ISOLATION AND CHARACTERIZATION OF THE UROTHELIAL LUMENAL PLASMA MEMBRANE

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ABSTRACT

The lumenal plasma membrane has been isolated from transitional epithelial cells (urothelium) lining the urinary bladder in sheep by a modified technique involving treatment with hypotonic thioglycolate. The isolated membranes, like those *in situ,* are distinguished morphologically by arrays of hexagonal particles (in plaque regions) separated by smooth interplaque regions. These plaque regions, specifically, can be isolated from the lumenal plasma membrane. Of the proteins constituting the lumenal plasma membrane, five were found to characterize the plaque regions and, in particular, the 33,000-dalton species appears to be most heavily concentrated in the sodium dodecyl sulfate-polyacrylamide gel pattern of the isolated plaque regions. Lipid analyses showed that there are approximately 0.93 mg of phospholipid and 0.27 mg of cholesterol for each milligram of protein, giving a value of 55% lipids and 45% proteins for the composition of the lumenal plasma membrane. The total sialic acid content was measured to be approximately 0.038 μmol/mg protein for the plasma membrane. Several plasma membrane marker enzymes were found to be associated with the lumenal plasma membrane fraction, but only the 5′-nucleotidase activity was found to be further enriched in the plaque region fraction. Amino acid analysis of the intrinsic proteins of the plaques indicated a polarity index of 45%.

The lumenal plasma membrane of the urinary bladder of most mammals including man (15) is characterized by its unique morphological features. The membranes lining the free surface of the bladder show in profile a scalloped or wavy appearance (31, 13), which results from alternating regions of the membrane having varying thicknesses: a concave (plaque) area of unusual thickness, 120 Å, alternates with a crested (interplaque) region having a more usual thickness, 80 Å (40). The unit membrane structure of the plaque region is asymmetrical, having a thinner inner leaflet (32, 43). The lumenal leaflet is about twice the thickness of the cytoplasmic one (40). A periodic structure has been observed in the plaque region of the membrane. According to Warren and Hicks (47), Hicks et al. (17), and Robertson (34) the apparent hexagonal lattice in the thick lumenal membrane of the rat urinary bladder contains particles consisting of 12 smaller subunits.

In contrast to plasma membranes generally, the lumenal plasma membrane of the mammalian urinary bladder appears to be relatively impermeable to water. The experiments of Hicks and co-workers (14, 17) demonstrated that the transitional epithelium acts as a barrier to the flow of water between the isotonic extracellular fluid of the body and the hypertonic urine. These authors con-

cluded that this barrier is a passive function of the bladder and is dependent on the integrity of the limiting surface membrane adjacent to the urine. Significantly, the loss of the characteristic surface membrane structure in cancerous transitional epithelium seems to parallel the increased permeability of the tissue (17). That the plaque regions of the lumenal membrane constitute the permeability barrier has been questioned by several investigators (32, 40, 21, 17) who emphasize the fact that even though the greater part of the total lumenal surface is made up of plaque regions, a smaller but significant portion of the surface area consists of interplaque regions.

The unusual morphological and biochemical properties observed for the limiting surface membrane of the urinary bladder have served as a stimulus for the isolation and better characterization of the lumenal membranes. In this paper the method used for the isolation was a modification of the thioglycolate procedure originally outlined by Chlapowski et al. (7), based on the chemical interaction of the epithelium with sodium thioglycolate. Exposure of the bladder membrane to thioglycolate solutions was shown to result in the destruction of the water-impermeability function (14) and to cause the dissolution of the fine cytoplasmic filaments normally attached to the plasma membrane (7). Refinement and increased standardization of this procedure employing thioglycolate have enabled us to isolate lumenal plasma membranes from sheep bladders in high quantity and purity to permit biochemical analyses. Furthermore, we have separated the plaque regions from the isolated lumenal plasma membranes and have attempted to partially characterize these specialized regions (plaques) of the lumenal membranes.

