Isolation, Morphology, and Composition of the Nuclear Membrane from Rat Liver*

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SUMMARY

Intact nuclear membrane from rat liver was isolated and purified from nuclear lysates by discontinuous gradient centrifugation. Two membrane fractions isolated at the interface density (d) 1.16 to 1.18 and d 1.18 to 1.20 g per cc have been characterized with respect to morphology and chemical and enzymic composition. Structurally, the purified nuclear envelope was composed of both an inner and outer leaflet possessing discrete pore complexes with an average diameter of 700 to 800 A. Each leaflet, which in itself represents a unit membrane, measured approximately 75 A thick. The chemical composition of nuclear membrane isolated at the interface d 1.16 to 1.18 g per cc was protein, 58.8%; lipid, 35.2%; carbohydrate, 2.9%; RNA, 3.1%; DNA, 0.0%. Similar values for membrane isolated at interface d 1.18 to 1.20 g per cc were protein, 62.3%; lipid, 27.7%; carbohydrate, 3.9%; RNA, 6.1%; DNA, 0.0%. Glucosamine was the only amino sugar detected in membrane hydrolysates. The lipid fraction was composed chiefly of phospholipid, cholesterol, and cholesterol esters. Glucose 6-phosphatase, rotenone-insensitive DPNH-cytochrome c reductase, and adenosine triphosphatase were found to be integral components of the nuclear membrane. The specific activities of glucose 6-phosphatase and DPNH-cytochrome c reductase were approximately 50% those found for the same enzymes in the cytoplasmic membrane. This finding strongly suggests that these activities are associated with only one of the leaflets of the nuclear membrane, presumably the outer.

A meaningful understanding of membrane structure and function is dependent largely upon a detailed knowledge of chemical and enzymic composition. This requires that pure material be examined so that the results may be interpreted unambiguously. Extensive studies on the erythrocyte membrane have provided a basis for understanding the dynamics of the red blood cell (1-3), while similar work performed on the cell (4-6), microsomal (7, 8), and mitochondrial (9-11) membranes has yielded valuable information dealing with chemical and biochemical interrelationships. Of the major membrane systems of the cell the nuclear membranes has received the least attention. This is evidenced by the conspicuous lack of information relating to the physicochemical and biochemical properties of purified nuclear membrane. Thus, it appears that a detailed study of the nuclear membrane is in order and that the results of such a study would be essential to a better understanding of nuclear activity and the role of the nucleus in the regulation of cellular processes. In this paper we present a method for the isolation and purification of the nuclear membrane from rat liver and report on the chemical and enzymic composition of this bilaminar, paucimolecular structure.

MATERIALS AND METHODS

Preparation of Nuclei

The method of Blobel and Potter (12) was adapted for large scale preparations using the Spinco SW 25.2 rotor. The nuclear pellet from each tube was suspended in 1.0 M sucrose-Buffer TKM† by vortex mixing, transferred to a 30-ml Corex centrifuge tube (No. 8445, Corning Glass Works, Corning, New York) and centrifuged at 3400 x g for 10 min in a Sorvall RC-3 equipped with the HG-4 rotor. After decantation, a final washing was performed with similar volumes of 0.25 M sucrose-Buffer TKM and the nuclei were collected by centrifugation at 750 x g for 3 min at 3°. The yield of nuclei based on the recovery of DNA was 50 to 70%.

Isolation of Nuclear Membrane (Fig. 1)

