<th>2 Hours</th>
</tr>
</thead>
<tbody>
<tr>
<td>400</td>
<td>4%</td>
<td>10%</td>
</tr>
<tr>
<td>4</td>
<td>4%</td>
<td>30%</td>
</tr>
<tr>
<td>0.04</td>
<td>6%</td>
<td>27%</td>
</tr>
<tr>
<td>0</td>
<td>6%</td>
<td>14%</td>
</tr>
</tbody>
</table>

* Approximate initial sample glucose concentration (grams per liter).
isotopes. The effect was prevented by use of a commercially available teflon disc insert in the vial cap.

Acids other than perchloric acid can also be used to solubilize tissues. Bartley and Abraham 6 reported solubilization of (14C)-tagged proteins in 88 per cent formic acid. An interested investigator might also wish to experiment with use of formic or other strong acids such as trichloracetic, trifloroacetic or phosphoric. Addition of hydrogen peroxide to decolorize may be necessary, as with the present system.

Details of the Method

A satisfactory method for preparation of biological samples is as follows: A 0.2-ml aliquot of fluid or up to 100 mg of tissue is placed in each counting vial. A 0.2-ml volume of 60 per cent perchloric acid is added and the contents are swirled. A 0.4-ml volume of 30 per cent hydrogen peroxide is then added and the contents are again swirled. Unless the perchloric acid is added first and mixed with the sample before adding peroxide, frothing occurs and prevents adequate mixing of reagents. Vials should be tightly capped to minimize evaporative loss of fluid during digestion. The vials can then be placed in a temperature-regulated warming oven at 70° C to 80° C for 30 to 60 minutes. The process of solubilization is accelerated by occasional agitation of the vials during digestion.

At the conclusion of warming, the vial content should be virtually clear and colorless. A fine precipitate may remain, but it is soluble in the final solution and has no effect on the counting efficiency. When animal tissues are solubilized, a flocculent precipitate may remain, especially in tissues with high lipid content. This precipitate also is soluble in the organic solvent system and causes no problem.

After samples are cooled, sample preparation is completed by addition of 6 ml of cellosolve (ethylene glycol monoethyl ether) and 10 ml of toluene phosphor solution containing 6 g PPO per liter of toluene. Slightly more cellosolve may be required to maintain a single phase solution if scintillation spectrometry is done at low temperature. POPOP cannot be used in the phosphor solution because it acquires a yellow color in acid solution and causes severe color quenching. POPOP adds little to counting efficiency under most liquid scintillation counting circumstances because modern quartz faced photomultiplier tubes transmit the 3650-A emission of PPO quite satisfactorily.

If a refrigerated spectrometer is used, samples must be allowed to reach thermal equilibrium (about 30–45 minutes) before they are counted. Despite the strong acidity in the digested material, some chemiluminescence may be observed when measuring (3H), but its decay half-time is short and it will usually disappear by the time temperature equilibrium is reached. We have not encountered chemiluminescence if the digested sample and phosphor solvent solutions were chilled to low temperature (−10° C) before mixing, but we consider this procedure unnecessary. The samples are moderately quenched so that for maximum counting efficiency, amplifier gain or high voltage on photomultiplier tubes must be increased. (See Chapter 31.)

The maximum relative merit [aqueous volume per sample times absolute counting efficiency for (3H)] in toluene-cellosolve is observed when the total aqueous volume in each sample vial is approximately 2 ml. This aqueous volume will be attained if a 0.5-ml blood or plasma sample or a 500-mg tissue sample is solubilized by wet oxidation and would thus appear to constitute an optimum sample volume. Oxidation of larger samples with larger volumes of oxidant increases the explosion hazard. No reliable methods exist to estimate this hazard, and safety can only be proved by trial. A few such samples have been prepared without incident, but more
experience is required and the practice is not recommended.

It should be noted that the proportions of blood, perchloric acid and hydrogen peroxide (1:1:2) are nearly the same as used by Cameron and are just sufficient to decolorize whole blood with normal hemoglobin concentrations. Fresh reagents should therefore be used. If packed red cells or whole blood with hematocrit above 60 per cent are measured, some yellow color, attributed by Cameron to ferric ion, may persist in the digested samples. He has suggested that this color can be eliminated with the reduction of ferric iron to the ferrous state through addition of 10 per cent aqueous hydroxylamine. We have found that color quenching in completed samples is minimal and that this step is usually unnecessary. If samples are incubated too long, the slight brownish color that may appear can be eliminated by adding slightly more H$_2$O$_2$. Table 2 summarizes the digestability of various types of samples by the perchloric acid-hydrogen peroxide method.

**SCINTILLATOR SOLUTIONS AND SAMPLE COMPLETION**

After samples have been digested and converted into clear aqueous solutions, a scintillator solution must be added. There are many possible combinations of primary and secondary solvents and solutes from which to choose. (See Chapter 2 and 3.) When the investigator has determined the size or volume of biological sample to be placed in each vial, he can calculate the final aqueous volume that will result after addition of perchloric acid (or other digesting acids) and H$_2$O$_2$. If a very small total aqueous volume is to be measured (5–10 lambda), it may be dissolved directly into organic solvent without resort to a secondary solvent, because water is slightly soluble in organic solvents. Such samples are not significantly quenched, and (3H) may be measured with 50–60 per cent absolute counting efficiency and more energetic β-emitters may be measured at nearly 100 per cent absolute counting efficiency.

**Phase Diagrams**

Much more frequently, the final aqueous volume will be in the range of 0.5 to 3 ml. In this case, a secondary solvent is usually required to achieve single phase solution. A series of phase diagrams shown (Figs. 1, 3, 5, 7, and 9) can be used to determine the proportion of secondary and primary solvents that produce single phase solutions with a given volume of water. These curves have all been generated to produce a final volume of 17 ml in each counting vial to facilitate comparison of solvent systems. Figure 1 shows these proportions for a toluene-methanol system. Figure 2 shows the relative merit (defined to be the aqueous volume per sample times the absolute counting efficiency observed for (3H) as a function of aqueous volume per sample. A maximum

<table>
<thead>
<tr>
<th>Material</th>
<th>Digestibility</th>
</tr>
</thead>
<tbody>
<tr>
<td>Plasma, whole blood, urine</td>
<td>Excellent</td>
</tr>
<tr>
<td>Soft Tissues</td>
<td></td>
</tr>
<tr>
<td>Liver, kidney, spleen, brain,</td>
<td></td>
</tr>
<tr>
<td>intestine, muscle, red blood</td>
<td>Very good</td>
</tr>
<tr>
<td>cells, feces, hair, lymphocytes</td>
<td></td>
</tr>
<tr>
<td>Solid Biological Material</td>
<td>Good; usually takes a little</td>
</tr>
<tr>
<td>Bone, teeth, chitin</td>
<td>longer than soft tissue</td>
</tr>
<tr>
<td>Solid Nonbiological Material</td>
<td></td>
</tr>
<tr>
<td>Millipore filter, Whatman #1</td>
<td>Very good</td>
</tr>
<tr>
<td>chromatographic paper</td>
<td></td>
</tr>
<tr>
<td>Silica gel (thin layer), ion</td>
<td>Not digestible with procedures</td>
</tr>
<tr>
<td>exchange resins</td>
<td>as used</td>
</tr>
</tbody>
</table>
DETERMINATION OF ISOTOPES BY WET OXIDATION

Fig. 1.—Proportions of toluene and methanol producing single-phase system as function of water content. Final volume is 17 ml.

relative merit of 5.5 is shown at an aqueous volume of 0.5 ml. Similar curves (Figs. 3 through 10) show the corresponding data for toluene-cellosolve, p-xylene-Triton X-100, toluene-Hyamine and toluene-NCS. Thus, if it is desired to measure a 3-ml aqueous volume (which Fig. 6 shows produces maximum relative merit), 8 ml p-xylene and 6 ml Triton X-100 are required to achieve single phase (Fig. 5).

Figures 1, 3, 5, 7 and 9 show the proportions for achieving a single phase at room temperature. Because of temperature-solubility dependence, slight adjustment may be necessary if samples are to be counted at low temperatures. The merit curves for toluene-methanol, toluene-cellosolve, and toluene-Hyamine were generated using toluene containing 6 g PPO per liter, but ideally the optimum phosphor concentration should

Fig. 2.—Relative merit for toluene-methanol mixtures as function of aqueous sample volume. Toluene contained 6 g/l of PPO. Wide window was used (0.5-10) on Packard Tricarb Liquid Scintillation Counter Model 3002. Gain settings are given in parentheses.

Fig. 4.—Relative merit for toluene-cellosolve mixtures as function of aqueous sample volume. Toluene contained 6 g/l of PPO. Settings as for Fig. 2. Abscissa values in parentheses show weights of tissue or biological fluid which, after wet oxidation, have final aqueous volumes shown below abscissa.
be determined for each proportion of water and solvent. If this were done, some additional variation in relative merit would be observed. We have found that absolute counting efficiency is not very sensitive to changes in phosphor concentrations near the optimum (about 4 g per liter final concentration) for this mixture, so that little improvement might be expected. The curves for p-xylene-Triton X-100 were obtained with p-xylene solution containing 10 g PPO per liter.

Biological samples prepared by wet oxidation contain many complex molecular and ionic species. These tend to increase chemical quenching substantially above that observed when water alone is solubilized into solvent phosphor solution (tap water was used to generate Figs. 1 through 12). For example, when 0.2 ml of whole blood is solubilized with perchloric acid and hydrogen peroxide, producing a final aqueous volume of 0.8 ml, the absolute counting efficiency observed in toluene-cellosolve is about 12 per cent. This corresponds to a relative merit of 9.6. If samples are prepared...
DETERMINATION OF ISOTOPES BY WET OXIDATION

Toluene

O.O NCS

(70)

(10)

(70)

observed. However, when p-xylene-Triton X-100 solution is used, almost complete chemical quenching is observed. This severe quenching is apparently produced by the wet oxidation reagents. A similar degree of severe quenching is observed when perchloric acid, hydrogen peroxide and water, instead of digested whole blood, are added to p-xylene-Triton X-100. Oxidizing reagents produce severe chemiluminescence in this solvent system. Therefore, this solvent system does not appear to be useful in measurement of wet oxidation digests of biological materials although it has great merit for measurement of noncolored aqueous solutions of small molecules.

Bray's solution cannot be used as a solvent system for perchloric acid hydrogen peroxide digested tissue samples either, because the residual oxidizing agents cause almost complete quenching. This is probably due to the extensive production of highly quenching peroxides in the p-dioxane solvent. The curve (Fig. 11) is included to compare its merits to those of other solvent systems for simple aqueous solutions.

Figure 12 shows the relative merit of with the same volume (0.8 ml) of tap water, the absolute counting efficiency is 16 per cent, corresponding to a relative merit of 13 (Fig. 4). Consequently, these curves serve only as a rough guide to establish optimal sample composition for tissue digests. Similar curves could be prepared for variable volumes of tissue digest per sample, but the aqueous volume per sample at which maximum relative merit results probably would not vary significantly from that observed for tap water alone.

Gel Scintillator Mixtures

In a variety of solvent systems (toluene-methanol, toluene-cellosolve, toluene-Hyamine, or toluene-NCS reagent) some additional quenching due to tissue digest products, in comparison to tap water, is observed. However, when p-xylene-Triton X-100 solution is used, almost complete chemical quenching is observed. This severe quenching is apparently produced by the wet oxidation reagents. A similar degree of severe quenching is observed when perchloric acid, hydrogen peroxide and water, instead of digested whole blood, are added to p-xylene-Triton X-100. Oxidizing reagents produce severe chemiluminescence in this solvent system. Therefore, this solvent system does not appear to be useful in measurement of wet oxidation digests of biological materials although it has great merit for measurement of noncolored aqueous solutions of small molecules.

Bray's solution cannot be used as a solvent system for perchloric acid hydrogen peroxide digested tissue samples either, because the residual oxidizing agents cause almost complete quenching. This is probably due to the extensive production of highly quenching peroxides in the p-dioxane solvent. The curve (Fig. 11) is included to compare its merits to those of other solvent systems for simple aqueous solutions.

Figure 12 shows the relative merit of
Insta-gel (Packard Instrument Co.). An Insta-gel sample containing more than 2 ml of water forms a translucent emulsion. The drop in relative merit shown for (H)-labeled water standards in volumes greater than 2 ml apparently results from internal self-absorption quenching within water droplets. When a second batch of Insta-gel was tested with a (H) water standard, the relative merit curve resembled the curve for tritiated toluene somewhat more closely. This variation suggests that individual batches should be tested for self-absorption before adding (H)-labeled aqueous solutions in volumes greater than 1.5–2 ml. The addition of wet oxidation reagents to Insta-gel does not produce the complete quenching that was observed in p-xylene-Triton X-100, although short-lived chemiluminescence is produced. When digested whole blood with a final aqueous volume of 2 ml is added to 15 ml of Insta-gel, a relative merit of 25 results. Hence this solution can be used satisfactorily with blood or tissue solubilized by wet oxidation.

Considering the difficulties with p-xylene-Triton X-100 and Bray's solution, and the rather poor relative merit in Hyamine-toluene, the Insta-gel, NCS-toluene and cellosolve-toluene solvent systems seem the most satisfactory of those tested for wet oxidation tissue digests.

**SPECIAL APPLICATIONS**

Many investigators have been searching for a simple method to assay paper strip chromatograms for tagged materials. Wet oxidation can be used for this purpose under many circumstances. Figure 13 shows such an application to thymidine methyl (H). Two 0.25-ml samples containing 60,000 dpm and 24,000 dpm, respectively, were chromatographed on 0.75-inch wide Whatman #1 filter paper using n-butanol-acetic

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**Fig. 11.**—Relative merit for Bray's solution as function of aqueous sample volume. Settings as for Fig. 2.

**Fig. 12.**—Relative merit for Insta-gel (Packard Instrument Co.) as function of aqueous sample volume. Settings as for Fig. 2.

**Fig. 13.**—Paper chromatogram of aged thymidine methyl(3H) solution.
DETERMINATION OF ISOTOPES BY WET OXIDATION

Acid-water (80:12:30). After drying, the strips were cut into 1-cm portions. Each portion was placed in a counting vial and digested with 0.3 ml, 70 per cent perchloric acid and 0.6 ml of 30 per cent H₂O₂. After two hours of incubation, 8 ml cellosolve and 10 ml of toluene-PPO solution (6 g/l) were added, and samples were counted. The resulting chromatogram shows several distinct peaks, confirming the fact that some decomposition had occurred in the old sample that was chromatographed.

Another application is the assay of polyacrylamide gel electrophoresis strips. Short segments of the strips may be placed in individual counting vials and the samples digested by wet oxidation as previously described. (See Chapter 27.)

ACKNOWLEDGMENT

The authors wish to express thanks to LTC David M. Ginsberg for helpful suggestions in the preparation of this manuscript.

REFERENCES

There are a number of reasons for the use of combustion techniques in the preparation of samples of \(^{3}H\) and \(^{14}C\) for liquid scintillation counting. The principle of this approach is to produce samples that are uniform in the sense that all the radioactivity is in the form of water or soluble carbonate. Jeffay has aptly called these “universal solutes.” Since these compounds are readily dissolved in quite efficient liquid scintillation solvents, this is a general answer to problems of sample solubility. Combustion also solves most problems of quenching due to the color or the chemical nature of the original sample material. It yields samples uniformly free of the problem of chemiluminescence. The principal drawback to this approach and the reason for its lack of popularity in the past has been that the majority of published methods are inordinately laborious and time-consuming. During the past few years there have been a number of developments that have greatly facilitated the application of combustion to the preparation of liquid scintillation counting samples.

The initial approaches to the use of combustion in sample preparation were largely borrowed, quiet directly, from the classical techniques of quantitative analytical chemistry. A number of papers reported on the use of variations of the combustion tube-furnace, on the sealed bomb or tube, and on wet combustion as applied to the preparation of liquid scintillation counting samples. These techniques were generally quite complicated and time-consuming.

Greatly improved facility for combusting the usual quantities of sample involved in liquid scintillation counting was offered by

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the oxygen flask combustion technique, which had been developed by Schöniger \textsuperscript{28} for quantitative analysis. Reports on the application of this general method for the preparation of samples of biological material containing tritium and radiocarbon for liquid scintillation counting were made almost simultaneously in 1961 by the groups from Lederle Laboratories \textsuperscript{14} and Sandoz at Basel.\textsuperscript{15} These studies indicated that the Schöniger combustion method could be rapid and quite simple for application to reasonable numbers of samples. A considerable number of variations and “improvements” on these early oxygen flask combustion techniques have appeared in the literature. Many of these have been reviewed in the publications by Jeffay,\textsuperscript{7} Ragland,\textsuperscript{10} Rapkin,\textsuperscript{11} and Kainz and Wachberger.\textsuperscript{12}

Our own group was responsible for one early variation which has proved so satisfactory that we have used it without modification for the past seven years.\textsuperscript{16,17}

**Oxygen Flask Combustion: The Basic Technique**

In the fall of 1961, with the help of Dr. Donald Buyske, then of Lederle Laboratories, our group in Chemical Pharmacology at the National Cancer Institute set up a technique for the oxygen flask combustion of biological samples for the determination of \(^{14}\text{C}\) and \(^{3}\text{H} \text{.} \text{) After a few months’ experience we introduced a number of modifications to the technique from that originally described by Kelly et al of the Lederle group, and we published the substance of these as a short communication the following summer.\textsuperscript{16} During the next seven years we continued to use this technique and have directly helped over a dozen other groups get started with this method.

The essence of the oxygen flask technique is to provide for the convenient combustion of numerous dried samples of tissues or organic compounds, with the quantitative collection of the resultant carbon dioxide and/or water for subsequent liquid scintillation assay of \(^{14}\text{C}\) or \(^{3}\text{H} \text{.} The practical problem is to suspend the dried sample material in the center of a confined quantity of oxygen and to provide for its ignition and its containment, suspended in the oxygen, with minimal loss of the heat until combustion is complete. It is then necessary to have an arrangement that permits the quantitative collection of the carbon dioxide and/or water into a measured volume of liquid scintillation counting solution.

The very significant contribution from the Lederle group was the standardization upon a 2-liter volume of oxygen and the demonstration that this could combust up to 300 mg of the usual dry organic material samples quantitatively and safely. We have never found it desirable to deviate either up or down from this sample size. We have sample bags that contribute only 50 mg of combustible weight to the system and leave 250 mg for the sample itself. In most instances this represents better than a gram of fresh, undried tissue. It might be stressed at this point that this technique is applicable only to samples in which the activity itself is in such a form that it is not volatile and will not be lost incidental to drying the sample preparatory to combustion.

Our technique employs an unmodified 2-liter heavy-walled Erlenmeyer filter flask as the combustion vessel. Such flasks are readily available, are inexpensive and tend to survive successive glass washings better than most of the modified versions that we have seen. For a number of years we conformed to the Lederle group recommendations and used a platinum-mesh sample holder on a platinum wire stalk, which in turn was carried by a glass rod through a rubber stopper in the neck of the flask. The use of an infrared ignition system permits this extreme simplification of the "flask head," because there is no need to provide for insulated, gas-tight electrical connections to an igniting filament or spark gap within
the flask as are employed in a number of other versions of this technique. Our "flask head" is simply a one-hole Neoprene No. 9 rubber stopper with a suitable length of Pyrex glass rod mounted in the hole of the stopper and attached to the stalk of the platinum sample basket. It is necessary to use Neoprene stoppers because standard black rubber laboratory stoppers are rapidly deteriorated by liquid scintillation counting solvents. Vinyl stoppers are also rapidly attacked by toluene. Silicone rubber withstands toluene very well but is a bit too slippery to stay in the flask mouth satisfactorily and is quite expensive. Closure of the side arm of the flask is simply effected with a 2-inch length of 5.0-mm O.D. × 1.6-mm wall silicone rubber tubing* and a pair of standard forceps, the jaws of which have been covered with short lengths of appropriate gum-rubber tubing to prevent their sharp edges from cutting the silicone tubing (Fig. 1).

The design of the sample basket is shown in Fig. 2. Kelly et al. described in detail the home fabrication of these platinum baskets from standard platinum-10 per cent iridium wire and the use of an electric spot welder. In a short communication we reported that we could make these baskets with the use of a Bunsen burner, tack hammer, and a pair of hemostats instead of the spot welder. The perimeter of the basket was made from a 12-cm length of 22-gauge platinum-iridium wire shaped into a square. The wire ends were held overlapping by clamping with a pair of forceps, heated to a dull-red glow in the burner flame, and

fused with the tap of a tack hammer against any convenient hard metal surface. The remaining wires for the basket were similarly welded into place using lengths of 22-gauge wire equally spaced, seven in each direction, to form a grid which could pass through the mouth of the 2-liter flask to be employed. This square grid was then mounted on a 15-cm length of 18-gauge platinum-iridium wire to serve as a center support post between the glass rod and the basket. On completion, the grid was folded across the center of its horizontal axis to form a trough in which samples could be placed. The wire stalk was finally bent at an angle which brought the platinum basket within about 2-cm of the wall of the flask with the sample head in position in the flask. The design represents a compromise between minimizing the mass of metal that takes away heat and still having a structure that will prevent samples from falling to the bottom of the flask and being extinguished.

Commercially made platinum baskets are available at a total cost for platinum and fabrication of about $60 per basket with mounting stalk.* For those with lean budgets, Conway has advocated the use of nichrome wire baskets.19 Nichrome wire is chemically more reactive than platinum so that it is possible that with certain sample materials, due to their chemistry or higher heat of combustion, some difficulty might be anticipated. (Any (3H) or (14C) that binds to the basket will be lost from quantitative recovery.) We have been successful in using the 16 mesh No. 24B and S nichrome wire gauze, that is readily available from most scientific supply houses, instead of resorting to special suppliers of 8-mesh gauze as reported by Conway.4

As indicated above, we have employed an infrared light beam for ignition of samples. This was chosen over the use of an igniter filament or spark within the flask for two reasons: (1) The use of a filament or spark requires a more complicated “flask head” because it must provide for two sealed, lead-in electric wires, and these must be connected to a power source for each ignition. (2) After a certain number of ignitions, which may be of the order of 100, platinum or nichrome filaments burn out. If this occurs during an ignition after the sample has been partially charred, the filament must be replaced and this sample will probably be lost. We found the commercially available Thomas-Ogg infrared igniter * to be effective and convenient. It permitted the use of the simplified “flask head” and gave several thousand ignitions before our first lamp burned out. Should a lamp burn out with a sample partially charred, a new lamp can be installed and the ignition completed without unstoppering the combustion flask. The combustion flask sold for use with this igniter is a specialized piece of glassware with a hemi-ball joint on the neck of the flask and with the flask head secured by a mechanical clamp. Apart from being more expensive than a Neoprene stopper, this design and those using standard taper glass joints, seem to us more hazardous since any excessively fast or large combustion may break the flask instead of popping the stopper as can occur with the Neoprene closure. The Thomas-Ogg igniter provides for aligning the flask and lamp and has provision for raising or lowering the infrared lamp in order to center the beam on the sample (Fig. 1). It also provides for tilting the flask toward the lamp so that the beam enters the flask at right angles to the flask wall.

Apart from providing the mechanics and electrical circuit for the ignition, the Thomas-Ogg igniter was designed to provide for containment of any explosive reaction and

* Baker Platinum Division, Engelhard Industries, Newark, N. J.

was accordingly constructed of rather heavy
gauge steel walls with a one-fourth-inch
thick acrylic plastic door. We must most
emphatically report that these seemingly
adequate design considerations have proven
insufficient in practice. Due to various
breaches in the procedure, there have been
several extremely violent explosions in
various laboratories. One such explosion
occurred in a laboratory at the National
Institutes of Health when ignition using a
Thomas-Ogg apparatus was attempted with
a flask which apparently contained residual
toluene. All of us who have had anything to
do with this technique of oxygen flask com-
bustion were appalled at the violence of
the explosion. The plastic door was shat-
tered, the steel walls were blown out, and
the heavy filter flask was reduced to powder.
Clearly this piece of equipment does not
afford adequate protection from an explo-
sion. The ignition switch, which in early
models was on the top of the cabinet, has
since been moved to about four feet down
the power cord.

In our own laboratory, we now conduct
all combustions with the Thomas-Ogg igniter
located inside a fume hood with a heavy,
shatterproof glass front window. With the
ignition switch located on the power cord,
the operator can stand back and to one
side at the time of ignition. Before any
readers decide that this is no technique for
them, may we hasten to reassure you that
in our laboratory more than 5000 combus-
tions have been done with no untoward
events, and in another group at NIH the
figure is well over 10,000. In the case of the
one explosion that occurred at NIH, it
seemed possible to reconstruct events to
indicate that there was a weak point in the
overall procedure. A used flask containing
residual toluene was too conveniently avail-
able to the technician who was responsible
for placing fresh samples into clean flasks
and flushing them with oxygen. Flask
loading and subsequent solvent aliquoting
and flask cleaning should be performed in
well-separated areas. Ours happens to have
been set up in different rooms and we de-
liberately keep it this way.

Samples for oxygen flask combustion are
normally prepared in small cellophane en-
vvelopes. These can be constructed from short
lengths of Visking tubing with the use of
Duco or similar household cellulose ester
cement to glue closed a short fold at the
bottom of the length of tubing, thereby form-
ing an envelope. Such Visking tubing en-
vvelopes are ideal and, by virtue of the semi-
permeable nature of the material, afford
rapid drying of fluid or wet solid samples.
However, they are laborious to make. Con-
siderable effort has been put into a search
for a commercial source of suitable enve-
lopes, and these are now available in the
form of rolls of folded cellophane strips with
crimped heat seals at 1-inch intervals*. Bags
can be cut from the roll of 2500 by a simple
transverse scissor cut through the middle of
the heat seal. One flap of the bag is slightly
longer than the other and permits the use of
a mouse-ear punch to produce a small hole
in the projecting flap so that it can be
mounted by a paper clip to an improvising
hanger of paper clips arranged on horizontal
rods on a ringstand. The bag can then
be pinched to open and it will accommodate
2 ml of fluid sample, homogenate, minced
tissue, powder and snips of chromatographic
paper. These commercial bags weigh ap-
proximately 50 mg and can accommodate up
to 250 mg of combustible dried material and
still be within permissible limits of the 2-
liter oxygen flask system. Drying in the cello-
phane bags is not as rapid as in the Visking
bags because cellophane has a waterproof
coating, which is essential to the heat sealing
but which does limit water loss to the path-
way through the open mouth of the bag. We
find that most biological samples of tissue or

* Combustion Envelopes from Ivers-Lee Co.,
Newark, N. J.
homogenate will go to dryness overnight or
in a couple of days if the bags are hung on a
rack in a hood where there is active air flow.
Drying can be further accelerated by the use
of an array of infrared heating lamps. It is
worth emphasizing that the limitation on
sample weight to 300 mg of combustible
material is due to the gas volume-pressure
considerations in this 2000-mi system. The
300-mg total weight can obviously be ex-
cceeded to the extent that a proportion of the
gross sample weight is contributed by incom-
bustible, inorganic salt.

As has been said before but cannot be
expressed too emphatically, volatile radio-
activity is lost incidental to this drying of
the samples. It is even difficult to retain
\((^{14}\text{C})\) in the form of carbonate despite ap-
propriate adjustment of fluid samples to an
alkaline pH. This is due to exchange of the
radioactive carbonate with atmospheric car-
bon dioxide. Another factor that has wor-
ried us is the possibility of bacterial degra-
dation of sample material and loss of activity
as a result of its conversion to carbon di-
oxide. At times when we have been working
with samples that contained radioactivity
in a compound that might be very suscep-
tible to bacterial decarboxylation, we have
added a few drops of liquid phenol to the
samples in bags as a bactericidal agent which
is fully combustible itself. With regard to
\((^{14}\text{C})\) in the form of carbonate, we have
found that the cellophane bags distintegrate
upon the addition of sodium hydroxide but
that they will withstand pH 12 borate buffer,
which should suffice to retain carbon dioxide
if drying can be done in an atmosphere free
of carbon dioxide as can be provided by an
appropriate desiccator. Fully dried samples,
expecially heat dried bags, tend to become
brittle, and the bags must be handled care-
fully to avoid mechanical loss of flakes of
sample material incidental to putting the bags
into the basket holders for combustion. A
wide variety of types of sample material
containing both \((^{11}\text{C})\) and \((^{3}\text{H})\) radioactiv-
ity have been combusted by ourselves and
other laboratories using this technique, with
essentially 100 per cent recovery of the
radioactivity so long as sample volatility is
excluded. We have had no difficulties from
the rather consistant quenching that has
been reported troublesome by some work-
ers.\(^{20-22}\) We presume this is a function of
how much excess oxygen remains in the
samples and therefore relates to the ex-
posure to air and alacrity with which sam-
ples are capped.

There are a few points worth mentioning
with regard to the combustion itself. This
technique does involve a combustion and
therefore there must be fuel to be comb-
busted. Some of the failures that we have
seen have been directly attributable to the
fact that the worker had a sample which had
insufficient combustible material to provide
the heat required for conversion of the sam-
ple to the desired carbon dioxide and water.
This means that when the sample size is
small, or when it is high in its inorganic salts
content, it may be desirable to add fuel. This
fuel may be in the form of a sucrose solution
to be evaporated with the sample or it may
be in the form of some snips of filter paper
put in the bag with the sample or may be
the sample deposited on the filter paper as
a few microliters of sample solution. Sam-
ples collected on Millipore filter generally
burn exceedingly well because of the cellu-
lose nitrate of the filter. We have found that
whole blood samples tend to sparkle at the
end of the combustion and have attributed
this to their iron content.

Since the infrared ignition depends upon
absorption of the light, it is necessary to
provide a black felt marker pen dot on the
sample bag or to include a small snip of
black paper in the bag to assure prompt
ignition. We might also belabor the seem-
ingly obvious. Oxygen is necessary and is
provided by putting a hose from a cylinder
in the neck of the flask for 10 seconds before
stoppering. One of the groups that set up our
technique had a series of failures due to omitting this step. In the same vein, one should not forget to have the clamp on the sidearm tube.

It is desirable to observe each combustion carefully for any signs of technical failure. If there is excessive smoking prior to the true ignition, particulate radioactivity may be deposited in the upper reaches of the flask and subsequently avoid solution in the solvent. Similarly, if any of the sample material falls or drips to the floor of the flask during the combustion, the process will be incomplete and quantitative recovery will not be obtained. With this technique as with any other method of combusting organic materials, one does encounter certain compounds which fail to combust quantitatively. This phenomenon is most frequently encountered with compounds having a high percentage of nitrogen or halogen. For this reason it is always good practice to determine with clean samples of a compound, that it is amenable to quantitative recovery.

Combustion of a sample is normally complete within less than one minute. As soon as the flame extinguishes, it is safe to remove the flask from any protective enclosure and set it aside for about five minutes to cool. The next step is the addition of the counting solvent. The composition of this depends upon whether one is dealing with (\(^3\)H) or (\(^14\)C). For the counting of (\(^3\)H) water we employ a solvent mixture of 30 per cent absolute methyl alcohol in reagent grade toluene with 4 g PPO and 100 mg POPOP per liter. Fifteen ml of this solvent has ample capacity to hold in solution at 0\(^\circ\)C the 0.18 ml of water that could arise from the combustion of 300 mg of carbohydrate sample. In practice, a volumetric pipette containing 15 mg of the solvent is attached to the silicone rubber sidearm on the flask after the five-minute cooling period and the clamp is released. Gas pressure in the flask is always negative with respect to atmospheric at this time and the solvent is drawn into the flask, following which the clamp is replaced on the sidearm tubing. The flask is swirled gently to distribute the solvent over the entire bottom and an inch or two up on the sidewalls. The upper walls of the flask and stopper should not be wetted. The flask is now placed in a cooling bath at \(-15\)\(^\circ\)C or lower with the cooling confined to the bottom inch of the flask and maintained for 15–20 minutes. We employ a mechanically refrigerated alcohol bath for this purpose, but it can equally well be done using a basin filled with a shallow layer of crushed dry ice in cellosolve. At the end of the condensing period an additional measured 3 ml of counting solvent is delivered again through the sidearm into the flask to rinse in any activity which may have sequestered in the solvent that wetted the sidearm initially. The flask is swirled to mix the solvents. The flask now contains 18 ml of solvent and can be set aside at room temperature for the subsequent removal of a 15-ml aliquot to be placed in a sample counting vial.

In the case of (\(^14\)C) combustions, including those in which there is both tritium and (\(^14\)C), a scintillation solvent of the composition shown in Table 1 is employed. Phenethylamine is a good, cheap absorbent for carbon dioxide and provides a higher counting efficiency than Hyamine and better carbon dioxide fixation than ethanolamine.\(^{28}\) The phenethylamine for this purpose must be of scintillation counting grade. This is now commercially available or can be pre-

<table>
<thead>
<tr>
<th>Phenethylamine</th>
<th>270 ml</th>
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<tr>
<td>Methanol</td>
<td>270 ml</td>
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<tr>
<td>Toluene</td>
<td>460 ml</td>
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<td>PPO</td>
<td>5 g</td>
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<tr>
<td>POPOP</td>
<td>100 mg</td>
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pared from standard material. We do this preparation quite simply by distilling it through a laboratory flash evaporating apparatus with the evaporating flask on a water bath at 95–100°C and with an aspirator water pump on 12°C colder water providing a vacuum of about 15 mm of mercury. Such a simple one-plate distillation yields crystal-clear phenethylamine of satisfactory quality for these purposes. If this is stored in full brown bottles in the refrigerator, it retains adequate performance characteristics for up to one year. The technique for the addition of the solvent for carbon dioxide absorption is exactly the same as that used for (3H) water absorption except that in this instance the absorption involves a chemical reaction rather than a condensation and can be permitted to occur at ice-water bath temperature instead of at −15°C. The supplemental sidearm rinse with 3 ml more solvent, which can be with or without the phenethylamine ingredient, is again imperative for quantitative recovery. In the case of either isotope and either solvent system, it is apparent that 15 ml out of 18 or five-sixths of the total combustion products wind up in the counting bottle.

An optional variation of technique is to prepare and use solvents without the PPO and POPOP scintillators in them and to provide the scintillators as a final step in the form of 1 ml of a 16-fold concentrate of PPO and POPOP in toluene added to the 15 ml of solvent in each sample bottle. This keeps the water-insoluble phosphors out of the combustion flasks and considerably facilitates the flask cleaning operation. We find that flask cleaning can be done very readily using a simple rinse with a warm-water solution of common laboratory detergent followed by a tap water rinse. Thorough drying is done in a glass drying oven or by inversion over a metal pipe delivering a jet of air. The air is heated while going through a few coils of the same pipe over a burner prior to delivery to the flask. Under no circumstances should any organic solvent such as acetone be used in a foolhardy effort to expedite drying of the flasks. The danger of a violent explosion from a residuum of such organic solvent in a flask is too great.

The technique presented here uses a minimum of specialized or expensive equipment. The glass components are as rugged as possible. The opportunity for a bigger combustion (euphemism for “explosion”) than is planned is at a minimum. The technique is not applicable to samples containing volatile radioactivity nor to samples whose net dry weight exceed about 250 mg. It is possibly not the technique of choice if your sample material is limited to 1–3 such as can be combusted within the final sample counting vial according to a recent publication. We would like to emphasize that almost any laboratory that is confronted with counting a variety of carbon and tritium labeled samples of biological origin will ultimately realize that combustion is an extremely useful technique. It is the answer to problems with color or insolubility of samples. It is a technique which any laboratory can adopt with a modest expenditure of money and effort.

**Peterson Automated Combustion**

We can proceed to the first description of what might be called “Second Generation of Oxygen Combustion Techniques” in liquid scintillation sample preparations. Early in 1966 a program was started at NIH to develop an automated combustion apparatus. After several planning sessions (with Dr. Sidney Siegel, Dr. Vincent Oliverio and myself), Dr. John Peterson, of the Biomedical Engineering Instrumentation Branch of the Division of Research Services at NIH, began work on the development of a practical device to permit the combustion of several hundred samples per day. About two years ago a promising prototype instrument had emerged and has been evaluated in the laboratory.
Two papers representing the work of Dr. John Peterson, Mr. Frank Wagner, Dr. Sidney Siegel, and Mr. Wilbert Nixon have recently been published, which describe the instrument and its performance.\textsuperscript{25,26}

\textbf{The Basic Apparatus}

Figure 3 shows the apparatus. Analogous to the bags used in the manual oxygen flask technique, the samples here are introduced in gelatin or Lexan poly-carbonate capsules. The capsule, containing up to 500 mg of dry sample, drops vertically downward into a furnace tube where it is combusted. Products of combustion are carried by the stream of oxygen into a condenser where they encounter an incoming stream of scintillation solvent. The dissolved combustion products in the solvent are delivered, at the bottom of the apparatus, into a liquid scintillation counting vial. To the left of the furnace assembly you see the oxygen gas flowmeter standing on top of the model K2/R Lauda refrigerated circulator which provides coolant to the condenser. To the right of the furnace tube assembly is a motor-driven burette to provide a measured volume of liquid scintillation counting solvent from the dark gallon jug at the extreme right. Figure 4 shows a diagram of the details of combustion tube and condenser. The blind-bored stopcock plug at the top serves to introduce the capsule without opening the top of the furnace tube to the atmosphere. Oxygen enters through the side tube just below the stopcock. The capsule falls through a guiding chimney to land on a bed of quartz chips maintained at a temperature of approximately 600\degree C. Here the sample bursts into flame and the pyrolysis products

\begin{figure}[h]
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\includegraphics[width=\textwidth]{Fig_3.png}
\caption{Peterson tritium sample combustion apparatus.}
\end{figure}

\begin{figure}[h]
\centering
\includegraphics[width=\textwidth]{Fig_4.png}
\caption{Diagram of Peterson tritium combustion apparatus.}
\end{figure}
are carried in the stream of oxygen down through an initial copper oxide catalyst to an hopalite (copper-manganese-mixed oxide) catalyst to complete the oxidation. The resultant carbon dioxide and water vapor leave the bottom of the furnace tube to enter the condenser. Here they meet a stream of liquid scintillation solvent being provided by a pump at a rate delivering 20 ml of solvent over the course of the two minutes required for the completion of the combustion. The combustion products condense at a temperature of 3°C and simultaneously dissolve in the scintillation solvent. This carries them down through the delivery tube into a standard liquid scintillation counting vial.

This constitutes the system for the determination of (°H) with the water derived from the sample being collected in a solvent consisting of 30 per cent methanol in toluene or any other cocktail a man may prefer. At the conclusion of such a combustion, radioactivity has been flushed from the combustion tube and condenser so that less than 0.1 per cent of the radioactivity remains to possibly contaminate the next sample. Approximately one minute is required to complete drainage of the solvent and to reload the solvent pump automatically before the next sample may be combusted.

(14C) Collection

Figure 5 shows the supplementary equipment needed for the collection of radiocarbon. For this purpose the water condenser below the furnace has been replaced by an outflow tube which connects directly with a carbon dioxide absorber column, seen to the right of the furnace. Figure 6 is a diagram of this apparatus. In this case the gaseous products of combustion are introduced at the bottom of a vertical condenser with which is mounted a spinner of twisted, stainless steel mesh. A stream of scintillation solvent containing phenethylamine enters at the top of the condenser and forms a continuous descending film on the inner wall of the condenser. The spinner assures mixing of the ascending gases and

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![Diagram of Peterson carbon-tritium absorption apparatus.](image-url)

---

"Fig. 5.—Peterson carbon-tritium combustion apparatus."
Dr. Nillo Kaartinen, at the University of Turku, Finland, was also developing an automated sample combustion technique. Very recently his design was incorporated in a commercial instrument to be marketed by the Packard Instrument Company, of Downers Grove, Illinois.

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vapors so that the carbon dioxide and water vapor are quantitatively absorbed in the descending solvent. This solvent discharges from the bottom of the apparatus into a liquid scintillation counting vial. As in the $^{(6)}$H combustion device, the solvent flow is programmed to deliver a volume of 20 ml over the course of the two-minute combustion.

The performance of this apparatus has been very good. Examples can be prepared at a sustained rate of one every three minutes. The radioisotope recovery seems to be essentially quantitative with a standard deviation on a series of replicates of approximately 3 per cent.

### Packard Sample Oxidizer

About the same time Dr. Peterson and his group were developing the previously described automated furnace combustion technique, Dr. Nillo Kaartinen, at the University of Turku, Finland, was also developing an automated sample combustion technique. Very recently his design was incorporated in a commercial instrument to be marketed by the Packard Instrument Company, of Downers Grove, Illinois.

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be delivered into a counting vial. This vial is supported in position on a pneumatic elevator.

In this apparatus, the principle is one of using an electrically heated platinum sample holder to ignite the sample material. The combustion is both contained and controlled by a laminar flow of oxygen gas entering at the base of the combustion chamber, surrounding the sample, and carrying the combustion products out the top into the condenser mechanism. Figure 9 shows the construction of the sample holder-igniter. Combustion is ignited and can be partially sustained to completion by the heat produced by an adjustable electric current through the spiral platinum coils of the heater. The heater is mounted on a circular block of Mycroy and is of such size, with respect to the base of the Pyrex combustion chamber, that it leaves a circumferential gap for the oxygen inlet, completely surrounding the sample. This entire assembly is on a pneumatically operated elevator. Figure 10 is a diagram of the system.

In operation, the sample elevator is lowered and the sample, as large as a gram or more, usually wrapped in paper tissue, is placed in the platinum carrier. The elevator is then pneumatically lifted. It maintains a seal to the bottom of the combustion chamber by a pressure of 25 psi for the duration of the combustion. This feature provides for effective relief of any overpressure occurring in the course of the combustion. One dual-purpose knob regulates the platinum heater temperature by an in-and-out movement and controls the quantity of oxygen flow by rotary movement. At the same time, as a safety feature, any oxygen flow has to be enabled by a simultaneous depression of a supplementary spring-loaded “oxygen-on” button. The operator initiates combustion as rapidly as possible, and by regulating oxygen flow and electrical heater current, can adjust the speed of combustion to achieve an even and complete oxidation of the sample. It should be noted that the glass combustion chamber is housed in an enclosure which is maintained at about 200°C by a circular electric heater at its base. This serves to prevent condensation of combustion products on the walls. In this apparatus the products of combustion are carried by the oxygen stream into a condenser, with no simultaneous admixture with counting solvent.

At the conclusion of the active combustion phase the operator releases the safety “oxygen-on” button and turns off the electrical sample igniter. At this point an automated program is started which does several things. It substitutes a current of nitrogen for oxygen to flush through the combustion chamber and condenser. It starts a pump which delivers a predetermined volume of scintillation counting solvent into the top of the condenser, where it and the nitrogen gas flush condensed combustion products into the counting vial,
which is now held by the operator under the discharge tip at the bottom of the condenser. The program also automatically re-loads the solvent pump and terminates the nitrogen flow after sufficient time has been allowed for collection of the entire sample under a nitrogen blanket in the counting vial. The operator promptly places the cap on the counting vial so that the sample is main-tained as free of oxygen as possible. This completes the preparation of a sample for tritium counting and the apparatus is ready for the next sample.

