

ISOLATION AND CHARACTERIZATION OF LUMINAL MEMBRANES FROM URINARY BLADDER

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ABSTRACT

A method is reported for the isolation of a highly purified fraction of urinary bladder membranes containing hexagonal plaques. The method uses zonal centrifugation as the final step of fractionation.

The purified fraction was characterized by its electron microscopic morphology, by its enzymatic profile, by quantitative and qualitative analysis of lipids and by the protein pattern obtained by electrophoresis in polyacrylamide sodium dodecyl sulfate gels. The fraction contains 65% lipids and 35% proteins. The major protein component has a molecular weight of 27,000 daltons.

Phospholipids are more than the 54% of the total lipid weight. Phosphatidylcholine, phosphatidylethanolamine, and phosphatidylinositol are the major phospholipids with 50%, 30%, and 7% of the total lipid phosphorus, respectively.

The glycolipid fraction is 10% of the total lipid weight and is formed by only two components, both sulfatides. Total cholesterol makes up 36% of the total neutral lipid fraction of which cholesterol esters constitute 6%. Glycoproteins are also found to be present in the fraction.

INTRODUCTION

The plasma membrane of the lumen and of the vesicles of mammalian urothelium contains an hexagonal array of subunits (Vergara, 1969). This array has the symmetry of the plane group $p6$ (Vergara et al., 1969; Hicks and Ketterer, 1969) which implies that each hexagon is made up of six identical subunits. At higher resolution the hexagons appear to be made up of 12 subunits (Warren and Hicks, 1970; Robertson, 1972) in which case the $p6$ symmetry would suggest that each hexagon is an aggregate of six pairs of subunits. The two members of each pair need not be identical. The membranes containing this structure will be referred to here as "hexagonal membranes."

Neither the function nor the structural relation-

ship between the components of the hexagonal membranes has been established. The isolation of a pure fraction and its chemical characterization are essential steps in clarifying these matters.

Two methods of separation of hexagonal plasma membranes have been previously reported (Hicks and Ketterer, 1970; Chlapowski et al., 1972). These were based on conventional methods of differential centrifugation and discontinuous sucrose gradient separation. The degree of purity of the final fraction obtained by these procedures has not been determined.

In this paper an improved method for obtaining hexagonal membranes is presented using zonal centrifugation as the final step in separation. When separated by this method, the hexagonal membranes adopt a distribution that overlaps to some

extent with contaminants such as fragments of smooth plasma membranes or mitochondrial membranes. Only a narrow density band contains a homogeneous population of hexagonal plasma membranes. The method reported here has proven to be highly reproducible, and relatively large amounts of highly purified hexagonal membranes have been obtained. This fraction has been morphologically characterized and its chemical components defined.

MATERIALS AND METHODS

Fresh supplies of porcine urinary bladder were obtained from a local slaughter house. The bladders were collected early on the morning of the day in which the separation was scheduled and were kept cool in an ice bucket before processing. All the following steps were performed at 4°C. The starting material consisted of 10–15 adult urinary bladders.

Obtaining the Pre-Purified Fraction

A partially clean fraction of plasma membranes was obtained by the following procedure which is based on a method devised by Fleischer and Fleischer (1969) for isolating liver plasma membranes. The bladders were separated from surrounding tissue, and their luminal cavities were flushed with an 0.9% solution of sodium chloride in 10 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethane sulfonic acid (HEPES) buffer at pH 7.5. The luminal surface of the urinary bladder was exposed and scraped with a blunt spatula.

The scrapings were collected in a 300-ml glass beaker containing 200 ml of a solution of 0.2 M sucrose in 10 mM HEPES buffer at pH 7.5 to which sodium thioglycolate had been added to a final concentration of 20 mM. The cells were gently agitated for 30 min in this medium and then homogenized in a Potter Elvehjem type homogenizer provided with a Teflon pestle, using 50 passes at a speed of 2,000 rpm. The resulting homogenate was filtered through a nylon net of 100- μ m mesh and centrifuged at 800 *g*. The pellet was used for separation of hexagonal membranes, and the supernate was differentially centrifuged to obtain other subcellular fractions (Fleischer and Fleischer, 1969).

20 ml of a suspension of the low speed pellet in 43% wt/wt sucrose containing 10 mM HEPES buffer at pH 7.5 and 1 mM MgCl₂ was placed in a 45 ml polyethylene centrifuge tube, and a two-step discontinuous gradient was formed by adding 25 ml of 0.25 M sucrose in 10 mM HEPES-MgCl₂ buffer at pH 7.5. After centrifugation at 26,000 *g* for 60 min, the material located in the interface was collected. This material was diluted 1:3 with chilled water containing 1 mM NaHCO₃, suspended by

means of a loose Dounce homogenizer, and spun at 12,000 *g* for 10 min. The resulting pellet containing hexagonal plaques, plasma membranes, and mitochondria, were suspended in 0.25 M sucrose-10 mM HEPES, containing 1 mM EDTA, and the sucrose concentration was adjusted to 38% by adding 60% wt/wt sucrose in 10 mM HEPES buffer containing 1 mM EDTA.

