STRUCTURE IN NUCLEATED ERYTHROCYTES

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ABSTRACT

The structure of the nucleated erythrocyte of frog and chicken has been investigated by electron microscopy and correlated with the distribution of haemoglobin and DNA-containing material determined by haem absorption and Feulgen staining in the light microscope. The nuclei of both species are found to contain haemoglobin which is continuous with the haemoglobin in the cytoplasm through holes or pores in the nuclear envelope. In addition the nucleus of the frog erythrocyte sometimes contains a single invagination which is lined by the nuclear envelope. The structure of the nuclear envelope and the pores and the organisation of the nucleus are similar to those already described for other somatic cells. Erythrocytes differ from the cells previously studied in that a continuity, via the nuclear pores, of chemical substance in the interior of the nucleus and in the cytoplasm can be directly demonstrated. This is due to the fact that the cytoplasm of erythrocytes is simple, consisting predominantly of haemoglobin, and that haemoglobin is easily recognised by its specific absorption. The static pictures obtained by electron microscopy have been supplemented by observations in phase-contrast of the changes in refraction of the cell contents due to the diffusion of the haemoglobin from the nucleus into the cytoplasm during haemolysis.

INTRODUCTION

In this paper the structure of the mature erythrocytes of frog (*Rana pipiens*) and chicken (*Gallus domesticus*) observed by electron microscopy has been correlated with the distribution of chemical substance, nucleoprotein and haemoglobin, obtained by light microscopy.

Experimental data on the structure of nucleated erythrocytes and the possible presence of haemoglobin within the nucleus are limited and sometimes misleading. According to a recent review by Swift (19) it has proved practically impossible to demonstrate pores in the nuclear envelopes of certain nucleated erythrocytes, whereas they are universally present elsewhere (excepting also (19) pyknotic nuclei and normoblasts). Stern *et al.* (17) found that chicken erythrocyte nuclei isolated by grinding in a non-aqueous medium contained about 19 per cent haemoglobin. According to Maximov and Bloom (12), erythrocyte nuclei of lower vertebrates do not contain haemoglobin. Wilkins and de Carvalho (24) observed in the light microscope the presence of granules of haemoglobin in nuclei of human erythroblasts; it has been suggested (23) that the particles were trapped from the cytoplasm in the nucleus during nuclear reconstruction.

Data obtained will show that nucleated erythrocytes conform to a common pattern. The nuclei contain haemoglobin, and pores similar to those described in other cell types have been found in the nuclear envelope. The extent to which the pores may provide a path for exchange of macromolecules between nucleus and cytoplasm is under discussion (7, 22, 19). What is interesting about

the nucleated erythrocyte is that, in contrast to the cells previously studied, a continuity of chemical substance between the cytoplasm and the interior of the nucleus, via the nuclear pores, can be directly demonstrated.

MATERIALS AND METHODS

The observations have been made on blood obtained from the heart of adult frogs, and of chicks several days old. Cells were examined microscopically in serum or in physiological salt solution (Ringer or Tyrode), after fixation, and during preparation for electron microscopy. Erythrocytes were also studied during haemolysis in physiological solutions either isotonic or hypotonic, or in saline containing a haemolysing agent. Fixation with OsO_4 was done in a 1 per cent solution buffered to pH 7.5 (14) and containing 45 mg./ml. sucrose (8), at about 0°C., for periods ranging from 10 minutes to 24 hours. Fixations were also performed in (*a*) a buffered 1 per cent OsO_4 containing 0.1 per cent $CaCl_2$ for 1 to 2 hours followed by a period of 2 hours in uranyl acetate before dehydration (16); (*b*) the same solution as in (*a*) but without the period in uranyl acetate; (*c*) buffered 1 per cent OsO_4 with Tyrode or Ringer at physiological concentration.

After fixation, cells were dehydrated in increasing concentrations of ethanol, and embedded in methacrylate (butyl 9 parts, methyl 1 part). In one experiment uranyl nitrate (21) was added to the methacrylate. Cells were also embedded in araldite (10).

Sections were obtained with a Huxley microtome, 0.5 to 1 μ in thickness for light microscopy, while for electron microscopy they were light-silver to dark-grey in colour before spreading with xylene vapour; staining was for 2 to 4 hours with 2 per cent uranyl acetate.

The distribution of total mass throughout the cell was deduced from observations and photomicrographs in monochromatic green light with the Zeiss neofluor phase-contrast objectives. The Smith-Baker and Zeiss interference microscopes were also used.

The distribution of haemoglobin was deduced from photomicrographs taken near the absorption maximum of the haem group at 4047 A. Violet light was isolated from a mercury arc by means of two filters, one a solution of iodine in carbon tetrachloride in 1 cm. path length, and the other a Zeiss BG12 filter to remove residual red light. The concentration of iodine was chosen with the aid of a small spectroscope so as to achieve the required spectral purity. Photomicrography was done with a Zeiss apochromat N.A. 1.3 and an aplanatic condenser.

The distribution of deoxyribose nucleic acid (DNA) was obtained from Feulgen staining. All photomicrographs were made at × 330 using Kodak microfile panchromatic 35 mm. film.

Electron micrographs were made on a Siemens microscope with double focussing condenser and an objective aperture of about 25 to 30 μ. Sections were mounted between a layer of collodion and a layer of carbon deposited by evaporation on the section. Primary magnifications of either 5,000 or 40,000 were employed.

Microdensitometry was carried out with a Walker (20) microdensitometer.

RESULTS

A. *Frog Erythrocytes*

1. *Intact Cells:* Erythrocytes approximate in shape a much flattened ellipsoid. As a result they usually orient in a thin layer so as to present the aspect seen in Figs. 1 and 2, where their appearance is seen to be similar whether the cells are freshly mounted in serum proteins or in Ringer's solution. In conventional phase-contrast the cytoplasm which contains haemoglobin appears dark and surrounded by a light halo, while the nucleus, also approximately ellipsoidal in shape, appears as a relatively light region. The nucleus is not homogeneous and contains darker areas.

It is well known that the appearance of objects in phase-contrast depends on refractive index and thickness (or the product, the so-called optical path) and also on size. Hence the refractive index of cells and components may sometimes be deduced from phase microscopy. The importance of refractive index lies in the fact that is is proportional to the concentration of substances other than water in the cell (see reviews (6, 9)). If the optical path difference due to the refracting (or diffracting) object is not too large, objects which contain a higher concentration than the medium in which they are immersed appear dark (in the conventional Zeiss phase-contrast) surrounded by a light halo, while the reverse holds for objects of relatively lower concentration. In particular, when the refractive index of the surrounding medium is equal to that of the object the latter appears with zero contrast, that is, it disappears.

Consequently it may be deduced that the refractive index and hence concentration of the erythrocyte cytoplasm is higher than that of the immersion medium, which fact is of course known.

The concentration of substance throughout the nuclear volume is lower, on average, than that of the surrounding haemoglobin. The nuclear detail (Figs. 3 to 5) is somewhat clearer after fixation in OsO_4 (buffered) and consists of relatively light bodies roughly cylindrical or ellipsoidal in shape with minor diameters of less than about 2 μ. These are subsequently referred to as nuclear bodies and are separated by darker, more refractile regions which are sometimes seen to be traversed by fine light threads. The nucleus of the frog erythrocyte is not a regular ellipsoid; a nuclear body often projects outwards (Figs. 3 and 4) and a single nuclear invagination located at one end of the long axis is frequently seen in the phase microscope. The discrete nature of the nuclear bodies can clearly be seen when they are simultaneously in focus, as in the flattened cell (Fig. 5).