There are several additional features and options to the Packard-Kaartinen apparatus. Provision has been made for the injection, by the push of a button, of a measured approximately 70 µl of water directly into the bottom of the combustion chamber. This water is preheated so that it is delivered in the form of steam and can be used as a means of “carrier” addition in the combustion of small samples, or “carrier” rinse of the combustion chamber between samples to reduce carryover of activity from sample to sample. The apparatus also provides for the storage of three separate containers of scintillation solvents with a front panel switch to permit selection of the one to be used at any time. All solvent reservoirs are provided with internal nitrogen bubblers and gas manifolds, so that they can be prepared and maintained free of oxygen. There is also supplementary equipment which per-mits the extension of this method to (14C) samples.

CONCLUSION

The two semiautomated techniques of sample combustion described above are very new and look most promising. They have certain similarities, such as the size of samples they handle and their speed of
sample preparation. In other respects they are quite different. The Peterson apparatus appears to require less operator attention and manipulation. The Kaartinen technique demands operator training and experience but by the same token provides for accommodation to special sample requirements. Kaartinen has emphasized the objective of high counting efficiency and achieves it in part by the virtual elimination of oxygen quenching. At this early date it is not appropriate or really possible to make any further evaluation, much less comparison, of these interesting new devices. Both need considerable independent evaluation, but both seem to have the potential for relieving most objections to the use of combustion as a technique of sample preparation.

ACKNOWLEDGMENTS

The authors are particularly indebted to Charlene Denham Adamson, Frank Wagner, Dr. Sidney Siegel, Wilbert Nixon, Dr. Daniel Zaharho, and Noreen Considine, who have contributed to the development and evaluation of the techniques reported.

REFERENCES

The application of labeled organic compounds to the solution of a wide variety of chemical and physical problems often requires accurate knowledge of the radiochemical purity of the compound to be used. More often than not, such knowledge then leads to efforts to purify the compound. For both the analytical and purification steps gas-liquid radiochromatography (GLRC) and preparative gas-liquid chromatography (PGLC) have proved to be very powerful tools in radiotracer applications.

The reason for such great reliance on GLC stems the fact that at normally used specific activities, the fraction of molecules tagged with the radionuclide is exceedingly small and is beyond the reach of most analytical tools. Table 1 illustrates the problem by presenting fractions labeled at a specific activity of 1 mCi/mMole for a number of commonly used isotopes.

Except with (³⁶Cl), the bulk of the molecules in such preparations is unlabeled. This poses not only a serious analytical problem, but an even more serious purification problem. For example, if a (¹⁴C)-labeled compound (1 mCi/mMole has a radiochemical purity of 75 per cent (i.e., 25 per cent of the radioactivity is present in a contaminant), the purification step will involve the removal of a contaminant present in 0.5 per cent concentration. While 99.5 per cent chemically pure, the compound is only 75 per cent radiochemically pure. The situation may be much worse with isotopes of shorter half-lives.

Purity of Labeled Compounds

Another consequence of this fractional labeling is the possibility of obtaining a labeled compound of extremely high chemical purity but low radiochemical purity. Such a situation usually arises for one of two reasons. (1) When a small amount of a labeled compound of high specific activity is diluted with a large amount of chemically
pure carrier, any radioactive contaminant remains at its original specific activity but is then present in very low mass concentration. (2) One of the reactants may contain an impurity that will react preferentially with the labeled precursor, producing a small amount of high specific activity contaminant in a much larger amount of the desired low specific activity product. The latter situation argues for obtaining highly purified starting materials, and PGLC has been extensively used for this purpose.

Some time ago we reported on a survey of a few commercially available hydrocarbons and would like to repeat some of the data here to illustrate the above problems. The analytical tool employed was GLRC with an ion chamber detector for radioactivity measurement. Two compounds were purchased and analyzed by GLRC, tetradecane-1-(14C) and 1-dodecene-1-(14C). Only the tetradecane was purified. Figure 1 shows the tetradecane analysis before and after purification and Fig. 2 shows the dodecene as received. Both figures clearly indicate the presence of radioactive contaminants present in trace amounts that have much higher specific activities than the parent compound. Following purification by PGLC, the GLRC chromatograms shown in fig. 1B demonstrate that the tetradecane-(14C) was of very high purity with no contaminating radioactive peaks. It is interesting to report that the 1-dodecene-1-(14C) was 99.92 per cent impure radiochemically and more than 99 per cent pure chemically. The foregoing results suggest that normal purification techniques (distillation, recrystallization) are often unsuitable for purification of such systems and demonstrate that preparative scale gas-liquid chromatography can be a most useful tool. Reports in the literature and our own experience over several years have supported this contention.

In this chapter we will discuss the present state of the PGLC technique as reported in the literature, and we will describe some of...
our experiences, hoping to set forth some helpful guidelines for the use of PGLC for radioactive compounds. Many developments in PGLC for nonradioactive compounds will obviously be of importance to radiochemists.

Purification of labeled compounds generally involves a range of 1 mg to 5g quantities of material. Thus, large-diameter columns (greater than one inch) are usually not required and will not be discussed. For most of the work discussed here, columns of one-fourth to three-eighths inch internal diameter have been used. There are some distinct advantages in the use of such columns aside from their higher efficiency compared to larger bore columns: (1) direct correlation with analytical GLC data and (2) low cost per column. The latter is not an insignificant point because contamination of GLC columns with radioactive compounds is not rare. A 20-foot-by-three-eighths-inch ID column may be replaced at a cost of $30-$75, a one-by-20-inch ID column for hundreds of dollars.

CONVERSION TO PGLC

If one uses small columns, the only modifications needed to analytical GLC units to make them preparative scale are methods of repetitive sample injection and fraction collection.

Sample injection systems for such units may be automated and commercial units * with this function are available. They operate either in programmed hypodermic suction/injection cycle as in the Varian Aerograph or by a pressure controlled injection system as described by Boer.5 For PGLC used in connection with radiotracer synthesis, such automation of injection is not normally required. However, where large sample workup is required following a tracer experiment, it would be quite attractive, and the prospective user should weigh his needs carefully before acquiring the appropriate GLC.

Greater differences among instruments and techniques of PGLC arise in the fraction collection step. The automatic systems may be divided into three types: (1) that of Boer ⁵ where the traps are fixed and a heated multiposition valve directs the effluent to any one of ten traps; (2) that of Varian Instrument Co., where the traps rotate on a turntable and the appropriate one is connected on command to the exit port; and (3) where both traps and exit port are fixed and the effluent is directed to the appropriate trap via a manifold with solenoid operated valves (F & M Scientific Co.). Our experience has been almost entirely with the rotating traps system, but we had occasion to use the Boer system for the separation of light hydrocarbons and found it quite satisfactory, giving excellent recoveries with the traps used (spiral, Fig. 4). Its major limitation lies in the limited temperature stability of the heated valve at about 200° C. A valve operating at greater than 300° C would be preferable. A priori it appears to us that a manifolding system might engender many problems due to contamination of the dead volume.

The design of the traps themselves if of major importance in PGLC and a considerable amount of literature is available on the subject. Quantitative recovery is highly desirable because of the cost of most radioactive preparations. The traps generally consist of three types: empty traps of varying design, packed traps using dry or wet packings, and electrostatic precipitators.

Borka and Privett ⁴ described one such precipitator consisting of a wire down the center of the trap as one electrode and a piece of aluminum foil around the outside as the other. High boiling esters, acids, and alcohols were tested. The effectiveness of the trap was measured by disappearance of

the effluent fog.* AC voltages required were 5500–6800; DC voltage was 8–12,000 depending on the compound. Clear evidence of decomposition was obtained from AC precipitation, none from DC. Recoveries were 90 to 96 per cent with 1-μl samples. Fish modified a Varian-Aerograph trap to serve as an electrostatic precipitator. Bierl et al. used traps made of short columns of cold GLC packing and reported recoveries of 80–100 per cent when the proper packing was used. Thus, methyl laurate was recovered to the extent of equivalent to 32 per cent on Poropak Q and 100 per cent on 5 per cent Carbowax 20M. However, recovery of the trapped fraction is usually inconvenient. Another method of minimizing fogging is the hot-cold trap which heats the incoming stream to melt the solid particles and then condenses the liquid on the trap walls. Such a trap is shown in Fig. 3. Verzele has studied a number of traps of different design and concluded that a double bulb trap of the type sold by Varian-Aerograph (Fig. 4A) was most efficient and that recoveries increase with elution temperature because the fraction is present in a high concentration. A more reasonable conclusion is that at low temperatures the losses were due to condensation inside the GLC. A comprehensive review of fraction collection methods has been presented by Perry. The reader is referred to this paper for an excellent survey.

**EXPERIMENTAL**

The work to be reported on below involves entirely the use of the Varian-Aerograph Autoprep Model A-700. In most cases, the operation was manual (injection periodically by syringe and manual trap changing). When automatic injection and fraction collection were required, the unit operated satisfactorily. The columns normally used were three-eighths-inch inside

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* This is a dubious criterion. We have found 95% recoveries with materials that produce fogs at the exit port of the trap.
diameter by 10–20-foot aluminum, and the array of column packings is shown in Table 2.

The above column packings cost about $30–$50 each, and for a modest sum one has available a wide variety of packings. Because the behavior of three-eighths-inch ID columns closely paralleled that of our one-fourth-inch ID analytical GLC column,1 data from the latter always guided the use of the former.

The packings in Table 2 cover a wide range in polarities and some (e.g., AgNO₃-ethylene glycol) were used for highly selective separations. Temperature ranges from ambient to greater than 250° C have been covered with materials such as SF-96, APZ-L, OV-1; OV-1 has been used up to 350° C without excessive bleed. Flow rates of about 100 ml/min have been generally used. This allows both efficient trapping and the emergence of peaks in a reasonable time (on the average, 30 minutes, but up to two hours). When long emergence times are involved, it is possible to overlap runs to save time. This has been done when fairly large volumes (greater than 5 ml) were being processed. Generally, injections of 0.1–0.3 ml were used with no serious loss in resolution. In practically all the work we have done, liquid N₂ was the coolant, even with the highest boiling compounds.

An essential modification to the Autoprep was the installation of an auxiliary heater very close to the tip of the exit orifice. It eliminated sample loss and contamination resulting from early condensation.

**Prior Stripping**

Contamination of the preheater, column, and other parts of the system poses a continual threat to PGLC. When the compound being purified is somewhat thermally unstable, it is impossible to avoid contamination, and scrupulous equipment cleanup after such a purification is required. Many

<table>
<thead>
<tr>
<th>Designation</th>
<th>Description</th>
<th>Source</th>
<th>Loading</th>
<th>Solid Support</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>SF-96</td>
<td>Methyl Silicone Fluid, General Electric</td>
<td>Applied Science Lab.</td>
<td>30%</td>
<td>42/60</td>
<td>Varian Aerograph</td>
</tr>
<tr>
<td>CBX-20M</td>
<td>Polyethylene Glycol MW ~ 20000</td>
<td>Varian Aerograph</td>
<td>30%</td>
<td>50/60</td>
<td>Anaprep A Analabs</td>
</tr>
<tr>
<td>HAM-18</td>
<td>Hallcomide M-18, N, N-Dimethylamide of Stearic Acid</td>
<td>Varian Aerograph</td>
<td>30%</td>
<td>50/60</td>
<td>Anaprep A Analabs</td>
</tr>
<tr>
<td>AgNO₃-EG</td>
<td>4.2M AgNO₃ in Ethylene Glycol</td>
<td>Prepared at ERC</td>
<td>40%</td>
<td>42/60</td>
<td>Varian Aerograph</td>
</tr>
<tr>
<td>FFAP</td>
<td>Free Fatty Acid Phase</td>
<td>Varian Aerograph</td>
<td>5%</td>
<td>45/60</td>
<td>Varian Aerograph</td>
</tr>
<tr>
<td>SF-96</td>
<td>Neopentylglycol Sebacate</td>
<td>Analabs</td>
<td>30%</td>
<td>50/60</td>
<td>Anaprep A Analabs</td>
</tr>
<tr>
<td>TCEP</td>
<td>1,2,3-Tris (2-cyanoethoxy) propane</td>
<td>Varian Aerograph</td>
<td>30%</td>
<td>50/60</td>
<td>Anaprep A Analabs</td>
</tr>
<tr>
<td>CBX-20M-TPA</td>
<td>CBX-20M terminated with terephthalic acid</td>
<td>Varian Aerograph</td>
<td>30%</td>
<td>50/60</td>
<td>Anaprep A Analabs</td>
</tr>
<tr>
<td>VAM-940</td>
<td>Versamid 940. General Mills</td>
<td>General Mills</td>
<td>30%</td>
<td>50/60</td>
<td>Anaprep A Analabs</td>
</tr>
<tr>
<td>APZ-L</td>
<td>Aprezon L</td>
<td>Varian Aerograph</td>
<td>20%</td>
<td>50/60</td>
<td>Anaprep A Analabs</td>
</tr>
<tr>
<td>PMPE</td>
<td>Poly-m-phenyl ether, 5 ring</td>
<td>Varian Aerograph</td>
<td>30%</td>
<td>50/60</td>
<td>Anaprep A Analabs</td>
</tr>
<tr>
<td>OV-1</td>
<td>High Temperature Methyl Silicone</td>
<td>Applied Science Lab.</td>
<td>5%</td>
<td>45/60</td>
<td>Varian Aerograph</td>
</tr>
</tbody>
</table>
preparations contain heavy ends in trace amounts that can deposit in the front end of the GLC system and slowly bleed radioactivity for weeks. This can be avoided by prior stripping in a separate, primitive GLC. We use a very simple GLC containing an eight-inch-by-three-eighths-inch packed column and a thermal conductivity detector and pass the entire sample through this column, trapping the volatiles for resubmission to the Autoprep. The short stripping column is discarded. This practice has served to reduce contamination incidents considerably.

Traps

We have used three types of traps shown in Fig. 4A, B and C. The first was supplied by Varian-Aerograph; the second was reported by Stevens and Mold to be effective for fogged effluents; and the third is a simple spiral trap with an expanded center portion. We have found the last to be surprisingly efficient for a wide range of compounds. Its application to low boiling compounds is shown in Table 3. Recoveries of low boilers are in the region of 90–100 per cent. A useful rule of thumb in the construction of such a spiral trap is that the residence time of a peak should be at least 15 s. Thus, at 100 ml/min helium effluent flow, the expanded body of the trap should be approximately 25 ml. The trap shown can hold about 4 ml before the lower exit port is covered.

The spiral trap is also effective for higher boilers. The recoveries of a homologous series of methyl esters of fatty acids are shown in Table 4 which includes the effect on purity of one pass through the Autoprep. Trapping efficiencies are high and purity is excellent.

Another indication of the broad spectrum of compounds we have worked with and of the trapping efficiency of the spiral trap is shown in Table 5. Note particularly the high efficiency of trapping for known foggers such as dotriacontane. Table 5 also shows one of the very few cases where the cavity trap was more efficient than the spiral trap.

### Table 3.—Compounds of High Volatility

<table>
<thead>
<tr>
<th>Compound</th>
<th>b.p.</th>
<th>Trapping Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>1-Butene</td>
<td>–5°</td>
<td>80–100%</td>
</tr>
<tr>
<td>Ethylene Oxide</td>
<td>13.5°</td>
<td>94%</td>
</tr>
<tr>
<td>1,1-Dimethylcyclopropane</td>
<td>20°</td>
<td>56%*</td>
</tr>
<tr>
<td>Iso-Pentane</td>
<td>28°</td>
<td>91–100%</td>
</tr>
<tr>
<td>1-Pentene</td>
<td>30°</td>
<td>95–98%</td>
</tr>
<tr>
<td>2-Methylpentene</td>
<td>35°</td>
<td>90%</td>
</tr>
<tr>
<td>n-Pentane</td>
<td>36°</td>
<td>100%</td>
</tr>
<tr>
<td>n-Propyl Chloride</td>
<td>46.4°</td>
<td>90%</td>
</tr>
</tbody>
</table>

* Varian-Aerograph cavity trap. Others, spiral trap.

### Table 4.—Efficiency of Spiral Trap for Methyl Esters

<table>
<thead>
<tr>
<th>Ester Methyl</th>
<th>b.p.</th>
<th>Before (%)</th>
<th>After (%)</th>
<th>Trapping Efficiency (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Propionate</td>
<td>80</td>
<td>99.0</td>
<td>99.9</td>
<td>94</td>
</tr>
<tr>
<td>Butyrate</td>
<td>102</td>
<td>99.4</td>
<td>99.8</td>
<td>93</td>
</tr>
<tr>
<td>Valerate</td>
<td>127</td>
<td>99.2</td>
<td>99.9</td>
<td>93</td>
</tr>
<tr>
<td>Hexanoate</td>
<td>130</td>
<td>98.8</td>
<td>99.9</td>
<td>93</td>
</tr>
<tr>
<td>Heptanoate</td>
<td>172</td>
<td>99.5</td>
<td>99.9</td>
<td>97</td>
</tr>
<tr>
<td>Octanoate</td>
<td>192</td>
<td>99.1</td>
<td>99.9</td>
<td>98</td>
</tr>
<tr>
<td>Decanoate</td>
<td>223</td>
<td>98.3</td>
<td>99.9</td>
<td>97</td>
</tr>
<tr>
<td>Laurate</td>
<td>148/18</td>
<td>99.4</td>
<td>99.6</td>
<td>98</td>
</tr>
<tr>
<td>Palmitate</td>
<td>196/15</td>
<td>99.5</td>
<td>99.9</td>
<td>95</td>
</tr>
<tr>
<td>Stearate</td>
<td>214/15</td>
<td>95.0</td>
<td>99.8</td>
<td>93</td>
</tr>
</tbody>
</table>
An attempt was made to purify some free carboxylic acids of varying volatility. For this we needed a column packing of 5 per cent, CBX-20M-TPA on Chromosorb G in an eight-inch-by-three-eighths-inch column. As seen in Table 6, only methylacrylic acid was quantitatively recovered; stearic and oleic acid were not. The loss of the fatty acids is believed to have been due to the metal surfaces of the system because of the high adsorptivity of such compounds.

The trapping efficiencies in Tables 3, 4 and 5 were determined on sample sizes of 200–1000 mg. We have had only limited experience with samples less than 100 mg, but generally these have also been recovered with good efficiency as shown in Table 7. Recoveries range from 88–100 per cent, which is quite satisfactory for this simple collection system.

The automatic trapping and collection feature of the Autoprep has occasionally been used. In one case we processed 100 ml of n-propyl chloride which was contaminated with 1 per cent of butyl chlorides, an unacceptable contaminant for the application to carbene insertion studies. The spiral trap was used with acetone-CO₂ as coolant and 1-ml injections made every 20 minutes. Twenty-four ml were collected every eight hours, and the final butyl chloride content was 0.001 per cent.

Resolution of Overlapping Peaks

While it is desirable to resolve GLC peaks completely for good product recovery and purity, it is not essential. Fractions resulting in overlapping peaks can on occasion be handled in a way to yield a

### Table 5.—Variety of Compounds Trapped

<table>
<thead>
<tr>
<th>Compound</th>
<th>Trapping Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>m-Heptane</td>
<td>98–100%</td>
</tr>
<tr>
<td>1-Heptene</td>
<td>100%</td>
</tr>
<tr>
<td>Toluene</td>
<td>98%</td>
</tr>
<tr>
<td>Styrene</td>
<td>100%</td>
</tr>
<tr>
<td>n-Butyl Chloride</td>
<td>98%</td>
</tr>
<tr>
<td>Methyl Stearate</td>
<td>95%</td>
</tr>
<tr>
<td>n-Butyraldehyde</td>
<td>96%</td>
</tr>
<tr>
<td>Benzylcyclohexane</td>
<td>95%</td>
</tr>
<tr>
<td>2-Octadecanone</td>
<td>91%</td>
</tr>
<tr>
<td>n-Propanol</td>
<td>93%</td>
</tr>
<tr>
<td>Allyl Alcohol</td>
<td>97%</td>
</tr>
<tr>
<td>Dimethyl Disulfide</td>
<td>97%</td>
</tr>
<tr>
<td>n-Hexadecane</td>
<td>98%</td>
</tr>
<tr>
<td>n-Dotriacontane</td>
<td>100%</td>
</tr>
<tr>
<td>1-Hexadecanol</td>
<td>94%</td>
</tr>
<tr>
<td>1-Octadecanol</td>
<td>87% (Cavity Trap)</td>
</tr>
<tr>
<td>1-Octadecanol</td>
<td>78%</td>
</tr>
</tbody>
</table>

### Table 6.—Results with Free Acids

<table>
<thead>
<tr>
<th>Acid</th>
<th>Recovery</th>
</tr>
</thead>
<tbody>
<tr>
<td>Methacrylic Acid</td>
<td>97%</td>
</tr>
<tr>
<td>Oleic Acid</td>
<td>26%</td>
</tr>
<tr>
<td>Stearic Acid</td>
<td>21%</td>
</tr>
</tbody>
</table>

### Table 7.—Trapping of Small Samples

<table>
<thead>
<tr>
<th>Sample</th>
<th>Efficiency</th>
<th>Final Purity</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 mg n-Heptane in 1 ml n-Hexane</td>
<td>100%</td>
<td>90%</td>
</tr>
<tr>
<td>10 mg n-Dotriacontane in 1 ml n-Octane</td>
<td>100%</td>
<td>99%</td>
</tr>
<tr>
<td>10–60 mg Methyl Stearate in 1 ml Benzene</td>
<td>95%</td>
<td>99%</td>
</tr>
<tr>
<td>10–60 mg Methyl Oleate in 1 ml Benzene</td>
<td>88%</td>
<td>99%</td>
</tr>
</tbody>
</table>
compound of excellent purity but always at a sacrifice in recovery. Some examples serve to illustrate this.

The first was the preparation of pure n-propanol (B.P., 97.8° C) from a mixture containing 3 per cent sec-butanol (B.P., 99.5° C). A 20-foot-by-three-eighths-inch column of HAM-18 was used and injections of 400 μl were made. Figure 5 shows the chromatogram and the use of overlapping injections to save time. The numbers correspond to the peaks from a particular injection. The trap was inserted and removed as shown; n-Propanol of 99.9 per cent purity was obtained in one pass.

The second example, a more difficult separation, involved the dl and meso diastereoisomers of 2,4 diacetoxy pentane. A 20-foot-by-three-eighths-inch column of 30 per cent NPGSE was used at 143° C with 200-μl samples. Figure 6 shows a typical chromatogram and the cut points and purities of each isolated fraction. The most difficult resolution was concerned with the isolation of pure 2- and 3-chloropentane from the commercial compounds of 70 per cent purity; the chief impurity was the other isomer. A 20-foot-by-three-eighths-inch column of 30 per cent SF-96 at 70° C was employed with injections of 200 μl. Figure 7 shows a typical chromatogram of commercial unlabeled 2-chloropentane; fraction 1 was isolated at the cut points shown (solid lines) and was found to be 92 per cent pure. It was then rechromatographed with results shown in the dashed-line chromatogram with cut points at the dashed lines. This second fraction was 98.4 per cent 2-chloropentane. Isolation of pure 3-chloropentane shown in Fig. 8 proceeded along similar lines with a purity of 98.3 per cent after two passes. (Cut points for 3-chloropentane were the same in both passes.)

Cleanup of complex reaction mixtures in radiotracer synthesis is often tedious and nonquantitative. PGLC frequently permits a one-step purification as used in the Wittig synthesis of 1-(14C)-labeled olefins. The final distillate usually consisted of the olefin.
Fig. 8.—Purification of 3-chloropentane.

(70%), benzene (25%), and several per cent of unreacted carbonyl starting material. Passage of the distillate through the Autoprep using a 20-foot-by-three-eighths-inch, 30 per cent TCEP column gave olefins (C₅-C₈) of 99.3-99.9 per cent chemical and radiochemical purity.

PROBLEMS AND LIMITATIONS

Contamination

The most serious problem with radioactive compounds in PGLC is contamination of the system. It is important to use a short stripping column (eight by three-eighths inches) in a primitive GLC unit to eliminate radioactive heavy ends which are troublesome contaminants in many purchased or stored samples. Because heavy ends vary in volatility and may migrate into the effluent, we have found it necessary to discard the short column after each injection rather than wait until the entire sample has been sent through. The short column reduces contamination because the sample emerges in a very short time, indicating that the practice of placing the stripping column ahead of the working column in prep GLC is dangerous, since one must wait until the chromatogram is completed before discarding the stripping section. Even with this precaution, contamination becomes a problem if the Autoprep is used for both high- and low-level radiotracer work. An example of this problem appeared following the preparation of 7 mMole of (³H)-methyl propionate at a specific activity of 0.25 mCi/mMole. In this reaction, (³H)-methanol was used in excess to ensure complete conversion of the propionyl chloride. A 20-foot-by-three-eighths-inch, 30 per cent HAM-18 column was used for the isolation of the pure ester from the reaction mixture.

Some time after completion of this project, we had occasion to concentrate a dilute solution of (¹⁴C)-labeled methanol of very low specific activity. Such concentrations are most conveniently made by PGLC and we used the above column. As is the case with low activity systems, some unlabeled methanol was first injected into the Autoprep, trapped and counted to determine if the background was reasonable. The results showed a high background which slowly declined with repeated injections:

<table>
<thead>
<tr>
<th>Injection</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>1st 300 µl</td>
<td>47,400 cpm</td>
</tr>
<tr>
<td>2nd 300 µl</td>
<td>21,400 cpm</td>
</tr>
<tr>
<td>3rd 300 µl</td>
<td>23,000 cpm</td>
</tr>
<tr>
<td>1 ml injection, no trapping</td>
<td></td>
</tr>
<tr>
<td>4th 300 µl</td>
<td>11,900 cpm</td>
</tr>
</tbody>
</table>

Spectrometric analysis of the activity in a Packard 3003 liquid scintillation spectrometer showed it to be mainly (³H). At this point the column was discarded and the Autoprep was disassembled and cleaned. Cleanup included the check valve on the He inlet, inlet tube, He line, column to detector block fitting, detector block and filaments, and outlet tube from detector block to traps. Following assembly and installation of a new column, background value for (³H) was only twice the true background (40 cpm).

Recovery

It is well known that compounds with very high boiling points, thermally unstable compounds, and highly reactive compounds are unsuited to purification by GLC. This does not mean that they are unsuited to
conventional analytical GLC, because if conditions are held constant, it is sometimes possible to obtain good precision in peak area measurements even though some of the sample is left on the column. However, such a condition is intolerable with radioactive compounds because the retained and/or degraded product can and usually does slowly bleed radioactivity into the effluent. Jansen and Baglan followed the recovery of trimethylsilyl derivatives of $^{14}$C-labeled sugars, cholesterol, and alcohols. About 25 per cent of the sugars was recovered: 80 per cent of the cholesterol derivative, and 25 per cent of the octadecanol-1 derivative. Radioactivity was found distributed throughout the column. However, even with such great losses, the results were reproducible, demonstrating that guidance for the PGLC by conventional analytical GLC would have been misleading.

A major uncertainty in Jansen's work was the failure to achieve radioactivity closure (to account for all the activity injected in the column). Whether the loss was due to inefficient trapping, production of untrapped degradation products (e.g., $^{14}$CO$_2$) or $\beta$-ray self-absorption by the column packing was not demonstrated. Analytical guidance by GLRC, wherein the activity injected was found to be much greater than the activity collected, would have better identified the problem.

Reactions in GLC

Chemical reactions may occur during passage through a GLC. The preparation of $^{14}$C-labeled 2-methyl-2-undecanol by dehydration with I$_2$ via

$$
\text{O} \quad \text{(14C)}
$$
$$
\text{C-C-C}_9 \rightarrow \text{C-C-C}_9 + \text{(14C)H}_2\text{MgI} \rightarrow \text{C-C-C}_9 \rightarrow \text{C-C-C}_9 \\
\Delta
$$

was studied. The analytical GLRC trace showed that the reaction mixture consisted of 80 per cent labeled olefin, 5 percent unreacted labeled alcohol, and 15 per cent unlabeled ketone. The Autoprep GLC showed no free alcohol but showed the other peaks. Additional experiments with the starting alcohol showed that quantitative dehydration occurred in the heated inlet system (231°C) of the Autoprep with up to 0.5-ml injection. Both olefin isomers were formed in the concentrations of 32 per cent for the 1-olefin and 68 per cent for the 2-olefin. It was also found that t-amyl alcohol dehydrated in the Autoprep to the extent of 20 per cent.

Isomerization

Double bond isomerization may also occur, depending on the column used. In attempting to purify some $^{14}$C-labeled methylenecyclohexane on a VAM-940 column, it was found that a second peak appeared in the preparative chromatogram which persisted on rechromatography of the purified parent peak. Isolation of the second peak showed it to be 1-methylcyclohexene as shown in Table 8 in which the extent of isomerization is a function of sample size, more severe with smaller samples. Substitution of the VAM-940 column with a TCEP column eliminated the reaction. One ex-

<table>
<thead>
<tr>
<th>Methylcyclohexane - methylenecyclohexane + 1-methylcyclohexane</th>
<th>Per cent Each Fraction in Column Effluent</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amount Injected (mg)</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>19.5</td>
</tr>
<tr>
<td>106</td>
<td>46.0</td>
</tr>
<tr>
<td>391</td>
<td>60.7</td>
</tr>
<tr>
<td>393</td>
<td>63.0</td>
</tr>
<tr>
<td>80.5</td>
<td>54</td>
</tr>
<tr>
<td>39.3</td>
<td>37.0</td>
</tr>
</tbody>
</table>

* 20-foot-by-three-eighths-Inch VAM-940, 95° C, Inlet 224° C.
planation is that the VAM-940 column was contaminated with some iodine from earlier work and that same free HI was responsible for the isomerization.

CONCLUSIONS

On the basis of several years' experience with preparative GLC for radioactive compounds, some general conclusions and recommendations can be made.

1. Column size of a preparative unit should be close to that of the analytical column for closest guidance on the conditions for best separation.

2. It is highly desirable to have a gas liquid radiochromatography system available for guiding PGLC work. Commercial radioactivity detectors are available based on proportional flow counting, ion chamber detection, and liquid scintillation counting of (14CO₂) and (3H₂). A conventional analytical GLC will provide no data on decomposition unless one keeps track of the activity injected and eluted.

3. Column and equipment contamination is an ever present threat. When the PGLC is to be used for low activity samples following high activity preparative work, injections of unlabeled carriers are required to purge the column and these must be counted to determine when the system is clean. If general contamination has occurred, replacement of the column and cleaning of the inlet and outlet systems is required.

4. Preparative samples should be stripped on a separate GLC using eight-by-three-eighths-inch packed columns that are immediately discarded after each injection. Attaching such stripping columns to the front end of the working column is a dubious practice that may lead to contamination.

5. The spiral trap described in this report is effective for the most of the samples we have tested, ranging from C₄ to C₃₂ hydrocarbons and up to C₁₈ esters and alcohols. A useful rule for determining the size of the expanded center portion is that its volume should be at least 25 per cent of the carrier gas flow per minute. According to the literature, quantitative trapping may also be achieved by using a GLC column packing in the trap, electrostatic precipitators using DC voltage and hot-cold traps aimed at melting the aerosol particles.

6. It is not essential that contaminating radioactive fractions be completely resolved for the isolation of radiochemically pure fractions. Judicious cutting of the fractions followed by rechromatography of the enriched fraction can result in satisfactory purity, but with a loss in yield.

7. The possibility of on-column chemical reactions should be borne in mind. Isomerization and dehydration reactions have been demonstrated. Preliminary chromatograms with the material under test should be made and any anomalous peaks carefully examined before committing a valuable preparation to the GLC.

8. The continued use of a preparative GLC, even with adequate precaution, is usually accompanied by a steady buildup of decomposition products in the front end of the system. Besides contributing radioactivity by slow pyrolysis, such decomposition products may also act as catalysts in on-column chemistry (e.g., acids, bases). Periodic cleanup is recommended.

REFERENCES

8. Pichat, L., Baret, C., Guermont, J. P., and
LIQUID SCINTILLATION RADIOASSAY OF THIN-LAYER CHROMATOGRAMS

F. Snyder

Procedures available for the radioassay of thin-layer chromatograms (TLC) have been reviewed in some depth. The methods include elution analysis, strip scanning, autoradiography, zone analysis, beta camera detection, and combustion analysis. Comparisons to strip scans, autoradiograms, and betagrams have shown that liquid scintillation radioassay is the most sensitive procedure for detecting low-energy β-emitters on thin-layer chromatoplates. The special scraping equipment developed for the procedure has provided the highest degree of resolution measurable on thin-layer chromatograms. This article gives a detailed account of the instrumentation, scintillation solutions, and techniques developed in our laboratories during the past decade for (14C) and (3H) liquid scintillation assay of thin-layer chromatograms.

Composition of Liquid Scintillation Solutions Recommended for TLC Radioassay

Special scintillation solutions must be used in conjunction with the radioassay of thin-layer chromatographic layers, since problems in quantitation are otherwise encountered. The solutions should contain either water for deactivation or gels for suspension of the adsorbent particles.

Solutions for Nonsuspension Assay

Dioxane-water solvent system: 7 g 2,5-diphenyloxazole, 0.3 g p-bis-2-(4-methyl-5-phenyloxazolyl) benzene, and 100 g naphthalene (Eastman) are diluted to one liter with reagent-grade dioxane. This solution is ready for use after it is mixed with water in the proportions of 15:3 (v/v).

The Medical Division, Oak Ridge Associated Universities, is under contract with the United States Atomic Energy Commission.

Fred Snyder, Ph.D.: Medical Division, Oak Ridge Associated Universities, Inc., Oak Ridge, Tenn.
Toluene - naphthalene - methylcellosolve-water solvent system: 4 g 2,5-bis-[5'-t-butylenzoxazolyl-(2')]-thiophene and 80 g naphthalene are dissolved in 600 ml toluene and 400 ml methylcellosolve. This solution is ready for use after it is mixed with water in the proportions of 30:1 (v/v).

Solutions for Suspension Assay

It is best to use systems that suspend particles when it is found that radioactive compounds remain on particles in the systems described above. Generally, a suitable solvent for this purpose is toluene, since such nonpolar solvents do not tend to elute compounds from the adsorbent particle. The scintillation solution 10,12 consists of 5 g 2,5-diphenyloxazole and 0.3 g p-bis-2-(4-methyl-5-phenyloxazolyl) benzene in one liter of reagent-grade Mallinckrodt toluene. This scintillation solution is used for making the suspending gel. Although several gel agents for suspension counting are available, our group has found Cab-O-Sil (finely divided silica available from the Cabot Corp.) to be satisfactory when mixed (4% w/v) with the toluene scintillation solvent.10 The addition of Cab-O-Sil has essentially no effect on the counting efficiency.

FACTORS AFFECTING LIQUID SCINTILLATION EFFICIENCIES IN TLC RADIOASSAY

Quenching

Most of the compounds used as the layer component in TLC, including inorganic oxides and organic matter such as ion-exchange resins or cellulose, have no effect on counting efficiencies in a liquid scintillation spectrometer. Metals (Ag, borate, arsenite) or organic materials (silicone), used to impregnate adsorbent layers, and iodine vapor or dichlorofluorescein and Rhodamine dyes, commonly used to visualize organic compounds on thin-layer chromatograms, are also nonquenchers. On the other hand, visualizing agents such as H₂SO₄ or alcoholic sprays (iodine in methanol) can cause severe quenching problems. Residual solvents on the layers can also cause quenching. However, pyridine and toluene, often used as chromatographic solvents and both difficult to remove, have no quenching properties. The polyvinyl alcohols and other organic binders and the thin support surfaces used in commercially available plates (plastics, aluminum sheeting) are not quenchers in the liquid scintillation systems described above. Although quenching of TLC components can be determined only empirically,19 some general information on specific components already tested has been presented in other papers.2,10 It is wise to determine the pulse-height spectra when testing possible quenchers, since minor adjustments in instrument settings can minimize or eliminate some of the quenching problems manifested by minor spectral shifts.

Self-absorption

Self-absorption losses due to adsorption of labeled compounds on the adsorbent layer can prevent quantitative radioassay.10,12 This problem can generally be prevented by using the scintillation solutions containing water, since they are polar enough to deactivate the adsorptive sites on these surfaces. We believe that such solutions make it possible for the scintillators to penetrate the crystal lattice of adsorbent particles. When a careful choice of scintillation solutions cannot eliminate self-absorption losses, it is often possible at least to reduce the size of the adsorbent particle to less than that of the distance traveled by the lowest energy of the β particle being assayed. The proportion of a labeled compound remaining on the particles after chromatography can easily be assessed by measuring the radioactivity of an aliquot of the scintillation fluid remaining in the supernatant after sedimentation of the particles by centrifugation or after filtration with microfilters. This measurement is compared to a
similar sample that is assayed in the absence of adsorbent.

An alternate choice is to add adsorbent to a vial containing an unseparated radioactive sample, previously assayed, to centrifuge it, and to redetermine the proportion of activity distributed in the solution and on the particles. The activity on the particles and the activity in solution can be assayed using the scintillation solutions described above. The efficiencies for each condition can be determined by relating to reference compounds and substituting known values into the equation

$$\text{total dpm} = \frac{\text{cpm on particles}}{\text{efficiency for particles}} + \frac{\text{cpm in solution}}{\text{efficiency in solution}}$$

Self-absorption losses can also be the consequences of the adsorption of labeled compounds directly on the inside glass surfaces of liquid scintillation counting vials.

(See Chapter 15.) This problem can be detected by the radioassay of a sample before and after the quantitative transfer from one counting vial to another. The problem with glass surfaces can generally be prevented by rinsing the vials with unlabeled carrier or, better yet, by using plastic vials. However, with plastic vials, one must guard against the loss of radioactivity because of labeled substances penetrating the plastic.

**TECHNIQUES AND INSTRUMENTATION FOR REMOVAL OF ZONES FROM CHROMATOGRAPHS**

Zones can be removed from chromatographic layers by scraping with (a) a razor blade in the hand, or (b) zonal scrapers, or by (c) cutting layers bonded to plastic or aluminum sheeting with scissors or similar cutting device. Zonal profile analysis, the assay of small reproducible zones along an entire chromatographic lane from origin to solvent front, can be carried out by simply scraping zones with a razor blade. For hand scraping, a special holder with marked gradations (Fig. 1) can be used so that relatively good reproducibility is possible. Quantitative, accurate, and reproducible zonal profile scans can be attained with special scraping instruments. The original instrument designed for this purpose was made in our laboratory from balsa wood, a rubber band, and a razor blade (Fig. 2). The razor blade was in a stationary position above a counting vial that could easily be replaced. A narrow chromatoplate (2 x 20 cm) was pushed along a guide edge so that the layer on the chromatoplate could be removed in increments of the investigator's choice. This relatively crude scraper served as a prototype for the high-precision zonal scrapers depicted in Figs. 3 and 4. Details of their operation, typical results obtained, and complete blueprints for each unit have been published. Brief descriptions, paraphrased from the articles, are included here for the manual and automatic models.
The manual scraper (Fig. 3) consists of a fixed blade mounted so that glass plates can be moved along a guide edge while the adsorbent falls from the blade's edge into a counting vial. The contact angle of the blade with the thin-layer plate is 45°, and it is held against the plate by a spring steel wire. Finger holes along the guide edge facilitate proper placement of the glass strip against the guide edge and the plate support. The movement of the chromatoplate is controlled by three gears (a modified Geneva drive) attached to a lead screw that permits 1-, 2-, and 5-mm increments to be scraped. A push-button control attached to the gears regulates the positioning of the gears so that one clockwise revolution of a crank moves the chromatoplate a distance of 1, 2, or 5 mm. After each collection of a zone of adsorbent, the blade assembly is gently tapped to remove any adsorbent adhering to the blade, the vial is removed, and a new vial is positioned for the next collection. A lever release on the carriage assembly attached to the lead screw permits free movement of the plate to accommodate its removal from the device.¹⁰

The design of the automatic zonal scraper and collector (Fig. 4) was based on that of the manual scraper. The carriage that holds the chromatoplate travels on guide rods and is moved by the intermittent rotation of a lead screw with its movement adjustable by a lever. The glass plate thus moves under a spring-loaded scraper blade in increments of 1 mm, 2 mm, or 5 mm. The scraper blade is constructed of tungsten-carbide. A special knob disengages the carriage from the lead screw allowing the carriage to be moved manually. Another knob permits manual rotation of the lead screw for initial positioning of the chromatoplate.

As the glass plate is intermittently pushed under the scraper blade, the adsorbent falls into counting vials, which are placed in a turntable tray * that holds 24 vials. The turntable on which the tray is placed moves intermittently and reciprocates with the movement of the glass plates. A solenoid, energized at the end of each scraping stroke, vibrates the plate behind the chromatoplate, causing any remaining loose adsorbent to fall into the vial.

The electrical circuit consists of a start and stop pushbutton switch, a maximum travel limit switch, a cycle switch, two control relays, a rotary stepping switch, a solenoid vibrator, and a 25-rpm gear motor. When 24 cycles are completed, the rotary stepping switch resets the control relays and stops the motor. Operation of the stop button or the maximum travel limit switch also resets the control relays. Return of the carriage to the starting position automatically lifts the scraper blade from the glass plate; convenient recesses in the guide bar aid in

* Available from Packard Instruments Sales Corp., Downer's Grove, Ill.
the easy removal and insertion of the glass plates.\textsuperscript{13}

The original zonal scrapers were designed to accept 2-\(\times\)-20-cm chromatoplates, since the narrow plates were easy to manipulate and the adsorbent did not have to fall a great distance into the vials after scraping. Chromatography on the narrow plates was limited to two lanes; one for chromatography of a standard and the other for chromatography of the sample to be scraped. Later a modification in the blade holder assembly for both the manual and automatic scrapers was introduced so that the plate size could range up to the standard end plate size, 5 \(\times\) 20 cm. These plates are wide enough to permit chromatographic development in four to five lanes. The new blade assemblies (Fig. 5) could regulate the position of the blade with respect to lane location. The width of the blades used can be varied from several millimeters to the width of the entire plate (5 cm).

Both manual \textsuperscript{*} and automatic \textsuperscript{†} commercial instruments based on the design of these updated models are available. Laboratories throughout the world have built similar units from our blueprints.\textsuperscript{14,15}

\textbf{DESCRIPTION OF RECOMMENDED PROCEDURE FOR ZONAL PROFILE ANALYSIS}

The following procedure is recommended for the preparation of zonal profile scans with the use of standard end plates (5 \(\times\) 20 cm).

1. Divide the chromatoplate into three or four lanes and draw a line across the width of the plate at the predetermined point of the solvent front. Use lanes 1 and 2 for the unknown sample to be analyzed (one for scraping and one for subsequent detection by charring) and lane 3 for a standard. Use lane 4 for preliminary radioassay of large areas for rapid analysis, autoradiography, additional standards, or additional experimental samples. The four samples chromatographed on the same plate provide reliable comparisons, since all components are resolved under identical chromatographic conditions.

