SUMMARY

Adult hen erythrocyte nuclei are isolated from cells or haemolysed in situ by acting on the plasma membrane with rotating knives or with non-ionic detergents. When the isolation medium contains magnesium ions (1 mM), sucrose (0.4 M) and Tris buffer (0.01 M, pH 7.5) called SMTOG (see text), the ultrastructure in thin sections through the condensed chromatin bodies, after staining with either uranyl-lead or phosphotungstic acid (PTA), is similar to that found in the intact cell. Hence it can be concluded that the 2 phases which comprise chromatin, the o- and the e-phase, survive nuclear isolation. These are so called because the structural units in chromatin are arranged at the surface of the nucleus into one or more layers and give rise to oddly (o) and evenly (e) numbered bands. The o-phase is also largely retained after extensive washing in 0.07 M NaCl as shown by electron microscopy and biochemical measurements; only 6% of the total nuclear protein is removed, a value small compared with the fractional amount of the chromatin protein calculated to lie in the o-phase, about 70%. After extensive washing in saline-EDTA there are structural changes in chromatin, but biochemical data show that the molecules in the o-phase are also largely retained; loss of protein amounts to between 5 and 11%.

These data suggest that the o-phase is a structural component of the chromatin bodies. They support the hypothesis that condensed chromatin is formed by folding superunit threads. These units consist of a central thread-like element about 17 nm diameter which stains preferentially with uranyl-lead and forms the e-phase, with an outer cylindrical shell forming the o-phase of total diameter about 28 nm.

The 5-10% proteins removed by salt washes are located exclusively in a particulate component, quite likely the chromatin. They have been examined by sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis. There are about 10 or more protein species, ranging in molecular weight from 21,000 upwards. The groups of large granules previously found in the nuclear sap of intact erythrocytes are shown to be associated with an amorphous or finely fibrillar body. During nuclear isolation and subsequent washes, we observed the fate of this large granule complex as well as that of the nuclear envelope and the filled cavities in chromatin.

INTRODUCTION

Earlier electron-microscope studies (Davies, 1968; Tokuyasu, Madden & Zeldis, 1968; Small & Davies, 1970) on several types of cell nuclei showed that when a thin section through condensed chromatin was treated with uranyl-lead a pattern of dots and dashes, width roughly 17 nm and separation roughly 28 nm, became visible, the areas in between taking up less stain. Davies, Murray & Walmsley (1974) showed that in a serial section stained with ethanolic phosphotungstic acid (PTA) the stain distribution was homogeneous. Hence it was concluded that the molecules in chromatin,
like the stain, are distributed between 2 phases. The thread-like regions which give rise to the dots and dashes are referred to as the e-phase because they occupy the evenly numbered bands seen near the nuclear envelope (Fig. 1, p. 118) in sections stained with uranyl-lead. The regions which stain less with uranyl-lead are referred to as the o-phase because they occupy the odd-numbered bands. It was concluded that, probably, the e-phase has a higher DNA-to-protein ratio than the o-phase. There is some uncertainty due to lack of data on the availability of binding sites for stain. There are 2 possible interpretations of these findings. First, that the o-phase represents merely a matrix material, for example a component of the nuclear sap in which thread-like structural units are embedded. The second hypothesis discussed in Davies et al. (1974), and based on observations made when chromatin is disrupted, envisages that the o-phase is part of the molecular structure of chromatin and forms an outer shell around the central element, diameter about 17 nm, previously referred to as the unit thread. These superunit threads are about 28 nm in diameter. A crucial question affecting the validity of the superunit thread hypothesis is whether the molecules forming the o-phase are easily removed from chromatin, as might be expected if the o-phase were a component of the nuclear sap, or whether they resist nuclear isolation and procedures known to remove loosely bound molecules from chromatin.

We have now studied the protein composition and ultrastructure of the chromatin bodies in chicken erythrocyte nuclei during the isolation of the nucleus and the preparation of chromatin. Our preparation of chromatin (see also Zubay & Doty, 1959; Dingman & Sporn, 1964; Hnilica, 1967; Fambrough, 1969; Elgin & Bonner, 1970) involves 3 major steps: (1) isolation of the cell nucleus so as to separate it from cytoplasmic organelles; (2) washing with saline (0.075 M) or saline-EDTA to remove any remaining nuclear sap and molecules loosely bound to chromatin; and (3) dispersal of the interphase chromosomes by hypotonic treatment, followed by shear to reduce the molecular weight so as to obtain soluble chromatin. We studied in particular the effects of steps 1 and 2 on the chromatin bodies as well as the other structural features of erythrocyte nuclei (Davies et al. 1974) including the filled cavities in chromatin and the large-granule complex.

A main conclusion is that the o-phase in chromatin is largely retained after the extensive washing designed to remove loosely bound molecules. In addition we studied, by SDS polyacrylamide gel electrophoresis, a group of proteins which constitute a relatively minor mass fraction of the nucleus but which appear to be located exclusively in a particulate component, that is chromatin and perhaps partly the large-granule complex. These proteins can be retained in erythrocyte nuclei isolated in media containing sucrose and magnesium, but are extracted upon washing with saline or saline-EDTA.

Chicken erythrocyte nuclei were chosen primarily for their relative uniformity and biochemical and ultrastructural simplicity. However, it can be shown (H. G. Davies & M. E. Haynes, manuscript in preparation) that there are basic underlying similarities in the structure of the interphase chromosomes irrespective of species, and the tissue of origin: nerve, muscle, epithelial and connective tissue.
Materials and Methods

Material

Adult hens were used, mainly Light Sussex breed. To check that the results, for example the presence of filled cavities and the large-granule complex were independent of breed, White Leghorns were also used. The results were independent of the breed.

