MEASUREMENTS OF SOME CELLULAR CHANGES DURING THE FIXATION OF AMPHIBIAN ERYTHROCYTES WITH OSMIUM TETROXIDE SOLUTIONS

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ABSTRACT

On average, 15 per cent of the total haemoglobin present in the blood of the newt *Triturus cristatus* was extracted during 45 minutes of fixation in Palade-Caulfield fixative. This extraction was reduced with fixatives buffered at pH 6.2 instead of pH 7.4. The addition of Ca^{++} ions to a final concentration of 0.01 M in the fixative completely suppressed haemoglobin extraction. The effect of the pH, and the presence or absence of Ca^{++} ions in the fixative, on the rate of haemoglobin extraction has been determined. During Palade-Caulfield fixation the average projected area of newt erythrocytes increased by 37 per cent, and after dehydration and embedding in Epon the average area was 25 per cent greater than that of the unfixed cell. Fixatives buffered at pH 6.2 and containing 0.01 M Ca^{++} ions caused cellular shrinkage, with the average projected area decreasing by 10 per cent in the fixative. This shrinkage continued during dehydration, and the final average area of the erythrocytes in Epon was 26 per cent less than that of the unfixed cells. Similar measurements with erythrocytes of *Amphiuma tridactylum* showed that after Palade-Caulfield fixation the average cellular area was increased by 45 per cent, and after dehydration and embedding in Araldite it was 36 per cent greater than that of the unfixed cell. The average nuclear area increased by 35 per cent during fixation but after embedding it was 26 per cent greater than that of the unfixed nuclei. With a fixative at pH 6.2 containing 0.01 M Ca^{++} ions, both the nucleus and the whole cell shrank during fixation. The nuclear area decreased by 20 per cent and the cellular area by 22 per cent. After dehydration and embedding in Araldite, the average nuclear area had decreased by 35 per cent and the cellular area by 40 per cent. It has been shown that OsO_4 fixation lowers the isoelectric points of haemoglobins and other proteins. This finding has been used in the interpretation of the observed cellular changes resulting from fixation.

INTRODUCTION

Despite the widespread use of OsO_4 as a fixative for electron microscopy, few quantitative studies of cellular changes occurring during such fixation have been made. This doubtless reflects the difficulty in finding useful parameters to measure. When tissues, free cells, or isolated nuclei in suspension are immersed in OsO_4 solutions, swelling generally occurs (Bloom and Friberg, 1956; Bahr *et al.*, 1957; Bahr *et al.*, 1958; Davies and Spencer 1962). This swelling can be countered by altering

the tonicity, pH, or ionic composition of the fixative vehicles. Changes in the weight and specific gravity of tissue blocks resulting from OsO_4 fixation have been measured by Bloom and Friberg (1956) and Bahr *et al.*, (1957). Bahr (1955) determined the uptake of OsO_4 by various tissues and the amount of organic material extractable from tissues after fixation. Recently, Kushida (1962) using sea urchin eggs, measured the volume changes during fixation with OsO_4, dehydration, and embedding in methacrylate and epoxy resins.

Before making a study of the fine structure of erythrocytes from *Triturus cristatus* and *Amphiuma tridactylum*, it was desirable to measure the cellular changes resulting from OsO_4 fixation. The following measurements were made: the absolute amount of haemoglobin extracted during fixation; the rate of haemoglobin extraction during fixation; the effects of pH, ionic composition, and tonicity upon the rate of haemoglobin extraction; and changes in the projected areas of the cells and nuclei during fixation, ethanol dehydration, and embedding.

OsO_4 is known to have a greater affinity for the basic amino acids than for the acidic ones (Bahr, 1954) and it destroys the basic properties of these amino acids (Baker, 1958, 1960). It has now been shown by zone electrophoresis that the isoelectric points of haemoglobin and other proteins are lowered by fixation with OsO_4. This fact has been used in the interpretation of the cellular changes observed and allows some general observations on OsO_4 fixation to be made.

MATERIALS AND METHODS

For all experiments with newts the blood was obtained from the heart of animals anaesthetised with MS 222 (Sandoz Ltd., Basel, Switzerland). There is considerable variation in the haemoglobin content per unit volume of blood obtained from different specimens; thus a mature male may have almost twice as much haemoglobin per unit volume of blood as a mature female. To reduce this variation to a minimum, immature females of the same size were used wherever possible. In the experiments with *Amphiuma tridactylum*, the animals were anaesthetised with MS 222 and blood was drawn from the tail.

Fixatives, Embedding Material, and Haemolysis Solution

Fixative A was that described by Davies and Spencer (1962), containing 1 per cent OsO_4 solution buffered with Veronal-acetate at pH 6.2, 0.24 M sucrose, and 0.01 M Ca^{++} ions added as calcium chloride. This fixative was modified by raising the pH of the buffer to pH 6.8 or pH 7.4, or by omitting the Ca^{++} ions. Fixation time was 1½ hours.

Fixative B was that of Palade (1952), containing a 1 per cent solution of OsO_4 buffered at pH 7.4 and 0.14 M sucrose (Caulfield, 1957). Fixation time was 45 minutes.

A solution of 1 per cent OsO_4 in glass distilled water at pH 6.2 was also used as a fixative. The fixation times were 45 minutes or 1½ hours.

The cells were dehydrated through a graded series of ethanol, 50 per cent, 70 per cent, and 90 per cent, and three changes in absolute alcohol, staying ½ hour in each. The embedding media were Epon (Luft, 1961), Araldite (Glauert and Glauert, 1958) and methacrylate. The fixation and dehydration were performed in bulk in a centrifuge tube. At each change the erythrocytes were spun down to a pellet, the fixative or alcohol was drawn off and replaced by the next in the series, and then the cells were resuspended. At each stage a small sample was removed for microscopy and the projected areas of these cells and their nuclei were measured. Since the size of these samples was very small compared with the total population, the cell numbers were effectively constant during fixation and dehydration.