2. *Sections: Light Microscopy:* Sections, 1 μ in thickness, of erythrocytes fixed in OsO_4 and embedded in methacrylate reveal (Figs. 6 and 7) further details of the nuclear structure. The nuclei contain irregularly shaped dark regions which interconnect with one another and often clearly appear as if connected to the cytoplasm by means of numerous dark cylindrical channels. Fig. 7 shows particularly clearly an invagination which seems to be characteristic of frog erythrocytes. At the other end of the section there is a slightly projecting nuclear body.

The composition and concentration of the material in the relatively dark regions within the nucleus were shown to be similar to those of the material in the cytoplasm, that is, haemoglobin, as follows. In a 1 μ section in phase-contrast (Fig. 8*a*) the dark regions of the nucleus and the cytoplasm indicate a relatively high mass per unit area. Observations in interference contrast confirm this. In bright-field with violet light (λ 4047 A) the contrast (Fig. 8*b*) is similar to that seen in the phase microscope, while at a wavelength for which there is no haemoglobin absorption (λ 5461 A) the cells show zero contrast (negligible absorption) when mounted in a medium of suitable refractive index. Hence we may conclude that the dark nuclear regions contain a haem group and, in order to account for the high concentration of substance, that it is conjugated to a protein, most likely in the form of haemoglobin.

Microdensitometry of the film negative (at λ 4047 A) has shown that extinction in the regions of nuclear haemoglobin is never greater than that in adjoining cytoplasm regions, is usually equal, and occasionally is less. These data are consistent with the hypothesis that the concentration of nuclear haemoglobin is similar to that found in the cytoplasm. Relatively lower values of extinction in the haemoglobin-containing regions of the nucleus could be accounted for by supposing that the section contained parts of non-absorbing nuclear bodies above and below the measured area. This effect is expected to decrease with section thickness and is in fact small in 1 μ sections.

After Feulgen staining (Fig. 8*c*) the relatively light regions of Figs. 8*a* and 8*b* become relatively dark, indicating that the nuclear bodies contain DNA.

3. *Sections: Electron Microscopy:* The quality of the sections when observed in the electron microscope was somewhat variable in different experiments (18 in number). There seemed to be no clear correlation with the fixation conditions (*a*), (*b*), and (*c*) described above, nor did uranyl nitrate appear to result in an improvement of the embedding. Certain facts could be established, however.

In sections stained in uranyl acetate, the nuclear area (Fig. 9) contains regions with relatively high electron-scattering power which appear to be finely particulate. Other regions within the nucleus have the same contrast and appearance as the cytoplasm which contains haemoglobin. These are presumably the regions which absorb violet light. In unstained cells in the electron microscope the relative contrast of nuclear bodies and haemoglobin-containing regions is reversed, the contrast being similar to that already noted in the unstained cells in the phase microscope. Preliminary rough measurements in the interference microscope kindly made by Dr. E. Baeckeland showed that, as a result of staining with uranyl acetate (2 per cent for 4 hours), the optical thickness of the nuclear bodies increased from about 15 per cent below to about 15 per cent above that of the haemoglobin-containing regions. Before staining, the particulate structure of the nuclear bodies is difficult to make out.

The cytoplasm of the apparently mature frog erythrocytes appears to consist predominantly of haemoglobin, but we have occasionally seen cytoplasmic organelles, for example, mitochondria. The appearance of the haemoglobin in thin

sections is variable and probably depends partly on the sharpness of the knife. It is common to find it looking spotted with dark areas about 0.05 μ in diameter (Fig. 9). These dark spots often have adjacent lighter areas, are sometimes aligned parallel to the knife edge, and sometimes occur in zones of sufficient size to be resolvable in the light microscope. A pile-up of haemoglobin is often found at the junction of the nuclear bodies with the haemoglobin (Fig. 9). Irregular patches could in fact be seen by phase-contrast microscopy on the surfaces only of thick sections, and the patches may therefore be assumed to be artifacts due to sectioning. The small spots may be a similar artifact produced by sectioning. The difficulty in obtaining good sections is not surprising. We may consider the volumes containing haemoglobin to consist of closely packed approximately spherical molecules, which are imperfectly held in space by polymerised methacrylate between their interstices. Under the impact of the knife edge these molecules easily slide around, resulting in dark spots with adjacent clearer areas from which material has been taken.

In the unstained sections (Figs. 10, 10*i*) haemoglobin can be seen within a nuclear invagination.

The envelope bounding the nucleus (Fig. 11) is similar to that found in other somatic cells and it appears as two dark lines separated by a space about 170 A wide. The clarity with which the envelope could be seen was variable and, as in other cell types, depended on section thickness, being clearest when the nuclear envelope was cut normally. An additional factor that probably reduces the visibility of the nuclear envelope in sections departing from the normal is the high density of the juxtaposed haemoglobin and the nuclear bodies. In Fig. 10, the nuclear invagination is also bounded by the nuclear envelope.

The DNA-containing regions which bound the outer perimeter of the nucleus are interrupted by channels of varying length which terminate in a hole (Fig. 9, arrow). The haemoglobin of the cytoplasm is seen to be continuous with that in the interior of the nucleus through the holes and channels. Other less well defined pores (Fig. 9) are present in the region where distortion occurs owing to pile-up of haemoglobin. The nuclear envelope does not stretch across the holes, but terminates at their edge, where the inner and outer components of the envelope sometimes appear joined. Thus in contrast to the single invagination the channels are numerous and not lined by a membrane or envelope. The apparent dimensions of the holes range from about 700 A to 1000 A.

4. *Haemolysing Cells: Light Microscopy:* During the haemolysis of the nucleated erythrocyte the

FIGURE 1

Fresh preparation of frog erythrocytes mounted in serum proteins. Phase-contrast, × 2,000.

FIGURE 2

Fresh preparation of frog erythrocytes mounted in Ringer's solution. Phase-contrast, × 2,000.

FIGURE 3

Frog erythrocyte after fixation in OsO_4-sucrose. Phase-contrast, × 2,000.