Isolation of nuclei by Virtis in SMTOG

Nuclei were isolated from erythrocytes by a procedure very similar to that developed by Zentgraf, Deumling & Franke (1969). The essential features are the use of high-speed rotating knives to allow removal of the plasma membrane in a sucrose-magnesium isolation medium. Blood (40-60 ml) from one animal was collected in 40 ml of medium (SMTOG) containing sucrose (0.4 M), MgCl₂ (1 mM), Tris buffer (0.01 M, pH 7.5), n-octanol (4 mM) and gum arabic (3 %). The bovine serum albumin (0.5 %) included in the medium originally designed by Kuehl (1964) was omitted since it adsorbed to nuclei, appeared in the SDS gels and hindered their interpretation. The anticoagulant heparin was avoided since it caused nuclei to swell (Davies & Spencer, 1962); presumably its negative charge resulted in dissociation of the histone from the DNA. Negatively charged detergents were similarly avoided and also positively charged ones, which cause chromatin condensation and bind to chromatin. Apparently however an anticoagulant, like heparin, can be included when processing whole blood cells without causing changes in nuclear morphology (Tanaka & Goodman, 1972). The blood-SMTOG mixture was centrifuged at 4000 rev/min for 5 min and the buffy coat removed by aspiration. The erythrocyte pellet was resuspended and centrifuged 3 times in 20 ml of SMTOG to remove serum proteins. After resuspension in 40 ml SMTOG the mixture was homogenized in the Virtis 45 (Arthur H. Thomas, Philadelphia). Hence they are called Virtis-SMTOG nuclei, or Virtis-nuclei. Treatment was at top speed, 110 V giving 40000 rev/min, for 3 periods of 30 s with 15-s intervals, followed by homogenization with 8 up-and-down strokes in a Potter Elvehjem homogenizer (clearance 1 x 10⁻⁴ in. (2.54 μm)) rotating at 950 rev/min, followed by filtration through 4 layers of muslin, followed by centrifugation at 4000 rev/min for 10 min. This procedure was repeated twice, the nuclear pellet being successively resuspended in 60 ml and then 80 ml of SMTOG. The final pellet was resuspended using the Virtis at top speed in 80 ml of sucrose (2.4 M), MgCl₂ (1 mM), Tris buffer (0.01 M, pH 7.5) and n-octanol (4 mM); then this suspension was layered on a 2-fold volume of a similar medium and centrifuged for 90 min at 75 000 g as to float off residual membranes. These pelleted nuclei were resuspended and washed 3 times with SMTOG to give SMTOG-washed Virtis-nuclei. Saline-EDTA washed Virtis-nuclei were obtained by resuspension of these SMTOG-washed nuclei in a 10-fold volume of NaCl (0.075 M), EDTA (0.024 M, pH 8.0) and stirred for 30 min. This step was repeated in a 5-fold volume.

All the above steps and those below were carried out as far as possible between 0 and 4 °C: the Sorvall RC2B centrifuge with the HB 4 rotor was employed throughout unless otherwise stated.

Haemolysis of cells and nuclei

Triton haemolysis in SMTOG. This method was chosen to remove cytoplasm and nuclear sap because it is less likely to damage nuclei than treatment with knives. It has the disadvantage that the few cytoplasmic organelles remaining in mature erythrocytes are retained within the detergent-modified plasma membrane. Blood from one animal was collected in SMTOG and treated to remove white cells and serum proteins as described under Isolation of nuclei. The erythrocyte pellet was then resuspended in a small volume of SMTOG and slowly added to a 10-fold volume of SMTOG containing the non-ionic detergent Triton X-100 at 0.5 % (w/v) with constant stirring. After 5 min the suspension was centrifuged at 3500 rev/min for 10 min and the resulting pellet resuspended and washed twice in SMTOG, first 20 ml then 10 ml, to give SMTOG-washed Triton-nuclei. When required the SMTOG-washed nuclei were further
washed with stirring in a 10-fold then a 5-fold volume of either saline (0.075 M NaCl) or saline-EDTA (0.075 M NaCl; 0.024 M EDTA, pH 8.0) for 30 min each time.

Saponin haemolysis in saline. The procedure was similar to that of Seligy & Miyagi (1969). Blood from one hen was collected in an approximately equal volume of SMTOG, mixed, and centrifuged at 4000 rev/min for 5 min and the buffy coat removed by aspiration. Cells were further washed twice in NaCl (0.14 M), phosphate buffer (0.015 M, pH 7.0) and haemolysed in a 10-fold volume of this solution with saponin (0.05%, w/v). By light microscopy, lysis appeared complete after 4 min and was allowed to continue for 30 min. After centrifugation for 4 min at 4000 rev/min the cells were washed twice, first in a 10-fold then a 5-fold volume of NaCl (0.14 M).

Preparation of isolated soluble chromatin

Isolated chromatin was prepared from salt-EDTA washed Virtis-nuclei, derived from 40-60 ml of blood, according to the procedure in Elgin & Bonner (1970). The washed nuclei were resuspended in 20 ml of Tris buffer (0.01 M, pH 8.0) with the Potter-Elvehjem homogenizer and centrifuged at 8000 rev/min for 10 min: in all there were 3 washes. The final pellet was resuspended in the same buffer and layered over a 2-fold volume of sucrose (1.7 M), Tris buffer (0.01 M, pH 8.0) and centrifuged for 2 h at 25000 rev/min in the SW27 rotor of the Beckman L 265B centrifuge. There was no visible supernatant. The pellet was again suspended in 20 ml of the same Tris buffer, so as to dissolve the sucrose and centrifuged at 10000 rev/min for 10 min. The pellet was resuspended in 20 ml of the Tris buffer and the gel sheared in the Virtis 45 homogenizer at 60 V for 90 s. The sheared chromatin was centrifuged for 30 min at 10000 rev/min and the supernatant used in the estimations of protein to DNA.

Determination of protein and DNA

Samples for determination of protein and DNA were obtained by precipitation of nuclei, soluble chromatin, or protein fractions with an equal volume of cold 10% perchloric acid (PCA). The precipitates were washed twice with cold 5% PCA followed by 2 washes each in ethanol:ether 1:1 and finally ether to remove lipid. For protein determination, the pellets were dissolved in 4 M deionized urea, 1% sodium dodecyl sulphate (SDS) with incubation for 20 min at 50 °C where necessary and protein was estimated by the method of Lowry, Rosebrough, Farr & Randall (1951) using, as a standard, bovine serum albumin (BSA) dissolved in the same solvent. For DNA estimation the washed precipitates were hydrolysed in 5% PCA for 20 min at 90 °C. Calf thymus DNA at a concentration of 1 mg/ml was hydrolysed simultaneously as a standard; the O.D. at 260 nm for a 1 mg/ml solution varied in several experiments in the range of 25-30. DNA was determined directly from the u.v.-absorbance at 260 nm of the acid hydrolysates and the results were checked by the diphenylamine assay of Burton (1956).

For estimation of the fraction of total nuclear protein extracted by saline-EDTA or saline, 0.2-ml samples of a homogeneous suspension of the SMTOG-washed nuclei were removed and centrifuged to remove any SMTOG-soluble material and the pellet was treated with 5% PCA, washed as described above, and the total protein content of these samples was estimated using the procedure of Lowry et al. (1951). A known volume (usually 10 ml) of the same suspension of nuclei was then pelleted and treated with saline or saline-EDTA as described earlier; the nuclei were again pelleted and estimations of the protein content of the supernatant were made by precipitating it with an equal volume of 10% PCA. The precipitate was washed to remove lipid as above and protein estimated by the Lowry et al. (1951) procedure. This saline-extractable fraction could then be expressed as a percentage of the total protein present in the original 10 ml suspension of nuclei.