2. Pipette aliquots (1-20 lambda) of samples directly on layers at the origin by using standard micropipettes, microsyringes, or disposable micropipettes. Use the same device to pipette an identical aliquot of the sample into a liquid scintillation vial containing no adsorbent so that you can determine recovery of the radioactivity from the chromatoplates. Since the adsorbent removes all of the sample from the pipettes or syringes, you need to pipette an identical aliquot into the vial for recovery determination and empty it completely. A small cellulose filter placed in the vial is helpful, but it is not essential if you are careful in the expulsion of the sample. You should remove traces of residual solvents from the samples placed in vials before you add the scintillation solution, since many of the solvents used in thin-layer chromatography are severe quenchers.

3. Place the chromatoplates in appropriate solvent systems for chromatographic development. The two-quart Mason jars or standard chromatographic chambers used for solvent development are available commercially.

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig5.png}
\caption{Modified blade assemblies for manual (left) and automatic (right) zonal scrapers.}
\end{figure}

* Available from Bio-Nuclear Corp., Oak Ridge, Tenn.
† Available from Analabs, Inc., North Haven, Conn.
4. After development, remove the chromatoplates from the jars or chambers and allow the solvent to evaporate. If necessary, you can use a heat gun or a vacuum chamber to speed this process.

5. Expose the adsorbent layer to iodine vapor (but not to alcoholic sprays of iodine) or dichlorofluorescein (the latter can also be impregnated in the layers) so that you can visualize the resolution of all compounds on the chromatographic layer. Designate areas of interest by marking the edge of the plate or standard lane so that you can readily identify zone numbers with appropriate reference compounds. Make a Polaroid picture of the chromatoplate at this stage.

6. Place the chromatoplate in the appropriate position of a zonal scraper so that you can scrape any lane in sequence, starting with the outer lane. The zones collected can vary from 1 mm wide to the length of the chromatographic lane; automatic scraping is generally done in 1-, 2-, or 5-mm increments. In some instances, using multiple zone widths is desirable. For example, you may scrape a portion of the lane into a single vial, scrape a second area at 1-mm increments, another area at 2-mm increments, and other areas at 5-mm increments. The number of each zone, starting about two zones below the origin, is identified by numbering the caps of the vials.

7. Fill the vials containing the zones of adsorbent and the vial containing the aliquot of samples used for recovery calculations with an appropriate volume (15 ml) of liquid scintillation solution. You can use an automatic pipetting device (Fig. 6) based on the turntable design of the automatic zonal scraper or a commercially available pipetter* to ease this task.

8. Place the counting vials in a liquid scintillation spectrometer for radioassay at the optimum conditions previously determined for the experimental sample and instrumentation.

9. Spray the unscraped portions of the plate (including lanes of duplicate sample, and standards) with concentrated H₂SO₄ and heat them on a hot plate or in an oven at 200°C. Compare the charred plate to the Polaroid of the iodine or dichlorofluorescein-exposed plate, to make sure that all organic compounds (including those that “tailed”) were properly detected by the less sensitive and more specific visualizing techniques. Make a Polaroid print of the H₂SO₄-charred plate for the record books, since prints can be important in the interpretation of the radioactivity data later.

10. Calculate the radioactivity data by adding the net cpm or dpm accumulated in all zones so that you can determine recovery, based on the vial containing the aliquot of total sample chromatographed. Recoveries should be within 95–105 per cent if you have assayed an adequate “statistical count.” Any departure from this range of recoveries indicates quenching, poor technique, instrument errors, or some other procedural problem that requires attention before you can assess any quantitative, and in some in-

Fig. 7.—Two-millimeter (14C)-zonal profile plotted automatically by Benson-Lehner electroplotter, standard glass chromatoplate with 250-μ layer of Silica Gel G. Chromatogram was developed in solvent system of hexane-diethyl ether-acetic acid (80:20:1, v/v/v). Peaks depict (14C)-activities associated with (14C)-labeled lipids by TLC.

stances qualitative, distribution of radioactivity. Plot the radioactivity in each zone on graph paper to construct the zonal profile of the radioactivity distribution along the entire chromatographic lane; organize the profile so that the distance from origin to solvent front is exactly the same distance as that you used for the original chromatoplate (Fig. 7). Calculate the activity associated with each activity peak along the profile as the percentage of the total activity recovered along the entire chromatographic lane. You can computerize these relatively tedious calculations and plottings.11,16

Zonal-profile analysis of plastic and aluminum chromatosheets generally requires a somewhat different approach. You can sometimes prepare zonal profiles of chromatosheets by merely taping the chromatosheet to a glass chromatoplate so that you can remove the layers in increments with the conventional zonal scrapers described. However, since the layers bonded tenaciously to the sheets tend to chip during scraping, cutting the lanes into appropriate zones with scissors, razor blade, paper cutter, or some other suitable cutting instrument is generally best. A typical zonal profile of such cuts is shown in Fig. 8. The plastic or aluminum backing does not affect the radioassay measurements.

Spot Area Scraping Technique for Liquid Scintillation Radioassay

Zonal-profile analysis is not recommended for two-dimensional chromatograms, although it is possible by using modified scrapers or special plates that can be cut into smaller sizes after chromatographic development. With two-dimensional chromatoplates, it is generally best to use a spot area scraping procedure for liquid scintillation radioassay. This procedure is often used for one-dimensional chromatograms as a matter of convenience.12 It consists of visualizing the resolved components, drawing an outline with a pencil or pointed stick around the areas to be scraped, and then scraping the area directly into a counting vial or transferring the scraped area with an aspirator tube to the counting vial for radioassay.

Fig. 8.—Manual plotting of 2-mm (14C)-zonal profile prepared by cutting 2-mm sections of commercially available aluminum chromatosheet (Brinkmann Instruments, Inc., Westbury, N. Y.). Development as in Fig. 7.
The percentage of the total radioactivity applied to a chromatoplate that is found in each spot can be calculated by the same procedure outlined previously for zonal-profile analysis. Homogeneity of radioactive components assayed by spot area scraping techniques should be checked by zonal-profile analysis of representative samples.\(^7\),\(^11\),\(^16\)

**DATA PROCESSING SYSTEMS FOR TLC RADIOASSAY**

Automated instrumentation and procedures, including computer programs, for transmitting, calculating, and plotting of radiometric data obtained from thin-layer chromatograms have been developed by our laboratory. These systems have used a phone-line hookup via the city telephone exchange to a card punch\(^13\) or, more recently, directly to a computer.\(^16\) The original phone setup required a data phone (Western Electric 401A) and a data receiver (Western Electric 401J), but the on-line system is now modified so that a regular phone replaces the data phone sets. The general description of the component parts of these systems has been published.\(^7\),\(^11\),\(^16\)

Once the samples are placed in the liquid scintillation spectrometer, identification information for a given batch of samples is transmitted by use of a manual keyboard located in the room housing the liquid scintillation spectrometers. The identification items are machine number, date, technician number, experiment number, sample range of batch, scan widths (including exceptions for varying the scan width at different locations on the chromatoplate), background samples, samples for calculation of recovery, efficiency (per cent in both channels), and isotope. The computer program will calculate percentage of recoveries, percentage of activity in each zone and peak areas, dual isotope data, statistical variations (standard deviations), and specific activities. A Benson-Lehner electroplotter is used to automatically plot the zonal profile scans obtained.

**MEASUREMENT OF MASS BY LIQUID SCINTILLATION QUENCHING**

The linear quenching of an isolated (\(^14\)C)-plastic scintillator source by a colored solution has been used as the basis for the development of a quantitative procedure for the determination of lipid mass.\(^18\) The actual arrangement of a liquid scintillation vial for such assays is shown in Fig. 9.

Any colorimetric procedure that is capable of absorbing photons in the region of 4000 Å (near the optimum sensitivity of most photomultipliers used in liquid scintillation spectrometers) can be used for mass measurements in conjunction with quenching of the special scintillation source. In our original communication on this subject, we used a slight modification of the H\(_2\)SO\(_4\)-
color procedure originated by Marsh and Weinstein \textsuperscript{19} for the measurement of lipids. The modified procedure consists of heating the lipid sample in a counting vial containing \( \text{H}_2\text{SO}_4 \) in an oven at 200\(^\circ\) C for one hour. After cooling and appropriate dilution, the isolated plastic scintillation source is inserted into the vial containing the colored solution and it is then assayed in the liquid scintillation spectrometer. The color intensity, which is linear with mass of lipid, linearly decreases counting efficiency.\textsuperscript{15} This new technique greatly extends the versatility of liquid scintillation instrumentation.

\textbf{REFERENCES}

Equilibrium density gradient centrifugation is a technique first described in 1957 by Meselson, Stahl, and Vinograd. Centrifuging a concentrated salt solution at a constant speed gives rise to a density gradient, in which the density increases with increasing distance from the center of the rotor. The density gradient attains equilibrium when the centrifugal force is balanced by back diffusion of the salt. Generally an aqueous solvent is used and the solute is a cesium salt, usually CsCl. If a macromolecule is present at a low concentration in the salt solution, it will move to a position in the gradient corresponding to its buoyant density, provided the initial density of the solution is close to that of the macromolecule. The macromolecules frequently examined by this technique are proteins, nucleic acids, viruses, subcellular particles, and synthetic polymers. The present discussion will deal primarily with deoxyribonucleic acid (DNA).

Quantitative considerations such as the time to attain equilibrium, factors determining the steepness of the gradient, distribution of the macromolecule at equilibrium, and the determination of the buoyant density of the salt solution and macromolecule have been described in detail. A wide variety of applications has evolved since the introduction of equilibrium density gradient centrifugation. Determination of the buoyant density of a macromolecule is a useful physical parameter which can be measured without destroying the molecule. Moreover, a macromolecule may be separated from other molecules of different densities, a procedure widely used in the purification of viruses. The technique may also be used to examine other properties of DNA, such as base composition, molecular
Fig. 1.—Ultraviolet absorption photographs (a) and microdensitometer tracings (b) of equilibrium density gradient centrifugation of DNA from E. coli at various times after addition of (14N) substrates to a (15N) labeled culture. Photographs were taken 20 hours after centrifuging at 44,770 rpm in Spinco model E ultracentrifuge. Density of CsCl increases to the right.

USE OF RADIOISOTOPES WITH DENSITY GRADIENTS

Labeling with β-emitting Isotopes

The use of radioisotopes in conjunction with equilibrium centrifugation in the preparative ultracentrifuge adds another dimension to this versatile technique. For example, (32P)-labeling of DNA can be accomplished by growing cells or virus in medium containing neutralized (32P). Labeling with (3H) and (14C) is accomplished by placing radioactive thymidine in the medium. After growth in medium containing the desired isotope, the labeled nucleic acid can then be purified from the cells or virus. After centrifugation, fractions are acid precipitated, collected on filters and counted in a toluene-based scintillation mixture. Alternatively, the fractions can be dissolved in a dioxane-naphthalene mixture, or suspended in toluene containing 30 per cent Triton X-100 and 10 per cent H2O. Dissolving the fractions has the advantage of higher counting efficiencies and permits correction for quenching of counting.

In our usual experiments, 0.1-ml aliquots of DNA-CsCl solution are collected in liquid scintillation vials and suspended in a toluene-Triton X-100-H2O-PPO scintillation mixture. (3H) is counted at 30 per cent efficiency and (14C) at 85 per cent. For double label experiments narrower windows are used to decrease spillover, and (3H) is counted at 26 per cent and (14C) at 60 per cent efficiency.

If unlabeled DNA is centrifuged to equilibrium and detected spectrophotometrically, at least 0.5 μg of DNA is required for analytical centrifugation and at least 10–20 μg is required for preparative centrifugation. Detection of radioactive nucleic acids, however, is a great deal more sensitive. 0.01–0.001 μg of DNA that has a specific activity of 200,000 dpm/μg can be centrifuged to equilibrium and detected by liquid scintillation counting.

In addition to allowing for the detection of very small quantities of DNA, radioisotopic labeling makes it possible to distinguish a particular species of DNA from another even when the latter is present in much greater quantity. Figure 2 shows the results of mixing a large amount of unlabeled DNA
with a small amount of radioactive DNA and centrifuging in the Spinco SW39 rotor (33,000 rpm for 62 hours at a starting density of CsCl of about 1.71 g/ml)\textsuperscript{15}. The three samples all contained 2.7 \( \mu \)g of \textit{E. coli} DNA with 520 cpm of \textsuperscript{14}C). Samples A-C contained, respectively, 15 \( \mu \)g, 45 \( \mu \)g, and 90 \( \mu \)g of mouse liver DNA. Especially note C in which only 2.7 \( \mu \)g of isotopically labeled \textit{E. coli} DNA can be easily distinguished from 90 \( \mu \)g of mouse liver DNA. Without the isotope, this DNA would not be detected, because its optical density profile would be completely masked by that of the mouse DNA.

\textit{Combination of Density and \( \beta \)-labels}

The use of density labels in conjunction with radioactive labels broadens the usefulness of radioisotopes in density gradient centrifugation. Stable isotopes such as \textsuperscript{2}H, \textsuperscript{15}N, and \textsuperscript{13}C and halides such as bromide can be incorporated into DNA and will increase its density.\textsuperscript{16-20} This altered density can be monitored by equilibrium centrifugation. The work of Kozinski is an example. In studying the replication of T4 bacteriophage, a double-stranded DNA phage, he made use of density labeling, radioactive labeling, and density gradient centrifugation.\textsuperscript{21} He was interested in following the transfer of DNA, the genetic material, from parent to progeny. T4, with its DNA labeled by \textsuperscript{32}P, was used to infect \textit{E. coli} B grown in medium containing 5-bromodeoxyuridine (BUDR) so that its DNA had a bromide density label. BUDR was kept in the medium after infection so that any new DNA synthesized would also be density-labeled. At various times after infection, DNA was extracted from the cells and centrifuged to equilibrium in a density gradient. The results are shown in Fig. 3. One can see the increase with time of \textsuperscript{32}P in density-labeled DNA, representing parent DNA being incorporated into progeny DNA isolated from purified progeny phage was then fragmented by sonication to one-fourth of its original size as determined by sedimentation velocity. This fragmented DNA was examined by equilibrium centrifugation as seen in Fig. 4. A large amount of DNA was examined (20 \( \mu \)g/ml) so that the optical density (O. D.) could be determined. Prior to sonication, the parental DNA (labeled by \textsuperscript{32}P) had banded at a density expected of bromide substituted progeny DNA. After sonication (Fig. 4), the parental DNA had a density expected of hybrid: one strand was parental, having no density label, and the other strand was density labeled and
I-30 I-0

Fig. 3.—Equilibrium centrifugation in Spinco SW39.L rotor, 35,000 rpm, 72 hours, of DNA extracted from T4 phage infected E. coli B. Parental phage DNA was labeled with (32P). Medium contained BUDR during infection.21 Therefore from the progeny. This indicated that parental DNA was integrated into progeny DNA as intact segments. Since parental DNA was a small fraction of progeny DNA, most of the DNA remained at the heavy density.

Differential Labeling

Two or more DNA species may also be distinguished by differential labeling, usually with (3H), (14C), and (32P). Double label experiments with or without spectrophotometry are generally performed, although triple labeling is employed occasionally.22 Thus, more than one DNA type may be examined in trace quantities and with high resolution.

An example is provided by some of our own work.23 We described a method for the density labeling of mammalian DNA with (15N). Previously, mammalian and infecting viral DNA could only be density labeled with halides. Since such labeling is toxic for the cell or virus, its usefulness is limited. Because mammalian cells and their viruses require complex media for growth, including essential amino acids, vitamins and serum, DNA cannot be labeled with (15N) simply by including (15N) HCl in the medium. Since the nitrogen of glutamine is a major source of the nitrogen in the purines and pyrimidines of DNA, the inclusion of amino-(15N), amide-(15N), L-glutamine in the medium results in DNA with a density of 0.010 g/ml higher than (14N)-DNA. Total (15N) substitution would result in a 0.014 g/ml difference. This density difference, although significant, is small compared to the 0.130 g/ml which can be attained with BUDR. Therefore the resolution of density gradient centrifugation

Fig. 4.—Equilibrium density gradient centrifugation (as in Fig. 3) of progeny T4 DNA after sonication. Parental DNA was labeled with (32P) and was associated with BUDR-labeled progeny prior to sonication.21
must be made maximal when ($^{15}$N) labeling is used. One method is the use of double labels. Figure 5 shows the results of equilibrium centrifugation of ($^{16}$N) HeLa DNA (peak B) with ($^{14}$N) HeLa DNA (peak A) in a CsCl solution of 1.700 g/ml density in an analytical ultracentrifuge at 44,770 rpm, 25° C., for 20 hours. This figure represents a microdensitometer tracing of an ultraviolet absorption photograph for this experiment. Peak C is a M. lysodeikticus DNA marker.

By contrast, Fig. 6 illustrates the result of centrifuging DNA labeled with ($^{15}$N) and ($^{14}$C) and DNA labeled with ($^{3}$H) in a preparative ultracentrifuge using the Spinco No. 40 rotor, 33,000 rpm, 25° C., for 60 hours. The increased resolution afforded by double labels and a fixed-angle rotor is evident. Two DNA preparations with different radio-isotopes may be observed separately even if they have very similar densities (Fig 7). Detection by O. D. or by a single isotope in this case would not indicate the presence of two different DNAs. 

**Conclusion**

Density gradient centrifugation has become extremely useful in the study of macromolecules. Radioisotopes and liquid scintillation counting have greatly broadened the scope of this technique. Among the benefits are higher sensitivity and resolution and the ability to study the kinetics of macromolecular synthesis and fate.
REFERENCES

Electrophoretic separations of protein and nucleic acids in gels of polymerized acrylamide have continued to gain in popularity with biochemists and clinical chemists since publication of two independent approaches to the technique by Ornstein, Davis and Raymond in 1964. Acrylamide gels are formed by introduction of acrylamide monomer

\[
\begin{align*}
\text{CH}_2 &= \text{CH} - \text{CO} - \text{NH}_2 \\
\text{NH}_2 - \text{CO} &\quad \text{NH}_2 - \text{CO} \\
R &\quad \quad \quad \quad R \\
\text{NH}_2 - \text{CO} &\quad \text{NH}_2 - \text{CO} \\
\text{CH}_2 - \text{CH} &\quad \text{CH}_2 - \text{CH}
\end{align*}
\]

to a cross-linking agent (N,N'-methylene-bis-acrylamide or ethylene diacrylate) and catalysts in aqueous solution. The resulting polymer consists of linear and saturated hydrocarbon chains cross-linked by methyl or ethyl groups (R).

The three-dimensional structure of the polymer presents a problem for the investigator if he wants to determine the radioactivity of macromolecules which have undergone electrophoretic fractionation. Particularly when (\(^3\)H) radioactivity is to be determined, the presence of acrylamide may prevent accurate counting by absorbing and backscattering \(\beta\)-particles. In liquid scintillation counting, interaction with the primary scintillator may

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THE LABELED SAMPLE

Table 1.—Counting of Acrylamide Gel Sections

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Solvent</th>
<th>References</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Autoradiography</td>
<td></td>
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<tr>
<td>2. Elution from the gel</td>
<td></td>
<td></td>
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<tr>
<td>3. Extruded gel sections</td>
<td></td>
<td></td>
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<tr>
<td>4. Permeation with toluene scintillator</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. After fixation</td>
<td></td>
<td></td>
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<tr>
<td>B. After partial hydrolysis (NCS) *</td>
<td></td>
<td></td>
</tr>
<tr>
<td>5. Dissolution of gels</td>
<td></td>
<td></td>
</tr>
<tr>
<td>A. Cross-linked with ethylene diacrylate 18</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrolytic Agent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. piperidine, hyamine</td>
<td>dioxane</td>
<td>Choules and Zimm,15 Groves et al.19</td>
</tr>
<tr>
<td>b. NH_4 OH (conc.)</td>
<td>dioxane</td>
<td>Spear and Raizman 10</td>
</tr>
<tr>
<td>c. 1N NaOH, NCS</td>
<td>toluene</td>
<td>Peacock and Dingman,21 Cain and Tinney,22</td>
</tr>
<tr>
<td>d. NCS</td>
<td>toluene</td>
<td>This chapter</td>
</tr>
<tr>
<td>B. Cross-linked with bis (N,N'-methylene-bis-acrylamide)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydrolytic Agent</td>
<td></td>
<td></td>
</tr>
<tr>
<td>a. H_2O_2 (0.1 ml), H_2O</td>
<td>dioxane</td>
<td>Tishler and Epstein 23</td>
</tr>
<tr>
<td>b. H_2O_2 (0.5 ml), hyamine</td>
<td>dioxane</td>
<td>Moss and Ingram 24</td>
</tr>
<tr>
<td>c. H_2O_2 (0.5 ml), NCS</td>
<td>toluene</td>
<td>LeBouton 25</td>
</tr>
<tr>
<td>d. H_2O_2 (0.1-0.2 ml)</td>
<td>BBS-3 † solubilizer, toluene</td>
<td>This chapter</td>
</tr>
<tr>
<td>6. Combustion (Schoniger)</td>
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</tbody>
</table>

* NCS Solubilizer, Amersham-Searle, Des Plaines, Ill.
† Bio-Solv solubilizer BBS-3, Beckman, Inc.

be almost entirely precluded or at least affected in an unpredictable manner.

Since we have had to deal with these problems ourselves, we have had occasion to evaluate various approaches to counting proteins and nucleic acids in gels (Table 1). In this paper we wish to share our impressions of these approaches, documenting some of them with experimental evidence. We also present several procedures we have found satisfactory.

**SECTIONING OF ACRYLAMIDE GELS**

Except for autoradiography of vertically sectioned gels, 6–7 a technique semi-quantitative at best, all of the other approaches listed in Table 1 require that the gel cylinder or slabs be horizontally sectioned (at 90° to the axis of separation). Table 2 summarizes the methods of sectioning gel cylinders employed by various investigators. It should be obvious that a gel cylinder or rod has significant advantages over a gel slab when a sectioning procedure is contemplated.

All four procedures listed in Table 2 have been employed in our laboratory. We have not been at all satisfied with the results of cassettes with either razor blades or wires. With soft gels (less than 10% acrylamide), the gels distort in the cassette so that the section size obtained is quite variable, and the portions of each section remain-

Table 2.—Sectioning of Acrylamide Gels

<table>
<thead>
<tr>
<th>Procedure</th>
<th>Equipment</th>
<th>Gel preparation</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. Razor blades</td>
<td>McIlwain chopper</td>
<td>Paraffin embedded</td>
</tr>
<tr>
<td>2. Fine wires</td>
<td>Rotary microtome</td>
<td>Frozen (CO_2) gels</td>
</tr>
<tr>
<td>3. Microtome procedures</td>
<td>Automatic gel-sectioning device</td>
<td></td>
</tr>
<tr>
<td>A. Equipment</td>
<td>McIlwain and Buddel26</td>
<td></td>
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<tr>
<td>B. Gel preparation</td>
<td>Gray and Steffensen 27</td>
<td></td>
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<tr>
<td></td>
<td>Gray and Steffensen 28</td>
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<tr>
<td>4. Gel extrusion</td>
<td>Loening,28 Bishop et al.,29 Bransome and Cadwgan 31</td>
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<td></td>
<td>Summers et al.,32 Maizel,33</td>
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</table>
ing on the blades or wires are difficult to re-
move and recover. Hard gels (more than 10% 
acrylamide) or softer gels which have been 
frozen are usually not cut through by such 
devices. We therefore recommend that a 
modified microtome or gel extruder be used.

Mr. Jules Lux of Savant Instruments was 
kind enough to let us compare recoveries of 
labelled acrylamide gel sections obtained with 
his company's version of the Maizel ex-
truder\textsuperscript{12,13} and a modified McIlwain chop 
per\textsuperscript{30,32,34} which is in everyday use in our 
laboratory. We prepared 7.5 per cent acryl-
amide solutions buffered to pH 8.9 with Tris-
HCl, adding bis (N,N'-methylene-bis-acryl-
amide) as a cross-linker and ammonium 
persulfate as a catalyst for polymerization. 
Guinea pig plasma proteins labeled in vivo by 
4,5(\textsuperscript{3}H) leucine (approximately 93,100 (\textsuperscript{3}H) 
dpm per 6-cm gel cylinder) were mixed with 
the solution. Partial dissolution of the 2.0-mm 
gel sections with NCS, one of the several 
techniques discussed below, was used to re-
lease the labeled protein from the gel matrix. 
Two sorts of variation between successive 
sections can be seen in Table 3 where repre-
sentative results have been summarized.

First there is the variation in the main por-

<table>
<thead>
<tr>
<th>Section</th>
<th>McIlwain Slicer</th>
<th>Maizel Extruder</th>
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<tr>
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<td>(\textsuperscript{3}H) Radioactivity: dpm/Section</td>
<td>(\textsuperscript{3}H) Radioactivity: dpm/Section</td>
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<td>1</td>
<td>3054</td>
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</tbody>
</table>
| 32      | 2440           | 11,247 (incom-
|           |               | plete)         |
| Total   | 90,320         | 78,103         |
| Mean (total) | 2913         | 2440         |
| SE     | ±43           | ±289           |
| Mean (6–30) | 2974         | 2170           |
| SE     | ±29           | ±43            |

Gels were labeled as described in the text. One gel was frozen with solid CO\textsubscript{2} powder and sliced. An identical 
gel was introduced into a barrel of the same internal diameter (0.6 cm) and extruded, with a flow rate of H\textsubscript{2}O 
calculated to yield approximately 30 sections per gel. Collections were timed and varied between 8 and 10 drops. 
(\textsuperscript{3}H) radioactivity was determined as described in the text. SE: Standard error of the mean.
tion of the gel (sections 6–30) which must be regarded as minimal in the sliced gel; the standard error of the mean is less than 1 per cent. In the extruded gel, collection per unit time was undoubtedly a source of error. With collection monitored by drop counter, the standard error (already close to that of the sliced gel) would certainly not have been larger.

The second source of variation is evident with both sectioning techniques. A variability in section size is always noted at either end of the frozen gel when it is sliced; the gel cannot be exactly positioned (on our equipment) at either the beginning or end of cutting. The problem was much more serious with the extruded gel. Initial sections were significantly less than the average because of an initial disproportion of gel extruded to the constant flow of diluent. The final sections of the gel, obviously much larger, indicate the reverse disproportion; indeed the last section could not be completely recovered from the extruder apparatus. This probably explains the lesser total recovery of (3H) protein: 84 per cent versus 97 per cent for the sliced gels. It is obviously necessary to position unlabeled gel on either end of a gel column containing radioactivity to achieve accurate fractionation with the extruder. The drawback to such a maneuver, however, is that it makes the radioactivity of a sectioned gel cylinder difficult to relate to a previously obtained densitometric scan.

DETERMINING PROTEIN RADIOACTIVITY IN ACRYLAMIDE GEL SECTIONS

We have carried out a large number of studies involving determinations of both (3H) and (14C) in RNA as well as in protein in acrylamide gel, but we will refer here only to experiments with (3H), the lowest energy β-emitter, and proteins, the molecules hardest to hydrolyze. Without question, the determination of protein (3H) is the most demanding of our techniques.

The principal drawback to the first method of determining gel radioactivity listed in Table 1, radioautography, has already been mentioned. At best it is semi-quantitative, and determination of the presence of low (3H) levels in parts of the gel may require several weeks of exposure.

Elution from the gel column with buffer has been successfully employed in preparative acrylamide gel electrophoresis (with apparatus supplied by several companies, including Canalco, and Buchler, Inc.). Diffusion of the unfixed sample, a problem even on the preparative scale, becomes a very inconvenient source of variability in analytical electrophoresis of small amounts of RNA or protein. In our hands, attempts at elution of either unfixed and unstained RNA or protein from gel sections with buffer have always yielded disappointingly low recoveries.

Raising the elution buffer pH to 9 is effective in removing RNA if the alkali-labile cross-linker, ethylene diacrylate, has been used. This might also be effective in removing unfixed proteins from gel sections, but it is not as effective if the gels (and proteins) have been fixed in acid or acid-alcohol and stained; much of the protein radioactivity will be absorbed either by the gel or by the protein itself. Ethylene diacrylate should not be used as a cross-linker for gels which will be run at a basic pH, e.g., the protein disc electrophoretic systems of Ornstein and Davis; gels will be unstable. The problem with bis cross-linked gels is of course even greater since the three dimensional structure of the polymer remains intact. The difficulty in eluting (3H) protein radioactivity even after gel extrusion can be seen in Table 4. Unless the pulverized gel was subjected to hydrolysis or combustion, the (3H) radioactivity determined was only a small fraction of that present in the gel. We can only reiterate the statement of Gray and Steffenson regarding (14C) protein: that solubilization of the gel is necessary for quantitation of radioactivity. Gel sectioning by the Maizel procedure should obviously be combined with a hydrolytic or combustion procedure if radioactivity is to be determined.
Gels were uniformly labeled with (el-I) plasma proteins: 93,100 of (acid precipitable) protein radioactivity.

* Scintillation mixtures were: 7 g 2,5 diphenyloxazole (PPO) per liter of toluene; 7 g PPO and 120 ml BBS-3 Solubilizer per liter of toluene; 7 g PPO and 100 g napthalene per liter of dioxane.

Low recoveries for procedures 4 and 5 are attributable to mechanical problems illustrated in Table 3 and are discussed in the text. These values should be compared to those from elution techniques 6 and 7.

In each case, samples were counted on a Beckman LS-150 scintillation counter and (3H) dpm were calculated from a quench correction curve comparing known standards to a (137Cs) external standard ratio.

NCS-Toluene

Permeation of gels with toluene scintillator after partial hydrolysis of the gel has been an effective method in our hands (Table 4).

Basch correctly points out that incubation with NCS at 65° C is effective when the gel section is translucent and swollen to 3-4 times its original volume. We have found that prolonged exposure to NCS at temperatures as low as 37° C will also suffice. As Hansen and Bush pointed out, the presence of water is necessary for the hydrolysis. The water content of gel sections is usually inadequate, so that water must be added. We recommend the following procedure: (1) Add 0.5 ml NCS and 0.05 ml H2O to a 1- to 2-mm section in a glass screw top scintillation vial. (2) Cover vial tightly and incubate at 50-60° C for at least 12 hours. (3) Cool to ambient temperature. (4) Add 10 ml toluene scintillator to vial. (5) Add 4 per cent aqueous ascorbic acid: 0.01-0.05 ml. (6) Do not count for 48 hours. The addition of ascorbic acid or SnCl2 (see Chapter 34) is desirable to decrease chemiluminescence in air-equilibrated samples. Waiting for 48 hours is also a good idea if low (3H) levels are to be determined. At least 24 hours are usually necessary for low levels of background chemiluminescence to subside. The problem of chemiluminescence is discussed at length in Chapters 32 to 34. 1N NaOH or hyamine are also effective hydrolyzers but are less desirable than NCS because they bring about considerably more chemical quenching.

When gels are cross-linked with ethylene diacylate, the procedure outlined above brings about complete dissolution of the acrylamide polymer and contributes much less chemical quenching to the scintillator than a piperidine, hyamine combination or 1N NaOH and NCS. (The NaOH is not really necessary.)

An attempt to use concentrated NH4OH to achieve partial hydrolysis of gels cross-linked with bis was not successful. NH4OH did act on ethylene diacylate cross-linked gels, but gel sections did not completely dissolve; they were fragmented into strands. (3H) protein radioactivity was less than one-third of that seen after the NCS procedure. As with the low recovery elution procedures mentioned above, the (3H) yield per gel section was unpredictable. We suspect that incubation with NH4OH may have produced another polymer: ß, ß', ß" Nitrilotrisopropionamide, which Subluskey has shown to be a major product of NH3 action on acrylamide. We further warn against its use, since even in samples where NH3 has been allowed to evaporate after hydrolysis, there is enough NH3 left to generate a yellow (quenching) color in a dioxane-naphthalene (100 g/l)-PPO scintillation mixture within several days.

### Table 4.—Comparison of Techniques for Releasing (3H) Protein Radioactivity from Acrylamide Gel Sections

<table>
<thead>
<tr>
<th>Sectioning Technique</th>
<th>Hydrolytic Agent</th>
<th>Solvent</th>
<th>% Recovery of dpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. McLlwain slicer</td>
<td>combustion</td>
<td>BBS-3, toluene*</td>
<td>98</td>
</tr>
<tr>
<td>2. McLlwain slicer</td>
<td>H2O2</td>
<td>BBS-3, toluene</td>
<td>96</td>
</tr>
<tr>
<td>3. McLlwain slicer</td>
<td>NCS</td>
<td>toluene</td>
<td>97</td>
</tr>
<tr>
<td>4. Maizel extruder</td>
<td>H2O2</td>
<td>BBS-3, toluene</td>
<td>92 f</td>
</tr>
<tr>
<td>5. Maizel extruder</td>
<td>NCS</td>
<td>toluene</td>
<td>84 f</td>
</tr>
<tr>
<td>6. Maizel extruder</td>
<td>none</td>
<td>BBS-3, toluene</td>
<td>7</td>
</tr>
<tr>
<td>7. Maizel extruder</td>
<td>none</td>
<td>dioxane</td>
<td>18</td>
</tr>
</tbody>
</table>

Gels were uniformly labeled with (3H) plasma proteins: 93,100 of (acid precipitable) protein radioactivity.

* Scintillation mixtures were: 7 g 2,5 diphenyloxazole (PPO) per liter of toluene; 7 g PPO and 120 ml BBS-3 Solubilizer per liter of toluene; 7 g PPO and 100 g napthalene per liter of dioxane.

† Low recoveries for procedures 4 and 5 are attributable to mechanical problems illustrated in Table 3 and are discussed in the text. These values should be compared to those from elution techniques 6 and 7.
H₂O₂-Solubilizer-Toluene

For bis cross-linked gels, we have had success in dissolving acrylamide polymer through incubation with 30 per cent H₂O₂. We advise the following procedure: (1) Add 0.1–0.2 ml H₂O₂ to a 1- to 2-mm section. (2) Heat in a scintillation vial with a foil-lined or plastic-lined cap at 50–60° C for at least six hours so that the gel is completely dissolved. (3) With (¹⁴C) samples, a center well with hyamine or phenylethylamine is necessary to trap (¹⁴C)O₂. (4) Cool to ambient temperature and mix thoroughly. (5) Add solubilizer such as BioSolv BBS-3, 3–4 times the volume. (6) Add 7500 units of catalase or more in a volume of 0.2 to 0.5 ml (versus chemiluminescence). Addition of the enzyme after the solubilizer decreases the violence of effervescence and avoids transient precipitation of enzyme protein. (7) Let the vial sit five to ten minutes to let effervescence subside before a toluene-scintillator solution is added. (8) If chemiluminescence is still suspected, more catalase can be added despite the presence of toluene. The vial is shaken and opened to allow released O₂ to escape. Any quenching effect of the additional catalase can be remedied by the addition of more toluene scintillator. (9) Let 72 hours elapse before counting. Benevenga et al.⁸ have shown that H₂O₂ treatment may liberate protein (¹⁴C) as (¹⁴C)O₂. Generally we suggest the NCS procedure outlined above for (¹⁴C) containing samples.

The tendency of peroxides toward chemiluminescence has been a considerable problem, particularly in determining small amounts of (³H) radioactivity. We have found that the enzyme catalase (obtained from Mann or Sigma) is extremely effective in removing the excess H₂O₂. As the good recovery seen in Table 4 indicates, it is not necessary to hydrolyse the protein itself as long as the protein is dispersed in solution. We have therefore omitted Hyamine²⁴ or NCS.²⁵ A white precipitate will form in NCS if it is exposed to free H₂O₂. We prefer the toluene-solubilizer combination to dioxane²³ because of the sensitivity of dioxane to the formation of chemiluminescent peroxides and because of toluene's superior efficiency. (See Chapter 2.)

Combustion of acrylamide sections by Schöninger techniques²⁶,²⁷ is of course an ideal method. Recovery is virtually complete (Table 4). There is no quench correction necessitated by introduction of the sample, but fixative such as trichloroacetic acid may be carried over in the combustion. Trichloroacetic acid carried over in a combustion process can produce color and/or chemical quenching; it may be displaced from acrylamide gels if they are soaked in 7 per cent acetic acid overnight. Quenching should always be assumed unless external standard ratios, for example, are similar to air equilibrated standards. (The quenching due to equilibrium of the scintillation mixture with air is quite reproducible.) The technique is Chapter 23. That acrylamide gel sections pelleted with Whatman No. 1 paper may be quantitatively analyzed for (³H) is shown in Table 4. Mr. William Grathwol of the Packard Instrument Company was kind enough to perform combustions of our sections of uniformly labeled gels (Tables 3 and 4) with an engineering model of the Packard Tritium Analyzer. Mahn and Lofberg in Chapter 22 mention that wet oxidation, a procedure we have not tried, may also be suitable.

CONCLUSIONS

We have reviewed the various procedures employed by others in sectioning acrylamide gels and for determining the radioactivity of gel sections.

Either an automatic slicer (of frozen gels) or an extruder seems satisfactory if some of the sources of sampling error for either procedure are properly taken into account. Extrusion alone will not permit quantitative determination of (³H) radioactivity. Further treatment is necessary to free molecules entrapped in the acrylamide polymer.
After considering published techniques for hydrolysis of acrylamide gels, we realized that simpler and more efficient procedures were needed. New hydrolytic procedures are outlined above. Carrying out these procedures or resorting to Schöniger combustion if the apparatus is available should resolve any difficulties in determining the radioactivity of proteins or nucleic acids separated in acrylamide gel.

REFERENCES

15. Terman, S.: Personal communication.
35. Grower, M. F., and Bransome, E. D., Jr.: This Volume, Chapter 36.
Each of the first three chapters of this book, in dealing with the basic mechanisms of liquid scintillation, contains a discussion of "chemical quenching" of $\beta$-energy and "color quenching" of scintillator fluorescence. The four chapters in this section represent a practical approach to the problem of nonuniformly quenched samples. Neary and Budd first give a coherent review of the causes of quenching, and then consider the divergence of chemical- and color-quench correction curves seen in samples labeled with $^{14}$C or $^{38}$s of higher energy. Their investigations have revealed that the divergence, when color quench is large, can be attributed to a statistical perturbation of the pulse height spectrum. This new information provides the investigator with additional reason to avoid samples which are intensely color-quenched if he hopes to quench-correct with channels-ratio, external standard, or external standard-channels ratio. At the same time the authors have shown how this divergence can be minimized.

In Chapter 29, Peng presents a somewhat different, but equally valuable, classification of quenching. He gives extensive descriptions of the various procedures for determining quench-correction curves. His discussion of his own rational approach to selecting channels for the channels-ratio technique satisfies a need which until now has gone unfilled. His definitions of "integral," "differential," and "balance-point" counting, and the relevance of the latter to external standardization deserve attention.

Cavanaugh, in Chapter 30, gives an extensive review of the external standardization method of monitoring quenching—currently the most popular method. He demonstrates the important sources of error in the external standard-channels ratio technique (ESR) and shows that the calculated dpm of a sample can, on occasion, be more accurate than the cpm. Practical considerations of how one should proceed in setting up ESR channels, and how ESR error can be calculated, are important additions to the literature.

There are two methods of automatic quench "correction" incorporated in recent liquid scintillation counters which utilize ESR to determine the amount of quenching in an unknown sample. One, discussed by Wang in Chapter 31, has been introduced by Beckman Instruments as "automatic quench correction" (AQC) in the LS-150 and LS-250 systems: comparison of the decreased ESR of a quenched sample to a "least-quenched" ESR calibrates an increase in photomultiplier tube gain which will restore pulse-height spectral distribution to "least-quenched" end
points. This approach greatly improves the statistics of double isotope counting of quenched samples (see Chapter 6) and allows constant-efficiency counting of $^{11}$C and higher energy $\beta$-s.

While the Beckman approach is a partial correction, the Packard Instrument Company has introduced a complete correction which does not improve the statistics of pulse height spectral distribution. Instead, the ESR and efficiency of a quenched sample are decreased to a pre-set numerical value on a quench-correction curve by the application of a magnetic field to the photomultiplier cathode. Since the counting efficiency at this ESR point is known, dpm may be calculated automatically. This system is referred to in Chapter 30; a full description can be obtained from the Packard Instrument Company in literature describing their 3380-554 system.
Chapter 1, 2 and 3 include discussions of the theoretical aspects of quenching. The two purposes of this chapter are to illustrate the intrinsic differences between color and chemical quenching and to present a theoretical model designed to explain the behavior of the colored scintillation sample. We consider it important to relate the significance of the difference in color and chemical quenching to practical scintillation counting of experimental samples.

Chemical Quenching

We will summarize briefly the processes discussed at length in Chapters 1-3. With the interaction of β-particle and solvent, the solvent molecule electrons are excited to singlet states. The energy of the singlet state may be transferred without radiation from solvent molecule to solvent molecule until a molecule of fluorescent solute is excited. The solute then emits photons of some wavelength. Chemical quenching can be thought of as any process, active within the sample, which reduces the energy transfer efficiency of some member or members of the set of compounds essential to the scintillation process. This set is usually composed of a solvent and a primary fluorescent material, and may include a secondary fluorescent material. We will consider that this process leaves the path of the photon light pulse through the sample-solvent milieu unaffected. It is possible to classify chemical quenching into five categories.

Acid quenching, a form of chemical quench, is the result of interaction of a proton with either the primary or secondary fluorescent material, which are relatively “good” organic bases. The influence of the proton on the permitted excitation states may be considered as an energy mismatch at the solvent-fluor interface.

Concentration quenching occurs when one member of the scintillation mixture, generally the primary fluor, reaches such a concentration that it interferes with energy transfer. This is similar to self-absorption in conven-
tional spectroscopy. An example is PPO above 8 g/l in toluene. However, when the sample's relative concentration increases, quenching increases. This effect can be considered as either concentration or dilution quenching.

*Dilution quenching* is the result of increasing the distance separating solvent molecules, thus reducing the probability of energy transfer. Any nonparticipant in the photon producing process can be considered to be a dilution quencher, but it should be noted that dilution reduces concentration quenching.

*Dipole-dipole quenching* can also be thought of as a chemical quenching where, due to the presence of some material, the solvent's $S_1$ energy is transferred via dipole-dipole interaction over a distance greater than the molecular dimensions to that material and subsequently dissipated as vibrational energy, and hence nonradiatively. Nitromethane and oxygen are examples of such materials.

*Capture of secondary electrons.* The presence of materials with high electron affinities also causes chemical quenching. Secondary electrons emitted from $\beta$-solvent interactions are captured, depriving other solvent molecules (along the probable $\beta$-path) of excitation energy. Halogenated hydrocarbons such as CHCl₃ or CCl₄ are good examples of this type of material.

Chemical quenching occurs before the production of photons and results in a reduction of the number of photons arriving at the photocathode of the photomultiplier tube. Chemical quenching also appears to be an exponential function of the concentration of the quenching agent.