The haemolysis solution contained 0.01 M Ca^{++} ions, 0.24 M sucrose, and 0.3 per cent saponin in distilled water (Davies and Spencer, 1962).

Spectroscopic Measurements

The spectroscopic measurements were made with a Perkin Elmer 137 UV spectrophotometer. Quartz 1 cm path-length cells were used in all the experiments. For measurements of the total haemoglobin, of the amount of haemoglobin extracted during fixation and the rate of haemoglobin extraction, a constant volume, 0.03 ml, of blood was used. The fixative and haemolysis solutions were at, or were corrected to, a constant volume, 2.5 ml, so that the amount of haemoglobin in these solutions was directly proportional to their measured optical densities and has been referred to in optical density units (OD units).

Measurement of Total Haemoglobin Present in 0.03 ml of Blood

0.03 ml of blood was added to 10 ml of haemolysis solution at 5°C in a centrifuge tube. After 5 minutes the ghosts were centrifuged down. The absorption spectrum of the haemolysate was then measured against a blank of haemolysis solution. The optical density of the solution at λ415 mμ was obtained from the spectrum. This experiment was repeated four times using separate animals and the average optical density at λ415 mμ was found.

Measurement of the Amount of Haemoglobin Extracted During Fixation

2.5 ml of the fixative were pipetted into a centrifuge tube and a layer of liquid paraffin was floated on the fixative. The paraffin prevents frothing when the blood is added to the fixative. The tube was then chilled to 5°C and the 0.03 ml of blood added. The blood and fixative were thoroughly mixed, care being taken to avoid any frothing and loss of cells in the paraffin layer. The tubes were allowed to stand in the dark at 5°C for the required time. When the fixation was complete, the cells were spun down at 3,500 RPM and 1 ml of the fixative supernatant was removed and diluted in 3 ml of water. The absorption spectrum, and hence the optical density at λ394 mμ, of the diluted solution was measured against a blank of glass-distilled water. This experiment was repeated with blood from five different animals.

Measurement of the Rate of Haemoglobin Extraction During Fixation

The fixations were done in a spectrophotometer cell which was maintained at constant temperature of 4 to 6°C. 2.5 ml of the fixative were pipetted into the cell and a layer of liquid paraffin added. Then 0.03 ml of blood was introduced and mixed with the fixative. The blank consisted of similar spectrophotometer cell containing fixative alone.

At known times after the beginning of fixation, the spectrophotometer cells were spun gently in a centrifuge tube causing the erythrocytes to sediment as a layer on the bottom of the cell leaving clear fixative above (care was taken to sediment the erythrocytes completely and hence avoid spurious optical density readings). The absorption spectrum of the fixative layer was then measured.

After each determination the erythrocytes were resuspended in the fixative by shaking.

Measurements on Solutions of OsO_4-Fixed Haemoglobin

A solution of oxyhaemoglobin was prepared by lysing saline-washed erythrocytes with distilled water. This solution was then denatured with OsO_4 and changes in the absorption spectra due to denaturation were determined. These solutions were also used to show that haemoglobin which has been fixed with OsO_4 obeys the Beer-Lambert law.

Measurements of the Optical Density of the Fixatives

The fixatives, A and B, were diluted 1:3 with water and their absorption spectra measured against a water blank. From these spectra the optical densities of the fixative solutions at λ394 mμ were measured.

Measurements of Projected Areas During Fixation

Phase contrast micrographs of unfixed erythrocytes in amphibian Ringer's, and of erythrocytes at various stages during fixation, dehydration, and after polymerisation in the embedding media, were taken on Kodak microfile Pan 35 mm film, in green light isolated by an interference filter; a Zeiss photomicroscope was used with an aplanatic condenser na 1.4. To prevent compression of the cells between slide and coverslip, a ribbon of paraffin wax of suitable thickness was used as a spacer (Bell, 1962). The coverslip was sealed onto the wax ribbon spacer with thick silicone grease. In all cases the cells were floating freely and did not adhere to the slide or coverslip. The final stage, embedding, was done on a glass slide with coverslip suitably supported with glass capillary tube to prevent any cellular compression. Tracings of the projected areas of the cells were measured by planimetry. Twenty-five to 50 cells were measured at each stage and the average values were taken. Since the population is uniform, no attempt was made to follow the size changes in individual erythrocytes.

Zone Electrophoresis

A Shandon horizontal strip apparatus was used with cellulose acetate paper (Oxoid) and Owen's acetate/barbiturate buffer at pH 8.6 and ionic strength of 0.1. A constant voltage of 110 volts was used. The samples were run for 2 hours at room temperature and, after drying, stained with nigrosine or ponceau S stains.

RESULTS

Some Properties of OsO_4-Fixed Haemoglobin

In the present experiments the amount of haemoglobin extracted during fixation was measured spectroscopically. Fixation denatures the haemoglobin and it was essential, therefore, to investigate the resulting change in the haemoglobin absorption spectrum and also to show that solutions of OsO_4-fixed haemoglobin obey the Beer-Lambert law.

When oxyhaemoglobin is fixed with OsO_4 the typical absorption maxima at λ540 mμ and λ570 mμ are lost and the Soret peak is shifted from λ415 mμ to λ394 mμ (Tooze and Davies, 1963). These changes indicate that the protein has been denatured but the haem prosthetic group is retained giving the Soret maxima. By taking equal volumes of a concentrated solution of oxyhaemoglobin, diluting them in either water or 1 per cent OsO_4 solution and then measuring the optical density

