Nuclear Membranes and Plasma Membranes from Hen Erythrocytes

I. ISOLATION, CHARACTERIZATION, AND COMPARISON*

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SUMMARY

The bird erythrocyte provides a cell system which comprises only two sorts of membranes, namely the plasma membrane and the nuclear envelope. As calculated morphometrically, both of these membranes are present in an almost 1:1 membrane surface ratio. Contamination with other types of membranes is a priori excluded in this cell. Purified fractions of plasma membranes and nuclear envelope membranes were isolated by using nondetergent methods, in which high speed rotating knife homogenization is combined with differential and gradient centrifugation steps. Nuclear membranes were separated from other nuclear constituents after high salt extraction of nucleoproteins and sonic oscillation, with or without an additional digestion with DNase. Purity and structural integrity of the fractions are shown in electron micrographs. The buoyant densities of nuclear membranes (\( \rho_{b} = 1.20 \)) and plasma membranes (\( \rho_{b} = 1.14 \)) are different. The gross compositions of intact cells and the nuclei, nuclear membrane, and plasma membrane fractions, with respect to lipids, phospholipids, cholesterol, protein, RNA, and DNA, are given, as well as corresponding recoveries. The nuclear membrane is distinct from the plasma membrane as shown in a much lower cholesterol-phospholipid ratio, in a higher content in protein, and a certain amount of DNA remaining firmly attached to it. In general, the properties of the bird erythrocyte plasma membrane agree with those known from the non-nucleated erythrocytes. Among the enzymes examined, the plasma membrane showed ATPase activity, including an ouabain-sensitive one. On the other hand, the nuclear membrane fraction was almost devoid of ATPase activity, but did show the activity of a typical "endoplasmic reticulum type enzyme," the NADH-cytochrome c reductase. While the nuclear fraction demonstrated a strong enrichment for NAD(PH)-pyrophosphorylase, this activity was not found in the plasma membranes or in the nuclear membranes, which indicates the low degree of nucleoplasmic protein contamination present in the nuclear membrane fraction. The differences between the only two membrane systems of this "dead end differentiated" cell type are discussed in context with the present concepts on the cytomembrane → plasma membrane polarization and diversity.

MATERIALS AND METHODS

Isolation Procedures

Freshly prepared blood from decapitated hens (RhodeSinder Huhn; about 4 months old; approximately 1000-g body weight) was immediately mixed with an at least 4-fold volume of the "minimum medium" described earlier (1, 2), which was 2% with respect to the anticoagulate Liquemin (Hoffmann-La Roche AG, Grunenbach, Baden). Erythrocytes were spun for 10 min at 300 \( \times \) g, and for enrichment and purification were washed three times at low centrifugal speed with the "minimum medium." Nuclei were isolated following a slight modification of the method described, starting with high speed rotating knives homogenization (1).

Plasma Membranes—Plasma membranes were then prepared from the supernatant of the first spin of the homogenate (3 min at 1,500 \( \times \) g) as follows. The supernatant was mixed with a 3-fold volume of 0.01 M Tris-HCl buffer, pH 7.2, which was 0.1 M with respect to KCl, and centrifuged for 15 min at 8,000 \( \times \) g (WKF ultracentrifuge P 50 K, Brandau, Germany). The pellet, consisting mainly of large pieces of plasma membranes...
contaminated by a variety of nuclear fragments, was designated as the “8,000 × g crude plasma membrane” fraction. The supernatant was further centrifuged for 3 hours at 110,000 × g, and the pellet thus obtained was washed twice in 0.01 M Tris-HCl buffer, pH 7.2, which was 0.05 M with respect to KC1. After every run the pellets were collected and resuspended by a few strokes with a tightly fitting Potter-Elvehjem Teflon-homogenizer. After final resuspension in a small amount of 0.01 M Tris-HCl buffer, pH 7.2, containing 0.05 M KC1, this fraction was layered on the top of a linear continuous 20 to 70% (w/v) sucrose gradient, which was 0.07 M with respect to KC1 and 0.01 M with respect to Tris-HCl buffer, pH 7.2. After 10-hour centrifugation at 80,000 × g in the swinging buckets of the WKF ultracentrifuge 3 × 60 ml rotor, the membrane material had accumulated in a somewhat diffuse band and was collected with conventional methods. After thorough suspension with a 4-fold volume of 0.01 M Tris-HCl buffer, pH 7.2, containing 0.05 M KC1, the membranes were finally spun for 2 hours at 110,000 × g and designated as “purified plasma membranes.”