Estimations of the amount of protein removed from saline-EDTA washed nuclei during the preparation of soluble chromatin were obtained by comparison of the 2 protein : DNA ratios.

SDS-polyacrylamide gel electrophoresis

Preparation of samples. Protein extracts were precipitated with an equal volume of cold 10% PCA. The precipitates were washed once each with 5% PCA, 1:1 ethanol:ether, and ether. The precipitates were taken up in 4 M urea, 1% SDS, 1% β-mercaptoethanol (MCE),
Electrophoresis. Electrophoresis was performed in cylindrical tubes (0.7 x 9 cm). The gels contained 8% cyanogum, 6 M urea, 0.1 M phosphate buffer (pH 7.1), 0.1% SDS, 0.1% tetramethylethylenediamine and 0.05% ammonium persulphate. The running buffer was 0.1% SDS, 0.1 M phosphate buffer (pH 7.1). Pre-electrophoresis was for 30 min at 6 mA per tube. The samples (20-50 μg protein) were applied to the top of the gel along with a bromophenol blue marker. Electrophoresis was performed at 6 mA per tube for 6 h, the running buffer being changed every 2 h. At the end of the run, gels were stained overnight in 0.1% Coomassie blue. Electrophoresis was performed at 6 mA per tube for 6 h, the running buffer being changed every 2 h. At the end of the run, gels were stained overnight in 0.1% Coomassie blue, in aqueous 50% methanol, 10% acetic acid. They were destained electrophoretically in aqueous 10% methanol and stored in this solution. Co-electrophoresis of protein standards of known molecular weight (bovine serum albumin, ovalbumin and cytochrome c) permitted molecular weight estimations of the proteins under investigation.

Preparation of chicken erythrocyte histones

Nuclei from 100 ml of chicken blood were prepared by the procedure described under isolation of nuclei. Instead of washing in SMTOG they were then washed twice in STKM medium, containing sucrose (0.25 M), Tris buffer (0.05 M, pH 7.5), KCl (0.025 M), MgCl₂ (5 mM); histones were extracted with 0.4 N H₂SO₄ and eventually converted to a dried powder according to the method of Panyim & Chalkley (1969). Histone samples were prepared for SDS-polyacrylamide gel electrophoresis as described on p. 116. When checked by the standard Panyim and Chalkley system for separation of histones (Panyim & Chalkley, 1969) the histones prepared by us from chicken erythrocyte chromatin gave a band pattern (not shown) which compared well with that found from the same cell type by Panyim, Bilek & Chalkley (1971).

Preparation of chick globin

Haemoglobin was first prepared from chicken erythrocytes by toluene extraction (Matsuda & Takei, 1963). Globin was then prepared from haemoglobin by acetic acid-HCl precipitation (Rossi Fanelli, Antonini & Caputo, 1958). The chicken globin was further purified on a CMC cellulose column using urea (8 M), phosphate buffer (0.01 M, pH 6.9) for elution (Clegg, Naughton & Weatherall, 1965). The column fractions containing globin were pooled and an equal volume of 20% trichloroacetic acid was added. After standing on ice for 1 h, the mixture was centrifuged at 8000 rev/min for 10 min. The globin pellet was prepared for electrophoresis as described on p. 118.

Electron microscopy and microspectrophotometry

For methods see Davies et al. (1974) and Small & Davies (1976). Briefly, fixation was in glutaraldehyde (3%) followed by OsO₄ (1%) in aqueous media of composition corresponding to the medium in which cells and nuclei were exposed prior to fixation. Whole cells were processed by the capillary tube method, whereas nuclei and haemolysed cells were spun at appropriate speeds in plastic centrifuge tubes and fixed in situ.

For microspectrophotometry, nuclei were fixed in glutaraldehyde only and examined, in unstained 1-2 μm thick sections cut with glass knives, on the Zeiss U.M.S.P.1.

RESULTS

Electron microscopy

The chromatin bodies. After haemolysis of whole cells in the Triton-SMTOG solution (see Methods) the remaining cellular structures did not alter after further washing in SMTOG. When stained with uranyl-lead (Fig. 6) no haemoglobin could be detected in the cytoplasm, and the regions between the chromatin bodies previously
occupied by nuclear sap were also empty apart from some threads and granules. The bulk of each chromatin body clearly showed a dot-dash pattern similar to that previously described in intact cells (see Fig. 1). There were also small areas in which the pattern was difficult to distinguish and presumably these corresponded to the cores, regions in which the nucleoprotein threads were either finer or more closely packed.

Fig. 1. A, schematic diagram showing the ultrastructure of a thin section through an isolated erythrocyte nucleus. In the chromatin bodies, uranyl-lead is distributed between 2 phases, relatively densely staining dots and dashes which are sections through thread-like regions and lesser-staining intermediate regions (not shaded). At the surface of the nucleus, shown enlarged at B, the threads are aligned in layers and give rise to densely staining even-numbered bands ($b_2$, $b_4$, ...) and less-densely staining odd-numbered bands ($b_1$, $b_3$, ...). In the interior the arrangement appears more random. Hence the less-dense regions are referred to as the o-phase and the material in the dots and dashes as the e-phase; note, however, that the even-numbered bands contain both e- and o-substance. fc, filled cavities; lgc, large granule complex; m, membranes; cores contain fc; im, inner membrane of nuclear envelope; r, ribosomes attached to a fragment of outer membrane of envelope. A, shows hexagonally packed superunit threads in which the o-phase is formed from the outer shells; $d$ is width of regions which stain intensely with uranyl-lead and form the e-phase; $a$ is the separation of the units shown in part end-on and also side-on. Data on intact 17-day embryo chick erythrocytes (Davies & Small, 1968) show $d$ averages 17.4 nm (15.0–20.0) and $a$ averages 28.0 ± 4.0 nm: measurements on isolated nuclei give similar values. (Davies et al. 1974). In marked contrast to the pattern after uranyl-lead, the chromatin stained nearly homogeneously after PTA (Fig. 7). There was a narrow zone of high density around the periphery of each chromatin body, similar to that described previously in some intact erythrocytes. The electron densities per unit thickness, proportional to the amount of stain taken up per unit volume were similar, about 0.32, after both uranyl-lead and PTA staining.

Washing in 0.075 M NaCl for about 1 h Triton-nuclei previously washed twice
with SMTOG resulted in no visible change to the dot-dash pattern after staining with uranyl-lead (Fig. 8) or to the homogeneous pattern after PTA (Fig. 9). These data show clearly that the 0-phase survives haemolysis in Triton and further exhaustive washes in SMTOG and in a saline solution. The 2 staining patterns are shown at higher power in Figs. 13, 14.