FIGURE 4

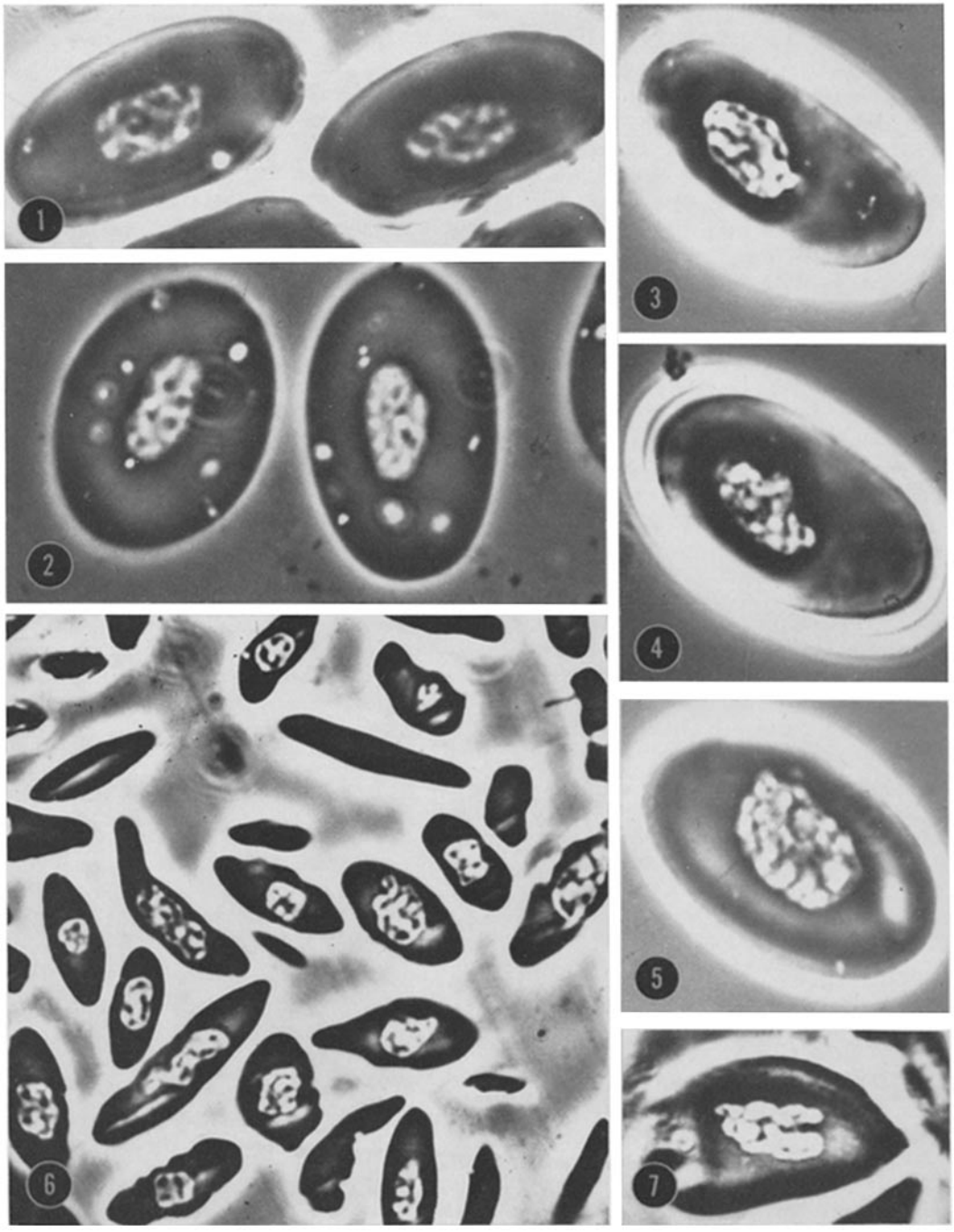
Same cell as Fig. 3 at different focal level.

FIGURE 5

Frog erythrocyte after fixation in OsO_4; flattened. Phase-contrast, × 2,000.

FIGURES 6 and 7

1 μ thick sections of frog erythrocytes, fixed in OsO_4-sucrose and embedded in methacrylate. Phase-contrast, × 2,000.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concentration of haemoglobin decreases owing to dilution with water, the shape and size of the cell remaining approximately constant. As a result the refractive index of the haemoglobin, which is initially higher, becomes equal to and finally falls below that of the nuclear bodies. These relative alterations of refractive index cause a series of interesting changes in the image of the cells in phase-contrast. These changes support the observations on intact cells which indicate that the nucleus contains haemoglobin which is at about the same concentration as that in the cytoplasm and apparently in continuity with it.

The observations were made on cells mounted between slide and coverslip in physiological or hypotonic Ringer's solution. In these solutions cells haemolyse slowly over a period of hours and do not undergo the changes in shape that occur when a haemolytic agent such as saponin is used. The same cell or group of cells was photographed in both phase-contrast and bright-field violet light. It is convenient to distinguish six stages of haemolysis corresponding to distinct appearances in phase-contrast. Cells in any one population haemolyse at different rates, so that each preparation contained cells at different stages. An individual cell was not followed through all stages of haemolysis, but single cells were seen to pass through several of the stages and these observations were used when assigning cells to a particular stage.

In the intact cell (stage I) the nuclear bodies are surrounded by haemoglobin of higher refractive index and hence they appear relatively light (Fig. 12*a* I). The cell appears in similar contrast in violet light where the nuclear bodies are non-absorbing (Fig. 12*b* I). In the initial stages (II, III) of haemolysis, the appearance of the cells in violet light (Fig. 12*b* II, Fig. 13*b*) does not alter appreciably, but with decrease in refractive index of the haemoglobin the visibility, in phase-contrast, of the nuclear bodies diminishes (stage II, Fig. 12*a* II). When the refractive index of the intra- and extranuclear haemoglobin equals that of the nuclear bodies (stage III) the nucleus almost disappears (Fig. 13*a*). Some residual variations of intensity in the image of the cell can be expected because of variations in thickness with resulting changes in optical path. Nuclear bodies, although almost invisible in phase-contrast, are plainly visible as non-absorbing regions in bright-field violet light (Fig. 13*b*).

With further haemolysis the refractive index of the haemoglobin falls just below that of the nuclear bodies (stage IV), which then appear as relatively dark regions surrounded by lighter haemoglobin-containing areas (Fig. 14*a* IV). The cytoplasmic haemoglobin, however, still appears dark relatively to the less refractile medium that surrounds it. Cells at stages IV and I look remarkably similar. The reversal in nuclear contrast at stage IV is indicated by a comparison of the images in phase-contrast and in bright-field violet light. The dark regions in phase-contrast (Fig. 14*a* IV) can be identified as the non-absorbing (light) regions in violet light (Fig. 14*b* IV) and hence as nuclear bodies. To facilitate the comparison, the nucleus of one of the cells at stage IV in Figs. 14*a* and 14*b* has been further enlarged in Figs. 15*a* and 15*b*.

At a later stage (stage V) the contrast within the nucleus increases and the nuclear bodies look dark against the light background of nuclear haemoglobin (Fig. 14*a* V). The reduced haemoglobin content is apparent when the violet light absorption is compared in cells IV and V in Fig. 14*b*.

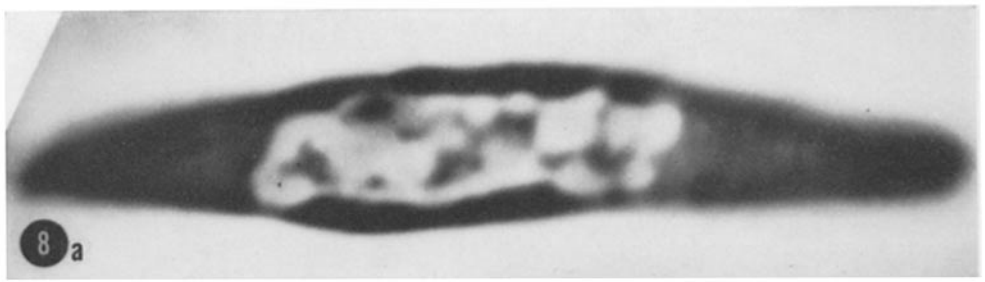
At stage VI the nuclear and cytoplasmic haemoglobin is completely removed and there is no absorption in violet light (Fig. 12*b* VI). In

FIGURE 8

8*a*. A 1 μ section of the same frog erythrocyte, fixed in OsO_4-sucrose and embedded in methacrylate. Phase-contrast, λ 5461 A.
8*b*. Same section as *a* in bright-field, λ 4047 A.
8*c*. Same section as *a*, Feulgen stained, in bright-field, λ 5461 A. All $\times$ 6,000.

