THE OCCURRENCE AND POSSIBLE SIGNIFICANCE OF HAEMOGLOBIN IN THE CHROMOSOMAL REGIONS OF MATURE ERYTHROCYTE NUCLEI OF THE NEWT *TRITURUS CRISTATUS CRISTATUS*

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ABSTRACT

Absorption measurements in violet light (λ 4047 A) of thin sections of mature newt erythrocytes have indicated the presence of a haem compound, probably haemoglobin, within the chromosomal regions of the nucleus. The amount is about 45 per cent of that in the cytoplasm. The possible significance of this nuclear haemoglobin in determining nuclear structure is discussed in terms of the electrostatic interaction of haemoglobin and nucleohistone.

In 1951 de Carvalho demonstrated the presence of haemoglobin within the nuclei of human erythroblasts, using what was thought to be a specific peroxidase reactivity of haemoglobin. Earlier workers (see Downey, 1938) had considered the possibility that haemoglobin was present in and elaborated by the nucleus. Unequivocal evidence for the presence of haemoglobin in the nuclei of chicken and goose erythrocytes was obtained by Stern, Allfrey, Mirsky, and Saetren (1952): erythrocyte nuclei were isolated by grinding in a non-aqueous medium and analysed in bulk. Wilkins and de Carvalho (1953) employing a method based on the violet light absorption of haem (Huggins, 1865; Thorell, 1947; Metcalf, 1951; Nomarski and Bessis, 1959) confirmed the presence of absorbing granules, interpreted as haemoglobin, within the nuclei of human erythroblasts. Subsequently, de Carvalho (1954), quoted by Grasso *et al.* (1962), using both microspectrophotometric and staining methods on human and rat erythroblast nuclei, interpreted his results as showing the presence and synthesis of haem within the nucleus. Uncertainty in interpreting the results of these two methods is due to the fact that violet absorption is given by both haem and haem conjugated to globin. Also free haem as well as haemoglobin has a peroxidase activity, the specificity of the reaction being dependent on the nature of the substrate (Lemburg and Legge 1949). O'Brien (1960), using a peroxidase technique similar to that of de Carvalho (1951), claimed to be specific for haemoglobin, showed that haemoglobin was present in the nuclei of embryonic chick erythroblasts and suggested that haemoglobin is first synthesised there. Wilt (1962) appears to have confirmed these observations.

Davies (1961) studied the distribution of haemoglobin in mature erythrocytes of frog and chicken by electron microscopy and microspectrophotometry. The results indicated that the nuclei contain pools of haemoglobin in direct connection with the haemoglobin of the cytoplasm *via* gaps in the nuclear chromatin and pores in the nuclear envelope. In an electron microscopic

study of the developing erythrocytes in mammalian fetal liver (Grasso *et al.*, 1962), dense material, interpreted to be haemoglobin, infiltrated the nuclear pores in late erythroblasts and normoblasts, and in some cases appeared to extend into the interchromatin regions.

The DNA of the nucleus in mature erythrocytes (Fig. 1) is concentrated in discrete regions, similar in density to mitotic chromosomes and previously referred to as nuclear bodies (8). In the frog erythrocyte these nuclear or chromatin bodies, or chromosomal regions, correspond roughly to the chromosome number (8). In this paper evidence will be presented that appears to demonstrate that the chromosomal regions themselves, in mature erythrocytes of the newt *T. cristatus cristatus*, contain a high concentration of a haem compound, probably haemoglobin. The function of haemoblogin is to combine reversibly with molecular oxygen. It is interesting that our observations lead to the suggestion that haemoglobin molecules may have a secondary role, that is, in the determination of nuclear structure in erythrocytes. It is suggested that, owing to their ionic properties, molecules of haemoglobin may interact with nucleohistone so as to determine, in part, the form of the nucleus of the mature erythrocyte and, possibly, the changes occurring during differentiation.

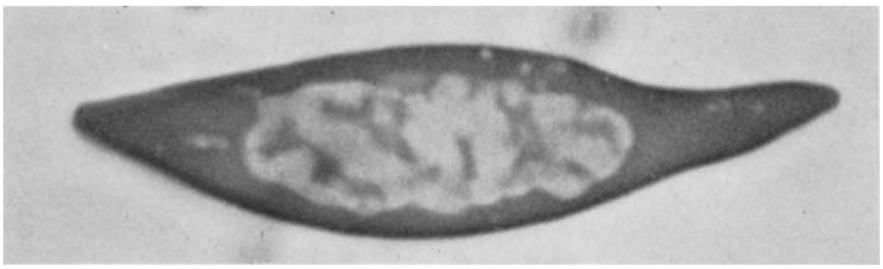


FIGURE 1

A 1-μ section of a newt erythrocyte photographed in bright field λ 4047 A. The cell has been treated with fixative B (see text) and embedded in Epon. The nuclear area contains light regions, the chromatin bodies, and dark regions, the pools of nuclear haemoglobin. × 1920.