15-ml portions of this suspension were placed in 30-ml polycarbonate tubes, and a new two-step discontinuous gradient was formed by adding 0.25 M sucrose in 10 mM HEPES with 1 mM EDTA. It was then centrifuged at 86,500 *g* for 60 min. The interface was again collected, resuspended in 0.25 M sucrose in 10 mM HEPES with 1 mM EDTA, and spun at 10,000 *g* for 10 min. The resulting pellet was washed in 0.25 M sucrose in 10 mM Tris-HCl buffer pH 7.5, spun down at 10,000 *g* for 10 min, resuspended in 47% sucrose in 10 mM Tris-HCl buffer pH 7.5 to a final volume of 70–80 ml, and stored in the cold room until ready to be processed in the zonal centrifuge for final purification.

Zonal Centrifugation

A highly purified fraction of hexagonal membranes was obtained by zonal fractionation in a Beckman Ti-14 rotor (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.) in a modified model L centrifuge that was equipped with an " ω^2t integrator" as described by Anderson et al. (1966). The continuous sucrose gradient was performed using a Gradient Former Device (Beckman Model 141 Gradient Pump). The gradient was 18–45% wt/wt sucrose and was 450 ml in length.

The sample was loaded at the periphery of the rotor using a syringe, and enough 55% sucrose was injected to clear the peripheral lines. The gradient formation and loading operations were performed under dynamic conditions with the rotor spinning at 3,000 rpm. After these operations were completed, the speed was raised to 20,000 rpm, and the run continued until $\omega^2t = 1.5 \times 10^{11}$. The rotor was unloaded by displacing the lighter part of the gradient centripetally by adding 55% wt/wt sucrose through the edge line. About 40 fractions, each 15 ml, were collected. The optical density was recorded at 280 nM with a Beckman-DB-G spectrophotometer (Beckman Instruments, Inc., Fullerton, Calif.) using a flow cell. The sucrose concentration of each fraction was determined with a Bausch and Lomb Abbe 3 L refractometer (Bausch and Lomb Inc., Analytical Systems Div., Rochester, N. Y.). The fractions were diluted 1:1 with chilled water containing 1 mM NaHCO₃ and centrifuged at 35,000 rpm for 30 min in a Beckman L-2-65-B ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.). The pellets were resuspended in chilled water con-

taining 1 mM NaHCO₃, and the centrifugation was repeated. The pellets were then suspended in 0.25 M sucrose which was neutralized with 0.1 M KOH to pH 7.0 for further studies. The yield of this separation was 2.9 mg of hexagonal membrane protein per gram of protein in the total homogenate.

Chemical Assays

PROTEIN DETERMINATION: Protein was determined by the method of Lowry et al. (1951), using bovine serum albumin as the standard.

PHOSPHORUS DETERMINATION: Total phosphorus was estimated by the method of Chen et al. (1956) as modified by Rouser and Fleischer (1967).

LIPID EXTRACTION AND SEPARATION: Lipids were extracted with 20 vol of chloroform-methanol 2:1 vol/vol containing 1 µg of 2,6-ditert-butyl-*p*-cresol (Butylated hydroxytoluene [BHT], Sigma Chemical Co., St. Louis, Mo.) per ml. The extract was passed through a Sephadex-G-25 chromatographic column (Pharmacia Fine Chemicals Inc., Piscataway, N. J.) to separate lipids from nonlipids. The lipids, eluted by chloroform-methanol (19:1 by vol) saturated with water, were then evaporated by means of a flash evaporator under reduced pressure to a moist residue and suspended in a small volume of chloroform.

Neutral lipids, glycolipids, and phospholipids were separated from the total lipid extract by means of a silicic acid chromatographic column (Unisil, Clarkson Chemical Co., Williamsport, Pa.), using as eluents chloroform, acetone, and methanol, respectively, according to Rouser et al. (1967). The excess of solvent was evaporated under nitrogen, and the moist residue was resuspended in 2 ml of chloroform-methanol 2:1 and stored at -20°C.

Thin-Layer Chromatography

The compositions of the phospholipid and glycolipid fractions were determined using two-dimensional thin-layer chromatography (Rouser et al., 1966). Thin-layer chromatographic plates, 250 µm in thickness, were prepared with a Desaga spreader (Desaga Gmb, Heidelberg, W. Germany). Adsorbent was prepared fresh for five plates by mixing 20 g of plain silica gel with 10% of magnesium silicate (Silica Gel, E. Merck, AG., Darmstadt, W. Germany, Distributed by Brinkmann Instruments Inc., Westbury, N. Y.) and suspended in 60 ml of distilled water.

Chloroform-methanol-ammonia (65:25:5 by vol) was used in the first dimension and chloroform-acetone-methanol-acetic acid-water (3:4:1:1:0.5 by vol) in the second. After passing nitrogen over the plate for 10 min, lipids were visualized by spraying the developed plates with concentrated sulfuric acid

plus 30% formaldehyde and then heated at 180°C. Phospholipids were determined by measuring the phosphorus content of the spots (Rouser and Fleischer, 1967) and identified by their relative migration.

In addition, phosphatidylethanolamine and phosphatidylserine were confirmed by their ninhydrin-positive reaction, and sphingomyelin and phosphatidylcholine by their Dragendorff-positive reaction (Bregoff et al., 1953). The glycolipid components were identified by their relative migration and by the Sulf Spray positive reaction (Sulf-Spray reagent, Supelco, Inc., Bellefonte, Pa.)

