EFFECTS OF FIXATION ON PROTEIN HISTOCHEMISTRY

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This work was undertaken to investigate some aspects of the effects of fixation of tissues on the histochemical demonstration of groups in protein or of specific proteins. During this study no critical inquiry was made concerning the formulation or the specificity of the histochemical methods used nor concerning the formulation of the reactions between the fixatives and the proteins in tissues. However, we concentrated on histochemical procedures with which we were most familiar on the basis of experience or of methodological development. We used in our initial studies the broad uncritical approach of simply testing some of the standard histochemical methods for groups in protein on a variety of tissues fixed in several ways, and some of these results will be reported. It became immediately apparent that no generalized conclusions could be reached by this sort of superficial work because of the number of variables implicit in the subject matter. Among the influencing factors we had to consider were the numerous types of fixative, the duration of fixation, the concentration of fixing ingredients, effect of temperature, the degree of penetration of fixative, the possible reactions between the fixative and the proteins in the tissues, the type of tissue used and size of block, the method of preparation of tissue sections, the thickness of the sections, the histochemical method used and the degree of technical experience with the method. Nevertheless we became particularly interested in three aspects of the study because the results seemed quite clear and could be better controlled. These were investigated in greater detail. The first involved the effect of several fixatives on the sulfhydryl groups of protein. The second was concerned with the effects of osmium tetroxide fixation on groups in protein. The third consisted of investigation of fixatives as histochemical reagents, taking advantage of differential solubility properties of protein to demonstrate certain specific proteins.

The following tissues of adult female albino rats were used in these experiments: parts of the central nervous, gastrointestinal, urinary, reproductive and endocrine systems as well as skin and a block section through the neck that included skeletal muscle, large blood vessels and nerves, thyroid gland, trachea and esophagus. These tissues were fixed overnight (14–18 hours) in the following fixatives: various concentrations of acetone, ethanol or trichloracetic acid, 10% formalin, 1% osmium tetroxide buffered to pH 7.6 with veronal acetate, 80% ethanol containing 1% trichloracetic acid, Bouin’s solution, Carnoy’s solution, Zenker-acetic, Zenker-formol, sublimate-formol and a modified Susa fluid containing acetic acid and formalin in physiological saline saturated with both mercuric chloride and picric acid. Some of the tissues were fixed in buffered osmium tetroxide for only 1 hour.

These tissues were dehydrated in ethanol in the routine manner appropriate to each fixative, cleared in chloroform, embedded in paraffin and sectioned at 7μ. These sections were stained with the following methods for groups in protein: sulfhydryl groups alone (2, 5, 26, 30), both sulfhydryl and disulfide groups (3), alpha-acylamido carboxyl groups (4), amino groups (32, 35) and acidophilia at various pH’s (28). Additionally, fewer experiments utilizing reagents that demonstrate a number of groups in protein such as phenolic hydroxyls, amines and certain heterocyclics were also used. These included the coupled tetrazonium method (11), the dinitrofluorobenzene method (9, 12), and the Millon reaction (27). These latter methods have been reviewed recently (9, 21); and from the practical standpoint several of them...
produced weak and inconsistent reactions, thereby limiting their usefulness for this study.

Finally, a method was developed for the histochemical demonstration of luteotrophic hormone (LTH) based upon the solubility characteristic of the anterior pituitary hormones by finding a fixative which dissolved all anterior pituitary hormones completely except LTH.