MATERIALS AND METHODS

Isolation of Lumenal Plasma Membrane

Fresh supplies of sheep urinary bladder were obtained from local slaughterhouses. The bladders were kept cool in an ice bucket during the transfer to the laboratory where they were processed immediately. After extraneous tissue was trimmed away, the bladders were inverted to expose the lumenal surface and were suspended from hemostats into a vigorously swirling solution of 0.02 M sodium thioglycolate, pH 7.4, for 10 min at room temperature. The lumenal surface of each bladder was then scraped with a scalpel into additional thioglycolate solution, and the combined solutions were centrifuged at 1,750 *g* for 10 min in the Sorvall centrifuge (Ivan Sorvall, Inc., Norwalk, Conn.), GSA rotor. The low-speed pellet, after resuspension and homogenization (in an Eberbach Con-Torque homogenizer, Eberbach Corp., Ann Arbor, Mich., using about 20 passes at a medium speed) in 0.02 M thioglycolate, was recentrifuged. The resultant pellet was suspended in 0.01 M sodium bicarbonate, pH 7.4, and layered over a discontinuous sucrose density gradient (10 ml of 0.75 M, 15 ml of 1.1 M, and 5 ml of 1.6 M sucrose) and centrifuged in the Beckman L2-65B ultracentrifuge (Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.), SW27 rotor, for 2 h at 25,000 rpm. The band at the 1.1 M interface was collected, diluted with distilled water, and concentrated by centrifugation. The material constituting the 1.1 M interface fraction was shown by electron microscope to be enriched in lumenal plasma membrane.

Isolation of Plaques

A dilute suspension of lumenal plasma membranes having a protein concentration of less than 5 mg per ml was mixed with an equal volume of 2% deoxycholate (DOC) in 0.01 M sodium bicarbonate pH 7.4 and allowed to sit at room temperature for 15 min with intermittent stirring. After centrifugation in the Sorvall SS-34 rotor at 12,000 rpm for 10 min, the resultant DOC pellet was suspended in the bicarbonate buffer.

Polyacrylamide Gel Electrophoresis

Electrophoresis on 5% polyacrylamide gels was run in 0.1 M sodium phosphate buffer, pH 7.2, containing 0.1% sodium lauryl sulfate (SDS) according to the directions of Berg (2) as adapted from Viñuela et al. (45). Protein samples were dissolved completely in 0.01 M sodium phosphate buffer, pH 7.2, containing 0.1% SDS, 0.14 M β-mercaptoethanol, and 10% (vol/vol) glycerol, and then heated at 65°C for 10 min before application to gels. The gels were run at 8 mA/tube, stained overnight in 0.25% (wt/vol) Coomassie Brilliant Blue in methanol-acetic acid-water (5:1:5, by volume), and destained electrophoretically in methanol-acetic acid-water (2:3:35, by volume). For visualization of glycoproteins and glycolipids the gels were stained by the periodic acid-Schiff (PAS) technique of Zacharius et al. (50). They were scanned for Coomassie Blue at 550 nm and for the PAS stain at 560 nm on a Beckman DU spectrophotometer with a model 2000 linear transport (Beckman Instruments, Inc., Fullerton, Calif.).

Analytical Methods

Protein was determined by the method of Lowry et al. (26). Lipids were extracted with 20 vol of chloroform-methanol (2:1, vol/vol) according to the description of Rouser and Fleischer (35), and total pospholipid phosphorus was estimated by the method of Chen et al. (6) as modified by Rouser and Fleischer (35). Cholesterol de-

terminations were performed as described by Leffler (24). Total sialic acid was assayed by the Warren thiobarbituric acid procedure (46) as modified by Bretscher (3) after hydrolysis of membrane samples for 1 h at 80°C in 0.1 M H_2SO_4. Sialic acid values were corrected for possible deoxyribose interference. The ribonucleic acid (RNA) content was determined by the orcinol reaction outlined by Mejbaum (28) after extraction and hydrolysis of the nucleic acids according to the procedure of Johnsen et al. (19). Amino acid analyses were performed on a Beckman 120 automatic amino acid analyzer (Beckman Instruments, Inc.). Cysteine and methionine were analyzed as cysteic acid and methionine sulfone, respectively, after performic acid oxidation of the protein as outlined by Eipper (8).