FIGURE 9

Electron micrograph of frog erythrocyte fixed in OsO_4-sucrose, embedded in methacrylate, and stained with uranyl acetate. The arrow indicates the site of a nuclear pore and channel. $\times$ 15,000.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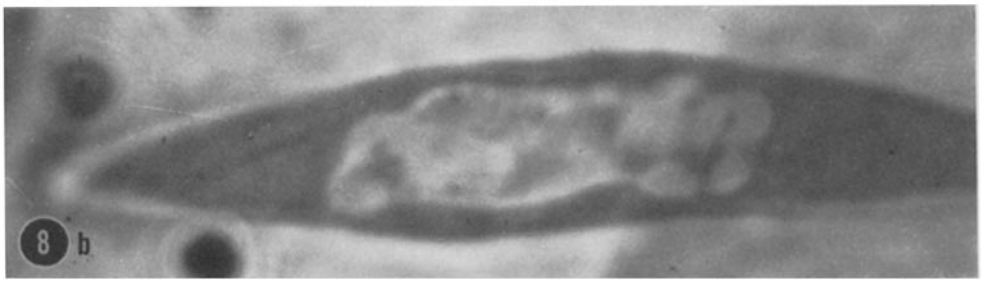
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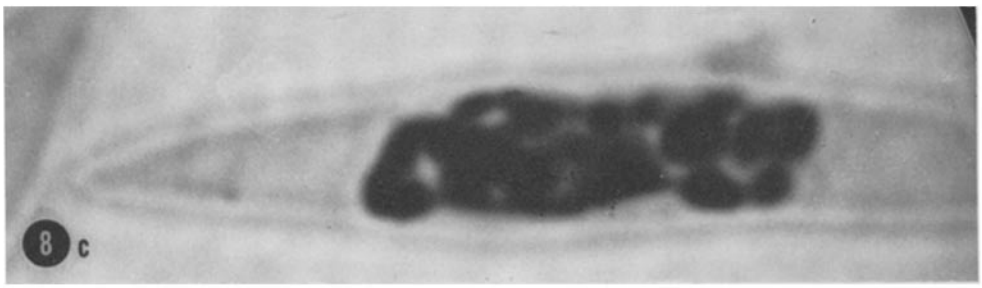
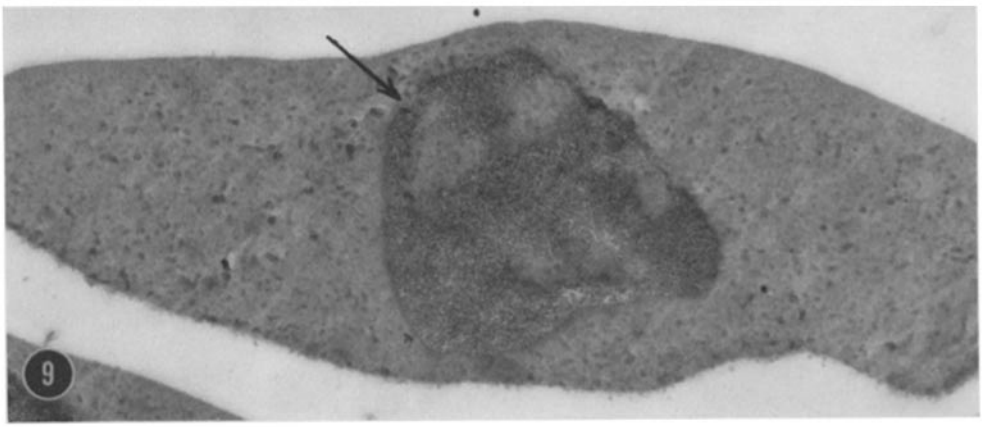

8 c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phase-contrast the nuclear bodies appear as highly refractile dark regions (Fig. 12*a* VI). The residual contrast within the nucleus (Fig. 12*b* VI) arises chiefly from so-called phase-contrast effects. These well known phenomena cause highly refractile objects in cells to be visible in bright-field illumination in the absence of real absorption. The plasma membrane (Fig. 12*b* VI) is rendered visible for this reason and appears as a double line, one dark, one light. Such effects are present in all the bright-field observations, but they are reduced at other stages where differences in refractive index are smaller and real absorption is present.

An attempt has been made in Fig. 16 to summarise and make clear the changes during haemolysis. The line separation is an indication of the relative concentration (dry substance per unit volume) of total substance (column *A*) and haemoglobin only (column *B*) deduced from phase microscopy and bright-field microscopy (λ 4047 A) respectively. An explanation is given in the description of the figure.

B. *Chicken Erythrocytes*

Fresh chicken erythrocytes observed in phase-contrast appear the same whether mounted in serum proteins (Fig. 17) or physiological Tyrode (Fig. 19). Apart from their smaller size they are very similar to frog erythrocytes, the nucleus appearing as a relatively light region containing darker areas. In a haemolysed cell nuclear bodies appear with reversed contrast (Fig. 18). In the erythrocytes fixed in buffered OsO_4 (Fig. 20), channels are clearly visible in the outer perimeter of the nuclei which apparently connect the interior of the nuclei with the cytoplasm. In thin sections (Fig. 21) the differentiation of the nucleus into light and dark regions is similar to that in frog erythrocytes. The shape of the erythrocyte nucleus of the chicken is more regular and more nearly ellipsoidal than that of the frog, and a nuclear invagination has not been found.

In the electron microscope (Figs. 22 and 23) narrow channels in the outer rim of chromatin, connecting the inside of nucleus and cytoplasm, can be clearly seen, especially after araldite embedding, when the contrast between nuclear bodies and haemoglobin is higher than after embedding in methacrylate. At the cytoplasmic junction of the channel there is a gap or hole in the nuclear envelope. The appearance of the gap is somewhat variable. In Fig. 24 the outer and inner parts of the nuclear envelope are not clearly joined and there is indication of a faint line extending across the gap.

In most preparations of chicken erythrocytes the nucleus was slightly retracted from the cytoplasm. This effect occurred much less frequently in preparations of frog erythrocytes. The space became clearly apparent, as shown by microscopic observations, during dehydration in 90 per cent alcohol. In photomicrographs showing this effect a narrow bridge of haemoglobin can also be seen connecting the nuclear channels with the cytoplasm (Figs. 22 and 23). The space between the nucleus and the cytoplasm seems to be formed between the two parts of the nuclear envelope (Fig. 24).

DISCUSSION

Since the discovery of nuclear pores there has been considerable discussion of the extent to which they permit direct interchange of material between the interior of the nucleus and the cytoplasm. In recent papers (7, 22, 19) the nature of pores has been further studied in the electron microscope and the possibility of transfer between