Nuclear Membranes—These membranes were isolated by two modifications of the method introduced by Franke et al. (3) for the isolation of nuclear membranes from mammalian liver.  

1. Nuclei obtained by the aforedescribed procedure (1) were suspended in a small amount of a 0.3 M sucrose solution, which was 0.135 M with respect to KC1 and 0.01 M with respect to Tris-HCl buffer, pH 7.2. Thereafter, the nuclei were fragmented by sonic oscillation (15 to 20 times at position 3 of the Branson Sonifier S 125; Branson Instruments, Danbury, Connecticut; 3 see each, with cooling intervals). The degree of disintegration was controlled with a phase contrast microscope. To this suspension a 30-fold volume of extraction solution I (0.3 M sucrose, 2 M KC1, 0.01 M Tris-HCl, pH 7.2) was added, and the suspension was kept at 4° for 10 hours during magnetic stirring. This was then centrifuged for 2 hours at 110,000 × g and resuspended afterward by several strokes with the Potter-Elvehjem homogenizer and was diluted with a 20-fold volume of extraction solution II (like Solution I but 1.5 M sucrose solution, which was 0.145 M with respect to KC1 and 0.01 M with respect to Tris-HCl buffer, pH 7.2). After 10-hour centrifugation at 80,000 × g in the swinging buckets of the WKF ultracentrifuge 3 × 60 ml rotor, the membrane material had accumulated in a somewhat diffuse band and was collected with conventional methods. After thorough suspension with a 4-fold volume of 0.01 M Tris-HCl buffer, pH 7.2, containing 0.05 M KC1, the membranes were finally spun for 2 hours at 110,000 × g and designated as “purified plasma membranes.”

2. In a modification of this method, the disintegrated and high salt-extracted membrane material was pooled and washed twice with 0.01 M Tris-Cl buffer, pH 7.2, containing 0.05 M KC1, and run for 2 hours in the ultracentrifuge at 110,000 × g. The pellet was then suspended in 0.01 M Tris-maleate buffer, pH 7.2, which was 5 mM with respect to MgCl2 and centrifuged for 2 hours at 110,000 × g. After repeating this step the pellets were incubated with DNase I (DNase I, EC 3.1.4.5.; Boehringer Mannheim) in a concentration of 50 µg per ml (0.01 M Tris-maleate buffer (pH 7.2), 0.005 M MgCl2) for 6 hours at 4° in a cold room (4). The subsequent centrifugation (2 hours at 110,000 × g) after dilution with a volume of 0.3 M sucrose solution, which was 0.135 M with respect to KC1 and 0.01 M with respect to McIlvaine’s citrate-phosphate buffer, pH 7.2, rapidly decreased the DNase activity. Further purifications followed the gradient procedure described under Paragraph 1.

In some experiments, especially those comparing the enzyme activities, the plasma membranes were treated with high salt solution prior to the sucrose gradient centrifugation, exactly in parallel with the nuclear membranes.

All operations were carried out at 4°.

Chemical Determination

After precipitation of the samples with cold 10% trichloracetic acid and four washes with 5% trichloracetic acid the lipids were extracted by one short extraction with cold methanol, immediately followed by three 10 min extractions with chloroform-methanol (2:1). The combined lipid extracts were washed once with an equal volume of 1% NaCl. Phospholipids and cholesterol were then determined directly in the chloroform-methanol extracts following the methods of Gerlach and Deuticke (5) and Clark, Rubin, and Arthur (6). Nucleic acids were extracted from the lipid extraction residue with hot 5% trichloracetic acid, followed by a trichloracetic acid wash. The residue obtained thereafter was combined with the material from the NaCl solution of the lipid wash and was used for the protein determination (7). Nucleic acids were estimated in the hot trichloracetic acid extract by using the orcinol (8) and the diphenylamine method (9). RNA values were corrected for the DNA influence in the orcinol reaction by using thymus DNA (Nutritional Biochemicals) as well as dATP (Boehringer Mannheim) for establishing the correction factor.

In special experiments, these determinations were carried out with suspensions of cells and nuclei, the total number of which was known from countings of aliquots in a Thomas counting chamber.

