

THE VARIATION IN THE STRUCTURE OF ERYTHROCYTE NUCLEI WITH FIXATION

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ABSTRACT

Observations have been made on the role of a divalent cation (calcium ion) during OsO_4 fixation of nuclei of frog erythrocytes, mainly after isolation from cells. The volume of the nucleus depends partly on the molecular interaction of charged macromolecules, is controlled by the ionic strength of the medium, and hence may be used as a guide in attempts to preserve structure. When the isolation and fixation media contain 0.01 M calcium at pH 6.3 the volume changes, in the light microscope, during processing are small. When the fixative does not contain these ions, reversible volume changes occur during fixation and dehydration. The chromatin of nuclei processed with minimal volume change appears, in the electron microscope, to contain fine dots and lines about 20 to 40 Å in diameter, relatively close together. The chromatin structure of nuclei in which volume changes have occurred consists of dense irregularly shaped patches, relatively far apart, and ranging in diameter from about 200 Å down to the limits of visibility (20 to 30 Å). It is suggested that the latter structure is a precipitation artefact.

INTRODUCTION

Several studies (*e.g.* De Robertis, 1956; Ryter and Kellenberger, 1958; Huxley and Zubay, 1960) on nuclei and chromosomes have indicated that divalent cations, for example calcium and uranyl ions, have an important effect in stabilising and staining the fine structure observable in the electron microscope. Calcium ions were also used by Schneider and Peterman (1950) to preserve nuclei during isolation.

In attempts, described in this paper, to elucidate the role of the divalent cations in determining resulting fine structure, we noted that the volume of erythrocyte nuclei after partial isolation by haemolysis is determined by the concentrations of ions in the medium. Such changes in the size and appearance of nuclei in the light microscope are very well known and depend on the ionic strength, the concentration of divalent cations, and the pH of the medium, as well as the dielectric constant of the solvent (*e.g.*, Lewis, 1923; Ris and Mirsky,

1949; Anderson and Wilbur, 1952; Philpot and Stanier, 1956, 1957). The basis of our approach was the idea that the volume of the nucleus depends on the molecular interaction of the charged macromolecules and hence may be used as a guide in attempts to preserve nuclear structure. By observing nuclei in the light microscope during hemolysis and preparation for electron microscopy, it has been possible to choose a concentration of divalent cations (calcium) and pH for which changes in nuclear volume are minimal. When the fine structure of nuclei processed with and without volume change are compared, large differences are observed.

These microscopic observations on partially isolated nuclei are part of an investigation of nuclear or chromosomal isolation for parallel studies by electron microscopy and x-ray diffraction. Some observations have also been made, for comparison, on the nuclei of intact cells.

We are aware that the nucleated erythrocyte is a specialised cell type in which the nucleus is probably non-functional. However, the nuclei of frog erythrocytes were chosen because their structure (Davies, 1961) is relatively simple and they can be obtained in large quantities in a well defined state for x-ray diffraction study. Wilkins, Zubay, and Wilson (1959) have already made some x-ray observations on nuclei of chicken erythrocytes. The intact frog erythrocyte approximates in shape to a flattened ellipsoid and the nucleus is similarly shaped. The nucleus contains hemoglobin which is continuous with that of the cytoplasm through pores in the nuclear envelope (10). When the hemoglobin has been removed from the nucleus, the remaining nuclear bodies (chromatin) are Feulgen positive, approximately equal in number to the chromosomes, and similar in refractivity to mitotic chromosomes. Consequently, in examining the fine structure of chromatin there is less likelihood of confusion arising from the presence of non-chromosomal material which occurs in large amounts in many interphase nuclei.

MATERIALS AND METHODS

MATERIAL

The observations were made on blood from the heart of adult frogs. Hemolysed cells were obtained by pipetting blood directly into hemolysis solutions at 0°C. When it was necessary to change the medium, cells were centrifuged for 4 minutes at 550 *g*.

STOCK SOLUTION

A stock solution (S) was made with a variable volume *x* ml 0.1 *N* HCl, 5 ml veronal acetate (0.14 *M* sodium barbital and 0.14 *M* sodium acetate), a variable volume *y* ml 1 *M* CaCl₂, 2.8 gm sucrose, made up to 25 ml with glass-distilled water. Three parts of this stock solution were mixed with one part of a second solution (*e.g.* fixative) to give the final required solution. The pH of the final solution was varied by altering *x*; for example, *x* = 5 corresponded to pH 7.4. The calcium-ion concentration of the final solution was varied by altering *y*; for example, *y* = 0.33 corresponded to 0.01 *M*. The final concentration of sucrose was 0.24 *M*.

HEMOLYSIS SOLUTIONS

Three parts of the stock solution (S) were mixed with one part of a 1.2 per cent solution of either a non-ionic detergent Renex (supplied by Honeywell & Stein, Ltd., London) or saponin (white saponin, sup-

plied by British Drug Houses Ltd.). The final concentration of detergent was, therefore, 0.3 per cent. An experiment in which the concentration of saponin was varied showed that 0.3 per cent (with *x* = 6.9, *y* = 0.33) was the minimum quantity which gave less than about 10 per cent non-hemolysed cells. In the final solution the sucrose was 0.24 *M*.