MATERIALS AND METHODS

The observations were made on mature erythrocytes obtained from heart blood of adult newts sp *Triturus cristatus cristatus* and also on haemoglobin obtained from the erythrocytes.

1. *Fixation, Embedding, and Sectioning*

The cells were fixed by pipetting blood directly into fixatives referred to previously as either A or B (Davies and Spencer, 1962). These two fixatives were used for the following reason. It has been shown (9) that erythrocyte nuclei isolated in a calcium-containing medium become swollen when subsequently immersed in a calcium-free fixative (B), but shrink upon dehydration with ethanol. In a calcium-containing fixative (A), followed by dehydration, such volume changes are not apparent. Although volume changes in intact cells were not investigated in detail, nuclei in intact erythrocytes were found to swell by about 25 per cent in volume during fixation in B. Since swelling and shrinking of nuclear chromatin might conceivably be accompanied by diffusion of haemoglobin, the effects of both fixatives A and B were studied.

Briefly, fixative A was similar to that used by Ryter and Kellenberger (1958) and contained 1 per cent OsO_4 in veronal acetate buffer, pH 6.3, containing 0.01 M $CaCl_2$ and 0.24 M sucrose. Fixative B was 1 per cent OsO_4 in veronal acetate buffer, pH 7.4, containing 0.14 M sucrose (Palade, 1952; Caulfield, 1957). After fixation and washing, the cells were embedded in agar, cut into 1 mm^3 blocks prior to processing in Epon (Luft, 1961) or methacrylate. For details of these processes see reference 9. Prior to dehydration, cells fixed in A were soaked in 0.25 M Versene for 2 hours since this improves section quality without apparently causing nuclear swelling or altering the fine structure (9).

Sections were cut on an A. Huxley microtome at nominal settings of 2, 1, 0.5, and 0.1 μ. The actual thickness of air-mounted specimens was measured on the Baker-Smith interference microscope with half-shade eyepiece. The refractive index (n) of Epon was 1.506 (one sample) measured on an Abbe refractometer.

2. *Quantitative Measurements on Sections*

The distribution of haemoglobin was deduced from photographs taken in violet light, λ 4047 A (30), isolated from a 250 watt Hg arc source by a filter combination of a 0.6 per cent solution of iodine in carbon tetrachloride at 2 cm path length, a Kodak Wratten filter 1A, and a Zeiss filter B.G.12. The sections were immersed in W.15 mounting medium (n = 1.5) manufactured by C. Zeiss. The photographs were taken with a Zeiss apochromatic objective NA 1.3 and an aplanatic condenser, at a magnification of × 250 on Kodak microfile Pan 35 cine film which was calibrated by inserting a Hilger rhodium step-wedge into the beam below the film. Film density was measured with a Walker microdensitometer (prototype of the instrument manufactured by Joyce-Loebl).

The absorption spectra in 1 μ diameter areas of the cytoplasm and chromatin bodies were determined in 1-μ-thick sections, using the newly designed microspectrophotometer of Walker and Chamberlain (28). These sections were immersed in glycerol.

Measurements of optical path differences in the nucleus and cytoplasm of erythrocytes were made with a Zeiss interference microscope, objective × 100, to which a C. Baker half-shade eyepiece had been attached. The cells had been previously fixed in fixative B and the methacrylate removed from the sections which were mounted in water.

The quantitative distribution of haem and the fine structure in the electron microscope have been compared in the same thin section of an erythrocyte as follows. A violet light micrograph was made of a thin (gold, about 1000 A) section of an erythrocyte embedded in Epon or methacrylate; the section was mounted on a grid coated with carbon and collodion, the section plus grid being immersed in W.15 and mounted in the usual way between slide and coverslip. Subsequently, the W.15 was removed by washing several times in distilled water and the section was stained in a 2 per cent uranyl acetate solution (½ to 4 hours) and then photographed in the Siemens Elmiskop I operated at 80 kv at a primary magnification of between 5000 and 40,000.

3. *The Absorption Spectra of Extracted Haemoglobin*

Erythrocytes were haemolysed in an equal volume of distilled water or a 0.3 per cent solution of saponin and the membranes and nuclei were removed by centrifugation. Drops of the haemolysate were smeared on glass slides. Some were exposed for 1 hour to OsO_4 vapour from a 2 per cent solution of OsO_4 during which time they dried. Other slides were allowed to dry in air. The absorption spectra were measured with a Hilger Uvispek.