**COLOR QUENCHING**

Color quenching can be thought of as the attenuation of photons which are emitted by the primary and/or secondary fluor. This attenuation is a primary function of absorptivity of the quenching molecule, of the concentration of the chromophore, and of the path length which a photon must transverse in order to be detected. Any compound which exhibits an absorption band within the emission band pass of the fluor will qualify as a color quencher. Changes in photon emission due to color quenching can, like chemical quenching, be characterized by the reduced number of photons arriving at the photocathode of the photomultiplier tube. With color quenching there is an additional parameter,
RELATIVE PULSE HEIGHT OR CHANNEL NUMBER

Fig. 2.—Color versus chemical quenching of (3H); differences between spectra at same levels of efficiency. Note slight dissimilarity over wide range of efficiency.

If the shape of the pulse height distribution resulting from a color quenched solution is compared with that of a chemically quenched solution, a shift is observed in which the color quenched pulse height distribution is spread out and somewhat depressed in comparison to the chemically quenched pulse height distribution. We have performed a number of experiments to illustrate this. An example of what one observes is shown in Figs. 1 and 2. The two samples had virtually identical counting efficiencies. The color quenched samples show evidence of the shift described above. Note the lower maximum and broader spectral shape and that the difference is more pronounced with the higher energy $\beta$, ($^{14}\text{C}$).

**EXPERIMENTAL**

Samples were prepared using a standard toluene cocktail containing 8 g/l Butyl PBD. The chemically quenched samples were prepared by adding the appropriate amount of nitromethane to each sample. A corresponding set of color quench samples were prepared by adding the dye Sudan Red. The ($^{14}\text{C}$) and ($^{3}\text{H}$) labels were added as the appropriately labeled toluene. Samples were in pairs with chemical and color quench adjusted to give identical efficiencies at several levels of quench. Efficiencies at each level were within $\pm 0.35$ per cent, when the nitromethane sample was compared to the corresponding color quenched sample in an integral counting channel with instrument conditions set for optimum counting efficiency. Table 1 shows the sample array.

Since it was our intention to relate relative pulse height to efficiency (or counts per minute to a constant amount of radioactivity), we put together the system shown in Fig. 3. This instrumentation enabled us to determine pulse height spectra with either linear or logarithmic amplification, thus permitting approximation of the basic electronics of most current liquid scintillation counters.

**Linear Spectra**

Pulse height spectra obtained with linear amplification are shown in Figs. 4 and 5. Linearity was verified by using a pulse generator whose pulse shape is very similar to an RCA 4501 V3 photomultiplier with each gain configuration used. Spectra were obtained by placing the sample in the counting well, selecting the appropriate gain and/or attenuation for the desired spectral region, and counting for $10^3$ seconds with a Nuclear Data 1100 Multichannel Analyzer. Data were read out on punched tape which was subsequently processed by an IBM 360 Model 50 where corrections for attenuation and gain were
applied and plot tapes prepared. Plots were prepared on a Cal-Comp Model 585.

Log Spectra

Pulse height spectra with logarithmic amplification were obtained under similar counting conditions at constant gain. Photomultipliers were operated in the linear mode and the EG & G amplifiers (Fig. 3) were replaced with the standard summing coincidence log convertor used in Beckman scintillation counters. Spectra obtained directly on a Mos-
Fig. 4.—Linear spectra of ($^3$H) at different counting efficiencies. Solid tracings represent chemically quenched spectra; dotted line, color quenched. As in Fig. 2, differences are small.

Fig. 5.—Linear spectra of ($^{137}$Cs) Compton electrons in presence of chemical and color quenching. (Compare with Fig. 4.) Amounts of quenching are indicated by different ($^3$H) efficiencies. As with ($^{14}$C), there is significant difference.
Table 1

<table>
<thead>
<tr>
<th>Label</th>
<th>dpm</th>
<th>Counting Efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td>(U4C)</td>
<td>226,970 ± 0.3</td>
<td>79.8%</td>
</tr>
<tr>
<td>Unquenched</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sudan Red</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. 79.8%</td>
<td>Counting Efficiency</td>
<td></td>
</tr>
<tr>
<td>3. 70.3%</td>
<td>Counting Efficiency</td>
<td></td>
</tr>
<tr>
<td>4. 58.3%</td>
<td>Counting Efficiency</td>
<td></td>
</tr>
<tr>
<td>(3H)</td>
<td>302,350 ± 0.3</td>
<td>42.4%</td>
</tr>
<tr>
<td>Unquenched</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sudan Red</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2. 42.4%</td>
<td>Counting Efficiency</td>
<td></td>
</tr>
<tr>
<td>3. 26.9%</td>
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<tr>
<td>4. 15.5%</td>
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<tr>
<td>(3H)</td>
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<tr>
<td>Nitromethane</td>
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<tr>
<td>5. 78.9%</td>
<td>Counting Efficiency</td>
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<tr>
<td>6. 69.6%</td>
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<tr>
<td>7. 57.8%</td>
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<td>(3H)</td>
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<td>Nitromethane</td>
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<td>5. 41.7%</td>
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<td>6. 27.2%</td>
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<tr>
<td>7. 16.0%</td>
<td>Counting Efficiency</td>
<td></td>
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</table>

ley Autograf X-6 plotter are seen in Figs. 1, 2, and 6. Figure 6 shows the log representation of unquenched (3H) and (14C). Figures 1 and 2 show the quenched spectra.

**Compton Spectra**

These were obtained by irradiating the sample with a 40-μCi (137Cs) source followed by a sample count for an equal time with the (137Cs) source removed. A subtraction of sample count contribution was made so that the pure (137Cs) compton spectra could be observed under the influence of both color and chemical quench as seen in Fig. 7.

![Fig. 6. Unquenched log spectra of (3H) and (14C).](image)

![Fig. 7. Log spectra of (137Cs) Compton electrons in presence of chemical and color quenching. (Compare with Fig. 5.)](image)
A Model to Explain the Difference

We propose the following model to rationalize the observed $\beta$-spectrum differences between chemical and color quenching: Consider each $\beta$ to be a point source of photons, each $\beta$ producing $N$ photons, where $N$ is proportional to the energy of the parent $\beta$. Let these $N$ photons be undirectional. They then follow some path $L$. (See Fig. 8.) If, along $X$, some path length less than or equal to $L$, there exists some number of chromophores $C$, then photons will be absorbed following the familiar Beer-Lambert relationship:

$$N = N_0 e^{-kx}$$  \(1\)

where $N$ is the number of photons arriving at the photocathode of the photomultiplier, $N_0$ is the number of photons at 0 path-length, $k$ is the molal absorptivity, $c$ is the molal concentration, and $x$ is the path length in centimeters. Since the number of photons $N$ is to be considered as a function of path length $x$, and the concentration of chromophores does not change ($\Delta c = 0$), we can let $c = 1$. Then Equation (1) can be reduced to

$$N(x) = N_0 e^{-kx}$$  \(2\)

Suppose five $\beta$s of energy $E$ cause photon emission at positions $X_0, X_1, X_2, X_3, \text{and} L$ in the cell or scintillation vial. At the photocathode of the photomultiplier tube, five light pulses would result in five electrical pulses of different magnitudes; $N_0 < N_1 < N_2 < N_3 < N_L$, where $N$ is proportional to pulse height. Thus Equation (2) gives a log distribution, as illustrated in Fig. 9.

Since $N_0 \neq 0 (N_0)$, the number of photons at zero path length, is directly proportional to the energy of a $\beta$-ray, $E$, then the distribution of $N$ as a function of $E$ and path length $x$ can be given as Equation (3):

$$N(E, x) = e^{-kx} \int_{E_0}^{E_{max}} N(E) dE$$  \(3\)

which can in turn be simplified to

$$N(E, x) = N_0 e^{-k \Delta x} + c$$  \(4\)

by letting $\int_{E_0}^{E_{max}} N(E) dE$

be proportional to $N_0$. Equation (4) then represents a distribution of all $N$ summed over $E_{max}/dE$ at intervals of $N(E, x)$ and is also clearly exponential in nature. If this formulation is correct, there must be an additional perturbation of the color quenched $\beta$-spectrum, and it must have a dependence on path-length.

The perturbation arises from this dependence and the statistical uncertainty resulting from it. The perturbation can be expressed by the coefficient of variation of $N(x)$: $\sigma$. When the pulse height distribution of a $\beta$-spectrum as seen by a perfect photomultiplier is perturbed by this $\sigma$, changes in spectral representations are similar to the difference of color-quench spectra from chemical-quench spectra seen in Figs. 1 and 2: the maxima are depressed and the upper ends of the spectra are.

\[ \text{Fig. 8.—Diagram of ideal sample and photomultiplier.} \]

\[ \text{Fig. 9.—Exponential distribution of photoelectrons over intervals of photoelectron path length in homogeneously quenched sample.} \]
Fig. 10.—Computer simulation of chemical- and color-quenched (³H) spectra.

Attenuated. Figures 10 and 11 give examples of such computed perturbations. We have approximated the color-quench perturbations noting that counting statistics are maintained by the secondary emission ratio of the photomultiplier and that the coefficient of variation of the computed β-spectrum as seen with a perfect photomultiplier could be expressed by Equation (5)

$$\sigma_s = \frac{2}{\eta g_1 E_{\text{max}}}$$

(5)

where η equals the number of photoelectrons per kilovolt of β energy; g₁ equals the gain

Fig. 11.—Computer simulation of chemical- and color-quenched (∈₁⁴) spectra.
on the first dynode, and $E_{\text{max}}$ is the maximum energy of the $\beta$.

Considering special cases of the path length $x$ for Equation (2), when $x = 0$, $N(x) = N_0$ (maximum); when $x = L$, $N(x) = N_0e^{-kL}$ (minimum). We can estimate the standard deviation to be

$$\sigma = \frac{1}{3} (N_0 - N_0e^{-kL})$$  \quad (6)

Then Equation (2) can be rewritten to solve for the total number of photons emitted:

$$N_T = \frac{N_0}{k} (1 - e^{-kL}) + c$$  \quad (7)

If $c$, the molal concentration of chromato-
phore, is ignored, the average photon yield per interval $\bar{N}$ is given by Equation (8):

$$\bar{N} = \frac{N_T}{L} = \frac{N_0}{kL} (1 - e^{-kL})$$  \quad (8)

Then Equation (6) can be written as

$$\frac{\sigma}{\bar{N}} = \frac{\frac{1}{3} (N_0 - N_0e^{-kL})}{\frac{N_0}{kL} (1 - e^{-kL})}$$  \quad (9)

and further reduced to

$$\frac{\sigma}{L} = \frac{kL}{3}$$  \quad (10)

If we further assume from the above that

$$\sigma_s = \sigma_L$$  \quad (11)

we are then able to use Equation (5) to relate gain on the first dynode ($g_1$) to the coefficient of variation of the $\beta$-spectrum. Solving for $g_1$, we get

$$g_1 = \frac{36}{\eta k^2 L^2 E_{\text{max}}}$$  \quad (12)

Assigning values for $\eta$ as indicated in Table 2 and letting $L = 20$ mm (approximately the diameter of a standard L. S. vial), $k$ = the molal absorptivity, and $E_{\text{max}}$ = 18.6 keV for ($^3$H), 156 keV for ($^{14}$C), we obtained the values for $g_1$ as indicated.

This approach to simulation of chemical quench is rational in view of the fact that no additional statistical perturbation, such as path length, is in force. To simulate color quench we introduce $\sigma$ at the source of the phototube statistics, the first dynode, using a programmed approximation of a perfect photomultiplier tube on an IBM 360-50 computer. Data generated were represented in x-y configuration by a Cal-Comp 585 Plotter. The expected deviation between color and chemical quench is observed. That the difference becomes more apparent with higher energy isotopes such as ($^{137}$Cs) and ($^{14}$C) supports the argument that the deviation is a statistical perturbation. The more photons produced, the better the intrinsic distribution statistics. Since color quenching is relatively independent of energy, its effect becomes much more apparent when other statistical perturbations decrease, as is the case with higher energy $\beta$-emitters.

Considering root mean square cumulation of errors may help to clarify this argument. The uncertainty of any point on a spectrum is the root mean square combination of many factors including the number of photons, photomultiplier cathode efficiency, first dynode multiplication factor, vial transmittance, instrument optics, and so on. This uncertainty or error is energy dependent, becoming larger at lower energies. This error ($e_x$) combines with the error or broadening introduced by color quench ($e_c$)

$$e_x \approx \sqrt{(e_{x,L})^2 + (e_c)^2}$$  \quad (13)

<table>
<thead>
<tr>
<th>Photo Electrons per kev ($\eta$)</th>
<th>Gain at First Dynode ($g_1$)</th>
<th>Color</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.7</td>
<td>5</td>
<td>$-$</td>
</tr>
<tr>
<td>1.36</td>
<td>5</td>
<td>2.0</td>
</tr>
<tr>
<td>1.09</td>
<td>5</td>
<td>0.9</td>
</tr>
<tr>
<td>0.68</td>
<td>5</td>
<td>0.29</td>
</tr>
<tr>
<td>0.34</td>
<td>5</td>
<td>0.07</td>
</tr>
</tbody>
</table>
In the case of $^3$H, there are few photons per event, and $\epsilon_{L\alpha}$ is relatively large and we see only slight difference between the color-quenched and chemically-quenched samples (Fig. 2). $^{14}$C and $^{137}$Cs present a different picture. Because of the larger number of photons per event, the $\epsilon_{L\alpha}$ uncertainty becomes less and the effect of color becomes the dominant effect (Figs. 1 and 7).

**PRACTICAL CONSEQUENCES**

The consequences to counting and the calculation of dpm from cpm of the redistribution of pulse heights due to color quenching are important if either channels ratio, channels ratio-external standard, or straight external standard methods for quench correction are used. In these cases one depends on changes in distribution of pulse heights to indicate quench changes. Consider a series of samples where there is both chemical and color quenching and in which there is always some chemical quench present with color quench. Quench calibration then becomes very uncertain, particularly when the ratio of chemical to color quench is not constant within the set. Then it is possible for the pulse height distribution of the external standard to be different despite identical counting efficiencies. The difference between chemical and color quench is relatively inconsequential in modern scintillation counters unless the color quench is large. The greater the color concentration, the worse the problem. Typical deviations are shown in Fig. 12, where the channels ratio versus per cent efficiency is plotted. A dramatic divergence can be seen on the part of $^{14}$C-labeled samples but only a small offset for $^3$H. When a calibration curve is generated by external standard-channels ratio, a slightly different divergence is seen.

It should be noted that while color and chemical quenching can be quite different if one uses modern counters and calibrates by external standard channels ratio, the differences can be held to less than 4 per cent even with heavily quenched samples.
29/A REVIEW OF METHODS OF QUENCH CORRECTION IN LIQUID SCINTILLATION COUNTING

C. T. Peng

In considering quench correction in liquid scintillation counting, it is necessary to differentiate between the different causes of quenching which contribute to the overall decrease in photon yield of the scintillation system. These causes may be attributable to nonfluorescent chemical compounds, to colored substances or to heterogeneity of the system. The results are respectively known as chemical, color, or "photon" quenching.

TYPES OF QUENCHING

Chemical Quenching

In chemical quenching, the fluorescence quantum yield of the scintillation system is decreased by the presence of nonfluorescent molecules which compete with fluor molecules for the excitation energy of the solvent molecules. From the point of view of energy transfer the liquid scintillation system is a donor-acceptor system; therefore, any process interfering with the donor-acceptor relationship leads to quenching. The fluor molecules, which act as acceptors, have a high fluorescence quantum yield and can degrade excitation energy by fluorescence emission whereas the nonfluorescent molecules convert this energy by molecular translational and vibrational relaxation into heat. This degradation of excitation energy by radiationless transition without enhancement of the photon yield is "chemical" or fluorescence quenching. In addition to energy transfer by resonance and exchange force, the nonfluorescent molecules can combine either with the solvent or the solute molecules to form encounter complexes. In static quenching, the encounter complexes consist of unexcited molecules, and the effect is to decrease the concentration...
of fluor molecules in the system. In diffusional (dynamic) quenching, excited solvent or solute molecules combine with the quencher molecules to form encounter complexes which then undergo reactions to yield deactivated products. Although quenching by energy transfer can be approximated by an exponential function of quencher concentration (see Chapter 28), quenching by formation of encounter complexes is not a simple function of quencher concentration. In the practice of liquid scintillation counting, scintillation systems are composed of intimate admixtures of liquid fluor and sample, thus rendering any material introduced into the system a potential quencher capable of participating in any of the quenching mechanisms described.

Color Quenching

A decrease in the scintillation counting efficiency can also be caused by a coloration of the scintillation system as a result of chemical or photochemical reactions or of incorporation of colored substances. Coloration diminishes the mean free path of photons and impairs the light collection efficiency of the multiplier photocathode. The diminution of photon transmission or the light-filtering effect, when not accompanied by chemical quenching, is dependent upon absorbance and is attributable to color quenching. (See Chapter 28.)

“Photon” Quenching

In “photon” quenching, the fluorescence quantum yield of the scintillation system per primary event is reduced. This type of quenching occurs mainly in heterogeneous samples such as in suspension counting and is attributable to adverse geometry and insolubility of added samples. It differs from quenching in homogeneous samples in that maximum interaction between the dissipated $\beta$-energy and the fluor is not achieved. Since the insoluble sample is dispersed in the system, the fineness of the dispersion determines the degree of self-absorption, especially when $\beta$-radiation from ($^{3}$H) is concerned. If the bulk of the dispersed phase can provide sufficient mass to stop the $\beta$-radiation and prevent interaction with the fluor, a lowering of the photon yield will result. As the degree of dispersion cannot be controlled from sample to sample, decrease in photon yield is therefore not predictable. The term “photon” quenching was previously used to indicate a reduction of the number of photons reaching the multiplier photocathode by masking a portion of the homogeneous sample vial with black tape.1

As the mechanisms for chemical, color and photon quenching differ, so do the methods for quench correction. In the following, these methods are categorically reviewed.

**QUENCH CORRECTION FOR HOMOGENEOUS SAMPLES**

The methods for quench correction in liquid scintillation counting for homogeneous samples are: (1) balanced quenching method; (2) channels ratio method; (3) dilution method; (4) external standardization method (external source, internal source); (5) extrapolation method; (6) internal standard method; (7) miscellaneous. Since these methods have been reviewed in articles published in 1964 and 1965,1,2 we will cover advances reported after that time.

**Balanced Quenching Method**

This method involves the use of a selected counting window centered around a threshold discriminator level so that positive and negative deviations from the unquenched counts are equally probable. Wright and Castle3 applied this method to counting ($^{14}$C) in scintillation systems containing chemical, dilution and color quenchers and observed that the balanced quenching method does not entirely eliminate the effect of quenching. Ross4 stressed the importance of selecting the proper width of counting window and the balanced quenching point for accuracy and outlined steps to achieve optimum conditions for the application of this method.
Channels Ratio Method

The basis of this method is the observed shift of the ratio of count rates in two counting channels in the presence of a quencher caused by diminution of the relative pulse heights. For counting \(^{14}\text{C}\) samples, the two channels can be selected to overlap each other. The ratio of the count rate in the narrow channel to that in the wide channel is plotted against the counting efficiency to yield an approximate linear correlation curve. For \(^{3}\text{H}\) samples, the ratio of count rates in two adjacent channels is used.

Many aspects of the channels ratio method have been examined and special emphasis has been placed on the construction of correlation curves. According to Herberg, a desirable correlation curve should be linear and of adequate slope, because linearity facilitates computation and recalibration, and adequate slope ensures sensitivity. In practice, these criteria can only be met by a systematic variation of gain, window width and baseline discriminator level. Since the monitor and the counting channels can be selected to have the same lower or the same upper channel limits or to have discordant upper and lower channel limits, correlation curves obtained under these conditions can assume different shapes. To achieve a proper and desirable curve, endless combination of instrument parameters may be necessary, and the lack of theory to guide the selection of optimum conditions will become very apparent.

From a consideration of simple quenching kinetics in liquid scintillation systems, Gibson and Gale derived an exponential relationship between relative counting efficiency and quencher concentration and applied the exponential form of the bias curve (a plot of counting efficiency versus bias level) to two-channel counting to yield the following equation:

\[ R_1 = R_0 (H_a)^{q_1/q_a} \]  

(See the Appendix for definition of terms.)

A plot of \( R_1 \) versus \( q_1 \) yields a straight line. Alternatively, if the bias level \((q_a \text{ and } q_b)\) of the two channels are so chosen that \( q_b = 2q_a \) which leads to \( q_1 = q_a \), \( R_1 \) is linearly dependent on \( H_a \). This method of defining the optimum condition for channels ratio has apparent limitations because of the lack of flexibility in the selection of channels ratio and the insensitivity of the correlation curve to small changes of quencher concentration.

A rational approach to the computation of channels ratio which has been reported by Peng is based on two instrument parameters, \( k \) and \( \gamma \), which are defined as follows:

\[ k = S_{01}/S_{02} \]  
\[ \gamma = \frac{\log S_{02} - \log S_2}{\log S_{01} - \log S_1} \]  

(See the Appendix for definition of terms.)

The value of \( k \) and \( \gamma \) can be obtained by counting an unquenched sample and a slightly quenched sample at baseline discriminator levels \( L_1 \) and \( L_2 \) in the integral mode of counting. The expression for channels ratio for \(^{14}\text{C}\) and for \(^{3}\text{H}\) given by Bush can be formulated in terms of \( k \), \( \gamma \), and quencher concentration \( C \) as follows:

\[ ^{14}\text{C}: \frac{L_1-L_2}{L_1-L_3} = 1 - \frac{1}{k} \exp (-[\gamma - 1]C/C_0) \]  
\[ ^{3}\text{H}: \frac{L_2-L_3}{L_1-L_2} = (k \exp ([\gamma - 1]C/C_0) - 1)^{-1} \]

Correlation curves of counting efficiency-versus-channels ratio can be generated by assigning arbitrary values to \( C/C_0 \) and plotting the right-hand term of Equation (4) or (5) against \( \exp (-C/C_0) \). The term \( \exp (-C/C_0) \) represents the relative counting efficiency. When multiplied by the counter efficiency, \( \epsilon \), for a given radionuclide, it yields \( \epsilon \cdot \exp (-C/C_0) \) as the absolute counting efficiency. The arbitrary value assigned to \( C/C_0 \) may vary from 0 to 1 corresponding to a range of zero to 63 per cent quenching.

The \( k \) and \( \gamma \) values are interrelated and characteristic of the spectrometer used. By
raising or lowering the discriminator levels and altering the window width, both \( k \) and \( \gamma \) can be made to vary with a limited degree of freedom from each other. The correlation curves that are generated by varying these two parameters arbitrarily will show different slopes and linearities, from which a desirable curve can be selected. The instrument settings can then be adjusted to reflect the appropriate \( k \) and \( \gamma \) values. The validity of the computed correlation curve can then be verified with a set of quenched standards.

This rational approach can yield \( k \) values from predetermined channels ratios for \(^{14}C\) and for \(^{3}H\) by using the limiting conditions (when \( C = 0 \)) of Equations (4) and (5), thus,

\[
(C.R.)^{(14C)} = \frac{k - 1}{k} \tag{6}
\]

\[
(C.R.)^{(3H)} = (k - 1)^{-1} \tag{7}
\]

Both \( k \) and \( \gamma \) are characteristic of a given correlation curve, and the knowledge of these values would allow the same curves to be reproduced on different spectrometers. Therefore, it may be imperative to report these values when quench correction by channels ratio method is made.

In computer-aided quench correction by channels ratio for single and dual isotope samples, Krivevsky, Zaveler and Bulkeley\(^9\) expressed the efficiency per channel as a polynomial of quencher concentration or as that of a selected channels ratio. The latter approach of expressing the counting efficiency in a given channel in terms of channels ratio was reported by Hendler\(^10\) for dual isotope samples of \(^{3}H\) and \(^{14}C\) and for \(^{14}C\) and \(^{32}P\) in a double-channels ratio method. For a series of quenched \(^{14}C\) samples, the experimental results obtained by fitting a polynomial of channels ratio were in agreement with theory, but for dual isotope samples containing \(^{3}H\) and \(^{14}C\) which were strongly quenched, the results for \(^{3}H\) became erratic. These findings emphasize the restrictions for application of the channels ratio approach to quench correction, that the count rates must be high enough to make the measurement of the channels ratio count accurate and the contribution of background rate negligible.

**Extrapolation Method**

Based on the observed exponential relationship between counting efficiency and quencher concentration, this method obtains the count rate of a sample in the absence of quenching, by extrapolation from a series of samples containing various amounts of the quencher. The method yields precise determinations when multiple counting samples are used.\(^11\) The subjection of a single counting sample to repeated addition of sample aliquots has been misinterpreted in some reports.\(^12\)\(^-\)\(^14\) It is not and has not been recommended. The exponential relationship between efficiency and quencher concentration is only valid if the following conditions obtain: the integral mode of counting is employed; quenching is not caused by formation of encounter complexes; and the property of the quencher is not altered at high concentrations by the formation of dimers or other complexes.

**External Standardization Method**

This method utilizes Compton recoil and conversion electrons from an external gamma source to determine the counting efficiency of the liquid scintillation system. The gamma ray stopping power depends upon the electron density of the absorber; therefore, the thickness of the counting vial, the average atomic number and the volume of the counting sample all become important factors in determining the count rate of the external standard. The volume dependence has been mitigated by the use of a compound source consisting of \((^{22}Ra)\) and \((^{241}Am)\) or by the substitution of the ratio of the count rates in two channels for the direct count rate of the source.\(^12\) Schrödt, Gibbs and Cavanaugh\(^13\) have discussed the merit of external standardization method in quench correction and have obtained a linear relationship between counting efficiencies of the external source and
the liquid scintillation sample. Because of the dissimilarity in the pulse height spectra, the accuracy of their method may ultimately depend on a matching of the spectrum of the external source with that of the isotope under study by means of instrumental attenuation.

Gibson and Gale suggested that for the measurement of relative counting efficiency, an external source can be used either in the integral or the differential counting mode. This selection is based on the bias curve obtained in their experiment which consists of a plot of counts per channel versus bias level and is composed of a flat portion at low bias levels and an exponential portion at high bias levels. The integral count rate of the external source is made at a high bias level which yields a value \( C_2 \) above bias \( x_2 \). A plot of \( \log C_2 \) versus \( \log H_0 \) at various concentrations of the quencher gives a straight line. At low bias levels, the differential count rate of the external source will increase with quenching. This represents a special case and its validity will depend upon the degree of contraction of the flat portion of the bias curve caused by quenching.

The use of an external standard for quench correction was systematically studied by De Wachter and Fiers with respect to the variation of instrument parameters. The conditions under which the gain was selected were guided by the criterion that the integral counting efficiency of the external standard \( N \) should approach as closely as possible an exponential function of the concentration of the quencher in accordance with the equation

\[
N = N_0 \exp \left( -q_{\alpha}C \right)
\]

(8)

(See the Appendix for the definition of terms.) From a similar equation relating the balance point counting efficiency, \( E \), of the sample, to quencher concentration in differential counting mode, a straight line can be obtained between \( \log E \) and \( \log N \) with a slope that is dependent on the \( \beta \)-energy, amplification and discriminator levels of the instrument.

The linear relationship of \( E \) versus \( N \) on log-log scale can be applied to quench correction by external standard when a single isotope is concerned. For samples containing two or three isotopes, a screening method for setting up the counting channels in the spectrometer is adopted, in which the high energy channel displays only counts from the high energy isotope while the low energy channel shows counts from both high and low energy isotopes. The linear log-log relationship between \( E \) and \( N \) holds for the isotopes in their respective channels, but the counting efficiency of the high energy isotope in the low energy channel at the balance point operation for the low energy isotope is linearly dependent on the logarithm of the counting efficiency of the external standard. In this manner, De Wachters and Fiers were able to extend the external standardization method for quench correction to samples containing three isotopes.

In the differential mode of counting the balance point shifts to a higher gain as the degree of quenching is increased. Since only samples that are counted in the balance point operation obey the exponential relationship between efficiency and quencher concentration defined above, it is necessary to monitor the shift of the balance point in relation to the count rate of the external standard and make appropriate adjustment of the gain accordingly. Calibration curves relating the count rate of the external standard to the gain obtained with standard quenching samples are used for this purpose. The requirement to adjust the gain of the instrument to balance point operation has limited the application of this method to samples of approximately equal quenching properties such as effluents from column chromatography and until recently has curtailed its use for samples of variable degrees of quenching. (See Chapters 30 and 31.)

**COLOR QUENCH CORRECTION FOR HOMOGENEOUS SAMPLES**

The methods reviewed above apply to chemical as well as color quenching with the
exception noted below. If a colored substance contributes also to chemical quenching, the separation of the total quenching effect into components will be difficult by the usual correction method. In a liquid scintillation system, the quenched pulse height spectrum caused by color differs in shape from that by chemical agents. For $\beta$-particles with energies higher than that of tritium, the dissimilar pulse height spectra will yield different correlation curves between counting efficiency and channels ratio. For intensely color-quenched samples containing ($^{14}$C), the correlation curves have been found to deviate significantly from chemical quench curves. This difference is either insignificant or non-existent with color-quenched ($^3$H) samples, probably owing to the small number of photons generated in the system which probably obliterates any existent distinction between the quenched pulse height spectra by color and by chemical agents. (See Chapter 28.)

**Spectrophotometric Methods**

The quench correction for colored samples can be carried out by spectrophotometry by measuring the absorbance of the scintillation solution at selected wavelengths. Many photomultiplier tubes have a spectral response (S-11) with peak sensitivity in the region from 420 to 520 nm. Iwakura and Kasida have evaluated the total absorbance of a scintillator solution by the formula

$$A = \frac{\sum A_i W_i}{\sum W_i}$$

(9)

where $W_i$ is the relative photocathode sensitivity within the spectral region $i$ nm, and $A_i$ the absorbance of light at the spectral region $i$ nm. They used 14 photosensitive cyanine dyes at concentrations from 0.5 to $10 \times 10^{-6} M$ to tint the scintillation solution. The total absorbance of each dye was obtained from component absorbances at 40-nm intervals throughout the sensitive spectral region (360–520 nm) of the photomultiplier cathode by the above equation. When the reciprocal of the total absorbance was plotted against counting efficiency, a straight line was obtained for ($^3$H) and an approximate linear relation for ($^{14}$C). A similar method was applied earlier by Herberg to the determination of quenching of background counts by color.

Ross has also reported a spectrophotometric method for color quench correction in which the per cent color quenching $Q$ is obtained from the absorbance of the solution at designated wavelengths by the following integral:

$$Q = K \int \lambda (\lambda) d\lambda$$

(10)

where $K$ is the quenching coefficient, $A$ the absorbance, and $\lambda$ the wavelength. The quenching coefficients at various wavelengths can be evaluated by using standard scintillator solutions containing different concentrations of the dye or dyes and solving the simultaneous equations generated from Equation (10). From a number of dyes studied in this manner, $Q$ values consistent with the observed ones were obtained, (with the exception of methyl red which was found unstable in the scintillation solution) at the concentration used.

**Isolated Internal Standard Method**

The use of spectrophotometry for the correction of color quench is a tedious task in practice, and an isolated internal standard has been recommended. This consists of a small glass ampule containing an unquenched scintillator solution spiked with the desired isotope to serve as a light source. Its intensity $A_q$ is measured by immersion in a given volume of the unquenched scintillator solution. To determine the degree of color quenching $Q$, this light source is inserted in a color-quenched sample and counted. The increase in count rate due to this light source in the sample is designated as $A_2$ and

$$Q = \frac{A_q - A_2}{A_q}$$

(11)
The observed radioactivity of the sample is corrected for color quench by multiplying it by the fraction $1/(1 - Q)$. The use of this method for color quench reveals that in liquid scintillation counting of biological specimens such as liver, blood and urine, the major fraction of the quenching encountered is caused by color.

"PHOTON" QUENCH CORRECTION FOR HETEROGENEOUS SAMPLES

The determination of efficiency in the liquid scintillation counting of nonhomogeneous samples faces many problems. Solid materials insoluble in the scintillation system can be counted in dispersed form on solid supports or in finely divided state as suspensions in the form of gel with the aid of aluminum stearate or a thixotropic agent such as Thixcin or Cab-O-Sil. The former category includes samples absorbed on ordinary or glass filter paper, and the latter includes those adsorbed on powdered silica gel. (See Chapters 19 and 20.) Chemical quenching in suspension counting is either absent or at a minimum for totally insoluble materials. In nonhomogeneous samples, the reduction of photon yield may be attributable to an adverse geometry ($2\pi$ versus $4\pi$) of isotope source in the system, self-absorption of $\beta$-ray energy in the nonscintillating phase, diminution of light transmission by the opacity of the gel system and so on. Among these effects, the self-absorption, being energy dependent, becomes most pronounced for $\beta$-particles from tritium. The $f$ value, defined as the ratio of suspension counting to homogeneous counting, also varies with specific radioactivity of the sample material. This dependence further complicates the process of correction for "photon" quenching.

Bush has employed a double-ratio technique to determine inhomogeneity of liquid scintillation solutions caused by adsorption, microscopic precipitation or phase separation of radioactive sample material. In this technique, the quenching characteristic of the solution is measured by the external standard ratio method while the "photon" quenching, due to the inhomogeneity of the scintillation system, is determined by the channels ratio method. In the absence of "photon" quenching, the external standard ratio and the channels ratio yield identical counting efficiencies; when disparate values are obtained, sample inhomogeneity is indicated. The double-ratio method only provides a qualitative differentiation, i.e., to determine whether the sample has been counted with true solution efficiency. It will not yield quantitative "photon" quench correction. A similar approach has been suggested, by Schrod et al.: to apply simultaneously the external standardization method and the channels ratio method to normalize the counting data of inhomogeneous samples by a correction factor. In view of the complexities of factors affecting the "photon" quench correction in nonhomogeneous samples, it is difficult to ascertain the general applicability of this method without much more extensive experimental verification.

The usefulness and versatility of the nonhomogeneous counting technique lies in the capacity to deal with large amounts of radioactive sample material in insoluble forms. In the absence of chemical quenching and when self-absorption is well defined as is the case with samples adsorbed on powdered silica gel, or solutions spotted on glass filter paper, nonhomogeneous sample counting can yield relatively reproducible results.

OTHER CONSIDERATIONS

The basis of quenching correction has been empirical in nature and much attention has been directed toward the significance of the exponential relationship between counting efficiency and quencher concentration. This relationship has been utilized for quench correction by the extrapolation method; computation of channels ratio through the use of $k$ and $\gamma$ values, and adjustment of instrument settings for channels ratio and external standardization methods.
It can be shown that such an exponential relationship can also be derived from theoretical considerations of quenching kinetics or from system parameters such as pulse height, amplification, bias level, fluor concentration, and energy transfer rate constants. The last approach lends more insight to the interrelation of factors that affect the quenching characteristics of the liquid scintillation system and can correlate many of the isolated observations with respect to quenching.

The equation derived by Kowalski, Anliker and Schmid, based on the mechanism of a binary organic scintillation system, relates the counting efficiency, $\epsilon$, to other system parameters as follows:

$$\epsilon = \epsilon_0 \exp \left\{ - \frac{1}{k_{tq}} \left[ \frac{1}{k_{tq}} (1 + \sigma_{ek} Y) \right] \cdot \frac{L_1}{b \cdot A} \cdot k \right\}$$

(See the Appendix for definition of terms.)

This equation expresses the efficiency as an exponential function of the concentration of the quencher when other parameters are held constant. The half-value quenching concentration $C_{1/2}$, for which the initial counting efficiency is reduced by a factor of 2, is given below:

$$C_{1/2} = \frac{q_{yo}}{L_1} \cdot \frac{b \cdot A}{(1 + \sigma_{ek} Y)} \cdot k \cdot \ln 2$$

The half-quenching concentration can be used as a figure of merit for comparison of various liquid scintillators in respect to their quenching resistance property. If other factors are kept constant, $C_{1/2}$ in Equation (13) is inversely proportional to $k_{tq}$ which is an intrinsic quenching property of the system. The $C_{1/2}$ value can also be used as an index for ordering the quenching property of compounds.

From Equation (13), the following correlations with the observed facts about quenching can be deduced:

1. Solvents with high $k_{tq}$ are preferred. At equal solute concentration, PPO (2,5-diphenyloxazole) in toluene can withstand quenching by carbon tetrachloride better than PPO in n-hexane, since toluene and p-xylene are more efficient solvents than n-hexane in energy transfer. (See Chapter 2.)

2. Solutes with high fluorescence yield, $q_{yo}$, are preferred. PPO ($\phi$:1.0) can resist quenching by carbon tetrachloride to a greater degree than anthracene ($\phi$:0.36). (See Chapter 3.)

3. When concentration quenching of the solute is negligible, $C_{1/2}$ is linearly dependent upon the solute concentration. Consequently, an increase in solute concentration should enhance the capacity of the scintillation system to resist quenching. The restoration of counting efficiency in a quenched system by high concentrations of naphthalene is a well-known example. A quenched solution can also be counted at higher efficiency with an increased concentration of primary solute in the liquid scintillator.

4. High gain and low discriminator level are factors necessary for maximizing the pulse height to suppress the effect of quenching. The dependence of $C_{1/2}$ value upon gain and bias level has already been noted.  

**Integral Versus Differential Counting**

The exponential relationship between efficiency and quencher concentration is only valid in the integral mode of counting. However, a similar relationship also holds in the differential counting mode provided that the efficiency is determined at the balance point. For quenched sample, diminution of the pulse height causes a shift of the balance point to a higher amplification. According to Kowalski et al., the gain at the balance point is a reciprocal function of the pulse height and is completely determined by the upper and lower discriminator levels defining the window width. The advantage of differential counting is the reputed high figure of merit, $E^2/B$ or $S^2/B$, which is determined with
an unquenched sample at its balance point. For a quenched sample, when the counting is performed at the balance point for the unquenched sample and not at its shifted position as recommended, the purported accuracy of this mode of counting becomes highly questionable. (See Chapter 31.)

The desirable condition for measurement of samples encompassing both $S/B >> 1$ and $S/B << 1$ is predetermined by maximum $S^2/B$ considerations, but for measurement of signal rates well above background (when $S/B >> 1$) with the highest precision, the signal strength should be maximized regardless of $S^2/B$ value. The erratic results obtained by Krichevsky et al. for $^{14}C$ in strongly quenched samples containing $^{14}C$ and $^{14}H$ by the channels ratio method when the count rate of $^{14}H$ fell to below $2 \times 10^6$ cpm (which is still well above the background rate) may point to the need for a reevaluation of the use of the differential mode of counting in the nonbalance point operation for quench correction in liquid scintillation counting.

**APPENDIX**

$R_1 = \text{Ratio of quenched bias curves (or count rates) at bias (or discriminator) levels } x_a \text{ and } x_b.$

$R_o = \text{Ratio of unquenched integral bias curves (or count rates) at bias (or discriminator) levels } x_a \text{ and } x_b.$

$H_o = \text{Relative counting efficiency of unquenched integral bias curve (or count rate) at bias (or discriminator) level } x_a.$

$q_o = bx_a k = \text{constant. Both } b \text{ and } k \text{ are constants.}$

$q_i = b(x_i - x_a)k = \text{constant.}$

$\exp = \text{exponent of natural logarithm.}$

$S_i = \text{Apparent specific count rate of a quenched sample measured in integral mode of counting at base line discriminator level position } i (i = 1 \text{ and } 2).$

$S_{ai} = \text{Specific count rate in the absence of quenching measured in integral mode of counting at base line discriminator level position } i (i = 1 \text{ and } 2).$

$L_i = \text{Discriminator level at position } i (i = 1, 2 \text{ and } 3).$

$C = \text{Sample concentration.}$

$C_{1/2} = \text{Half-value concentration of a quenched sample that will reduce the count rate to half its initial value by quenching.}$

$q = \text{Quenching constant. It equals } 0.693/C_{1/2}.$

$C_o = 1/q = 1.44 C_{1/2}. C_o \text{ is the reciprocal quenching constant for the counting channel having the lowest discriminator level as base line.}$

$N_o = \text{External standard count rate at zero quencher concentration.}$

$N = \text{External standard count rate at quencher concentration } C.$

$q_{ex} = \text{A constant dependent upon the quenching substance and the instrument parameters.}$

$\epsilon_a = \text{Integral counting efficiency.}$

$\epsilon_{bo} = \text{Integral counting efficiency at } [Q] = 0.$

$[Q] = \text{Quencher concentration.}$

$[Y] = \text{Solute concentration.}$

$k_{txq} = \text{Transfer rate constant from solvent molecule } X \text{ to quencher molecule } Q.$

$k_{tqy} = \text{Transfer rate constant from solvent molecule } X \text{ to primary solute molecule } Y.$

$k_{ix} = \text{Rate constant of internal conversion of solvent } X.$

$k_{iy} = \text{Rate constant of internal conversion of solute } Y.$

$k_{eiy} = \text{Rate constant of concentration quenching of solute } Y.$

$k_{fy} = \text{Rate constant of fluorescence emission of solute } Y.$

$q_{sp} = k_{fy}/(k_{fy} + k_{iy}) = \text{Molecular quantum yield of fluorescence of solute } Y.$

$\sigma_{cy} = k_{eq}/(k_{fy} + k_{iy}) = \text{Factor of concentration quenching.}$

$A = \text{Amplification or gain of the counter.}$

$b, k = \text{Constants, related to } \beta\text{-energy and instrument factors.}$
REFERENCES


In scintillation counting, the efficiency, certainly the most troublesome unknown can be obtained only by inference. The primary standards for liquid scintillation counting are NBS (National Bureau of Standards) standardized tritiated water, tritiated toluene, and so on. Tritiated water, for example, the primary (3H) standard of activity, had to be standardized by an "absolute counting method" (in the true sense of the word, there is no such thing) or at least by a method for which biases and errors could be accurately accounted to be no more than 1 per cent. (3H) toluene has been standardized with respect to (3H) water by an internal standardization technique. (See Garfinkel et al. for further details.) Provided a reasonable effort is made to calibrate working standards with respect to the primary NBS standards, one can keep track of the input and output dpm in experiments. Often in biological experiments, it may be necessary to determine only the ratio (cpm output/cpm input) which suffices unless counting efficiency varies from counting input to counting output.

To be on the safe side, however, it is always desirable to know the dpm of the material counted. Internal standardization is now generally regarded as too time-consuming and troublesome for routine use in efficiency determinations and is also wasteful of radioactivity. The easier methods (channels ratios and external standardization) have their own unique pitfalls and short-comings (see Chapter 29), but for routine use they are generally preferred over the internal standardization method and are presently much used.

It is my purpose, therefore, to discuss in some detail the errors involved in quench correction using the AES (Automatic External Standard) ratio. Our treatment is also applicable, with some modification, to the gross AES count and channels ratio methods as well.