Enzyme Assays

All enzyme assays were conducted at 37°C unless otherwise specified. 5′-Nucleotidase activity was assayed according to Touster et al. (42) except that the reaction was stopped at the end of a 15-min incubation by the addition of dilute silicotungstic acid, and the mixture was analyzed for the release of inorganic phosphate by the method of Martin and Doty (27) as described by Lindberg and Ernster (25). Phosphodiesterase I and *N*-acetyl-β-D-glucosaminidase activities were measured as outlined by Touster et al. (42); a 5-min preincubation time and a 15-min incubation time were used; the reactions were initiated by the addition of *p*-nitrophenyl 5′-thymidylate and *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide, respectively. Adenosine diphosphatase (ADPase) activity was estimated as described by Perdue (30) with a 15–30-min incubation time, and the release of inorganic phosphate was analyzed as outlined by Lindberg and Ernster (25). Mg^{++}, Na^+, K^+-dependent adenosine triphosphatase (ATPase) activity was determined by the method of Post and Sen (33), and the inorganic phosphate released was analyzed by the Martin and Doty procedure (27) as described by Lindberg and Ernster (25). Nicotinamide adenosine dinucleotide phosphate (NADPH)-cytochrome *c* reductase and succinate-cytochrome *c* reductase were assayed at room temperature according to the spectrophotometric methods of Sottocasa et al. (39).

Electron Microscopy

All tissues and pellets destined for sectioning were fixed in Karnovsky's fixative (12), postfixed with osmium tetroxide (OsO_4), stained "en bloc" with uranyl acetate (20), embedded in Epon, sectioned with a diamond knife on an MT-1 Porter-Blum microtome, and observed with a Philips 300 electron microscope at 60 kV.

Materials

Thioglycolic acid, acrylamide (Eastman X5521), *N,N′*-methylene bisacrylamide (Eastman 8383) and ammonium persulfate (A-682) were purchased from Fisher Scientific Co., Pittsburgh, Pa. Coomassie Brilliant Blue R (B-0630) was bought from Sigma Chemical Co., St. Louis, Mo. Purified SDS was obtained from Gallard-Schlesinger Chemical Mfg. Corp., Carle Place, N. Y. Epon 812 epoxy resin was bought from Ladd Research Industries, Inc., Burlington, Vt. All other chemicals were of reagent grade and were purchased from commercial sources.

RESULTS

Isolation of Plasma Membrane Using Thioglycolate

It was shown by Chlapowski et al. (7) that when the lumenal surface of the urinary bladder epithelium is exposed to hypotonic thioglycolate, the surface membrane continuum ruptures in a nonuniform fashion, and, as a result, large pieces of plasma membrane become detached and, being freed of cytoplasmic attachments, appear to escape into the lumen. These authors suggested that thioglycolate acts primarily to destroy the system of fine cytoplasmic filaments which form associations with the cytoplasmic surface of the plasma membrane. Further, it was stated by these authors (7) that the thioglycolate treatment does not uniformly affect all cells and should be considered a rather mild procedure.

Advantage was taken of the effect of thioglycolate to isolate the lumenal plasma membrane. The procedure as described by Chlapowski et al. (7) was modified and expanded to include physical scraping of the lumenal surface of the inverted bladder. First, the lumenal surface was exposed to 20 mM thioglycolate under conditions of vigorous mechanical stirring at room temperature for 10 min, so that segments of the plasma membrane could become detached and removed into the thioglycolate solution. Since not all of the loosened membrane would float freely away from the bladder, a more substantial and consistent detachment of the lumenal plasma membrane could be guaranteed by physical scraping after exposure to the thioglycolate.

Table I documents the effect of scraping on the yield of lumenal plasma membrane in the 1.1 M interface fraction. Exposure to thioglycolate as a lone treatment netted a yield of approximately 5 mg of protein after 200 bladders were processed. However, when the thioglycolate treatment was followed by scraping, the yield was increased to approximately 29 mg of protein, in spite of a 50% reduction in the number of bladders processed.

TABLE I

Effect of Scraping on Yield of Membrane Fractions

No. of bladders	Treatment	Yield (mg protein)	
		1.1 M Interface	DOC Pellet
200	Thioglycolate	4.9	0.63
100	Thioglycolate and scraping	29.3	3.25

Inverted sheep bladders were exposed to 20 mM thioglycolate under standard experimental conditions of vigorous mechanical stirring at room temperature for 10 min. Where stated, thioglycolate-treated bladders were scraped with a scalpel along the lumenal surface to assist in detachment of loosened surface membrane fragments. The lumenal plasma membrane was isolated in the 1.1 M interface fraction, and the plaque regions were isolated in the DOC pellet.