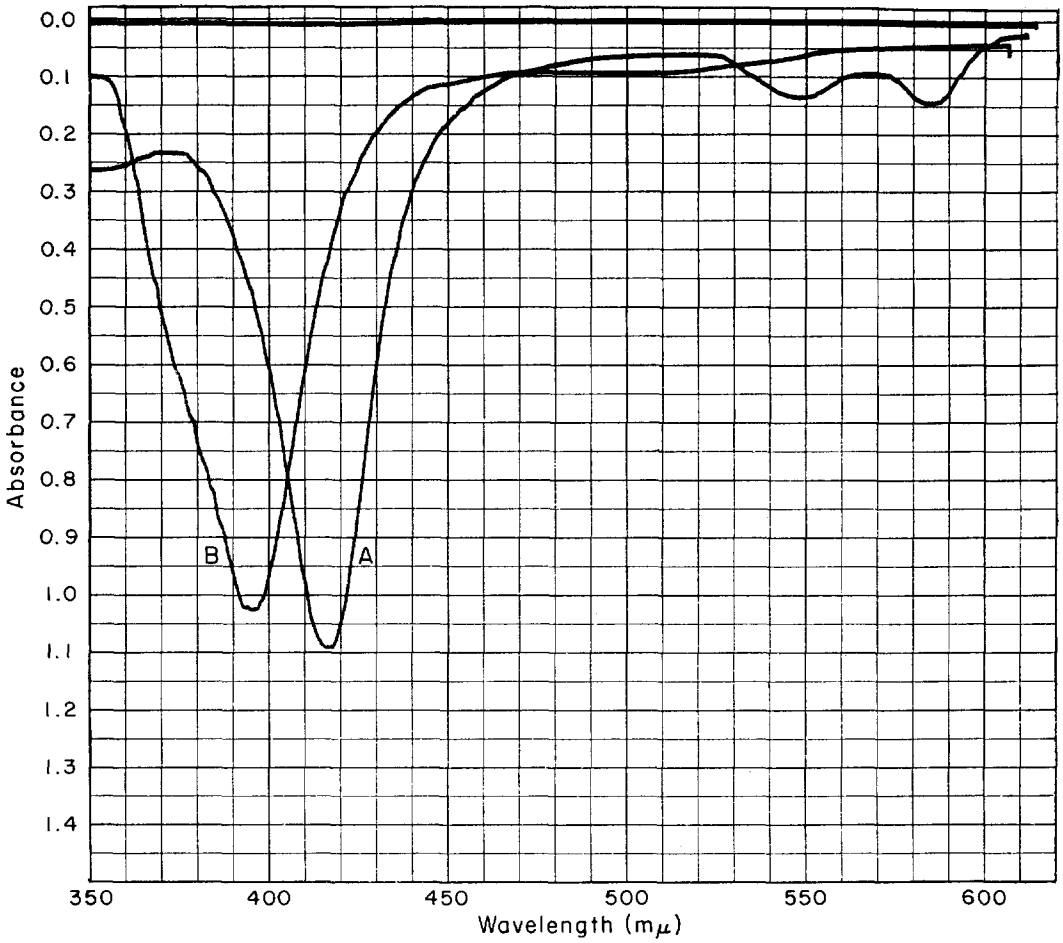

FIGURE 1 The absorption spectra of solutions of equal concentration of newt oxyhaemoglobin (*A*) and OsO_4-fixed haemoglobin (*B*). The wavelength of the Soret maxima has shifted as a result of fixation, but the optical densities at the maxima are very similar.

at the Soret maximum, it has been shown that, although the wavelength of the maximum shifts on denaturation, the optical density at the maximum changes very little. Effectively 1 OD unit of oxyhaemoglobin measured at λ415 mμ yields 1 OD unit of denatured haemoglobin measured at λ394 mμ (see Fig. 1).

The absorption spectra of solutions of denatured haemoglobin at various concentrations were measured. The optical densities at λ394 mμ and λ370 mμ were plotted against the concentration protein. The graphs show that, at the concentrations measured, these solutions obey the Beer-Lambert law and the method is valid (see Fig. 2).

The change in isoelectric point of newt haemoglobin resulting from OsO_4 fixation has been determined. Native newt haemoglobin has an isoelectric point in the range pH 6.9 to 7.2 (Gratzer and Allison, 1960). The isoelectric point of the fixed haemoglobin, determined approximately by titration, is in the range pH 5.0 to 5.2. It has also been shown, by zone electrophoresis, that OsO_4 fixation lowers the isoelectric point of other proteins (see Fig. 3). The significance of this finding is discussed later.

The Amount of Haemoglobin Extracted During Fixation

To measure the amount of haemoglobin extracted after fixation, the absorption spectrum of the fixative supernatant was measured against a water blank and the absolute optical density at

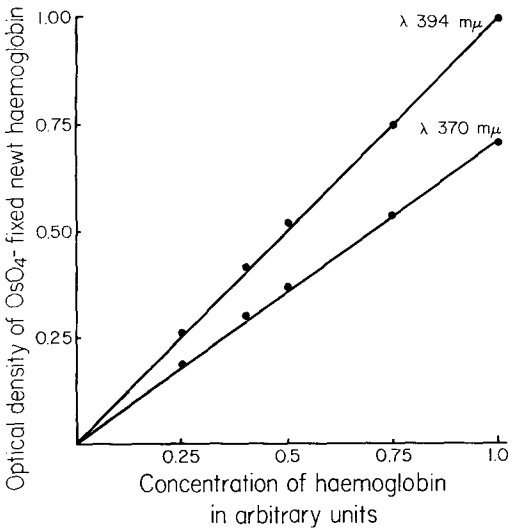

FIGURE 2 The optical densities at λ394 mμ and λ370 mμ of solutions of OsO_4-fixed newt haemoglobin are plotted against the protein concentrations of the solutions. The straight line graphs passing through the origin indicate that the fixed haemoglobin obeys the Beer-Lambert law.

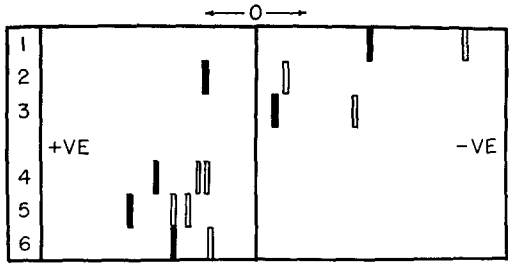

FIGURE 3 The zone electrophoretic migrations, at pH 8.5, of native and OsO_4-fixed haemoglobins and other proteins are shown. The open bars indicate unfixed proteins and the solid bars the fixed proteins. The origin is marked *O* and the direction of migration is shown by the arrows. The proteins were numbered as follows: *1*, salmon sperm protamine; *2*, DNAse; *3*, trypsin; *4*, *Triturus cristatus* haemoglobin; *5*, *Amphiuma tridactylum* haemoglobin; and *6*, human haemoglobin A. In all the experiments the migrations after OsO_4 fixation indicated that the isoelectric points of the proteins had been lowered.