Neutral lipids were analyzed by one-dimensional thin-layer chromatography on alkaline plates (silica gel with 10% magnesium silicate resuspended in 60 ml of 0.05 N KOH), using *n*-hexane-diethyl ether (70:30 by vol) as a solvent. Cholesterol ester was quantified by collecting the silica gel of the corresponding spot visualized with iodine vapor. The silica gel was suspended in chloroform-methanol (2:1) and then shaken and centrifuged at 5,900 × *g* for 10 min. The procedure was repeated twice. Cholesterol ester was subsequently determined in the supernate after evaporation to dryness. The weights of neutral lipids, glycolipids, phospholipids, and total lipids dried in vacuum were determined by using a Cahn electrobalance (Cahn Instruments Div./Ventron Corp. Paramount, Calif.).

Enzyme Assays

Succinic cytochrome *c* reductase was assayed by the method of Slater and Planterose (1960). DPNH cytochrome *c* reductase was assayed by the method of Dallner et al. (1966). ATPase was determined by the method of Post and Sen (1967).

Polyacrylamide Gel Electrophoresis

Electrophoresis in polyacrylamide gels containing 0.1% sodium dodecyl sulphate (SDS) was performed by the method of Fairbanks et al. (1971) in a vertical electrophoresis cell. To 0.3 ml of membrane suspension containing 1 mg of proteins per ml were added 5 µl of β-mercaptoethanol and 0.1 ml of 20% SDS. The mixture was immediately heated in boiling water for 10 min, cooled at room temperature, and mixed with 0.1 ml of 50% glycerol containing 50 µg of Pyronine Y per ml.

5–50 µl of sample was applied in each slot and run for 30 min at 25 mA. When the samples penetrated the upper part of the gel, the electrophoresis was continued at 90 mA for 8 h. The gels were calibrated by placing in one slot a mixture of standard proteins containing 2 mg per ml of ovalbumin, bovine serum albumin, ribonuclease, catalase, and chymotrypsinogen. The gels were stained with Coomassie Blue and destained with 5% acetic acid.

Electron Microscopy

NEGATIVE STAINING: Samples were stained with 1% phosphotungstate at pH 7.0.

THIN SECTIONS: Samples of the membrane suspensions were diluted with 0.25 M sucrose in 10 mM HEPES buffer at pH 7.5 and centrifuged in cellulose nitrate tubes at 84,000 *g* using the 50 rotor in the model L-2-65B Beckman ultracentrifuge. The pellets were fixed and dehydrated without being removed from the centrifuge tubes. Fixation was done with 5% glutaraldehyde in 0.1 M cacodylate buffer for 1 h. Dehydration proceeded to absolute alcohol, at which point the pellets were trimmed out of the centrifuge tube and transferred to a Petri dish containing absolute alcohol.

Under the stereoscopic microscope, the pellet was cut into small parts, carefully detached from the cellulose nitrate tube, and placed in propylene oxide. Infiltration and embedding in Epon 812 proceeded as usual (Luft, 1967). Thin sections were cut on a Reichert microtome (C. Reichert, sold by American Optical Corp., Buffalo, N. Y.), stained with uranyl acetate and lead citrate, and examined in an AEI 801 electron microscope.

RESULTS AND DISCUSSION

Fig. 1 is a protein and sucrose concentration profile obtained after zonal fractionation. Plasma membranes, mitochondria, and microsomes are widely spread in the continuous gradient. Purified hexagonal plaques are located in a narrow zone between 28 and 32% wt/w sucrose. Although hexagonal membranes were detected by electron microscopy in almost all the fractions, contamination of small vesicles was usually found in a concentration lower than 28%, and fragments of smooth plasma membranes and mitochondria were seen at above 32% sucrose (see Fig. 1).

This method of separation is based on the fact that after zonal centrifugation, a large part of the total population of hexagonal membranes is located in a contaminant-free density band which can be separated from the bands containing mitochondria and smooth plasma membranes. Negative staining of the fractions located in the range of 28–32% sucrose showed some contaminants, usually small fragments of smooth tubular membranes with no hexagonal pattern (Fig. 2). This material represents only a negligible part of the isolated membranes.

Fig. 3 shows a thin section of a purified membrane pellet in which the characteristic asymmetric profile of the membrane leaflets is observed in

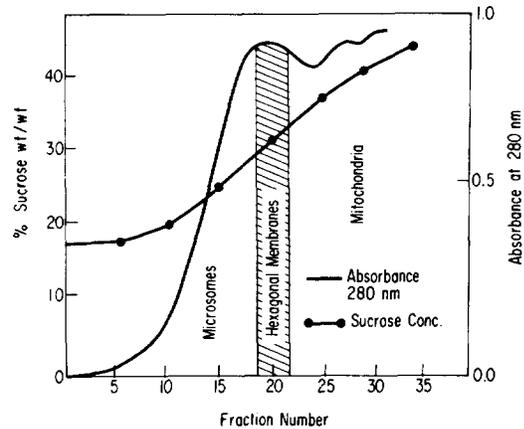


FIGURE 1 Separation of hexagonal membranes of porcine urinary bladder urothelium in a sucrose (19–40% wt/wt) gradient in a zonal rotor. Hexagonal membranes are located in the narrow zone between 28 and 32% sucrose. Small vesicular components are present at concentrations less than 28% sucrose and mitochondria membranes and fragments of smooth plasma membranes in concentrations higher than 32%.

perpendicular section. The hexagonal array is seen in oblique and face view. Mitochondrial membranes and smooth plasma membranes were not detectable in this fraction.