To each nuclear pellet (approximately 16 mg of protein) contained in a 30-ml Corex centrifuge tube were added 10 ml of 0.05 M Buffer TKM. The tube was positioned vertically in an ice bath, and the microtip of the Sonifier cell disrupter W 140-1 (Heat Systems, Melville, New York), was placed approximately 1 cm above the bottom of the tube. Nuclei were disrupted by sonic treatment at a setting of 6.5 for 10 to 15 sec. A smear was made of the suspension for examination with a phase microscope to determine the extent of lysis; additional sonic treatment was performed if more than 30% of the nuclei remained intact. Care was taken not to overtreat since the nuclear membranes is quite susceptible to sonic disintegration. When adequate...
breakage of nuclei had been achieved, solid potassium citrate was added with mixing to a final concentration of 10% w/v. This step resulted in the solubilization of much of the nucleoplasm which otherwise adhered to the nuclear membrane and complicated the purification procedure. Examination of the citrate-treated sonic extract under the phase microscope revealed structures resembling empty nuclei (nuclear ghosts) and some intact nuclei (approximately 20 to 30% of the original number). The lysate was centrifuged at 39,000 \( \times g \) for 45 min at 3\(^\circ\) C in a Sorvall RC-2 centrifuge, and the slightly opalescent supernatant was discarded. The pellet was suspended in 3 ml of sucrose-buffer TKM-10% citrate solution of density (d) 1.22 g per cc by vortex mixing and transferred to a cellulose nitrate centrifuge tube to fill the Spinco SW 25.1 rotor. An additional 7 ml of sucrose solution (d 1.22 g per cc) was used as a rinse and added to the cellulose nitrate tube. The membrane suspension was overlaid, using a peristaltic pump, in a stepwise manner with 7 ml each of sucrose-buffer TKM solutions containing 10% citrate with densities of 1.20, 1.18, and 1.16 g per cc. Densities were measured with a pycnometer at 25\(^\circ\) C. The discontinuous gradient was developed by centrifugation at 100,000 \( \times g \) for 10 to 14 hours at 3\(^\circ\) C. Nuclear membrane was isolated at interfaces of d 1.18 to 1.20 g per cc and d 1.16 to 1.18 g per cc by lateral puncture using a syringe and 18-gauge needle. The respective membrane fractions from the gradient were diluted with 2 volumes of 0.05 M Buffer TKM containing 10% potassium citrate and centrifuged at 100,000 \( \times g \) for 60 min. For electron microscopy, portions of the pellet were fixed directly.

**Preparation of Cytoplasmic Membrane**

Rat liver microsomes were prepared from fasted Holtzman rats (150 to 200 g) as described by Murray, Sass, and Pitot (13). The crude pellet obtained from the 100,000 \( \times g \) centrifugation was suspended in 15 volumes of sucrose-buffer TKM-10% citrate of d 1.22 g per cc and sonically treated to obtain an even suspension. The sonic extract was overlaid with a discontinuous sucrose gradient and centrifuged for 1.20 g per cc for 45 min at 3\(^\circ\) C in a Sorvall RC-2 centrifuge. An additional 7 ml of TKM-citrate, d 1.22 g per cc was added with mixing to a final concentration of 10% w/v. The crude pellet obtained from the 100,000 \( \times g \), 12 hours, 3\(^\circ\) C centrifugation was suspended in 3 volumes of sucrose-buffer TKM containing 10% potassium citrate and centrifuged at 100,000 \( \times g \) for 60 min. For electron microscopy, portions of the pellet were fixed directly.

**Electron Microscopy**

A suspension of nuclei or membrane was fixed for 2 to 5 hours in freshly prepared 1% glutaraldehyde in 0.05 M cacodylate buffer (pH 7.4) containing 0.2 M sucrose. After centrifugation, the pellet was fixed in buffered 1% OsO\(_4\) according to the method of Sabatini, Bensch, and Barnett (14). The pellets were dehydrated in ethanol-water solutions of increasing concentrations of ethanol and embedded in Epon and Araldite (15). Thin sections were mounted on carbon-covered grids and post-stained with lead citrate (16). Specimens were examined with the Hitachi IIIB or C electron microscope at 75 kv accelerating voltage.

**Chemical Analyses**

**Protein**—The biuret (17) or Folin (18) procedures employing a standard ovalbumin crystallized four times were used for the estimation of protein.

**Nucleic Acid**—RNA determinations were performed by the method of Munro and Fleck (19). It was assumed that 32 \( \mu \)g of RNA per ml would have an absorbance of 1.00 at 260 m\(\mu\). DNA was measured by the diphenyldiamine reaction after perchloric acid hydrolysis (0.5 M HClO\(_4\) at 70°) (20). Salmon testis DNA (Worthington) was used as a standard.

**Lipid**—Analyses were performed on lipid extracts prepared by the method of Polich, Lees, and Sloane Stanley (21). Total phosphorus and organic extractable phosphorus were determined by the procedure of Bartlett (22). Phospholipid was calculated by assuming 25 \( \mu \)g of phospholipid per \( \mu \)g of lipid phosphorus (23). Cholesterol was assayed by the method of Glick, Fell, and Sjolin (24).

**Carbohydrate**—Membranes were first treated with pancreatic and T\(_1\) ribonuclease before being analyzed for carbohydrate. Neutral sugars in membrane hydrolysates (1 N HCl, 100°, 6 hours) were determined by the phenol-sulfuric acid method of Dubois et al. (25) using an equinormal mixture of galactose and glucose as a standard. For the analysis of glucosamine and
galactosamine, membrane was hydrolyzed with 3 N HCl for 10 hours in a sealed, evacuated (less than 50 µ of mercury) tube at 110°C. The liberated amino sugars were determined quantitatively in the 60-cm column of the amino acid analyzer (Spinco 120C).