Equal volumes of the haemolysate were diluted either with water or an equal volume of an aqueous 0.1 per cent solution of OsO_4. After 1 hour at room temperature the absorption spectra of both solutions were determined with a recording Perkin Elmer spectrometer.

RESULTS

In a 1-μ-thick section photographed in violet light (Fig. 1) the erythrocyte nucleus appears as a relatively light region containing dark areas. These dark areas, which look roughly similar in density to the cytoplasm, are the pools of nuclear haemoglobin which are continuous with the cytoplasm *via* pores in the nuclear envelope. The light areas, which are Feulgen-positive and contain DNA, are sections through the chromosomal regions. Microdensitometry shows that these have an appreciable violet light absorption, about 40 to 50 per cent that of the cytoplasm. Absorption within the chromosomal regions might indicate the presence of haem in these regions of the living cell or might be due to (1) over- and underlying haemoglobin; (2) diffusion of haemoglobin into the regions during preparation, especially fixation; (3) a large light scattering by the chromosomal regions outside the collecting aperture of the objective, simulating real absorption, or an increased light absorption resulting from the interaction of OsO_4 with chromatin (Davies, 1954); (4) blurring of the image due to diffraction, whereby the absorption in neighbouring haemoglobin-containing regions spreads into the chromosomal regions. We attempted to eliminate possibilities (1) to (4) as follows.

1. The ratios of violet light absorption in the nuclear body to that in the cytoplasm in erythrocytes fixed in fixative B were measured in a series of sections of decreasing thickness (Fig. 2, open circles). The thinnest section was about 0.1 μ (1,000 A) thick, small compared with the average dimensions of the chromatin bodies. The size of these bodies was determined roughly from phase contrast micrographs of erythrocytes haemolysed in a solution similar to fixative A except that the OsO_4 was replaced by a 0.3 per cent saponin solution (9). The chromatin bodies range in size from about 0.65 to 1.4 μ (see histogram in Fig. 2). If the absorption in the section through the body were due to over- and underlying haemoglobin the absorption ratio would fall to zero with decreasing section thickness. In fact, the ratio decreases to a finite value, which shows that the

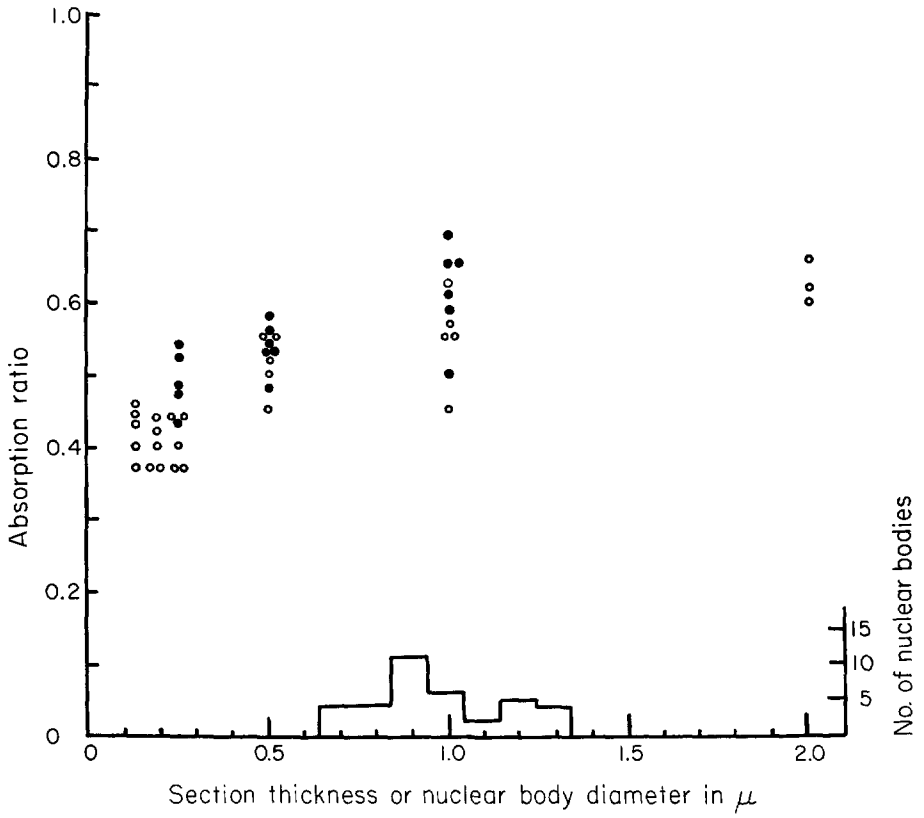


FIGURE 2

The ratio of the absorption in the chromatin bodies to that in the cytoplasm of the newt erythrocyte λ 4047 A (left hand ordinate) is shown as a function of section thickness (abscissa). Erythrocytes were fixed in solution A (●) or B (○) (see text) and embedded in Epon. Each point is the mean value of three determinations at different sites in one cell. The histogram shows the distribution of the chromatin body diameter.