Enzyme Assays

Phosphohydrolase—The activities of these enzymes were determined by measuring the released inorganic phosphate, according to either Fiske and SubbaRow (10) or Martin and Doty (11). Both methods gave nearly the same results under our test conditions. As described for each enzyme, 0.9 ml of the reaction mixture was first incubated at 37° for 5 min before starting the reaction by addition of 0.1 ml of membrane suspension (about 1 mg of protein per ml) in 0.25 M sucrose solution in Tris-HCl buffer (20 mM, pH 7.0). After incubating the mixture at 37° for 15 min, the reaction was stopped by adding 1.0 ml of cold 10% trichloracetic acid solution. The precipitate was then spun for 5 min at 1200 × g in the cold, and 1.0 ml of the clear supernatant was analyzed for inorganic phosphate.

The reaction mixtures (final concentrations) included the following. ATPase (ATP phosphohydrolase, EC 3.6.1.4): 5 mM ATP, 5 mM MgCl2, 100 mM NaCl, 10 mM KC1, 50 mM Tris-HCl buffer, pH 7.4. In order to measure the ATPase activity stimulated by Mg++ alone, the reaction mixture additionally contained 10-4 M ouabain. 5'-Nucleotidase (5'-ribonucleotide phosphohydrolase, EC 3.1.3.5): 10 mM AMP, 5 mM MgCl2, 50 mM Tris-HCl buffer, pH 7.4. Glucose 6-phosphatase (6-gluc-
NADH cytochrome c reductase activity (NADH2:cytochrome c reductase, EC 1.8.1.7)—The rate of cytochrome c \((\text{Fe}^{3+})\) reduction was measured according to Mahler (13) and enzyme activity was calculated using a molar extinction increment of 27.7 \(\text{mM}^{-1} \text{cm}^{-1}\) (14).

Succinate dehydrogenase (Succinate:2-(p-Iodophenyl)-3-(p-nitrophenyl)-5-phenyltetrazolium oxidoreductase, EC 1.3.99.1)—This activity was measured by reduction of iodophenyl nitrophenyltetrazolium as described by Pennington (15).

Cytochrome c oxidase (Cytochrome c oxidoreductase, EC 1.9.3.1)—Cytochrome c \((\text{Fe}^{3+})\) was reduced by bubbling hydrogen gas through a 1% solution of cytochrome c in 10 mM phosphate buffer, pH 7.0, in the presence of plastin charcoal. The catalyst was then removed by low spin centrifugation and the rate of oxidation was determined according to Smith (16).

Protein was measured with the method of Lowry et al. (17) with crystalline bovine serum albumin (Serva GmbH, Heidelberg) as standard. All experiments were carried out with two controls—without substrate and without enzyme—and for each assay two parallels were run. Biochemical reagents were obtained from Boehringer Mannheim, except for ouabain, which was purchased from E. Merck, Darmstadt. All chemicals were of the highest purity available.

**Electron Microscopy**

Samples of freshly prepared blood were allowed to drop into 0.1 mM cacodylate-buffered 4% glutaraldehyde (pH 7.2) for a 30-min fixation at 40, 20, or 4°C. After thorough washes with ice-cold cacodylate buffer, the cells were postosmicated with 0.1 M cacodylate-buffered 2% OsO4 for 2 hours. Samples of the fractions were fixed with 0.1 M cacodylate-buffered (pH 7.2) 4% glutaraldehyde and 2% OsO4, either sequentially or simultaneously (18) for 30 min, and were then postosmicated for 2 hours with 0.1 M cacodylate-buffered 2% OsO4. Some samples were only fixed with 0.2 M cacodylate-buffered 2% OsO4. After dehydration through graded ethanol solutions and propylene oxide, the fractions were embedded in Epon 812. Thin sections were cut on a Reichert ultramicrotome OmU2 (Fa. Reichert, Vienna, Austria, or American Optical Corporation, Buffalo, New York) with diamond or glass knives, and were double stained with uranyl acetate (4% aqueous solution, 5 min) and lead citrate (19).

The relative values of volume and surface of the membranes of each mature erythrocyte were determined by using the point lattice method (compare References 20, 21, and 22). Values given in this study are means from 100 cells.

The fractions were also examined by using the negative staining method (2% phosphotungstic acid, adjusted to pH 7.2). All micrographs were taken with a Siemens Elmskop IA, by using the double condenser illumination.