FIXATION AND EMBEDDING

1. Fixative A was obtained by mixing 3 parts of S to 1 part of 4 per cent OsO₄. In S the pH was 6.3 (*x* = 6.9) and *y* was 0.33. In the final solution, veronal acetate was 0.023 *M*, the HCl 0.015 *N*, the calcium ion 0.01 *M*, and sucrose 0.24 *M*. Fixation times varied from 2 to 24 hours.
2. Fixative B was essentially that of Palade (18); *i.e.* 5 ml. 2 per cent OsO₄, 5 ml veronal acetate buffer, pH 7.4 (made by mixing 5 ml. veronal acetate, 5 ml. 0.1 *N* HCl, 2.5 ml. H₂O), plus 0.5 gm sucrose (0.14 *M*) (8). In the final solution, the veronal acetate was 0.03 *M* and the HCl 0.02 *N*. Fixation time was 1 to 2 hours.
3. After hemolysis, cells were washed in a solution containing 3 parts of S (*x* = 6.9, *y* = 0.33) to 1 part distilled water (washing solution A). Cells were also washed in this solution after treatment in fixative A. After fixation in B, cells were washed in a solution similar to B minus osmium tetroxide (washing solution B).
4. After washing, the cells were suspended in a drop of 2 per cent agar (25) made up in either washing solution A or B. To facilitate handling of hemolysed nuclei, 0.2 per cent methyl green was added to the agar. When cooled, the agar suspension was cut into 1 mm³ blocks for subsequent treatment.
5. Some agar blocks were either soaked in washing solution A, or in washing solution A plus 0.5 per cent uranyl acetate (0.013 *M* uranyl) prior to dehydration (25). Others were dehydrated directly in the ethanols, 30, 50, 70, 90, 95 per cent, (5 to 10 minutes in each), and 100 per cent (3 changes, total 1 hour).
6. Dehydrated agar blocks were embedded in either
 - (a) Methacrylate: ½ hour in 1 part ethanol to 1 part methacrylate (butyl 9; methyl 1); 1½ to 24 hours in methacrylate plus 2 per cent benzoyl peroxide; about 24 hours in the mixture at 48°C.
 - (b) Araldite (Glauert and Glauert, 1958) except that the longer times in Araldite mixtures with and without accelerator were doubled.
 - (c) Araldite (Luft, 1961).

In some experiments, cells were not embedded in agar but were processed by repeated centrifugation.

SECTIONING

Sections were cut with a Huxley microtome, 1 μ in thickness for light microscopy and light-silver to dark-

grey in colour before being spread with xylene vapour for electron microscopy. They were stained for 2 to 4 hours in uranyl acetate; a few sections were stained with lead hydroxide (Watson, 1958). Methacrylate sections were mounted on collodion films and a layer of carbon deposited on the section by evaporation. Araldite sections were mounted on a film of collodion plus carbon.

MICROSCOPY

For phase contrast microscopy, Zeiss Neofluor objectives, NA 1.3 or 0.65, and an aplanatic condenser were used. Photomicrographs were taken at either $\times 330$ or $\times 132$ on Kodak Microfile Pan 35 mm film with mercury monochromatic green light isolated by an interference filter.

Optical part differences were measured with a Baker interference microscope and half-shade eyepiece.

Electron micrographs were made on a Siemens Elmiskop I with a double condenser, at 80 kv, objective aperture 50 μ , and primary magnification of $\times 5,000$ or $\times 40,000$.

RESULTS

A. Nuclei in Hemolysed Cells

HEMOLYSIS

The object of the first experiment was to determine a hemolysis medium in which the volume of the nucleus did not change appreciably either during or after hemolysis. Nuclei were observed in the phase microscope during hemolysis in two series of media in which either the concentration of calcium ions or the pH was separately varied. In the first series, the pH was 7.4, and the concentrations of calcium ion were zero, 0.001, 0.0025, 0.005, and 0.01 M. At all concentrations of calcium, loss of hemoglobin was rapid. In the intact cell the nucleus contains discrete nuclear bodies and is said to be in the condensed state. During hemolysis without calcium ions the nuclear bodies immediately became swollen and merged into an almost homogeneous ellipsoid, the area of which was about the same as that of the nucleus in the intact cell. In this state the nucleus is said to be extended (22). With time, the nucleus appeared more homogeneous and the volume continued to increase. When isolation was in 0.001 M calcium the nuclear bodies were swollen to a variable degree: they began to merge into one another. At all the higher concentrations of calcium ion the nucleus contained separate and discrete nuclear

bodies. Rough measurements with an ocular micrometer showed that the dimensions of the nucleus were similar to those of the intact cell. Figs. 1 and 3 show nuclei in the condensed and extended states, respectively.

In the second series, the concentration of calcium ions was 0.01 M and the pH was decreased to 6.3. Nuclei isolated in 0.01 M calcium ion looked the same at pH 6.3 and 7.4. Nuclei also looked similar immediately after isolation in either Renex or saponin. In the experiments on fixation, the observations were made on nuclei isolated in 0.01 M calcium at pH 6.3.

As discussed below, the volume changes of nuclei are explicable in terms of electrostatics. Consequently, non-ionic detergents were chosen in preference to ionic detergents which were found to have very different effects. Similarly, the use of heparin to prevent clotting was avoided, since this molecule is negatively charged and at concentration 0.05 per cent caused nuclei to swell.

In cells hemolysed with Renex or saponin, the plasma membrane was visible in phase contrast (Fig. 1).

FIXATION OF NUCLEI

When hemolysed condensed nuclei (Fig. 1), isolated in 0.01 M calcium at pH 6.3, were placed in fixative B containing no calcium, they began slowly to expand. After 1 hour, about 50 per cent of the nuclei were in the extended state, whilst in others the nuclear bodies appeared swollen (Fig. 3). This difference may be attributed to a slow variable rate of release of calcium ions. In these experiments the time between isolation and fixation, and hence the period the nuclei were exposed to calcium ions, was short, about 5 to 10 minutes. The osmium-treated nuclei were subsequently dehydrated; at concentration of between 70 and 95 per cent ethanol, all the expanded nuclei had recondensed (Fig. 5). In the recondensed nuclei the nuclear bodies were similar in shape to those of the original isolated cell, but it was difficult to find out if the individual bodies had returned to their original volume.

Similar changes were observed in the nuclei of leucocytes (Figs. 2, 4, 5). Upon treatment in B fixative the condensed chromatin became swollen. This appearance was reversed during dehydration.