Another sample of the same SMTOG-washed nuclei was washed with 0.075 M NaCl, 0.024 M EDTA for 2 successive periods of 0.5 h. When whole cells were observed in the phase-contrast microscope, the chromatin bodies were seen to have swollen and this appearance is retained in thin sections (Fig. 10) examined in the electron microscope. There also the chromatin bodies are seen to have swollen and have often filled the space previously occupied by the nuclear sap. A dot-dash pattern in the chromatin is now as apparent after PTA as after uranyl-lead stain (compare Figs. 10 and 11). Concomitant with the swelling, real non-staining spaces appear within the chromatin due to the dots and dashes having separated. After either uranyl-lead or PTA stain the dots and dashes, when separated, appear larger than those in saline-washed nuclei and are about 25–30 nm in width (see Davies et al. 1974). But areas still remain in which the packing is fairly close and the pattern of staining and lesser-staining regions is preserved.

After haemolysis of cells in saponin-saline, the ultrastructure of the nucleus (not illustrated) is very similar to that after Triton-SMTOG. Evidently, the 2 phases in condensed chromatin are as well retained when nuclei are exposed directly to saline as when the chromatin is first treated with sucrose and divalent cations at 1 mM (SMTOG) and then treated with a salt solution.

After isolation of erythrocyte nuclei by the Virtis-SMTOG method their ultrastructure was very similar, as expected, to that already reported by Zentgraf et al. (1969). Either before or after further washing with SMTOG, the dot-dash pattern with lesser-staining intermediate areas (Fig. 15) was visible after uranyl-lead stain. After treatment with PTA the pattern could not be distinguished, but the electron density per 100 nm was considerably lower than in the similarly stained nucleus haemolysed in Triton SMTOG. The reason for this is not clear but it implies less change in the packing of the molecules and a greater similarity to the intact cell (Davies et al. 1974). A variable proportion of Virtis-SMTOG nuclei in our preparations was damaged. Either the nuclei were fragmented or the threads comprising chromatin had begun to separate. Davies et al. (1974) reported that after the first treatment with the Virtis homogenizer, in one count about 25% of the nuclei were severely disrupted. These nuclei are largely removed during subsequent steps but nuclei which are less damaged evidently remain in the final preparation.

When SMTOG-washed Virtis-SMTOG nuclei were treated with either saline or saline-EDTA the resulting electron-microscope appearance was very similar to that already reported for similarly treated Triton-SMTOG nuclei.

**Cavities in chromatin bodies.** Scattered throughout the chromatin bodies of the intact cell are roughly circular spaces up to about 150 nm in diameter (Davies et al. 1974). These spaces are sections through roughly spherical cavities and their contents appear more-or-less amorphous after staining with uranyl-lead. The contents of these cavities...
are retained in nuclei obtained by treatment in Triton, saponin or Virtis and are still present after washing in saline. They are more difficult to distinguish after staining with PTA (Figs. 7, 9). In one sample prepared from Triton-nuclei prior to SMTOG wash, counts in the electron microscope established that in about 25% of the nuclei there were numerous cavities in the chromatin bodies. Microspectrophotometric analyses were made on sections about 2 μm in thickness. In none of 20 traces was the smallest inflexion detected in the spectra at 410 nm, the absorption maximum of haemoglobin after glutaraldehyde fixation. This method for detecting haemoglobin is very sensitive and was used (Small & Davies, 1970) on sections 0.5 μm in thickness, through intact amphibian erythrocytes to show that the concentration of haemoglobin in condensed chromatin is about 10% of that in the cytoplasm. In 2-μm sections, proportionally smaller amounts can be measured. We conclude that there is negligible haemoglobin present and the result strongly suggests that the material in the cavities is not haemoglobin. In the swollen masses of chromatin found in Triton or Virtis-nuclei treated with saline-EDTA, stained with either uranyl-lead or PTA, there are circular areas (Figs. 10, 11) containing material which no doubt corresponds to the contents of the cavities. It is difficult to be sure but their numbers may be less than in samples of the same preparation treated in saline alone.

Davies et al. (1974) described spherical bodies, up to 0.4 μm in diameter, located between the chromatin bodies in some preparations of erythrocyte nuclei after the first treatment with the Virtis. These bodies which may contain haemoglobin are absent from the final Virtis preparations.

The large-granule complex. In intact erythrocytes from adult hen one or two prominent groups of large granules, heterogeneous in size and structure and up to about 30 nm in diameter, were observed lying in the nuclear sap area between the chromatin bodies (Davies et al. 1974). These groups of granules (Figs. 6, 12) occur with about the same frequency as do nucleoli in many somatic cells. In both Triton and Virtis-nuclei each group was very commonly seen to have associated with it an amorphous, or finely fibrillar body (Figs. 6, 8) which stained to the same extent as the contents of the cavities in the chromatin bodies. This amorphous component was first seen in haemolysed cells and only later was it found (Davies et al. 1974) in the intact cell where it is usually not visible due to it taking up stain to approximately the same extent as the nuclear sap in which it is immersed.

After further washing of Triton-nuclei in saline the large-granule complex is preserved, although the granules sometimes stain less intensely. The complex is also retained after further treatment with saline-EDTA (Figs. 10, 11).

Plasma membrane and nuclear envelope. After treatment with Triton, the plasma membrane collapses, lying close to the nucleus and its structure is disordered (Fig. 7). The visibility of this membrane is higher when stained with PTA than after uranyl-lead (Figs. 6, 7). The outer membrane of the nuclear envelope is not visible after Triton treatment, but there are a few disordered membrane-like fragments at the surface of the nucleus where there may be a few polysomes presumably also derived from the outer membrane. The inner membrane of the envelope also cannot be distinguished.
Ultrastructure and biochemistry of chromatin

After haemolysis in saponin-saline the plasma membrane did not collapse and the outer component of the nuclear envelope is seen to have lifted away and to be lying some distance from the nucleus. The inner component of the nuclear envelope, not normally visible in the intact cell because it stains to the same extent as the \( b_1 \) layer, can now be seen.

The plasma membrane, which is evidently resistant to the action of detergents, is removed by Virtis treatment. However, in agreement with the results of Zentgraf et al. (1969) fragments remain of the outer component of the nuclear envelope and these sometimes have attached ribosomes. Treatment with 0.5% Triton, at the 2.4 M sucrose stage in the Virtis procedure, was not effective in removing all these fragments. The visibility of the inner nuclear membrane in the Virtis-nuclei was variable. For example, after the treatment with Triton, just mentioned, the inner membrane was visible in some cells (Fig. 16) and not in others (Fig. 15). Washing Virtis-nuclei with saline-EDTA often made the inner membrane visible due, we assume, to swelling of chromatin and a lowering in the stain uptake in the \( b_1 \) layer.

