The Action of Fixatives on the Ultra-violet-absorbing Components of Chick Fibroblasts

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With three plates (figs. 1, 2, and 15)

SUMMARY

1. This paper describes an investigation by ultra-violet spectrometry of the physical and chemical changes caused by treating interphase chick heart fibroblasts, grown in tissue culture, with a variety of fixatives. The same cells, before and after fixation, were photographed either at 265 and 313 mμ or at a series of wavelengths required for an absorption spectrum.

2. The nucleus of the living interphase cell, apart from nucleoli, is nearly homogeneous, and non-specific light loss measured by the optical extinction at 313 mμ (E_{313}) is not detectable. After fixation the nuclear material is redistributed into filamentous and rounded structures. These nuclear structures are least apparent after osmium tetroxide, methanol freezing-substitution, and freezing-drying; may be more marked after 10 per cent. neutral formalin; and are very marked after Carnoy fixation. E_{313} is measurable in a fixed nucleus and the ratio of optical extinctions E_{313}/E_{265} is always less than about 15 per cent. E_{265} increases as a result of the shrinkage of the nuclear area which ranges from about 8 to 16 per cent. for osmium tetroxide, about 5 to 35 per cent. for neutral formalin, about 19 to 47 per cent. for Carnoy, and about 20 to 40 per cent. for freezing-substitution. Apart from occasional cracks in cells, freezing-substitution and freezing-drying preserved nuclear and cytoplasmic structure as well as osmium tetroxide.

3. (i) Total nuclear extinction is defined as average nuclear extinction times nuclear projected area. Decreases in total nuclear extinction at 265 mμ, after fixation in Carnoy and neutral formalin, range from 10 to 35 per cent. and may be attributed to losses of absorbing material. After formalin fixation there is a decrease of approximately 40 per cent. in the total cytoplasmic extinction at 265 mμ. The method of correcting for non-specific light loss and other possible sources of error are discussed.

(ii) Osmium tetroxide itself absorbs in the ultra-violet and 'stains' the cell, thereby increasing the optical extinction. The staining reaction was studied by fixing films of protein, fat, and nucleic acid.

(iii) After methanol freezing-substitution the total extinction of the nucleus increases slightly.

4. The ratio of optical extinctions E_{265}/E_{280} for the living nucleus is about 2·0 and by applying the methods of curve analysis due to Caspersson it is concluded that the amount of 'standard protein' in the nucleus is small compared with the amount of nucleic acid. After all methods of fixation E_{265}/E_{280} decreases to about 1·3 and similarly it is concluded that the fixed nucleus contains about ten to twenty times as much 'standard protein' as nucleic acid. This curious result is discussed and several possible explanations suggested. It is concluded that the derivation of relative amounts of protein and nucleic acid by analysis of the absorption spectra of either living or fixed cells should be treated with caution.

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Contents

Introduction

ULTRA-VIOLET microspectrometry pioneered by Caspersson (1936, 1950) is used to determine the content of nucleic acids and certain proteins in cells. The aim of such measurements is to relate amounts and concentrations of the absorbing substances in various parts of the *living* cell with the changes occurring during cell metabolism. For many reasons (Caspersson, 1950) such measurements have often been made on fixed cells; this introduces the possibility of errors due to physical and chemical changes caused by fixation.

The purely morphological changes due to fixation have been investigated in tissue cultures where the same cell can be observed in the living and undamaged state and again after fixation. From a study of the action of a variety of fixatives on chick fibroblasts observed with dark ground microscopy (Strangeways and Canti, 1927) and salamander macrophages with phase contrast microscopy (Buchsbaum, 1948), it was concluded that osmium tetroxide, either alone or combined as in Maximow's fluid, produced the least change in the appearance of the cell. The method of freezing-drying was not investigated.

The chemical changes due to fixation may be studied in several ways. One

method is to fix material in bulk and then examine the fixing fluid for new chemical substances. Sylvén (1951) has analysed the chemical fluids (Carnoy, formalin, &c.), used in fixing various organs of the rabbit and has concluded that there is a loss of 10–30 per cent. of the total tissue mass of fresh organs. However, by this method the precise region of the cell from which the losses occur cannot be determined.

Another method, often used in cytochemistry, is to compare, with the same technique (staining, for example), cells fixed in a variety of ways. Thus Nurnberger, Engström, and Lindström (1952) have used ultra-violet and X-ray microabsorption techniques to compare the ventral horn cells of the spinal cord of the adult cat after fixation in Carnoy, formalin, and by freezing-drying, and they discuss the difficulties of interpreting their data in terms of the nucleic acid and protein content of the cell. However, in the absence of measurements on the living cells there is no way of excluding the possibility that each fixative introduces a common artifact. By using tissue cultures this particular difficulty has been avoided and this paper describes an investigation by ultra-violet microspectrometry of physical and chemical changes caused by treating interphase chick fibroblasts with a variety of commonly used fixatives.

Errors in microspectrometry

Errors arise not only in recording the light intensities in the image of the object under the microscope, but also in relating these light intensities to the amount of absorbing material in the object. The numerous discussions of these latter errors have been reviewed by Davies and Walker (1953). Some are caused by a heterogeneous distribution of material in the object as is often brought about by fixing the relatively homogeneous living cell. It is important therefore to enumerate these errors, since changes in distribution may cause alterations in the optical extinction of the object, which might be wrongly interpreted as changes in the amount of absorbing material.

(i) Fixation is often accompanied by shrinkage and hence the concentration of material in the cell increases. If Beer's law does not apply, then the change in concentration will lead to an apparent change in the total amount of absorbing material per cell.

(ii) The optical extinction of the cell in the ultra-violet is due partly to real absorption by specific chemical substances and partly to light lost by scattering, &c., outside the collecting aperture of the objective. This non-specific light loss is usually assessed by the optical extinction at 313 mμ, at which wavelength the nucleoprotein has no real absorption. Caspersson (1942) suggested that the real absorption can be determined by subtracting from the observed optical extinction the optical extinction due to scatter calculated by extrapolation from the value at 313 mμ by using Rayleigh's law. This law holds for scattering particles whose size is about one-tenth of the wavelength of light and less, and states that the turbidity or loss of light due to scattering varies inversely as the fourth power of the wavelength. For several reasons

this law of scattering cannot be satisfactorily applied to cells. In this investigation limits are set to the value of the real absorption at the wavelength 265 mμ by assuming that the light scatter either varies inversely as the fourth power of the wavelength (λ^{-4}) or is independent of the wavelength (λ^{0}). It is likely that the true value lies somewhere in between.

(iii) The optical extinction of a volume in which the material is heterogeneously distributed may be less than if the same quantity of material were distributed with no variation in concentration throughout the same volume. This is commonly called the 'distributional error' and it can be shown (e.g. Glick, Engström, and Malmström, 1951) that the magnitude of the error increases with the optical extinction of the absorbing structures in the cell. An experimental approach to this problem has been adopted in this investigation.

(iv) As a result of glare, that is, light scattering by surfaces in the objective, &c., a fraction of the light energy from each point in the object plane is spread all over the image plane. This causes the measured optical extinction of the object to be lower than it really is and the magnitude of the error increases with the optical extinction. Such an increase in optical extinction usually occurs as a result of shrinkage due to fixation. Hence glare might cause an apparent decrease in the total amount of absorbing material per cell after fixation.

Experiments

Material

Fragments of heart from 11 to 12-day old chick embryos were grown in a fluid medium by a modification of the hanging drop method devised by H. B. Fell for cytological studies. After 24–48 hours' growth, when the explant was surrounded by a single layer of cells, the 0·1 mm. quartz coverslip was mounted on the culture chamber, a stainless steel ring (1 mm. thick and 20 mm. diameter) sealed with a 0·5 mm. quartz slide. (For details see Davies, 1952.)