Sources of Error in Counting

The main object of liquid scintillation counting is to obtain the absolute radioactivity or disintegrations per minute (dpm)
of an isotope in a sample. All isotopes count with less than 100 per cent efficiency, depending on the quench level of the sample-scintillator mixture and a host of other factors such as photomultiplier tubes, windows and amplifier gain, each with a varying efficiency. The objective is two-fold: to obtain counts per minute for the sample and to determine the efficiency with which it is counted. After these steps are completed, we compute

\[ \text{dpm} = \frac{\text{cpm}}{\text{efficiency fraction}} \]

Let \( A \) stand for activity in dpm, \( c \) stand for cpm, and \( e \) stand for efficiency fraction \( E \) (efficiency = 100\% \( \times \) \( e \)).

Then

\[ A = \frac{c}{e} \]  

(1)

Taking the total differential, we have

\[ dA = \frac{dc}{e} - \frac{cde}{e^2} \]  

(2)

Now \( dA \) alone is conceptually useless, but \( dA/A \) is the fractional error and is a useful expression:

\[ \frac{dA}{A} = \frac{dc}{c} - \frac{de}{e} \]  

(3)

Up to this point we have purposely treated these quantities as if they were not statistical variables, for no one could argue with the output of a perfect counting instrument which followed Poisson statistics exactly. Real liquid scintillation counters, however, fall short of perfection for a variety of reasons.

1. Vials used in counting are not perfect. Glass vials have the same inhomogeneities peculiar to the glass tubing from which they are fabricated. Thicker glass on one side of a vial, or even over a small part of its surface, distorts the optical path of light emitted from the liquid. The distorted light path may cause more light to fall on a less sensitive part of the photocathode of a photomultiplier tube in a given orientation of the vial, and less light in another. A given orientation may cause more light to be reflected from the walls back into the liquid, further attenuating the light in a different orientation this distortion may cause no light loss. Plastic vials likewise have the inhomogeneities peculiar to the process by which they are made.

2. The gains of photomultipliers vary with time. These gain changes can be minimized by employing a lower gain in the tubes. This approach, however, necessitates increasing electronic gain with the consequent penalties of sudden bursts of electronic noise and high and unstable backgrounds. (See below regarding PMT normalization.)

3. Other factors include geometric reproducibility, lateral and vertical, of sample versus PMTs, electronic gain stability, high voltage stability, effects of power line transients, effects of dead time, effects of induced phosphorescence, droplets of scintillator-sample solution clinging to the cap or the vial walls, shifting of filter paper samples within vial, and so on.

If we could eliminate all of these effects we still would expect to have a significant per cent standard error in counting a single sample (loading the sample in a random manner) of the order of about 1.5 per cent.

Let us consider in particular the sources of error in the most common methods of efficiency determination:

*Internal standardization.* Provided that the added "spike" does not change the quench level, that we accurately pipette it into the vial, that we mix it adequately and reinsert the vial in the same orientation, and that we carry out these steps immediately after counting the sample, internal standardization should be an ideal means of efficiency determination. The sources of error mentioned above (1, 2 and 3) should be the same with or without the "spike." Such conditions are hard to fulfill, and most researchers prefer that the counting be done automatically and without interruption, (without demands on their time during counting). Groups of samples are generally counted three or so cycles and the results are averaged; the averaging, in effect, reduces err-
ors due to variations in orientation, positioning, gain, high voltage, decay and temperature. With internal standardization, there is a time lag and efficiency determination necessarily follows the last count. The possibility that long-term variations in high voltage, temperature, or gain might have occurred in the meantime, negating some of the benefits of averaging over multiple cycles, is usually ignored.

Channels ratios are problematic in that the efficiency determination error is related to the sample Poisson counting error. Channel settings may have to be changed if isotope is changed, and dual-isotope quench correction becomes statistically hazardous if isotope ratios vary over wide limits. (See Chapters 6 and 29.) Otherwise, many of its features are equivalent to those of external standardization.

External standardization is currently the most popular method of efficiency determination. It is employed in basically two forms: net or gross counts of an external standard, and net external standard ratio. The basic difference is that employing the ratio results in less volume dependence of the quench correction curves. Otherwise, an error study is equally applicable to both.

Errors in External Standardization

Let us consider a typical external standardization quench correction curve for $^3$H in a toluene-scintillator solution, as shown in Fig. 1. The basic curve is determined by counting samples of known dpm and then plotting the efficiency of counting in a given window versus the external standard ratio. It is possible to choose windows that will give straight-line curves over a limited quench range but rarely, if ever, over a wide quench range, no matter what geometry or isotope is used. Frequently it is found, depending on the choice of a few (3, 5 or 7) quenched samples, that one can draw a straight line through the points, but the line will usually depart markedly from linearity if the quench correction range is extensive.

The external standard is usually counted either prior to or immediately after the sample count. The efficiency determination is made without changing the vial orientation and position.

From Equation (3) we can see that any effect on sample counting and external standard counting will be in the same direction; there is a correlation between the two errors. Therefore, if an efficiency determination is made for each AES ratio determination and the corresponding counts (cpm) of the sample is divided by that efficiency, the resulting dpm should have a smaller error than either the sample cpm or the external standard ratio alone. This can be confirmed by plotting histograms for the cpm, AES ratio, and computed dpm for a single sample counted over a series of cycles. (There will be more about this below.)

Influence of Efficiency

If the level of counting efficiency had no effect on the error of the efficiency determination, the relationship between $\Delta R$ (a difference in ratio) and efficiency would be linear and would arise from the zero intersect (Fig. 2). If the Poisson error ($\sigma_R$) of the ratio were plotted against the AES ratio, again with no effect of the actual efficiency level on error considered, the theoretical relationship (based on Poisson statistics) shown in Fig. 3 would pertain. Experimental $\sigma_R$s from the Packard 3375 and 3380 Tri-Carb Systems are also shown. Note that they conform fairly closely
Fig. 2.—Theoretical relationship between $\Delta R$ and efficiency at 1 per cent error level.

Fig. 3.—Error of AES ratio as function of ratio $\delta R$ is indicated by the ordinate. Solid line is theoretical error ($\sigma R$) if independence of efficiency level is assumed. Broken line represents errors determined experimentally with Packard 3375 and 3380 systems.

to the theoretical curve. Note also that as the AES Ratio ($R$) approaches zero, that the Poisson error $\sigma R$ also approaches zero. Although the per cent standard error ($\sigma R/R \times 100$) becomes infinite at zero $R$, it is still possible to obtain a less precise but still reasonably accurate dpm value near zero $R$ provided that the efficiency of counting the isotope is significantly above zero. What this means practically can be seen in Figs. 4 and 5 which show typical percent standard errors of dpm for single determinations made with a Packard Model 3380 and Model 544 absolute activity analyzer for groups of samples having 100 different quench levels, spanning ratios from 1.0000 down to .0000. Note that the per cent standard error of dpm calculation significantly increases as $R$ decreases, principally because the divisor in the error computation ($\sigma R/R$) decreases. The differences between the ($^3$H) and ($^{14}$C) errors relate partly to respective differences from the dynamic range of the external standard and partly to other factors. (Please see Appendix for a more thorough discussion of the statistics of the AES ratio.)

Influence of PMT Normalization

Provided PMTs were normalized for gain at the time quench correction curves were determined, we can discover how much error to expect in dpm calculation if the tubes drift $\Delta R_N$ out of normalization. This error is dependent, of course, on the specific window chosen to count the isotope. The usual procedure for choosing a window for ($^3$H) is to

Fig. 4.—Per cent standard error of ($^3$H) dpm versus AES ratio. Dual isotope model Packard 3380-554. Single 1-minute counts of $10^6$ dpm.

Fig. 5.—Per cent standard error of ($^{14}$C) dpm versus AES ratio. One-minute counts of $10^6$ dpm. (See Fig. 4.)
set a wide window and adjust gain until the count rate is maximal for an unquenched sample. This procedure leads to shifts of the correction curve for (3H) as shown in Fig. 6. The normalized curve is the same one shown in Fig. 1. From this curve, it is apparent that the standard error (\(\frac{\Delta R}{R}\)) of the AES ratio for normalization (i.e., at a ratio 1.000) is approximately 0.11 per cent. We suggest three successive readings (in the range 1.000 ± 0.0030) to indicate if the PMT's are normalized. If the true AES ratio was outside the range 1.0000 ± 0.0050, the probability is small that one would obtain three successive readings within the range 1,000 ± 0.0030. We have found that the statistics of the AES ratio on a blank sample are such that (\(\frac{\Delta R}{R}\)) varies between 0.11 per cent and 0.20 per cent and averages 0.15 per cent. Sometimes when instruments display a drift of PMT gain that makes \(\frac{\Delta R}{R}\) appear larger, one practical solution to this problem is to keep a constant counting load on the instrument. If this fails, a change of PMTs is the only solution.

To see the effect of a change in normalization on counting, refer to Figs. 7 and 8 showing the effect of a change (drift of one PMT by ± 0.0100 in normalization) in PMT gain on (3H) efficiencies in two commonly used (3H) windows:

\[ N = \text{Narrow (25-700; 100\% gain) used for double label counting; } W = \text{Wide (25-1000; 64\% gain) used for single label counting.} \]

(Also see the example given by Wyld in Chapter 5.) The usual problem is drift of one PMT. If both drift, and each drifts in the opposite direction, the effects tend to cancel. If both drift in the same direction, the effects tend to be additive. If both drift in the same direction, a drift of .0050 of each will cause an equivalent error in efficiency determination.

Let us return to the problem of quench correction, even for samples that are prepared almost identically. The AES ratio is correlated with the counting efficiency through the quench correction curve. A drift of PMTs causes a change in AES ratio and a change in counting efficiency, so that if dpm are computed by referring to the (AES ratio-isotope efficiency) quench correction curve to obtain
the efficiency, these errors are generally reduced. The computed dpm data may then be more accurate than the cpm data. If the ratio for the unquenched sample has drifted $\Delta R_N$, the ratio for all quenched samples has drifted approximately $\Delta R_N \cdot R$. Then the standard error of the ratio becomes

$$\sqrt{\left(\frac{(\Delta R_N \cdot R)^2}{R^2}\right)^2 + \frac{\sigma R^2}{R^2}}$$

(4)

where $\sigma R$ is the Poisson error (as above) and $\Delta R_N \cdot N$ is a bias caused by the drifting of the tubes. If we include the Poisson error for counting the sample, we can calculate the overall theoretical error for a single determination of dpm:

$$\frac{\sigma \text{dpm}}{\text{dpm}} = \sqrt{\left(\frac{(\sigma R)^2}{R^2}\right)^2 + (\Delta R_N)^2 \left(\frac{de}{dR}\right)^2 R^2 + \frac{1}{\text{cpm} \cdot T}}$$

(5)

(where $T$ is time in minutes, $de/dR$ is the slope of the efficiency fraction versus the AES ratio curve at the ratio $R$, and $\Delta R_N$ is the error in normalization. Since we have already noted that in our experiments the error of the ratio averages about 0.15 per cent, we can substitute $(0.0015 \sqrt{R})$ for $(\sigma R/R)$ in Equation (5) and solve it for the dpm error.

**EXPERIMENTAL VERSUS THEORETICAL ERROR**

In practice, a 0.6 per cent standard error for $^3$H dpm has been achieved for a single determination several times the amount that Equation (5) would indicate, assuming no normalization error and an accumulation of 900,000 counts. Clearly the discrepancy must lie in the incomplete cancellation of some non-Poisson variables, by the efficiency determination. This difference in error can be reduced in practice but requires multiple cycles of AES and sample and considerable attention to procedural details. For example:

1. The normalization standard must always be similarly oriented toward the PM tubes for normalization, or an averaging over many orientations must be carried out.

2. Normalizations must be performed often enough (to keep $\Delta R_N$ small) to detect any significant error of normalization.

3. The turntable must be kept clean and free of dirt and lint. All vials should be wiped clean with lint-free cloth or tissue before insertion on the turntable. Some LSC systems have vial cleaners as standard equipment.

4. The elevator top should be wiped clean often with lint-free tissue or cloth.

5. The counting load should be steady, i.e., when not counting samples, count standards. This keeps PMTs operating under a constant load. Always use the AES, whether needed or not. This, too, keeps a steady load on the PMTs.

6. Because it affects almost all quench correction parameters, probably the most serious factor of all is the variation from vial to vial in optical distortion, a function of orientation. This variable alone sets a limit on the accuracy with which one sample may be compared to another by means of single determinations. Any sort of progress toward optical uniformity will thus diminish quench correction error.

A more detailed treatment, in which the statistical errors are not considered independent, reveals that Equation (3) is more appropriate to practical counting situations than Equation (5).

To illustrate how gain changes in the PMTs of a Packard 3375 or 3380 system can be partly offset by a related change in the quench correction parameter $R$ (the AES Ratio), consider a change in $R_n$, the normalization ratio of one of the tubes or a combined change of both tubes. The true efficiency will change by $de$, because of the drift of the PMT away from its normalized gain, denoted by $dR_n$, the independent variable:

$$de = \frac{de}{dR_n} dR_n$$

(6)
We quench correct by the quench correction curve, so we have

\[ de = \frac{\partial e}{\partial R} dR - \frac{dR}{\partial R} dR_n \]  

(7)

Then if \( R_n \) is the only independent variable, we have

\[ \frac{de}{e} - \frac{de}{e} = \frac{dR_n}{R} \left( \frac{de}{dR_n} - \frac{\partial e}{\partial R} dR_n \right) \]  

(8)

So if

\[ \frac{de}{dR_n} = \frac{\partial e}{\partial R} dR_n = 0 \]  

(9)

we have a complete cancellation of this effect to the first order. The general case is that Equation (9) is zero for higher energy isotopes when one sets windows to exclude very-low-energy events. For wide-window \(^3\)H counting, there is only partial cancellation, and PMT drift is only partially offset. Figure 7 shows\((de/dR_n)\) graphically for \(^3\)H counting and Fig. 8 shows the quench corrected \(^3\)H dpm error computed from Fig. 7 using the quench correction from curves such as those shown in Figs. 9 and 10. Note that there is an overall improvement for each window in Fig. 8. Over a restricted portion of the quench range it is possible that \((de/dR)\) might be

![Graph showing quench correction curve for \(^3\)H single label (wide channel).](image)

**Fig. 9.**—Quench correction curve for \(^3\)H single label (wide channel).

**Fig. 10.**—Volume independence of quench correction. Dual label curves and \(^{14}\)C \(\rightarrow\) \(^3\)H spillover are indicated.

![Graph showing volume independence of quench correction.](image)
entirely independent of \( \delta e/\delta R_n \) in which case quench correction would introduce a larger error than the non-quench-corrected efficiency would exhibit. However, we are presently considering only one parameter \( R_n \) out of the many parameters that quench correction compensates (for example, vial orientation variations, volume and temperature). The improvement in accuracy usually makes quenching correction worthwhile whenever accuracy is required, even over a narrow quenching range.

There are several recently developed instruments that provide automatic quench-corrected dpm as printout data. With these instruments as well as with any computer program, overall performance should be judged over a wide quenching range with a large number of samples of different quench levels to exhibit any biases inherent in the particular approach used to accomplish the quench correction.

**The Benefits of Averaging**

It is common practice in liquid scintillation counting to average cpm data. For example, a given sample is cycled three times in an LSC system. The counts per minute are averaged, the AES ratios are averaged, and dpm or relative cpm are computed. If one were to examine the cpm or ratio data separately, he would find that both varied by \( \pm 2 \) per cent, far in excess of what one would expect by Poisson statistics. Yet by averaging the counts per minute and the ratios, the resultant dpm or relative cpm are far more accurate than one would expect, based on the measured \( \sigma \)s of cpm and \( \sigma R/R \). Thus, if \( 1/s \) is the standard error actually measured for a single determination of cpm, three countings should yield a standard error:

\[
\frac{1}{\sqrt{2}} \cdot \frac{1}{s}
\]

and three determinations of \( R \) should only reduce the standard error in \( R \) \( (\sigma R/R) \) to

\[
\frac{1}{\sqrt{2}} \cdot \frac{\sigma R}{R}
\]

Consequently, the dpm error should not be less than

\[
\frac{\sigma d\text{pm}}{d\text{pm}} = \sqrt{\frac{1}{2s^2} + \frac{s^2_{de} (\delta e)^2}{2R^2} R^2} \equiv e^2 (10)
\]

yet almost invariably it is far smaller than this value. Some measure of what is lost in counting cpm and ratios separately is regained by averaging and then performing the quench correction, a result that might be expected with nonindependent or correlated random variables. Some of the correlations of random variables are happenstance while other correlations are designed into counting systems; for example, the AES ratio is essentially independent of sample vial volumes.

The results obtained by using a quench correction instrument such as the Packard 3380 with the absolute activity analyzer indicate that averaging still plays an important role in the reduction of non-Poisson variate errors when external standardization is used as the quench correction parameter, even though quench correction with external standardization achieves significant non-Poisson variate error reduction in a single determination. Whether the averaging is done before quench correction or after with a quench corrected dpm printout should theoretically make little difference.

**Volume Independence**

Because both efficiency factor and AES ratio vary with volume, it is logical to arrange the geometry and gamma sources so that the quench-corrected dpm value is volume-independent. Assuming no Poisson or curve reading errors,

\[
\left( \frac{s_{de}^2}{e_t^2} \right) = \left( \frac{\delta e_t}{e_t} - \frac{\delta e_s}{e_s} \right) \frac{1}{j} (11)
\]

Since \( v \) is to be considered the independent
variable, the error differentials can be written as Equations (12) and (13):

\[
de_{i} = \frac{de_{i}}{dv} dv \\
de_{e} = \frac{de_{e}}{dv} dv
\] (12)

\[
de_{i} = \frac{de_{i}}{dv} dv \\
de_{e} = \frac{de_{e}}{dv} dv
\] (13)

To make Equation (11) vanish, i.e., to nullify any error contribution from \( dv \), it is necessary that

\[
\frac{1}{e_{i}} \frac{de_{i}}{dv} dv - \frac{1}{e_{e}} \frac{de_{e}}{dv} dv = 0
\] (14)

Assuming that no errors other than those contributed by volume affect this determination,

\[
\left( \frac{1}{e_{i}} \right) = \left( \frac{1}{e_{e}} \right)
\]

and

\[
\frac{dv}{e} \left( \frac{de}{dv} - \frac{de}{dv} \right) = 0
\] (15)

so that Equation (16)

\[
\frac{de}{dv} - \frac{de}{dv} = 0
\] (16)

is the required condition for volume independence of dpm determination. Here \( \frac{de}{dv} \) is simply the change in counting efficiency of the isotope with respect to volume, \( \frac{de}{dv} \) is the slope of the quench correction curve, and \( \frac{dv}{e} \) is the change of ratio with respect to volume. The task then is simply to make sure that

\[
\frac{1}{e} \left( \frac{de}{dv} - \frac{de}{dv} \right) = 0
\] (17)

or is very small for both \( (^{3}H) \) and \( (^{14}C) \) throughout the entire quench range for reasonable windows. Since early experiments, it has been recognized that positioning directly underneath the vial was adequate for a low-energy gamma. In our (Packard) systems we have chosen the high-energy gamma \( (^{226}Ra) \) to obtain a wide quench correction. We have added a low-energy gamma \( (^{241}Am) \) to act as a correction factor. Equation (18) applies well to wide \( (^{3}H) \) and \( (^{14}C) \) windows over a volume range of 8 to 20 ml and for narrow (double isotope) windows of 10 to 20 ml. (See Fig. 10.)

**Poisson Statistics of Automatic External Standardization in Models 3375 and 3380 Tri-Carbs**

The statistics of the AES ratio are derived in the Appendix. A graph of \( ^{c}R \) versus \( R \) is given in Fig. 11. Because both external standard discriminators are above \( (^{3}H) \) at any quench level, only pulse overlapping of \( (^{3}H) \) counts over \( (10^{7} \text{ dpm}) \) can affect the lower energy channel. \( (^{14}C) \) at any quench level can only contribute to the error of the lower energy channel because the higher energy channel is above \( (^{14}C) \) at any quench level. \( ^{c}R \) is plotted versus \( R \) for \( 1 \times 10^{8} \text{ dpm} \) \( (^{14}C) \) in Fig. 11. \( ^{c}R \) is approximately two times the “no-activity” case for \( 10^{7} \text{ dpm} \) \( (^{14}C) \). For isotopes of higher energy than \( (^{14}C) \) such as \( (^{35}Cl) \) or \( (^{32}P) \) the statistics are more involved because the isotopes contribute to both external standard (G and H) channels. Good results should nevertheless be obtained for all isotopes of less than \( 10^{7} \text{ dpm} \). Errors \( [^{c}R \text{ and } (^{c}R/R)] \) can theoretically be computed for \( Rs \) of 1.0, 0.9, 0.8, etc., to 0.0 with Equation (18):

\[
\frac{^{c}R}{R} = \left( \frac{G^{2} - GH}{G^{2}} \right) + \left( \frac{G^{2} + H^{2} - 2GH}{G^{2}H^{2}} \right) e^{H^{2}}
\] (18)

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**REFERENCES**

APPENDIX
STATISTICS OF THE AES RATIO

It is well known that the combination of two random variables such as counts in a channel over different time intervals $n_1$ over $t_1$ and $n_2$ over $t_2$ leads, on addition, to

\[ n_1 + n_2 \rightarrow \text{mean} \]  
\[ n_1 + n_2 \rightarrow \text{variance} \left( \sigma^2 \right) \]

since the distribution is Poisson, and, on subtraction, to

\[ n_1 - n_2 \rightarrow \text{mean} \]  
\[ n_1 + n_2 \rightarrow \text{variance} \left( \sigma^2 \right) \]

and since they are independent random variables,

\[ \sigma_1^2 + \sigma_2^2 = \sigma^2 \]

Now suppose we have two independent chan-
nels, G and H, as shown in Fig. 12 and consider the statistics of the ratio (H/G = R). Since

\[ \frac{dH}{G} = \frac{dH}{G} - \frac{H \, dG}{G^2} \]  

then

\[ \left( \frac{dH}{G} \right)^2 = \left( \frac{dH}{G} \right)^2 + \frac{H^2}{G^4} (dG)^2 - \frac{2H}{G^2} dH \, dG \]  

Following the same limiting procedure as before, we obtain the variance of the ratio:

\[ \frac{\sigma_H^2}{G} = \frac{\sigma_H^2}{G} + \frac{H^2}{G^4} \sigma_G^2 \]  

is zero. The fractional standard error in the ratio is

\[ \frac{\sigma_R}{R} = \sqrt{\frac{\sigma_H^2}{H^2} + \frac{\sigma_G^2}{G^2}} \]  

Suppose we have two overlapping channels instead, as shown in Fig. 13 and again consider the statistics of the ratio (H/G) determined by counting the external standard in each channel simultaneously. We now return to Equation (7) where we let X = H, Y = G, and Z = H ∩ G to make d(X - Z) independent of dZ and d(Y - Z). Then

\[ d \left( \frac{H}{G} \right)^2 = \left[ d(X - Z) + dZ \right]^2 \]

\[ + \frac{H^2}{G^4} \left[ d(Y - Z) + dZ \right]^2 \]

\[ - \frac{2H}{G^3} [d(X - Z) + dZ][d(Y - Z) + dZ] \]  

Following the same limiting procedure as before, we obtain the variance of the ratio:

\[ \frac{\sigma_H^2}{G} = \frac{\sigma_H^2}{G} + \frac{1}{G^2} \left[ \sigma_{X-Z}^2 + \sigma_{Y-Z}^2 \right] \]

\[ + \frac{H^2}{G^3} \left[ \sigma_{X-Z}^2 + \sigma_{Y-Z}^2 \right] - \frac{2H}{G^3} \sigma_{X-Z} \]

\[ + \left[ \frac{G^2 + H^2 - 2GH}{G^4} \right] \sigma_Z^2 \]  

If we take the limit as \( n \to \infty \), we have, by the standard definition of variance,

\[ \sigma_R^2 = \frac{\sigma_H^2}{G^2} + \frac{H^2}{G^4} \sigma_G^2 \]  

Since G and H are independent, the mean value of the limit of the term

\[ \frac{1}{n} \sum_{i=1}^{n} \left( \frac{H_i - \overline{H}}{G} \right)^2 \]

is zero. The fractional standard error in the ratio is

\[ \frac{\sigma_R}{R} = \sqrt{\frac{\sigma_H^2}{H^2} + \frac{\sigma_G^2}{G^2}} \]  

Fig. 13.—Overlapping ES channels. Area of intersection I can be indicted by logic symbol G ∩ H.
and the standard error:

\[
\frac{\sigma_R}{R} = \sqrt{\frac{\sigma_R^2}{R^2}}
\]

\[
= \sqrt{\frac{\sigma^2 \chi - \bar{z}}{H^2} + \frac{\sigma^2 \chi - \bar{z}}{G^2} + \frac{G^2 + H^2 - 2GH}{G^2H^2} \sigma_z^2}
\]

Now suppose \( G \) counts 10^6 cpm and \( H \) counts 10^5 cpm:

\[
G^2 + H^2 - 2GH = 0
\]

The last term in Equations (12) and (13) vanishes. By comparison with Equation (9) of this appendix, the “independent” random variable case, we see that the variance as well as standard error for a ratio of overlapping channels counts is less than that for independent channels, provided they are counting simultaneously.
Quench Compensation by Means of Gain Restoration

C. H. Wang

In liquid scintillation counting, various methods have been developed to measure the extent of quenching, thereby providing one with necessary corrections for loss of counting efficiency. (See Chapter 29.) These methods, while useful, do not compensate or minimize the phenomenon of quenching. It is well known that with a quenched sample, the spectrum of a β-emitter shifts toward the lower energy end when measured by the liquid scintillation counting process. Such a shift not only reduces the efficiency of liquid scintillation counting but also compresses spectra of concurrent β-emitters and forces them closer, particularly those with low E_{max} such as (14C) and (3H). The quenching effect on β-spectra is shown in Figs. 1 and 2 for (3H) and (14C), respectively. The problem one may encounter in counting (3H) and (14C) simultaneously in a double radioisotope experiment is shown in Fig. 3. With a heavily quenched sample, virtually all of the quenched (14C) spectrum resides in the (3H) counting channel, constituting a serious problem for obtaining good results in a dual-label experiment. In recent years, an alternate approach has been developed to combat the problem derived from quenching of the liquid scintillation process. The approach is derived from an examination of the nature of β-spectra as measured by a liquid scintillation counter equipped to present signals in logarithmic function.

Effect of Amplification on β-spectrum

The spectra of (14C) at various levels of pulse amplifications are given in Fig. 4 in which the number of detectable pulses of (3H) is plotted against pulse height at various gains. The β-spectra of (14C) at various levels of pulse amplifications are given in Fig. 5. It is noted that as amplification decreases, the endpoints of the β-spectra gradually shift toward the lower pulse height and display increasingly sharper
peaks, very much resembling the effect of quenching.

In the case of $(^1{}^4$C), the counting efficiency, represented by the integrated area under the curve, remains essentially the same with moderate decreases in pulse amplification. Further reduction in amplification resulting in loss of counting efficiency gives a situation analogous to the case of a severely quenched sample. With $(^3{}^H)$, the situation is basically the same except that, even when pulse amplification is slightly lowered, the counting efficiency is significantly reduced. This is understandable inasmuch as $(^3{}^H)$ is a $\beta$-emitter with very low $E_{\text{max}}$. Many $\beta$-particles at the lower end of the continuous spectrum are barely energetic enough to produce detectable pulses in the liquid scintillation process, even with greatest pulse amplification. This situation accounts for the drastic reduction in $(^3{}^H)$-counting efficiency by the presence of even small amounts of quenching agent.

**Gain Restoration of Quenched Samples**

It follows that if one applies greater pulse amplification in counting a quenched sample, one should be able to restore, to a large extent, the distorted shape of a quenched $\beta$-spectrum to a shape comparable to that of an unquenched sample. The gain restoration of spectra of quenched samples is illustrated in Figs. 6 and 7 for the radioisotopes $(^1{}^4$C) and $(^3{}^H)$, respectively, and in Fig. 8 for double radioisotope counting. In these experiments, the pulse amplification was increased so that the endpoints of the quenched spectra were restored to those seen...
Fig. 2.—Effect of quenching on $^{14}$C spectrum as measured by liquid scintillation process (see legend Fig. 1).

Fig. 3.—Effect of quenching on $^{14}$C and $^{3}$H spectra in dual radioisotope counting.
Fig. 4.—($^3$H) spectrum at various pulse amplifications. Numerals in increasing order refer to increasing pulse amplification (see legend Fig. 1).

It should be noted that in multiple radioisotope experiments the extent of additional pulse amplification required to restore the endpoint of one $\beta$-spectrum at a given quencher concentration is the same for other concurrent $\beta$-emitters.

It should be obvious that when the endpoint of a quenched ($^{14}$C) spectrum is restored, the original loss in counting efficiency of ($^{14}$C) is not fully compensated. With ($^3$H), gain restoration of spectrum does not improve counting efficiency to any significant extent, as indicated by the relative areas.

Fig. 5.—($^{14}$C) spectrum at various pulse amplifications (see legend Fig. 4).

Fig. 6.—Gain restoration of quenched ($^{14}$C) spectra. Beckman Model LS-250 AQC system was set for restoration of ($^3$H) endpoint.
under the plots marked Q and UQ (Fig. 8), respectively. Nevertheless, endpoint restoration offers a great advantage for counting ($^3$H) and ($^{14}$C) in double radioisotope experiments. With a quenched sample, the spectrum of ($^{14}$C) is compressed toward the lower energy end, thereby shifting a major portion of the pulse from the ($^{14}$C)-counting channel to the ($^3$H)-counting channel (Fig. 3). To count these two radioisotopes separately, one has to lower the channel-separation discriminator. This is a tedious operation and necessitates the determination of the proper setting of the discriminators for each of the quenched samples. In contrast, adequate separation of the ($^{14}$C) and ($^3$H) spectra is provided by gain restoration with the same discriminator setting for either unquenched or quenched samples.

To make full use of the gain restoration phenomenon in automatic liquid scintillation counting, mechanisms must be provided in the counter to perform two operations. First, the effect of quenching in each of the counting samples must be precisely determined. This can be accomplished by the use of automatic external standard calibration since the external standard ratio is a direct indication of the extent of quenching in the counting sample. Second, the measured extent of quenching must be coordinated with the pulse amplification mechanism so that an appropriate increase in pulse amplification can be executed for spectrum restoration. Figure 9 shows the relationship be-

Fig. 7.—Gain restoration of quenched ($^3$H) spectra. AQC set for restoration of ($^3$H) endpoint.

Fig. 8.—Unquenched and restored ($^{14}$C) and ($^3$H) spectra.
between the external standard ratios of a series of quenched samples and the necessary pulse amplifications brought about by increasing the anode potential of the photomultiplier.

**Automatic Quench Correction (AQC)**

The essentially linear function observed is in accordance with theoretical considerations and accounts for the basic concept underlying the design of the gain restoration mechanism in the Beckman LS-150 and LS-250 liquid scintillation systems. The required increase in pulse amplitude in this system is accomplished by increasing the potential on the last 11 dynodes of the photomultiplier as a linear function of the external standard ratio. This design concept was chosen to take advantage of the reproducibility and stability with which anode potential can be controlled. The cathode to first dynode potential is held fixed so that the changes in the amplification do not affect the phototube efficiency.

Beckman's automatic quench compensation (AQC) system employs two digital-to-analog converters and appropriate numeric registers. The external standard ratio for the unquenched sample, for which the system was set up, is stored in one register (RUQ). The external standard ratio for the counting sample is stored in the other register (RQ). The difference between the two numbers is converted to an analog signal constituting the input to a very stable operational amplifier, the gain of which can be varied, depending on the slope of restoration required. The output of this amplifier is passed through a linear gate and then added to the reference potential of the high voltage power supply control amplifier. The function of the gate is to turn off the restoration circuit when AQC is not desired or during the external standard cycle. The latter is necessary to obtain a value that is referenced to the unquenched case. In actual operation, the master gain (and discriminators, if necessary) is adjusted for proper spectral distribution on unquenched samples. The external standard ratio is measured on the unquenched sample and stored in RUQ, and a heavily quenched sample is placed in the system. An external standard is taken. The ratio is automatically loaded into RQ and the AQC is automatically activated. The AQC gain is then adjusted to give the desired distribution. Since the external standard and restoration always follow a set function, the external standard ratio can be used in the normal matter for efficiency correction as a function of quench. Correlation curves for efficiency determination are of course prepared with AQC activated.

*Effects on Background*

In addition to facilitating the counting of individual radioisotopes in double labeling experiments, gain restoration also offers several other advantages to the counting of single radioisotopes. First, when a quenching agent is present in a counting sample, not only the $\beta$-spectrum but the spectrum of background radiation undergoes shifting to the lower energy end. The phenomenon is
QUENCH COMPENSATION BY GAIN RESTORATION

Fig. 10.—Unquenched, quenched and restored spectra of background radiation as measured by liquid scintillation (Beckman Model LS-250 system).

Illustrated in Fig. 10. Such a shift results in a higher background counting rate in the (3H) channel, reducing the figure of merit in the (3H)-counting operation. With gain restoration, the background in the (3H) channel is significantly reduced. Second, gain restoration provides better reliability in applying the external standard ratio method for the calibration of the counting efficiency. As shown in Fig. 11, the slope of the plot of external standard ratio versus (14C)-counting efficiency in the narrow (14C) channel is much milder when the spectrum is restored. The milder slope minimizes the inherent errors in correlating the external standard ratio to counting efficiency.

**Constant Counting Efficiency**

The compensation of the quenching effect by means of gain restoration can also be used to devise procedures to count (14C) and other β-emitters at a constant counting efficiency over a wide range of quench. Such a procedure is making use of the balance point method and the flat spectrum counting method. To illustrate the conceptual approach underlying such procedures, a series of quenched (14C) samples was first counted with the conventional setting of the (14C) channel. The gain restoration mechanism (AQC system) of the counter was then activated. In the present case, the mechanism was deliberately set to over-restore the endpoint of the quenched (14C) spectra by 80 divisions on a discriminator scale of 1000 divisions. What happened is illustrated in Fig. 12. The same series of samples was then counted with the automatic gain restoration mechanism activated and the discrimina-

Fig. 11.—Relationships between (14C) counting efficiency and external standard ratio; effect of gain restoration.
tor settings of ($^{14}$C)-counting channel set at 320 divisions for the lower limit and 1000 divisions for the upper limit. The lower limit discriminator was deliberately set at a higher level than that conventionally used for ($^{14}$C) counting. Such an arrangement naturally would reduce the counting efficiency of ($^{14}$C) to some extent, because a portion of the ($^{14}$C) spectrum was located in the area below the lower discriminator, but this unused portion of the ($^{14}$C) spectrum constituted a reservoir of counts that could be moved into the counting channel by gain over-restoration to compensate loss of counting efficiency in the ($^{14}$C)-counting channel due to quenching. The net result was that when concentration of quenching reagent increased, the counting efficiency in the ($^{14}$C)-counting channel remained the same. The counts in a window below the lower discriminator decreased, reflecting the effect of quenching.

Without gain restoration, the counting efficiency reduced from 60 per cent to 16 per cent as the concentration of quenching agent increased (Table 1). When the counter was operated in the gain over-restoration mood, the number of counts observed in the counting channel remained the same throughout the series of quenched samples. These results illustrate not only the conceptual soundness of the gain restoration operation but the reliability of the gain restoration mechanism.

![Fig. 12.—Over-restoration of ($^{14}$C) spectrum for constant efficiency counting.](image)

**Table 1. Counting of ($^{14}$C) at Constant Efficiency by Gain Over-restoration**

<table>
<thead>
<tr>
<th>Concentration of Quenching Agent</th>
<th>Counting Rate and Efficiency</th>
<th>Without Gain Restoration</th>
<th>With Gain Restoration</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>observed cpm</td>
<td>counting efficiency (%)</td>
<td>observed cpm</td>
</tr>
<tr>
<td>None</td>
<td>61,176</td>
<td>60</td>
<td>60,851</td>
</tr>
<tr>
<td>0.05 ml CHCl₃</td>
<td>52,151</td>
<td>51</td>
<td>61,895</td>
</tr>
<tr>
<td>0.1 ml CHCl₃</td>
<td>42,509</td>
<td>42</td>
<td>60,672</td>
</tr>
<tr>
<td>0.2 ml CHCl₃</td>
<td>35,683</td>
<td>35</td>
<td>62,383</td>
</tr>
<tr>
<td>0.3 ml CHCl₃</td>
<td>27,792</td>
<td>27</td>
<td>60,613</td>
</tr>
<tr>
<td>0.4 ml CHCl₃</td>
<td>21,676</td>
<td>21</td>
<td>60,248</td>
</tr>
<tr>
<td>0.5 ml CHCl₃</td>
<td>15,769</td>
<td>16</td>
<td>59,468</td>
</tr>
</tbody>
</table>

Sample composition: Toluene and PPO (6.0 g per liter) containing ($^{14}$C) with total radioactivity of 102,000 dpm. Counting procedure: Beckman Model 250 system with discriminator set at 320 — infinite divisions and AQC set to provide over-restoration of ($^{14}$C) spectrum to the extent that restored endpoint is 80 divisions beyond endpoint of unquenched spectrum. Counting was carried out in sufficient time to provide data with no greater than 1 per cent standard deviation.

**REFERENCES**


In recent literature explicitly devoted to liquid scintillation counting, there is little information on the cause, detection, or elimination of unwanted long-term phosphorescence in scintillator-solvent-sample mixtures. Hercules in Chapter 32 summarizes what is currently known of chemiluminescence mechanisms. He provides some completely new information on model systems, and reviews much previous work that most liquid scintillation counter users have been blithely unaware of. Of particular relevance in liquid scintillation counting is his discussion of the involvement of molecular oxygen and peroxide decomposition in chemiluminescent reactions. In addition, the luminol reaction, referred to in Chapter 11 as a means for microchemical assay, is analyzed in detail.

In Chapters 33 and 34, Kalbhen, and Bransome and Grower, review the practical aspects of chemiluminescence in counting samples; they make new observations and recommendations for its elimination.
Ever since Radziszewski demonstrated in 1877 that light emission could be produced by the oxidation of simple organic compounds, the production of light by chemical reactions has fascinated many chemists. One can recall the use of luminol chemiluminescence as a demonstration in Freshman chemistry or demonstration of the oxalyl chloride reaction at the New York World's Fair. Unfortunately fascination with chemiluminescence has not been matched by an equivalent amount of productive curiosity and even today relatively little is known about chemiluminescence mechanisms. This article will summarize some recent work that has begun to shed light on certain chemiluminescence mechanisms and will point out areas where there are still many unanswered questions.

Chemiluminescence is best viewed as the process of producing an excited electronic state by the energy from a chemical reaction. It can simply be regarded as one of several mechanisms whereby molecules dissipate energy produced by a chemical reaction. In other words, chemiluminescence is an alternative to the usual thermal dissipation mechanisms which increase translational and vibrational energy of a system at the expense of electronic excitation. There are certain restrictions placed on those reactions which can show chemiluminescence, since few known reactions produce light. The definition of the requirements for light production by chemical reactions is one of the major concerns of chemiluminescence research today.

Originally it was thought that oxygen was ubiquitous in chemiluminescence, since at one time all known systems involved reaction with either oxygen or peroxide. Recent discoveries have shown that oxygen is not essential. In chemiluminescence produced by simple electron transfer reactions; light emission does not involve, and is quenched by, oxygen. Figure 1 shows some representative reactions known to produce light, some involving oxygen or peroxide and some not. One is immediately impressed with the diversity of reactant structures and types of reac-

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Fig. 1.—Examples of chemiluminescent reactions. Reactants, emitters and reaction conditions are shown.

The use of chemiluminescence for the quantitative assay of various inorganic and organic chemicals.

**Criteria for Observing Chemiluminescence**

If a chemical reaction is to produce light, a number of requirements must be met: sufficient energy for excitation; the presence of some chemical species capable of forming an excited electronic state; the presence of some emitter to give off excitation energy; a rapid rate of chemical reaction; and a reaction coordinate system favoring production of an excited state over direct ground-state formation.

Before discussing each of these requirements in detail, it is necessary to define the chemiluminescence yield, $\phi_{el}$. The chemiluminescence yield is the ratio of the number of photons emitted by a system divided by the number of molecules reacted. For the reaction $A + B \rightarrow C + h\nu$. 
The chemiluminescence yield can be considered as the product of two separate quantum efficiencies, one for chemical production of an excited state, \( \phi_{es} \), and one for efficiency of emission from that state, \( \phi_r \); i.e., \( \phi_{et} = \phi_{es} \cdot \phi_r \). Since generally chemiluminescence emission involves fluorescence by some species, \( \phi_r \) is considered the fluorescence efficiency of the emitter. A low value for the quantum yield of chemiluminescence does not necessarily imply inefficient excited state production by a chemical reaction but may merely reflect a small value for the fluorescence efficiency. Likewise, structural effects on chemiluminescence intensity can be due to two different factors. The rate of production of the excited species is sensitive to all steps in the chemical sequence prior to its formation, and hence to structural effects on the kinetics of each step. The rate of light emission from an excited state is also sensitive to structural changes, but not necessarily in the same way as the chemical steps. Therefore it is not usually meaningful to measure the overall chemiluminescence efficiency but the really significant parameter is the probability of excited state production, \( \phi_{es} \). Unfortunately very few reliable estimates of this quantity are available in the literature.

**Energy Considerations**

It is clear that if light is to be emitted by a chemical reaction, sufficient energy must be provided by that reaction. For example, if blue light, \( \lambda = 450 \text{ nm} \), is to be emitted, a minimum energy of 63.5 kcal/Mole (2.75 eV) must be provided; for green light, \( \lambda = 500 \text{ nm} \), 57.1 kcal/Mole (2.48 eV) are necessary; for red light, \( \lambda = 600 \text{ nm} \), 47.6 kcal/Mole (2.07 eV) are required. This large energy requirement restricts the types of chemical reactions that can produce chemiluminescence. The making and breaking of hydrogen bonds, for example, would not be expected to give chemiluminescence in the visible region of the spectrum.

The necessity for sufficient excitation energy is further illustrated in Fig. 2. The energy necessary to excite the fluorescent product, \( C \), is indicated by the arrow linking the curves \( C + D \) and \( C^* + D \). For the nonchemiluminescent reaction with a fluorescent product, going from the reactants curve, \( A + B \), to an excited state of the product, \( C^* + D \), is endothermic while ground state production, \( C + D \), is exothermic by an amount \( \Delta H \). In this situation the reactants have no alternate pathway to direct ground state formation since sufficient energy for excitation is lacking. In the middle curve of Fig. 2, curves \( A + B \) and \( C^* + D \) cross so that production of an excited state is exothermic as well as production of the ground state. Under these circumstances it is possible for the chemical reaction to be chemiluminescent depending on the relative activation energies for production of the ground state, \( \Delta H^* \), and for production of the excited state, \( \Delta H^{**} \). These considerations will be discussed further as they relate to reaction coordinates.