Table I summarizes the enzymatic profiles of hexagonal membrane fractions isolated at 30, 32, and 34% sucrose and of other subcellular fractions. The hexagonal membrane fractions show an enzymatic profile typical of plasma membranes with a high Na- and K-dependent ATPase which are sensitive to ouabain, low specific activity for the mitochondrial enzyme succinate cytochrome *c* reductase, and low specific activity for the microsomal enzyme DPNH cytochrome *c* reductase. Concomitant with the appearance of mitochondrial membranes in the fraction isolated at 34% sucrose, there is an increase in the value for succinate cytochrome *c* reductase and DPNH cytochrome *c* reductase. The Na⁺- and K⁺-stimulated ATPase activity is four to five times greater in the hexagonal membrane fraction than in the total homogenate, or in the mitochondrial and microsomal fractions. This ATPase activity is 34% inhibited by 1×10^{-5} M ouabain. In contrast, inhibition of only 4% was found in crude plasma membrane fractions. These findings support the idea that the hexagonal membrane might be involved in the active transport of sodium by transitional epithelium (Wickham, 1964).

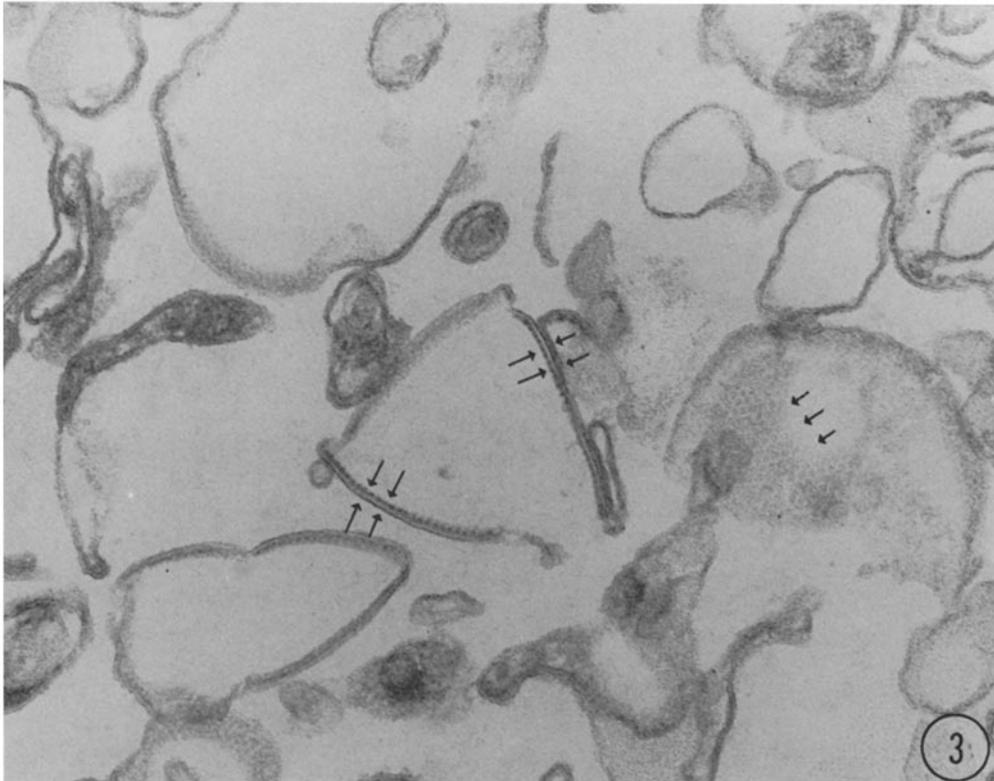
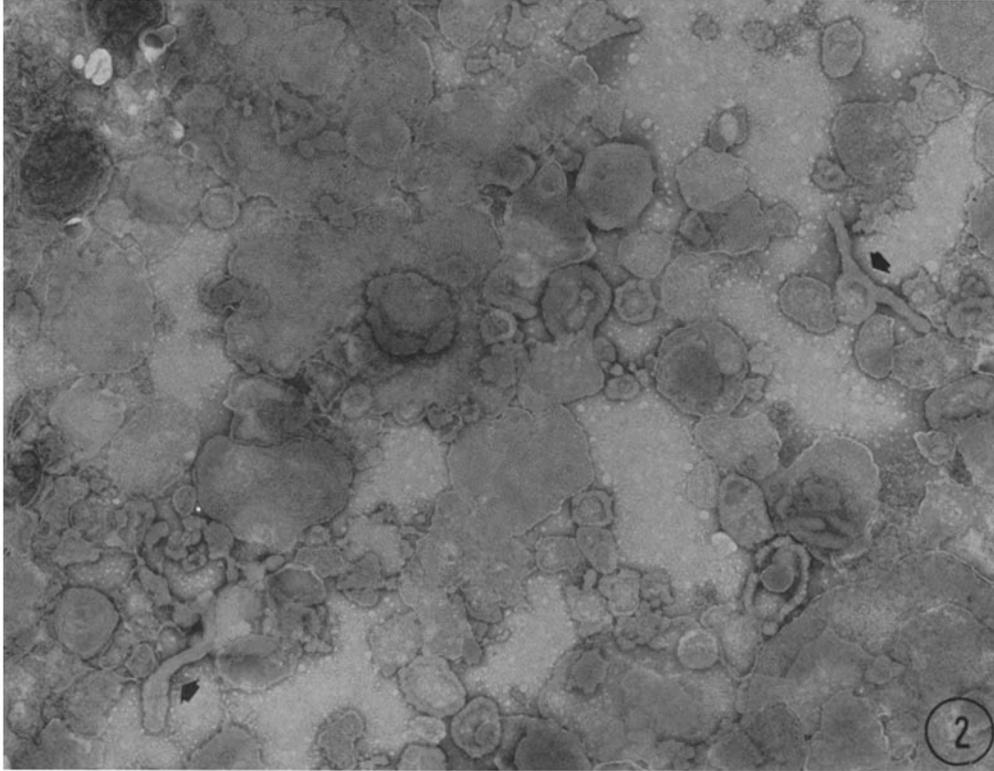