The effect of fixatives on the demonstration of sulfhydryl groups by various methods was studied in detail, with several surprising results. None of the common, nonmetal-containing or oxidizing fixatives appeared to inhibit the demonstration of sulfhydryl groups. Formalin, for example, reacts with the thiol groups of protein to form thioazolidine carboxylic acids (13, 23), but these products are unstable and are easily and rapidly reversible to the free sulfhydryls in neutral aqueous solutions at room temperature (fig. 6). Theoretically, acid-containing fixatives should be preferable since the acid pH prevents the oxidation of sulfhydryl groups by molecular oxygen, a reaction that is catalyzed by traces of heavy metals (8). However, we could not find any difference in the distribution or intensity of staining of tissues fixed in neutral, dilute ethanol (80%) alone and those fixed in dilute ethanol in the acid range (pH 4.5 with either acetic, tri-chloroacetic or hydrochloric acid). As would be expected, the intensity of the reaction for sulfhydryls varied directly with the amount of protein retained, provided the sulfhydryl groups were not blocked by the fixative (figs. 5, 6). Surprisingly, fixatives which contained heavy metals (sublimate-formol) or oxidizing agents (Zenker-acetic), which retain more protein, did not completely inhibit the staining of sulfhydryl groups for some of the methods used (fig. 7). Fixation of tissues overnight in a saturated solution of mercuric chloride reduced the intensity of the reaction for sulfhydryl groups with 2,2'-dihydroxy-6,6-dinaphthyl disulfide (2), X-(4-hydroxy-1-naphthyl)-maleimide (26, 30) as reagents, especially the former; but it completely blocked the staining reaction produced with a naphthol-containing mercaptide-forming agent (5). This raises the possibility that some powerful reagents such as maleimides or the isomaleimides are capable of replacing the heavy metal in a mercaptide. Similarly, the blockade of sulfhydryl groups by mercaptide-forming agents should be looked upon as an easily reversible blockade and should not alone be held as a rigid criterion of specificity. Reducing groups or weak alkali alone will reverse this blockade of the heavy metal, and this forms an important property of mercaptides (8). In addition, this evidence suggests a histochemical parallel for that frequently noted in the biochemical literature: that methods for quantitatively measuring sulfhydryl groups give different results depending on the type of reaction employed, mercaptide-forming, alkylating or oxidizing reactions. Differences also occur as the result of the effect of different denaturing agents on proteins. This has resulted in protein-bound sulfhydryls being divided into "free, sluggish and masked" varieties (8); and the results presented here may indeed provide a histochemical counterpart.

Similarly a weakly positive reaction for sulfhydryl groups occurred after Zenker-acetic fixation with some reagents, indicating that these groups in protein were not completely blocked by the mercuric salts in the fixative or were not oxidized by the acidic solution of chromate (fig. 7). The free sulfhydryls could probably be accounted for on the basis of weak oxidative activity plus steric factors in the arrangement of the sulfhydryl groups. If the sulfhydryl groups are separated from each other so far as to make impossible the disulfide bridge, oxidation will not take place.

Methods for both sulfhydryls and disulfide groups of protein were successful after a greater variety of fixatives. No complication was introduced by mercaptide formation with mercuric chloride because the mercaptides formed were readily dissociated by the reducing agents and by the high hydroxyl ion concentration that were necessary for reducing disulfide groups. The possible oxidative activity of chromates likewise was without final effect since the disulfides produced would be reduced in the method along with the naturally occurring disulfides. Therefore, with almost all the fixatives, the intensity of the combined sulfhydryl and disulfide reaction appeared proportional to the ability of the fixative to retain protein. With a single reagent as fixative, more reactive protein was retained by increasing the concentration of the fixative. This was especially true of those tissues whose cells contained a secretory protein product. However, it should be pointed out that some proteins are clearly soluble in some concentrated fixatives, or become
Figures 1-3. Rat cerebellum stained for alpha-acylamido carboxyl groups after three different fixatives. Figure 1 is taken from 3', trichloracetic acid fixed material; Figure 2, 10'; formalin; and Figure 3, sublimate-formol. Since none of these fixatives block the carboxyl groups, the differences in intensity of the reaction depicted probably represent the differences in the ability of the fixatives to retain protein. X 300