Apparatus

The ultra-violet microspectrometer was especially designed for work on living cells and full details of its construction and mode of operation have been given elsewhere (Walker and Davies, 1950). Briefly, it had the following features. The cells could be observed continuously with visible phase-contrast illumination, provided by an auxiliary optical train, while growing and dividing on the warm stage of the microscope. Without changing the objective an ultra-violet photomicrograph at one monochromatic wavelength between 248 and 313 mμ could be taken rapidly in about 1 second after accurate focusing in visible phase-contrast. A series of photomicrographs at ten selected ultra-violet wavelengths could be taken in about 90 seconds. The objective was a lithium fluoride, fused-quartz achromat of numerical aperture N.A. 1·24, focal length 2·2 mm., designed by R. J. Bracey of the British Scientific Instruments Research Association; the condenser was a reflecting

system with N.A. 0·6 and 40 per cent. central obstruction (Seeds and Wilkins, 1949). The shadow of a calibrated rhodium step-wedge adjacent to each photomicrograph was used to calibrate the film at each wavelength. At a magnification of × 114 on Kodak Microfile Pan 35 mm. film exposure times were about 1 second at 265 mμ; film was developed in Kodak D.19.b for 4 minutes at 20° C.

Measurements

Two or three cells, with a clear area around them to facilitate accurate densitometry were photographed in each culture at wavelengths 313 and 265 mμ. The culture was then rapidly removed from the microscope, fixed in one of the ways described below, and two further photomicrographs taken of each cell. Measurements were mainly confined to the area occupied by the interphase nucleus (see *Discussion*, I (i) (*a*)). The average optical extinctions at 313 and 265 mμ were computed from six to ten traces taken across each nucleus with a recording microdensitometer constructed by P. M. B. Walker (1953). The aperture in the densitometer corresponded to a circle 0·2–0·4μ diameter in the cell. The error in recording optical extinction in a single trace was ±0·005, owing to uncertainty in the background density of the cell, and the error in the average extinction was correspondingly less. The *total extinctions* of the nuclei (excluding the nucleoli) were determined from the products of average optical extinction and projected nuclear area.

The total amount of material absorbing at 265 mμ in the living nucleus was determined from the expression $E_{265}.A/k$ mg., where E_{265} is the average optical extinction, A the area in cm.^{2}, and k an extinction coefficient equal to the optical extinction of the nuclear material at a concentration of 1 mg./c.c. in a path length of 1 cm. k was chosen as 20, which is the value for a dilute solution of desoxyribose nucleic acid (DNA) and amounts of nuclear material were expressed as 10^{-12} gm. DNA. This procedure has been arbitrarily adopted for convenient comparison with the results of other workers for the DNA content of cell nuclei. However, in these living nuclei absorbing substances other than DNA, and with different extinction coefficients, may be present.

Measurements of total amounts of cytoplasmic material tend to be inaccurate and laborious owing to the presence of refractile structures and the uneven outline. Such measurements were only attempted before and after formalin fixation.

The complete series of photomicrographs required for an absorption spectrum were taken of only one cell per culture before and after fixation. Absorption spectra were constructed from the average optical extinctions along the same line in the images of the cell at the different wavelengths. To increase the accuracy, the average extinction of three traces each separated by approximately 1μ, was used.

Living cells were also observed and photographed in media of different hydrogen ion concentrations. The coverslip bearing the cells was separated

from a quartz slide by two narrow glass strips 0·5 mm. thick. The ends were left open for irrigation with either Tyrode solution or a 1 per cent. solution of sodium chloride to which hydrochloric acid had been added to reduce its pH to 4·6; the solutions were drawn through by filter paper.

In model experiments films of protein, nucleic acid, and fat, were spread evenly on quartz slides cut to fit the Hilger photoelectric spectrophotometer. These films were allowed to dry in air and absorption spectra were recorded before and after fixing them in certain ways.

Fixation

The coverslip bearing the cells was first washed in Tyrode solution at 37° C. for about 15 seconds to remove the liquid medium in which the cells were grown. This medium contains proteins which would be precipitated by certain fixatives, thereby obscuring cell detail and making quantitative measurements difficult. Cells observed in Tyrode for periods of up to half an hour remained perfectly normal in appearance so that the preliminary washing does not upset the cells. The cells were then fixed in one of the following ways:

(i) With 10 per cent. neutral formalin-Ringer for 10 minutes (solution prepared by diluting commercial formalin 1:9 with Ringer; the commercial material was neutralized by shaking with powdered calcium carbonate); washed ½ to 1 hour; immersed in water or glycerol.

(ii) With Carnoy's original solution, freshly prepared 1 part of glacial acetic acid and 3 parts of absolute alcohol, for 10 minutes; washed twice with 90 per cent. alcohol and either taken down through the alcohols to water and measured in water or glycerol or immersed in glycerol from absolute alcohol.

(iii) By exposure for 2 minutes to the vapour of a freshly prepared 2 per cent. solution of osmium tetroxide; washed ½ to 1 hour, immersed in water or glycerol; some cells were bleached by Mayer's chlorine method (in Bolles Lee, 11th ed., p. 242) for periods of up to 2 hours.

(iv) By freezing-substitution (Simpson, 1941). After the cells had been washed in warm Tyrode, the surplus solution was drawn off with filter paper. The coverslip was then mounted on a brass U-shaped holder either with or without a copper plate, about ¼ mm. thick, backing it. The holder was then rapidly immersed and stirred in a bath of propane cooled with liquid nitrogen.

FIG. 1 (plate). Photomicrographs of interphase chick heart fibroblasts in tissue culture, taken with an objective of N.A. 1·24, condenser N.A. 0·6, λ 265 mμ.

A, living cell.

B, after fixation in 10 per cent. neutral formalin; mounted in water.

C, living cell.

D, after fixation in the vapour of osmium tetroxide; mounted in water. Shows a general increase in light-absorption, especially by the nucleus and centrosphere.

E, cell fixed in 10 per cent. neutral formalin, showing a bleb (arrow).

F, cell fixed directly in methanol; mounted in glycerol. Shows marked precipitation throughout the cell.