The effect of increasing thioglycolate concentration on the yield of membrane protein is presented in Fig. 1. When the concentration of thioglycolate in the suspending medium was increased from 10 to 20 mM, there appeared a corresponding increase in the amount of material removed from the bladder lumenal surface and recovered in the low-speed pellet. The amount of protein recovered in the 1.1 M interface fraction (lumenal plasma membrane fraction) also followed a corresponding increase in yield. Further increase in the thioglycolate concentration to 50 mM did not augment the yield of membrane protein; rather, the recoveries with 50 mM thioglycolate solution paralleled those obtained with 20 mM thioglycolate. At each of the thioglycolate concentrations the yield of 1.1 M interface protein remained as a constant percentage (approximately 3.6%) of the low-speed pellet protein value.

A comparison of different thioglycolate treatments is presented in Fig. 2. Exposure to 20 mM thioglycolate followed by scraping of the lumenal bladder surface proved to be the most productive treatment for obtaining the best yield of isolated plasma membrane. When the lumenal surface of the bladder was exposed to 20 mM thioglycolate for an increased 30-min time period, rather than 10 min (but without scraping), an increase in the yield of lumenal plasma membrane was also obtained. Most interesting, however, was the observation that the yield of plasma membrane (in the 1.1 M interface fraction) as a percentage of the protein removed from the lumenal surface (low-speed pellet) was greatly enhanced. Under standard conditions of thioglycolate treatment at room temperature for 10 min with or without ensuing scraping, approximately 3.5 to 5.5% of the low-speed pellet was recovered in the 1.1 M interface fraction. When the bladder was exposed to 20 mM thioglycolate for 30 min (without ensuing scraping), approximately 14% of the low-speed pellet was recovered in the 1.1 M interface fraction. It would appear that exposure to thioglycolate in itself tends to selectively remove the lumenal surface membrane.

The treatment of increased exposure (30 min or more) to thioglycolate (without scraping) may prove to be an alternate method for isolation of the urothelial lumenal membranes. Our current studies were conducted on plasma membranes which had been exposed to thioglycolate for a shorter time period (10 min) (followed by scraping) to minimize any possible effect of treatment upon the inherent plasma membrane structure. We are, therefore, presently attempting to isolate the lumenal plasma membrane in the absence of thioglycolate so that any effect of thioglycolate can be evaluated in comparative studies. Also, comparative studies will be carried out with membranes isolated after thioglycolate treatment for

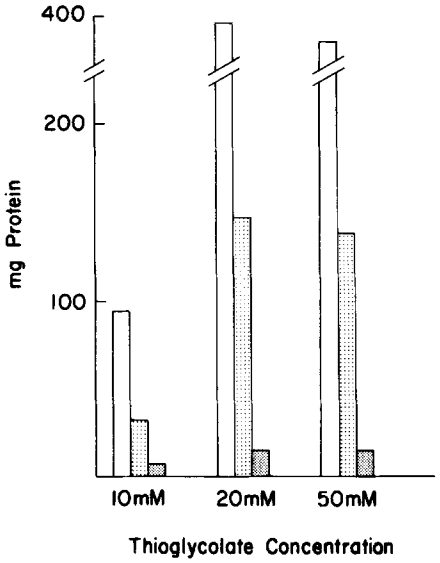

FIGURE 1 Effect of increasing thioglycolate concentration on the yield of membrane protein. 50 inverted sheep bladders were suspended over a thioglycolate solution of given concentration at room temperature for 10 min. After the thioglycolate treatment, the lumenal surface of each bladder was scraped with a scalpel, and the scrapings were added to the thioglycolate solution. Standard conditions were followed to isolate plasma membrane fractions: □ crude homogenate fraction (low-speed pellet), ▨ the lumenal plasma membrane (1.1 M interface) fraction × 10 and ▩ the plaque (DOC pellet) fraction × 10.