Moreover, samples of freshly prepared blood were immediately dropped into a Ringer solution, which was 2% with respect to Liquemin, and were centrifuged for 10 min at 300 \(\times\) g. Blood cells were incubated for 15 min in a Ringer solution, which was 10% with respect to glycerol, followed by a 20-min incubation in Ringer solution, which was 20% with respect to glycerol. Small drops of this suspension were mounted on specimen holders and frozen rapidly in liquid Freon 22. All of the specimens were frozen-etched in a Balzers apparatus BA 360 M applying 1 min of etching at \(-100^\circ\) (cf. References 23 and 24).

**RESULTS**

Electron Microscopic Morphometry—The cell and nuclear surfaces were determined from sections through fixed cells (Fig. 1) according to the methods outlined. For calculation of the membranous surface of the nuclear envelope the value for the nuclear surface was multiplied by 2. Corrections for the nuclear area appeared not to be necessary, since roughly half of the nuclear area was compensated by the pore walls and, in addition to that, the erythrocyte nucleus has only 6% of its surface occupied by pores (for details see Footnote 1 and compare, e.g., References 25 and 26). The morphometrical measurements revealed that in the hen erythrocyte 54.2% of the total cellular membrane material is constituted by plasma membrane, with nuclear membranes representing 45.8%. Other membranes such as from mitochondria, lysosomes, mitochondrial extrusion bodies, or endoplasmic reticulum type cisternae were never found to exceed 1% of the total membrane material in the erythrocytes, and were introduced almost exclusively by immature erythrocyte stages (27) and some nonerythrocytic cellular contaminations, which occurred only very infrequently in our erythrocyte preparations after the initial washes. In order to rule out the possibility that any membranous structures present in the erythrocyte might have been overlooked as a result of inadequate fixation and staining procedures, the freeze-etch technique was also used (compare, e.g., the problems with the detection of the yeast promitocondria (28)). This technique, however, did not reveal any considerable membranous components, except the nuclear envelope and the plasma membrane (Fig. 2).

Buoyant Density and Electron Microscopic Appearance of Fractions—Under the conditions of the present study the 8000 \(\times\) g crude plasma membranes (Fig. 4), as well as those membranes purified through one further 0.01 M Tris-buffer (pH 7.2) wash (Fig. 5) and those sonically disrupted and purified in the sucrose gradient (Fig. 6) sediment as “right side out” vesicles, derived from the fragmented erythrocyte ghost (29, 30). The hemoglobin contamination of the fractions was somewhat variable, but appeared moderate as indicated by their pale pink color. After all of the procedures described under “Materials and Methods,” including the high salt treatments, the plasma membranes exhibited, in addition to the membranous sheet, distinct fibrillar material covering their inner surface (Figs. 7 to 9), as has also been reported for cytochrome membranes from diverse sources (31–33) and which has recently been brought in connection with the Cu\(^{2+}\)-dependent ATPase activity of human erythrocyte ghosts (34). At higher magnifications this inner surface-associated material sometimes gives the impression of making up a lamellar network in a distance of about 50 A from the true membrane (27), and some nonerythrocytic cellular contaminations, which occurred only very infrequently in our erythrocyte preparations after the initial washes. In order to rule out the possibility that any membranous structures present in the erythrocyte might have been overlooked as a result of inadequate fixation and staining procedures, the freeze-etch technique was also used (compare, e.g., the problems with the detection of the yeast promitocondria (28)).

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FIGS. 1 AND 2. Survey electron micrographs of hen erythrocytes, as revealed after fixation (40° sequential glutaraldehyde-OsO₄) embedding-section procedures (Fig. 1) and after freeze-etch preparation (Fig. 2). In Fig. 2, the plasma membrane and the cytoplasm are nearly cross-fractured, whereas at the nucleus the fracture runs along the nuclear membrane. With both types of preparations only minor membranous components beside the plasma membrane and the nuclear envelope can be identified (small arrows). Pores can be seen in the nuclear envelope. × 14,000.