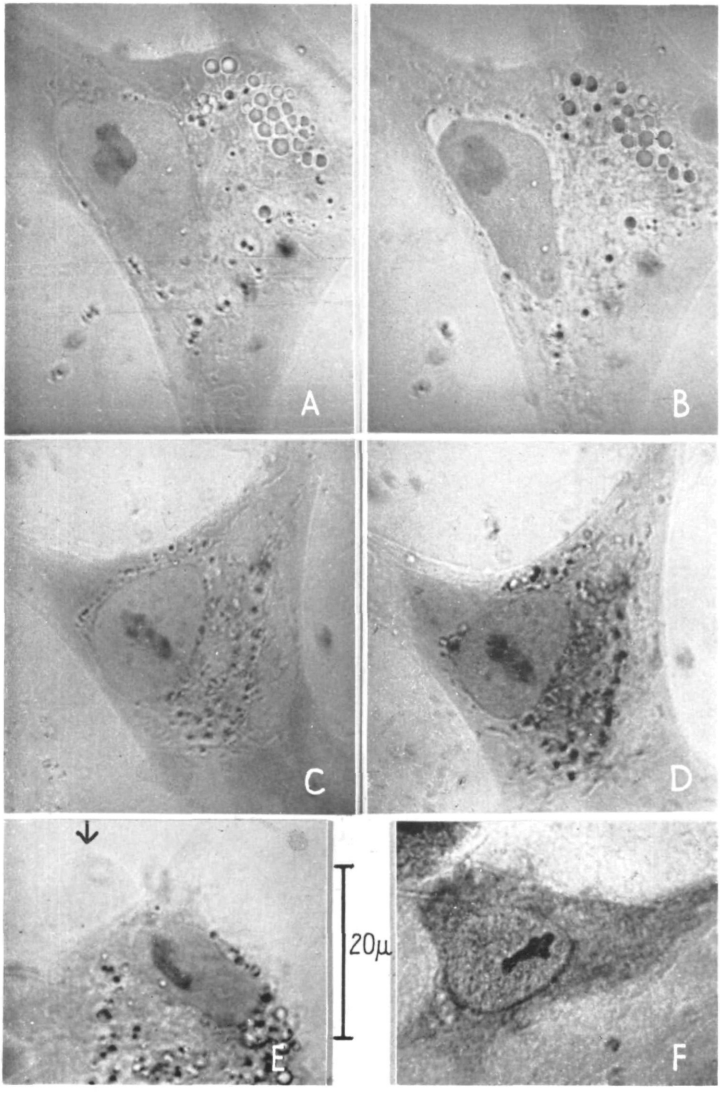

FIG. 1

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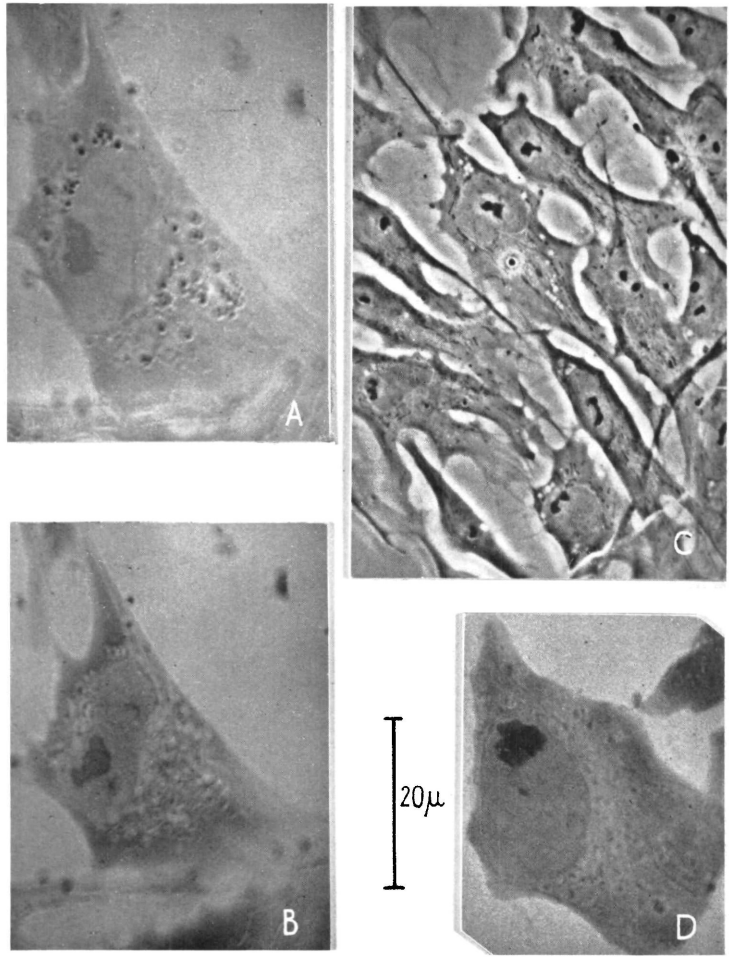

FIG. 2

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Propane, which remains liquid at the temperature of liquid nitrogen, was suggested by Bell (1952). The mounted coverslip was rapidly transferred to a large glass tube (about 200 c.c.) of methyl alcohol (BDH solvent methanol) previously immersed in a cold bath of solid carbon dioxide and industrial spirits. The tube was removed to a lagged container and allowed to warm up to room temperature, which took ½ to 1 hour; loss of water occurred somewhere between −78° C. and room temperature. The cells were immersed in doubly distilled glycerol.

(v) Some cells were examined after freezing-drying in an Edward's commercial freezer-drier modified to take the 1-inch square coverslip. They were cooled as in freezing-substitution and dried in a vacuum of approximately 0·005 mm. for 2 days at about −40° C. Temperatures were measured by a copper-constantan thermocouple in contact with the copper plate backing the coverslip. The temperature of the coverslip was raised to about 20° C. before breaking the vacuum so as to prevent condensation of water on to the cells, which were then immersed in doubly distilled glycerol.

Results

The living interphase cell

The morphological features demonstrable in photomicrographs at 265 mμ of the living interphase cell (figs. 1, A and C, 2, A, also 15, A (see p. 450)) will be enumerated briefly. The nucleus, apart from one or two nucleoli, and an occasional chromocentre, is a nearly homogeneous structure; sometimes filamentous bodies which lie near the limit of resolution may be seen. In the cytoplasm, which is homogeneously absorbing, the centrosphere can often be distinguished as a less absorbing region adjacent to the nucleus. Usually this area is the site of an aggregation of thin filamentous mitochondria, which, together with the fat-droplets, are the most conspicuous structures in the cytoplasm.

Ultra-violet absorption spectra of the living nucleus (figs. 3, A, 5, A, 7, A, 10, A and B) have an absorption maximum in the region 265 mμ. A negligible optical extinction can be detected at 313 mμ; a slight non-specific light loss occurs at the boundaries of nucleolus and nucleus and these regions are excluded from the measurements. The shape of the absorption spectrum between 270 and 280 mμ varies from cell to cell. Some (fig. 3, A) show a plateau in the region 270–275 mμ, whilst others (figs. 5, A, 7, A) show a plateau

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Fig. 2 (plate). Photomicrographs of interphase chick heart fibroblasts in tissue culture. The optical arrangement was the same as in fig. 1, except that fig. 2, C was taken with a Beck phase-contrast objective, N.A. 0·85, focal length 4 mm., λ 546 mμ.

A, living cell.

B, after fixation by methanol freezing-substitution. Shows some cracks.

C, phase-contrast photomicrograph of cells fixed by methanol freezing-substitution. Shows some cracks.

D, cell fixed by freezing-drying; mounted in doubly distilled glycerol.

The scale refers to A, B, and C; D is at one-half of this magnification.

in the region 275–280 mμ. It would be interesting if the stage in interphase were partly characterized by the shape of absorption spectra. However, it is not possible to attach much significance to small variations in these spectra owing to the inaccuracies of the photographic method of recording and to the limited number of wavelengths employed.

When two substances with known absorption spectra are present in a mixture then the relative amounts of the substances may be determined by

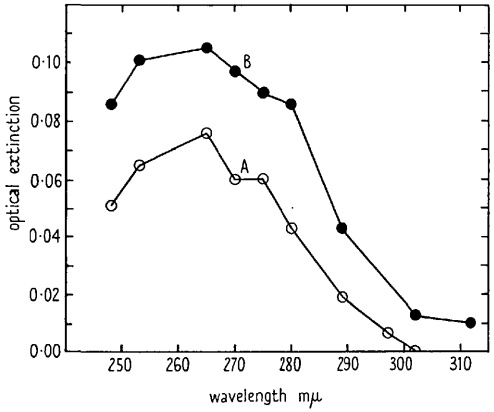

FIG. 3. Absorption spectra of the nucleus of the living cell (A), and after fixation in 10 per cent. neutral formalin (B). Cell mounted in water.

analysis of the absorption spectrum of the mixture. This well-known method has been used by Caspersson (Caspersson and Santesson, 1942) to determine the relative amounts of nucleic acid and 'standard protein' in the cell from the ratio E_{260}/E_{280}, where E_{260}, E_{280} are the optical extinctions at 260 and 280 mμ. The 'standard protein' has a certain assumed content of the amino acids tyrosine and tryptophane. For nucleic acid alone, E_{260}/E_{280} is 1·83 and when protein is present the ratio decreases. For technical reasons the ratio E_{265}/E_{280} has been recorded in these investigations but the values will not differ so significantly from those at the first-mentioned wavelengths as to affect the limited conclusions drawn. The ratios E_{265}/E_{280} for the nuclei of several living cells (table 1, col 3: see page 456) give a range of values with an average approximately 2·0. If the equations of Caspersson and others (1942) were applied to interpret this value, then it would be concluded that the amount of protein in the nuclei of these living cells is small compared with the amount of nucleic acid. This result is discussed later especially in relation to the marked change in the ratio E_{265}/E_{280} which occurs as a result of fixation.

Formalin fixation

After fixation with 10 per cent. neutral formalin a finely filamentous and granular structure can usually be distinguished in the nucleus (fig. 1, B). The projected area of the nucleolus sometimes increases but that of the nucleus invariably decreases; in the nuclei measured the decrease ranges from 5 to 35 per cent. Changes in the thickness of the nuclei were not measured. The cytoplasm usually remains homogeneous but occasionally a fine precipitate is formed in some regions; mitochondria can still be seen.