**SDS gels**

The compositions of the various protein fractions removed from washed whole erythrocytes, analysed by SDS polyacrylamide gel electrophoresis, are shown in Fig. 2. The initial haemolysate (Fig. 2B) from cells treated with Triton-SMTOG, which contains cytoplasm and nucleoplasm, shows only one major band due to haemoglobin and several minor bands which are haemoglobin oligomers, formed during gel preparation procedures, as is frequently observed. We know these bands to be due to haemoglobin and its oligomers because they all coincide in position with the bands found from a purified chicken globin (Fig. 2A) and because calculations of the molecular weights of the various minor bands indicate that they are globin multimers. A subsequent SMTOG wash removes more haemoglobin (Fig. 2C). A second SMTOG wash (Fig. 2D) removes yet more haemoglobin and 2 additional protein species, just detectable at dotted lines in Fig. 2D, not detectable in the haemolysate and first wash. In a subsequent saline-EDTA wash these 2 protein species appear in an increased proportion and about nine more distinct high-molecular-weight bands (Fig. 2E) which are not found in the nucleo-cytoplasmic fraction (Fig. 2B). There is still (Fig. 2E) an intense band due to haemoglobin: the main new band corresponds to a protein of molecular weight 21 000 (dot in Fig. 2E). Molecular weight was calculated from the expected linear plot relating relative mobility with the known molecular weight of standard proteins. When the nuclei were washed with saline alone the composition of the protein fraction (Fig. 2F) was identical to that of the saline-EDTA wash.

The compositions of the various protein fractions removed from nuclei isolated by the Virtis-SMTOG method are shown in Fig. 3. We did not attempt to obtain a fraction corresponding to the cytoplasmic and nuclear sap as with Triton, because we supposed that the considerable nuclear disruption in the first Virtis treatment, reported by Davies et al. (1974) might contribute proteins whose retention depended on maintaining nuclear ultrastructure. In the first SMTOG wash the major protein is haemoglobin (Fig. 3A) with a smaller proportion of the protein with a molecular
weight of 21,000 and a number with higher molecular weight. In the second and third SMTOG washes (Fig. 3B, c), the proportion of haemoglobin decreases with a concomitant increase in relative amount of protein with molecular weight 21,000 and above. In the saline-EDTA wash (Fig. 3D) there is very little haemoglobin and as in the Triton-nuclei, the major band is at 21,000 molecular weight. Fig. 3E is chicken globin and Fig. 3F various proteins (see text to Fig. 3) of known molecular weight used as standards.

Table 1. Estimation of the percentage of total nuclear protein soluble in saline-EDTA or saline solution

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Nuclei: how isolated</th>
<th>% of protein removed in 1st wash</th>
<th>% of protein removed in 2nd wash</th>
<th>% of protein soluble in saline-EDTA (total)</th>
<th>% of protein soluble in saline (total)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Virtis</td>
<td>2.4^d</td>
<td>2.6^d</td>
<td>5.0</td>
<td>—</td>
</tr>
<tr>
<td>2</td>
<td>Virtis</td>
<td>5.0^d</td>
<td>0.6^d</td>
<td>5.6</td>
<td>—</td>
</tr>
<tr>
<td>3</td>
<td>Virtis</td>
<td>3.2^d</td>
<td>0.6^d</td>
<td>3.8</td>
<td>—</td>
</tr>
<tr>
<td>4</td>
<td>Virtis</td>
<td>3.6^d</td>
<td>1.1^d</td>
<td>4.7</td>
<td>—</td>
</tr>
<tr>
<td>5</td>
<td>Triton</td>
<td>9.95</td>
<td>1.35</td>
<td>11.3</td>
<td>—</td>
</tr>
<tr>
<td>6</td>
<td>Triton</td>
<td>8.27^d</td>
<td>1.03^d</td>
<td>9.3</td>
<td>—</td>
</tr>
<tr>
<td>7</td>
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<tr>
<td>Average</td>
<td></td>
<td></td>
<td></td>
<td>10.8</td>
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</table>

Nuclei were isolated in Virtis-SMTOG and washed 3 times in SMTOG, or haemolysed in Triton X-100 and washed twice in SMTOG. Proteins extracted in 2 further washes of 30 min each in either saline-EDTA or saline, are shown in cols. 3, 4 and the totals in cols. 5, 6; the values are expressed as a percentage of total nuclear protein. ^d indicates duplicate measurements.

Fragments of outer nuclear membrane sometimes with attached ribosomes remained in our experiment using 0.5 % Triton on Virtis-nuclei, although their number may have been reduced. Nevertheless, because of the known efficacy (see Discussion) of Triton in removing outer membrane and the small sample observed by us in the electron microscope which may have been atypical, we studied the SDS gel pattern of saline-EDTA washes of nuclei either treated or untreated with Triton in the SMTOG washes (Fig. 4A, B). The fact that they are indistinguishable from one another supports the view that the bands are not derived from ribosomes attached to the nuclear envelope. A comparison of Fig. 4C with Fig. 4A, B shows, not surprisingly, that none of the protein species in the saline-EDTA washes correspond to histones.
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The saline-EDTA soluble proteins extracted from Triton-nuclei and Virtis-nuclei are very similar, except that an additional 2 proteins (lines in Figs. 3D, 4 and 5) are found in the Virtis-nuclei.

Of course, these gels give no indication of the amount of protein removed, only the relative proportions in a given wash. Measurements of the percentage of total nuclear protein removed during either the saline or the saline-EDTA washes are shown in Table 1. When Triton-nuclei are washed with saline-EDTA the bulk of the protein

Table 2. Protein:DNA ratios of saline-EDTA washed Virtis-nuclei and of soluble isolated chromatin

<table>
<thead>
<tr>
<th>Expt. no.</th>
<th>Washed nuclei</th>
<th>Isolated chromatin</th>
<th>% of protein lost during preparation of chromatin</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>1.29×</td>
<td>0.83</td>
<td>36</td>
</tr>
<tr>
<td>2</td>
<td>0.90×</td>
<td>0.88</td>
<td>2</td>
</tr>
<tr>
<td>3</td>
<td>0.96×</td>
<td>0.66</td>
<td>31</td>
</tr>
<tr>
<td>Average</td>
<td>1.06</td>
<td>0.81</td>
<td>25</td>
</tr>
</tbody>
</table>

*4 indicates duplicate measurements.*

is removed in the first half hour, and the total loss is about 11%. Saline alone appears to be somewhat less effective in removing proteins; about equal amounts are removed in the successive half hours and the total removed is only 6%. Similarly for Virtis-nuclei, after saline-EDTA wash the bulk of the protein is removed in the first treatment. The total amount extracted is only half that for Triton-nuclei, about 5%.