ratio of haem within the nuclear body to that in the cytoplasm is about 0.4. At the greater values of section thickness, over and underlying haemoglobin is included in the section and this causes the ratio to increase above 0.4. Also, the variation in the amount of haemoglobin included is, no doubt, partly responsible for the spread in the values of the absorption ratio at any one thickness.

From observations in phase and interference contrast it is known that the concentration of total dry substance in the chromatin body is less than that in the cytoplasm. Hence, the concentration of a haem compound in the nuclear chromatin must be greater than 40 per cent (dry weight). The ratio of optical path difference in the cytoplasm to that in the nuclear chromatin was measured in sections after fixation in solution B and was about 1.3 (15 cells measured). This is approximately the ratio of the total concentration of solids. Hence, a rough value for the fractional dry mass of haem compound in the nuclear chromatin is 50 per cent.

2. Observations (9) on isolated erythrocyte nuclei have shown that nuclei swell during Palade (B) fixation and shrink during dehydration. However they do not swell or shrink in this way when treated with fixative A. Data on volume changes of nuclei in intact cells are limited but the changes are smaller, about 25 per cent increase in volume after treatment with fixative B. Such a change might conceivably be accompanied by diffusion of haemoglobin into the chromosomal region. Another series of measurements were, therefore, made on nuclei in intact cells after treatment with fixative A in which volume

changes are very unlikely. The results (Fig. 2, closed circles) are similar to those obtained when fixative B was used, except that the ratio has increased to about 0.50 at the smaller section thickness; this may be attributed to the different changes in volume during the two types of fixation.