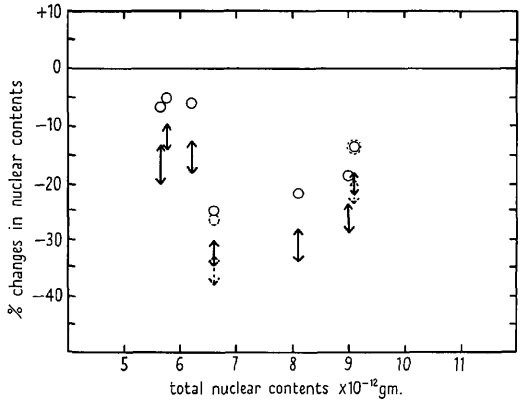

FIG. 4. Per cent. changes in the total extinction at 265 mμ of the nuclei after fixation in 10 per cent. neutral formalin. Abscissae: the total amount of absorbing material in the living nuclei expressed as DNA. Ordinates: per cent. changes relative to the living cell (zero ordinate). For cells immersed in water the changes are shown by empty circles, non-specific light loss being neglected; by the upper arrows light loss being corrected according to λ^0, and by the lower arrows when corrected according to λ^{-4}. The dotted circles and arrows show corresponding changes for some cells immersed in glycerol from water.

A characteristic feature is the formation of 'blebs', balloon-like structures containing material faintly absorbing at 265 mμ; they sometimes remain attached to the cell (fig. 1, E) but are usually lost when the culture is washed. Such blebs, formed during formalin fixation, have been noted by others (Buchsbaum, 1948; Crawford and Barer, 1951). After fixation a reduction in the optical extinction at 265 mμ of the cytoplasm is apparent even in photomicrographs, and under positive phase contrast the cytoplasm appears less dark.

The total amount of cytoplasmic material absorbing at 265 mμ was found to have decreased on an average by approximately 40 per cent. after fixation; six cells were measured. Clearly some if not all of the loss in absorption occurs by bleb-formation.

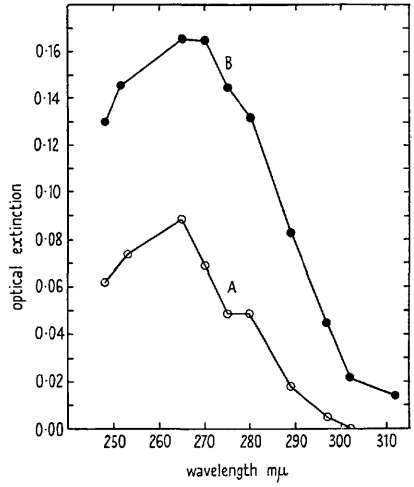

FIG. 5. Absorption spectra of the nucleus of the living cell (A), and after fixing in Carnoy (B). Cell mounted in glycerol.

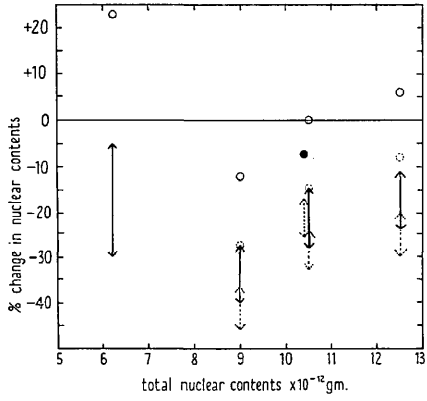

FIG. 6. Per cent. changes in the total extinction at 265 mμ of nuclei after fixation in Carnoy. Abscissae: the total amount of absorbing material in the living nuclei expressed as DNA. Ordinates: per cent. changes relative to the living cell (zero ordinate). For cells immersed in water the changes are shown by empty circles, non-specific light loss being neglected; by the upper arrows when light loss is corrected according to λ^0, and by the lower arrows when corrected according to λ^{-4}. The dotted circles and arrows show corresponding changes for some cells immersed in glycerol from water and the full circle and dotted arrow for a cell immersed in glycerol after absolute alcohol.

After fixation the optical extinction of the nucleus at 313 mμ becomes measurable and is attributed to a non-specific loss of light. The ratio of the optical extinctions at 313 mμ and 265 mμ, E_{313}/E_{265}, in the fixed cell can be conveniently used as a measure of this non-specific loss; it is about 7 per cent. As a result of shrinkage the optical extinction of the nucleus at 265 mμ increases markedly as shown in the comparative absorption spectra (fig. 3, A and B). After fixation there is a significant drop in the ratio E_{265}/E_{280} to a value which is nearly independent of the way in which the optical extinctions are corrected for non-specific light loss (table 1, cols. 4, 5, 6: see page 456).

In fig. 4 the difference between the total nuclear extinctions of the living and fixed cells are expressed as percentages of the total extinctions of the living nuclei. The circles denote the changes when the non-specific loss is not taken into account and the upper and lower arrows the change when the non-specific loss is assumed to vary as λ^0 or λ^{-4} respectively. The ratios E_{313}/E_{265} in the fixed cells are given approximately by subtracting the values for the upper arrows and the corresponding circles; the exact values could be calculated by simple algebra. There is very little further change when cells are immersed in glycerol (dotted data). It is concluded that as a result of fixation there is a decrease in the total nuclear extinction ranging from 10 to 35 per cent.

Carnoy fixation

After fixation in Carnoy the material in the nucleus is redistributed into granules (fig. 15, B, p. 450); shrinkage of the nuclear area ranges from 20 to 47 per cent. The nucleolus shrinks, the mitochondria are destroyed and the cytoplasm becomes granular. The non-specific light loss is now greater than after formalin fixation; E_{313}/E_{265} ranges from 10 to 14 per cent. The ratios E_{265}/E_{280} (table 1) deduced from the comparative absorption spectra (fig. 5, A and B) show a similar change to that found after formalin fixation.

Fig. 6 shows that there is a measurable decrease in the nuclear non-specific light loss when cells are transferred from water to glycerol, but little further change in the total extinction. It is concluded that the decrease in total nuclear extinction is similar in magnitude to that after formalin fixation.

Osmium tetroxide fixation

(i) *Fixation of cells.* When fibroblasts are fixed in the vapour of osmium tetroxide there is little morphological change. Any nuclear detail is somewhat enhanced and the area of the nucleus shrinks by 8–16 per cent., the cytoplasm remains homogeneous and the mitochondria are well preserved. However, a general increase in the optical extinction at 265 mμ is apparent even in the photomicrographs (fig. 1, D). Fat-droplets become highly absorbing and the centrosphere stands out as a more absorbing region; mitochondria also may be more easily distinguished. After fixation the absorption spectrum of the nucleus (fig. 7, B) shows a relatively large increase in optical extinction at 313 mμ, and a continual increase towards shorter wavelengths.

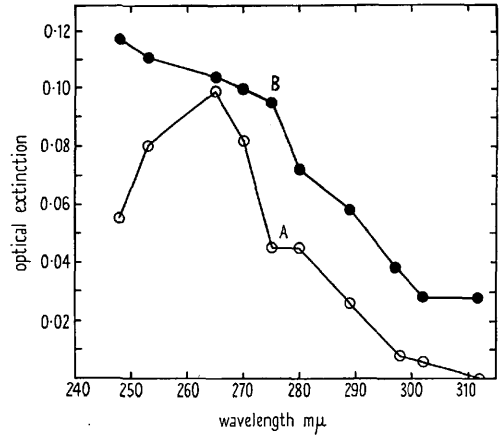

FIG. 7. Absorption spectra of the nucleus of the living cell (A), and after fixation in the vapour of osmium tetroxide (B). Cell mounted in water.