contaminations are widely diminished when the fraction is further purified (Figs. 5 and 6). Plasma membrane fractions, especially when fixed with sequential use of glutaraldehyde and OsO₄, repeatedly showed “black dots” along the membranes (e.g. Fig. 6). This is an effect which is often observed in section work with isolated plasma membranes, be they from erythrocytes (34) or from other sources (e.g. Fig. 8 of Reference 35). Nuclear and plasma membranes are distinct in their densities (Fig. 3). The nuclear membrane banding was frequently sharper than that of the plasma membranes. Purification of the membrane material through a continuous sucrose gradient was a very effective step. In experiments in which nuclear membrane material was voluntarily mixed with a small sample of plasma membranes, the latter separated from the nuclear membranes by accumulation at a higher position in the gradient tube. Thus, the nuclear membrane fraction routinely prepared after the methods given can be regarded as being of high purity, concerning possible plasma membrane contamination. The plasma membranes obtained in the preparative sucrose gradient showed a mean peak density of $\rho_{20} = 1.142 \pm 0.024$, whereas the nuclear membranes were as dense as $\rho_{20} = 1.198 \pm 0.037$ (for comparison with mammalian liver material see, e.g., References 3, 36, and 37). The nuclear fraction used in preparing the nuclear membranes was of the same high purity as shown in our previous publication (1). The nuclei were very weakly pink, and in single experiments were almost gray-white. Preliminary determinations of the hemoglobin content within these nuclei showed values much lower than expected from the literature of the so-called “nuclear (chromatin) hemoglobin” (38-43), the existence of which is rather doubtful in the authors' opinion (see also Reference 44). The nuclear membrane fraction prepared therefrom is shown in Figs. 10 to 14. DNA (or chromatin, or both) remnants in fibrillar form always remain attached to the membranous sheets or vesicles. The nuclear membranes showed a remarkably higher variability in their densities in different preparations, which is most likely a consequence of the chromatin and DNA fibrils remaining more or less firmly bound to the membranous fragments. The DNase procedure seemed to produce slightly lighter and less DNA-contaminated membranes. Even repeated treatments with 2.0 M and 1.5 M KCl (and in a few experiments also with NaCl) combined with sonic oscillation steps could not separate these DNA fibrils from the membrane (Figs. 10, 12, and 13), nor did the DNase treatment bring about a separation (Fig. 11). Similar experience has recently been made with nuclear membranes from rat, pig, and bovine liver by different authors (3, 4). The clumps presumed to be of chromatin origin tend to unravel into very thin ~30 Å (Fig. 13) strands after prolonged high salt extraction. Extensive sonic oscillation led to an increase in the number of smaller vesicles, on account of larger cisternal fragments (Fig. 13). Negative staining preparations of the nuclear membrane fractions showed a variety of membranous components, from large cisternal pieces down to very small vesicles or single membrane sheet fragments (or both) (Fig. 14). In some of the larger fragments, pores could be recognized (Fig. 14, inset; for details compare Footnote 1).
Figs. 4 and 5. Typical thin section surveys of the 8000 × g crude plasma membrane fraction, as obtained after direct fixation (Fig. 4) and after one additional low salt Tris buffer wash prior to fixation (Fig. 5). The plasma membrane-derived vesicles show "right side out" orientation. Arrows denote contaminants of predominantly chromatin nature. The fraction shown in Fig. 5 is of greater purity. C, contaminating vesicle including some cytoplasmic material. Fig. 4, × 21,000; Fig. 5, × 26,000.
Thin fibrils, presumed to represent chromatin-derived membrane-anchored DNA strands, were also regularly observed in association with the fragments (Fig. 14, arrows).

**Gross Compositions of Cells and Fractions**—For the intact cells, as well as for the various fractions, the percentages and the characterizing ratios of nucleic acids, proteins, and lipids are given in Tables I and II. The data for the nuclear fraction are generally identical with those previously reported from our laboratory for chicken erythrocytes (1), with one exception, i.e. the RNA content. More refined determination schedules, in which the DNA background of the Burton reaction has been corrected, as indicated under “Materials and Methods,” yielded a lower RNA content. On the other hand, we found, in some contradiction to other authors (42), always a low but significant amount of RNA in the erythrocyte cell, which is preferentially located in the nucleus, as is shown by the recovery consideration given in Table III. Since RNA synthesis has nearly totally ceased in mature erythrocytes (44-47), such “structural” nuclear RNA might be supposed to fall into the category of the so-called “chromatin-bound” RNA (48-50). The nuclear membranes are characterized by a relatively high content in protein (for liver material see Reference 3). They have a phospholipid to
FIGS. 10 to 13. Thin sections through final nuclear membrane fractions from hen erythrocytes, with (Fig. 11) and without (Fig. 10) DNase treatment. Small clumps or fibrils of (heterochromatin) DNA have remained closely attached to the inner membrane. The association of the membrane-bound DNA is presented in more detail in Fig. 12 (arrows). During prolonged sonic oscillation (Fig. 13) the nuclear envelope material tends to break down into smaller vesicles. However, the membrane-attached DNA matter still remains (e.g. upper left of Fig. 13).