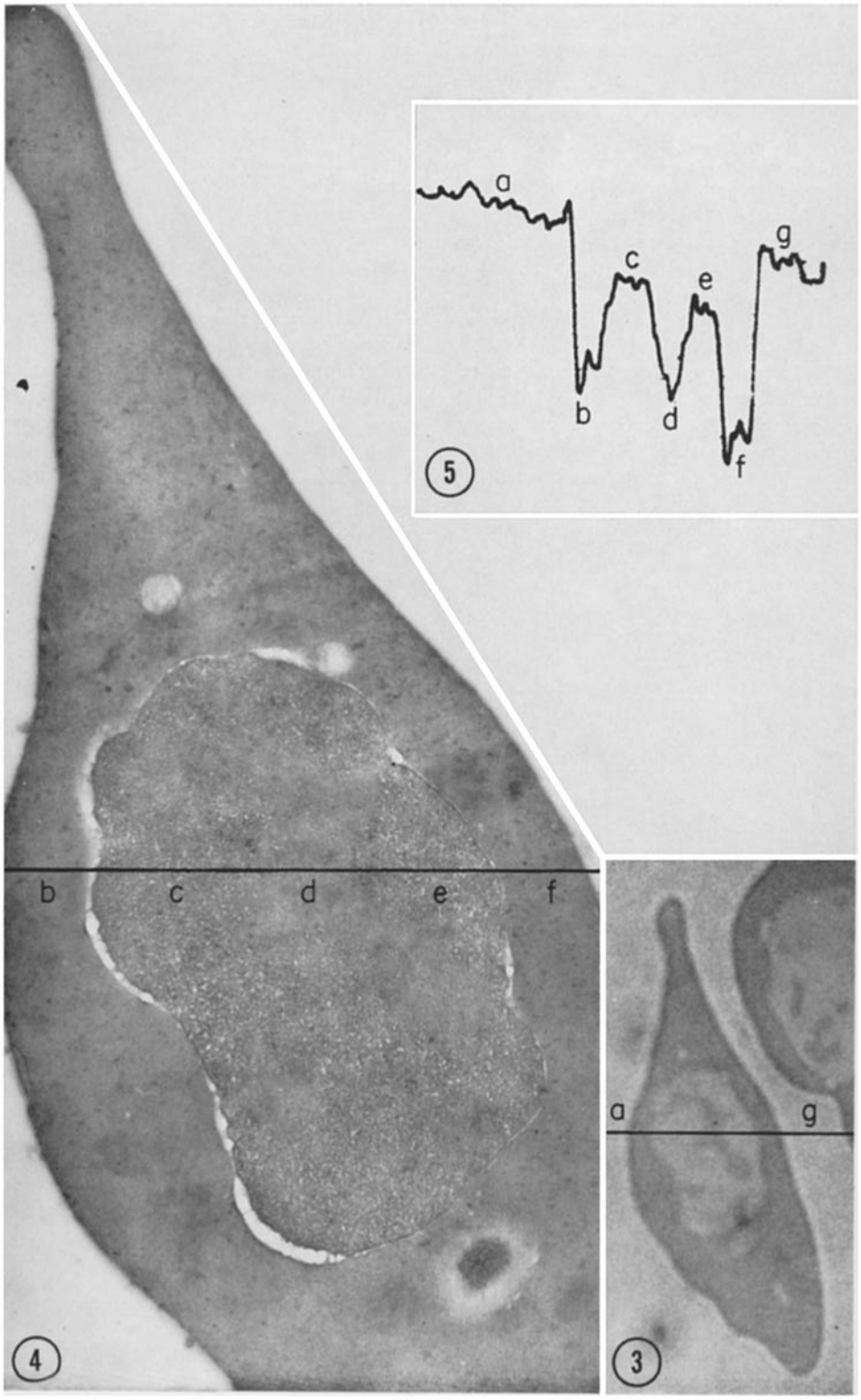
3. The apparent absorption within the chromatin bodies might be due to light scattered, by the chromatin bodies, outside the collecting aperture of the objective, rather than a true haem absorption. This was tested in another amphibian, frog *Rana pipiens*, for which blocks of Epon-embedded isolated nuclei were available. These nuclei had been haemolysed in 0.3 per cent saponin in 0.01 M $CaCl_2$ and fixed in A. In violet light micrographs of thin sections of this material the image of a chromatin body appeared, above focus, as a light central region surrounded by a dark rim. Below focus the effect was reversed. These so called phase effects are well known to occur in small refractile objects and make it difficult accurately to evaluate the magnitude of any additional apparent absorption. However, a rough estimate was calculated from measurements of the areas in densitometer traces across images close to focus. It was found that the apparent absorption in the chromatin bodies of isolated nuclei was small (< 5 per cent) compared to that of the same thickness of chromatin bodies in nuclei of intact cells. A similar result was obtained after fixation in B. These tests appear to exclude significant light scatter by the chromatin bodies of intact cells since the scattering is likely to increase upon isolation. Phase effects are smaller in the chromosomal regions of the intact cell due to the surrounding refractile haemoglobin. These observations also show that osmium tetroxide does not interact with chromatin so as to increase the absorption at λ 4047.

4. Theoretical calculations on bodies of the smallest size for which absorption measurements can be made with 1 to 2 per cent accuracy give values of about 3 to 4 times the wavelength (λ) of the light employed, for an objective aperture of about 1.3 (Caspersson 1936). Wilkins (1950) using a different approach calculated that measurements can be made on bodies of size about 1 λ, if somewhat larger errors are acceptable. The chromosomal regions of newt erythrocytes range from about 1.5 λ (λ approx. 0.4 μ) to 4 λ. Consequently, accurate results can be expected if the larger chromosomal regions are measured.

We also experimentally investigated the errors in measuring small objects. The method relies on the assumption that the cytoplasm of the newt erythrocyte contains a constant concentration of haemoglobin confined to volumes of such a shape that in thin sections a strip of variable width is occasionally presented. An example is shown in the electron micrograph Fig. 4. The assumption that the erythrocyte cytoplasm contains a constant concentration of haemoglobin can be checked by densitometry of the electron micrograph and dimensions can be determined very accurately. When the violet light micrograph Fig. 3 of the same thin section was measured at widths of 1 μ and upwards, negligible change in light absorption could be detected. These data confirm that measurements on such absorbing bodies of about 1 μ, *i.e.* 2.5 λ, in diameter can be made accurately in our experiments. Furthermore, it appears that the method might be extended to study experimentally the lower limit of size for which accurate measurements of absorption can be made in the light microscope.