Figure 4. Rat skin fixed in osmium tetroxide for 1 hour and stained with methylene blue at pH 3. Distal parts of the hair shafts, epidermis (top) and skeletal muscle (bottom) are intensely basophilic indicating that the sulfhydryl and disulfide groups are oxidized during fixation to cysteic or sulfonic acids. X 75

Figures 5-7. Rat kidney stained for sulfhydryl groups after three different fixatives. Figures 5 and 6 were taken from 80', ethanol and 10'; formalin fixed material, respectively, and demonstrate the difference between the two fixatives in retaining protein that contains sulfhydryl groups. Figure 7 is from a kidney fixed in Zenker-acetic and demonstrates that free sulfhydryls are available for staining despite the chromate and mercuric ions in the fixative. Mercuric crystals are not removed in an effort to equalize the treatment of sections. X 300
All photomicrographs are sections of blocks of rat pancreas fixed in osmium tetroxide for one hour.

Figures 8 and 9. Figure 8 represents the reaction for both sulfhydryl and disulfide groups, and Figure 9 for alpha-acylamido carboxyl groups. Both figures indicate the three types of results obtained. In the outer zone (bottom) the tissue is overfixed, reacts negatively for groups in protein, but is stained with osmium. An intermediate zone (middle) is fairly well fixed and shows a mixed osmiophilia and a weak reaction for the above groups. The inner zone (top) is poorly fixed, as though by alcohol, and contains the most intense reaction for the groups in protein, × 100.

Figures 10, 11, 12. Enlargement of three areas routinely seen in the osmium-fixed sections representing different degrees of fixation, cytological preservation of structure, and reaction for alpha-acylamido carboxyl groups. The most intense histochemical reaction occurs in the poorest fixed portion (Figure 12) whereas a negative reaction occurs in the well-fixed or slightly overfixed portion (Figure 10). Figure 12 shows the transition between these two zones, × 300.
PLATE III
Figs. 13–18
soluble during dehydration, rehydration or staining in aqueous solutions.

That osmium tetroxide will blacken organic substances was first shown in 1804 by Tennant (29), and this fixative was introduced into histology nearly a century ago (1). Since then, it has been reiterated that osmium tetroxide, of all histological fixatives, causes the least disruption of cell structure (16, 22) and at present finds its greatest use as a fixative for electron microscopy (20). In the present experiments the most clear-cut result was that none of the tissue fixed in osmium tetroxide overnight showed a positive reaction for any of the groups in protein. While bleaching with dilute peroxides removes the dark color of reduced osmium compounds, it does not restore any of the reactions. Two methods gave positive staining results, but these were not due to groups in protein. In the case of the alloxan or ninhydrin-Schiff reaction (35) (fig. 18), it was shown that staining was due to the reduction of carboxyls by the oxidizing activity of the fixative, since tissues fixed in osmium tetroxide overnight reacted similarly with leukofuchsin without previously being reacted with either alloxan or ninhydrin. Furthermore, the areas of staining, i.e., cartilage matrix, mast cell granules, connective tissue ground substance etc., are sites known to contain either glycoproteins or mucopolysaccharides which are Schiff positive after other means of oxidation (periodic or performic acids). These positive sites were negative with the method for amino groups utilizing 2-hydroxy-3-naphthaldehyde as reagent (32) (fig. 17). Additionally, when tissues were fixed in osmium tetroxide for one hour, these Schiff positive areas were not so marked. The oxidation of 1–2 glycols by osmium tetroxide was first shown by Creige in 1936 (10) and more recently by Wolman (34). Moreover, the carboxyls, either native or produced by the oxidation with osmium tetroxide, are stable (1, 33). The other positive reaction occurred with the tetrazolium method (3). However, the sites of reaction were the same as those that were Schiff positive. This finding reemphasizes the fact that tetrazolium salts are not specific for sulfhydryls and reduced disulfides (3), but will react with aldehydes, ketols and other reducing groups.