In fixative A, isolated condensed nuclei did not swell and remained condensed during fixation and dehydration. After treatment in fixative A for 4

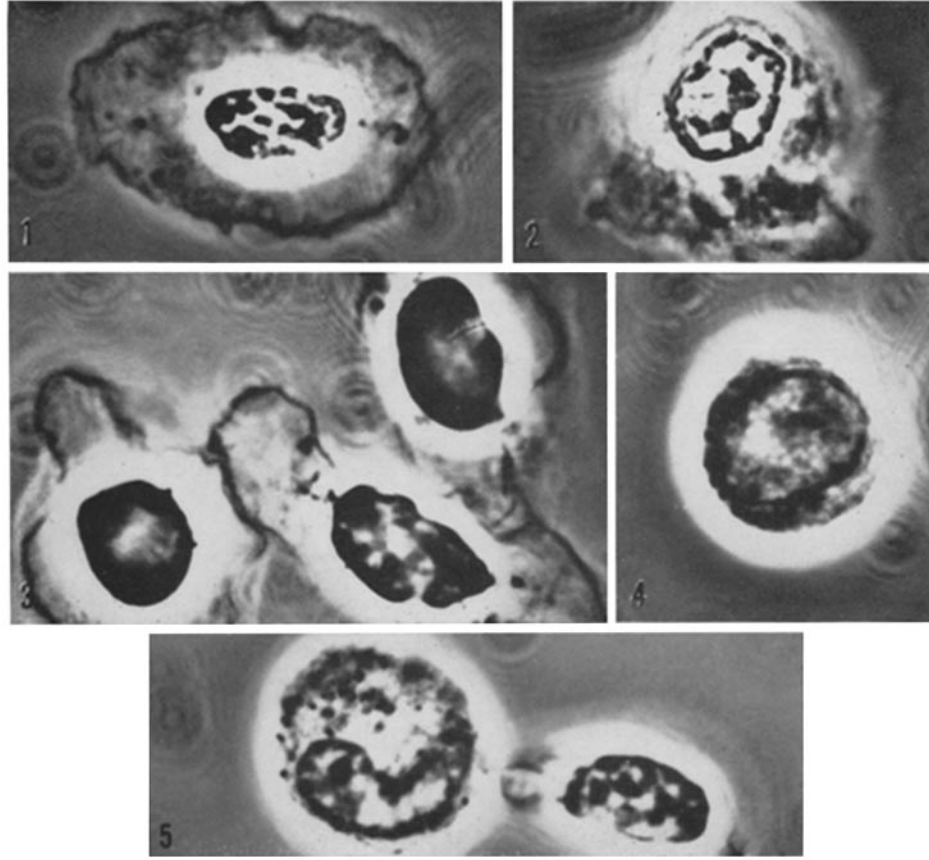


FIGURE 1

Frog erythrocyte hemolysed and mounted in a medium containing saponin (0.3 per cent), sucrose (0.24 M), calcium chloride (0.01 M), at pH 6.3. The nucleus is condensed. Phase contrast, $\times 2,000$.

FIGURE 2

Frog leucocyte similarly treated to cell in Fig. 1, showing a condensed nucleus. Phase contrast, $\times 2,000$.

FIGURE 3

Frog erythrocytes previously hemolysed in the same medium as in Fig. 1, now in Palade fixative (OsO_4 1 per cent, sucrose 0.14 M, pH 7.4). Two nuclei are in the extended state; a third is partially extended. Phase contrast, $\times 2,000$.

FIGURE 4

Frog leucocyte after treatment in same medium as in Fig. 1, now in Palade fixative. The nucleus is in the extended state. Phase contrast, $\times 2,000$.

FIGURE 5

Frog erythrocyte and leucocyte. Isolated in condensed state; treated in Palade fixative (1 hour); recondensed by dehydration in alcohol-series; photographed when in 95 per cent ethanol. The nuclei of both red and white cells are recondensed. Phase contrast, $\times 2,000$.

hours, nuclei did not subsequently expand even when treated with versene (0.25 M) for 2 hours.

ELECTRON MICROSCOPY

Apart from the fine structural detail within the nuclear chromatin, hemolysed cells fixed in both A fixative and B fixative, referred to as A type and B type cells, respectively, look similar at low power (Figs. 7, 8). The sites previously occupied by nuclear hemoglobin appear as empty spaces con-

and sheet- or rod-like structures became visible. Ill defined structures corresponding to them are seen in the electron micrographs (Fig. 7).

At low power the B nucleus appears particulate (Fig. 8), with a darker rim at the periphery. This variation is attributed to differences in shrinkage during dehydration. In contrast, the A nucleus is relatively homogeneous (Fig. 7).

At higher power (Figs. 11, 12) there is a striking difference between the appearances of the two

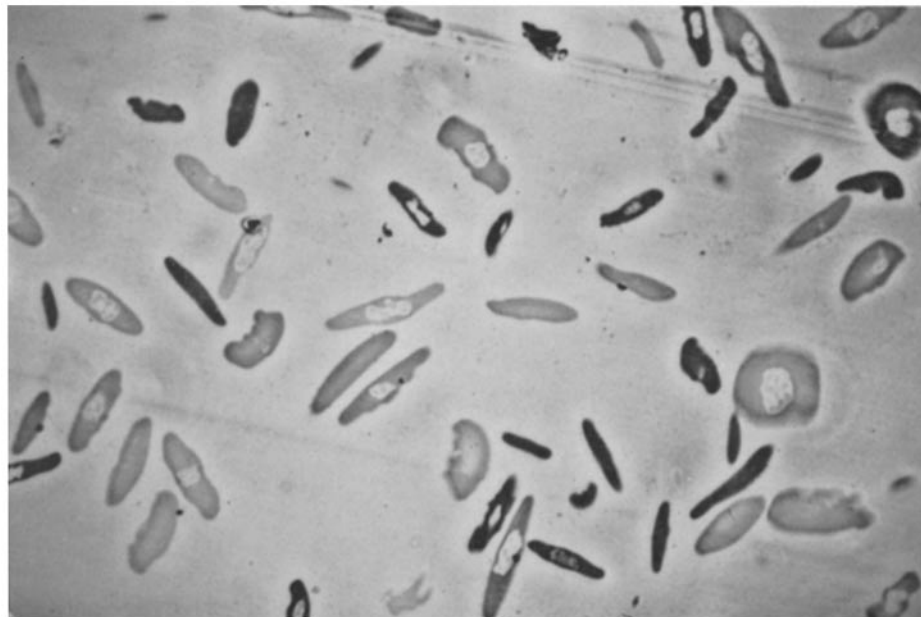


FIGURE 6

Phase contrast micrograph of a 1μ thick section of a mixture of two batches of intact frog erythrocytes separately treated in A fixative and B fixative, and mixed after dehydration, when in 100 per cent ethanol (see text). The mixture was processed in Araldite (17). Two classes of cells, with either light or dark cytoplasm, can be distinguished; these can be identified as the B and A type, respectively. $\times 400$.

nected to the empty cytoplasm by gaps in the nuclear chromatin. Empty regions also occur within the nuclei of leucocytes subjected to hemolysis medium. Presumably, these sites were occupied by the so called soluble proteins of the nucleus which are relatively easily lost during isolation (Allfrey, Mirsky, and Stern, 1955). In addition to the remains of the plasma membrane, a second membrane which is part of the nuclear envelope is loosely wrapped around the nucleus. In phase contrast when the refractive index of the cytoplasm was lowered during hemolysis, granules

types. Without attempting to infer the nature of the three-dimensional structure which gives rise to the two-dimensional image, it can be seen that the B micrograph (Fig. 11) shows irregularly-shaped patches of variable diameter ranging from about 200 A downwards. Numerous filamentous structures are also visible with variable widths from about 200 A down to the limits of visibility, about 20 to 30 A. Dimensions of about 100 A are common. The electron-opaque elements are separated by spaces, the size of which depends on section thickness; in dark-grey sections the space may

exceed 500 Å. This characteristic feature is absent from the A structure.