An interesting possibility presents itself if one considers an excited state lying at slightly higher energy than is available from the reaction, based on simple thermodynamic considerations. Such a situation is represented in the right hand of curve Fig. 2. At room temperature the value of \( kT \) is 0.5961 kcal/Mole (0.02585 eV) which means that small amounts of energy can be provided to a chemical reaction by thermal activation. Because chemiluminescence is the observation of an absolute light intensity, it is possible to detect reactions with very low efficiencies. If \( \Delta H^{**} \) is smaller than \( \Delta H^* \) (Fig. 2), the rate for the light-emitting pathway will be greater than for the radiationless pathway and chemiluminescence will be very efficient, although the rate of light emission might be low. Even if \( \Delta H^* < \Delta H^{**} \), light production by an endothermic mechanism would still be possible. If one assumes virtually no activation energy for the reaction \( A + B \to C + D \) and an
activation energy of 13 kcal/Mole for $A + B \rightarrow C^* + D$, a chemiluminescence efficiency of about $10^{-4}$ would still be possible, assuming $C^* \rightarrow C + h\nu$ has unit probability. This would be sufficient energy to excite an emitter having a 0,0 transition at 440 mÅ, by a chemical reaction calculated by simple thermodynamics, to produce light at 550 mÅ. This means blue light could be produced by a reaction in itself energetic enough to produce only yellow light.

**Excited States and Reaction Rates**

In a chemiluminescent reaction some species must be present which are capable of receiving the excitation energy produced by the reaction. The energy region of most chemiluminescent reactions corresponds to electronic excitation energies of aromatic hydrocarbons and their derivatives (for example, flavors used in liquid scintillation counting). The species to be excited initially must be directly involved in the chemical reaction since the reaction is the only mechanism for transforming chemical energy to electronic excitation. The additional necessity of having a low-lying excited state available explains why most known chemiluminescent systems involve reactions of large organic molecules. A reaction between sodium hydroxide and hydrochloric acid may be sufficiently energetic, but there are no excited states available to acquire the reaction energy.

In addition to the necessity for formation of an excited state, the state must also be emissive, or capable of transferring its excitation energy to an emitter. For example, the oxalyl reaction produces chemiluminescence characteristic of an added fluoroscer, as does the thermal decomposition of dibenzalperoxide. In both cases the reactions are not chemiluminescent by themselves (or only weakly chemiluminescent); addition of an energy acceptor causes bright light emission.

Compounds of similar structure can show dissimilar chemiluminescence characteristics.
Luminol is strongly chemiluminescent while the unsubstituted phthalhydrazide is not. The phthalhydrazide shows oxidation reactions similar to those of luminol, but the product of the luminol reaction, 3-aminophthalic acid, is fluorescent while the phthalic acid produced from phthalhydrazide is not.

The requirement for a rapid reaction rate in the production of chemiluminescence is a practical one. Detectors such as the human eye or a photomultiplier tube respond to photon flux, i.e., photons cm⁻² sec⁻¹. Discovery of chemiluminescent reactions depends on the emission of a reasonable number of photons in a given time interval. Even a 100 per cent efficient chemiluminescent reaction would go unnoticed if it emitted one photon per fortnight.

Reaction Coordinates

Marcus⁷ has analyzed chemiluminescence from simple electron transfer reactions and the discussion here is based largely on his treatment. In chemiluminescent reactions one must consider the relative probabilities of the reactants giving the ground state product directly versus formation of an excited state. This probability is directly related to the free energy of the reaction and to the relative free energies of activation. The center curve in Fig. 2 serves as an illustration. The reactants, A + B, have a choice of going directly to the ground state product, C + D, or the excited product, C* + D, with subsequent emission of light. Marcus⁷ has shown that the free energy of activation in electron transfer reactions is related to the free energy difference between the product and reactant curves. If the free energy difference is large, the energy of activation for the reaction will likewise be large. If we neglect entropy effects, Fig. 2 indicates that for most systems excited state production will be more likely than a direct return to the ground state, since the free energy change involved in excited state formation is smaller. Such an idea is based on the assumption that the curves for A + B and C* + D cross not far above their minima, that there are small configurational changes between the ground state of the reactants and the excited state of the product. If this is not true, the curves will be displaced horizontally from each other. Even though the free energy difference for going from the reactants to an excited state of the product will still be small, the large configurational change will cause the energy of activation to become large and chemiluminescence will be less favored than a direct path to the ground state. This is a major consideration in chemiluminescence; the configurational changes must be favorable. Unfortunately it is also one of the most difficult of the necessary parameters for chemiluminescent reactions to evaluate a priori.

In the chemiluminescence of luminol, it is likely that decomposition of a bridged peroxide intermediate results directly in the formation of an excited state of the product, 3-aminophthalic acid. Simple bond-energy calculations indicate sufficient energy is available from such a decomposition. The mechanism probably involves concerted breaking of the C-N and O-O bonds of the intermediate, as shown in Fig. 3. As the newly-formed carboxyl groups change their geometry to conjugate with the ring, the charge density of each group will be greater than that of a normal carboxyl group already conjugated with the ring. The lowest excited singlet state of 3-amino-phthalic acid is probably a charge-transfer state with electron density on the carboxyl groups greater than in the ground state. Therefore the intermediate configuration produced by the peroxide decomposition will be more similar, electronically, to the excited state of 3-amino-phthalic acid than to the ground state. Such a situation should greatly favor excited-state production (and chemiluminescence) over ground-state formation.

Investigation of Chemiluminescent Systems

On the basis of the foregoing remarks, there are several questions that need to be answered
for any chemiluminescent system. What is the nature of the emitting species? Is the emitting species formed by a chemical reaction or is it present and unchanged during the course of the reaction? How does the emitting species get excited—directly by the chemical reaction or by energy transfer from some other excited species? What is the exact nature of the step that causes the conversion of chemical energy into electronic excitation? Several chemiluminescence systems will be summarized below to see how well these questions are answered. Unfortunately, for no system are there unambiguous answers to all of these questions at the present time.

CHEMILUMINESCENT SYSTEMS

In this section selected examples of chemiluminescent reactions will be used to illustrate current thinking about the mechanisms of these reactions and about chemical excitation mechanisms in general.

The excited state of an emitter can be generated directly by the chemical reaction; that is, the chemical excitation step and the last step of the chemical sequence are the same. The chemical reaction providing excitation energy does not necessarily have to be the main chemical reaction in a system but may be a side reaction. Excitation may also occur by energy transfer from an initially excited species to an emitter. The initially excited species may also be an emitter and therefore emission from more than one species may be

![Fig. 3: Geometrical relationships in decomposition of luminol peroxide intermediate.](image1)

The chemical reaction providing excitation energy does not necessarily have to be the main chemical reaction in a system but may be a side reaction. Excitation may also occur by energy transfer from an initially excited species to an emitter. The initially excited species may also be an emitter and therefore emission from more than one species may be

![Fig. 4: HMO diagrams for photoexcitation in aromatic hydrocarbons.](image2)
Radical-Ion Reactions

Chemiluminescence can be produced by the reaction of cation radicals and anion radicals generated alternately at electrodes as shown by reactions 1, 2 and 3 of Table 1. A detailed review of electrochemically-generated chemiluminescence will soon be available.

The basis of light production by radical ion reactions can be seen from the simple HMO diagram shown in Fig. 4. The highest filled and lowest empty molecular orbitals of the aromatic hydrocarbon, \( A \), are designated \( \pi \) and \( \pi^* \), respectively. Photoexcitation corresponds to moving one electron from \( \pi \) to \( \pi^* \) to form the excited singlet state, \( 1A^* \), which will emit its characteristic fluorescence. The radical anion \( A^- \) is obtained by adding an electron to the \( \pi^* \) orbital while the radical cation is produced by removing an electron from the \( \pi \) orbital as shown in Fig. 4. When the anion and cation react, an electron will be transferred from \( \pi^* \) of the radical anion to \( \pi^* \) of the radical cation to produce an excited state of the hydrocarbon, rather than return to the ground state directly. This simple mechanism is the basis of the chemical excitation process of the aromatic radical ion reactions.

Chemiluminescence from radical ion reactions is most conveniently studied by a combined electrochemical and photometric apparatus, such as that shown in the block diagram of Fig. 5. The electrochemical part of the apparatus is located on the left and the photoelectric detection part on the right. The cell contains a conventional three-electrode system used for cyclic voltammetry. Light is generated at the indicator electrode located near the photodetector. The counter electrode is isolated both optically and chemically from the cell, so that processes occurring at this electrode do not contaminate the bulk solution. The potentiostat imposes a potential on the indicator electrode relative to the reference electrode by varying the potential of the counter electrode to compensate for the IR drop of the solution. Potentiostatic control is particularly important in chemiluminescence studies since reactions are usually carried out in organic solvents where the IR drop is high. The wave form generator can drive the potentiostat to apply a particular potential-time curve to the solution. Usually an XY recorder is used to record current-voltage curves.

The light emitted from the indicator electrode is picked up by the photomultiplier tube powered by a regulated high voltage power source.
supply. The signal from the photomultiplier tube is amplified and recorded. Usually the voltage of the potentiostat defines the $X$-axes of both the current and light intensity recorders so that for an individual voltage scan one can record both current-voltage and light intensity-voltage curves.

Using the apparatus shown in Fig. 5, one obtains curves similar to those of Fig. 6 for the chemiluminescence produced by rubrene radical ions. Curve A is a triangular-wave cyclic voltammogram obtained for rubrene in dimethylformamide (DMF). The dashed curve shown in Part A is the residual current curve obtained under the same conditions. On the cathodic cycle at $-1.37$ volts the rubrene radical anion is generated and on the anodic cycle at $+1.07$ volts one generates the rubrene cation. Curve B is a plot of light intensity vs potential scanned between the limits of $+1.1$ and $-1.5$ volts, light emission being observed on each half cycle after the first half cycle. Curve C is the light emission observed when only the radical cation is generated, produced by reaction between the cation and the solvent.

The emitter in the radical ion chemiluminescence reaction is clearly the excited singlet state of the aromatic hydrocarbon as can be seen by comparison of the fluorescence spectrum of rubrene with the radical-ion chemiluminescence spectrum shown in Fig. 7. This has been observed not only in the case of rubrene but for a variety of other compounds as well. Chemiluminescence from radical-ion reactions has been observed for a large number of compounds including aromatic hydrocarbons substituted aromatic hydrocarbons and a variety of heterocyclics.

A generalized mechanism for radical-ion chemiluminescence is summarized by the reactions shown in Table 1. Using this mechanism, Feldberg has presented a computerized solution of the boundary value
The double-potential-step method is an electrochemical technique where the electrode is maintained at a potential generating one species for a given time interval (e.g., at −1.35 volts on the cathodic wave of rubrene) and then switched rapidly to the potential of another species (e.g., +1.1 volts for rubrene oxidation). Under these conditions the radical ions generated during the two steps react in the diffusion layer and the light intensity is given by

\[
\log \omega = -1.45 \left( \frac{t_2}{t_1} \right)^{1/2} + 0.71 \tag{1}
\]

where \( t_2 \) is the duration of the second pulse, \( t_1 \) the duration of the first pulse, \( F \) is the value of the Faraday, \( \phi \) the fluorescence efficiency of the emitter, \( I \) the current during the first pulse, and the product \( FP \) the light intensity in units of Einsteins per second. Equation (1) is valid only under the conditions of \( k_3 t_2 C > 10^6 \), where \( C \) is the bulk concentration of the hydrocarbon; i.e., reaction 3 in Table 1 is diffusion controlled. Equation (1) requires that a plot of \( \log \omega \) versus \( (t_2/t_1)^{1/2} \) yield a straight line having a slope of −1.45 if the reaction is diffusion controlled. We have confirmed Equation (1) for rubrene in several solvents and have demonstrated that \( k_3 > 10^8 \) Ms\(^{-1}\).

It is interesting to know that several preliminary estimates of the quantum efficiency of radical-ion chemiluminescence have been made, but the most reliable approximations are those of Watne\(^{10}\) who utilized a coulometric approach. He determined the Einsteins emitted per coulomb of electricity consumed and observed that the quantum efficiency of rubrene in benzonitrile varied depending on the direction of generation. When the rubrene radical anion was generated first, a value of 0.006 was obtained, but when the radical...
cation was generated first, the efficiency increased to 0.015.

Originally it was thought that radical-ion reactions would be useful in producing excimers under conditions where they could not be formed by photoexcitation, and this has recently been reported. Excimer reactions have therefore been included in Table 1. Parker and Short have also observed excimer emission for 9,10-dimethylanthracene in DMF, but the conditions of their experiments were such that excimers could have been formed by the normal process, $^1\text{Ar}^* + \text{Ar} \rightarrow ^1\text{Ar}^*\text{Ar}^*$, rather than by reaction 8 of Table 1. We and other workers have shown that several suspected cases of excimer formation can be attributed to impurities produced in the solution by decomposition of radical ions. At present there is no clearly confirmed case of excimer formation by the reactions shown in Table 1.

So far the energy requirements of radical-ion reactions have been discussed in terms of producing an excited singlet state of the emitter. There is no reason why only singlet states should be formed in chemical reactions; triplet states are also possible. Several authors have considered that chemiluminescence could be produced in situations where insufficient energy is available to form an excited singlet state directly, if triplet states were produced first and triplet-triplet annihilation occurred subsequently. Such a process is indicated by reactions 5 and 6 of Table 1. Triplet-triplet annihilation is a well-known phenomenon and represents an interesting possibility of a major mechanism in chemiluminescence reactions.

In the interpretation of electrochemical data to determine whether or not sufficient energy is available for excited singlet state formation from radical ion reactions, the magnitude of the $T\Delta S$ term applied to the electrochemical data becomes important. The magnitude of this term has been estimated to be about 0.2 eV by several workers and has recently been confirmed by measurement. If electrochemical data indicate that insufficient energy is available for direct excited singlet state formation, the maximum possible quantum efficiency of the reaction will be limited, with the needed energy being supplied by $kT$. This will require comparable slowing of the reaction rate, but since chemiluminescent reactions of low quantum efficiency are readily detected, it is not unreasonable to expect that the energy provided by $kT$ might be significant in some cases.

Several results reported indicate that chemiluminescence does occur when insufficient energy is available for direct formation of an excited singlet state. One example is the reaction of the rubrene radical cation with dimethylformamide, amines and water. Similar results were observed for perylene and 9,10 diphenylanthracene where the energy deficiency was even greater than for rubrene. Although part of the energy discrepancy can be accounted for by irreversible electrode reactions, it is likely that chemiluminescence is indeed produced by reactions between solvent oxidation intermediates and the radical cation followed by triplet-triplet annihilation. Weller and Zachariasse have produced fluorescence by reactions between radical anions and Wurster's blue perchlorate in dimethoxyethane, a situation where, based on electrochemical data, insufficient energy was available for excited singlet state formation. Of special interest is the reaction between Wurster's Blue cation and the chrysene anion. The energy required for formation of the chrysene singlet is 3.43 eV, but the cation-anion reaction can provide only 2.66 eV. The energy of the chrysene triplet is 2.44 eV, indicating sufficient energy would be available to form the chrysene triplet. Emission spectra observed for the chrysene-Wurster's Blue reaction showed a chemiluminescence matching the fluorescence spectrum of chrysene to within $\pm 0.01 \mu$ on each of four vibronic bands. Bands at longer wavelengths were observed in the chemiluminescence spectrum with peaks at 1.98,
These bands show a close correlation to the vibronic peaks observed in the phosphorescence spectrum of chrysene at 1.95, 1.80 and 1.66 μ⁻¹. Because of the close correlation between the structured peaks, the identification of the emitter as the triplet state of chrysene is probably a valid one and probably represents the only confirmed observation of triplet emission in a radical-ion reaction. Because chrysene phosphorescence is not normally observed in fluid solution, if the triplet concentration is high enough that phosphorescence can be seen as a result of chemiluminescence, it is quite reasonable to assume that triplet-triplet annihilation occurs with a high efficiency.

A combination of theoretical and experimental studies has produced additional evidence that triplet-triplet annihilation occurs in electrochemically generated chemiluminescence. Feldberg has computerized a solution of the boundary-value problem for the occurrence of triplet-triplet annihilation in radical-ion reactions. Although it is not possible under normal conditions to distinguish between direct singlet excitation and triplet-triplet annihilation, the latter can be detected if a triplet quencher is present during the reaction. When significant triplet quenching occurs, the normalized light emission, ωb, is

\[ \log \omega_b = -2.90(t_r/t_f)^{1/2} - \log \beta + 1.42 \]  

(3)

where \( t_r \) and \( t_f \) are as defined in connection with Equation (1). The terms \( \omega_b \) and \( \beta \) are complex. For definition the reader is referred to the original paper. Equation (3) requires that a plot of \( \log \omega_b \) versus \((t_r/t_f)^{1/2}\) yield a slope of -2.90 when triplet quenching occurs. When triplet quenching is absent, Equation (3) reduces to Equation (1). When the reaction changes from triplet quenching to no triplet quenching, a changing slope would be expected to vary between the limits of -2.90 and -1.45.

Chang et al. have shown that the electrochemiluminescence of rubrene can be produced when insufficient energy is available to form an excited singlet state. Reaction of the rubrene radical anion with p-phenylenediamine radical cations and reaction of the rubrene radical cation with semiquinone anions show large energy discrepancies as indicated in Table 2. For example, rubrene oxidation occurs at +0.88 V versus SCE while naphthoquinone reduction occurs at -0.72 V. This gives a net energy of only 1.60 V available from the radical ion reaction, although the rubrene excited singlet state requires 2.39 V.

Figure 8 shows a plot of \( \log \omega \) versus \((t_r/t_f)^{1/2}\) for rubrene over a wide range of \( t_f \) values. For \( t_f > 1 \) s, the plots are linear with average slopes of -1.45 as predicted by Equation (1). At small values of \( (t_r/t_f)^{1/2} \) the derivation from linearity (such as for the

<table>
<thead>
<tr>
<th>Table 2.—Energetics of Electron Transfer Reactions</th>
</tr>
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<tbody>
<tr>
<td>E/V</td>
</tr>
<tr>
<td>-----------------------------------------------</td>
</tr>
<tr>
<td>Rubrene</td>
</tr>
<tr>
<td>9,10-Diphenyl anthracene</td>
</tr>
<tr>
<td>9,10-Dimethyl anthracene</td>
</tr>
<tr>
<td>Tetracene</td>
</tr>
<tr>
<td>Naphthoquinone</td>
</tr>
<tr>
<td>N,N,N',N'-tetramethyl p-phenylene diamine</td>
</tr>
</tbody>
</table>

\( p \)-benzoquinone (-0.60 V), anthraquinone (-0.80) are also used as donors. \( p \)-phenylene diamine is also used as acceptor (+0.24 V).
curve $t_f = 9.2 \text{ s}$ is consistent with subsequent computer calculations. For values of $t_f < 1 \text{ s}$, the slope increases until at $t_f = 0.043 \text{ s}$ it attains a value of $-2.90$. According to Equation (3), a slope of $-2.9$ is expected when triplet-triplet annihilation occurs in the presence of a triplet quencher. Although no triplet quencher was intentionally added to the solutions, Chang et al.\textsuperscript{22} interpreted their measurements on the basis of quenching of the rubrene triplet by the rubrene cation and anion radicals. Such quenching would depend on the relative probability of a rubrene triplet encountering a radical ion as opposed to another triplet after it was formed in the diffusion layer. Computations have shown that when $t_f$ is small, the volume element of solution in which the radical-ion reaction occurs is small, and it is probable that a triplet will migrate out of this region before it can encounter another triplet. It is therefore likely to encounter a radical ion and be quenched. When $t_f$ is large, the reaction zone becomes thicker, and it is more probable for a triplet to encounter another triplet than to diffuse out of the reaction zone. Although these observations provide evidence for the occurrence of triplet-triplet annihilation in chemiluminescence, it has not yet been possible to compare the relative contribution of singlets formed directly to that of the annihilation reaction.

Ruthenium Chelates

Electron transfer to species other than hydrocarbon radical cations can produce chemiluminescence if the reaction product is fluorescent. Two systems illustrating such a reaction are the reduction of Ru(III) chelates by hydrazine and the reduction of radical cations of aromatic amines.\textsuperscript{24} The first type of system can be written as

$$ML_{n}^{+} + \text{red} \rightarrow ML_{n}^{+} + h\nu$$  \hspace{1cm} (4)

The complexes studied had ruthenium as the metal ion ($n = 2$) and 2,2'-bipyridine 5-methyl-o-phenanthroline, 5,6-dimethyl-o-phenanthroline and 3,5,6,8-tetramethyl-o-phenanthroline as ligands. Either aqueous base or hydrazine served as the reductant. The chemiluminescence emitted from these reactions was identical to the fluorescence to the Ru(II) chelates under photoexcitation.

The overall reaction for the second system is given by

$$RNH_{m}^{+} + \text{red} \rightarrow RNH_{m} + h\nu$$  \hspace{1cm} (5)

The compound was 1,6-diaminopyrene, either as the radical cation ($m = 1$) or the dication ($m = 2$). Either hydrazine or sodium naphthalenide could be used as the reductant. Although the chemiluminescence did not exactly match the fluorescence spectrum of the amine, the similarity was sufficient to in-
dicate that diaminopyrene was the primary emitter and either an impurity generated by the reduction reaction or an excimer could account for the small discrepancy in the observed spectra.

A detailed investigation of the mechanism of the reaction between the ruthenium (III) bipyridine chelates and hydrazine has been carried out. Using thermochemical and electrochemical data, we can calculate that about 1.4 to 1.5 eV are available from the reaction. On the basis of spectral data, about 2.1 eV are necessary to produce an excited singlet state, indicating that there is a discrepancy between the energy available from the reaction and the energy necessary for excitation. We are not compelled to invoke an energy doubling mechanism, however, because the ruthenium (III) bipyridine-hydrazine reaction is complicated. Chemiluminescence could result from reactions involving intermediate species. There is evidence that the emitting state in ruthenium (II) bipyridine chelates is a charge-transfer triplet rather than a singlet state, a state of affairs consistent with a high degree of spin-orbit coupling induced by the presence of a high-atomic-number atom such as ruthenium. Therefore, the chemiluminescence produced by reductions of ruthenium (III) chelates may be more correctly termed phosphorescence than fluorescence.

The kinetics of the chemiluminescent reaction between the Ru (III) chelate and hydrazine have been studied in some detail. The following mechanism is adequate to account for the kinetics observed at low hydrazine concentrations:

\[
\text{Ru (III)} + \text{N}_2\text{H}_4 \rightarrow [\text{Ru (II)}]^* + \text{N}_2\text{H}_3 \quad (6)
\]

\[
2\text{N}_2\text{H}_3 \rightarrow \text{N}_2 + \text{NH}_3 \quad (7)
\]

\[
\text{Ru (III)} + \text{N}_2\text{H}_3 \rightarrow \text{N}_2\text{H}_2 \quad (8)
\]

\[
\text{Ru (III)} + \text{N}_2\text{H}_2 \rightarrow [\text{Ru (II)}]^* + \text{N}_2 \quad (9)
\]

\[
[\text{Ru (II)}]^* \rightarrow \text{Ru (II)} + h\nu \quad (10)
\]

Ligands are omitted in the formulae. Production of ground-state Ru (II) complexes has been omitted in the above sequence but occurs for reactions 6, 8 and 9. Reaction 8 does not produce excited states directly. Reaction 6 is about 1 per cent efficient and reaction 9 about 99 per cent efficient. Using the above rate data, computer-simulated chemiluminescence-time curves matched exactly with curves recorded on a stopped-flow spectrophotometer at low hydrazine concentrations. At higher hydrazine concentrations using the above data, the curves could be fitted qualitatively but not quantitatively, indicating that there was probably an unknown reaction occurring at high concentrations that did not occur at low hydrazine concentrations.

The Ru (III)-hydrazine system is of particular interest because it is easily amenable to mechanistic studies. The nature of the emitter is now known, as are details about the emission process. The reaction pathway is understood both in terms of chemical steps that occur in dilute solution and those that produce excited states and is noteworthy because more than one reaction in the system produces an excited state. Simple thermodynamic considerations suggest that direct production of the emitting excited state in the chelate is not possible, but because of the complex reaction mechanism such calculations are of questionable validity. This particular system illustrates the necessity for using thermodynamic calculations only in those situations where both the oxidant and reductant undergo simple one electron transfer reactions.

The Luminol Reaction

Although in the literature there are a number of reports that imply the occurrence of energy transfer in the luminol system, the best documented example is given by White and Roswell. They coupled strongly fluorescent functional groups to the hydrazide function, by means of methylene bridges, so that energy transfer could occur from the part of the molecule excited by the chemical reaction to
the fluorescent part. The two compounds studied that showed strong intramolecular energy transfer were:

In 90 per cent dimethyl sulfoxide (DMSO) the chemiluminescence of (I) and fluorescence of the solution after reaction show peaks at 425 and 440 m\(\mu\). Under the same conditions, 9,10 diphenylanthracene also shows fluorescence at 425 and 440 m\(\mu\). Compound (II) shows chemiluminescence and fluorescence after reaction at 440 m\(\mu\), the wavelength of the fluorescence maximum of N-methylacridone in the same solvent. Using 10\(^{-4}\) \(M\) solutions of N-methylacridone and 4-methyl phthalic hydrazide, no energy transfer could be observed. Clearly the compounds represent an example of intramolecular energy transfer in chemiluminescent reactions.

If a peroxide intermediate is formed in the process of a chemiluminescent reaction, it is quite conceivable that its decomposition could yield an excited state if the correct geometric relationship existed between the excited state produced and the intermediate. Although no example of such a decomposition reaction has been completely confirmed, the work of White et al.\(^{27,28}\) indicates that this is probably the case for luminol. It was confirmed that 3-aminophthalic acid (3APA) was the emitter in the luminol reaction\(^{28}\) and was produced by a reaction of the stoichiometry (luminol + \(O_2\) + 2 Base \(\rightarrow\) 3APA + \(N_2\) + \(h\nu\)) in DMSO-water mixtures. The reaction rate was found to be first order in luminol, base and oxygen. It was also observed that \(^{18}O_2\) was incorporated symmetrically into the two carboxyl groups of the 3-aminophthalic acid.\(^{27}\) This led White et al. to postulate the following mechanism for the luminol reaction:

\[
\begin{align*}
\text{LH}_2 + \text{OH}^- & \rightarrow \text{LH}^+ + \text{H}_2\text{O} \\
\text{(100\% complete)} & \\
\text{LH}^+ + \text{OH}^- & \Leftrightarrow \text{L}^2\text{O}_2^+ + \text{H}_2\text{O} \quad \text{(12)} \\
\text{L}^2\text{O}_2^+ & \rightarrow \text{L}^2\text{O}_2^{-} + \text{N}_2 + \text{hv} \quad \text{(14)}
\end{align*}
\]

where \(\text{LH}_2\) is luminol, \(\text{LH}^+\) singly ionized luminol, \(\text{L}^2\text{O}_2^+\) doubly ionized luminol, and \(\text{L}^2\text{O}_2^{-}\) the luminol peroxide intermediate. We have conducted stop-flow kinetic studies, the results of which are consistent with the above mechanisms, i.e., two bimolecular steps preceding a unimolecular decomposition reaction.\(^{29}\) At present it is not possible to determine definitely whether reaction with base or oxygen is the primary step in the three step sequence. However, preliminary observations have been consistent with the interpretation that base rather than oxygen is involved in the first step of the luminol reaction in dimethylsulfoxide.

In addition to the kinetic data, other considerations point to the decomposition of an intermediate to yield an excited state directly. The chemiluminescence efficiency was measured\(^{28}\) to be about 0.05 in DMSO-water mixtures. In the same medium the quantum efficiency of fluorescence for 3APA is 0.05-0.10 and in the chemical reaction about 90 per cent of the luminol is converted to 3APA. This establishes the lower limit on the quantum yield of excited state formation as 0.5. Such a value rules out any energy doubling mechanism like triplet-triplet annihilation. This is consistent with the fact that the decomposition of the proposed peroxide intermediate to yield nitrogen would be quite exothermic and could supply sufficient energy to excite the 3-aminophthalic acid singlet state.

Lucigenin

Lucigenin is one of the well-known classical chemiluminescent reactions. It has been
known for some time that N-methyl acridone (NMA) is the primary emitter responsible for the blue chemiluminescence seen in the lucigenin reaction and that the chemiluminescence of lucigenin can show a green luminescence band, the presence or absence of which depends on environmental conditions. By energy transfer studies and cyclic voltametry techniques, it has been possible to show that one of two emitters is responsible for the color changes observed and that energy transfer between N-methyl acridone and one of the emitters is the excitation mechanism for the green chemiluminescence.

The electrochemical reduction of lucigenin in nonaqueous solvents is irreversible and the final reduction product is dimethylbiacridine (DBA):

\[
\text{CH}_3
\]

(DBA)

the latter having been identified by NMR ultraviolet and mass spectra. The pertinent electrode reactions are:

\[
\text{Luc}^{++} + e^- \leftrightarrow \text{Luc}^+ \]  \hspace{1cm} (15)
\[
\text{Luc}^+ + \text{solvent} \rightarrow \text{DBA} \]  \hspace{1cm} (16)

Oxygen in nonaqueous solvents is also reduced at platinum electrodes to superoxide ion, \( \text{O}_2^- \), and thus two possible reduction reactions can occur in the lucigenin system. However, it has been shown that the only light-producing reaction occurs between lucigenin and superoxide ion.\(^{30}\)

When DMF, DMSO or acetonitrile (AN) were used as solvents, a chemiluminescence emission spectrum having two bands was observed, while when ethanol was used as a solvent, only a single band was found. In ethanol the single band corresponded exactly with the fluorescence of N-methylacridone (NMA), as did the short wavelength band in the other three solvents. In AN, the long wavelength band corresponded to the fluorescence of lucigenin, while in DMF and DMSO, the second band corresponded to the fluorescence of DBA. Figure 9 shows a typical example of fluorescence and chemiluminescence curves recorded for lucigenin in DMF. These curves are consistent with the idea that NMA is initially formed in an excited state by reaction with superoxide ion and that either DBA or lucigenin acts as a secondary emitter after accepting the NMA excitation energy by an energy transfer process.

It was observed\(^{30}\) that NMA transfers excitation energy to either lucigenin or DBA at about the same rate. Which of these compounds will be the secondary emitter will depend on their relative concentrations and fluorescence efficiencies in the particular solvent. In DMSO, for example, lucigenin has \( \phi_f = 0.08 \), whereas DBA has \( \phi_f = 0.58 \); therefore, secondary emission in DMSO corresponds to the fluorescence of DBA. At the start of the chemiluminescence reaction in DMSO, little DBA is present and the chemiluminescence spectrum is identical to the fluorescence spectrum of NMA. As the reaction proceeds, DBA is produced and the in-

\[ \text{Fig. 9.--Spectra in DMF. (A) fluorescence of } 1 \times 10^{-4} \text{ M NMA; (B) fluorescence of } 1 \times 10^{-4} \text{ M DBA; (C) ECL spectrum at beginning of electrolysis; (D) ECL spectrum after 10 minutes of electrolysis.} \]
tensity of the long wavelength band increases. In AN, the \( \phi_f \) for lucigenin is 0.72, while for DBA, \( \phi_f = 0.40 \). The long wavelength emission in the beginning of the reaction corresponds mostly to the fluorescence of lucigenin, while as the reaction proceeds, some fluorescence due to both lucigenin and DBA can be noted. In ethanol, lucigenin has \( \phi_f = 0.09 \) and DBA is insoluble. Hence, neither of these compounds is able to act as a secondary emitter and only the blue emission of NMA is observed.

The question arises as to whether the energy transfer mechanism operating in the lucigenin system is of the long range dipole-dipole type or whether the transfer mechanism is trivial, i.e., emission by NMA, absorption by the acceptor and subsequent emission by the acceptor. We have performed calculations of the probability of energy transfer by the trivial and dipole-dipole mechanisms for lucigenin, DBA and NMA in all three solvents and have found that dipole-dipole energy transfer (Förster type) is the predominant mechanism at lucigenin concentrations greater than about \( 5 \times 10^{-4} \) M.

**Systems Related to Singlet Oxygen**

Another possibility of energy transfer in chemiluminescence and one of particular relevance to liquid scintillation counting occurs in the reaction between hydrogen peroxide and chlorine. When only the reactants are present, weak red chemiluminescence is observed, but when energy acceptors are added, light emission (characteristic of acceptor fluorescence) is produced. It has been shown that the red (634 m\( \mu \)) emission observed for the hydrogen peroxide-chlorine reaction is from a dimer of singlet oxygen formed by the following chemical reaction:

\[
H_2O_2 + Cl_2 \rightarrow ^1O_2^* + 2 HCl
\]

(17)

\[
^1O_2^* + ^1O_2^* \rightarrow 2(^3O_2)^*
\]

(18)

The energy levels of the monomeric and dimeric states of singlet oxygen are shown in Fig. 10, in which the dimer levels are given for the 0,0 bands. Since singlet oxygen dimers are long-lived in solution, they provide an array of electronic states that might be produced by the hydrogen peroxide-chlorine reaction. If an energy acceptor is added to a solution, energy transfer from a singlet oxygen dimer to the acceptor could be responsible for light emission. For example, the \( 2(^1\Delta_g) \) state corresponds to 44.8 kcal/Mole and the \((^1\Delta_g + ^1\Sigma_g^+)\) state provides 59.8 kcal/Mole of excitation energy; both are capable of exciting visible fluorescence.

In addition to observing the emissive \( 2(^1\Delta_g) \) state at 634 m\( \mu \), Khan and Kasha have observed emission from the \((^1\Delta_g + ^1\Sigma_g^+)\) state in solution, establishing that this state is also a potential donor for chemiluminescence reactions. The possibility exists that energy

![Fig. 10.-Electronic energy levels of molecular oxygen and singlet oxygen dimers. 0,0 levels are shown. Levels for several organic acceptors are shown as indicated.](image-url)
transfer can occur not only from the levels indicated in Fig. 10 but from levels containing 1 or 2 quanta of vibrational energy as well. Khan and Kasha have proposed that energy transfer from singlet oxygen dimers to emitters in solution may be a general mechanism for chemiluminescence. A number of known systems support the idea of Khan and Kasha: anthracene, acridine, eosin, and fluorescein have been excited as acceptors in chemiluminescent reactions. Very often in other chemiluminescent systems, the light emission observed is that of an unaltered acceptor molecule or the emission may be characteristic of the starting material rather than one of the reaction products. As is apparent from Fig. 10, the $2(1^+\Sigma_g^+)$ state would have sufficient energy to excite the fluorescence of anthracene and the $(1\Delta_g + 1^+\Sigma_g^+)$ state could excite the fluorescence of eosin. Further support for the proposal of energy transfer from singlet oxygen as a general mechanism comes from some chemiluminescent reactions that show the sort of squared dependence on either oxygen or peroxide which would be required for formation of a dimer from two excited oxygen molecules. The very low quantum efficiencies of many reactions adds credence to this interpretation. The singlet oxygen dimer mechanism is particularly apropos because it allows a simple interpretation for chemiluminescence produced by a side reaction unrelated to the main reaction occurring in the system.

Kurtz reported red chemiluminescence from the reaction of alkaline hydrogen peroxide and chlorine in the presence of violanthrone. Violanthrone (III) is a red, chloroform-soluble, vat dye having the structure shown below:

![Structure of Violanthrone](image)

Violanthrone has a high quantum efficiency of fluorescence, and the energy level of its lowest singlet state relative to the excited states of oxygen dimers is shown in Fig. 10.

Because a high concentration of singlet oxygen is produced by the hydrogen peroxide-chlorine reaction and the ability of singlet oxygen dimers to transfer energy, one might anticipate that energy transfer from singlet oxygen would excite violanthrone. Recent work in our laboratory, however, has shown that the mechanism of energy transfer from a singlet-oxygen dimer is inconsistent with the experimental results: chemiluminescence under steady-state conditions showed first-order dependance on both dye and peroxide concentrations. For the singlet-oxygen mechanism, a second-order dependence on peroxide would be required.

It has also been shown that violanthrone is not the emitting species in the chemiluminescent reaction but trichloroviolanthrone formed by a reaction of violanthrone with chlorine. The reaction does not involve the violanthrone radical cation as was originally proposed by Kurtz. The high intensity of the chemiluminescent reaction is due to the high fluorescence efficiency of trichloroviolanthrone and to its inertness to oxidative destruction by chlorine.

As seen in Fig. 10, the 0,0 level of violanthrone (and trichloroviolanthrone) lies slightly above the 0,0 level of the $2(1\Delta_g)$ state of the oxygen dimer. In an energy transfer mechanism involving singlet oxygen, either an excited vibrational level of the $2(1\Delta_g)$ state or the $(1\Delta_g + 1^+\Sigma_g^+)$ state would have to be responsible for energy transfer. If this were true, Rhodamine B should be excited readily by the vibrationally excited level of the oxygen dimer in the $2(1\Delta_g)$ state, and rubrene and eosin should be excited if transfer occurred from the $(1\Delta_g + 1^+\Sigma_g^+)$ state. Because the intensity of chemiluminescence from these latter compounds is lower than that observed from violanthrone and because there is first-order dependence on peroxide concentration, serious doubts may be cast on the occurrence of energy transfer from a singlet oxygen dimer in the violanthrone system. The following
mechanism, however, is consistent with the experimental observations on the violanthrone system:  

\[
\begin{align*}
\text{HO}_2^- + \text{Cl}_2 & \rightarrow \text{O}_2^* + \text{Cl}^- \quad (19) \\
\text{V} + \text{O}_2^* & \rightarrow \text{V} - 00 \\
& \quad \text{rate determining} \quad (20) \\
\text{V} - 00 & \rightarrow \text{V}^* + \text{O}_2 \\
& \quad \text{fast} \quad (21) \\
\text{V}^* + \text{O}_2^* & \rightarrow \text{O}_2 + \text{V}^* \\
& \quad \text{fast spin exchange} \quad (22) \\
\text{V}^* & \rightarrow h\nu \\
& \quad \text{fluorescence} \quad (23)
\end{align*}
\]

where V represents trichloroviolanthrone. Peroxide formation in reaction (20) would be rate determining. Although violanthrone does not form a stable peroxide that can be isolated, it is not unreasonable to expect that it would form a transient peroxide in solution. The spin exchange reaction shown in Equation (22) would probably be fast relative to the peroxide reactions, accounting for the observed first-order dependence on peroxide. The mechanism proposed in reactions 19 through 23 is consistent with recent studies on the luminescence of violanthrone excited by singlet oxygen generated in a microwave discharge.\(^\text{32}\)

Kurtz\(^\text{33}\) has reported chemiluminescence from the thermal decomposition of dibenzalperoxide in the presence of fluorescent compounds. We have studied the thermal decomposition of this and similar peroxides\(^\text{36}\) and have found the chemical reaction essentially to be quantitative:

\[
\begin{align*}
\text{C}_6\text{H}_5 & \quad \text{O}_2 \quad \text{R} \\
\text{O} & \quad \text{C} \\
\text{C} & \quad \text{O}_2 \\
\text{C}_6\text{H}_5 & \quad \text{O}_2 \\
\text{R} & \quad \text{C}_6\text{H}_5 \\
\end{align*}
\]

\[
\begin{align*}
\text{C}_6\text{H}_5 & \quad \text{R} \\
\text{O}_2 & \quad \text{C} \\
\text{C} & \quad \text{O}_2 \\
\text{C}_6\text{H}_5 & \quad \text{O}_2 \\
\text{R} & \quad \text{C}_6\text{H}_5 \\
\end{align*}
\]

\[
\begin{align*}
\text{2} \text{C} = \text{O} + \text{O}_2
\end{align*}
\]

where \(\text{R} = \text{H}, \text{C}_6\text{H}_5, \text{C}_6\text{H}_4\text{Cl}\). In the case of dibenzalperoxide where benzaldehyde is the reaction product, the aldehyde cannot be isolated quantitatively because some of it is destroyed, apparently by oxidation during the reaction. There are three possible mechanisms whereby the energy of peroxide decomposition can be transferred to the fluorescent acceptor:

**Singlet oxygen dimer mechanism:**

\[
P \rightarrow 2 \phi\text{CHO} + \text{O}_2^* \quad \text{peroxide decomposition} \quad (25)
\]

\[
2 \text{O}_2^* \rightarrow 2(\text{O}_2^*) \quad \text{singlet oxygen dimerization} \quad (26)
\]

\[
2(\text{O}_2^*) + \text{A} \rightarrow 2 \text{O}_2 + \text{A}^* \quad \text{energy transfer} \quad (27)
\]

**Intermediate peroxide decomposition:**

\[
P \rightarrow 2\phi\text{CHO} + \text{O}_2^* \\
\quad \text{peroxide decomposition}
\]

\[
\text{O}_2^* + \text{A} \rightarrow \text{AO}_2 \\
\quad \text{intermediate peroxide formation} \quad (28)
\]

\[
\text{AO}_2 \rightarrow \text{A} + \text{O}_2 \quad \text{triplet formation} \quad (29)
\]

\[
\text{2A} + \text{O}_2^* \rightarrow \text{A}^* + \text{O}_2 \\
\quad \text{spin exchange} \quad (30)
\]

**Triplet-singlet energy transfer:**

\[
P \rightarrow \phi\text{CHO} + \phi(\text{CHO})^* + \text{O}_2 \\
\quad \text{decomposition to form aldehyde triplet} \quad (31)
\]

\[
\phi(\text{CHO})^* + \text{A} \rightarrow \text{A}^* + \phi\text{CHO} \\
\quad \text{energy transfer} \quad (32)
\]

Where P is dibenzalperoxide and A is the energy acceptor.

Table 3 shows the singlet energy levels for some acceptors that produce fluorescence during peroxide decomposition, indicates whether or not the endoperoxide is known, and gives the overlap with excited states of potential energy donors. On the basis of preliminary studies,\(^\text{24}\) it is not possible to distinguish positively between the three mechanisms presented above, but some considerations indicate that two of the possible
mechanisms listed cannot be applicable to all of the compounds studied.

Of the six compounds listed in Table 3, perylene showed the brightest chemiluminescence. If the singlet oxygen dimer mechanism were operative, perylene would have to be excited by the \((1A_g + 1\Sigma_g^+)^*\) state. On the basis of the observed relative intensities of the singlet-oxygen-dimer emission bands,\(^{32}\) it is unlikely that perylene, if singlet oxygen dimers were involved, would be exceedingly bright while emission from acceptors such as fluorescein, TCV and coronene is much weaker. There are also difficulties with the intermediate peroxide decomposition mechanism. For example, fluorescein is not known to form any sort of intermediate peroxide at room temperature or at low temperature. The triplet-singlet energy transfer mechanism is most consistent with the brightness of the compounds listed in Table 3 because energy transfer could occur from a triplet state of benzenaldehyde and would thus depend on spectral overlap of the specific compounds. This mechanism also avoids an energy doubling, since the energy of the benzenaldehyde triplet is either equal to or greater than the energy of the singlet state of all of the acceptors except phenanthrene. Related work in our laboratory has demonstrated that triplet-singlet energy transfer can occur in the benzenaldehyde-violanthrone and benzophenone-perylene systems as well as in others.\(^3\) Although this must be regarded as a tentative conclusion, it appears that the triplet-singlet transfer mechanism is the one most generally applicable to the peroxide decomposition reactions studied.