FIGURE 2 Purified pellet of urinary bladder hexagonal membranes negatively stained with 1% neutralized phosphotungstic acid. The typical hexagonal pattern is clearly seen. (Contaminants (arrows) are smooth tubular membranes.) $\times 35,000$.

FIGURE 3 Thin section from a pellet isolated from zonal centrifugation at 30% wt/wt sucrose, showing asymmetrical hexagonal membranes in perpendicular (arrows), and face view. $\times 40,000$.

TABLE I
Enzymatic Profiles of Hexagonal Plasma Membranes, Total Homogenate, Mitochondria, Microsome and Plasma Membrane Fractions Isolated from Porcine Urothelium

| Fraction | $\mu\text{g P/mg protein}$ | Succinate cytochrome <i>c</i> reductase* | DPNH cytochrome <i>c</i> reductase* | ATPase† Mg-Na-K-dependent | Ouabain Sensitivity‡ |
|---------------------|----------------------------|--|-------------------------------------|---------------------------|----------------------|
| Homogenate | 35.3 | 0.032 | 0.700 | 0.135 | |
| Mitochondria | 17.9 | 0.386 | 0.181 (29.6%) | 0.185 (64.3%) | |
| Microsomes | 47.0 | 0.042 | 1.525 | 0.156 | |
| Plasma membranes | 23.0 | 0.052 | 0.015 | 0.610 | 4.0 |
| Hexagonal membranes | | | | | |
| 30% | 28.6 | 0.003 | 0.102 | 0.650 | 29.4 |
| 32% | 29.4 | 0.004 | 0.182 | 0.627 | 34.0 |
| 34% | 30.2 | 0.010 | 0.373 | 0.610 | 34.2 |

The percentages express inhibition by rotenone and oligomycin, respectively.

* Expressed as micromoles of cytochrome *c* reduced per minute per milligram of protein.

† Expressed a micromoles of Pi released per minute per milligram of protein.

‡ Inhibition by 10^{-5}M ouabain (%).

|| Mg ATPase.

TABLE II
Lipid Composition of Porcine Urinary Bladder Hexagonal Membranes

| | Mean | SD* |
|---|-------|------|
| $\mu\text{g total phosphorus per mg protein}$ | 28.03 | 1.24 |
| $\mu\text{g lipid phosphorus per mg protein}$ | 22.30 | 5.06 |
| | | |
| mg total lipid (weight) per mg protein | 2.03 | 0.57 |
| mg phospholipids (weight) per mg protein | 1.10 | 0.27 |
| $\mu\text{g lipid-phosphorus per mg phospholipids}$ | 21.10 | 6.40 |
| | | |
| % phospholipids weight in total lipid weight | 54.34 | 6.04 |
| % glycolipid weight in total lipid weight | 10.45 | 3.54 |
| % neutral lipid weight in total lipid weight | 35.21 | 3.42 |
| % total cholesterol in neutral lipids weight | 35.80 | 2.23 |
| % cholesterol ester in total cholesterol | 6.56 | 1.86 |
| | | |
| Molar ratio cholesterol phospholipids | 0.47‡ | |

* Mean and SD of six different preparations.

‡ The value 775 was used as an average molecular weight for phospholipids and 386 for cholesterol.

Lipid Analysis of the Purified Fraction

Table II summarizes the lipid composition of the purified fraction. The data were obtained from six different preparations of membranes isolated by zonal centrifugation.

A lipid-protein ratio greater than one was obtained in all the samples studied. The mean value, 2.03 ± 0.57 , gives a value of 65% lipids and 35% proteins for the composition of hexagonal plasma membranes. As mentioned below,

small amounts of triglycerides and fatty acids were detected in our extracts. These were considered to be preparation artifacts or contaminants. Even if the presence of these components is neglected, the lipid-protein ratio remains greater than one. There are 1.10 mg of phospholipids and 0.16 mg cholesterol for each mg of protein. The most conservative figure for this lipid-protein ratio would be 1.26. The high ratio of lipid to protein is in agreement with the low density exhibited by this fraction. At

isopycnic equilibrium, the hexagonal plasma membranes are located in a band corresponding to a density of 1.13 g/ml.

The molar ratio of cholesterol to phospholipids was found to be 0.47. This value does not differ from the ratios found for several plasma membranes (Pfleger et al., 1968; Korn, 1969). Two molecules of phospholipids are expected to be present in hexagonal plasma membranes for each molecule of cholesterol. The ratio of lipid phosphorus to phospholipid is 21 $\mu\text{g}/\text{mg}$. This is close to the value found for other kinds of plasma membranes, and indicates that nothing unusual should be expected in the length of the fatty acid paraffin chains of the phospholipids (Zambrano et al., 1971).