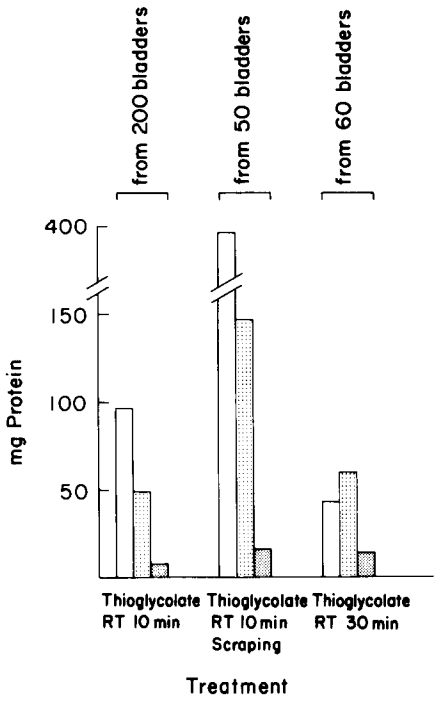

FIGURE 2 Effect of different thioglycolate treatments on yield of membrane protein. Inverted sheep bladders were suspended from hemostats into a swirling solution of 20 mM thioglycolate pH 7.4. This thioglycolate treatment was conducted under three sets of conditions: (*a*) 10 min at room temperature, (*b*) 10 min at room temperature followed by scraping of the bladder lumenal surface, or (*c*) 30 min at room temperature. □ low-speed pellet, ⊡ 1.1 M interface × 10, ⊠ DOC pellet × 10.

30 min (without scraping) to assess not only the effect of reagent upon membrane structure but also the degree of contamination within each type of isolated membrane preparation.

Morphology of the Lumenal Plasma Membrane of Sheep Bladder

The morphology of the urothelium of sheep closely resembles that previously described for other mammals. The free surface is shown in Fig. 3*a*, which illustrates the scalloped profile of the lumenal membrane lining the bladder. Concave plaque regions alternate with crest-shaped interplaque regions. Within plaques, the membrane, approximately 120 Å thick, has a thicker outer leaflet (Fig. 3*b*), while in the interplaque regions the membrane measures approximately 80 Å in diameter and has leaflets of equal thickness. In favorably oriented sections, the outer lumenal leaflet of the plaques displays a periodicity due to the presence of the hexagonal array of particles with a 160-A center-to-center spacing (Fig. 3*c*).

Morphology of Isolated Plasma Membrane

Electron micrographs show the 1.1 M interface fraction to be enriched in lumenal plasma membrane, an example of which is shown in Fig. 4*a*. A scalloped profile can be seen. Plaque (P) and interplaque (I) regions can be discerned. The membrane preparation appears clean and devoid of attached cytoplasmic filaments, which *in situ* can be seen underlying the untreated plasma membrane (40). The unit membrane structure and the dimensions of the isolated plasma membrane resemble those of untreated membrane. In tangential section a striated pattern can often be discerned, indicating the presence of a regular array of particles also observed in untreated membranes. At higher magnification of the 1.1 M interface fraction (Fig. 4*b*), the asymmetrical unit membrane of the plaque region can be visualized more clearly. The outer leaflet (single arrow) is thicker than the inner one, and the inner leaflet (double arrows) is free of attached filaments.

Isolation of Plaque Regions from the Plasma Membrane Fraction

Plaque regions are those regions of the plasma membrane which have an enhanced membrane thickness distinguished by an asymmetry in the constituent membrane leaflets. They were isolated from the plasma membrane by detergent treatment. The 1.1 M interface fraction was subjected to an equal volume of 2% DOC at room temperature for1 h and then centrifuged at 20,000 *g* for 15 min. The resultant "DOC pellet" was found to be enriched in plaque regions. It would appear that upon exposure to DOC many lumenal membranes are disrupted along interplaque areas. Triton-X-100 could replace DOC as effectively. Treatment with detergent for increased lengths of time was found not to be necessary, as the same yield of DOC pellet was obtained after 15 min (10.5% of the 1.1 M interface protein) as after 60 min (11.1% of the interface protein).

The use of sonication to obviate detergent treatment in the isolation of plaque regions has not proved to be successful. Fragmentation of the lumenal plasma membrane by controlled enzymatic