### Grignard Reagents

Often chemiluminescence is the result of a reaction incidental to the course of the main reaction. Such a mechanism is probably responsible for much of the low-level light emission observed in organic oxidation reactions and possibly in many peroxide decomposition reactions. Chemiluminescence research on such systems is hampered by the inability of investigators to record accurate spectra and, hence, inability to characterize the emitter. Such a reaction recently studied in detail\(^{38}\) is the chemiluminescence from the oxidation of aryl Grignard reagents. The chemiluminescence spectra reported in the literature generally showed little correlation with the structure of the Grignard. It was particularly puzzling that oxidation of a compound like phenylmagnesium bromide would result in an emitting species having a spectrum entirely in the visible region. These discrepancies were shown to arise from the fact that earlier workers obtained spectra for Grignards in concentrated solutions (about 1 M) and considerable distortion of the spectrum had been observed due to self-absorption.

Chemiluminescence from the oxidation of phenylmagnesium bromide in dilute solu-

---

Table 3. Energy Levels and Overlap of Possible Donors and Acceptors in Chemiluminescence of Dibenzalperoxide

<table>
<thead>
<tr>
<th>Acceptor</th>
<th>(E_s) (kcal M(^{-1}))</th>
<th>Overlap with (\phi) CHO</th>
<th>2((1\Sigma_g))</th>
<th>((1\Sigma_g^+ + 1\Sigma_g^+)^*)</th>
<th>2((2\Sigma_g^+))</th>
<th>Forms Endoperoxide</th>
<th>Relative Intensity</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenanthrene</td>
<td>76</td>
<td>poor</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>good</td>
<td>yes</td>
</tr>
<tr>
<td>9,10-Diphenylanthracene</td>
<td>73</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>fair</td>
<td>yes</td>
</tr>
<tr>
<td>Coronene</td>
<td>70</td>
<td>good</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>fair</td>
<td>yes</td>
</tr>
<tr>
<td>Perylene</td>
<td>65</td>
<td>very good</td>
<td>-</td>
<td>poor</td>
<td>-</td>
<td>yes</td>
<td>very bright</td>
</tr>
<tr>
<td>Fluorescein</td>
<td>57</td>
<td>good</td>
<td>-</td>
<td>good</td>
<td>-</td>
<td>no</td>
<td>bright</td>
</tr>
<tr>
<td>Tetrachloroviolanthrone</td>
<td>47</td>
<td>poor</td>
<td>good</td>
<td>-</td>
<td>-</td>
<td>yes</td>
<td>weak</td>
</tr>
</tbody>
</table>

* Overlap between lowest triplet state of \(\phi\) CHO and lowest singlet state of acceptor.
† Overlap between the particular singlet oxygen dimer state with the lowest singlet state of the acceptor.
‡ Based on relative darkening of photographic plates.
Identification was accomplished by isolation of the product and comparison of its UV, IR and mass spectra with those of an authentic sample. The mismatch on the long-wavelength side of the spectrum shown in Fig. 11 is attributed to a small amount of some fluorescent product other than p-terphenyl. It is interesting to note that in the solvent system studied, p-terphenyl accounts for essentially 95 per cent of the light emission produced by the chemiluminescent reaction even though it accounts for only about 0.1 per cent of the starting material. The 0,0 band of p-terphenyl lies at about 310 mμ, corresponding to an energy of about 92 kcal/Mole. This means that the chemiluminescence reaction associated with the oxidation of phenylmagnesium bromide is one of the most energetic chemiluminescent reactions known to occur in solution.

On the basis of simple energetics and energy transfer experiments, it was shown to be unlikely that p-terphenyl served merely as an acceptor for the energy of some more energetic species produced in the chemiluminescent reaction. It was observed that adding biphenyl to a phenylmagnesium bromide solution gave no increase in chemiluminescence, nor did adding p4erphenyl to the reaction mixture as oxidation was occurring. However, by adding p-terphenyl to the Grignard solution prior to oxidation, the light intensity increased as a function of p-terphenyl concentration. This implies that p-terphenyl reacts in the Grignard solution before oxidation to produce a species that can produce excited p-terphenyl when oxidized. Two possible types of reactions could account for these results: metallation and ionization. Metallation reactions between p-terphenyl and phenylmagnesium bromide could produce the p-terphenyl Grignard:

\[ \phi \text{MgBr} + \phi-C_6H_4-O \rightarrow C_6H_5 + \phi-C_6H_4-C_6H_4\phi \text{MgBr} \]  

Oxidation of the p-terphenyl Grignard and reaction with the solvent could produce p-
terphenyl hydroperoxide that could decompose to give an excited state. This is quite consistent with many known chemiluminescent reactions in which peroxide decomposition is responsible for the light-emitting step.

Ion formation could also occur in Grignard solutions as follows:

$$\phi \text{MgBr} + \phi - C_6H_5-O \rightarrow C_6H_5^- + [\phi - C_6H_5-O]^{-}\text{MgBr} \quad (34)$$

which would give rise to the $p$-terphenyl radical anion. Reaction of the radical anion with either oxygen or peroxide during the oxidation process could produce light. Again it is well known that oxidation reactions involving peroxides and aromatic radical anions will produce light emission characteristic of the hydrocarbon. Unfortunately it is not possible to distinguish between these two mechanisms at present, although the latter is consistent with all observations to date on the oxidation of phenylmagnesium bromide.

ACKNOWLEDGMENTS

I wish to thank Professors David Roe, Jack Chang, Richard Bardsley, Glyn Short, Robert Lansbury, Fred Lytle, Kenneth Legg, John Gorsuch, T. C. Werner, Tony Vaudo, Bruce Watne and Seth Abbott who made major experimental contributions to the work discussed in this article.

REFERENCES


Chemiluminescence in liquid scintillation counting has been called the *bete noir*. It has caused a great deal of trouble and has contributed to errors in quantitative determination of radioactive material by the liquid scintillation technique. In the previous chapter, Hercules summarized recent advances in the knowledge of the physical chemistry of chemiluminescence. His discussion of the role of oxygen and peroxides is quite germane to the practical problems discussed here.

The “photoluminescent effect” was first attributed to proteins reacting with Hyamine 10-X. In recent years the causes of the unwanted chemiluminescence reactions have been somewhat better understood. We shall deal here with chemiluminescence only as it occurs in liquid scintillation samples. To suggest means of avoiding it, we will outline what some of the specific factors are. In the next chapter, Bransome and Grower present some additional methods recently developed in their laboratory for the purpose of detecting and avoiding chemiluminescence.

**EXPERIMENTAL**

Among the many substances recommended and applied in liquid scintillation counting techniques, we investigated possible chemiluminescence effects with the following solvents: toluene, dioxane, ethanol, and methylglycol; the following solutes: naphthalene, PPO (2,5-diphenyloxazole), POPOP (2,2′-phenylene-bis [5-phenyloxazolyl] benzene), BBOT (2,5-bis [5′-t-butyl-benoxazolyl-(2′)]-thiophene), a mixture for nonaqueous samples of 4 g PPO and 50 mg POPOP in 1000 ml toluene (Solution A); and the following mixtures for aqueous samples: 2.6 g PPO, 80.0 mg POPOP, 300.0 ml toluene, and 500.0 ml methylglycol (Solution B); 4 g PPO, 100 mg POPOP, 1000 ml toluene, and 1000 ml ethanol, (Solution C); 120 g naphthalene, 4 g PPO, and 75 mg POPOP in 1000 ml dioxane (Solution D);
Butler cocktail); and 4 g BBOT (Ciba), 600 ml toluene, 400 ml methylglycol, and 80 g naphthalene (Solution E; BBOT cocktail).

As bleaching agents we used hydrogen peroxide (30%) and benzoyl peroxide (saturated solution in toluene). All reagents were of "analytical grade."

For many years investigators have observed spurious counts and high background levels in liquid scintillation counting, especially when radioactive biological material solubilized by strong organic or inorganic bases was measured. Various basic agents for hydrolysis and solubilization of biological materials (such as amino acids, polysaccharides, nucleic acids, proteins and entire tissues have been described. The following frequently used substances have been used in the present investigations: aqueous or alcoholic solutions of potassium or sodium hydroxide; a methanolic solution of Hyamine 10-X,9 (p-(di-isobutylcresoxyethoxyethyl)-dimethylbenzyl-ammonium chloride monohydrate; Rohm & Haas) a solution of NCS in toluene (NCS = mixture of organic quaternary ammonium bases in the molecular weight range of 250 to 600; Nuclear Chicago).

The chemiluminescence measurements were performed with a Nuclear Chicago liquid scintillation counter three-channel system, Model 725.

**BASIC SOLUBILIZERS**

In our experimental procedure for quantitative determination of the intensity of chemiluminescence reactions, 1.0 ml of basic solubilizing agent was added to 10.0 ml of scintillation cocktail or solvent in the counting vial and the light impulses were measured 30 seconds later in a liquid scintillation counter, over a period of six seconds at a temperature of 10° C. If not otherwise mentioned, the high voltage of the photomultipliers of the instrument was adjusted in the same manner as for (14C) counting.

Relative chemiluminescence intensities are listed in Table 1. It can be seen that dioxane and the dioxane-containing cocktail gave highest chemiluminescence values.

**Rate of Decay**

An example of the duration and decay of chemiluminescence (Fig. 1) indicates that the count rates are initially very high and fall exponentially over several orders of magnitude within the first 30 minutes but remain elevated for at least several hours over the background baseline.

**Influence of Temperature**

Because some liquid scintillation counters are operated with a refrigerated system while others run at ambient temperature,12 it was of interest to know the influence of temperature on the degree of chemiluminescence. For the investigation of the temperature dependence, 10.0 ml of Solution D (Butler cocktail) and 0.5 ml Hyamine were mixed and measured at different temperatures. The results are shown in Fig. 2 and demonstrate that the chemiluminescence reaction is more intensive at higher temperatures.

**Influence of Solute**

As we could demonstrate in our experiments listed in Table 1, the chemiluminescence reaction at 10° C in two scintillation mixtures containing 1.0 ml Hyamine 10-X (counts on log-scale).
Fig. 2.—Influence of temperature on intensity of chemiluminescence reaction.

Chemiluminescence in cpm

<table>
<thead>
<tr>
<th>Temperature in Centigrades</th>
</tr>
</thead>
<tbody>
<tr>
<td>8</td>
</tr>
<tr>
<td>12</td>
</tr>
</tbody>
</table>

10 ml Butler - Cocktail + 0.5 ml Hyamine

The chemiluminescence reaction in the tested solvents was always more pronounced when the solvents contained scintillator solutes. To investigate the effect and participation of solutes on the chemiluminescence reaction, we measured the light intensity of a mixture of dioxane and Hyamine with increasing amounts of solutes dissolved in dioxane. An example of our results is shown in Fig. 3 in which the increase of chemiluminescence with increasing naphthalene concentrations is demonstrated.

Although a mixture of naphthalene and Hyamine alone did not emit any light, there was a significant increase in chemiluminescence depending on the amount of naphthalene dissolved in dioxane. These findings indicate that naphthalene itself may not react with Hyamine but that it makes the dioxane more transparent (see Chapters 1 and 3) so that the photomultiplier of the counter will "see" more light impulses. Naphthalene and scintillator solutes may also improve the energy transfer efficiency of the solvents.3 (See Chapters 1 and 3.)

Table 1

<table>
<thead>
<tr>
<th>Scintillation Cocktails and Solvents (10 ml)</th>
<th>Solubilizing Agents (1.0 ml)</th>
<th>Counts/0.1 min.</th>
</tr>
</thead>
<tbody>
<tr>
<td>Solution A</td>
<td>Hyamine</td>
<td>29</td>
</tr>
<tr>
<td>Solution A</td>
<td>NCS</td>
<td>5140</td>
</tr>
<tr>
<td>Solution A</td>
<td>2-N KOH</td>
<td>14</td>
</tr>
<tr>
<td>Solution B</td>
<td>Hyamine</td>
<td>230</td>
</tr>
<tr>
<td>Solution C</td>
<td>Hyamine</td>
<td>210</td>
</tr>
<tr>
<td>Solution D</td>
<td>Hyamine</td>
<td>500,410</td>
</tr>
<tr>
<td>Solution D</td>
<td>NCS</td>
<td>600,514</td>
</tr>
<tr>
<td>Solution D</td>
<td>2-N KOH</td>
<td>10,324</td>
</tr>
<tr>
<td>Solution E</td>
<td>Hyamine</td>
<td>949</td>
</tr>
<tr>
<td>Solution E</td>
<td>NCS</td>
<td>121</td>
</tr>
<tr>
<td>Solution E</td>
<td>2-N KOH</td>
<td>79</td>
</tr>
<tr>
<td>Toluene</td>
<td>Hyamine</td>
<td>167</td>
</tr>
<tr>
<td>Dioxane</td>
<td>Hyamine</td>
<td>2800</td>
</tr>
<tr>
<td>Dioxane</td>
<td>NCS</td>
<td>13,887</td>
</tr>
<tr>
<td>Dioxane</td>
<td>2-N KOH</td>
<td>29</td>
</tr>
<tr>
<td>Methyglycol</td>
<td>Hyamine</td>
<td>30</td>
</tr>
</tbody>
</table>

PEROXIDES

Investigations about the cause and the physicochemical nature of chemiluminescence in liquid scintillation counting have made it clear that organic peroxides may react in an alkaline medium to produce emission of light.14 (See Chapter 32.) Neither proteins nor other substances from biological samples appear to be involved in this phenomenon. To demonstrate that chemiluminescence is attributable to peroxides present in dioxane and toluene as contaminants, these solvents were shaken with small amounts of hydrogen peroxide and measured after the addition of Hyamine.4,5 The result was an intense stimulation of light emission. As reported by Winkelman and Slater,6 the increase of chemiluminescence is even more pronounced when benzoyl peroxide rather than hydrogen peroxide is used.

During sample preparation for liquid scintillation counting, solutions of digested tissues, blood or urine in Hyamine or NCS very
Chemiluminescence reactions in liquid scintillation mixtures are of very low energy and for this reason may interfere even more in \((^3H)\) counting. To demonstrate this, we measured a mixture of 1.0 ml benzoyl peroxide, 1.0 ml NCS, and 15.0 ml Solution D (Butler cocktail) at a high voltage setting of the counter for \((^{14}C)\) and \((^3H)\). As seen in Fig. 5, the chemiluminescence is much higher when measured in the \((^3H)\) range, although the decay curves are quite similar in shape. Examples given by Bransome and Grower in the following chapter involve chemiluminescence in the \((^3H)\) range.

**CONCLUSION**

Because of our experimental results and of the investigations of other researchers,\(^6,18\) we would like to suggest consideration of the following to eliminate, minimize or avoid chemiluminescence in liquid scintillation counting. The user of this counting technique should observe the following points:

1. In contrast to dioxane which is notorious for the formation of peroxides on contact with air, toluene-based scintillation mixtures generally contain much less peroxides and preferably should be used, especially

![Fig. 4. Chemiluminescence decay curves of liquid scintillation mixtures containing benzoyl peroxide and methanolic KOH (counts on log-scale).](image)
in connection with basic solubilizers such as NaOH, KOH, Hyamine and NCS.

(2) The use of peroxides as bleaching agents should be avoided in basic solubilizers.

Fig. 5.—Chemiluminescence curves of mixture of 1.0 ml benzoyl peroxide, 1.0 ml NCS, and 15.0 ml Butler Cocktail measured at (14C) and (3H) settings of counter (counts on log-scale).

(3) If biological samples that have been digested in basic agents are used, the samples should be neutralized to pH values equal to or lower than 7.0 by the addition of acid. Acid may increase quenching and may not always be sufficient to eliminate chemiluminescence.

(4) All counting samples of a pH higher than 7.0 should be checked for chemiluminescence to avoid counting errors. If chemiluminescence is found in the prepared counting sample, it is advisable to store the sample at room temperature or higher until the luminescence has decayed to a tolerable level.

(5) Because dioxane-containing scintillation solutions have great merit in determining low-energy isotopes in aqueous solution and by the addition of naphthalene, considerably reduce the quenching effect of water (see Chapter 7), it is sometimes desirable to use dioxane mixtures. In this case, I recommended using an acid solubilizing agent such as the Beckman Bio-Solv BBS-2,2° or other methods for the hydrolysis of biological material. (See Chapters 22 and 23.)

REFERENCES

DETECTION AND CORRECTION OF CHEMILUMINESCENCE IN LIQUID SCINTILLATION COUNTING

E. D. BRANSOME, JR., AND M. F. GROWER

In Chapter 33, Kalbhen described the problem of chemiluminescence encountered in liquid scintillation counting of low-energy betas. In Chapter 32, Hercules reviewed what is now known of the physicochemical basis of chemiluminescence. There is a rather extensive terra incognita between these two discussions, since there are as yet no data to identify precisely which of the different chemical mechanisms of chemiluminescence operate under different counting conditions. Certainly a major source of chemiluminescence in the energy range of the $^3$H β interaction with primary scintillator must be the formation of singlet oxygen systems, a process at least partially dependent on the presence of peroxides and the presence of an alkaline pH in the aqueous component of the scintillator solution. We can offer no additional insights into the nature of the problem, but we can present a method of detecting chemiluminescence in liquid scintillation counting samples and offer some new methods of eliminating it. Several of these approaches have not been reported before. Because the major portion of the chemiluminescent spectra we have encountered is in the $^3$H range and because many projects in our laboratory involve determinations of low levels of $^3$H radioactivity, our illustrations will be confined to $^3$H counting. It seems worth reiterating that chemilumines-

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cence, if appreciable, will contribute spurious counts per minute above the \((^3\text{H})\) endpoint [e.g., to a spectral range selected for \(^{(14}\text{C})\) radioactivity alone]. In Chapter 33, Kalbhen used a \(^{(14}\text{C})\) channel for his studies.

**Selected Examples of Chemiluminescence**

Chemiluminescence is one of the problems in the two hydrolytic procedures we have suggested (Chapter 27) for the preparation of radioactive samples in acrylamide gels. In addition to the examples given by Kalbhen, we can give examples of chemiluminescence in the \(^{(3}\text{H})\) range generated by an air-equilibrated alkaline toluene-scintillator solution (Fig. 1) and by \(\text{H}_2\text{O}_2\) (Fig. 2).

**Alkaline Chemiluminescence**

Figure 1 illustrates three findings we have found to be of general relevance:

1. By ignoring the advice of Hansen and Bush\(^1\) that the amount of water may be rate-limiting for hydrolysis by NCS, we did not allow the initial hydrolysis of the acrylamide ester cross-link and of the protein samples to proceed to completion. Hydrolysis was still taking place at a low rate when the samples were first counted. The chemical reaction and the presence of oxygen constituted ideal circumstances for chemiluminescence. If hydrolysis is still taking place, chemiluminescence will take place also. (See the discussion of Grignard reactions in Chapter 32.)

2. This point was reviewed by Kalbhen and is well known to all who have encountered chemiluminescence in their scintillation counting samples: the spurious radioactivity generated by chemiluminescence decays over time. The liquid scintillation counting of samples again after several days have elapsed is the standard method of detecting the presence of chemiluminescence. (We report an additional method below.)

3. The total counts per minute in the \(^{(3}\text{H})\) range of a chemiluminescent sample has no predictable relationship to the actual \(^{(3}\text{H})\) or \(^{(14}\text{C})\) radioactivity of the sample. (Compare the two dpm profiles of "protein" radioactivity in Fig. 1.)

---

**Fig. 1.** Chemiluminescence in \(^{(3}\text{H})\) range attributable to hydrolysis, alkalinity and oxygen. A bis cross-linked acrylamide gel cylinder was sliced and partially hydrolysed in NCS after \(^{(3}\text{H})\) \((\text{leucine-labeled})\) cytosol protein of guinea pig adrenal cortex was separated in it by electrophoresis. (See Chapter 28 for details of procedure.) Solid line indicates absorption (optical density) of separated amido-schwarz stained proteins. Broken lines indicate dpm \(^{(3}\text{H})\) "radioactivity" calculated (see Chapter 36) from the cpm in the \(^{(3}\text{H})\) channel. Alternating short and long dashes denote \(^{(3}\text{H})\) "count rate" of each of 30 2-mm gel slice samples four days after addition of NCS and insufficient \(\text{H}_2\text{O}\) to permit complete hydrolysis. Additional \(\text{H}_2\text{O}\) and NCS were then added to each vial. Radioactivity without chemiluminescence (obtained one month later) is represented by lower broken line.
Fig. 2. Chemiluminescence in (3H) range attributable to H₂O₂. Proteins of guinea pig adrenal cortex cytosol were separated in acrylamide gel under electrophoretic conditions slightly different from Fig. 1. Each 2-mm slice was incubated in 0.5 ml H₂O₂ at 60°C for 16 hours, dissolved in 15 per cent Bio-Solv solubilizer BBS-3 0.7 g per cent PPO in toluene (see Chapter 27 for this procedure). Solid line indicates absorbence (compare Fig. 1) Alternating broken line indicates "radioactivity" (mostly chemiluminescence) three days after addition of toluene. Lower line represents true (RH) dpm obtained several weeks after addition of 7500 units of catalase to filled vial.

Peroxide Chemiluminescence

The peroxide chemiluminescence shown in Figs. 2 and 3 was encountered after dissolution of acrylamide polymer by H₂O₂ and addition of toluene-PPO scintillator and BBS-3 solubilizer for one of the samples shown in Fig. 2. Note that the counts per minute actually increased to a maximum between days 11 and 12. The simultaneous dpm increases calculated suggest what was observed: there was no significant change in the external standard channels ratio (ESR; see Chapters 29 and 30) used to monitor changes in counting efficiency. The rise in chemiluminescence must be ascribed to the exhaustion either of a quenching reaction preventing chemiluminescence (e.g., a triplet quencher; see Chapter 32) or of a less probable mechanism interfering with the energy transfers involved in the chemiluminescent effect.

The decay after day 12 in this and other samples (not shown) appeared to be exponential. A background of 4–5 cpm attributable to chemiluminescence was still present as long as six months later. The addition of excess catalase on day 17 (for the procedure, see Chapter 27) removed all chemiluminescence in less than 72 hours. In the experiment referred to in Figs. 2 and 3, catalase was added to the toluene scintillator-solubilizer-sample mixture instead of the initial solubilizer-sample-mixture as in the recommended procedure. When the enzyme protein was added after toluene, an increase in quenching of (3H) radioactivity occurred. There was a significant drop in ESR which could be partially corrected for by the addition of more toluene to the vial. We did not determine a quench correction curve for background (versus ESR) of the samples represented by Fig. 3, nor did we count samples (after day 21) long enough to obtain standard errors of 5 per cent or less. These errors of omission may serve to explain the divergence of dpm from cpm on day 1 in Fig. 3. The dpm and cpm curves were parallel from day 36 on.

Detection of Chemiluminescence

The principal point in Fig. 3 that we wish to emphasize is the comparison of the dpm calculated from cpm obtained in a wide (3H) channel and dpm calculated from a narrow (3H) channel. We employed slope equations for the relationship between ESR of (137Cs) Compton electrons and (3H) efficiency as outlined in Chapter 36 to determine dpm:

\[
\text{dpm} = \frac{\text{Net cpm}}{\text{efficiency}}
\]

Because each quench correction relationship is dependent on the particular part of the β-energy spectrum discriminated by a channel
Fig. 3.—Basis of monitoring chemiluminescence. Counts and of dpm (3H) protein sample taken from electrophoretic fractionation shown in Fig. 2 Location of sample is indicated by number 3 below abscissa in Fig. 2. Here ordinate represents apparent (3H) radioactivity. Solid line indicates cpm in wide (3H) window (maximum efficiency 63%); broken line with wide dashes, dpm calculated from wide-window counts per minute; line with small dashes, dpm calculated from narrow-window [maximum (3H) efficiency 40%] cpm. Arrows indicate addition of catalase at day 17. (See text for further details.)

(whether it is wide or narrow), a ratio of deviations per minute from two channels

\[ \frac{\text{dpm narrow}}{\text{dpm wide}} = 1 \]

should be obtained if the \( \beta \) assumed in the quenched standards used to construct the quench correction curves is all that is being counted. Figure 3 shows that when chemiluminescence is present,

\[ \frac{\text{dpm narrow}}{\text{dpm wide}} > 1 \]

We have taken advantage of the asymmetry of the energy spectrum of chemiluminescence in the (3H) range to detect its presence: photelectrons are skewed toward the lower limit of detection. Thus any pair of wide-versus-narrow windows can be used to monitor for chemiluminescence as long as the endpoint in the narrow window is significantly below the endpoint of the \( \beta \)-spectrum (as long as the downward shift of energy distribution in quenched samples has not made cpm from the \( \beta \) equal in the two channels). Because the dpm channels ratio is used, one can monitor a chemiluminescence contribution to the counts per minute of either isotope in a sample labeled with two isotopes.

When chemiluminescent counts are first encountered in a sample-scintillator mixture, they are a product not only of the slowly decaying chemical reactions referred to above but of several relatively short-lived photoluminescence phenomena. The intensity of such “early” chemiluminescence (not shown in Fig. 3) is usually greater and the energy distribution broader so that the (dpm narrow:dpm wide) ratio may be close to unity. In this situation chemiluminescence is obvious: the count rate is significantly decreased when the sample is counted again, even if the delay is brief.

There are several other methods of detecting the presence of very large amounts of chemiluminescence. Davidson\(^2\) has suggested that switching off the coincidence circuit on the scintillation counter will result in a disproportionate increase in phototube singles rate because chemiluminescent counts are actually accidental coincidences of single photon events. In some scintillation counters the incorporation of a delay line allows detection of chemiluminescence. With the delay line, coincident counts are eliminated and only singles remain.\(^8\) Unfortunately, neither of these approaches is helpful in the detection of moderate or low levels of chemiluminescence, although single-tube counting is applicable to analytical uses of chemiluminescence. (See Chapters 11 and 12.)
Methods for Correcting Chemiluminescence

As the illustrations in this chapter and the preceding two chapters amply document, the appearance of chemiluminescence is favored, among other things, by (1) the presence of molecular O₂ in the sample (see Chapter 1 also); (2) the presence of peroxides; (3) the presence of an active chemical reaction (e.g., hydrolysis); and (4) alkaline pH of the aqueous phase, particularly if phenolic compounds are present (see Chapters 19 and 33).

A number of procedures are helpful in coping with the problem of chemiluminescence. A list of practical measures to be considered along with those of Kalbhen (Chapter 33), is necessarily incomplete but has proved quite useful in our laboratory:

1. Combustion of samples to H₂O and CO₂ by an oxygen flask technique.
2. N₂ or argon flushing of samples followed by air-tight sealing.
3. For peroxides, the catalase method as mentioned above.
4. Care that emulsifiers or solvents are not contaminated with phenols or quinones.
5. In samples subjected to hydrolysis, care that the reaction is complete.
6. In alkaline samples, acidification to pH 7 or below. This desirable procedure may be accompanied by considerable additional quenching. If it is, we favor procedures 7 or 8 below (compare Chapter 33).
7. Addition of several drops (0.01–0.05 ml) of 5–15 per cent ascorbic acid to the sample-scintillator solution. The mechanism of action of this antioxidant has not been investigated. It is probably acting as an O₂ scavenger.
8. Addition of several drops of SnCl₂ in 0.1 N HCl. Newman⁴ recommends that 4 g SnCl₂ be taken up in 10 ml of concentrated HCl and then made up to 100 ml with H₂O. He warns against using the solution if it becomes opalescent and strongly suggests storage in a glass bottle impermeable to O₂. We have noted that both SnCl₂ and ascorbic acid addition to toluene-NCS solutions improved the counting efficiency. Presumably oxygen uptake by either compound relieves O₂ quench, partially imitating the flushing of a sample with N₂ or argon.
9. Letting chemiluminescence decay over time. The price of this maneuver is often infinite patience.

We agree with Moghissi (Chapter 7) in warning against considering that counting at low temperatures is a solution to the practical problem of chemiluminescence. Chemiluminescent counts per minute do tend to diminish in a refrigerated scintillation counting system, but they do not disappear. We have also found that cold storage apparently prolongs the life of some of the reactions engendering chemiluminescence, whereas heating may decrease it.

What is important is that the investigator recognizes that he has encountered chemiluminescence in his samples. The dpm channels-ratio technique outlined above is flexible and quite sensitive, particularly if the counting error of a sample is 7 per cent or less. It provides an easy and rapid procedure for determining for each sample whether these uninvited and unpleasant but nonetheless interesting photons are being counted.

REFERENCES

As Spratt points out in Chapter 35, there are a number of published programs for batch processing of liquid scintillation counter output. He briefly reviews several, devised in his laboratory, which are currently in widespread use. In Chapter 37, Litle describes a similar off-line program used through a computer terminal. He also describes the Beckman LS-16 system, a small on-line computer which can process output from up to four instruments, also on a terminal-time sharing basis. As mentioned in Chapter 4, several individual scintillation counting systems manufactured by Intertechnique and Philips include on-line dpm computation ability.

Small computer-calculators, inexpensive and generally useful in the laboratory as calculators, also have enough capacity for computation to be employed for calculation of dpm from single or double isotope samples. Grower and Bransome in Chapter 36 show how three currently available systems can be programmed for such use. In addition, they review the rationale and practice of curve-fitting quenched standard data by the least-squares method: the method used in the majority of computer programs for quench-correction, spillover correction, etc. Some readers new to the scintillation counting field may also be unfamiliar with the statistical procedures in general use.
It has now been more than 12 years since I had the pleasure of attending the Liquid Scintillation Counting Symposium held at Northwestern University in August 1957.\textsuperscript{1} It is also about 15 years since I first became associated with one of the earliest commercial instruments for internal liquid scintillation counting. As shown by Birks\textsuperscript{2} and by the other contributors to this volume, the developments in liquid scintillation spectrometry during the intervening years have been quite rapid. When I started using the technique, one sat in front of the instrument, placed the individual sample in the chamber, set the time for the appropriate time, manually recorded the count rate at the end of the time interval, removed the sample and inserted the next sample—and this continued until one was too sleepy to remain in the laboratory. Now our laboratory personnel load the counter with 200 samples, set the appropriate dials, fill the key punch hopper with cards, return two days later to send the punch deck to the computer center and refill the counter with the next batch of samples. These developments have emanated from the many advances in both liquid scintillation instrumentation and data-handling technology. It is impossible to give a complete review of advances in data-handling technology and their many applications in this brief presentation, but I will attempt to review some of the aspects of data handling in more or less chronological order. Some of the procedures used in our own laboratory will be used as examples.

**HISTORICAL DEVELOPMENTS**

As shown in Table 1, the techniques of quench correction and the methods of data presentation and collection have changed over the years in conjunction with changes in the basic instrumentation of liquid scintillation spectrometry. The introduction of rotary sample changers and printers meant that the instruments could be continuously utilized without the necessity of personnel constantly being present. When the continuous sample chain was introduced, there was also substan-
tial upgrading of electronic components to better realize the possibilities of differential pulse-height analysis by different channels. This meant that double isotope counting in the same sample and the channels-ratio method of quench correction could be vastly improved. Printing calculators and the initial efforts at on-line collection of data in machine-readable form were also introduced at about this time. Much of the impetus for updating the methods of data collection and computation with the continuous sample chain spectrometers emerged from the fact that these instruments had various "sample reject" features and could be "programmed" in regard to the manipulation of various sample groups. When external standardization was commercially introduced, built-in computers and on-line desk top calculators followed shortly thereafter. Such features as the ability to preselect counting intervals on a statistical basis have also contributed to data-handling technology in this area.

**CURRENT EXAMPLES**

Many examples of off-line computation programs for liquid scintillation spectrometry have now appeared, and additional programs have been described in material from commercial sources. This is no surprise since once the data are in machine-readable form, there are infinite ways of handling them. The various programs available use punch cards or paper tape and are used on diverse hardware from a variety of computer manufacturers. It would be quite impossible to review all of the proposed systems today. Instead, I have chosen to discuss off-line computation as used in our own laboratory. Such examples should serve to emphasize many of the primary advantages of off-line computation. These programs are undoubtedly of interest to the scientific community, because over 150 of our source decks have now been obtained by other users and we have yet to hear a complaint.

Three basic programs have been used. They have been used for counting ($^3$H) and ($^{14}$C) in the same counting runs but not for counting ($^3$H) and ($^{14}$C) in the same sample. MANDPM determines dpm from 3-cycle counting, from output on manually punched cards. The printer strips from the three cycles are aligned and the data from the pertinent channel are punched onto one card; i.e., 200 cards for 200 samples. AUTDPM was programmed to accommodate similar information but from cards automatically punched by having a key punch interfaced with the spectrometer; i.e., 1000 cards for 200 samples. AUTDPM was programmed to accommodate similar information but from cards automatically punched by having a key punch interfaced with the spectrometer; i.e., 1000 cards for 200 samples in five counting cycles. The usual basic computations for these two programs are shown in Table 2. Both programs produce a punched answer card for each sample having a cumulative total of 1500 counts from the first count run. These samples are then "spiked" with internal standard and counted again (Table 2). The first-count answer deck is submitted with the second-count data. The program computes dpm as before but this time proceeds to match up the appropriate spiked and non-

<table>
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<tr>
<th>Quench Correction</th>
<th>Basic Instrumentation</th>
<th>Data Collection</th>
</tr>
</thead>
<tbody>
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<td>Internal standardization</td>
<td>Each sample manually inserted</td>
<td>Tube display and manual collection</td>
</tr>
<tr>
<td>Channels ratio</td>
<td>Rotary sample changer with elevator</td>
<td>Tube display plus printers</td>
</tr>
<tr>
<td>External standardization</td>
<td>Continuous sample chain and background subtraction</td>
<td>Tube display plus on-line computation (printing calculator or built-in computer)</td>
</tr>
<tr>
<td></td>
<td>External standard pellet</td>
<td>Tube display plus on-line collection in machine readable form (punch cards or punched paper tape)</td>
</tr>
</tbody>
</table>

Table 1.—Developments in Data Handling
spiked sample data and performs the quench corrections.

Our more recent program, AUTAES, is based on AUTDPM but incorporates automatic external standardization. An appropriate number of sealed vials containing the same number of dpm but with variable quench are counted along with the samples. The relationship between the fraction of dpm "seen" in the sample (with the AES pellet out) versus the cpm in the AES channel (with the AES pellet in) is that of a polynomial computed for each run (Table 2). The program applies this polynomial equation to each sample in order to accomplish the appropriate quench correction. The computer output from the AUTAES program is shown in Figs. 1–4. Figure 1 is a logbook page giving title information, background, efficiencies, data on quenched standards (later plotted), and the quench correction polynomial printed in both the "E" and the "F" format so that all significant figures of the polynomial are available. The order of presentation of the data on any given line is the degree of the polynomial, the correlation coefficient, the constant, and the coefficients of increasing powers of x. Figure 2 shows the plot of the fraction seen (ordinate) versus cpm in the AES channel with the pellet in (abscissa) for the quenched (³H) standards that determine the polynomial. Figure 3 shows the same information from the quenched (¹⁴C) standards. Figure 4 shows the final portion of the output with the quench-corrected information available for use. The two columns on the right are disposed of when this final data sheet is cut to 8½-by-11-inch notebook size.

This description of AUTAES is far from complete, but details need not be discussed here. Let me only mention that, for instance, the user has the option to designate acceptable upper and lower limits for quench corrections and upper and lower limits for the polynomial constant and each coefficient of the powers of x. This means that the investigator has the ability to define his own acceptable performance limits.

Advantages of Off-line Computation

Many of the items to be considered when contemplating computer utilization (Table 3) are already apparent from the foregoing example, just as they would be from other off-line computational techniques. Reliability, speed and cost are often considered together because of their mutual pertinence. Reliability is excellent as compared to manual
AUTAES TEST DECK

****** DATA FOR LOG BOOK ******

RED BACKGROUND (TRITIUM) = 15.47 CPM FOR 30.00 MINUTES
GREEN BACKGROUND (CARBON) = 26.67 CPM FOR 30.00 MINUTES
BLUE BACKGROUND = 17.53 CPM FOR 30.00 MINUTES

TRITIUM EFF = 0.27134 CARBON EFF = 0.74881 NUMBER SAMPLES = 34

AES BLUE CPM(X) AND PER CENT SEEN(Y) FOR TRITIUM

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TRITIUM COEFFICIENTS

1  0.998873E 00  0.34979OE-01  0.579135E-05
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3  0.999998E 00  0.505528E-01  0.405677E-05  0.300545E-10  -0.123507E-15

AES BLUE CPM(X) AND PER CENT SEEN(Y) FOR CARBON

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</table>

CARBON COEFFICIENTS

1  0.966271E 00  0.172790E 00  0.575495E-05
2  0.999382E 00  0.478086E-01  0.117065E-04  -0.364185E-10
3  0.999895E 00  0.269757E-01  0.135066E-04  -0.644514E-10  0.111175E-15

Fig. 1.—Logbook page: calibration data.

Computation, while speed of computation is not necessarily that impressive if the investigator cannot obtain good turnaround time. However, batch processing with reasonable turnaround is usually adequate for most users of liquid scintillation spectrometers.

Costs can only be compared in the locale of the user. It certainly cannot yet be considered to be extremely cheap, but analysis of personnel time, concern for reliability, and advantages of machine-readability will probably make it competitive in most circumstances. This is particularly true when one does not have to sustain the costs of program development but can initiate such efforts with already available programs.

Although current liquid scintillation instrumentation is quite sophisticated and reliable, off-line computation allows one to perform instrumentation checks by multiple cycling of samples without any difficulty. This splitting-up of total counting time for each sample can still be of benefit but can be a problem when using built-in calculator-computers because in the latter situation one still has to average the data manually. Machine-readability can also be a very important advantage for off-line computation. For example, machine-readability has allowed us to incorporate editing routines in our programs. If there is a malfunction in the spectrometer-key punch operation so that the data are punched in the
wrong card column or an improper character is punched, a program error message identifying the card will be printed. Data storage capabilities are obvious because the data can be stored on cards or tape and manipulated in various fashions. It can be readily transformed and combined with other data by machine techniques to yield specific activities, concentrations, or units of compound per unit of tissue or body weight. Such information can also be displayed by sophisticated or simple plotting techniques, many of which can be readily incorporated into the original counting programs. Since the data are in machine-readable form, they can also be easily analyzed by statistical programs. In the utopian situation, it could actually be possible to proceed from counting data collection to

Table 3.—Considerations for Computer Utilization

<table>
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<tr>
<th>Reliability of computation</th>
<th>Speed</th>
<th>Cost</th>
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<td>Instrumentation checks via multiple cycling</td>
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<td>Data storage</td>
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<td>Editing capabilities</td>
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<td>Data storage</td>
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<tr>
<td>Recent advances</td>
<td>Increased remote input access</td>
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<td>On-line access</td>
<td>Time-sharing</td>
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Fig. 2. —(3H) quench correction curve.
publication without ever having to record any specific data manually.

**FUTURE DEVELOPMENTS**

Recent technological advances portend even more in the near future. Remote input stations are becoming more common so that more investigators are having easier access to large computer facilities. Time-sharing and on-line access have not yet arrived for many of us, but these capabilities will also make computation by means of a central facility perhaps even more tempting than it is today. (See Chapter 37.)

**REFERENCES**


**Table 1**: Measurement of Radioactivity with Some Useful Graphs and Nomographs. (continued)

<table>
<thead>
<tr>
<th>Sample</th>
<th>Tritium CPM</th>
<th>Carbon DPM</th>
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Fig. 4.—The dpm output.
The measurement of β-radioactivity with liquid scintillation spectrometers has advanced remarkably in the past few years. New instruments combine low background, high sensitivity, and the ability to count many samples automatically. External standardization is available for monitoring decreased counting efficiency due to quenching. (See Chapters 28–31.) The relative radioactivity (counts per minute or cpm) of properly prepared samples can be determined quickly and simply. Determining the absolute radioactivity (disintegrations per minute or dpm) of samples is now often the most tedious aspect of liquid scintillation counting. This is particularly true when a sample is labeled with two isotopes [e.g., (3H) and (14C)]. Because the channels used to count the isotopes must overlap to some extent, the contribution (spillover) of each isotope to the counts per minute of the other isotope must be corrected for before dpm may be calculated.

\[
dpm = \frac{\text{net cpm}}{\text{efficiency}}
\]

(1)

\[
(\text{net cpm} = \text{raw cpm} - \text{background} - \text{spillover})
\]

(2)

If the radioactivity of more than several samples is to be determined, or the activity of more than one isotope is to be determined on the same samples, manual calculation becomes extremely tedious and time consuming even if ingenious methods of recalling constants from desk calculator registers are used. Therefore automatic procedures for handling liquid scintillation counting data have become desirable.

At the present time there are several scintillation counters available which perform an
on-line computation of dpm after the instrument user has entered calibration
data.\textsuperscript{3-5} Off-line computation, however, offers much more power and flexibility. The investigator may increase the counting capacity of his laboratory through purchasing two scintillation spectrometers for little more than the cost of a single unit possessing computation ability. Off-line facilities are available for all of the other data-handling and statistical chores of a busy laboratory. Computation tied to the scintillation counter is either for a single purpose or limited to a few supplementary programs. We find the on-line use of a scintillation counter difficult to justify unless it is fully utilized as in the LS/16 scheme proposed by Litle in Chapter 37.

In Chapter 35, Spratt reviews the use of a large digital computation facility for data handling. There are also a number of problems encountered in handling data with large digital computers.\textsuperscript{6-10} Consultation may be needed if there is a novel data format or if there are any special requirements in data handling. An interface between the scintillation counter and a card puncher or time-sharing facilities for access to the computer must be obtained. Cards may have to be delivered to a computation center and then returned some time later after the data have been processed, and such facilities may not be available or economical for an individual investigator.

Within the past several years, a number of small, desk-top laboratory computers have been introduced; with these, computation of single and double isotope data is inexpensive and convenient. They are also accessible to the many other needs of a majority of laboratories. We will comment on the use of three such devices: the Olivetti Programma 101,\textsuperscript{11} the Wang 380 system,\textsuperscript{12} and the Hewlett-Packard Model 9100 A.\textsuperscript{13} Counting data may be entered by means of a keyboard or entered automatically with the first two machines if the output of a scintillation counter can be coded in ASCII symbols for a tape punch. Data tape is read in a sequence specified by a separate control tape on an interfaced double tape reader (e.g., the Beckman Omega Master Control Module\textsuperscript{14} with the Olivetti) or in a sequence specified by a program already in the computer (the Wang).