The result of brief fixation in osmium tetroxide produced a mixed effect that obviously was dependent on the depth of penetration of the fixative and the tissue examined. Although small blocks of tissue were used (approximately 0.5 cm in at least one diameter), the periphery of the blocks was either unreactive, or very weakly reactive, for any of the methods for groups in protein (figs. 8, 9). A subjacent intermediate zone was variably but usually more reactive than the periphery, and the central zone usually was strongly reactive. For example in the pancreas (Figs. 10–12) the outer portion of the block was overfixed and negative for carboxyl as well as other groups in protein. An intermediate zone was well fixed and was weakly reactive, and an inner zone was poorly fixed and strongly reactive. This inner zone was similar in cytological appearance and reaction intensity to tissues which had been fixed in dilute ethanol. In other tissues like the skin, the reactions were moderate throughout; and, in the mucosa of the intestine, the reactions were negative throughout. How-

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**PLATE III**

**Figs. 13–18**

Figures 13–16 represent the results of experiments on the demonstration of LTH in the anterior pituitary glands of female adult rats. Figure 13 is from a control pituitary fixed in sublimate-formol. Only the acidophiles react intensely for alpha-acylamido carboxyl groups. Figure 14 is from a pituitary gland extracted in 0.5% TCA and fixed in sublimate-formol. The extraction which removed all the hormones except LTH caused a marked decrease in the number of acidophiles that stain. Figure 15 is from a pituitary gland of a lactating rat, three days postpartum, fixed in sublimate-formol. There are no acidophiles, which are larger and more reactive for carboxyl groups than in the non-pregnant controls. Figure 16 is from a pituitary gland of a lactating rat, three days postpartum, extracted with 0.5% TCA before fixation in sublimate-formol. Although all hormones other than LTH have been extracted, the similarity between Figures 15 and 16 suggests that all or almost all the acidophiles contain LTH in this animal. X 300

Figures 17 and 18, Rat tracheal cartilage fixed in osmium tetroxide. Figure 17 is from a section that was stained for amino groups with naphthaldehyde as reagent. Although the chondrocytes are osmophilic, no reaction has occurred in the ground substance. Figure 18 is from a section stained for amino groups with the ninhydrin-Schiff method. The false positive reaction in the ground substance is due to the production of aldehydes by the oxidation of glycol groups in mucopolysaccharides by osmium tetroxide. X 300
ever, it is clear that, if the duration of fixation is
that commonly used in electron microscopy, os-
mium tetroxide will react with most, but not all, of
these groups in protein in the best fixed portion of
the block.

In addition it was established that during
fixation with osmium tetroxide sulfhydryl and
disulfide groups are oxidized to cysteic or sulfonic
acids on the following basis: ordinarily, the distal
portions of the hair shaft, rich in disulfides, and
the region of horny transformation, rich in
sulfhydryl groups, are not basophilic. After
fixation in osmium tetroxide for an hour or more,
those regions were strongly basophilic, even
when the pH of the staining mixture was reduced
to low acid ranges (Fig. 4). This rapid oxidation
of reducing compounds containing sulfur was
first demonstrated chemically before the turn of
the century and has recently been extended to
common biological materials containing sulfhy-
dryls (1).

It has been reported that osmium tetroxide
does not block carboxyl groups of proteins (33).
This might account for some of the staining with
basic dyes after this fixation (acidophilia being
completely abolished); the remainder being due
to the production of sulfonic acids described
above and to nucleic acids which are unaffected
by this fixation (1). However, the specific method
for alpha-acylamido-carboxyl groups (4) was
completely negative after osmium tetroxide
fixation. If the carboxyl groups remain intact
after this fixation, destruction of the acylamido
group alpha to the carboxyl groups would result
in the observed negative reaction. The alteration
of the alpha acylamido group could pre-
sumably occur by destruction of the properties
of the electrophilic nitrogen necessary for the
histochemical reaction since it has been shown
that osmium tetroxide has a special affinity for
nitrogenous groups that does not depend on
oxidative capacity (17).