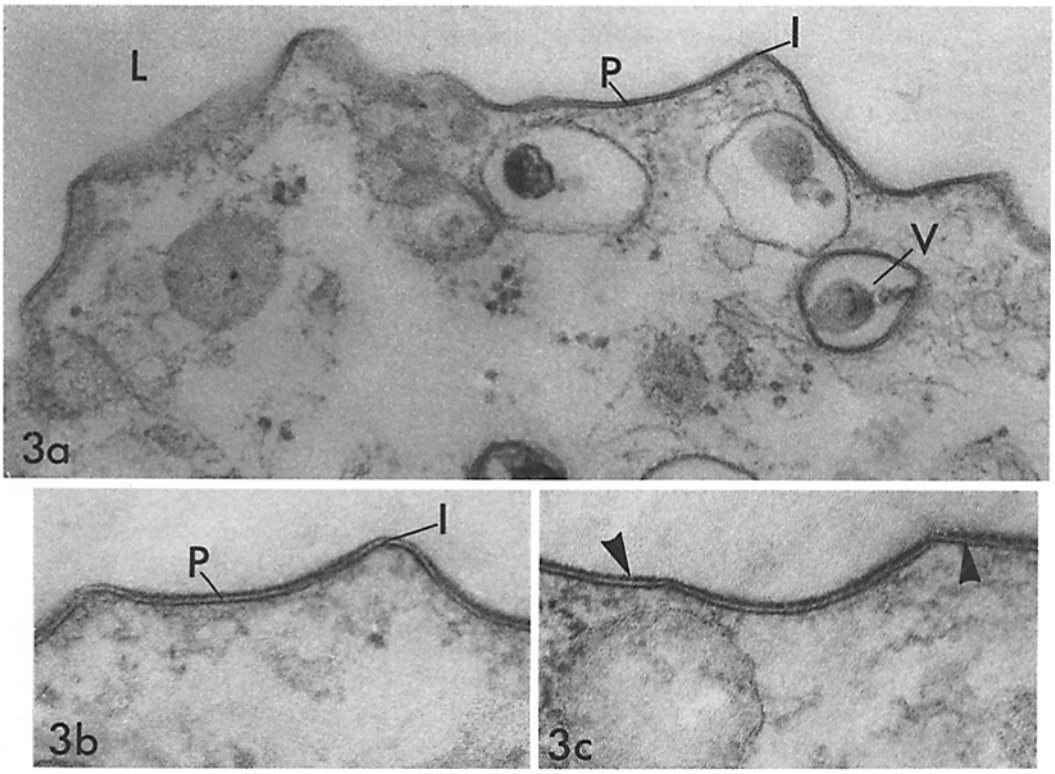

FIGURE 3 Normal sheep bladder. (*a*) Plasma membrane of epithelial cell lining the lumen (*L*) displays concave plaque regions (*P*), ~120 A thick, alternating with interplaque regions (*I*) forming crests, ~80 A thick. Spheroidal cytoplasmic vesicles (*V*) with similar membrane structure are also present. × 69,500. (*b*) The thicker plaque (*P*) and alternating thinner interplaque regions (*I*) are seen more clearly. × 142,000. (*c*) In favorable sections, the lumenal leaflet of the plasma membrane (arrowheads) displays a particulate substructure due most probably to the presence of arrays of hexagonal particles (40) in plaque areas. × 142,000.

digestion, such as that obtained with phospholipase, trypsin, etc., is being planned to obtain a preparation of membrane plaque regions that has not been exposed to detergent action.

When examined in thin section (Fig. 5), the DOC pellet comprised many small pieces of membrane which appeared to be predominantly plaque regions. Small curved pieces, seemingly individual plaques, are often seen. In addition, angles of vesicles with sharp bends in their membranes at approximately 90° often occur, as if rejoining of plaque areas has taken place after removal of most of the interplaque regions. The profile of the unit membrane of the plaque regions retains its characteristic thickness (Fig. 6). The asymmetry of the unit membrane structure observed *in situ* is preserved in this DOC preparation enriched in plaque regions.

Characterization of the Isolated Membrane Proteins

Fig. 7*a* demonstrates the polyacrylamide gel electrophoretic pattern of the isolated plasma membrane. The 1.1 M interface fraction exhibited a number of bands after SDS-5% polyacrylamide gel electrophoresis and subsequent staining with Coomassie Blue. The R_f of each band was measured relative to the migration of the tracking dye, bromophenol blue. The approximate molecular weight assigned to a band was obtained from a standard curve relating proteins of known molecular weight and their corresponding mobilities. No attempt was made to correct the apparent molecular weight for nonprotein components. Thus, the true molecular weights of certain protein species, for example, those containing carbohydrate moie-