FIGURE 10

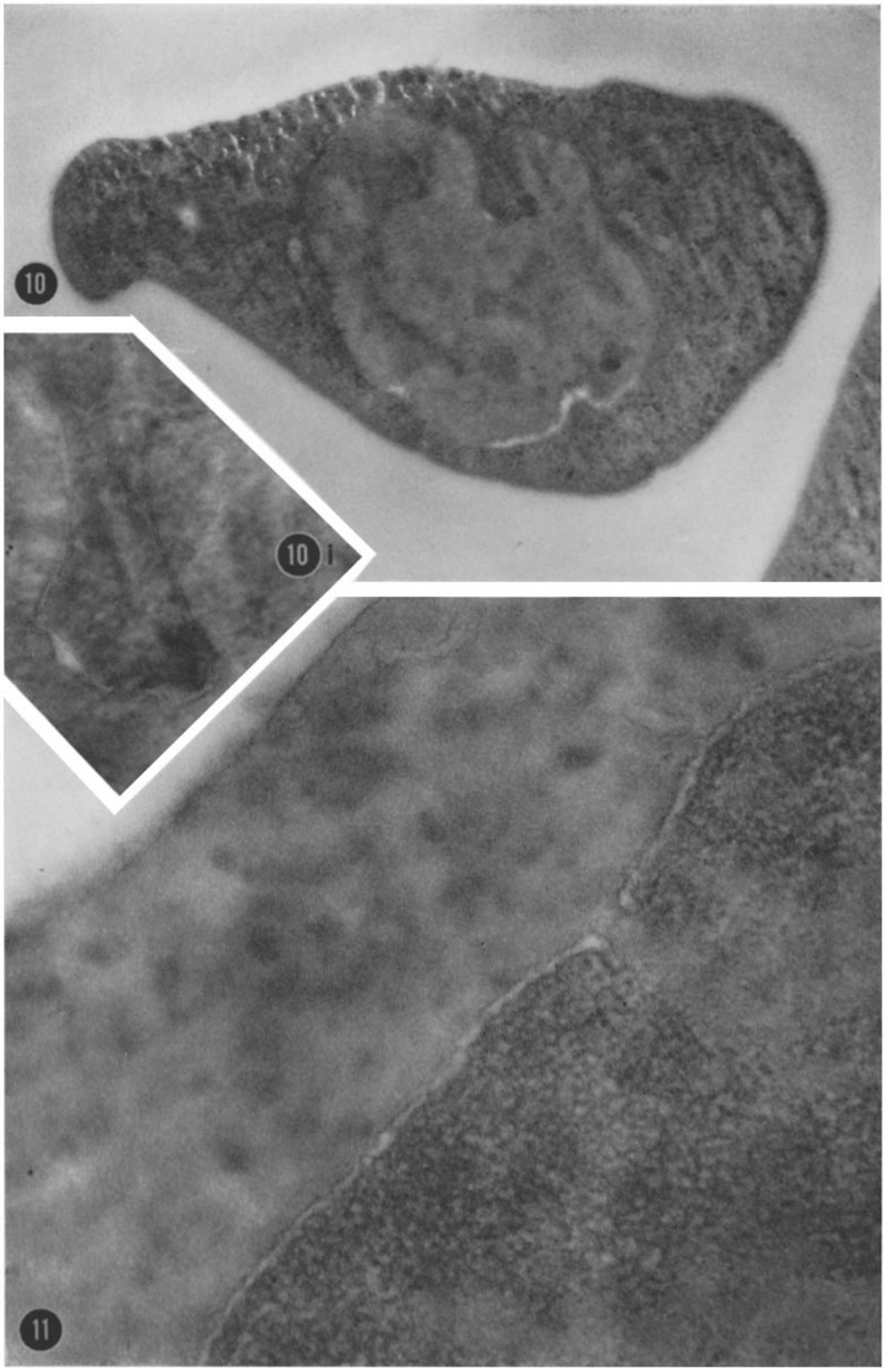
Electron micrograph of unstained section of frog erythrocyte. × 15,000.

FIGURE 10 *i*

Enlargement of nuclear invagination of Fig. 10 to show bounding nuclear envelope. × 40,000.

FIGURE 11

Electron micrograph of frog erythrocyte, showing nuclear envelope and pore. × 80,000.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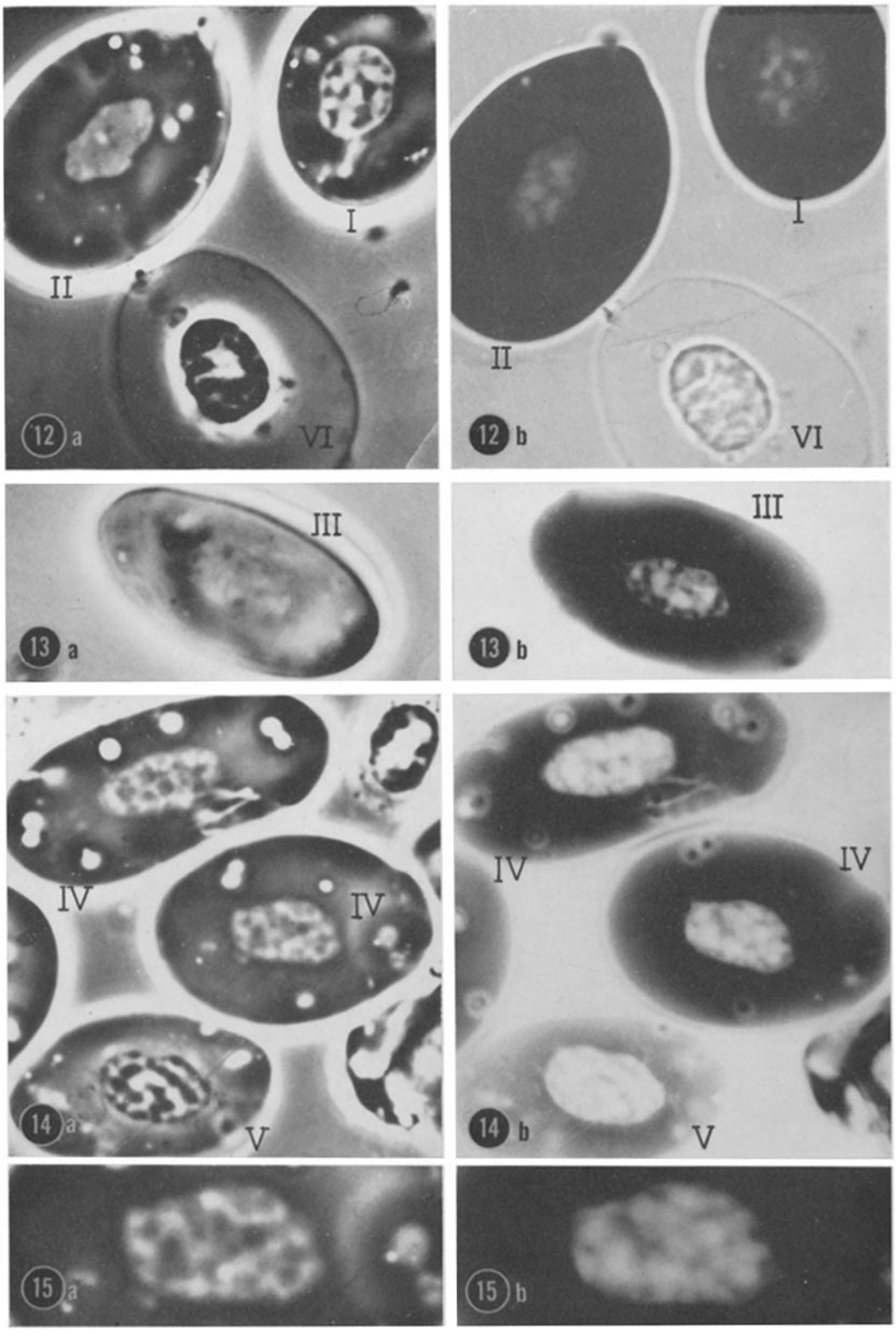
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12 a
I
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14 a
IV
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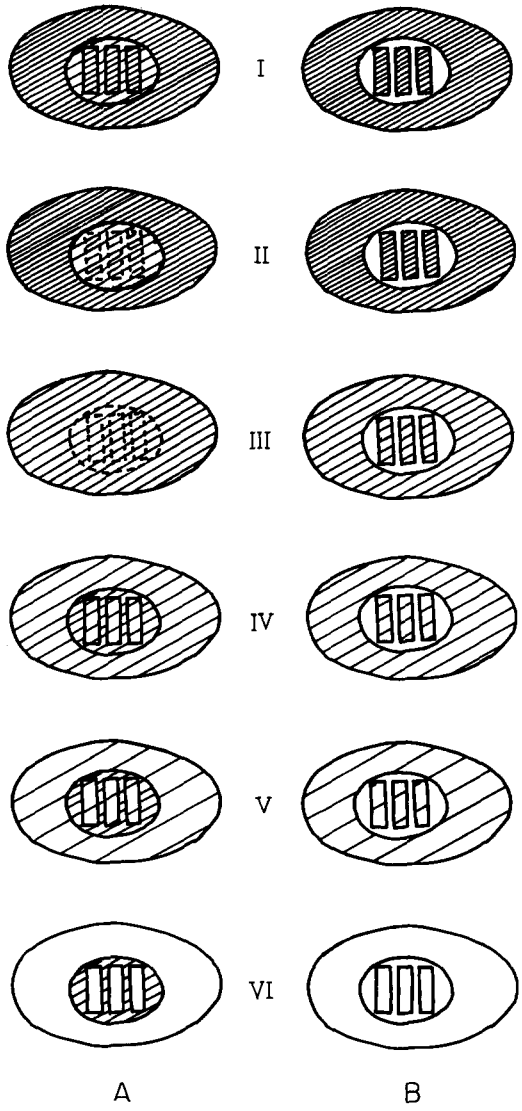
V
14 b
15 a
15 b