Ratios similar to those in Fig. 2 were obtained on sections (about 0.1 μ thick) of erythrocytes in a spleen sinus after fixation in B and embedding in Epon and in erythrocytes from blood after fixation in B and embedding in methacrylate. In another series of measurements which gave similar ratio values, the same section was photographed first in violet light and then in the electron microscope (Figs. 3 and 4). In the electron microscope the chromosomal regions are fibrillar or granular whilst the haemoglobin of the nuclear pools and cytoplasm is relatively homogeneous. The fine structure along lines in the violet light micrograph traced in the microdensitometer (Fig. 5) could be examined in the electron microscope. It was possible to exclude the influence of small regions of haemoglobin occurring within the chromosomal regions but which were not apparent in the light microscope. The chromosomal absorption is not, therefore, due to the presence of pools of haemoglobin just below the limits of resolution of the light microscope. Similarly, uncertainties due to the possible influence of occasional shrinkage, causing non-absorbing gaps between nucleus and cytoplasm, could be eliminated by directly comparing the light and electron micrographs.

The trace (Fig. 5) demonstrates what is frequently observed, that the concentration of haem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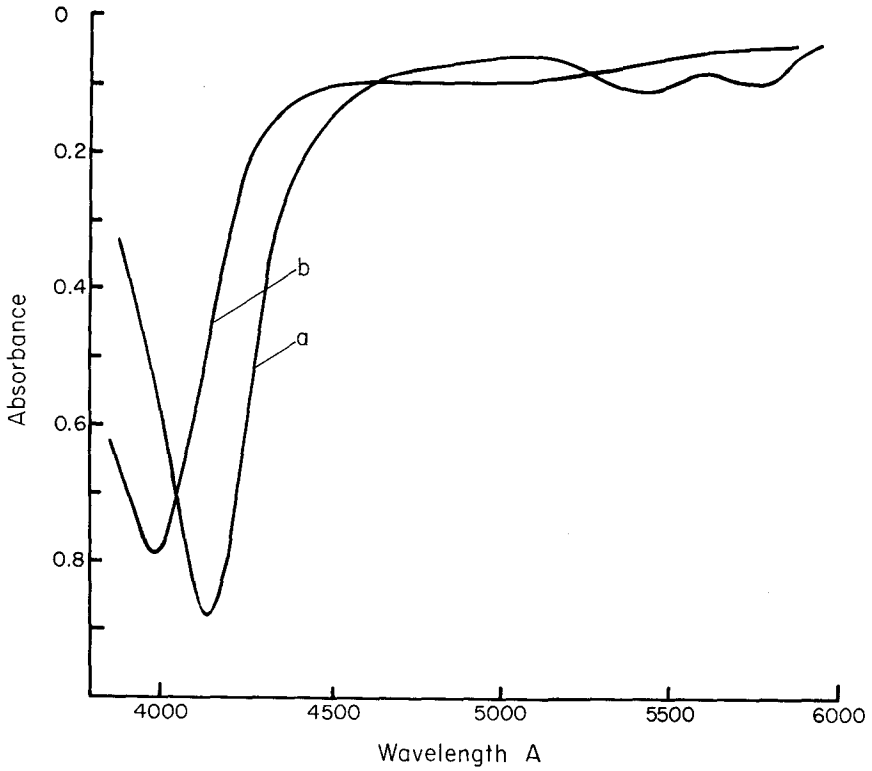

FIGURE 6

The absorbance (ordinate) is shown as a function of wavelength (abscissa) in (*a*) a smear of haemoglobin solution air-dried, and (*b*) a similar smear dried while exposed to OsO_4 vapour. The main effect is to shift the position of the Soret band from about λ 4150 A to about λ 4000 A.

in a nuclear pool is similar to that found in the cytoplasm.

The Effect of OsO_4 on Haemoglobin

Because it was essential to measure the absorption of thin sections and desirable to correlate electron micrographs and violet light micrographs of the same section, it was necessary to fix and embed the cells. OsO_4 was chosen as the fixative because it gives good preservation of fine structure. To see whether treatment with OsO_4 greatly alters the absorption spectrum, smears

FIGURE 3

A 1,200-A-thick (gold) section of a newt erythrocyte photographed in bright field, λ 4,047 A. The cell has been treated with fixative B (see text) and embedded in methacrylate. × 3,500.

FIGURE 4

An electron micrograph of the same section as in Fig., 3 stained in uranyl acetate. × 12,500.

FIGURE 5

A densitometer trace across the lines shown in Figs. 3 and 4. The deflection in the cytoplasm corresponds to an optical density of about 0.05.