λ394 mμ was obtained from the spectrum. At this wavelength the optical density of the supernatant is the result of two components, the extracted denatured haemoglobin and the OsO_4 of the fixative.

A and B fixatives are effectively 1 per cent solutions of OsO_4. The optical density of a 1 per cent solution of OsO_4 at λ394 mμ was measured. If it is assumed that none of the OsO_4 is removed from the fixative during fixation, this value can be used for the contribution of the OsO_4 to the absolute optical density of the fixative supernatant at λ394 mμ, and hence the absolute optical density of the extracted haemoglobin can be found. Less than 10 per cent of the OsO_4 was, in fact, removed from the fixative solution by the blood during fixation and the error in the spectroscopic measurements of haemoglobin caused by this OsO_4 uptake was, therefore, negligible. The results for fixative B, using five separate animals, are shown in Table I.

It has been shown that 1 OD unit of oxyhaemo-

TABLE I

The Extraction of Haemoglobin During Fixation with Fixative B

Animal number	OD at λ394 mμ of B fixative supernatant diluted 1:3 with H_2O	OD at λ394 mμ of B fixative supernatant corrected for OsO_4 contribution	OD at λ394 mμ of B fixative supernatant corrected to 2.5 cc of solution
1	0.49	0.39	1.56
2	0.26	0.16	0.66
3	0.36	0.26	1.04
4	0.29	0.19	0.76
5	0.32	0.22	0.88

Average OD of the fixative supernatant after 0.03 ml blood was fixed in 2.5 cc of B fixative = 0.98.

The haemoglobin extracted during B fixation can be expressed as a percentage of the total haemoglobin present. The average optical density of the total haemoglobin in 0.03 ml of newt blood diluted in 2.5 cc of haemolysis solution is 6.69 measured as oxyhaemoglobin at λ415 mμ (see Table II).

TABLE II

The Measurement of Total Haemoglobin in 0.03 *ml of Blood*

Animal number	OD of total haemoglobin in 0.03 ml of blood at λ415 mμ diluted to 2.5 cc of solution
6	7.08
7	6.75
8	6.80
9	6.12

Average OD of total haemoglobin in 0.03 ml blood diluted in 2.5 cc of haemolysis solution = 6.69.

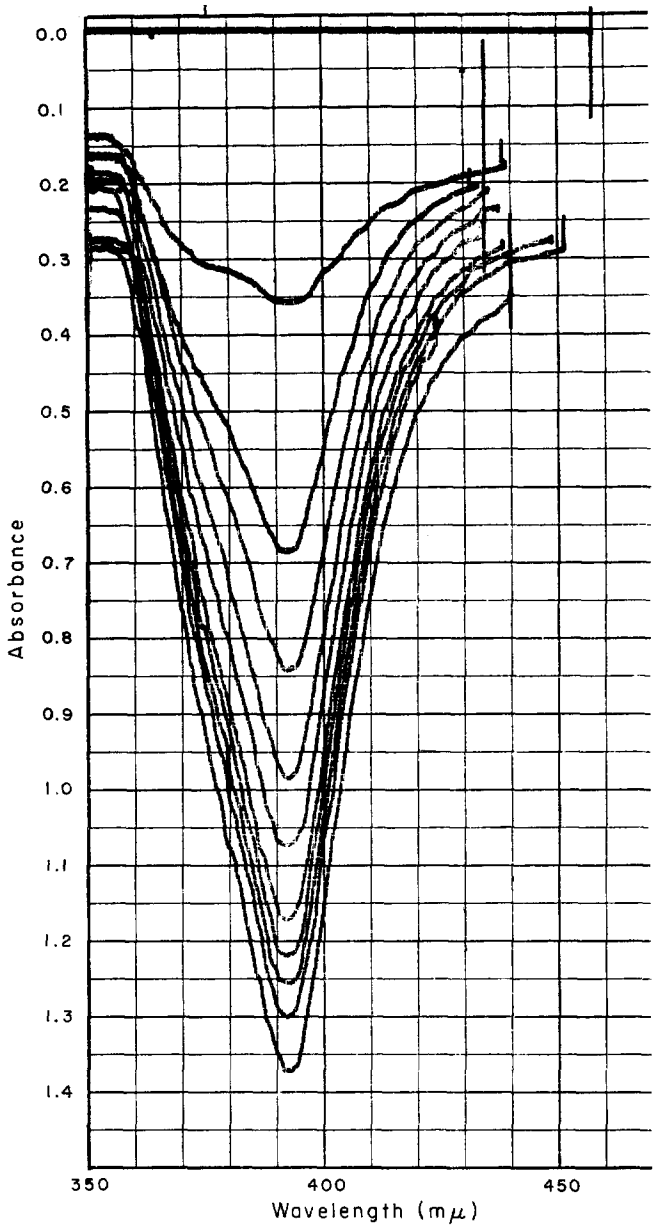

FIGURE 4 The absorption spectra, in the Soret band region, of fixative B measured at various times during fixation of 0.03 ml of newt blood in 2.5 ml of fixative, at 5°C, are shown. The spectra shown were recorded 6, 15, 24, 34, 47, 61, 71, 84, 98, and 112 minutes after the beginning of the fixation, respectively.

globin when fixed yields approximately 1 OD unit of denatured haemoglobin. Hence the percentage extraction of haemoglobin =

$$\frac{\text{OD of extracted haemoglobin } (\lambda 394\ \text{m}\mu) \times 100}{\text{OD of total oxyhaemoglobin } (\lambda 415\ \text{m}\mu)}$$

The average total amount of haemoglobin present is 6.69 OD units (see Table II) and the average amount of haemoglobin extracted during B fixation is 0.98 OD units; the highest extraction was 1.56 OD units, and the lowest extraction 0.66 OD units. These values correspond with an average extraction of 14 per cent of total haemoglobin present, while the highest and lowest extractions measured were 20 per cent and 10 per cent of the total, respectively.