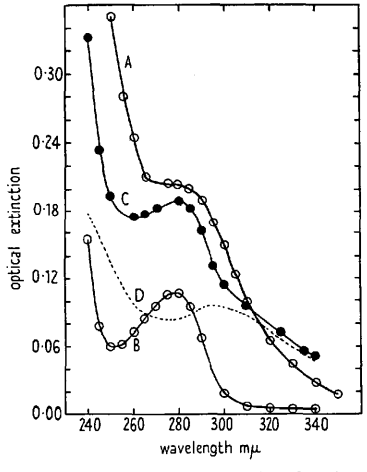

FIG. 8. A, absorption spectrum of a 0·005 per cent. solution of osmium tetroxide (1 cm. path length). B, absorption spectrum of an air-dried film of egg-white. C, the same film after exposure to the vapour of a 2 per cent. solution of osmium tetroxide for 2 minutes. D, absorption spectrum of reduced osmium tetroxide obtained by subtracting curve B from curve C.

(ii) *Model experiments.* The possibility that the fixative itself stains the cell, that is, combines with the cell substances to produce additional absorption, was tested by model experiments. Osmium tetroxide in dilute solution absorbs appreciably (fig. 8, A) and a film of egg-white exposed to its vapour showed a marked increase in ultra-violet absorption (fig. 8, B and C). The spectrum (fig. 8, D) obtained by subtracting curve *B* from curve *C* is different from that of osmium tetroxide and probably represents a reduced form. Films of gelatin and bacon fat showed similar increases in absorption after exposure to the vapour of osmium tetroxide and by subtraction similar spectra were obtained for the reduced form of osmium tetroxide. However, a film of thymus nucleic acid similarly exposed showed no change in absorption. Apart from explaining the general increase in ultra-violet absorption of the fixed cell, these observations may also be helpful in the interpretation of electron micrographs where the contrast may be partly due to differential staining after fixation with osmium tetroxide.

The ratio E_{265}/E_{313} for the reduced form of osmium tetroxide was found to be approximately 1·1. This value was used to correct the optical extinction of the cell at 265 mμ from the optical extinction at 313 mμ. The results obtained for the changes in total nuclear extinction are described in detail in the text to fig. 9. The optical extinction of the cell increases after washing with water for half an hour; this may be due to a slow reaction between the fixative and the cell. The nuclear extinction at 313 mμ was unchanged as a result of immersion in glycerol and could not be completely removed by bleaching. There was fair agreement between the corrected decreases in total nuclear extinction for cells after washing and after bleaching. These results indicate in a general way how the absorption of the nucleus varies after fixation with osmium tetroxide vapour, but the method used in correcting for the additional absorption is probably not very reliable.

Freezing-substitution

(i) *Fixation of cells.* After fixing by freezing-substitution with methyl alcohol the nucleus either appears homogeneous or contains fine structure near the limits of resolution of the ultra-violet microscope. The ground substance of the cytoplasm also is well preserved and often appears homogeneous although a fine reticular structure may sometimes be seen; the mitochondria and fine cytoplasmic processes appear almost unaltered (fig. 2, B). There is an overall shrinkage of the cell, that of the nuclear area ranging from 20 to 40 per cent. The tissue culture explant also contracts and this results in a gap between it and the sheet of cells. Cracks also appear running through the cells. However, only a small fraction of the cells are cracked and the appearance of the fixed cells shown in the phase-contrast photomicrograph (fig. 2, C), is very lifelike.

Cells fixed by freezing-substitution contrast sharply in appearance with cells fixed directly in methyl alcohol (fig. 1, F) where an intense precipitate is

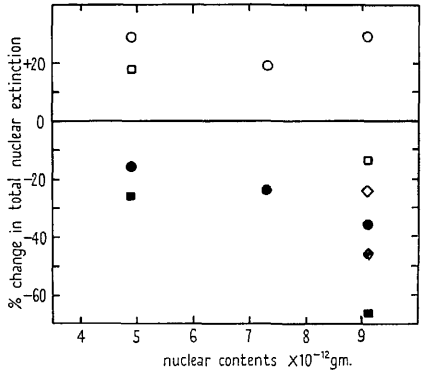

FIG. 9. Per cent. changes in total extinction at 265 mμ of the nuclei after fixation in the vapour of osmium tetroxide. Abscissae: the total amount of absorbing material in the living nuclei expressed as DNA. Ordinates: per cent. changes relative to the living cell (zero ordinate). The optical extinction at 265 mμ is corrected from the extinction at 313 mμ by using $E_{265}/E_{313} = 1{\cdot}1$, obtained from fig. 9, D. The empty and full squares indicate the uncorrected and corrected values respectively before washing. The empty and full circles indicate the uncorrected and corrected values respectively after washing. The empty and full diamonds indicate the uncorrected and corrected values respectively after attempts to bleach the cell.

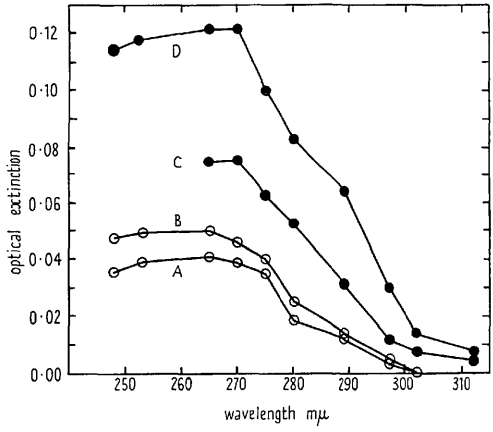

FIG. 10. Absorption spectra of the nucleus of living cells (A, B) and the corresponding spectra (C, D) after methanol freezing-substitution; cells mounted in doubly distilled glycerol. A and C correspond.

seen throughout the cell and the mitochondria are mostly obliterated. Presumably this is the result of diffusion currents, absent during freeze-substitution, which sweep the material in the cell to new positions (Bell, 1952).

The first step in freezing-substitution, the attempt to vitrify the cell, is the same as that in the better-known freezing-drying method of Altmann and Gersh, reviewed by Bell (1952). One important difficulty sometimes encountered in freezing-drying has been the formation of ice crystals within the cell, giving

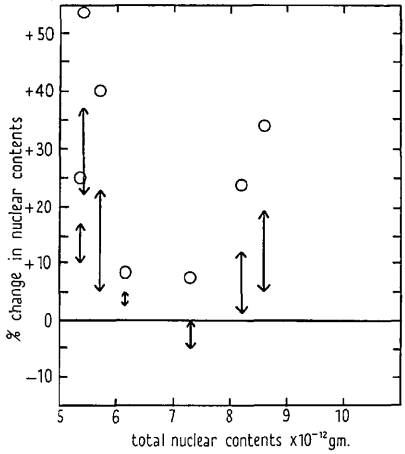

FIG. 11. Per cent. changes in the total extinctions at 265 mμ of nuclei fixed by methanol freezing-substitution; cells in doubly distilled glycerol. Abscissae: the total amount of absorbing material in the living nuclei expressed as DNA. Ordinates: per cent. changes relative to the living cell (zero ordinate), are shown by empty circles, non-specific light loss being neglected, and by the upper and lower arrows when light loss is corrected according to λ^0 and λ^{-4} respectively.

rise to redistribution of material around the boundaries of the crystals. Such crystals can arise either through imperfect vitrification during cooling or through devitrification before or during dehydration. The ice crystal artifact was noted during early attempts at freezing-substitution in the present investigation and was traced to devitrification of the cells during transfer from the propane to the cooled methyl alcohol. The coverslip bearing the cells has only a small thermal capacity and when in air quickly warms up beyond the devitrification temperature of the cell. A thin copper plate at the back of the coverslip, although it hindered cooling, usually prevented noticeable ice crystals from forming, but despite this precaution a small proportion of the cells (probably about 10 per cent.) localized in zones of the culture often showed a marked reticular structure.

The non-specific light became measurable after fixation, the ratio E_{313}/E_{265} ranging from 3 to 12 per cent. Comparative absorption spectra of the nucleus showed a decrease in the ratio E_{235}/E_{280} (table 1). The total nuclear extinctions at 265 mμ (fig. 11) increased after fixation and the possibility of the fixative staining the cell was tested.