The nuclear structure in the A type (Fig. 12) is much more uniform. The micrographs are difficult to interpret but appear to show closely packed dots and lines of dimensions (widths) near the

When comparing the cutting properties of blocks in which fixed cells had been subsequently soaked in either washing solution A (*i.e.* 0.01 M calcium) or washing solution A containing in addition 0.013 M uranyl acetate, it was noted that the latter were more prone to a chatter artefact.

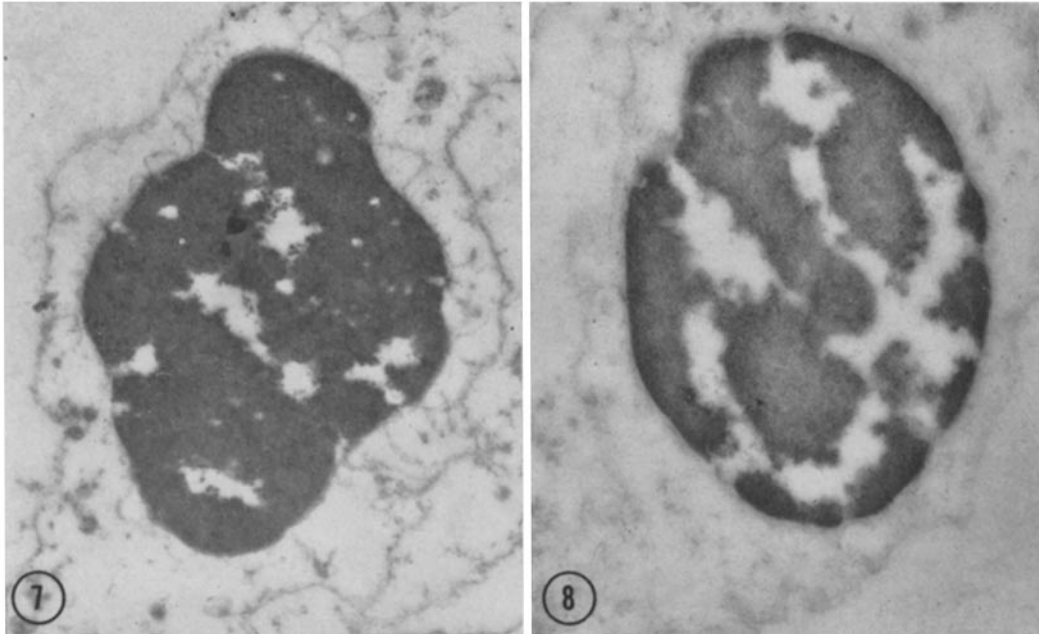


FIGURE 7

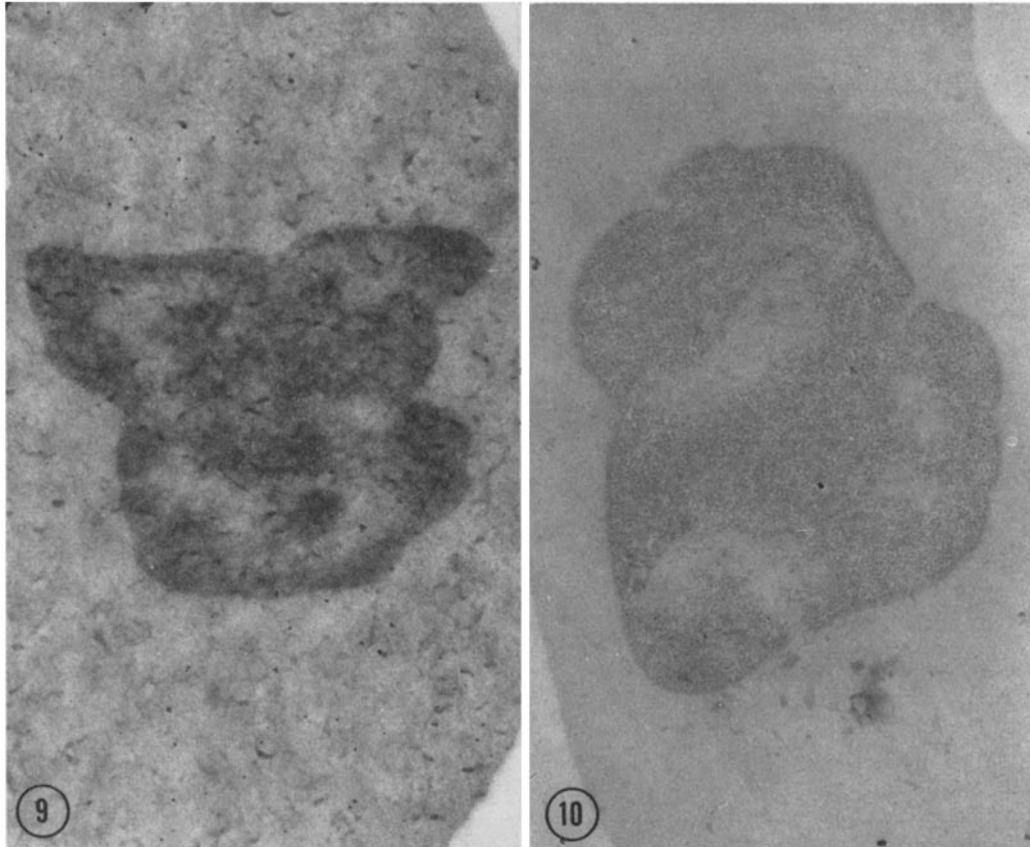
Electron micrograph of hemolysed frog erythrocyte treated in A fixative (23 hour), washing solution A (2 hour), embedded in methacrylate. The volume changes during preparation are minimal: compare with Fig. 8. Section stained in uranyl acetate, $\times 15,000$.