When these experiments were repeated with

fixative A, there was no detectable extraction of haemoglobin during the 1½ hours of fixation. Similarly, Davies (unpublished results) found that the erythrocytes of fish lost a large amount of haemoglobin during fixation with fixative B. This loss was reduced by lowering the pH of the fixative buffer or by adding 0.02 M Ca^{++} ions to the fixative.

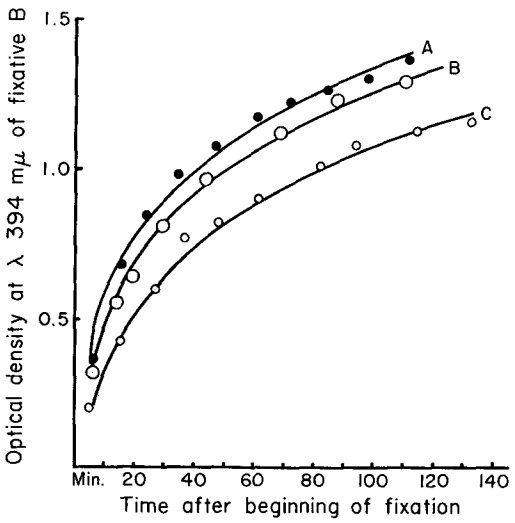

FIGURE 5 The optical densities at λ394 mμ, of B fixative, have been plotted as a function of time after the beginning of fixation of newt erythrocytes. *A* is drawn from Fig. 4. *B* and *C* are from experiments with two other newts.

Measurements of the Rate of Extraction of Haemoglobin During Fixation

In the previous experiments the absolute optical density of the haemoglobin that had been extracted during fixation was measured at the end of the process. By performing the fixation in a spectrophotometer cell it is possible to measure the optical density of the fixative at any time during fixation and hence estimate the rate of extraction.

Fig. 4 is the result of an experiment in which the absorption spectra in the Soret band region of fixative B, at 5°C, were measured at various time intervals after the beginning of fixation of 0.03 ml of newt blood. The fixative optical density at λ394 mμ has been plotted as a function of time (see Fig. 5, *A*). Two other experimental results have also been plotted (Fig. 5, *B* and *C*). As in the previous experiments, there is considerable experimental variation because different animals have been used for each experiment. At 45 minutes, the usual time used for the B fixation of amphibian erythrocytes, the extraction, and hence fixation, is not complete. Even after 100 minutes of fixation, extraction is continuing, though slowly. A simple interpretation of these results is that initially all the haemoglobin is unfixed and extractable, therefore

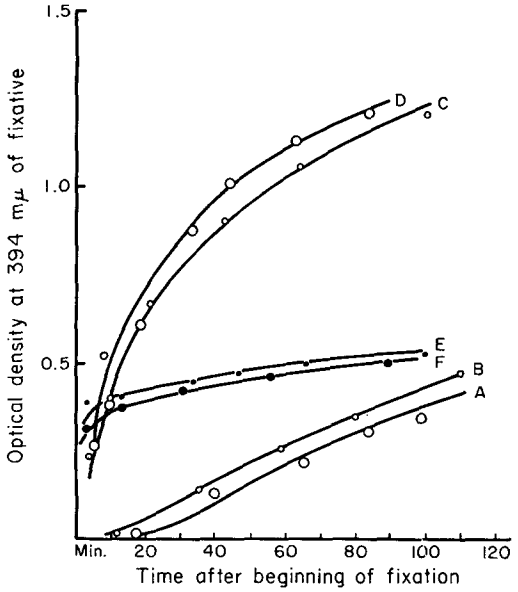

FIGURE 6 The optical densities at λ394 mμ, of 2.5 ml of B fixative, pH 6.2, and of A fixatives and of a 1 per cent solution of OsO_4 in glass-distilled water have been plotted as a function of time after the introduction of 0.03 ml of blood. A is the result when B fixative buffered at pH 6.2 is used. *B* shows the result when fixative A, buffered at pH 6.2, but without Ca^{++} ions is used. *C* and *D* show the results obtained with fixative A buffered at pH 7.4 and without Ca^{++} ions; two separate animals were used in these two experiments. *E* and *F* are the results obtained with fixations in a 1 per cent solution of OsO_4 in glass-distilled water at pH 6.2.

the initial rate of extraction is high. As fixation continues, less haemoglobin is available for extraction and the rate decays.

Davies and Spencer (1962) noted that when intact frog erythrocytes were fixed in fixative B a very small fraction of the cells was immediately haemolysed. In the present experiments the high initial rate of extraction was doubtless in part due to complete haemolysis of a small percentage of the total erythrocyte population. However, since ex-

traction continues even after 100 minutes, the experiment shows that haemoglobin was being extracted from the rest of the cells.

The experiments were repeated with fixative A at pH 6.2; there was no evidence of any extraction of haemoglobin. When the cells are introduced there is a slight increase in the optical density of the fixative solution but this is non-specific and probably results from the interaction of OsO_4 with plasma proteins.

Factors Affecting the Rate of Haemoglobin Extraction During Fixation

The two fixatives used have very different effects upon erythrocytes. To investigate whether it is the low pH or the presence of 0.01 M Ca^{++} ions in fixative A that stabilises the erythrocytes and prevents both the immediate total haemolysis of a small percentage of the cells and some haemoglobin extraction from the rest, a series of experiments were performed with modified fixatives.