Excellent resolution of the two amino sugars was obtained on a 19-cm column of PA-35 resin using the 0.2 n citrate buffer, pH 5.25, but the hydrolysates contained an unidentified ninhydrin-reactive component that eluted 1.5 ml before galactosamine; this necessitated the use of a longer column.

Sialic acid was measured by the thiobarbituric acid method of Warren (26).

**Enzyme Assays**

DPNH-cytochrome c reductase (EC 1.6.99.3) was assayed by the method of Mackler and Green (27); all incubations were carried out at 30°C using a thermostated Beckman DB-G spectrophotometer. Glucose 6-phosphatase (EC 3.1.3.9) activity was measured in a 1.0-ml system containing 8 µ moles of glucose 6-phosphate (Sigma) in 0.05 M potassium citrate buffer, pH 6.5. Adenosine triphosphatase (EC 3.6.1.3) activity was also measured in a 1.0-ml system containing 5 µ moles of ATP and 2 µ moles of succinate in 0.05 M Tris-HCl buffer, pH 7.5, containing 0.05 M MgCl₂. In the case of both phosphatases, incubations were conducted for 37°C for 15 min. The enzyme reactions were terminated by the addition of silicotungstic acid and inorganic phosphate determined by a modification of the Martin-Doty procedure as described by Lindberg and Ernster (28).

**RESULTS**

**Isolation Procedure**—Several methods were investigated for the disruption of nuclei. These were exposure to hypotonic conditions, vigorous homogenization in a Dounce homogenizer with a tight-fitting pestle, and sonic disruption. Only the last procedure yielded results which permitted the isolation of the nuclear envelope on a large scale. The phosphorus and nucleic acid content of nuclei used as starting material for the preparation of nuclear membrane is given in Table I. The nuclear fraction accounted for 2.9% of the total RNA and less than 0.25% of the DPNH-cytochrome c reductase activity present in the homogenate. It is important to note, however, that the specific activity of DPNH-cytochrome c reductase was quite constant from one nuclear preparation to another. Examination of the nuclei with the phase and electron microscope indicated freedom from cytoplasmic contamination and revealed the typical double membrane nature of the nuclear envelope (Fig. 2). The sonic disruption step was most crucial. Excessive ultrasound degraded the nuclear membrane into extremely small fragments which were difficult to isolate and resulted in low yields. Adequate disruption of the nuclei produced the same end result but for the reason that membrane was not released. Consequently, in this procedure, we elected to use conditions which permitted the isolation of nuclear ghosts from the discontinuous sucrose-citrate gradient. Photomicrographs taken at two different stages of the isolation procedure illustrate this point (Fig. 3). A, B, C, and D are representative of those structures seen in sonically treated nuclear suspension to which potassium citrate was added. Intact nuclei appeared as dense, well-defined spherical bodies (Fig. 3A), while nuclei with a broken envelope and in the process of losing nucleoplasm appeared swollen and considerably less dense (Fig. 3, B and C). A nucleus that had lost the bulk of its nucleoplasm appeared as a collapsed spheroid (Fig. 3D) and has been termed a nuclear ghost. Structure representative of those isolated from the gradient at the interface of d 1.16 to 1.18 g per cc and d 1.18 to 1.20 g per cc is shown in Fig. 3, E and F. A larger percentage of nuclear ghosts was obtained at the interface of d 1.16 to 1.18 g per cc than in the region of higher density. The relative proportions of membrane found at the two interfaces varied and probably reflected the difficulty in precisely reproducing the conditions of sonic treatment. The yield of membrane ranged between 6 and 9% based on total nuclear protein.

**Morphology of Isolated Nuclear Membrane**—Examination of the nuclear membrane under the electron microscope revealed several characteristic features (Figs. 4 and 5). Sheets of membrane and large vesicles are present which have a bilamellar structure; the two layers represent the inner and outer membranes of the nuclear envelope. At high magnification, ribosomes were occasionally noted to be distributed on one of the membranes of the envelope, presumably the outer leaflet. For complexes known to be integral components of the envelope as abundant and may be observed in transversal sections or sections taken perpendicular to the surface of the pore. Frequently in transverse section, the inner and outer membranes appear to converge and form a single electron-dense line (diaphragm) which is bordered by electron-opaque material. In such instances, the diaphragm spanning the diameter of the pore is approximately the same thickness as either of the two leaflets, which individually measure approximately 75 A thick. The section of the pore making up the electron-opaque region is of particular interest since it contributes significantly to the over-all thickness of the pore complex. In the transversal view, pores appear as annuli with particularly electron-dense periphery. The diameter of the pore complex is in the range of 70 to 800 A. These values are in good agreement with measurements performed on intact nuclei obtained from the newt, the frog, and the starfish (29).