GLYCOLIPIDS: These compounds form 10% of the total lipid extracts and give conspicuous double spots in the thin-layer chromatograms. The spots give positive reaction for sulfatides (Fig. 4). Although the role of sulfatides in the molecular structure of plasma membranes is not understood, it is interesting that the sulfatransferase activity has been found located in Golgi membranes isolated from rat kidney (Fleischer and Zambrano, 1973). The early relationship between the Golgi apparatus and the hexagonal plaques during the differentiation of urothelium superficial squamous cells, and the probable role of the Golgi apparatus in the turnover of the plaques (Hicks, 1966; Koss, 1970), provide a possible explanation for these observations.

NEUTRAL LIPIDS: Fig. 5 shows a thin-layer chromatograph of the neutral lipid fraction. Cholesterol represents 35% of the total lipids and about 36% of the neutral lipids. Triglycerides and fatty acids form the main part of the remaining neutral lipids. Fatty acids derive most probably from hydrolysis of phospholipids during the isolation procedure. At present there is no good explanation for the presence of the small amount of triglycerides found in our neutral lipid extract. They are not found in the lipid analysis of other plasma membranes, and we prefer to consider them contaminants rather than structural components of the hexagonal membranes.

PHOSPHOLIPIDS: Table III summarizes the quantitative composition of phospholipids and Fig. 6 shows the separation of the phospholipids by two-dimensional thin-layer chromatography. Phosphatidylcholine and phosphatidylethanolamine form 80% of the total amount of phospholipids. Sphingomyelin, phosphatidylinositol, and

phosphatidylserine are the other important phospholipids, being 16% of the total. Phosphatidic acid, lysophosphatidylcholine, and lysophosphatidylethanolamine form less than 3% of the total phospholipid extract.

Phosphatidylcholine is responsible for 50% of the total amount of phospholipids. Sphingomyelin, by contrast, accounts for only 5% of the total lipid phosphorus. Plasma membranes from several sources have a sphingomyelin value of 15–16% of the total phospholipids. These chemical coincidences also suggest that the luminal plasma membrane of the urinary bladder may be derived from the Golgi complex (Fleischer and Fleischer, 1971).

Cardiolipin was not detected in the thin-layer chromatograms, supporting the evidence from morphological and enzymatic studies as well as from lipid analysis that no important mitochondrial contaminants are present in the purified fraction. The small percentage of lysocompounds can be explained as a breakdown of phosphatides after the long procedure leading to membrane isolation.

Electrophoresis in SDS-Polyacrylamide Gels

Eight bands were observable on SDS-polyacrylamide gels between the limits of 100,000 and 15,000 daltons (Fig. 7). Two major bands are located around 27,000 daltons (bands 6 *a* and 6 *b*), and a less dense one is located in the region of 55,000 daltons (band 4).

If each hexagon is formed of 12 globular particles as has been suggested by Warren and Hicks (1970), the subunit molecular weight could be about 27,000. The observation of two bands at this position may indicate the presence of a pair of chemically distinct polypeptide chains as is suggested by the $p6$ symmetry. The 55,000 band could then be interpreted as being due to some of the intact pairs which have resisted denaturation during the preparation. There is no way to test this suggestion until the particulate components can be isolated and characterized independently of the membrane.

Other faint bands are located in the region of 60,000 (band 3), 70,000 (band 2), and 100,000 (band 1) daltons. Small molecular weight glycoproteins are also seen in the electrophoretic pattern. These migrate to a region of 15,000 daltons and are detected by a positive reaction with periodic acid-Schiff.

Our purpose has been to isolate hexagonal mem-

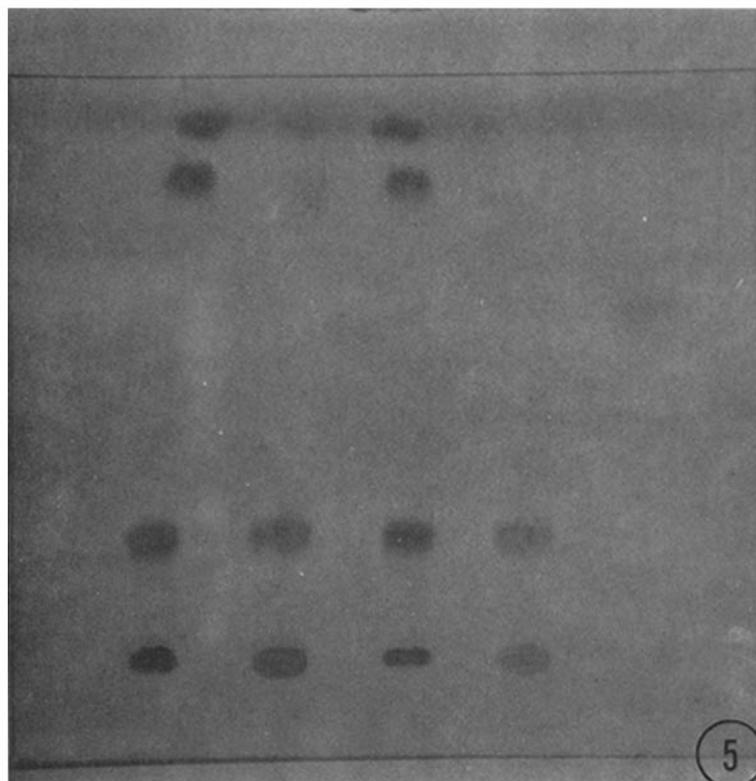
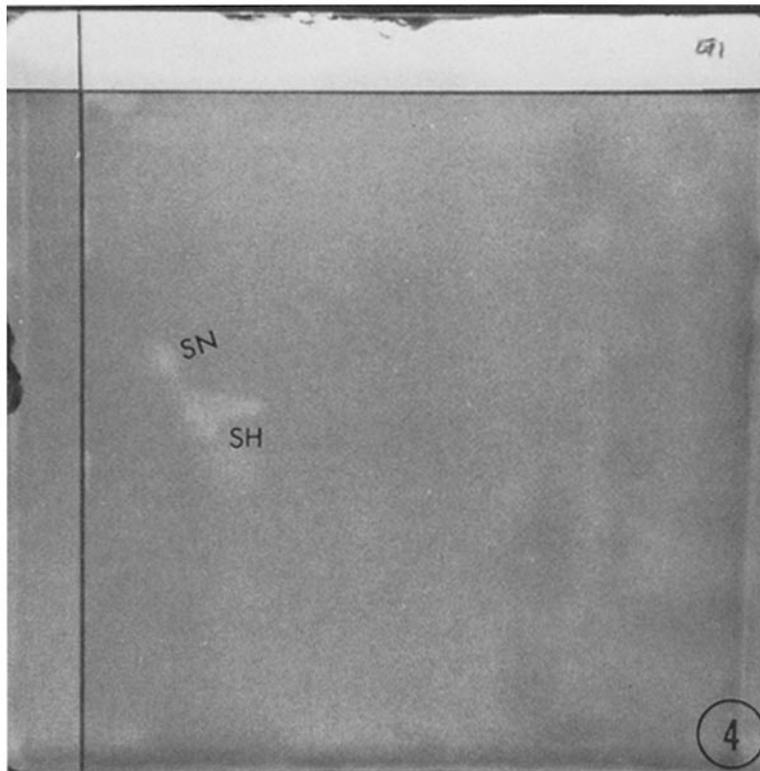