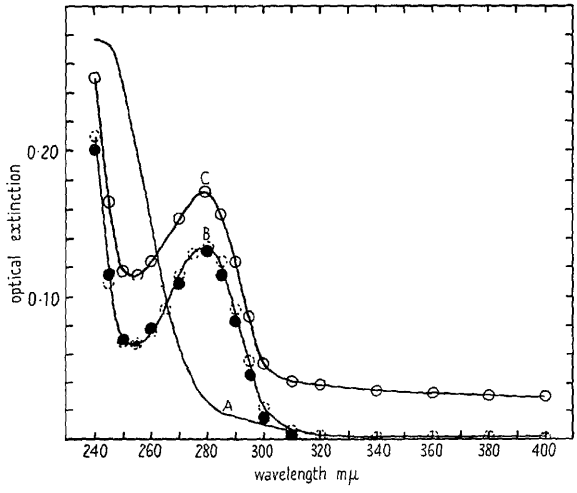

FIG. 12. A, absorption spectrum of 0·25 per cent. solvent methanol (1 cm. path length). B, absorption spectrum of air-dried film of egg-white, broken circles. C, absorption spectrum of the film after methanol freezing-substitution. The full circles on spectrum B are obtained by correcting C for non-specific light loss.

(ii) *Model experiments.* The solvent methanol had a high ultra-violet absorption (fig. 12) and the optical extinction of a film of egg-white increased after freezing-substitution (figs. 12, B and C). The fixed and dried film was cracked so that it no longer looked transparent in ordinary light and the increase in extinction was attributed to light scatter. The extinction at wavelengths greater than 313 mμ was found to vary inversely as the wavelength and the remainder of the spectrum was corrected by extrapolation. The corrected extinctions (full circles) lie, within the limits of experimental error, on the original spectrum for egg-white.

In a similar experiment a film of ribonucleic acid mixed with egg-white was fixed by methanol freezing-substitution (fig. 13, A and B). This film was much thinner, exhibiting interference colours, and there were no cracks in the fixed and dried film. The increased extinction was small, approximately the same at all wavelengths, and must be attributed to non-specific light loss in the

denatured egg-white. These experiments make it less likely that the increase in total nuclear extinction is due to the fixative staining the cell.

Freezing-drying

After freezing-drying, the tissue culture mounted in glycerol is similar in appearance to a culture after alcohol freezing-substitution. The nucleus of the

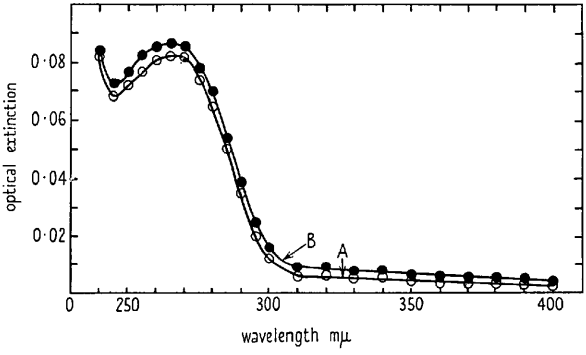

FIG. 13. A, absorption spectrum of a film obtained from a mixture of egg-white and ribonucleic acid. B, the same film after methanol freezing-substitution, showing a slight increase in non-specific light loss.

well-fixed cell either appears homogeneous or contains a very fine reticular structure. Well-preserved mitochondria lie in a cytoplasm in which structure is often difficult to detect (fig. 2, D). Cells sometimes show cracks and occasionally a marked reticular structure due to the formation of ice crystals.

Frozen-dried cells appear to be less firmly attached to the coverslip than chemically fixed cells. Thus the edges of cells mounted in glycerol were sometimes seen to have curled away from the coverslip and the explant also became detached from the glass, usually when the glycerol was dropped on to the frozen-dried culture. The loss of the explant, a convenient landmark, made it difficult to find the same cells after fixation. Principally for this reason no results are presented here on the change in total nuclear absorption after freezing-drying.

A typical absorption spectrum (fig. 14) of the nucleus of a frozen-dried cell showed a significantly lower ratio E_{265}/E_{280} (table 1) than is characteristic of the living cell.

The effect of varying the pH of the culture medium

Lewis (1923) has described how homogeneous nuclei of cells in tissue culture become filled with refractile structures when the pH of the medium

surrounding the cells is reduced to about 4·6. When the pH is returned to normal the material in the nucleus, under certain circumstances, reverses to the homogeneous state. Measurements of the total nuclear extinction at each of these three stages would seem to be a good way of checking the magnitude of any error due to distribution (see Introduction (iii)). This experiment was suggested to the writer by A. E. Mirsky, who has observed similar reversible changes in the distribution of material in isolated nuclei (Ris and Mirsky, 1949).

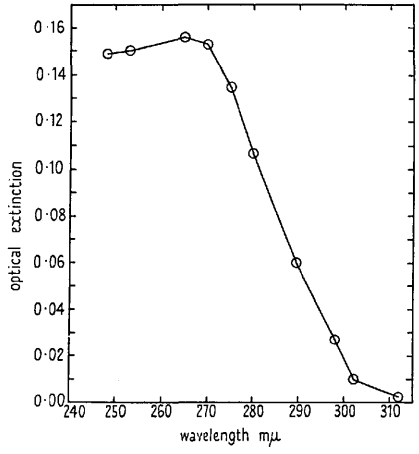

FIG. 14. Absorption spectrum of the nucleus of a cell frozen-dried and mounted in doubly distilled glycerol.

Cells were photographed at 265 mμ (fig. 15, C) and 313 mμ immediately after the protein medium had been washed away with Tyrode. The Tyrode was then replaced by the saline at pH 4·6; 5–10 minutes later the movement of the mitochondria had ceased. In about ½ to 1 hour, during which time the culture was occasionally re-flooded with the acid medium, the material in the nucleus aggregated into refractile threads and granules. The cells were immediately photographed at 313 and 265 mμ (fig. 15, D) and then flooded

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FIG. 15 (plate). Photomicrographs of interphase chick heart fibroblasts in tissue culture Optical arrangements as in fig. 1.

A, living cell.

B, after fixation in Carnoy; cell mounted in glycerol. There is marked precipitation throughout the cell.

C, living fibroblast immersed in Tyrode.

D, same cell as C at pH 4·6; the nucleus is full of threads and granules.

E, same cell after subsequent immersion in Tyrode, when the nucleus reverts to its previous homogeneous state.

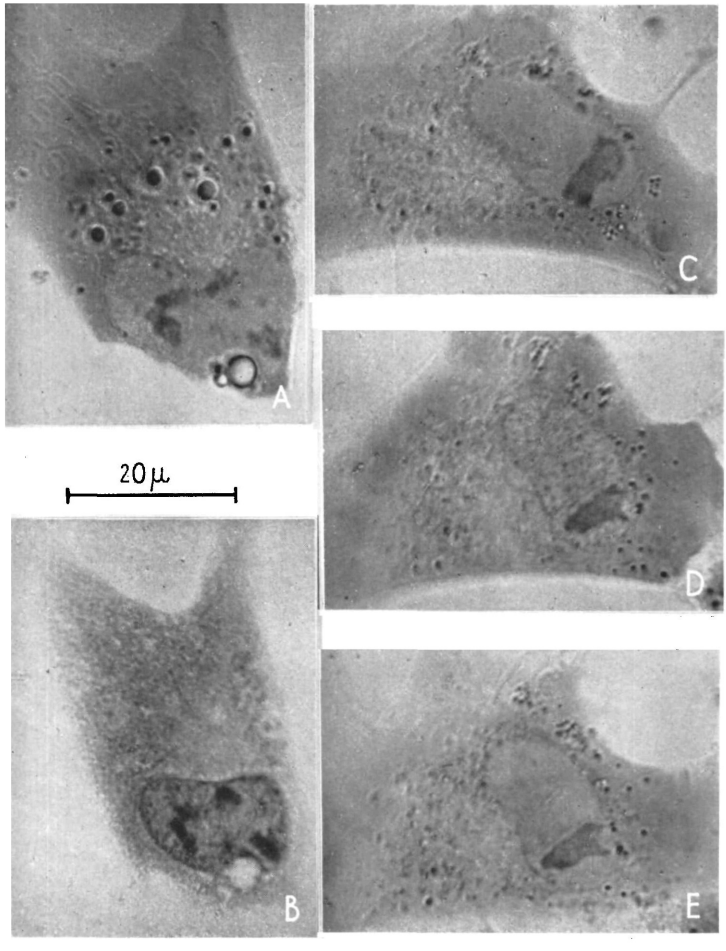

FIG. 15

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with Tyrode. Immediately, and in a most striking manner, the nucleus reverted to its homogeneous state (fig. 15, E).