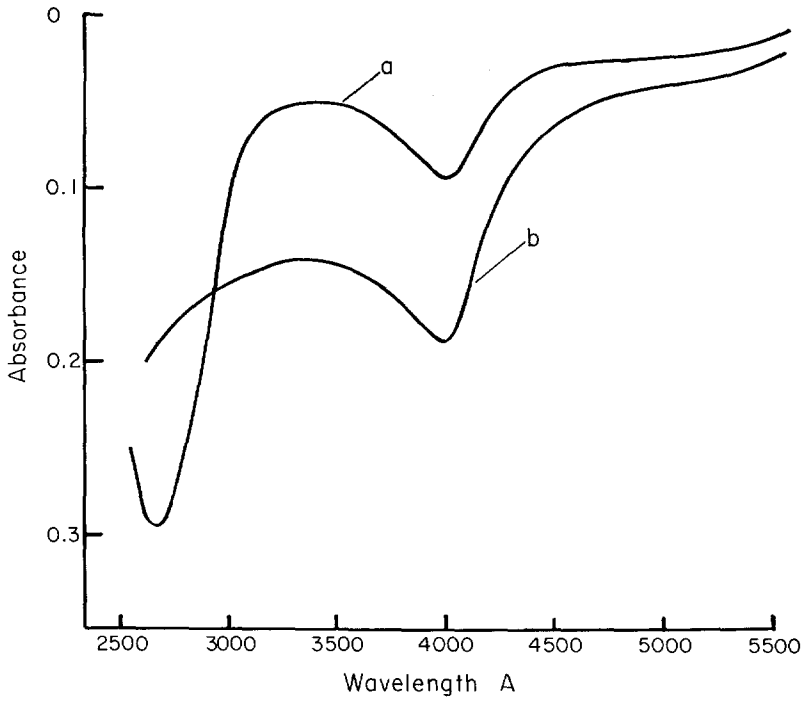

FIGURE 7

The absorbance (ordinate) of an area 1 μ in diameter is plotted as a function of wavelength (abscissa) in (*a*) a chromatin body and (*b*) the cytoplasm in a 1-μ-thick section of an erythrocyte, fixed in solution B and embedded in Epon.

and solutions of haemoglobin were measured before and after treatment. The effect of OsO_4 vapour on smears of haemoglobin is shown in Fig. 6, *a* and *b*. Before treatment with OsO_4 vapour, freshly isolated haemoglobin shows a pronounced absorption band at λ 4150 A, the Soret band, with smaller maxima at about λ 5400 A and λ 5760 A. After reaction with OsO_4 vapour, from a 2 per cent solution, smears of haemoglobin show no absorption maxima at λ 5400 A and λ 5760 A and the Soret band absorption is shifted to about λ 4000 A. At λ 4047 A the optical density is not appreciably changed. This comparison cannot be made accurately since the thickness of the smears was not exactly the same; repetition with other pairs of smears gave similar results. Treatment of a dilute aqueous solution of haemoglobin with a 1 per cent aqueous solution of OsO_4 gives a slightly different result. The Soret absorption maximum is shifted from λ 4150 A to about λ 3920 A, while absorption at λ 4047 A is 30 per cent less after OsO_4 treatment. At the longer wavelengths, λ 5400 A and λ 5760 A, the absorption maxima are eliminated as they are when smears are treated with OsO_4 vapour. These results suggest that a wavelength λ 4047 A is suitable for determinations of haemoglobin in cells after OsO_4 treatment.

The observations on smears and solutions were confirmed by the measurements on thin (1 μ) sections of OsO_4-fixed and embedded erythrocytes. The absorption spectrum of the cytoplasm (Fig. 7 *b*) is similar in shape to the OsO_4-treated smear; the position of the absorption maximum is also at about λ 4000 A. The absorption spectrum of the chromosomal region (Fig. 7 *a*) also has an absorption maximum at λ 4000 A; the absorption maximum at about λ 2600 A is due to DNA. This spectrum supports the conclusion that the chromosomal region has a real haem absorption.

It has been shown independently (Trapp, L., unpublished, 27) that the nucleus of *intact* erythrocytes of an amphibian has an absorption spectrum in visible light similar to that of haemoglobin.

DISCUSSION

Our observations indicate that there is a high concentration of a porphyrin or iron-porphyrin (haem) compound within the chromatin bodies of the mature erythrocytes of *T. cristatus cristatus*, about 45 per cent of that in the cytoplasm.

Thorell (1955) has shown that the violet light absorption of erythrocytes is unlikely to be due to free porphyrin, since observations on the fluorescence of developing erythrocytes showed a negligible pool of free porphyrin indicating rapid conversion of porphyrin to haem. Similar results on mature erythrocytes were obtained by Keller and Seggel (1934).