FIGURE 8

Electron micrograph of a hemolysed frog erythrocyte treated in B fixative (1 hour), embedded in methacrylate; section stained in uranyl acetate. The nucleus has expanded and recondensed during fixation. The periphery of the nucleus is more condensed than the interior, which appears more particulate than the nucleus in Fig. 7. Both sections were "dark grey." $\times 15,000$.

limits of visibility, roughly about 20 to 30 Å in a faint background. In one experiment, the close packing was similar in hemolysed condensed nuclei treated in A fixative alone or with a further 2 hours' treatment in washing solution A plus 0.013 M uranyl acetate. The closely packed appearance is similar after both methacrylate embedding (Fig. 12) and Araldite embedding (Fig. 13). Neither did treatment with Versene (0.25 M for 2 hours) after a long period (4 hours) in fixative A, affect the appearance of the A structure.

These conclusions are based on 19 experiments; in this context one experiment refers to one set of experimental conditions, which were varied, or repeated. Three were B fixation and 16 were variations or repetitions of A fixation. In 4 experiments the A type cells had a less homogeneous structure than that shown in Fig. 12, but it never approached the coarseness shown in Fig. 11. We assume this was due to uncontrollable variations in embedding, etc. The A type fine structure (Fig. 12) was never observed after B fixation.



FIGURES 9 AND 10

Electron micrographs from the same section (silver colour) of a block containing a mixture of A and B type cells embedded in Araldite (17) (see text); stained in uranyl acetate. $\times 22,000$. A comparison of the photographic densities of the cytoplasm with the small region of clear field identifies Figs. 9 and 10 as A type (dark cytoplasm) and B type (light cytoplasm), respectively. The nuclear-chromatin appears relatively more uniform in Fig. 9 than in Fig. 10.

B. Nuclei in Intact Cells

FIXATION

For comparison, a few observations were made on the nuclei of intact cells, to exclude the possibility that the process of isolation caused the difference between the A and B structure. For example, if lipid were present in chromatin it might be disrupted during isolation and result in a different response to fixatives with and without calcium.

When whole blood, containing intact cells, was pipetted into a relatively large volume of B fixative (zero calcium), the nuclei did not expand in such a dramatic way as did previously hemolysed nuclei similarly treated. A very small fraction of

the cells, however, began to hemolyse immediately and in these the nuclear bodies swelled to the extended state. In cells oriented with the smallest axis of the ellipsoid parallel to the optic axis, a comparison was made between the nuclear projected areas in photomicrographs of cells in serum, and in B fixative, respectively. The results showed an increase in area after about a half-hour in B fixative, corresponding to a volume increase of less than 25 per cent. This figure is inaccurate because of the large spread in nuclear areas, and more measurements would be needed to establish the magnitude of the difference. Heterochromatic regions of leucocytes also became noticeably less refractile in phase contrast during B fixation, presumably owing to swelling.

MICROSCOPY

Cells fixed by methods A and B were compared in the light microscope. In this experiment, intact cells were fixed in B fixative (1½ hours), and also in A fixative (1½ hours) followed by a period (2 hours) in washing solution A. After dehydration, A and B type cells were either mixed in 100 per cent alcohol and embedded in methacrylate and Araldite or were separately embedded.

In a 1 μ thick section of the mixture, two cell types could be clearly distinguished in phase contrast (Fig. 6), one having a relatively darker cytoplasm than the other. In a histogram (Fig. 15) of the optical path differences of the cytoplasm in randomly chosen cells, two modes are apparent. The lower mode belongs to the B type cells. This was demonstrated by measurements (Fig. 15, dotted line) from a block containing only these cells. Optical path difference, which is proportional to the concentration of hemoglobin, was measured in methacrylate-embedded cells from which the methacrylate had been removed. The concentration in the two modes was found to differ by a factor of two. In these sections the nuclei were not measured because of their small area and the presence in them of hemoglobin.

In electron micrographs also, two distinct cell types could be distinguished. Cells with light cytoplasm had a particulate nuclear structure (B type) at a magnification of 22,000 (Fig. 10), whilst in cells with a dark cytoplasm (Fig. 9) the nucleus appeared relatively uniform (A type). Apparent differences in packing can arise merely from differences in section thickness, and in the observations on isolated nuclei these were avoided, as far as possible, by comparing sections of the same colour-thickness. However, such estimates of thickness are inaccurate. In the experiment on intact cells

the two types could be examined side by side in the same section and uncertainty due to relative differences in thickness could be excluded.

Because of the close-packing of the hemoglobin molecules and the relatively small volume-fraction occupied by embedding material it was difficult to obtain good thin sections of the A type cell. In Fig. 9, distortion of the section produced by cutting is apparent; both hemoglobin and chromatin are affected. The B type cells were correspondingly easy to section, and at high-power (Fig. 14) the structure of the nuclei was similar to that of the hemolysed nuclei similarly fixed. Structure could occasionally be seen in the electron-opaque patches. For example, circular patches 100 A in width with hollow centres were observed; these resemble sections through tubules.

DISCUSSION

Volume Changes—Contribution of Osmotic and Electrostatic Phenomena

Changes in the volume of cells during fixation are well known. Bahr, Bloom and Johannisson (1958) showed that the volume of chicken erythrocytes and ascites cells increased during fixation in osmium tetroxide. For example, in 1 per cent OsO₄ and 1.5 per cent dextran made up in Tyrode, the volume of erythrocytes increased by about 40 per cent. The swelling could be prevented by the addition of suitable concentrations of sucrose and dextran. These workers concluded that the chief factor governing swelling was osmotic. Bahr, Bloom, and Friberg (1957) have also shown that tissues swollen after fixation in osmium tetroxide shrink when subsequently dehydrated in ethanol.