FIGURE 16

Column *A* shows the concentration of total dry substance deduced from phase-contrast microscopy; column *B* shows the concentration of haemoglobin indicated by bright-field microscopy at λ 4047 A. The nucleus is the region enclosed by the smaller ellipse, and the rectangular regions within it represent haemoglobin. The number of diagonal lines roughly indicates the magnitude of the concentration.



Stage I:

A. The concentration in the nuclear bodies is less than that of the haemoglobin.

B. The nuclear bodies contain no haemoglobin, while the concentration of haemoglobin is approximately the same in nucleus and cytoplasm.

Stage II:

A. The concentration of haemoglobin within the nucleus only slightly exceeds that of the nuclear bodies, reducing their visibility in phase-contrast.

B. Here and in subsequent stages, III B to VI B, the concentration of haemoglobin is decreasing.

Stage III:

A. The concentration and hence refractive index of the haemoglobin equals that of the nuclear bodies, and the nucleus disappears.

B. The nucleus shows differential contrast in violet light, the nuclear bodies being non-absorbing.

Stage IV:

A. The concentration of haemoglobin is now less than that of the nuclear bodies, the intranuclear haemoglobin being relatively light in phase-contrast.

B. The appearance in violet light is little changed from stage III B.

Stage V:

A. The haemoglobin is considerably diluted, the chromosomes appearing as dark bodies in phase-contrast.

B. The haemoglobin is considerably diluted (*cf.* Fig. 14*b* V).

Stage VI:

Corresponds to complete haemolysis.

A. Only the chromosomes appear highly refractile.

B. The contrast is almost zero.

FIGURE 12

12*a*. Frog erythrocytes in phase-contrast. Stages I, II, and VI.
12*b*. Same cells as *a* in bright-field, λ 4047 A. All × 2,000.

FIGURE 13

13*a*. Frog erythrocyte in phase-contrast. Stage III.
13*b*. Same cell as *a* in bright-field, λ 4047 A. All × 2,000.

FIGURE 14

14*a*. Frog erythrocytes in phase-contrast. Stages IV and V.
14*b*. Same cells as *a* in bright-field, λ 4047 A. All × 2,000.

FIGURE 15

15*a*, 15*b*. The lower of the two cells at stage IV in Figs. 14*a* and 14*b* further enlarged to facilitate comparison.

nucleus and cytoplasm discussed. Swift (19) concludes, with special reference to RNA, that there is no evidence for the transfer of resolvable material. A similarly realistic opinion is held by Watson (22). From detailed electron microscope observations on tissues of rat he believes that the pore is a complex structure and that any passage of material which may take place through it is probably controlled and not a random process.

We will not discuss here the various physiological experiments which test the permeability of the nuclear envelope, the results being contradictory. References to these are given in the interesting paper by Merriam (13), who has shown that isolated oocyte nuclei are permeable to large macromolecules, a conclusion at variance with that of Harding and Feldherr (11), who investigated the semipermeability of *intact* oocyte nuclei.

Erythrocytes differ from the cells previously studied in that a continuity of chemical substance between the cytoplasm and the interior of the nucleus, via the nuclear pores, can be directly demonstrated; the interchange of nuclear and cytoplasmic haemoglobin seems to be a random process, that is, controlled by simple diffusion. The evidence for this is summarised below and the structure of erythrocytes is compared with that of other somatic cells.

In the electron microscope haemoglobin has not been identified by a specific method, although regions within the nucleus exhibit the same spotted appearance, previously discussed, as the cytoplasm. This is seen in the low power electron micrographs (*e.g.*, Fig. 23). The identification, however, depends on the specific absorption. Although a complete absorption spectrum is not yet available, the light-absorption of certain of the nuclear regions is similar to that of the cytoplasm, and the high concentration of dry substance in both volumes indicates that the haem group is combined with a protein.

The continuity between nucleus and cytoplasm of the erythrocytes via the nuclear pores seems obvious in the electron micrographs. The fact that the concentrations of nuclear and cytoplasmic haemoglobin are apparently equal further indicates that there is continuity and hence free diffusion between the two haemoglobin-containing volumes. It might be argued that this equality is merely accidental, that there is perhaps a thin membrane covering the pores which prevents free diffusion and is not visible in the electron micrographs owing, for example, to its having evaporated from the section at the high beam intensities that are used at high magnifications. (This possibility has been noted by Watson (22) in a discussion of the relative visibility, at low and high magnifications, of membrane-like material across the nuclear pores in other cells.) The

FIGURE 17

Fresh preparation of chicken erythrocytes mounted in serum proteins. Phase-contrast, × 2,000.

FIGURE 18

Haemolysed chicken erythrocyte mounted in serum protein, showing relatively dark nuclear bodies. Phase-contrast, × 2,000.

FIGURE 19

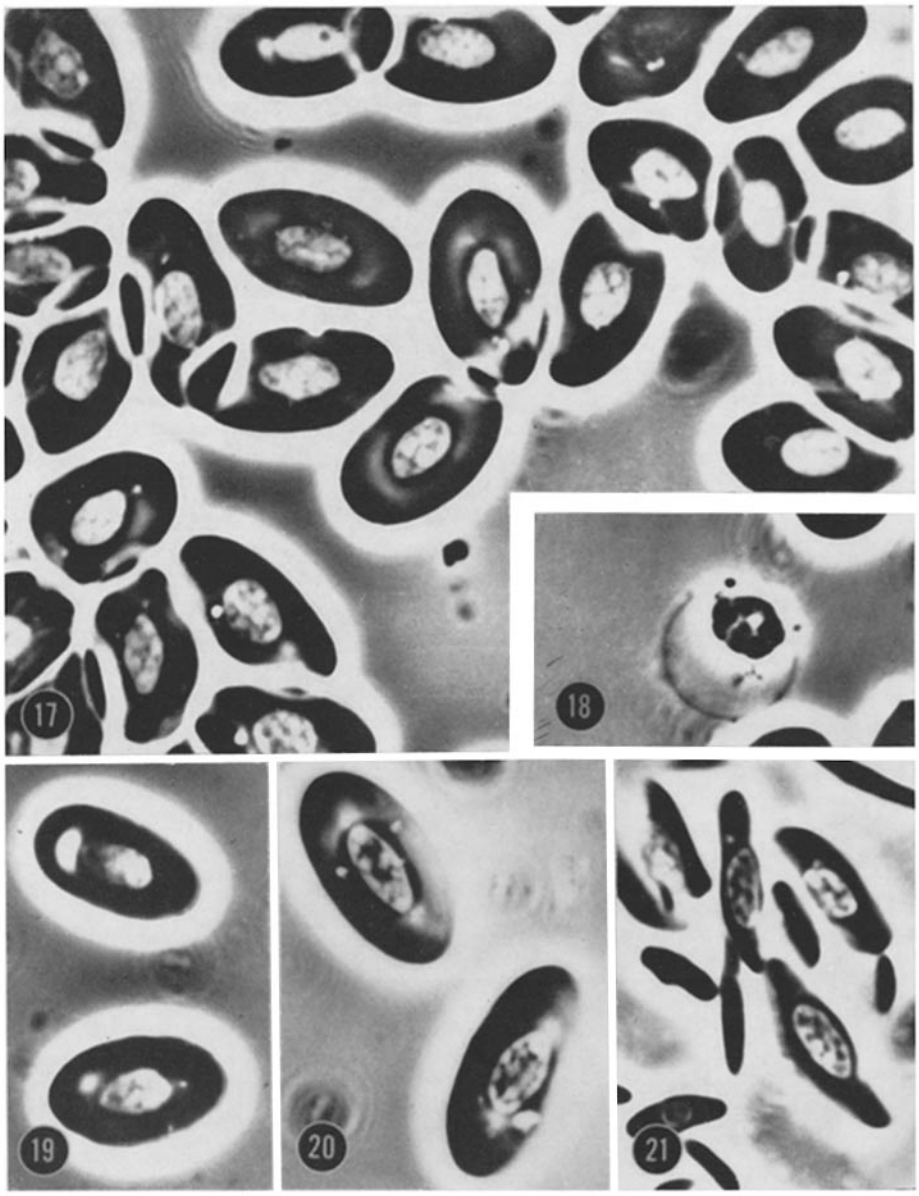
Fresh preparation of chicken erythrocytes mounted in physiological Tyrode. Phase-contrast, × 2,000.