In summary it appears that, given sufficient
time of fixation, osmium tetroxide will react and
block at least the sulfhydryl, disulfide, phenolic,
hydroxy, carboxyl, amino, and certain hetero-
cyclic groups in protein. The present observa-
tions may provide a histochemical counterpart
to many of the classical histological observations
on the penetration and fixation with osmic acid,
and could account for the well-known finding
that osmium-fixed tissues stain poorly with
routine histological methods, especially with
acid dyes. There are few free, native, reactive
groups for dye binding.

It is possible to employ fixatives as histo-
chemical reagents to aid in the demonstration of
specific proteins. These methods entail the use
of the physical characteristics of proteins such as
differential solubilities. Previously, histochemical
methods for demonstrating the glycoprotein
hormones (follicle stimulating, luteinizing and
thyrotropin) of the anterior pituitary gland based
on the combined use of differential protein solu-
bility, histochemical staining and bioassay were
developed and their usefulness shown by various
applications (7). In essence, the methods de-
pened on in vitro testing of the precipitation of
the six purified anterior pituitary hormones. A
reagent was found that would precipitate all the
hormones, biologically intact, so that they
could be bioassayed and their presence proven quanti-
tatively; and another reagent was found which
would precipitate the hormones irreversibly so
that they could be stained by histochemical
methods. Application of these findings to intact
pituitaries of rats characterized the normal gland
and equated the bioassay and histochemical
results. Further in vitro testing was instituted,
and a reagent was found that would precipitate
five and dissolve one hormone. When this reagent
was applied to normal glands and then refixed
for bioassay or for histochemical procedures, the
bioassayed glands showed five hormones com-
pletely present and one completely absent; and
comparison of histochemically stained sections of
extracted and unextracted glands revealed the
sites of the extracted hormone by the absence of
staining. Similarly, another solvent was found
that would dissolve two hormones and precipi-
tate four and so on, allowing the demonstration
of the sites of glycoprotein hormones.

Similar methods were developed for studying the
simple protein hormones of the anterior pituitary,
adrenocorticotropin, somatotropin, and luteto-
pin (6).

The simplest of these studies is one that con-
cerns luteotropin (LTH). A histochemical
method was found that would demonstrate LTH
in that a fixative was found that precipitated it
and dissolved all the other anterior pituitary
hormones. This fixative was 0.5% trichloracetic
acid (TCA). It was first found by titrating solu-
tions of simple protein hormones with TCA that
only LTH precipitated immediately on addition of trace amounts whereas, the other hormones required much larger amounts for precipitation. When normal, female rat pituitary glands were fixed in 0.5% TCA and refixed in absolute acetone, it was found by bioassay that LTH was retained completely (no difference in LTH content in comparison with acetone-fixed or fresh pituitary glands) whereas, all the other hormones were absent as revealed by bioassay. When such pituitaries were refixed in a fixative that precipitated irreversibly the hormones for histochemical procedures, it was found that some of the acidophiles, which are positive for disulfide or carboxyl groups, were reactive (fig. 14). It should be noted here that in the normal unextracted pituitary gland only acidophiles react intensely for disulfide (18), carboxyl (4) (fig. 13) and amino groups (24); basophiles or chromophobes react negligibly or not at all. Although this might appear to be a circular argument of demonstrating a hormone by methods which are restricted to the staining of one cell type, we felt on reasonable grounds since the glycoproteins were already proven to be in basophiles, and it appeared logical to use histochemical methods for demonstrating groups in protein to demonstrate these groups in the simple protein hormones. Physiological experiments appeared to prove our contention since the pituitary glands of pregnant and lactating rats fixed in 0.5% TCA showed an increase in the size, number and intensity of staining of some of the acidophiles, the peak being reached at 2–3 days postpartum (fig. 16). In fact almost all the acidophiles that are present in the gland at the later stage gave evidence of containing LTH (figs. 15, 16). These results correlate with the LTH content of the gland as determined by bioassay and indicate that the acidophile is the site of LTH elaboration in the rat.