The effect of the pH of the fixative on the haemoglobin extraction was determined with new fixatives, a B fixative buffered at pH 6.2 instead of pH 7.4 and an A fixative buffered at pH 6.8 or pH 7.4 instead of pH 6.2. With the B fixative at pH 6.2 there is no extraction during the first 20 to 30 minutes of fixation but thereafter extraction begins and continues slowly (see Fig. 6, *A*). This result is in striking contrast with that obtained with fixative B at pH 7.4. The experiment shows that extraction in the absence of Ca^{++} ions is very pH-sensitive and is greater at the higher pH. It also shows that at pH 6.2 all the erythrocytes are stable and there is no immediate haemolysis of a small percentage of the cell population.

In the reciprocal experiments with A-type fixatives at pH 6.8 and pH 7.4, the result was surprising because there was no haemoglobin extraction in either case. This shows that the Ca^{++} ions completely suppress extraction whether the pH is at 6.2, 6.8, or 7.4.

To confirm these results a fixative B at 7.4, containing 0.01 M Ca^{++} ions, and A fixatives at pH 6.2 and 7.4 without Ca^{++} ions were used. As expected, there was no haemoglobin extraction with the fixative B containing Ca^{++} ions. Fixative A at pH 6.2 without Ca^{++} ions gave a result identical, within the limits of experimental variation, with that obtained with fixative B buffered at pH 6.2 (see Fig. 6, *B*). Extraction is small and only begins after 20 to 30 minutes of fixation. In contrast, the A-type fixatives without Ca^{++} ions at pH 7.4 gave a result similar to that obtained with normal B fixation (see Fig. 6, *C* and *D*).

The results of fixation with a 1 per cent solution of OsO_4 in glass distilled water at pH 6.2 are shown in Fig. 6, *E* and *F*. When the amount of haemoglobin extracted is taken as a criterion of fixation, it seems that a simple 1 per cent solution of OsO_4 is better than fixative B. Malhotra (1963), using pancreatic and kidney tissues, similarly concluded that it was not necessary to buffer OsO_4 solutions in order to obtain good preservation.

In the absence of any buffer ions or sucrose the erythrocytes are subjected simultaneously to an osmotic shock and to the fixative. The amount of haemoglobin extracted in the initial minutes of fixation with a 1 per cent aqueous solution of OsO_4 is 0.4 OD units (see Fig. 6, *E* and *F*). Microscopic observations show that immediately after fixation not more than 5 per cent of the erythrocytes are completely haemolysed, and this accounts at least in part for the initial high liberation of haemoglobin. The spectroscopic measurements show that there is a subsequent gradual extraction of haemoglobin from the remaining erythrocytes although under the microscope these appear stable.

The following conclusions can be drawn from all these experiments:—

1). Erythrocytes are stabilised and haemoglobin extraction is reduced when a fixative at low pH is used.

2). 0.01 M Ca^{++} ions in the fixative completely suppress extraction of haemoglobin and stabilise all the erythrocytes irrespective of the pH within the range used, whereas the monovalent Na ions at the concentration in the fixative buffer do not have a stabilising effect.

3). The difference in concentration of sucrose in the two types of fixative used does not have any significant effect upon the extraction of haemoglobin.

Changes in Cellular Volume During Fixation, Dehydration and Embedding

The literature contains several descriptions of changes in volume during OsO_4 fixation of erythrocytes. Zeiger (1949) found that frog erythrocytes fixed in 2 per cent OsO_4 in amphibian Ringer's swelled by 8 per cent, while Davies and Spencer (1962) using fixative B showed that the

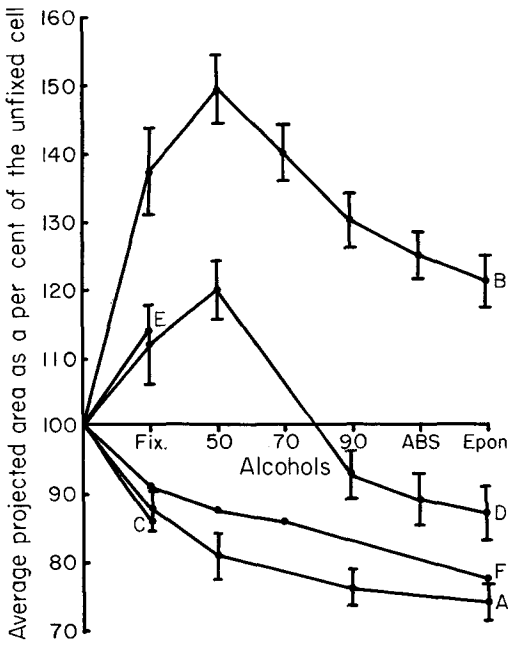

FIGURE 7 This figure shows the average projected cellular areas of newt erythrocytes after fixation in various fixatives, and during ethanol dehydration and Epon embedding. The average areas are given as a percentage of the unfixed cell which is taken as 100 per cent. The results obtained with fixative A are shown in *A*; fixative B, at pH 7.4, in *B*; with fixative B, at pH 7.4 and containing 0.01 M Ca^{++} ions, in *C*; with fixative B buffered at pH 6.2, in *D*; and those obtained with fixative A, at pH 6.2 without Ca^{++} ions, in *E*. Standard deviations are given in *A*, *B* and *D*. *F* shows the average projected area of newt erythrocyte nuclei after fixation in fixative A and during ethanol dehydration and Epon embedding.

nuclei of frog erythrocytes swelled by about 25 per cent. Bahr *et al.* (1958) describe a 40 per cent swelling of chick erythrocytes when fixed in 1 per cent OsO_4 in Tyrode solution containing 1.5 per cent dextran.

Because the standard haematological methods for determining cellular volumes give information only about the whole cells and cannot be applied to their nuclei, they were not used. In the following experiments the changes in projected area of the whole cells and their nuclei were measured to indicate the direction and approximate magnitude of the changes in volume. During these experiments the erythrocytes and their nuclei maintained their characteristic shape and were in no way constricted by the glass surfaces of the slide and coverslip. This observation supports the view that projected area and volume changes occur in the same direction. The blood and fixative proportions were identical with those used in the measurements of haemoglobin extraction. By altering the pH of the fixative and by adding or omitting Ca^{++} ions, the effect of these factors was investigated (see Fig. 7).