protein ratio of 0.187, which is far lower than the comparable value for the plasma membrane (0.330). One of the most striking differences between nuclear and plasma membranes is the more than 100% higher ratio of free cholesterol to phospholipids in the latter. The nuclear membranes are associated with a small but resistant percentage of either nucleic acid. The amount of RNA and DNA attached to the nuclear membranes was found to be rather variable from one experiment to another, and even prolonged sonic oscillation, high salt, or nuclease treatments did not eliminate this nucleic acid “contamination” of the membrane fraction.

An unexpected but interesting finding is that a certain amount...
of RNA also remains firmly associated with the plasma membrane fraction. This RNA moiety could possibly be truly "membrane-bound" RNA (compare, e.g., References 51 to 54). The 8000 x g crude plasma membrane fraction is, with respect to most characteristics, somewhat intermediate between the nuclear and the plasma membrane fractions.

From the recovery calculation of Table III, which is based on a DNA value of 1.72 per erythrocyte (i.e. almost 30% lower than the "classic" early value of Vendrely (55)), it is apparent that a certain loss of nuclear membrane material occurs during the isolation of the nuclei: only 11% of the cellular phospholipids are recovered with the nucleus instead of a value of about 80%. As would be expected from the relative amounts of the two membraneous components present within the cell. This loss of membraneous material is, at least partially, caused by the relatively brutal "rotating knife" homogenization procedure that they are not recovered in the 2-hour collecting spin of the plasma membranes. However, the membranes is at least partially due to a progressive inactivation.

Enzyme Activities—In general, the enzyme activities measured with the hen erythrocyte homogenate, as well as with the various subcellular fractions, were rather poor, especially when compared with those found in corresponding probes from mammalian liver material studied in parallel (compare References 3 and 56). The homogenate and all fractions studied were totally devoid of glucose 6-phosphatase activity and did not show any activity in succinate:iodophenyl:nitrophenyl:phenyltetrazolium-reductase or cytochrome c oxidase. The total lack of cytochrome oxidase within this cell which does not possess functional mitochondria is especially noteworthy in discussions of the so-called "nuclear membrane respiratory activities" (57–61).

Table IV shows some enzyme activities found with our hen erythrocyte fractions. Both Mg++-dependent ATPase and (Na+·K+)-activated Mg++-dependent ATPase can be detected in the plasma membranes, but there is little or no activity of these enzymes within the nuclei or nuclear membrane fractions. The values for the plasma membranes are in good correspondence with data obtained from mammalian erythrocyte ghosts by other authors (e.g., References 39 and 62 to 64). Adenosine monophosphate is hydrolyzed only to a slight extent, and the existence of an AMPase within erythrocyte plasma membranes is not as yet clear (with respect to nucleated erythrocytes, compare the results of Tooze with amphibias (65)). Such low values in 5'-nucleotidase activity could be due to an unspecific phosphomonoesterase (66, 67).

With NADH-cytochrome c reductase activity the enrichment behavior appeared just opposite to that of the ATPases. This oxidoreductase, which in other cell systems is considered as characteristic for cytomembranes of the endoplasmic reticulum type (reviewed in Reference 68; for nuclear membranes see Reference 69), was significantly enriched with the nuclei and nuclear membranes, respectively. The loss of activity during the preparation steps from the nuclei to the nuclear membranes is at least partially due to a progressive inactivation. Moreover, prolonged centrifugation of the nuclear extracts for a further 5 hours at 110,000 x g yielded a pellet containing membraneous material with high NADH-cytochrome c reductase activity. This indicates that a considerable part of the nuclear envelope material is fragmented to vesicles and sheets so small that they are not recovered in the 2-hour collecting spin of the routine preparation.