**Chemical Composition of Nuclear Membrane**—Analyses performed on both the high and low density membrane fractions indicate only minor differences in composition (Table II). Membrane isolated at the interface of d 1.18 to 1.20 g per cc had a reduced lipid content and an approximately 2-fold increase in RNA. Both of these changes parallel an increase in density. The variation in RNA content most likely represents a random loss of ribosomes from the nuclear surface during sonic disruption and exposure to citrate-containing media.

It is interesting to note that glucosamine was the only amine sugar found in membrane hydrolysates; however, if galactosamine were present at less than 5% the level of glucosamine, it was
Fig. 2 (upper). Electron micrograph of a rat liver nucleus illustrating the inner and outer leaflets of the nuclear envelope.

Fig. 3 (lower). Phase-contrast micrographs depicting stages in the isolation of nuclear membrane. Structures A through D are typical of those observed after sonic treatment of a nuclear suspension and the addition of potassium citrate to a final concentration of 0.5% w/v. E and F illustrate nuclear ghosts isolated directly from the sucrose-citrate gradient. Final magnification in each case, x 1250.

not have been detected. Although no DNA was found associated with the nuclear membrane, the conditions of the isolation procedure could have removed noncovalently bound chromatin. Thin-layer chromatography of lipid extracts of the nuclear membrane revealed phospholipid and cholesterol (free and esterified) as the major lipid components. Triglycerides and free fatty acids were present only at extremely low levels.

The ratio of lipid phosphorus to protein for the membrane (Table III) was 4 to 5 times greater than the corresponding value for intact nuclei (Table I). The yield of membrane based
Fig. 4 (upper). Electron micrograph of nuclear membrane isolated at the interface $d$ 1.16 to 1.18 g per cc. The enclosed area has been enlarged and appears as Fig. 5.

Fig. 5 (lower). Enlargement of the enclosed area of Fig. 4. Arrows point out both transverse and tangential sections of pore complexes.
.. the recovery of lipid phosphorus from the nuclear fraction was in the range of 24 to 36%. Membrane which had been digested with pancreatic and T, ribonuclease contained approximately 25% of its total phosphorus in a form which was not extracted with CHCl3-CHOH (2:1). In the case of nuclei, non-lipid phosphorus accounted for 91% of the total phosphorus.

Enzymes Associated with Isolated Nuclear Membrane—Enzymic analyses performed on the nuclear membrane, intact nuclei, and cytoplasmic membrane are summarized in Table IV. Adenosine triphosphatase, glucose 6-phosphatase and ribonucleic-insensitive DPNH-cytochrome c reductase are known to be integral components of the endoplasmic reticulum and are frequently used as marker enzymes when testing nuclear fractions for cytoplasmic contamination. Both glucose 6-phosphatase and DPNH-cytochrome c reductase were found in nuclei at approximately one-twentieth the specific activity obtained for the cytoplasmic membrane. The purified nuclear membrane exhibited a 7- to 10-fold increase in specific activity over the nuclear fraction for these two enzymes. Nuclear membrane isolated at the d 1.16 to 1.18 g per cc interface contained glucose 6-phosphatase and DPNH-cytochrome c reductase at specific activities 49% and 42% that of the cytoplasmic membrane respectively. The same values for nuclear membrane isolated at the d 1.18 to 1.20 g per cc interface were 68% and 42%, respectively. The finding that glucose 6-phosphatase is an integral component of the nuclear membrane is in direct opposition to the report of Zbarsky et al. (30).

The adenosine triphosphatase activity of the nuclear membrane was 1.6 to 2.1 times greater than that noted for intact nuclei, and it represented 16 to 22% the specific activity found for the cytoplasmic membrane. No stimulation of ATP hydrolysis was noted on the addition of Na+ or K+ ions. In order to obtain reproducible analyses for both phosphatases it was necessary to perform all assays immediately after their isolation. Storage at ice bath temperature for 1 to 2 hours or freezing and thawing resulted in almost complete loss of adenosine triphosphatase and glucose 6-phosphatase activity. The same enzymes associated with the cytoplasmic membrane did not exhibit this extreme lability. Nuclei and membrane preparations could be stored at 0° for at least 5 hours without detecting a decrease in DPNH-cytochrome c reductase activity.