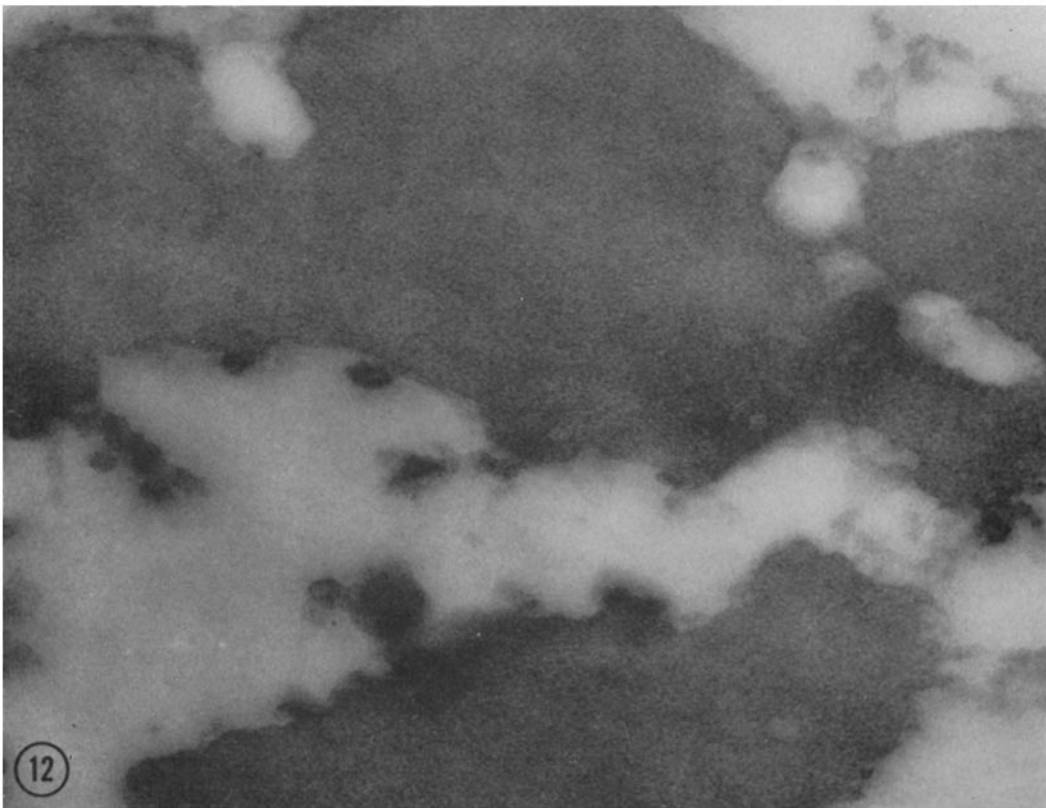
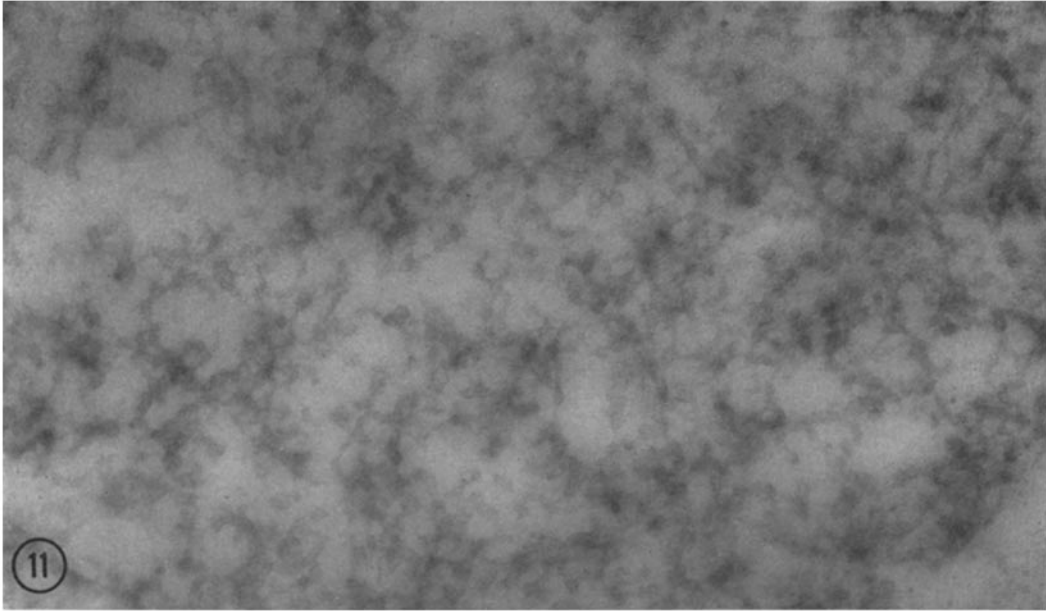
In contrast, the volume changes in the nuclei of hemolysed erythrocytes are due mainly to

FIGURE 11

Electron micrograph of a frog erythrocyte nucleus isolated in the condensed state; subsequently treated in B fixative (1 hour); embedded in methacrylate; stained in uranyl acetate. \times 120,000. The nucleus has recondensed in ethanol and should be compared with Fig. 12.

FIGURE 12

Electron micrograph of a frog erythrocyte isolated in the condensed state; subsequently treated in A fixative (4½ hours), followed by 2 hours in washing solution A; embedded in methacrylate; stained in uranyl acetate. \times 120,000. This treatment results in a more uniform appearance (A type nucleus) than that shown in Fig. 11 (B type nucleus).



electrostatic factors. Osmotic factors must have a negligible role in hemolysed cells since both the plasma membrane and the nuclear envelope, which contains pores of up to about 100 Å in diameter (10), are permeable to large hemoglobin molecules. Sucrose was included in the isolation medium in order to prevent an osmotic shock which might have led to swelling of the nucleus,

only in isotonic sucrose (Allfrey, Mirsky, and Osawa, 1957).