During the preparation of soluble chromatin from saline-EDTA washed nuclei prepared by the Virtis-SMTOG method, a comparison of the protein:DNA ratios (Table 2) suggests a loss of about 25% of the protein.

DISCUSSION

The o-phase and the superunit thread hypothesis

When adult hen erythrocyte nuclei are isolated in a medium containing sucrose and magnesium ions by methods that involve damage to the plasma membrane by either detergents or mechanical fragmentation, the resulting staining patterns in chromatin after uranyl-lead and PTA are similar to those found in the intact cell. The dot-dash pattern after uranyl-lead more closely resembles that seen in the 4-day chick reticulocyte than the erythrocytes of adult hen where the pattern has a low visibility. We assume this is due to slight changes in packing of the constituent molecules resulting in increased visibility of the structural units. The 2 different staining patterns after uranyl-lead and PTA are also retained after extensive washing in 0.07 or 0.14M saline. Hence the 2 phases, the o- and e-phases, deduced (Davies et al. 1974) from
these patterns, survive these 2 procedures. The same conclusion, that the regions
which stain less with uranyl-lead, or o-phase, survive washing in saline, can also be
deduced from the fact that the amount of protein removed, about 6 % (Table 1, p. 122),
is small compared with an estimate of the amount of protein occupying the o-phase.
To make this we assume that dots and dashes occupying the e-phase have widths of
roughly 17 nm and separation 28 nm, when it can be shown (Small & Davies, 1970)
that the fractional volume occupied by the o-phase is roughly 0.7. In the electron
micrographs the o- and e-phases stain approximately equally with PTA and if it is
assumed that PTA stains only the protein (Silverman & Glick, 1969) then it follows
that the o-phase contains about 70 % of the protein. This may be an overestimate since
uptake of the stain PTA may be limited by a closer packing of the molecules in the
e-phase (Davies et al. 1974). After washing in saline-EDTA, because of the redistribu-
tion of material, it is not possible to deduce from electron microscopy that the 2 phases
are retained. However, it follows that the bulk of the o-substance is preserved since
the measured loss of 5-11 % (Table 1) is small compared with the figure of 70 %.

It is generally supposed (Zbarsky & Georgiev, 1959; Wang, 1963; Murray, 1965)
that washing isolated cell nuclei in either saline (0·14 m) or saline-EDTA removes
nuclear and presumably cytoplasmic sap as well as molecules loosely bound to
chromatin. If we accept this then it follows that the o-substance must be firmly bound
to the remainder of the chromatin. This evidence provides a firm basis for the hypo-
thesis put forward in the previous paper, that chromatin consists largely of folded
superunit threads (Fig. 1 B), so called to distinguish them from the previously described
unit threads which give rise to the dots and dashes in uranyl-lead stained sections.
These latter form the inner element of the superunit which has an outer cylindrical
shell of material which gives rise to the o-phase. Most probably the e-phase has a
higher ratio of DNA to protein than the o-phase (Davies et al. 1974).

Because the o-phase resists washing in saline and saline-EDTA it seems most
likely, as we have suggested, that the o-phase forms part of the structure of condensed
chromatin. Of course nuclei do contain molecules, for example enzymes which are
insoluble in physiological saline but soluble in 1·0 m NaCl (Siebert & Humphrey,
1965; Fambrough, 1969). But it seems unlikely, considering the minimal metabolism
of the condensed chromatin in erythrocytes, that the o-phase consists of enzymes
and other molecules concerned with nuclear activity.

The question of whether the o-phase survives the processes involved in the isolation
of soluble chromatin is not resolved. The data in Table 2 (p. 123) indicate that there
is an appreciable loss of protein, about 25 %, when saline-EDTA washed nuclei are
converted into soluble chromatin. Hypotonic treatment of saline-washed haemolysed
nuclei is known to lead to the production of finer threads, about 5-10 nm in diameter
or less (Brasch, Seligy & Setterfield, 1971; Davies et al. 1974). Such a large structural
change might lead to altered interaction of charged groups which could cause loss of
protein. However, data of Brasch et al. (1971) are in disagreement with ours. They
showed that when saline-washed haemolysed nuclei are disrupted in water and then
recondensed in saline (0·14 m) the changes in protein content do not vary by much
more than 10 %. Evidently judging by the spread in their results (± 10 %) and the
wide range in protein to DNA ratios reported by different workers on the same type of nucleus there are problems in making very accurate measurements of this ratio. Problems inherent in estimating the amount of DNA in mixtures of protein and DNA have been discussed by Eberhard (1972) and Munro & Fleck (1968). Estimations of protein in the presence of DNA which forms the basis of Table 1 appear to be less affected by error. Further studies of protein composition are needed to examine the possibility that proteins may be lost as a result of configurational changes occurring during the preparation of chromatin, and to begin to understand how the histone and non-histone proteins are distributed between the 2 phases.

The saline-extractable chromatin proteins and SDS gels

When nuclei are obtained by Triton-haemolysis in SMTOG they retain a group of proteins which can be subsequently extracted by washing with saline or saline-EDTA (Fig. 2F, E). These saline-extractable proteins are most likely located in a particulate component of the nucleus and not the nuclear sap or cytoplasm, for the following reason. The initial haemolysate, Fig. 2B, which consists of cytoplasm and nucleoplasm contains only haemoglobin which can be seen by comparing Fig. 2B with the gel for purified globin, Fig. 2A. Apart from the faint bands in Fig. 2D, the proteins also resist extraction in 2 further washes in SMTOG (Fig. 2C, D). It could be argued that the nucleoplasm is only a small fraction of the total cytoplasmic volume and hence that if the saline-extractable proteins are present in nucleoplasm they would escape detection. This does not appear to be a valid consideration since the saline-extractable proteins were not present even in gels heavily overloaded with initial haemolysate and also electron micrographs show that the volume of the nuclear sap is not negligible compared with the cytoplasm. Further, the nuclear sap and cytoplasm are probably in continuity in erythrocytes as judged by the equal concentrations of haemoglobin in the two compartments (Davies, 1961; Fawcett & Witebsky, 1964; Tooze & Davies, 1967). Hence absence of molecules from the cytoplasm indicates their absence from the nucleoplasm as well. It is difficult to be quite sure where in the nucleus these saline-extractable proteins are located, but neither the large-granule complex nor the contents of the cavities in chromatin appear to be the main source, since these structural features are largely retained after the saline washes. We suppose that the proteins are located in the major particulate component, namely the chromatin bodies, and hence they are called saline-extractable chromatin proteins. Johns & Forrester (1969) and Comings & Tack (1973) have discussed the possibility that, during isolation procedures, proteins not normally present in chromatin may become adsorbed from the nuclear sap or cytoplasm. Clearly this possibility does not apply to the saline-soluble proteins described here.