In fig. 16 the changes in total nuclear extinction at 265 mμ are relative to the cell when first in Tyrode. With the appearance of refractile structures in the nucleus the non-specific light loss becomes measurable and the changes in total extinction are indicated by the upper and lower arrows according to whether the non-specific loss varies as λ^0 or λ^{-4}. The small decrease in total

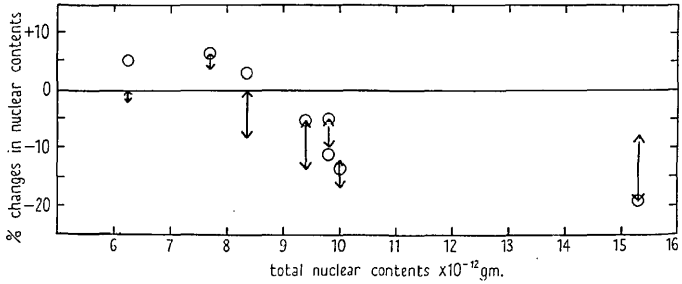

FIG. 16. Per cent. changes in total nuclear extinction at 265 mμ of the nucleus of the living cell during reversible changes brought about by altering the pH of the medium around the cell. Abscissae: total amount of absorbing material expressed as DNA. Ordinates: per cent. changes relative to the living cell when first in Tyrode (zero ordinate). The changes at pH 4·6 are shown by the upper and lower arrows when non-specific light loss is corrected according to λ^0 and λ^{-4} respectively. The circles show the changes when the cells are re-immersed in Tyrode and the nucleus once again homogeneous; there is a relatively small residual non-specific light loss for which correction was made according to λ^{-2}.

extinction appears to increase in magnitude with the amount of material in the nucleus. After the cells have been re-flooded with Tyrode (circles) there is hardly any change relative to the cell in acid medium.

DISCUSSION

We must first consider the possibility whether the changes observed after fixation may be due in part to ultra-violet radiation damaging the cell. It was shown (Davies, 1952) that the dosage required for one photomicrograph at 265 mμ in the apparatus used may disturb the metabolism of the cell and that the series of photomicrographs required for a complete absorption spectrum would lead to eventual damage to the cell. However, Walker and Davies (1951) showed that no change in the total nuclear extinction could be detected after about fifteen photomicrographs at 265 mμ taken at intervals over a period of 3 minutes. In the present experiments the dosage was less and cells were fixed immediately after irradiation. Also, the low value for E_{265}/E_{280} was observed in fixed cells which had not been previously irradiated in the ultra-violet. Hence it is concluded that the changes observed after fixation are not in any way due to irradiation of the cells by ultra-violet light.

I. *Total amount of absorbing material in the nucleus*

(i) *Changes in total amount after fixation.* When chick fibroblasts are fixed in formalin and Carnoy the total nuclear extinction at 265 mμ decreases. This change might be due to an actual loss of absorbing substances but other factors complicate this obvious interpretation.

(*a*) The optical extinction in the area occupied by the nucleus of the living interphase cell is due partly to the nucleus itself and partly to the layers of cytoplasm above and below it. Conceivably, shrinkage of the nucleus without a corresponding cytoplasmic shrinkage might cause some decrease in the total extinction of the area occupied by the nucleus. It has so far proved impossible to measure the contribution made by the cytoplasm to the absorption of the nuclear area, but there are reasons, summarized by Walker and Yates (1952), for believing that this contribution might be small. It seems likely that the changes in the total extinction of the nuclear area due to differential shrinkage will be small.

For the sake of brevity, measurements of optical extinction in the area occupied by the nucleus have been, and will be, referred to as measurements of the nucleus. It is to be understood that an indeterminate fraction of the measured extinction, probably small, is due to over- and under-lying cytoplasm.

(*b*) The heterogeneity produced by fixing the cell might result in a decrease in the total nuclear extinction (Introduction (iii)). Theory indicates that the errors due to distribution are likely to be small compared with the changes actually observed since (1) the cells have a low optical extinction ($< 0{\cdot}2$), and (2) densities are recorded with an aperture that is small compared with the area of the nucleus. The experiments in which the pH of the medium around the cells was varied may be interpreted to support this conclusion. Thus, in the rapid reversal from the heterogeneous to the homogeneous state of the nucleus there is on the average a negligible change in the total extinction at 265 mμ. Conceivably the first drop in total nuclear extinction, which is, in any case, less than that observed during fixation, is due to an actual loss of absorbing material during the long period, up to 1 hour, in acid medium. Also, results obtained after freezing-substitution show that the distributional error is small. A few cells were measured, the heterogeneity of which was comparable with that of Carnoy-fixed cells. They showed no decrease in total nuclear extinction at 265 mμ.

(*c*) The non-specific light loss due to the heterogeneity of the fixed cell also introduces some uncertainty in the results. However, in these fixed nuclei E_{313}/E_{265} is never very high and is usually less than 15 per cent. Hence the changes in the total extinction of the nuclei of chick fibroblasts due to fixation are not seriously affected by the method used to correct for non-specific light loss; the greatest uncertainty is in cells fixed with Carnoy's fluid.

In other types of cells (Caspersson, 1950, Nurnberger and others, 1952), E_{313}/E_{265} is often much larger, ranging from 30 to 50 per cent. Conceivably this might be due partly to a higher ratio of light-scattering substances

(proteins, &c.) to light-absorbing substances (nucleic acids, &c.). Much of the material is in the form of sections and it is possible that further treatment after fixation increases non-specific light loss. It has been noted, for example, that there is an unusually high optical extinction at 313 mμ in fibroblasts fixed by freezing-substitution and left overnight in the methyl alcohol. This has been attributed to a hardening of the proteins by the alcohol, resulting in sharper gradients of refractive index which increase light scatter (Caspersson, 1950).

(*d*) There is no way of assessing whether any of the observed changes are due to failure of Beer's law. The magnitude of the glare was determined by photographing an opaque object (Wilkins, 1950), the measured optical extinction of which was about 1·0 at 265 mμ. The procedure described by Davies and Walker (1953) was used and it was concluded that there was a linear relationship between the measured and true optical extinctions over the range encountered in this investigation. Hence no error was introduced by glare.

(*e*) It has been shown (Kunitz, 1950) that digestion of DNA with desoxyribonuclease causes an increase of about 30 per cent. in the optical extinction at 260 mμ; this increase generally precedes the liberation of free acid or the formation of acid soluble split products. Conceivably fixation also might change the structure of the DNA and hence alter its absorption. This may be a reason for the increase in the total nuclear extinction after freezing-substitution.

It has been concluded that the experimentally observed decreases in total nuclear extinction ranging from about 10 to 35 per cent. after formalin and Carnoy fixation roughly represents the magnitude of an actual loss of absorbing substances from the nucleus. If there were an increase in absorption due to changes in the structure of the DNA on fixation, then the loss of absorbing substances would be greater than that indicated by this range of values. The approximate agreement between the values of total nuclear extinction before and after methanol freezing-substitution, corrected for light scatter according to λ^{-4}, is regarded as fortuitous.