Fixative A (see Fig. 7, *A*) causes shrinkage which continues during dehydration and embedding, whereas fixative B (see Fig. 7, *B*) causes swelling which continues in 50 per cent alcohol and is followed by shrinkage in the higher alcohols and during embedding. A similar pattern of volume changes occurs during B fixation and dehydration of isolated erythrocyte nuclei of frog (Davies and Spencer, 1962) and of nuclei and chromosomes of HeLa and Krebs ascites cells (Richards and Davies, 1963). Despite swelling in lower alcohols, the ultimate effect of dehydration is to cause shrinkage. The results of Seki (1937 *a*, *b*) and Bahr *et al.* (1957) show that all the common cytological dehydrating agents cause shrinkage. In the present experiments the transfer from absolute alcohol to Epon caused little change in projected area, some 2 to 3 per cent decrease. This is in agreement with the work of Kushida (1962). Attempts were made to measure the projected areas of cells embedded, between slide and coverslip, in methacrylate, but the shrinkage of the plastic was so great that it caused clumping of the cells and no satisfactory measurements could be made.

With modified A and B fixatives the projected area changes during fixation were measured. The addition of 0.01 M Ca^{++} to fixative B caused the cells to shrink (Fig. 7, *C*) and resemble those obtained after normal A fixation. Further changes during dehydration, etc., were not measured. Fixative B buffered at pH 6.2 (Fig. 7 *D*) caused less swelling than fixative B at pH 7.4 (compare curves B and *D* in Fig. 7). After dehydration and embedding, the average projected area was 88 per cent that of the unfixed cell. Fixative A at pH 6.2 without Ca^{++} ions caused swelling during fixation (Fig. 7, *E*) by an amount similar to that with fixative B at pH 6.2 (compare curves *E* and *D* in Fig. 7).

The changes in nuclear projected area, with fixative A, were also measured (see Fig. 7, *F*). The nucleus shrinks in fixative and alcohols by an extent almost equal to the shrinkage of the whole cell. It was impossible to make measurements in

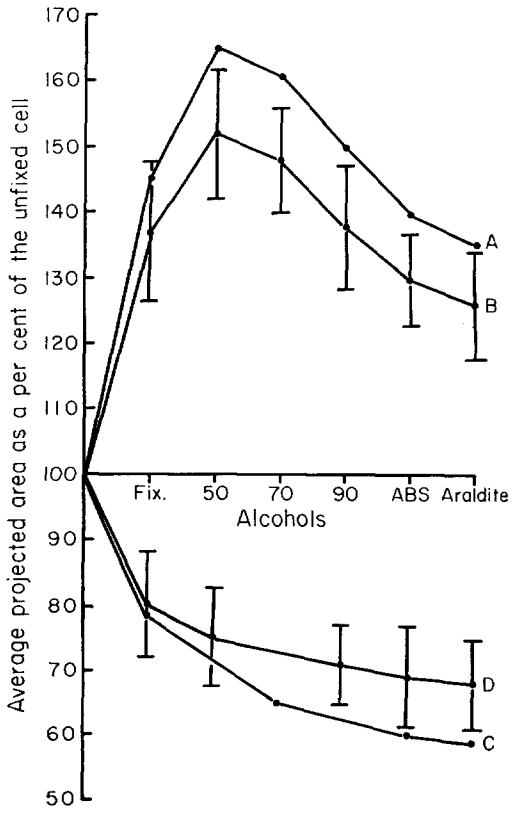

FIGURE 8 The average projected areas of the nucleus and whole cell of *Amphiuma* erythrocytes after fixation in A or B fixatives, and during ethanol dehydration and Araldite embedding are shown as a percentage of the unfixed erythrocyte. *A* and *B* show, respectively, the average projected cellular area and the average projected nuclear area after B fixation. *C* and *D* show, respectively, the average cellular and nuclear projected areas after fixation with fixative A. Standard deviations of the average nuclear projected areas are given in *B* and *D*. The standard deviations of the average projected areas of the whole cells are of similar magnitude and have been omitted for the sake of clarity.

70 per cent and 90 per cent alcohols because the refractive index of the nucleus matched that of the cytoplasm, so that the nucleus could not be seen in the phase contrast microscope.

Similar experiments were done on erythrocytes of *Amphiuma tridactylum* which are the largest of any vertebrate (Noble, 1931). The cell's long axis is about 65 μ and the nucleus is correspondingly large, so that measurements of the whole cell and the nucleus can be made with more accuracy than in the erythrocytes of other amphibia.

Fixative B causes cellular swelling which continues in 50 per cent and 70 per cent alcohols, but in the higher alcohols shrinkage occurs (Fig. 8, *A*). The nucleus swells and contracts by an almost equal amount (Fig. 8, *B*). Fixative A causes cellular (Fig. 8, *C*) and nuclear (Fig. 8, *D*) shrinkage which continues during dehydration. The *Amphiuma* erythrocytes, after both A and B fixations, were embedded in Araldite. There is little (3 to 5 per cent) shrinkage in this embedding medium. It is noticeable that the changes occurring during the fixation of *Amphiuma* erythrocytes are in the same direction as, but of greater magnitude than, those in newt erythrocytes.

From these results and the previous measurements of haemoglobin extraction, it can be concluded that the conditions that favour cellular swelling also favour haemoglobin extraction and that, conversely, conditions causing shrinkage also tend to suppress haemoglobin extraction. They also show that both nuclear and cellular volume changes are controlled by the same factors.

DISCUSSION

OsO_4 reacts strongly with histidine and with proteins containing high proportions of this amino acid (1). The other two basic amino acids, lysine and arginine, are reactive when present in proteins, and since OsO_4-fixed proteins lack affinity for acid dyes it seems likely that the basic properties of these amino acids are blocked by fixation, although the mechanism of blockage is unknown (Baker, 1958, 1960). It has been shown that OsO_4 lowers the isoelectric point of proteins (see Fig. 3), presumably by reacting preferentially with their basic amino acids.