TABLE III
Phospholipid Composition of Porcine Urinary
Bladder Hexagonal Membranes

| Phospholipid class | Percent of Total* Lipid-Phosphorus |
|------------------------------|---------------------------------------|
| Phosphatidylcholine | 50.60 ± 7.25‡ |
| Phosphatidylethanolamine | 30.10 ± 3.15 |
| Sphingomyeline | 5.69 ± 0.69 |
| Phosphatidylinositol | 7.21 ± 0.24 |
| Phosphatidylserine | 3.39 ± 0.37 |
| Phosphatidic acid | 0.72 ± 0.16 |
| Lysophosphatidylcholine | 1.41 ± 0.07 |
| Lysophosphatidylethanolamine | 0.40 ± 0.29 |

* Phosphorus recovered from thin-layer chromatography plates was 97.48 ± 2.31 .

‡ Standard deviation of six thin-layer chromatography plates from six different separations.

branes from mammalian urinary bladder epithelium in quantity and purity which are suitable for chemical analysis without using extractive agents. The procedure of fractionating a plasma membrane-rich preparation by zonal centrifugation seems to fulfill these requirements. Hexagonal membranes (of a high degree of purity) were obtained at densities 1.12–1.13 g/ml after isopycnic zonal separation in the continuous sucrose gradient. Observations of such material by negative staining and by thin sectioning confirmed the high purity of the preparation. These criteria were complemented by the absence of cytochrome *c* reductase activity in the membrane fraction and by the high concentration of Na⁺- and K⁺-dependent ATPase.

The high proportion of lipids (65%), unexpected for a membrane bearing closely packed globular

subunits thought to be protein in 75% of the membrane area (Stachelin et al., 1972), suggests that some of the lipid may be associated with the subunits in the hexagonal array. Indeed, the fact that these hexagonal membrane plaques contain closely packed intramembrane particles of a type widely seen by the freeze-fracture method in the fracture faces of membranes in general suggests that the study of the isolated plaques may contribute to a better understanding of the general nature of such intramembrane particles. It has been widely assumed that such intramembrane particles, at least in many instances, represent protein residing inside the lipid bilayer cores of membranes. In the particular case of the hexagonal membrane plaques isolated and studied by us, if the particles had been purely protein, we would have expected the percentage of protein in the purified membrane fraction to have been higher as compared with other purified membrane fractions. Instead, it is lower. This suggests that these particular intramembrane particles are likely to be mainly lipid rather than protein. The results further suggest that the lipid in the particles may be more densely packed than the lipid in the non-particle-containing membrane bilayers. This alone may provide enough difference from the non-particulate lipid bilayer regions to account for the building up of metal in particulate form on the membrane fraction faces. The protein interacting with the lipid polar heads and conceivably to some extent with some of the lipid nonpolar chains (Deamer, 1970) would, according to this interpretation, be the agent responsible for the specific lipid organization. The similarities in the lipid classes of the hexagonal membrane and the lipids extracted from Golgi membranes emphasizes the

FIGURE 4 Separation of glycolipids by two-dimensional thin-layer chromatography. The absorbent and solvent systems were the same as for the separation of the phospholipids fraction. 200 μ g of glycolipid were used in each chromatogram. Abbreviations: SN, sulfatide with normal fatty acid; SH, sulfatide with hydroxyfatty acid. Sulfur compounds show up as brilliant white spots after visualization with Sulfspray reagent.