The nuclear bodies are known to contain DNA and histone as well as, in chicken at least, a second protein equal in quantity (Stern, Allfrey, Mirsky, and Saetren, 1952) to the histone but of unknown significance. A rough explanation of the volume changes can be given in terms of the following

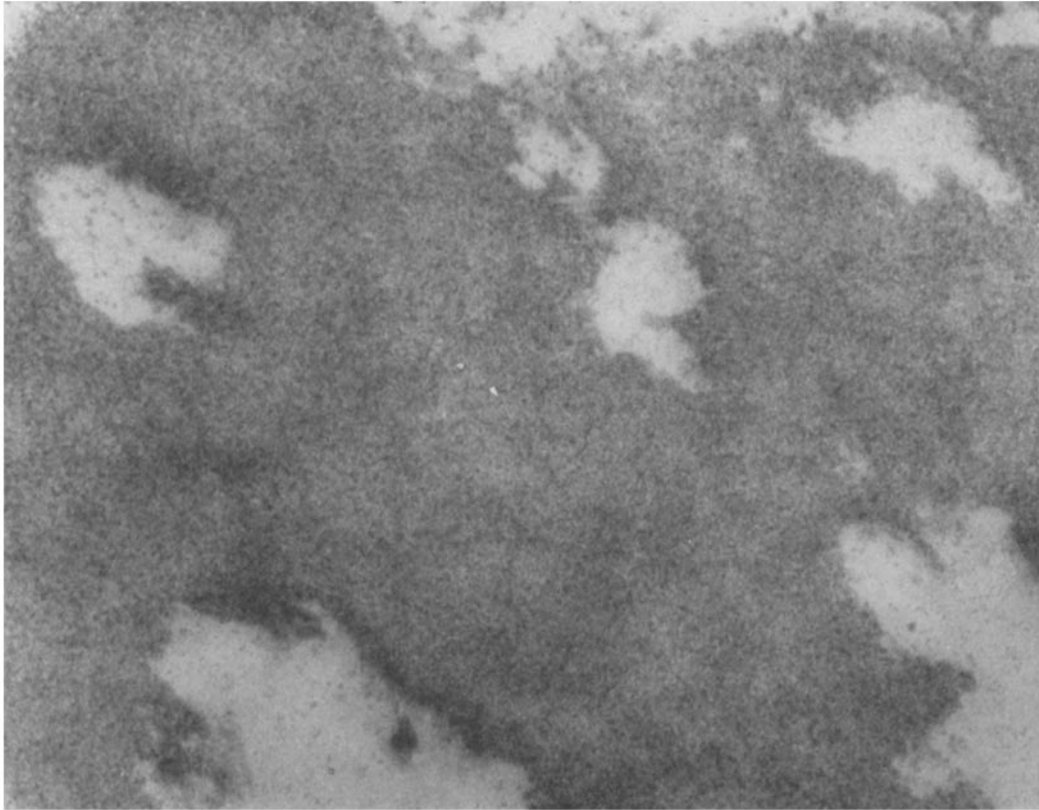


FIGURE 13

Electron micrograph of a frog erythrocyte isolated in the condensed state; subsequently treated in A fixative (19 hours), followed by 2 hours in 0.013 M uranyl acetate in washing solution A; embedded in Araldite (17); stained with lead hydroxide. $\times 120,000$. The close-packing is similar to that observed after methacrylate embedding (Fig. 12).

during the release of nuclear hemoglobin, followed by subsequent shrinkage. Although volume changes in nuclei may appear reversible in the light microscope, it is not known whether they are truly reversible at a molecular level, and, therefore, they were avoided as far as possible during isolation. The washing solution A was kept at 0.25 M sucrose because other nuclei, those of thymus, have been shown to be functionally active

model of the nuclear chromatin. We assume in the model that the DNA phosphate groups which are ionised and carry a negative charge are only partially neutralised by the positively charged histone molecules (see also Peacocke, 1960, and reference 21). The molecular configuration assumed depends *in part* on this net negative charge which causes nucleohistone aggregates to repel one another. In the intact erythrocyte the

nucleohistone is in a condensed state because, we may assume, the net negative charge is more or less neutralised by positively charged ions in the environment. It is possible that the ionic environment produced by hemoglobin is important. Apparently, conditions change upon isolation at physiological pH so that the nuclei expand. Addition of calcium ions, however, neutralises the

Heparin was avoided in the isolation medium because it caused nuclei to expand independently of the calcium ion concentration. Heparin, which is negatively charged, competes with DNA for binding to histone (Kent, Hichens, and Ward, 1958). Removal of histone from DNA leaves the negatively charged DNA free to expand.

Our observations on swelling and shrinking of

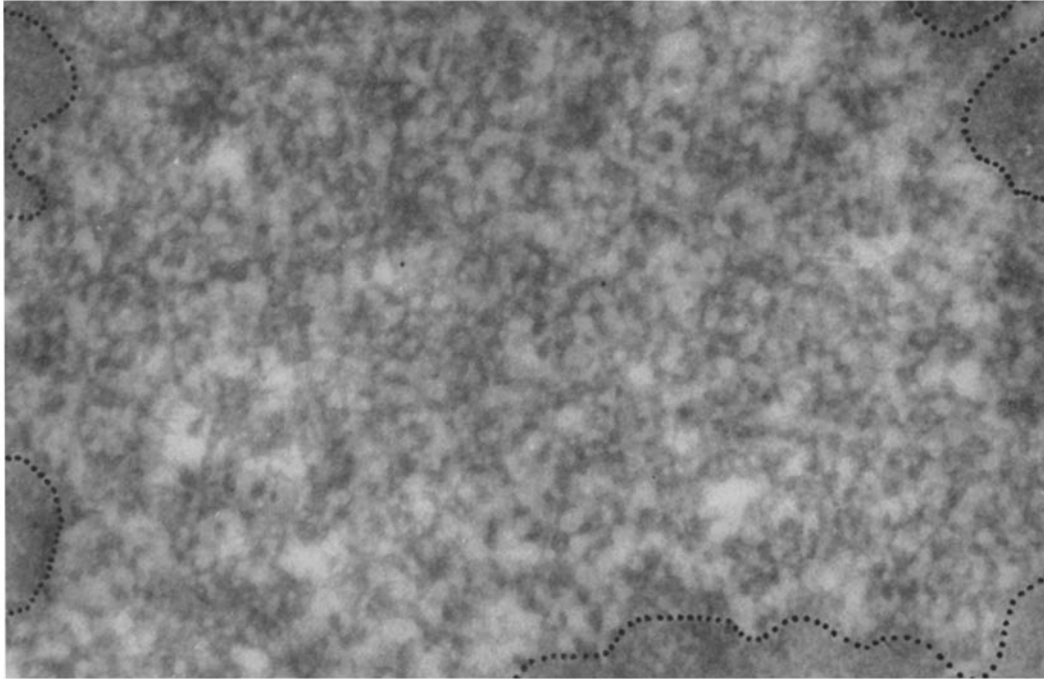


FIGURE 14

Electron micrograph of nuclear chromatin in an intact frog erythrocyte treated in B fixative (1 hour); embedded in methacrylate; stained in uranyl acetate. $\times 120,000$. The chromatin consists of separated irregularly shaped electron-opaque patches. A comparison with the cytoplasm (not shown) indicates that the denser regions enclosed by the dotted lines contain hemoglobin.