Solutions of newt haemoglobin upon fixation darken and gel. At the same time, the isoelectric point is lowered from the range pH 6.9–7.2 to about pH 5.0–5.2. As well as altering the properties of haemoglobin, the OsO_4 affects the erythrocyte plasma membrane. Little is known about the permeability of this membrane after fixation, but from the present experiments the conclusion can be drawn that it is relatively impermeable to the haemoglobin and permeable to Ca^{++} ions. Similarly, the experiments of Davies and Spencer (1962) also showed that OsO_4-fixed frog erythrocytes were permeable to Ca^{++} ions.

Since fixatives A and B are buffered at pH 6.2 and pH 7.4, the fixed haemoglobin gel carries a net negative charge because the pH's of both

fixatives are above its isoelectric point. The fixed erythrocyte consists essentially of a negatively charged hydrophilic haemoglobin gel surrounded by a membrane permeable to water and cations but virtually impermeable to the protein. Two sets of forces, electrostatic interactions of the charged protein particles and osmotic forces, might be expected to cause swelling or shrinkage. In the absence of cations the protein chains in the gel would repel each other because they all carry a net negative charge which gives rise to a double layer repulsive force. The magnitude of this repulsive force would depend upon the conditions of the medium in which the erythrocyte is placed, and would be reduced by cations that can penetrate the erythrocyte membrane; divalent cations are 10 to 100 times more effective than monovalent cations (Verwey and Overbeek, 1948). Lowering the dielectric constant of the medium, or bringing its pH to the isoelectric point of the fixed haemoglobin, would also diminish these repulsive forces. For any given set of conditions swelling would continue until the coulombic repulsive forces were balanced by Van der Waal's attractive forces. Verwey and Overbeek (1948) and Bungenberg de Jong (1948) have given detailed descriptions of such properties of hydrophilic colloids. The second type of force acting on the haemoglobin gel is that resulting from the establishment of a Donnan equilibrium. Cations present in the fixative would accumulate in the erythrocyte owing to the fixed negatively charged groups on the protein gel; this produces an increased osmotic pressure which causes the gel to swell. A model system of this type was described by Proctor and Wilson (1916).

The volume changes that occur when erythrocytes are placed in various fixatives can readily be interpreted in these terms. With fixative B the cells swell because the repulsive potential on the fixed haemoglobin is not greatly reduced by the Na^+ ions, which are present in the buffer, and since these ions accumulate in the cell there is an osmotic swelling. Fixative B buffered at pH 6.2 instead of the usual pH 7.4 caused less swelling. This is to be expected, because at pH 6.2 the net negative charge on the gel is less than at pH 7.4, and so both sets of forces causing swelling are reduced.

With any fixative containing 0.01 M Ca^{++} ions the erythrocytes shrank. These divalent ions are very effective in reducing the double layer repulsive force. They probably cross-link the separate, negatively charged protein chains in the gel and this must offset any increase in osmotic pressure due to the Donnan equilibrium. The result is a net cellular shrinkage. These cells do not subsequently swell during dehydration in the graded alcohols. This shows that the Ca^{++} ions are firmly bound to the gel and are not washed off by the alcohols. The shrinkage in the higher alcohols is again the result of water being replaced by ethanol.

The erythrocytes of *Amphiuma tridactylum* show greater swelling or shrinkage during fixation than those of *Triturus cristatus*. This was expected since both the native and fixed haemoglobins of *Amphiuma* have lower isoelectric points than the haemoglobins of the newt (see Fig. 3, and Dessauer *et al.*, 1957) and consequently the electrostatic and Donnan osmotic forces which contribute to cellular volume changes are greater in *Amphiuma* erythrocytes than in newt erythrocytes.

The erythrocyte nucleus swells or shrinks during fixation, as does the whole cell. The nuclei of newt erythrocytes contain haemoglobin both in pools within the nuclear envelope and within the chromosomal regions. (Tooze and Davies, 1963) The nuclei of *Amphiuma* erythrocytes contain pools of haemoglobin and also may have haemoglobin in the chromosomal regions. After fixation this nuclear haemoglobin would have a net negative charge. Fixation would probably affect the charge distribution on the nucleohistone of the chromosomal regions. The positively charged groups on the basic nucleoproteins are thought to neutralise the majority if not all of the negatively charged ionised phosphate groups on the DNA (Mirsky and Pollister, 1946; Mirsky and Ris, 1951; Peacocke, 1960). Because OsO_4 reacts preferentially with the basic amino acids and blocks their basic groups but does not react with DNA (Bahr, 1954; Davies, 1954), fixation would cause an increase in the net negative charge on the nucleohistone. After fixation, therefore, the erythrocyte nucleus contains negatively charged haemoglobin and nucleohistone molecules and so would be subject to the same osmotic and electrostatic forces as the erythrocyte cytoplasm. This explains why the changes in nuclear volume during fixation are qualitatively similar to the volume changes of the whole cell, although the magnitude of the changes may well differ because the net negative charge on the nucleohistone is likely to be different from that on the haemoglobin.

The observation that OsO_4 lowers the isoelectric

point of proteins has general significance and provides some explanation of why OsO_4 is a poor nuclear and chromosomal fixative. Robbins, (1961), Davies and Spencer (1962), and Richards and Davies (work in preparation), in studies on the fixation of HeLa cells, frog erythrocytes, and Krebs ascites cells, respectively, have shown that, in the absence of divalent cations, OsO_4 solutions cause chromosomal and nuclear swelling. They interpreted this swelling in terms of the mutual repulsion of charged nucleohistone macromolecules. The lowering of the isoelectric point of histones after fixation with OsO_4 would contribute to an increase in a net negative charge on the nucleohistone which would cause chromosomal swelling. It appears therefore that, in the absence of stabilising factors such as divalent cations, OsO_4 solutions do not fix chromosomes but, on the contrary, make them unstable.

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