### TABLE II

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<th>Component</th>
<th>Composition at interface</th>
<th>d 1.16-1.18 g/cc</th>
<th>d 1.18-1.20 g/cc</th>
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<td>0.09</td>
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### TABLE III

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<th>Quantity measured</th>
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<th>d 1.16-1.18 g/cc</th>
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<tr>
<td>Total phosphorus</td>
<td>25.9 ± 3.0 µg</td>
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<td>Lipid phosphorus</td>
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<td>Phospholipid</td>
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<td>Cholesterol</td>
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### Table IV

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<th>Fraction</th>
<th>Glucose 6-phosphatase</th>
<th>DPNH-cytochrome c reductase</th>
<th>ATPase</th>
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<tr>
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<td>µmoles Pi released/min/mg protein</td>
<td>µmoles cytochrome c reduced/min/mg protein</td>
<td>µmoles Pi released/min/mg protein</td>
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<tr>
<td>Nuclear membrane</td>
<td>3.82</td>
<td>0.381</td>
<td>0.433</td>
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<td>d 1.16 to 1.18 interface</td>
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<tr>
<td>d 1.18 to 1.20 interface</td>
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<td>Nuclei</td>
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<td>Cytoplasmic membrane</td>
<td>7.79</td>
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Enzymic data for nuclear membrane, nuclei, and cytoplasmic membrane

**DISCUSSION**

One of the main objectives of this study was to develop an isolation procedure for nuclear membrane that would permit the unequivocal identification of the purified membrane as being nuclear in origin. The isolation of nuclear ghosts directly from the sucrose-citrate gradient and the subsequent demonstration that these structures were composed of a double membrane with intact pore complexes constitute the major evidence establishing this point. Nuclear membrane was found to localize at the interfaces of d 1.16 to 1.18 g per cc and d 1.18 to 1.20 g per cc. Although these two fractions differed in density and composition, they exhibited almost identical morphological features. The cytoplasmic membrane in the same discontinuous gradient system migrated to the air-buffer interface and thus presumably has a buoyant density of less than 1.16 g per cc. This is a most significant result since it eliminates the possibility of even minor contamination of the nuclear membrane with endoplasmic reticulum. It is interesting to note that purified nuclei had a ratio of lipid phosphorus to DNA phosphorus of 0.11. Gurr, Finean, and Hawthorne (31) have calculated that this would correspond to four monolayers of lipid around a single nucleus. The reasonably close correspondence between the diameter of the pore complex of the rat liver nuclear membrane (700 to 800 A) and the same measurement performed on intact nuclei of such diverse species as the frog, newt, and starfish (20) indicates an interesting morphological uniformity. The diaphragm

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that bridges the pore does not appear to be a simple continuation of the inner and outer leaflets but is most likely a discrete structural unit within the membrane.

In gross composition, the nuclear membrane is quite distinct from the erythrocyte membrane, which contains 49.2% protein, 43.6% lipid, and 7.2% carbohydrate (3). The erythrocyte membrane contains both glucosamine and galactosamine in a molar ratio of approximately 2:1 while only glucosamine was detected in the nuclear membrane. The significance of the variation in hexosamine composition of membranes is obscure; however, as detailed information is accumulated regarding the levels and distribution of amino sugars in membranes, an understanding of their role in structure and function will be forthcoming. Comparatively, the intact red blood cell ghost also contains about 12 times more sialic acid than its nuclear counterpart. Lipid phosphorus was found to represent approximately 75% of the total membrane-bound phosphorus (Table III). This observation suggests the presence of a phosphorus-containing compound that is resistant to ribonuclease digestion and is not extracted with chloroform-methanol (2:1). RNA which was an integral part of the membrane would satisfy these criteria. This is in contrast to the plasma membrane, in which practically all the membrane phosphorus consists of phospholipid phosphorus (4, 6). The ratio of cholesterol to membrane-bound phosphorus was 0.42 and 0.30 for the low and high density nuclear membrane fractions, respectively, while values of 0.74 and 0.38 (4) have been reported for the plasma membrane. It is interesting to note that the density of the nuclear membrane is reasonably close to the value of 1.185 to 1.194 g per cc reported for the plasma membrane isolated from the same tissue (6) even though there are significant differences in composition.

Ultrastructural studies performed on thin sections of intact cells have indicated that regions of the outer nuclear membrane are continuous with the endoplasmic reticulum. Convincing biochemical data linking these two structures have been lacking. Our enzymic data, showing that both rotenone-insensitive DPNH-cytochrome c reductase and glucose 6-phosphatase are present in the nuclear membrane at approximately 50% the specific activity noted for these enzymes in the cytoplasmic leaflet is biochemically distinct from the outer leaflet. It is also suggested by these data that the inner leaflet of the nuclear envelope, most likely that bridges the pore, does not appear to be a simple continuation of the inner and outer leaflets but is most likely a discrete structural unit within the membrane.

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