If the quench correction curves for series of quenched standards are linear, simple slope equations are derived from calibration data. If a graphical representation of efficiency

![Fig. 1.—Quench correction curves for (\textsuperscript{3}H) efficiency versus external standard ratio (ESR) Beckman LS-150. Wide (\textsuperscript{3}H) window registered maximal (\textsuperscript{3}H) efficiency of 63 per cent and background of 27 cpm for “figure of merit” [(E\textsuperscript{3}/B = 150). Narrow (\textsuperscript{3}H) window efficiency was 45 per cent and background 22 cpm [(E\textsuperscript{3}/B) = 92]. Range of ESR was 0.865–0.000 (least quenched to most quenched). Values for quenched toluene series (Beckman, Inc.) are shown for wide window (broken line) and narrow window (long dashes). For comparison, a more severely quenched series of sealed dioxane standards (100 g naphthalene/l-PPO 5 g/l) is shown. Solid lines indicates wide (\textsuperscript{3}H) window and alternating long and short dashes indicate narrow (\textsuperscript{3}H) window. Reproduced with the permission of Academic Press.](image-url)
versus external standard cpm. An external standard ratio, or channels ratio indicates an unsatisfactory linear fit, a quadratic equation for the quench correction is employed. Simple computation programs are then used to determine dpm. They can be tailored to the output of any scintillation counter that employs external standardization or channels ratio to monitor quenching.\textsuperscript{15,16}

Programs for an interpolation approach to quench correction have been advocated.\textsuperscript{11} We do not recommend this approach because the correction of cpm to dpm is then extremely dependent on the accuracy of two points. If one of the quenched standard samples were miscalibrated, a considerable error would ensue. The least squares approach tends to minimize an error of an individual data point. The use of slope equations to calculate quenching rather than an interpolation procedure also increases the speed of computation.

**EXPERIMENTAL**

**Counting Samples**

The series of quenched (\textsuperscript{14}C) and (\textsuperscript{3}H) standards used were in argon-flushed sealed vials. They were obtained from Beckman Instruments, Inc., Fullerton, California, and the Nuclear Chicago Corp., Des Plaines, Illinois:

(\textsuperscript{3}H) Toluene Standard \#845B, 226,000 dpm on 9/29/67 (Beckman).

(\textsuperscript{3}H) Toluene Quenched Series \#7, 199,000 dpm on 10/31/66 (Beckman).

(\textsuperscript{14}C) Toluene Standard \#845B, 116,500 dpm on 9/29/67 (Beckman) and \#181330, 53,400 dpm on 8/10/66 (Nuclear Chicago).

(\textsuperscript{14}C) Toluene Series \#17, 97,000 dpm on 11/4/66 quenched with chloroform (Beckman) and Series \#180060, 231,500 dpm on 6/20/66 quenched with carbon tetrachloride (Nuclear Chicago).

The Beckman vials (10 ml) contained 5 g/l of 2,5-diphenyloxazole (PPO) and 0.3 g/l of 1,4-bis-2,4-methyl-5-phenoxazolyl-benzene (dimethyl POPOP) in toluene. The Nuclear Chicago vials contained 6 g/l PPO and 0.75 g/l POPOP. These standards provided the data for Figs. 1-4. Additional series of unsealed quenched (\textsuperscript{3}H)- and (\textsuperscript{14}C)-containing samples made up in our laboratory from similar scintillation mixtures have been quenched with aqueous solutions of sucrose and NaCl (dioxane solvent) or with proteins and nucleic acids in Biosolv BBS-3 solubilizer (Beckman, Inc.) (toluene solvent) and hydrolysates of proteins, nucleic acids, stool samples, and Acrylamide gels (colored or uncolored) in NCS solubilizer (Nuclear Chicago Corp.). Unless counting efficiency was quite low,
less than 10 per cent for (3H), the quenching agent seemed to be immaterial. (See Chapters 28 and 29.) A significant change in scintillator composition (e.g., from toluene to a dioxane scintillator mixture or from a dioxane-naphthalene scintillator to Bray's mixture 17) necessitated determination of a new quench correction curve.

**Scintillation Counters**

To illustrate our approach, we have used three different liquid scintillation counters: Beckman Models LS-100 and LS-150 and Nuclear Chicago Model 6824. In the Beckman instruments the Compton effect of a (137Cs) external γ source was counted in two separate channels. After subtraction of sample radioactivity, an external standard channel ratio (ESR) was determined and employed to monitor quenching (Figs. 1–3). The channel discriminators on each instrument used for differential counting were calibrated so that

![Fig. 4.—Effect of gain on quench correction curve shape. (14C)-toluene samples were counted on Nuclear Chicago Model 6842 liquid scintillation spectrometer. External standardization with (226Ra) was by means of cpm accumulated in a single channel. Difference in gain settings for (14C) channel is specified by A (attenuation D, fine adjustment 2.0) and B (attenuation E, fine adjustment 7.50). Dynamic range was 20:1 and background 35 cpm. Solid lines connect observed data points. Alternating long and short dashes show quadratic equation. For “A” settings and Equation (7), A = 0.30112; B = 0.00872; C = -0.000038; and χ² = 0.220. For “B” settings, A = -0.16895; B = 0.00826; C = -0.000027; χ² = 0.383. Reproduced with the permission of Academic Press.](image-url)
(³H) efficiencies and backgrounds were similar but the dynamic range of the external standard ratio in the two Beckman machines was different. In the Nuclear Chicago instrument, amplification was linear rather than logarithmic. External standardization was carried out with a (²²⁶Ra) source and accumulated in a single channel of Compton electron cpm (Fig 4).

Computer-calculators

The Olivetti Programma 101 desk-top computer is programmed (up to 120 steps) by punching in the program or by inserting a single magnetic program card. The data used by the calculator in the programs we utilized were the sample number (for identification purposes), the ESR, and the raw cpm found in each channel. The Beckman Omega Master Control Module allows rapid automatic entry of the necessary data. It utilizes a short loop of punched paper editing tape specifying only the entry of desired information from a data tape punched with scintillation counter output. When lengthy programs are used, the editing tape can be used to enter numerical constants, thereby saving program steps or freeing storage registers.

The Wang calculating system, which we examined for liquid scintillation data applications, consisted of a Model 380–2 programmable keyboard, a Model 362 electronic package with the capacity for 12 whole storage registers, a Model 379–19 data editor for reading paper tape punched in ASCII numerical data, and an IBM Selectric typewriter output with a 379–5/7 control unit. Programs of variable length (up to 640 steps per track) are accommodated by a cartridge of two-track magnetic tape. The Wang tape editor reads all numerical data printed on the tape. Program steps on the magnetic tape are used to specify which numbers are to be used for calculations and which are to be disregarded. If the program required has more constants than storage capacity of the electronic pack-

age, the additional constants may be entered as program steps.

The Hewlett-Packard calculator Model 9100 A can be equipped with an electrostatic printer and has a magnetic core memory. There are 19 accessible registers as well as some intrinsic trigonometric and logarithmic functions. Three registers are utilized for numerical display; two are for data storage. Fourteen registers can be used for program storage or additional data storage and will accommodate up to 196 program steps, but each register used for data storage reduces the number of available program steps by 14. A magnetic card accommodating up to 196 steps on each side can be used to insert prerecorded programs (as with the Olivetti). When we examined this system, Hewlett-Packard did not manufacture a tape editor for this computer, and data could only be entered manually. Now a new attachment, the 2570 A instrumentation coupler, is available; it will allow attachment of currently available tape readers to the Model 9100 A. The data tape input must be in a proper format or a tape editor (in a double tape reader such as the Beckman Omega) must be programmed to specify which of the data on the tape will be entered.

DETERMINATION OF QUENCH CORRECTION CURVES

Series of quenched samples containing known amounts of radioactivity were counted and the efficiencies

\[
\text{cpm} - \text{background} \over \text{corrected dpm at time of counting}
\]

plotted against ESR or ES counts per minute. We have employed two methods of determining quench correction curves:

1. Generation of linear quench correction curves by least squares analysis after the data has been divided into groups by drawing approximate quench correction curves. With this approach the curves can be expressed as

\[
y = b(x - \bar{x}) + \bar{y}
\]
This equation can be reduced to the form
\[ y = bx + c \]
when there is a single linear quench correction curve and where \( y \) = efficiency to be calculated; \( \bar{y} \) = reference efficiency; \( x \) = external standard value or channels ratio of sample; \( \bar{x} \) = reference external standard value or channels ratio; \( b \) = slope of quench correction curve; \( c = \bar{y} - b\bar{x} \) (a computed constant). The slope \( b \) of the quench correction curve is obtained by least squares analysis of the data obtained from quenched standard series. For example,
\[
b = \frac{\sum_{i=1}^{n} (x_i - \bar{x})(y_i - \bar{y})}{\sum_{i=1}^{n} (x_i - \bar{x})^2}
\] (5)
which reduces18 to the formula used in the program:
\[
b = \frac{\sum_{i=1}^{n} x_i y_i - n\bar{x}\bar{y}}{\sum_{i=1}^{n} x_i^2 - n\bar{x}^2}
\] (6)

2. When quench correction curves cannot be reduced to linear equations without significant error, we have found that the quadratic equation
\[ y = A + BX + CX^2 \] (7)
usually generates an accurate curve. A least squares fit of observed values for efficiency \( (y) \) versus ES or ESR values \( (x) \) is used to generate the elements of the following normal equations:
\[
\Sigma y = An + B\Sigma x + C\Sigma x^2
\] (8)
\[
\Sigma xy = A\Sigma x + B\Sigma x^2 + C\Sigma x^3
\] (9)
\[
\Sigma x^2y = A\Sigma x^2 + B\Sigma x^3 + C\Sigma x^4
\] (10)
The coefficients \( A, B \) and \( C \) can be determined with the solution of these three simultaneous linear equations in three unknowns by forming a determinant and solving it by Cramer’s rule. (The manufacturers of the three machines discussed have in their program libraries19,20 the appropriate subroutine programs. We have not found it necessary to use polynomials of higher order to obtain accurate curve-fitting or to assume that quench correction curves are parabolic functions of the form
\[ y = ax^b \] (11)
It should be noted, however, that programs are available for deriving these more lengthy functions.19,20

DPM OF SINGLE ISOTOPE SAMPLES:
LINEAR QUENCH CORRECTION CURVES

The quenched standards series of \(^3\text{H}\) and \(^{14}\text{C}\) standards described above were counted on a Beckman LS-150 scintillation counter with discriminator settings and ESR dynamic range set up so that quench correction curves were linear. Wide channels were employed for single isotope counting, which embraced most of the energy spectrum of the \( \beta \)-emissions being counted. Narrow channels were chosen to minimize the spillover factor of the lower energy to the window for the higher energy isotope when doubly-labeled samples were counted. Since the dpm of each standard were known, efficiency versus ESR could be calculated. The data were graphed (Figs. 1 and 2) so that the suitability of a linear fit to the observed quench correction curve or the necessity for a fit of higher order to a more complex curve could be assessed. Since the observed quench correction curves were almost linear, an accurate slope \( b \) could be calculated with a program for linear least squares analysis [Equation (6)]. Mean values of efficiency \( \bar{y} \) and ESR \( (x) \) of the standard series could be used for reference points in a program for the solution of an unknown efficiency \( (y) \) given its ESR \( (x) \) with either Equation (3) or (4). These values of \( b, \bar{x}, \) and \( \bar{y} \) or \( b \) and \( c \) [see Equations (3) and (4)] were entered at appropriate points in programs for calculating cpm \( \rightarrow \) dpm. Figures 5 through 9 are examples of such programs on each of the three desk-top computers.
Fig. 5.—Example of Olivetti-Omega program. Calculation of dpm from cpm of single isotope given external standard ratio (ESR) and counts per minute of sample when quench correction relationship is linear. Values for x, y, and b (see Table 1) are stored in registers E, D/, and E/, respectively. Constant background cpm, stored in D, are appropriate when quench variation between samples is small. Punched paper tape with data in ASCII code is read for sample number, ESR, and cpm as specified by editing tape.

DPM OF SINGLE ISOTOPE SAMPLES: COMPLEX QUENCH CORRECTION CURVES

With some liquid scintillation counting systems, particularly those with linear amplification, it is difficult to manipulate gain controls, discriminator settings, or the external standard dynamic range to obtain a set of almost linear quench correction curves as seen in Figs. 1 and 2. If one user of a scintillation counter calibrates the instrument so that quench correction for a particular isotope or scintillation mixture is linear, the curves for other counting conditions will often be complex. Figure 3 shows (3H)-toluene quench correction curves obtained with the Beckman LS-100 ESR dynamic range calibrated so that while (3H)-dioxane curves were linear (not on graph), the other curves would not be. The resulting curves show acceptable counting efficiencies. Because both toluene and dioxane were counted in this machine, it was decided to use the same calibration for both types of samples, but one graph of linear coordinates would obviously not provide an adequate fit for either curve in...
Fig. 6.—Example of Wang program. Calculation of dpm from cpm of single isotope given external standard ratio (ESR) and cpm of sample when quench correction relationship is linear. In this program, \( m = b \) (in Table 1) and \( b = c \) (in Table 1). Single data tape is read for all numeric information; computer program specifies what data format is to be employed. Reproduced with the permission of Academic Press.
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Additional commands for graphical output of LSC 150 data

Record program on Channel #2

Constants to be entered on Channel #1 are:

- \( n \) number of lines to be printed before machine double spaces
- \( D \) = DPM increment

---

Fig. 7.—Program for graphical output of dpm that can be added after Step 57 of program in Fig. 6. (See Fig. 8.)
Two approaches were possible: the data could be separated into two groups, one above a breakpoint of \( \bar{x} \) (ESR reference) and one below it, or the curve could be generated with a quadratic equation. The low Chi square values for differences between observed and theoretical curves show that either approach was suitable. If the former (breakpoint or double linear) approach was employed, the two linear equations describing the curves had to be solved for the value of \( \bar{x} \) at the intersection of the straight lines. A testing procedure then had to be inserted in the program to test the observed external standard \( x \) against the reference external standard \( \bar{x} \) so that the efficiency \( y \) could be calculated. For example, the Olivetti program shown in Fig. 5 can easily be modified to the double linear approach by the insertion of four instructions between steps 10 and 11 and one between steps 11 and 12:

1. Between 10 and 11: (a) /V: conditional jump. If \( x - \bar{x} \) (step 10) = 0 or less, the next step is executed. If \( x - \bar{x} > 0 \), the program jumps to A/V. (b) F/X is substituted for step 11, multiplying \( b \) for the first curve (the slope of higher ESR values entered in F register) by \( \bar{x} - x \). (c) D/Z: unconditional jump to EZ where the program will continue. (d) A/V: reference point for /V, allowing execution of step 11: \( b \) of lower curve multiplied by \( \bar{x} - x \).

2. Between 11 and 12: EZ reference point for DZ. Continue with program.

The slope \( b \) used in Equation (3) was quite different if the ESR of an unknown sample was below \( \bar{x} \) rather than above it. This type of program is appropriate to other laboratory measurements. As an example, we have used it in converting absorbance to protein content in quantitative colorimetry. The quadratic approach obviated the need for testing \( x \) against \( \bar{x} \) in the computation program. A quadratic equation also accounted for the complexity of all of the observed quench correction curves.

In scintillation counters with linear rather than logarithmic amplification or with low maximal efficiencies, considerable variation in channel gain and attenuation may be neces-
Fig. 9.—Example of Hewlett-Packard 9100 A program. Calculation of dpm from cpm of single isotope given external standard ratio (ESR) and cpm of sample when quench correction relationship is linear (compare Fig. 6). Constants entered are derived from values (similar to those in Fig. 6) of \(^{(1)}\text{H}\) efficiency and ESR in wide window obtained with quenched \(^{(1)}\text{H}\)-toluene series.

An illustration of the effect of variations in gain on the shape of a quench correction curve is provided by \(^{(14}\text{C})\)-toluene data obtained on the Nuclear Chicago Model 6842.21 (See Fig. 4.) A reasonable double linear fit can be made to the observations at “B” settings. If samples were counted at maximal efficiency at “A” settings, however, this was no longer true. Only the quadratic equation gave an adequate fit to the observed quench correction values. Figure 10 shows a Wang program for quadratic fit of single label quench correction.

**DPM OF DOUBLE ISOTOPE SAMPLES**

The procedures used for calculating the dpm of doubly-labeled samples were no different from those above. They involved combinations of the computation programs for single labels after determination of the net cpm of each isotope by utilizing the spillover factor for the cpm contribution of one isotope in the channel used to determine the other. The spillover correction and background are subtracted from the gross cpm to get the net cpm from which dpm are determined. Table 1 lists constants derived from the narrow \(^{(1)}\text{H}\) and \(^{(14}\text{C})\) channels of a Beckman LS-150 system (Figs. 1 and 2). The channels were used for a program for \(^{(3)}\text{H}\) and \(^{(14}\text{C})\) double labeling. Figure 11 shows an Olivetti program for a doubly labeled sample with linear quench correction curves. Other examples have been published by Wang, Inc. A more thorough...
**Fig. 10.**—Example of (Wang) program using quadratic equation for quench correction. Constants A, B, and C were obtained by second-order regression analysis. Program is for manual data entry (compare Fig. 6).
Fig. 11.—Example of Olivetti-Omega program for doubly-labeled samples using linear quench correction curves of form \( y = bx + c \). Background cpm in Channel 1 is entered in register D; background in Channel 2, register D/. Slope (b) constant for isotope \( \alpha \) (lower energy) spillover into Channel 2 is entered in register E. Slope of quench correction curve for isotope \( \beta \) is entered in E/. Other constants (see Table I) are generated within program.
With Equation (4) \( y = bx + c \); \( y \) = efficiency; \( x \) = ESR; \( c \) = calculated constant; for S.O.F. calculation, \( y = S.O.F. \); \( x = ESR \). Values of \( x \) and \( y \) are also given for the original Equation (3) \[ y = b(x - x_0) + y_0 \].

Important when (al-I) cpm are much higher than \((^{14}C)\). If samples are low in radioactivity and if there is much range in ESR, an ESR versus background curve should also be obtained.

discussion of spillover of one isotope into the channel used to count another is provided by C. H. Wang in Chapter 31.

Spillover of low-energy cpm into the channel for the higher energy may be significant. We have found that achieving a \((^{14}C)\) channel completely free of \((^{3}H)\) cpm required an undesirable loss of \((^{14}C)\) efficiency and that calculation of reverse (A to B) spillover can be important when samples with small amounts of \((^{14}C)\) and relatively large amounts of \((^{3}H)\) radioactivity are counted. Therefore, we have sometimes included a low-to-high spillover factor in our double label computation programs. The slope constants to be used for spillover-factor correction are determined just as the quench correction constants are: by least squares analysis for a straight line [Equation (6)] if the quench correction curve is linear and by a quadratic if it is complex [Equation (7)].

**Table 1.**—Constants Derived from Figs. I and 2 for Computing DPM* of \((^{3}H)\) and \((^{14}C)\) Doubly-labeled Samples (Toluene)

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<th>( y )</th>
<th>( c )</th>
<th>( b )</th>
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<td>0.5791</td>
<td>0.3581</td>
<td>0.3776</td>
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<td>(10% constant: AQC on)</td>
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<td></td>
<td></td>
</tr>
<tr>
<td>((^{3}H)) narrow</td>
<td>0.6778</td>
<td>0.2724</td>
<td>-0.3270</td>
<td>0.8844</td>
</tr>
<tr>
<td>((^{3}H) \rightarrow (^{14}C)); S.O.F. †</td>
<td>0.6915</td>
<td>0.0025</td>
<td>0.0138</td>
<td>-0.0164</td>
</tr>
</tbody>
</table>

* With Equation (4) \( y = bx + c \); \( y \) = efficiency; \( x \) = ESR; \( c \) = calculated constant; for S.O.F. calculation, \( y = S.O.F. \); \( x = ESR \). Values of \( x \) and \( y \) are also given for the original Equation (3) \[ y = b(x - x_0) + y_0 \].
† Important when \((^{3}H)\) cpm are much higher than \((^{14}C)\). If samples are low in radioactivity and if there is much range in ESR, an ESR versus background curve should also be obtained.

**SUMMARY**

We have presented simple, rapid procedures for calculating the absolute radioactivity of samples labeled with one or two isotopes, given the counts per minute from liquid scintillation counting and an external standard ratio or external standard count rate. Data were entered by keyboard or paper tape into small off-line laboratory computers. The information for calculating efficiency of counting from an external standard cpm or ratio data was derived from a linear least squares analysis or from a second order regression analysis carried out with these same computers (which were also available for general laboratory computation and statistical analysis). Systems examined included the Olivetti Programma 101 with a Beckman Omega double tape reader, the Wang 380, and the Hewlett-Packard 9100 A. Similar machines, some with increased capacities for program length and for data storage, will soon be available.22

**REFERENCES**

ON- AND OFF-LINE DATA REDUCTION FOR LIQUID SCINTILLATION COUNTING

R. L. LITTLE

In Chapters 35 and 36, Spratt, and Grower and Bransome, respectively, have discussed batch processing of liquid scintillation counting data by a large digital computer and the use of small calculator-computers. Considerations of data reduction for liquid scintillation counting and for all analytical chemistry have also been strongly influenced by two additional developments of the last few years: (1) the advent of the so-called minicomputer, typically a low-priced computer with 2–4 K (K = 1024) 12- or 16-bit words of memory and an assortment of peripheral devices that permit easy interfacing with analytical instrumentation, and (2) the rise of the commercial time-sharing services. The subscriber to such services has access to a large and powerful computing system that may be many miles away. He communicates with it over ordinary telephone lines by means of a teletype or typewriter terminal. The result of these two developments has been to enhance the investigator’s capabilities for data reduction in two areas: on-line systems where a small computer can handle acquisition and reduction of data in real time from one or several analytical instruments, and off-line data reduction where easy access to a large computer affords a means for sophisticated and/or lengthy calculations. Two systems for liquid scintillation data reduction recently developed in our laboratories provide good illustrations of the techniques involved.

OFF-LINE SYSTEM (LSDRS)

In developing an off-line system, we decided to treat the various types of calculations (calibration, dpm, single- or dual-label counting error) as options to be selected from one general program rather than maintain a number of distinct programs. The selection of these options and the input of specifications was to be done by question-and-answer conversation under program control. This method of operation greatly reduces the amount of knowledge and understanding of the time-sharing system required of the user. In practice, the user creates

---

a data file from the paper tape produced by his scintillation counter and then calls and executes the program LSDRS (Liquid Scintillation Data Reduction System). It was decided that the output generated by the system should be, insofar as possible, in a final form with 11-inch numbered pages, identification and column headings. Operation of the system is illustrated in Figs. 1–10 which reproduce actual output generated during the execution of LSDRS.

Calibration Mode

Figure 1 shows the "conversation" for carrying out a calibration. Items 1 and 2 (name, date and title) allow the user to enter identification information. This and all other input is written into a specifications file available to all subprograms of the system. The "Yes" answer to Item 3, "Is this a calibration?", causes the program to enter the calibration mode and determines the subsequent questions. Item 4, "Is this single label?" is self-explanatory. Item 5, "Select Channel . . . ," determines the instrument channel to which the next group of inputs apply. Standards' data, as input to Items 1–5, are entered next. From the standardization date and half-life (which may be in years, days or hours), the calibration parameters are calculated on a time-independent basis. The standardization date is given to the minute, allowing the program to be used for relatively rapidly decaying isotopes. The sample numbers identify the standards in the data set. These can occur anywhere but must occupy contiguous locations. Since a dual-label calibration has been specified in this example, another channel selection is made and standards' data entered for a second isotope. Note that a "high energy-low energy" distinction between the two isotopes is unnecessary because calibration and calculations are done on a "dual-spill" basis. Input to Item 6, "Background sample numbers," locates the background standards, while Item 7, "Time at which . . . ," establishes reference time zero. A final input requested by "Do you want to change any input?" allows the user to correct any error by entering the appropriate item number. In the example, a "No" answer is given and the program proceeds to the actual data processing. The message "Reading data file" is given as the program begins scanning the raw data file. During this phase the data are identified as to type (cpm or counts) and instrument, and a standard format data file is created. The calculation and output subprogram is then chained and the corresponding message printed.

The output from this calibration run is shown in Fig. 2. Following the identification heading are the coefficients for the least-squares polynomial fit of the several parameters \(X = \text{external standard ratio or channels ratio}\). The degree of the polynomial is chosen by the program and is the lesser of 3 and \(n/2\) where \(n\) is the number of standards. For the background a linear fit was used \((C = D = 0)\) because only two background standards were used. These coefficients are written into the specifications file and are used for all subsequent calculations until a new calibration is made. Also written into the file but not printed is the rms (root mean square) coefficient of variation of the fit for error analysis. The final output is a plot of spillovers (see Chapters 29 to 31) and efficiencies-versus-standard ratio. This allows a quick visual evaluation of the calibration results.

The DPM Mode

In the dpm mode, counting data are processed using the current calibration data to give deviations per minute. Input required to set up a run are shown in Fig. 3. Items 1–3 are shown in Fig. 1. The "No" input to Item 3 causes the program to select the dpm mode and generates output showing the status of the current calibration file. This allows the user to verify the appropriateness of the calibration data to the counting data at hand. Input to Item 4 selects the start of the data block in the data file (which might contain other data
LSDRS

2. EXPERIMENT TITLE? CALIBRATION NO. 3
3. IS THIS A CALIBRATION? YES
4. IS THIS SINGLE LABEL? NO
5. SELECT CHANNEL A OR B OR C? A
   FOR CHANNEL A:
   I. ISOTOPE? TRITIUM
   II. STANDARDIZATION DATE MO, DAY, YR, HR
       E.G., 7, 14, 1968, 0830? 6, 1, 1967, 1200
   III. DPM ON 6/ 1/1967
        ? 98500
   IV. HALF-LIFE (EG. 4500 Y)? 12.26 Y
   V. SAMPLE NO.'S
       FROM? 1
       TO? 8
   SELECT ANOTHER CHANNEL? B
   FOR CHANNEL B:
   I. ISOTOPE? CARBON-14
   II. STANDARDIZATION DATE MO, DAY, YR, HR
       E.G., 7, 14, 1968, 0830? 6, 1, 1967, 1200
   III. DPM ON 6/ 1/1967
        ? 21000
   IV. HALF-LIFE (EG. 4500 Y)? 5500 Y
   V. SAMPLE NO.'S
       FROM? 9
       TO? 15
6. BACKGROUND SAMPLE NO.'S
   FROM? 16
   TO? 17
7. TIME AT WHICH COUNTING BEGAN(MO, DAY, YR, HR)? 1, 15, 1969, 150

DO YOU WANT TO CHANGE ANY INPUT? NO

READING DATA FILE

ENTERING CALIBRATION PROGRAM

Fig. 1.—Conversation for dual-label calibration (LSDRS). In this and subsequent examples, alphanumeric information on each line through "?" was program output. Remainder of line was entered by user and constitutes input.
**LIQUID SCINTILLATION DATA REDUCTION SYSTEM**

**BECKMAN INSTRUMENTS, INC./DATA SYSTEMS GROUP**

CALIBRATION CURVES FOR:

ROBERT L. LITTLE 3/1/1969
CALIBRATION NO. 3

\[ Y = A + B \cdot X + C \cdot X^2 + D \cdot X^3 \]

<table>
<thead>
<tr>
<th>BACKGROUND:</th>
<th>A</th>
<th>B</th>
<th>C</th>
<th>D</th>
</tr>
</thead>
<tbody>
<tr>
<td>CH A</td>
<td>8.441</td>
<td>30.434</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>CH B</td>
<td>26.699</td>
<td>-23.062</td>
<td>0.000</td>
<td>0.000</td>
</tr>
</tbody>
</table>

TRITIUM:

| EFF'Y | 0.115 | -0.469 | 2.957 | -2.071 |
| SPILL  | 0.097 |      |      |       |

CARBON-14:

| EFF'Y | 0.498 | 0.217 | 0.356 | -0.300 |
| SPILL  | 0.097 | 0.516 | 1.290 | 0.957 |

CARBON-14 PER CENT:

<table>
<thead>
<tr>
<th>RATIO</th>
<th>0</th>
<th>10</th>
<th>20</th>
<th>30</th>
<th>40</th>
<th>50</th>
<th>60</th>
<th>70</th>
<th>80</th>
<th>90</th>
<th>100</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>PER CENT</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>+</td>
</tr>
<tr>
<td>+</td>
</tr>
</tbody>
</table>

Fig. 2.—Dual-label calibration: output (LSDRS).
ON- AND OFF-LINE DATA REDUCTION

LSRS

2. EXPERIMENT TITLE? DEMONSTRATION NO.
3. IS THIS A CALIBRATION? NO

***CALIBRATION TABLE STATUS***
ROBERT L. LITTLE 3/15/1969
CALIBRATION NO. 3
DUAL LABEL CALIBRATION
WITH TRITIUM IN CHANNEL A
AND Carbon-14 IN CHANNEL B
4. SAMPLE NO. AT WHICH DATA BEGINS? 2B
5. DO YOU WANT DPM PROJECTED? NO
6. DO YOU WANT DPM SCALED? NO
7. DO YOU WANT ISOTOPE RATIO? NO
8. TIME AT WHICH COUNTING BEGAN DATE, TIME? 3/15/1969, 1500

DO YOU WANT TO CHANGE ANY INPUT? NO

READING DATA FILE

ENTERING CALCULATION PROGRAM

Fig. 3.—The dpm mode (LSDRS).

types, data of other investigators or standards). Input to Items 5, 6 and 7 select the various calculations available in addition to dpm. In this example, none of these is chosen, and the output produced is shown in Fig. 4. Because projection was not desired, dpm were calculated as of counting time. The columnar output gives sample numbers (in this data set duplicate counts were made) and dpm for each isotope. The quantity in square brackets following each dpm value is the coefficient of variation (2 σ) expressed as a per cent of the calculated dpm.

In the remaining examples, the effect of choosing various options is shown. Figure 5 shows the isotope ratio option. In response to the “Yes” input to Item 7, the program determines which of the two possible ratios is desired. Output is shown in Fig. 6. The scaling option is shown in Fig. 7 (Item 6). This allows dpm of each isotope to be divided by an arbitrary constant, thus giving results in microcuries, milligrams and so on. Because isotope ratio was also selected (Item 7), the output gives the ratio of the scaled values (Fig. 8). The dpm projection in time is shown in Figs. 9 and 10. The radioactivity in dpm may be calculated as of a past or future time. In this case, deviations per minute were projected approximately one tritium half-life into the future. Figure 9 also shows how an input can be changed.

ON-LINE SYSTEM (LS16)

The on-line system was designed to accept the formatted bed output normally transmitted from an LS counter to its teletype, process the data and output results on a remote teletype not necessarily located at the LS counter. (See Fig. 11 for a schematic of the system.) Because data become available so infrequently (periods of about 1 minute in the fastest case), it is possible to share the computer among four counters (LS) and perform some background processing. The system is designed to allow each counter to operate independently of the others. Thus, the particular mode of operation and type of instrument on a given data processor channel can be different.

In practice, the user communicates with the system by means of the teletype. At any time, by striking a key on the teletype, he may do one of the following: display the current operating parameters, change these parameters or remove his data channel from the system. The required parameters are I, instrument type (here the Beckman LS 100 or 200 series); M, mode of operation; and N, single-double label indicator. The modes of operation are: idle mode (M = 0) in which the system ignores the data channel; dpm mode (M = 1) in which deviations per minute are calculated using current calibration tables; and calibrate mode (M = 2) in which the first samples processed are assumed to be standards. (In our laboratory, the sequence is: 2 background, 6 isotope 1, 6 isotope 2.) Calibration tables are set up and dpm mode is entered automatically. Echo mode (M = 3) produces standard LS counter output, useful for setting up and checking out the system. Plot mode (M = 4) produces a bar graph output useful for rapid scanning of samples.
**LIQUID SCINTILLATION DATA REDUCTION SYSTEM**

**BECMAN INSTRUMENTS, INC. / DATA SYSTEMS GROUP**

**EXPERIMENT:**

DEMONSTRATION NO. 1  
ROBERT L. LITLE 3/15/1969

USING CALIBRATION DATA OF:

CALIBRATION NO. 3  
ROBERT L. LITLE 3/1/1969

**DPM AS OF 1/15/1969**

<table>
<thead>
<tr>
<th>DPM TRITIUM</th>
<th>DPM CARBON-14</th>
</tr>
</thead>
<tbody>
<tr>
<td>20 90049.9 [2.1]</td>
<td>20515.2 [2.3]</td>
</tr>
<tr>
<td>20 89838.3 [2.1]</td>
<td>20436.6 [2.3]</td>
</tr>
<tr>
<td>21 89289.5 [2.3]</td>
<td>20916.0 [2.2]</td>
</tr>
<tr>
<td>21 89412.9 [2.3]</td>
<td>20896.3 [2.2]</td>
</tr>
<tr>
<td>22 87950.6 [2.7]</td>
<td>20562.1 [2.2]</td>
</tr>
<tr>
<td>22 87711.1 [2.7]</td>
<td>20538.7 [2.2]</td>
</tr>
<tr>
<td>23 88933.1 [2.1]</td>
<td>20699.0 [2.3]</td>
</tr>
<tr>
<td>23 89154.1 [2.1]</td>
<td>20631.4 [2.3]</td>
</tr>
<tr>
<td>24 89836.1 [2.1]</td>
<td>20768.6 [2.3]</td>
</tr>
<tr>
<td>24 89205.2 [2.1]</td>
<td>20442.1 [2.3]</td>
</tr>
<tr>
<td>25 88257.7 [2.1]</td>
<td>20616.4 [2.3]</td>
</tr>
<tr>
<td>25 88046.1 [2.1]</td>
<td>20640.9 [2.3]</td>
</tr>
<tr>
<td>26 82066.9 [2.2]</td>
<td>17194.7 [2.2]</td>
</tr>
<tr>
<td>26 81454.3 [2.3]</td>
<td>17035.1 [2.5]</td>
</tr>
<tr>
<td>27 74539.8 [2.5]</td>
<td>19684.0 [2.3]</td>
</tr>
<tr>
<td>27 73359.4 [2.5]</td>
<td>19478.8 [2.3]</td>
</tr>
<tr>
<td>28 84707.2 [2.3]</td>
<td>20477.4 [2.5]</td>
</tr>
<tr>
<td>28 84274.6 [2.3]</td>
<td>20794.2 [2.5]</td>
</tr>
<tr>
<td>29 89617.7 [2.3]</td>
<td>20656.6 [2.5]</td>
</tr>
</tbody>
</table>

**Fig. 4.**—The dpm mode: output (LSDRS).
CONCLUSION

I would like to reiterate that a number of very useful data reduction systems (particularly off-line systems) have been devised for LS data reduction. The two discussed here are presented as typical of recent advances in current practice. I have intentionally omitted discussion of the oldest and probably most widely used off-line systems, those using batch processing and those utilizing the so-called programmable calculators. These have been discussed in Chapters 35 and 36.

\[\text{Note: Figures 6-13 follow.}\]
## EXPERIMENT:

**DEMONSTRATION NO. 2 (ISOTOPE RATIO)**

**ROBERT L. LITTLE**

**USING CALIBRATION DATA OF:**

**CALIBRATION NO. 3**

**ROBERT L. LITTLE 3/1/1969**

### DPM AS OF 1/15/1969

<table>
<thead>
<tr>
<th>DPM TRITIUM</th>
<th>DPM CARBON-14</th>
<th>RATIO CARBON-14/TRITIUM</th>
</tr>
</thead>
<tbody>
<tr>
<td>29 0.90499  [ 2.1]</td>
<td>20515.2 [ 2.3]</td>
<td>.2278E+00 [ 3.1]</td>
</tr>
<tr>
<td>20 0.89338  [ 2.1]</td>
<td>20436.6 [ 2.3]</td>
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</tr>
<tr>
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<td>20916.0 [ 2.2]</td>
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<tr>
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<td>.2337E+00 [ 3.2]</td>
</tr>
<tr>
<td>22 0.87950  [ 2.7]</td>
<td>20562.1 [ 2.2]</td>
<td>.2338E+00 [ 3.5]</td>
</tr>
<tr>
<td>22 0.87711  [ 2.7]</td>
<td>20538.7 [ 2.2]</td>
<td>.2342E+00 [ 3.5]</td>
</tr>
<tr>
<td>23 0.88933  [ 2.1]</td>
<td>20699.0 [ 2.3]</td>
<td>.2327E+00 [ 3.1]</td>
</tr>
<tr>
<td>23 0.89154  [ 2.1]</td>
<td>20631.4 [ 2.3]</td>
<td>.2314E+00 [ 3.1]</td>
</tr>
<tr>
<td>24 0.89836  [ 2.1]</td>
<td>20768.6 [ 2.3]</td>
<td>.2312E+00 [ 3.1]</td>
</tr>
<tr>
<td>24 0.89205  [ 2.1]</td>
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<td>25 0.88257  [ 2.1]</td>
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</tr>
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<td>25 0.88046  [ 2.1]</td>
<td>20640.9 [ 2.3]</td>
<td>.2344E+00 [ 3.1]</td>
</tr>
<tr>
<td>26 0.82066  [ 2.2]</td>
<td>17194.7 [ 2.2]</td>
<td>.2095E+00 [ 3.1]</td>
</tr>
<tr>
<td>26 0.81454  [ 2.3]</td>
<td>17035.1 [ 2.5]</td>
<td>.2091E+00 [ 3.4]</td>
</tr>
<tr>
<td>27 0.74539  [ 2.5]</td>
<td>19684.0 [ 2.3]</td>
<td>.2641E+00 [ 3.4]</td>
</tr>
<tr>
<td>27 0.73359  [ 2.5]</td>
<td>19478.8 [ 2.3]</td>
<td>.2655E+00 [ 3.4]</td>
</tr>
<tr>
<td>28 0.84707  [ 2.3]</td>
<td>20477.4 [ 2.5]</td>
<td>.2417E+00 [ 3.4]</td>
</tr>
<tr>
<td>28 0.84274  [ 2.3]</td>
<td>20794.2 [ 2.5]</td>
<td>.2467E+00 [ 3.4]</td>
</tr>
<tr>
<td>29 0.89617  [ 2.3]</td>
<td>20656.6 [ 2.5]</td>
<td>.2305E+00 [ 3.4]</td>
</tr>
</tbody>
</table>

Fig. 6.—Isotope ratio: output (LSDRS).
1. NAME AND DATE? ROBERT L. LITTLE  
2. EXPERIMENT TITLE? DEMONSTRATION NO. 3 (SCALING)  
3. IS THIS A CALIBRATION? NO  

***CALIBRATION TABLE STATUS***  
ROBERT L. LITTLE, 3/1/1969  
CALIBRATION NO. 3  
DUAL LABEL CALIBRATION  
WITH TRITIUM IN CHANNEL A  
AND CARBON-14 IN CHANNEL B  

4. SAMPLE NO., AT WHICH DATA BEGINS? 20  
5. DO YOU WANT BPM PROJECTED? NO  
6. DO YOU WANT BPM SCALING? YES FOR TRITIUM  
   BY WHAT VALUE? 9000  
   FOR CARBON-14  
   BY WHAT VALUE? 20500  
7. DO YOU WANT ISOTOPE RATIO? YES  
   IS THE RATIO (TRITIUM / (CARBON-14)) ACCEPTABLE? YES  

DO YOU WANT TO CHANGE ANY INPUT? NO  

READING DATA FILE  
ENTERING CALCULATION PROGRAM  

Fig. 7.—Scaling (Item 6) (LSDRS).
EXPERIMENT:
DEMONSTRATION NO. 3 (SCALING)
ROBERT L. LITLE

USING CALIBRATION DATA OF:
CALIBRATION NO. 3
ROBERT L. LITLE 3/1/1969

DPM AS OF 1/15/1969

| DPM TRITIUM DIV BY 9000.00 | DPM CARBON-14 DIV BY 20500.00 | SCALED RATIO TRITIUM/CARBON-
|---------------------------|-------------------------------|---------------------------
| 20 .1001E+02 [ 2.1]       | .1001E+01 [ 2.3]              | .9998E+01 [ 3.1]         |
| 20 .9982E+01 [ 2.1]       | .9969E+00 [ 2.3]              | .1001E+02 [ 3.1]         |
| 21 .9921E+01 [ 2.3]       | .1020E+01 [ 2.2]              | .9724E+01 [ 3.2]         |
| 21 .9935E+01 [ 2.3]       | .1019E+01 [ 2.2]              | .9746E+01 [ 3.2]         |
| 22 .9772E+01 [ 2.7]       | .1003E+01 [ 2.2]              | .9743E+01 [ 3.5]         |
| 22 .9746E+01 [ 2.7]       | .1002E+01 [ 2.2]              | .9727E+01 [ 3.5]         |
| 23 .9881E+01 [ 2.1]       | .1010E+01 [ 2.3]              | .9786E+01 [ 3.1]         |
| 23 .9906E+01 [ 2.1]       | .1006E+01 [ 2.3]              | .9843E+01 [ 3.1]         |
| 24 .9982E+01 [ 2.1]       | .1013E+01 [ 2.3]              | .9853E+01 [ 3.1]         |
| 24 .9912E+01 [ 2.1]       | .9972E+00 [ 2.3]              | .9940E+01 [ 3.1]         |
| 25 .9806E+01 [ 2.1]       | .1006E+01 [ 2.3]              | .9751E+01 [ 3.1]         |
| 25 .9783E+01 [ 2.1]       | .1007E+01 [ 2.3]              | .9716E+01 [ 3.1]         |
| 26 .9119E+01 [ 2.2]       | .8388E+00 [ 2.2]              | .1087E+02 [ 3.1]         |
| 26 .9050E+01 [ 2.3]       | .8310E+00 [ 2.5]              | .1089E+02 [ 3.4]         |
| 27 .8282E+01 [ 2.5]       | .9602E+00 [ 2.3]              | .8628E+01 [ 3.4]         |
| 27 .8151E+01 [ 2.5]       | .9502E+00 [ 2.3]              | .8578E+01 [ 3.4]         |
| 28 .9364E+01 [ 2.3]       | .1014E+01 [ 2.5]              | .9231E+01 [ 3.4]         |
| 29 .9958E+01 [ 2.3]       | .1008E+01 [ 2.5]              | .9882E+01 [ 3.4]         |

Fig. 8.—Scaling: output (LSDRS).
ON- AND OFF-LINE DATA REDUCTION

NAME AND DATE: ROBERT L. LITTLE
EXPERIMENT TITLE: DEMONSTRATION NO. 2 (ISOTOPE RATIO)
IS THIS A CALIBRATION? NO
CALIBRATION TABLE STATUS
DUAL LEVEL CALIBRATION WITH TRITIUM IN CHANNEL A
AND CARBON-14 IN CHANNEL B
SAMPLE NO. AT WHICH DATA BEGINS? 20
DO YOU WANT DPM SCALED? NO
DO YOU WANT ISOTOPE RATIO? YES
IS THE RATIO TRITIUM/(CARBON-14) ACCEPTABLE? NO
TIME AT WHICH COUNTING BEGAN (DAY/MONTH/yr)? 3/15/1969
DO YOU WANT TO CHANGE ANY INPUT? NO

ENTERING CALCULATION PROGRAM

Fig. 9.—Projection (Item 5). Note change of input for Item 7 (LSDRS).

Fig. 10.—Projection: output. Example of second-page output (LSDRS).

Fig. 11.—System LS/16 schematic. TTO is optional teletype at computer for background processing.

Fig. 12.—LS/16 operation. Mode was changed from echo to plot between sample numbers 54 and 55 and changed back following sample number 65.
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