FIGURE 5 Thin-layer chromatography of neutral lipids. Neutral lipids were chromatographed in a solvent containing *n*-hexane-diethyl ether (70:30 by vol). 40 and 20 μ g of lipid (column 2 and 4, respectively) were spotted and compared with a standard 40 μ g and 20 μ g total neutral lipid (column 1 and 3). The standard mixture contained equal amounts of palmitic acid, cholesterol, tripalmitin, and cholesterol palmitate in this sequence from origin (fatty acid) to front. The plate was charred at 180°C for 30 min after spraying with H₂SO₄-30% formaldehyde.

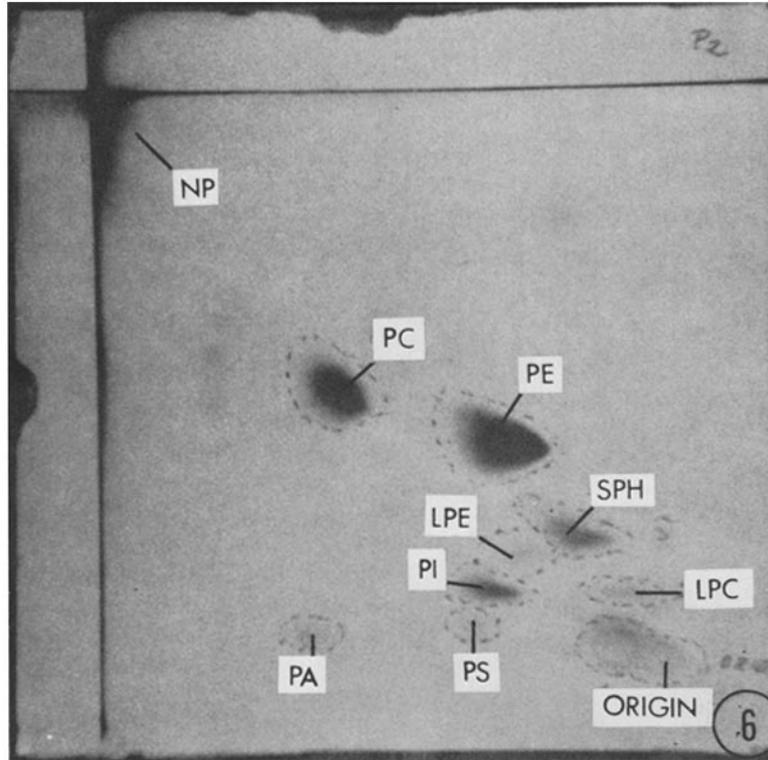


FIGURE 6 Separation of phospholipids by two-dimensional thin-layer chromatography. The absorbant was plain silica gel with 10% Mg silicate. Chloroform-methanol-ammonia (65:25:5 by vol) was used in the first dimension and chloroform-acetone-methanol-acetic acid water (3:4:1:1:05 by vol) in the second. Abbreviations: *NP*, nonpolar lipids; *PE*, phosphatidylethanolamine; *PC*, phosphatidylcholine; *SPH*, sphingomyelin; *PI*, phosphatidylinositol; *PS*, phosphatidylserine; *LPC*, lysophosphatidylcholine; *LPE*, lysophosphatidylethanolamine; *PA*, phosphatidic acid.

relationship between both structures suggested on morphological grounds.

The high concentration of Na^+ and K^+ ATPase would be a surprising finding if the membranes were part of the permeability barrier mechanism in transitional epithelium. The role of the membrane ATPase in the proposed active transport of ions (Wickham, 1964) and in the maintenance of the transepithelial potential difference exhibited by the mammalian urinary bladder has yet to be defined.

A study of the chemical composition of the luminal plasma membrane of the rat bladder has been recently published by Ketterer et al. (1973). No quantitative analysis of lipids or enzymatic profile as criteria of purity was reported, probably due to the "small amount of material obtained from rat epithelia."

Our method which yields about 2 mg of membrane protein in each preparation allows the characterization of one and the same sample by multiple assays and thus a better chemical and morphological correlation. It is not possible, so far, to make inferences about the function of the hexagonal membranes. More detailed information about the chemical components of these unique membranes is essential to further experimental approaches.

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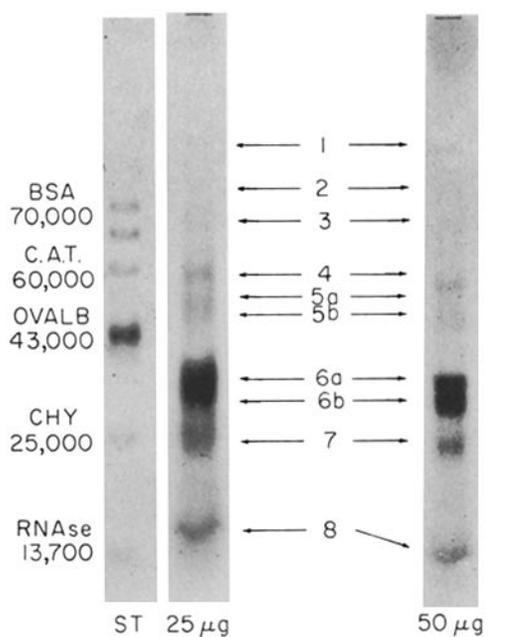


FIGURE 7 SDS polyacrylamide gel electrophoretic profiles. ST, standard proteins; 25 μ g, 25 μ g of membrane protein; 50 μ g, 50 μ g of membrane protein; BSA, bovine serum albumin; CAT, catalase; OVALB, ovalbumin; CHY, chymotrypsin; RNase, ribonuclease.

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