FIGURE 20

Chicken erythrocytes after fixation in OsO_4-sucrose. The channels in the peripheral nuclear chromatin leading from the interior of the nucleus into the cytoplasm can be clearly seen. Phase-contrast, × 2,000.

FIGURE 21

A 1 μ section of chicken erythrocytes fixed in OsO_4-sucrose. Phase-contrast, × 2,000.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observations during haemolysis do not support such a contention, however, and are consistent with the idea that there is free diffusion of haemoglobin between nucleus and cytoplasm. Diffusion of haemoglobin from the nucleus is indicated by the fact that the contrast in the nuclear bodies decreases, becomes zero, and finally reverses. We have not measured the rate at which haemoglobin diffuses into the cytoplasm, although ways of doing this suggest themselves. Diffusion through the plasma membrane and out of the cell is a very slow process under the conditions of our experiments. The data indicate that diffusion within the cell is by comparison rapid, and this might be checked quantitively by densitometry of violet-light photomicrographs of thin sections of cells, fixed at the various stages of haemolysis. Our results do not prove that diffusion of haemoglobin during haemolysis occurs only via the pores in the nuclear envelope, but we think this to be most likely.

What is striking about the general organisation of the nucleus of erythrocytes is that in certain fundamental respects it is remarkably similar to that of the nuclei of other somatic cells which have been studied. Watson (22) summarises his observations on nuclei of tissues of the rat as follows:

"The nucleoplasm of cells when viewed in osmium fixed material consists of masses of moderate density separated by regions of lower density. Near the nuclear envelope the low density portion of the nucleoplasm is disposed in cylindrical channels about 1200 A in diameter which course inwards towards the centre of the nucleus and gradually anastomose and lose their identity in the more generalised regions of low density. At the nuclear envelope each of these channels is associated with one of the pores."

This division of the nucleoplasm into two zones is exactly what has been found in erythrocytes. As a matter of detail the regions of low density referred to by Watson are replaced in the case of the erythrocyte by regions of high density containing haemoglobin. Swift (18) had previously observed, in nuclei other than erythrocytes, the channels of low-density material extending from the pores at the nuclear envelope and had suggested that the relatively high-density material surrounding the channels was chromatin. We have shown that the regions surrounding the channels in erythrocytes contain DNA.

The question arises as to how the pores that have been found in the nuclear envelope of erythrocytes compare with those in other cell types. The general picture that has emerged from studies of the latter is as follows. The pores are circular in shape, of the order of 500 to 1000 A in diameter, and are formed by the junction of the inner and outer membranes of the nuclear envelope. The nuclear pore sometimes appears to be crossed by material of density comparable with that of the nuclear membranes, and it has been considered that this might correspond to a diaphragm which might perhaps control the passage of molecules. An alternative explanation of the appearance of this structure has recently been suggested (7, 22), *viz.*, that it represents merely a strip of pore margin which has been included in the section, and does not correspond to a membrane across the pore. It is further argued that the thickness of the section is comparable with the dimensions of the pore and hence it should be rare to obtain a section that does not include such pieces of nuclear membrane above and below the pore centre as seen in the photomicrograph.

FIGURE 22

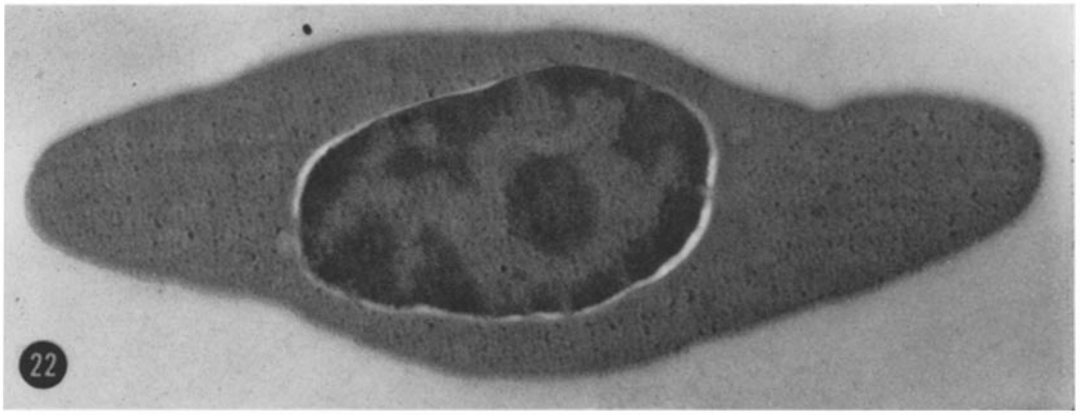
Electron micrograph of chicken erythrocyte fixed in OsO_4-sucrose, embedded in methacrylate, and stained in uranyl acetate. × 15,000.