Certain other features of the gels require explanation. The SMTOG washes of the Triton-SMTOG nuclei are largely free of the saline-extractable proteins whereas this is not so for the SMTOG washes of Virtis-SMTOG nuclei (Fig. 3A, B, C). We know that after the first Virtis treatment about 25% of the nuclei contain chromatin bodies which have become decondensed (Davies et al. 1974) and that the final preparation contains a variable proportion of nuclei in which the chromatin bodies consist of
threads which have begun to separate. We suggest that non-extractability with SMTOG is dependent on the preservation of the original ultrastructure and that the presence of the proteins additional to haemoglobin in the SMTOG washes of Virtis-nuclei are the result of nuclear damage during preparation. A feature of the gels which is not understood are the 2 additional bands found only in Virtis-SMTOG nuclei.

The average value of 11% for the amount of protein extractable by saline-EDTA from Triton-SMTOG nuclei probably represents an upper limit for the amount present. The saline-extractable proteins contain a band due to haemoglobin (Fig. 2E) which varied in relative amount in different experiments. Since haemoglobin is largely removed from the chromatin bodies during haemolysis (see Results), a likely source is partially haemolysed erythrocytes scattered in variable numbers throughout the different preparations. A lower limit of 5% for protein extractable by saline-EDTA is obtained from the data on Virtis-nuclei. Fig. 3D shows a gel pattern which contains no haemoglobin. This lower value is also consistent with the loss of these proteins from Virtis-nuclei during the previous SMTOG washes. The fact that more protein can be removed from Triton-nuclei by saline-EDTA than saline alone is consistent with the suggestion made earlier that extractability is structure dependent, since treatment in saline-EDTA results in greater structural change.

There are several earlier studies of proteins extractable from nuclei by salt at 0.14 M, or in Tris buffer at 0.05-0.1 M, pH 7.2-7.6 (Barton, 1960; reviews in Hnilica, 1967, and Fambrough, 1969), but how they relate to the proteins described here has not been evaluated. Siebert & Humphrey (1965) showed by isolating nuclei in non-aqueous media that one group of proteins, soluble in 0.14 M NaCl and consisting of globular proteins and enzymes, is present in both nucleus and cytoplasm at similar concentrations. These and other experiments support the idea that there is a relatively free diffusion of globular proteins between nucleus and cytoplasm. However, the extent to which they are partitioned between condensed chromatin and nuclear sap is less clear. Exchanges of protein between nucleus and cytoplasm have been reviewed by Goldstein (1970). What is special about the saline-soluble proteins described here in chicken erythrocytes is that they appear to be confined to a particulate component, probably chromatin, and are absent from nuclear sap and cytoplasm. Their behaviour is different from the saline-soluble protein, haemoglobin, which from microspectrophotometry is largely released from the chromatin bodies during Triton-SMTOG haemolysis.

The nuclear envelope

Several workers (Dounce & Lan, 1943; Fisher & Harris, 1962; Hymer & Kuff, 1964) have used detergents in methods for isolating the cell nucleus and there are also studies of their action on the inner and outer membranes of the nuclear envelope (Hubert, Favard, Carasso, Rosencwaig & Zalta, 1962; Blobell & Potter, 1966; Holtzman, Smith & Penman, 1966; Barton, Kisielski, Wassermann & Mackevicius, 1971; Tata, Hamilton & Cole, 1972; Laval & Bouteille, 1973). Most have reported that the bulk of the outer membrane can be removed and with it the attached ribosomes. There is less certainty
about whether the inner membrane is retained. Hubert et al. (1962) were among the first to study the action of detergents on rat liver nuclei isolated first in a sucrose-magnesium medium. Their micrographs show that the inner membrane is retained and that the outer membrane is largely but not completely removed.

When chicken erythrocyte nuclei were treated with saponin the outer membrane of the nuclear envelope was seen to be lifting away from the nucleus. In Triton-haemolysed erythrocytes the outer membrane is not visible at its usual site, but it is not possible to say whether it has lifted away because the plasma membrane has in many places, confusingly, collapsed on to the nucleus. Also the disorganization of membrane structure, illustrated in the micrograph of the plasmalemma in Fig. 7, makes it difficult to be sure about the actual fate of membranes after detergent treatment. However, we know that Triton does not remove the inner membrane from chicken erythrocyte nuclei, under the conditions in our experiments although it may not always be visible due to the bilayer having taken up stain to a similar extent.

We were particularly concerned to show that ribosomes which are attached in appreciable numbers to the outer membrane in erythrocytes from adult hen did not contribute to the saline-extractable protein. In the Virtis-nuclei the great bulk of the outer membrane is removed and a further fraction may have been eliminated by the treatment in Triton. The fact that the SDS gels obtained from these nuclei were very similar to those from Triton-haemolysed nuclei shows that ribosomes and indeed other cytoplasmic organelles remaining in the haemolysed cells, do not contribute appreciably to the saline-extractable protein.

The cavities and the large-granule complex

An intriguing structural feature of the chromatin bodies in the nuclei of chicken erythrocytes is the large number of approximately spherical cavities containing a nearly amorphous material. The variation in their number from cell to cell and in different animals (Davies et al. 1974) suggests that they may be related to nuclear metabolism. In our previous studies on erythrocytes from various amphibia, Rana pipiens, Triturus cristatus, Amphiuma and Necturus (Davies, 1961; Tooze & Davies, 1967; Small & Davies, 1970), these cavities were not so apparent. However, cavities are present in the telophase chromosomes of dividing erythroblasts in Triturus cristatus (Davies & Tooze, 1966). Nuclear bodies in erythrocytes resemble and are no doubt closely related to the condensed mitotic chromosomes. In our observations on chicken erythrocyte nuclei we have been reluctant to accept the possibility that the cavities do not contain nuclear sap, but microspectrophotometry indicates that haemoglobin is not present. They may contain RNA (Davies et al. 1974).