NAD+-pyrophosphorylase, which is widely regarded as a non-membrane-bound nucleoplastic enzyme (71–74) and which was in 1956 first measured by Malkin and Denstedt (75) for nuclei-enriched fractions from chicken erythrocytes, showed a marked enrichment with the nuclei, but was almost absent with the nuclear membranes. This result shows that: (a) NAD+-pyrophosphorylase is not constitutively associated with the nuclear membrane, a finding which agrees with our measurements with liver nuclear subfractions; and also with its occurrence in nuclei isolated with the use of organic solvents (76); (b) NAD+-pyrophosphorylase can be almost totally extracted with high salt treatment from nuclei, and might serve as a marker enzyme for nucleoplastic contamination of nuclear membrane fractions. According to this concept, our nuclear membrane

**Table I**

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<tr>
<th>Protein</th>
<th>Phospholipids</th>
<th>Cholesterol</th>
<th>Other lipids</th>
<th>RNA</th>
<th>DNA</th>
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<td>% of dry weight</td>
<td></td>
<td></td>
<td></td>
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<td>Nuclei...</td>
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<td>0.3</td>
<td>0.1</td>
<td>2.3</td>
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<tr>
<td>Nuclear membranes</td>
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<td>13.0</td>
<td>2.5</td>
<td>1.2</td>
<td>4.0</td>
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<tr>
<td>Plasma membranes</td>
<td>60.3</td>
<td>19.0</td>
<td>10.5</td>
<td>3.0</td>
<td>6.6</td>
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**Table II**

Protein, nucleic acid, and lipid ratios (w/w) from hen erythrocytes and subcellular fractions

<table>
<thead>
<tr>
<th>Protein</th>
<th>Nucleic acid</th>
<th>Lipid ratios (w/w)</th>
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<tr>
<td>Phospholipids to protein</td>
<td>Cholesterol to phospholipids</td>
<td>RNA to protein</td>
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<tr>
<td>DNA to RNA</td>
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<tr>
<td>Intact cells</td>
<td>0.015</td>
<td>0.369</td>
</tr>
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<td>Nuclei</td>
<td>0.023</td>
<td>0.222</td>
</tr>
<tr>
<td>Nuclear membranes</td>
<td>0.187</td>
<td>0.198</td>
</tr>
<tr>
<td>8000 x g crude plasma membranes</td>
<td>0.227</td>
<td>0.510</td>
</tr>
<tr>
<td>Purified plasma membranes</td>
<td>0.330</td>
<td>0.540</td>
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</table>

**Table III**

Contents of hen erythrocyte whole cells and isolated nuclei

Results given as picograms per cell and nucleus, respectively; mean values from three determinations.

<table>
<thead>
<tr>
<th>DNA</th>
<th>RNA</th>
<th>Protein</th>
<th>Phospholipids</th>
<th>Cholesterol</th>
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<tr>
<td>Cell</td>
<td>1.72</td>
<td>0.19</td>
<td>50.50</td>
<td>0.75</td>
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<tr>
<td>Isolated nucleus</td>
<td>1.70</td>
<td>0.13</td>
<td>3.02</td>
<td>0.08</td>
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<td>Recovery in nuclear fraction (%)</td>
<td>98.8</td>
<td>65.4</td>
<td>6.0</td>
<td>10.7</td>
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</table>

* F. D. Jarasch, unpublished results.
8000 X g crude plasma membranes remains bound to the nuclear membrane through the harsh clear membranes (for detailed discussion, see Reference 3). This and the total absence of a nucleoplasmic enzyme such as NAD+-pyrophosphorylase. The observation that some DNA and RNA cytochrome c reductase, respectively. The nuclear membrane cholesterol to phospholipid ratio, a higher content of protein, nucleoplasm or nuclear envelope apparently can be excluded by the absence of NADf-pyrophosphorylase and of the NADH-cytochrome c reductase activity typical for endoplasmic reticulum type membranes in many other cell systems, and is characterized by the absence of ouabain-sensitive ATPase activity and a relative richness in protein. In addition, electron microscopy suggests that it is slightly thinner than the plasma membrane.

Whether there is any turnover of the constituents of the two membrane systems at this late “dead end differentiation” stage appears to be no rapid exchange of membrane character through the cytoplasmic sap phase. This shows that membrane differentiation once established in a cell can be rather stable. Whether there is any turnover of the constituents of the two membrane systems at this late “dead end differentiation” stage remains to be clarified.

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