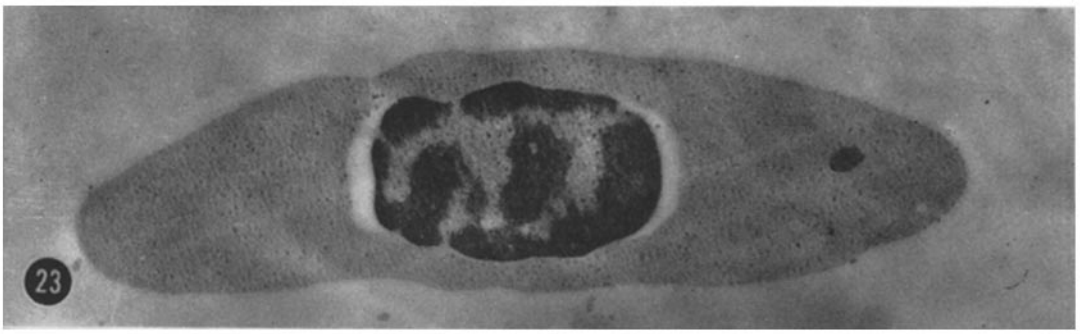
FIGURE 23

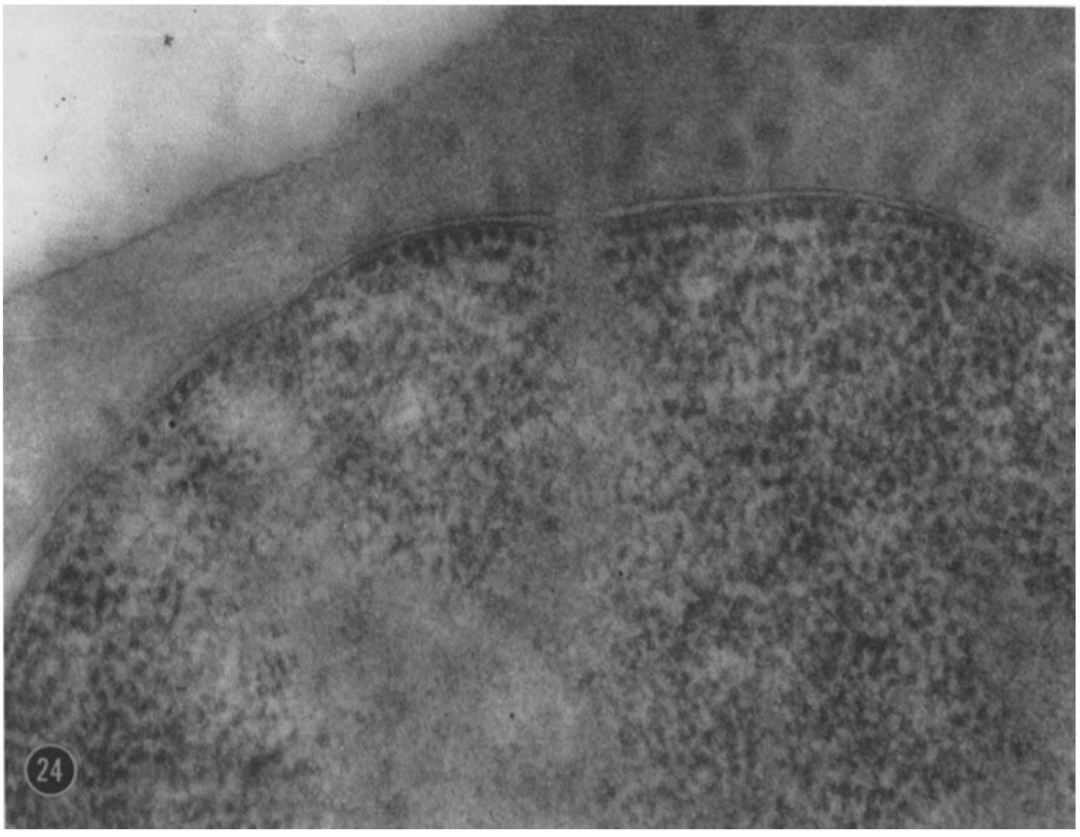
Electron micrograph of chicken erythrocyte fixed in OsO_4-sucrose, embedded in araldite, and stained in uranyl acetate. × 15,000.

FIGURE 24

Electron micrograph of chicken erythrocyte fixed in OsO_4-sucrose, embedded in methacrylate, and stained in uranyl acetate. On the left where the nucleus and cytoplasm have retracted the two parts of the nuclear envelope are seen to be separated. × 80,000.


22


23


24

Other features of pore structure vary between cell types and in general are not so clearly defined. Briefly the findings are as follows. The nuclear envelope of *Amoeba proteus* consists of a double membrane with pores formed by the junction of the two membranes similar to that found in mammalian cells, but superimposed on this is a system of well defined hexagonal prisms precisely oriented with respect to the pores above them (15). Observations have been made on amphibian and echinoderm oocytes (*e.g.* (25), (1)) which have led to the suggestion of a tube-like structure passing through the pore, extending a short distance into nucleus and cytoplasm, and itself made up of microcylinders. According to André and Rouiller (5), however, in arachnid oocytes there is no tubular structure, but what appears to be one is merely dense granular material passing through the pores and collecting round their edges. Watson (22) concludes from his careful study of pores in mammalian cells that there is an extrusion through the pore forming a well defined cortex on the cytoplasmic side, and that this cytoplasmic cuff is the principal contributor to the annuli seen in sections tangential to the nuclear envelope. We consider that the contribution made by the pore edge to these annuli may have been underestimated.

Occasionally granules have been seen near the centre of the pore, but not consistently, and hence they may be accidental.

To summarise, our present findings, showing a gap in the nuclear envelope associated with a channel in the chromatin, are identical with the well established features of the nuclei and pores described in various mammalian cells. Though it seems obvious that the pores in the nuclear envelope of such mammalian cells provide a means for simple diffusion between nucleus and cytoplasm of macromolecules other than those bound to large cytoplasmic organelles, there is no proof that this does occur, and the idea is not generally accepted. One obvious reason for this is that electron micrographs present merely static pictures from which it is difficult to adduce dynamic processes. In the case of the erythrocyte the evidence from electron microscopy is supplemented by measurements of the concentration of a specific marker (haemoglobin) in the nuclear and cytoplasmic compartments, together with observations during the dynamic process of haemolysis. The erythrocyte, however, is a differentiated cell which lacks much of the cytoplasmic organisation found in other differentiated cells. There seem to be no suggestions about the possible function of the nucleus in these erythrocytes, and, of course, in mammals the functional erythrocyte does not contain a nucleus. Observations on erythrocytes, although very suggestive, cannot be generalised. What is clearly desirable is a specific demonstration of whether or not there is free diffusion of each particular component of interest (*e.g.*, RNA) in other cell types with a more complex cytoplasmic organisation than that of erythrocytes.

The nature of the so-called nuclear bodies in erythrocytes is in question. They contain DNA and must obviously be derived from chromosomes. The concentration of material within them is much higher than that generally found in interphase somatic nuclei and is comparable with that in mitotic chromosomes. Furthermore, there is reason for believing that the bodies are not chromatin which has randomly condensed into a variable number of clumps, but that each body is derived from an individual chromosome. Anderson and Norris (4) recently showed that about 20 discrete bodies occur in the nuclei of *Amphiuma* erythrocytes, which is of the same order as the chromosome number, 24. A rough count of the number of bodies in the flattened nucleus of the frog erythrocyte (Fig. 5) shows that the number, roughly about 20, is also approximately the same as the diploid chromosome number, 26. In order to make precise counts, however, better preparative techniques are needed.

Another indication of the nature of the nuclear bodies is obtained from chemical analyses. The ratio of protein to DNA (P/D) in chicken erythrocyte nuclei isolated in non-aqueous media is 2:1 (allowance having been made for the haemoglobin content (17). This may be compared with the P/D of 5.5 (3) for chicken kidney nuclei and indicates that the bodies are protein deficient as compared with functional somatic nuclei. It is known, however, that up to about half of the nuclear protein of functional nuclei (the amount depends on nuclear type and functional state) is easily removed from nuclei and does not appear to be part of the chromosome structure (Allfrey *et al.* (2), page 449). Almost certainly erythrocytes have lost these nuclear proteins. It would be more interesting, however, to compare the P/D ratio in nuclear bodies with that in the mitotic (*e.g.*,

metaphase) chromosomes of the same species. These data, which would help establish the relationship between nuclear bodies and chromosomes, is not available. At higher resolution than is needed to demonstrate the existence of nuclear pores, the nuclear bodies appear to consist of threads or ribbons ranging in size from about 100 A downwards. These will not be discussed here.

Preliminary observations on other species suggest that the general pattern of organisation of nucleated erythrocytes may be similar throughout the animal kingdom.

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