The violet light absorbing material, that is haem, within the nuclear pools can be interpreted as haem conjugated to globin (haemoglobin) for the following reasons. The concentration of haem, measured by violet light absorption, is apparently the same in the nuclear pools as in the cytoplasm, small differences being attributable to experimental error caused by the size of the pools which are small relative to the chromosomal regions. Although precise measurements have not yet been made in the interference microscope, the optical path difference and hence total dry mass in the pools is very high, comparable to that of the cytoplasm. Furthermore, the electron microscope shows direct continuity between the nuclear pools and the cytoplasm (8), the structure of the two regions being similar with similar electron opacity. We cannot be certain that the haem within the chromosomal regions is conjugated to globin, because the above considerations cannot be applied. However, it seems most likely that the haem is conjugated to globin because the chromosomal regions are bathed in nuclear pool haemoglobin. It should be possible to test for the absence of free haem in erythrocytes by chemical analysis.

If, as seems probable, the chromatin bodies contain haemoglobin rather than haem, the significance of haemoglobin in the chromatin bodies may be considered. In mature nucleated erythrocytes the DNA-containing regions, the chromatin bodies, exist in the so called condensed state. (This term was used by Ris and Mirsky (1949) in a discussion of the action of ions on interphase nuclei. We suggest that this state is due, at least in part, to the presence of haemoglobin within the chromatin. This view is based on the known behaviour of erythrocyte nuclei during haemolysis and the changes in their morphology with ionic environment (9), as well as on the known ionic properties of haemoglobin. When erythrocytes are haemolysed in a medium of low ionic strength, the loss of haemoglobin is associated with swelling of the nuclear bodies which assume the so called extended state. This swelling can be eliminated by increasing the concentration of ions in the medium. These volume changes during nuclear isolation can be roughly accounted for by the following model (9). We assume that the DNA phosphate groups are not completely electrostatically neutralised by the positively charged histone molecules. Experimental data on this point are conflicting (Peacocke, 1960). In the intact erythrocyte the nucleohistone molecules lie fairly close together (the condensed state), presumably because the net negative charge is more or less neutralised by positively charged ions in the environment. These ions are lost during nuclear isolation and the net negative charge on the nucleohistone causes electrostatic repulsion with the nucleohistone molecules relatively far apart (the extended state). It was earlier suggested (9) that the ionic environment of the haemoglobin might help to keep the nucleus of the intact cell in a condensed state. This suggestion is now made more plausible by the finding that the chromatin bodies actually contain a high concentration of haemoglobin. Electrochemical studies (for a discussion, see Haurowitz and Hardin, 1954) have shown that, in the vertebrates that have been investigated, haemoglobin does combine with phosphate ions, neutralising their negative charge. The slightly basic properties of the globins depend on their high content of histidine. To take a specific example (Adair and Adair, 1934), the isoionic point of carboxyhaemoglobins from sheep erythrocytes is pH 7.6 whilst the isoelectric point in phosphate buffer varies from pH 7.16 to 6.7, owing to the combination of protein and phosphate anions. Variations due to the effect of gas combination with haemoglobin are small, the isoelectric point of haemoglobin, oxyhaemoglobin, and carboxyhaemoglobin being similar.

Not only will phosphate ions combine with haemoglobin but it has been shown that DNA itself will combine with globin from ox-blood, and this has been made use of in a method for fractionating DNA (Crampton, Lipshitz, and Chargaff, 1954).

Adair and Adair (1) showed that in a phosphate

buffer at pH 7.0 1 molecule of haemoglobin neutralises about 3.8 equivalents of phosphate ion. It will be assumed that the chromatin bodies contain equal quantities of DNA and histone and that the molecular weight of haemoglobin is about 68,000. These data give an upper limit of about 3 per cent for the fraction of DNA which might be neutralised by the haemoglobin inside the chromatin, assuming that all the net positive charge on the haemoglobin is available. Although conditions inside the erythrocyte are more complex than in this system, this calculation gives a rough indication of the order of magnitude of the phenomenon.

During erythropoiesis the nucleus of the developing erythrocyte undergoes a gradual condensation, during which time the concentration of haemoglobin in the cytoplasm increases. A study which is being made of the distribution of haemoglobin in differentiating and dividing erythroblasts indicates that haemoglobin is present in the nucleus during development: It is suggested that the changes in nuclear structure may be due, in part, to the interaction of haemoglobin with nucleohistone.

Finally, in sperm heads and mitotic or meiotic chromosomes the DNA is in a condensed state, *i.e.*, highly concentrated and closely packed. In this form it is relatively metabolically inactive. Similar considerations apply to the nuclei of mature erythrocytes. Also the amount of protein being synthesised in the mature cell is small. It is, therefore, permissible to ask whether the increase in the end-product, haemoglobin, which is associated with the condensation of chromatin during erythropoiesis, may itself act as a type of feed-back mechanism for switching off protein synthesis as the cell approaches maturity. Some of these suggestions are being experimentally tested.

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