Tokuyasu et al. (1968) in a study on phytohaemagglutinin (PHA) stimulation of human small lymphocytes, also noted small round spaces in the condensed chromatin of the untreated cell. By serial sectioning, these spaces were shown to be isolated from the nuclear sap and they may be related to those found by us in chicken erythrocytes. In small lymphocytes the number of spaces increased after 4 h of stimulation with PHA and it was suggested that they might act as centres for the decondensation of chromatin which results from stimulation.
In isolated rat liver nuclei Barton et al. (1971) depict cavities in chromatin which are strikingly similar in shape and stain content to those described by us in chicken erythrocytes. However, Barton et al. (1971) show them in sections close to the nuclear periphery where they supposed, as we did in comparable sections, that the circular areas and their contents were part of the pore annulus complex. However, in erythrocytes similar filled cavities occur throughout chromatin, including the interior of the nucleus. These structures which appear to be common to the chicken erythrocyte and the hepatic cells of the rat liver need further study to elucidate their nature and possible role in nuclear metabolism.

Granules of various sizes sometimes shown to contain RNA have been described by numerous workers in many types of cell nuclei. What is striking about the group of granules in the chicken erythrocytes is their common association with an amorphous component which cannot usually be seen in the intact cell, due to the similarity in stain uptake between it and the nuclear sap. This complex, which might be a general feature of cell nuclei, discernible in the erythrocyte especially after haemolysis because of the relative simplicity of this cell, resembles the nucleolus in that there is an amorphous or finely fibrillar component and a granular component. The granules in the complex are much larger and more heterogeneous in size than those in the nucleolus which latter have been shown to be derived from the fibrillar component (Das, Micou-Eastwood, Ramamurthy & Alfert, 1970). Details of the chemical composition of the 2 components are needed before considering this interesting parallel further.

We thank Professor M. H. F. Wilkins, F.R.S., for his continued interest, Mrs Y. Buchner for preparing the specimens for electron microscopy, Mr D. Back for microspectrophotometry and Mrs F. Collier and Mr Z. Gabor for help in preparing the plates. We also thank the members of Dr H. J. Gould’s group for assistance and discussion of the biochemical aspects.

REFERENCES


M. E. Walmsley and H. G. Davies


(Received 21 May 1974)

Fig. 2. SDS-polyacrylamide gel electrophoresis patterns from Triton-SMTOG cells, except A which is chicken globin standard (loading 30 µg); B, initial haemolysate (20 µg); C, 1st SMTOG wash (30 µg); D, 2nd SMTOG wash (31 µg); E, saline-EDTA wash (40 µg); F, saline wash (33 µg); a major band at dot (.) in E and F has a molecular weight of 21 000; 2 bands just detectable at dotted lines in D are not seen in 1st wash.

Fig. 3. SDS-polyacrylamide gel electrophoresis patterns from Virtis-SMTOG nuclei. A, first SMTOG wash (loading 20 µg); B, 2nd SMTOG wash (~20 µg); C, 3rd SMTOG wash (14 µg); D, saline-EDTA wash (25 µg) in which the major protein at (.) has a molecular weight of 21 000; E, chicken globin (5 µg); F, standards (15 µg): 1, bovine serum albumin (66 000), 2, ovalbumin (46 000), and 3, cytochrome c, (12 400). The lines in D indicate 2 proteins which are not present in the saline-EDTA washes of Triton-SMTOG nuclei (compare 3D with 2E); the 21 000 mol.wt. band is at dot (.) in 3A–D.

Fig. 4. SDS-polyacrylamide gel electrophoresis patterns from Virtis-SMTOG nuclei. A, saline-EDTA wash from nuclei previously washed in SMTOG (loading 32 µg); B, saline-EDTA wash from nuclei previously washed in SMTOG + 0.5% Triton X-100 (32 µg); C, chicken histones (10 µg). The 21 000 mol.wt. band is at dot (.) in 4A and B. The lines point to 2 bands not present in Triton-SMTOG nuclei.

Fig. 5. An enlarged photograph showing the pattern in the saline-EDTA wash of Virtis-SMTOG nuclei. The 21 000 mol.wt. protein is at dot (.) and the bands, not present in the comparable wash of Triton-nuclei, are at lines.
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[Image of gel electrophoresis results with lanes labeled A to F and numbered images 2, 3, 4, and 5]
Fig. 6. Electron micrograph of adult hen erythrocyte haemolysed in Triton-SMTOG (see methods) stained with uranyl-lead; large granule complex at double-headed arrow; filled cavities at arrows. The plasma membrane can just be detected, as at m. × 25 000.

Fig. 7. Electron micrograph of adult hen erythrocyte haemolysed in Triton-SMTOG, stained with PTA; filled cavities can just be distinguished at arrows; plasma membrane at m. × 41 000.
Fig. 8. Electron micrograph of adult hen erythrocyte haemolysed in Triton-SMTOG, after extensive washing in 0.075 M NaCl, stained with uranyl-lead; the amorphous component of the large-granule complex is at double-headed arrow; arrows point to filled cavities. ×40000.

Fig. 9. Electron micrograph of adult hen erythrocyte haemolysed in Triton-SMTOG, after extensive washing in 0.075 M NaCl, stained with PTA; arrows point to filled cavities; membrane at m. ×27000.
Fig. 10. Electron micrograph of adult hen erythrocyte haemolyzed in Triton-SMTOG, after extensive washing in saline (0.075 M), EDTA (0.024 M, pH 8.0), stained with uranyl-lead; large-granule complexes, at double-headed arrows; contents of cavities at arrows. x 34,000.

Fig. 11. Electron micrograph of adult hen erythrocyte haemolyzed in Triton-SMTOG, after extensive washing with saline-EDTA, stained with PTA: large-granule complex at double-headed arrow; cavity contents at arrows; membrane at m. x 31,000.
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Fig. 12. Electron micrograph of adult hen erythrocyte showing large-granule complex, stained with uranyl-lead; chromatin at c. × 100 000.

Fig. 13. Electron micrograph of adult hen erythrocyte haemolysed in Triton-SMTOG, washed in saline (0·075 M), stained with uranyl-lead. × 90 000.

Fig. 14. Electron micrograph of adult hen erythrocyte haemolysed in Triton-SMTOG, washed in saline (0·075 M), stained with PTA. × 90 000.

Fig. 15. Electron micrograph of adult hen erythrocyte nucleus isolated in Virtis-SMTOG, and treated with Triton X-100 (0·5 %), stained with uranyl-lead. Bands b2, b3, b4 can just be distinguished but the inner membrane of the nuclear envelope is not visible. × 108 000.

Fig. 16. Electron micrograph of adult hen erythrocyte nucleus isolated in Virtis-SMTOG and treated with Triton X-100 (0·5 %), stained with uranyl-lead; this nucleus is adjacent in the section to the one shown in Fig. 15. A decrease in stain intensity in band b1 allows the inner membrane of the nuclear envelope to be seen (arrowhead); bands b2, b4 are indicated by lines. × 108 000.