repulsive effect of the negative charges and the nuclei either recondense if previously expanded, or can be isolated in the condensed state. Nuclei may also be recondensed by substituting ethanol for water or by addition of salt at physiological pH. Monovalent ions are about one hundred times less effective than divalent ions in preventing swelling in gels of isolated thymus nucleoprotein (Ambrose and Butler, 1953). A similar relationship between the effects of ions of different valencies is found in the case of lyophobic colloids (Verwey and Overbeek, 1948).

nuclei suggest that requirements for fixation may be different in different cell types. In erythrocytes the nuclear bodies appear to be in a permanently condensed state, similar to mitotic chromosomes, but dissimilar to the DNA-containing regions of many interphase cells. It is known (22) that, at acid pH or in the presence of ions, chromosome-like bodies or condensed regions can be produced in such interphase cells, and hence we should not expect fixative A to be suitable for preserving these interphase nuclei.

The results reported show that treatment with

OsO₄ alone does not prevent volume changes occurring in nuclei in response to changes in the ionic strength or dielectric constant of the medium. Thus, nuclei treated briefly with 0.01 M calcium ion during isolation extend when placed in OsO₄, due presumably to loss of calcium ions from the nuclei. Also, when these extended nuclei that have been treated with OsO₄ for 1 hour are put into a medium of lower dielectric constant, containing ethanol, they recondense. This result is not entirely unexpected since DNA does not combine chemically with OsO₄ (Bahr, 1954; Davies,

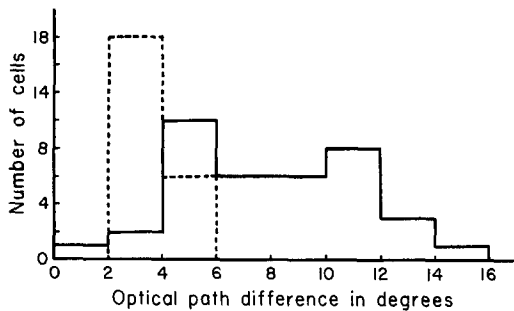


FIGURE 15

Histogram showing the distribution of optical path differences in the cytoplasm of frog erythrocytes randomly chosen from a mixture of cells fixed separately in A and B fixative, but embedded together (see text). The dotted line shows the distribution in a second 1 μ thick section from a block containing only B type cells, also Araldite-embedded (17). Optical path differences in degrees are measured relative to the clear embedding medium.

1954). However, treatment of condensed nuclei with OsO₄ plus calcium (0.01 M) for about 2 hours results in a stable state in which nuclei apparently cannot be re-extended even by treatment with a chelating agent (0.25 M Versene).

Comparison with Other Electron Microscope Observations

The results on erythrocyte nuclei are, in several respects, very similar to those of Ryter and Kellenberger (1958) on bacterial nuclei. These workers treated bacteria in fixatives containing 1 per cent OsO₄, at pH's 6.0, 7.2, and 8.0, and various concentrations of calcium ions. In a fixative containing 0.01 M calcium ion at pH 6.0, followed by subsequent treatment in 0.01 M calcium or pref-

erably 0.013 M uranyl acetate in addition to calcium ions, a homogeneous fine fibrillar appearance of the nucleus was obtained. In contrast, in the absence of calcium or uranyl ions, a coarse nuclear structure was found. These two states of the bacterial nucleus are very similar to the A and B structures found in erythrocytes. It is not known with certainty whether the bacterial nucleus contains protein in addition to DNA or whether protein, if present, is combined with DNA. Although a protein was isolated from *E. coli* in association with DNA (Zubay and Watson, 1959), x-ray diffraction photographs showed (Wilkins and Zubay, 1959) that the isolated material resembles a mixture of DNA and protein. Consequently, it is perhaps surprising that the conditions that result in a relatively homogeneous structure in erythrocyte nuclei are similar to those already reported by Ryter and Kellenberger to produce a homogeneous fine structure in bacterial nuclei.

The results on erythrocyte nuclei show that discrete filaments or possibly tubules varying in diameter from about 200 A downwards can be produced in the chromatin of higher organisms by what appears to be an improper fixation, accompanied by volume changes. Since different nuclei may require different fixation conditions, it is difficult to relate our results to those of others on different cell types in which various molecular aggregates of dimensions of about 100 A have been observed. It has been clearly shown that DNA and protein molecules can sometimes aggregate *in vivo* into supramolecular structures, as, for example, in spermatogenesis where lamellae may be observed (Ris, 1959). We consider, however, that the separated 100 A structures we observe after B fixation of erythrocyte nuclei are an artefact that arises when extended nuclei shrink upon treatment with ethanol. This artefact may be similar to, but finer than, the coarse precipitate produced when many interphase nuclei are fixed directly in absolute ethanol. The latter precipitate is visible in the light microscope. When nuclei are stabilised against volume change, the relative positions of the molecules are maintained, leading to a more uniform A structure. This probably represents a closer approximation to the original structure.

Since this work was completed, Robbins in an interesting paper (Robbins, 1961) has demonstrated the swelling of nucleoli and metaphase chromosomes, and also an increased homogeneity of the nucleoplasm in HeLa cells fixed in hypotonic

1 per cent OsO₄. He has also shown, in agreement with our observations on isolated nuclei, that swelling during fixation can be eliminated by the addition of ions; altering the dielectric constant of the medium also prevented swelling. Huxley and Zubay (1961) have also commented on the role of calcium ions during the preparation of isolated nucleohistone fibers for electron microscopy. In the absence of calcium ions, there was inadequate penetration of the embedding medium which was attributed to the material's becoming too densely packed during fixation and dehydration to permit

infiltration of the embedding medium. This effect was avoided when the fixative contained 0.01 M calcium chloride.

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