With regard to the effects of different fixatives on tissues and their subsequent staining with the majority of histochemical methods used, few safe generalizations can be made as yet. However, the most striking overall result was the finding that, with the exception of osmium tetroxide, no fixative used completely inhibited any of the reactions.

Although it has been shown that formaldehyde will react with amino, amido, peptide, imino, guanidyl, indole, hydroxyl, and carboxyl groups of protein (13, 15, 31), tissues fixed by this means always showed a positive reaction for groups in protein. In the case of the demonstration of protein-bound amino groups with naphthaldehyde as the reagent (32), although some reaction took place it was markedly reduced in intensity. Fixatives containing other reagents in addition to formalin (Zenker-formol) gave approximately the same results as 10% formalin alone, but fixative in Zenker-acetic gave a very strong reaction; thus indicating that the presence of formalin as such as the significant factor in the degree of reaction for amino groups. It is probable that the amino groups available for histochemical staining after formalin fixation were produced during the processes of dehydration of tissue blocks since many of the combinations of formalin with tissue protein are reversible by the simple process of washing. The alloxan or ninhydrin-Schiff (35) and dinitrofluorobenzene (11) methods which at least reveal amino groups were less seriously affected by formalin fixation, but the latter method was partially inhibited by fixatives containing dichromate or mercuric salts.

Although fixatives containing chromates react with carboxyl groups (15), a moderately positive reaction was obtained with the method (4) for these groups. This may be explained by the assumption that simple salt formation between the metal radical and carboxyl groups would not interfere, since the method depends upon production of a ketone on an acylamido group alpha to such carboxyl groups by treatment of sections with acetic anhydride in pyridine. However, if the primary reaction between chromium complexes and carboxyl groups is followed by some coordination with amino and hydroxyl groups (14), one might expect a diminution in the reaction. This, in fact, was noted, since Zenker-formol and Zenker-acetic fixed tissues reacted with less intensity than 89% alcohol fixed ones despite the former reagents having been shown by other methods to precipitate more proteins irreversibly than dilute ethanol. No reduction in the intensity of the carboxyl reaction was noted with sublimate-formol (figs. 1–3) in spite of the reactivity of mercuric ions with carboxyl groups (21). However, this metallic ion differs from chromium in not forming complexes capable of binding together adjacent protein chains. Methods which demonstrated at least phenolic hydroxyl groups
were most seriously inhibited by fixatives containing chromate or mercuric salts. This would be expected from the known reactions of these compounds with proteins (14, 15, 21, 33).

We feel as a result of this work that, for each of the protein methods to be studied or, for that matter, for the study of reactive groups in a particular protein or tissue, knowledge of the effect of fixation is required. It does not suffice merely to accept the use of a rather unreactive fixative because all fixatives at least result in chemical or physico-chemical changes, attended by changes in the structure of the protein and the number of available groups. Some of the unreactive fixatives, such as acetone or alcohol, fail to precipitate many proteins, especially in dilute (less than 90%) concentrations.

In choosing a fixative, it frequently becomes a matter of selecting the one that precipitates a specific protein, or proteins in general, irreversibly and yet does not block the reactive groups that will label the protein. For a specific protein, this can be best assayed by in vitro testing of model systems with the purified protein. However, frequently the selection of the fixative will be at best a compromise between denaturation and precipitation of proteins, availability of reactive groups and the cytological or morphological effects. As a result, it can be concluded that, although there are optimal fixatives for the demonstration of groups in protein, it can hardly be accepted that there is one ideal fixative for the histochemical study of proteins or the groups in protein. Much obviously depends on the protein, the tissue, the chemical groups which serve for the visualization of the protein and the histochemical methods employed. The claims made for alcohol (25), inert fixatives (14), or even "freeze-drying" (12) will be countermanded by the specific requirements of the individual investigation.

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