(ii) *Nature of the absorbing substances in the nucleus.* The range of values obtained for the contents of the nuclei of living chick fibroblasts, expressed as DNA, is $5–15 . 10^{-12}$ gm., in agreement with the extensive data of Walker and Yates (1952). These workers have drawn attention to the fact that these values are considerably higher than the DNA content of diploid differentiated cells from the same species. Thus, using ultra-violet microspectrometry, they obtained $3·0 . 10^{-12}$ gm. for the DNA content of the chick erythrocyte, in fairly good agreement with the value $2·5 . 10^{-12}$ gm. obtained by bulk biochemical measurements (Davidson and Leslie, 1950). Walker and Yates have shown that some of the increase in ultra-violet-absorbing material in the interphase nucleus is due to the synthesis of DNA in preparation for the next mitosis. But as this would be expected to yield a range of values approximately $3–6 . 10^{-12}$ gm. it was concluded that absorbing substances other than DNA are present in the interphase nucleus. In view of these results several fixed nuclei were digested with ribonuclease and it was found that on average the

total nuclear extinction was reduced by about 50 per cent.; the nucleoli were not included in the measurements. However, such a high value for the ribonucleic acid content of the interphase nucleus cannot be accepted without more extensive investigation.

We can at present only speculate as to the nature of the material lost on fixation with formalin and Carnoy. It is likely that unbound molecules of low molecular weight originally present in the nucleus, or produced by fixation, would diffuse away during fixation. Indeed Walker and others (1952) have suggested that the substances lost during fixation are nucleotides of low molecular weight which are precursors of DNA. It is interesting that these small molecules, if they are present, do not diffuse away during freezing-substitution. Clearly chemical analyses of the fixing fluids are needed to determine the precise nature of any substance lost by fixation. This would require the use of much larger numbers of cells than were available in this investigation.

II. *The absorption spectra of the nucleus*

(i) *The living nucleus.* It will be recalled that E_{260}/E_{280} for isolated DNA is approximately 1·83 and that when protein is present the ratio decreases. On this basis it was concluded that the amount of protein in the nuclei of these living cells is small compared with the amount of nucleic acid. However, the absorption spectrum of the nucleus appears to differ from that of isolated DNA since (1) E_{265}/E_{280} is often greater than 1·83, and (2) there are bumps on the spectra in the region 270–280 mμ. A typical spectrum for an air-dried film of nucleic acid was obtained when calibrating the apparatus (Walker and Davies, 1950); E_{265}/E_{280} was approximately 1·7.

Results (Davies, Wilkins, Chayen, La Cour, 1954) for the total dry mass of the living nucleus obtained by interferometry do not agree with those obtained by microspectrometry, which shows a relatively low protein content. The method makes use of the fact that the mass of material other than water in a cell may be obtained from the optical path difference of the cell, a measurement which is conveniently made by interference microscopy. The dry masses obtained for the nuclei of several living fibroblasts were about 30.10^{-12} gm. and this is to be compared with the total contents absorbing at 265 mμ ranging from 5 to 15.10^{-12} gm. Hence it would be concluded from these combined measurements that the amount of protein is several times greater than the total amount of absorbing material.

(ii) *The fixed nucleus.* One change in the absorption spectra of the nuclei common to all the fixatives employed is the marked drop in E_{265}/E_{280} (table 1) from about 2 to 1·3, a decrease outside the limits of experimental error. From the equations of Caspersson and Santesson (1942) it appears that in contrast to the relatively lower amount of 'standard protein' in the living nucleus, the amount of protein in the fixed nucleus is about an order of magnitude greater than the amount of nucleic acid. Some comments will be made on the factors which might conceivably change the shape of the absorption spectrum and hence produce this result.

(*a*) The errors in microspectrometry are usually a function of optical extinction and, since shrinkage causes the extinctions of the living and fixed cells to differ, often widely, this might cause a change in shape of the absorption spectrum in the manner observed. For the reasons stated in the Discussion, I (i), however, these errors are small in these cells and hence will not produce the marked change observed. Any loss of substances absorbing like DNA, after formalin and Carnoy fixation, would be expected to change E_{265} and E_{280} in such a manner as to leave the ratio unchanged. After freezing-substitution, when there is no decrease in total nuclear absorption at 265 mμ, E_{265}/E_{280} decreases to a similar value.

(*b*) Conceivably, substances with a relatively high optical extinction at 280 mμ might diffuse into the nucleus from the cytoplasm. However, a similar decrease in E_{265}/E_{280} was recorded in the cytoplasm of cells fixed by freezing-substitution (table 1).

(*c*) The ratio E_{265}/E_{280} is unaffected by the way in which the correction is made for non-specific light loss. However, changes in light scatter resulting from changes in the refractive indices of the substances in the cell with wavelength have previously been ignored. If the dispersion in the region of the nucleic acid absorption band were anomalous, the refractive index of the cell and, hence, the light scatter, might be relatively greater at 280 than at 265 mμ. This would result in a relative increase in optical extinction at 280 mμ which would cause a decrease in the ratio E_{265}/E_{280}. Measurements of the refractive indices of nucleic acids and proteins at absorbing wavelength are required to test this possibility, which, however, seems an unlikely one owing to the low value of the non-specific light loss measured by the optical extinction at 313 mμ.

(*d*) The method of curve analysis is based on the supposition that the absorption spectrum of nucleic acid and protein in combination in the cell can be obtained by simple addition of the absorption spectra of the individual substances in solution outside the cell. However, the absorption spectrum of one substance is often altered by combination with another or by changes in the degree of polymerization, &c. Hence different spectral shapes may be produced by, and might eventually be used to elucidate, differences in the state of nucleic acids and proteins in cells. The results suggest that the derivation of relative amounts of nucleic acid and protein from absorption spectra of the living or the fixed cell must be viewed with caution.

III. *Comparison of fixatives*

Although fixation with osmium tetroxide results in little change in cell structure, it is unsuitable for quantitative measurements due to the staining by the fixative. Likewise, formalin is contra-indicated since it results in large losses of cytoplasmic absorption. Freezing-drying has long been suggested as the most suitable method of preserving cells for ultra-violet microspectrometry (Caspersson, 1940). This investigation shows that after freezing-drying, as after freezing-substitution, (1) the appearance of the cell is very lifelike,

(2) the material in the cell may be preserved in a nearly homogeneous state and hence the light scatter and distributional errors are at a minimum, and (3) the absorption spectrum of the nucleus is altered. Shrinkage after freeze-substitution will affect the measurements of concentration but not of total amounts of substances in the cell. After freezing-drying, cells have a low non-specific light loss as a result of swelling of the cell proteins in the doubly distilled glycerol in which they are mounted; this eliminates sharp changes in refractive index (Caspersson, 1940). Conceivably, proteins precipitated with alcohol will be less likely to swell in glycerol and hence it is likely that, in general, non-specific light loss will be greater in cells fixed by freezing-substitution. Measurements have yet to be made to see if freezing-drying preserves total nuclear extinction.

Freezing-substitution has the advantage that it is a much quicker method than freezing-drying. Also, cells after freezing-substitution are immediately available for further measurements in aqueous media, for example, after enzyme digestion. It seems likely that for such measurements there will be little to choose between a tissue culture cell fixed by alcohol freezing-substitution and a frozen-dried cell fixed by alcohol.

I wish to thank Professor J. T. Randall, F.R.S., and Dr. Honor B. Fell, F.R.S., for encouragement and suggestions. I am grateful to Dr. P. M. B. Walker for the use of his densitometer, to Miss S. F. Jackson and Miss J. Harvey, who made the tissue cultures, and Miss F. Ticehurst for preparing the plates. I wish to acknowledge helpful discussion with colleagues at King's College.

TABLE I

Fig. no.	*Fixation*	*Region of cell*	*Living* E_{265}/E_{280}	*Fixed*		
				E_{265}/E_{280} *uncorr.*	E_{265}/E_{280} *corr.* λ^0	E_{265}/E_{280} *corr.* λ^{-4}
3, A and B	Formalin	Nucleus	1·65	1·21	1·29	1·23
..	,,	,,	2·20	1·17	1·17	1·16
5, A and B	Carnoy	,,	1·85	1·23	1·26	1·22
7 A	Osmium	,,	2·2	..	..	..
10, A and C	Freezing-substitution	,,	2·0	1·46	1·50	1·49
10, B and D	,,	,,	2·18	1·43	1·47	1·46
..	,,	Cytoplasm	1·84	1·30	1·34	1·32
14	Freezing-drying	Nucleus	..	1·46	1·46	1·46

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