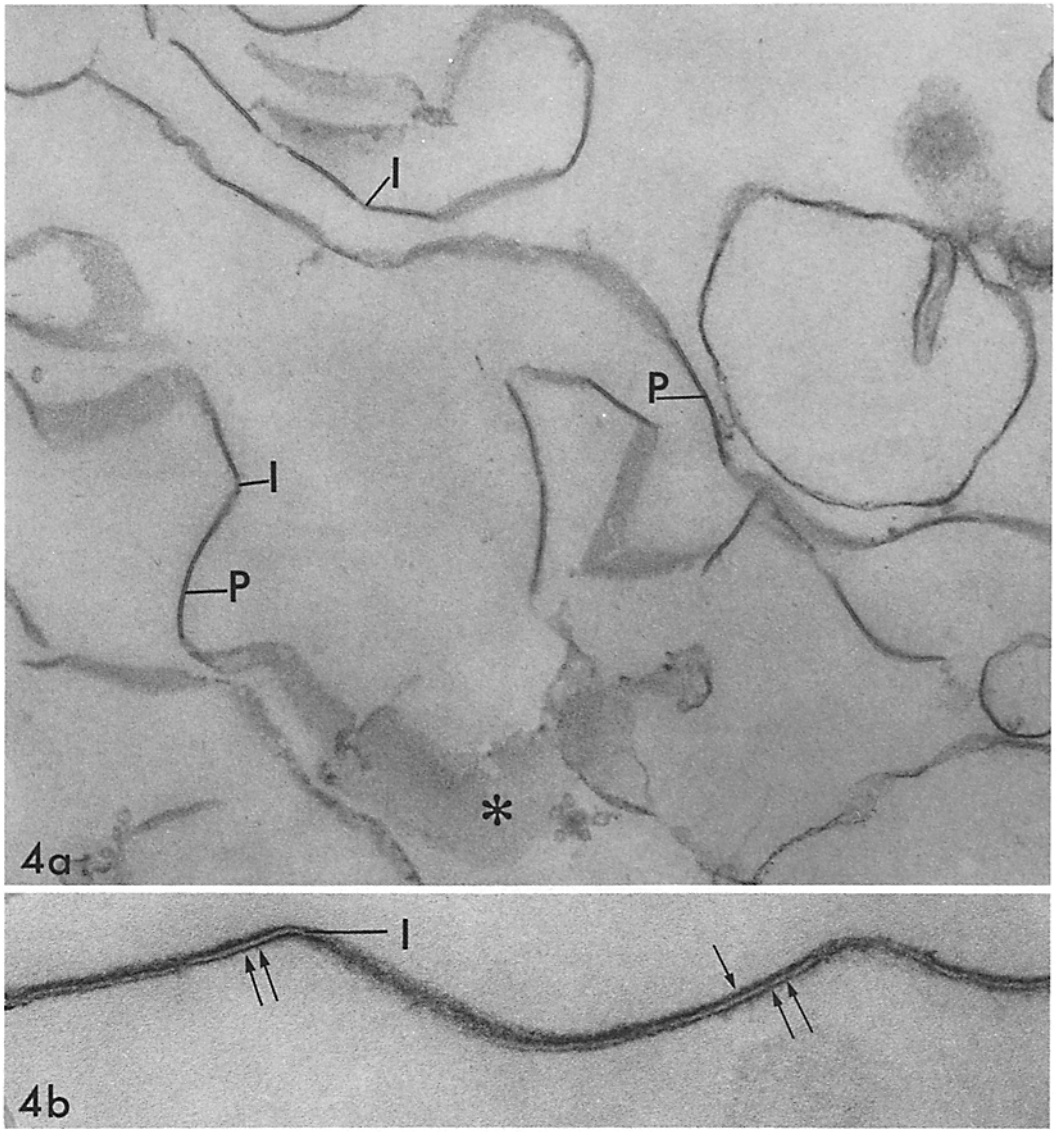

FIGURE 4 (*a*) Lumenal plasma membrane from sheep bladder, isolated by the thioglycolate method. Electron micrograph shows a typical field of pellet collected from 1.1 M interface. Exposure to thioglycolate has resulted in removal of cytoplasmic filaments, and the membranes look "clean." Plaque (*P*) regions can be identified easily, and interplaque (*I*) regions can also be seen. A striated pattern (asterisk) in tangential sections reveals the presence of arrays of particles in the plaque regions. × 37,600. (*b*) A similar preparation at higher magnification. The thicker outer leaflet (arrow) of the plaque regions may be distinguished clearly. The thinner inner leaflet (double arrows) is devoid of attached fine filaments. Interplaque (*I*) regions are also visible. × 237,400.

ties, are in all probability lower than the apparent molecular weight values assigned to them throughout this investigation. Thus, for the 1.1 M interface fraction the major band having an R_f of 0.57 represented a protein with the approximate mol wt of 62,000. Also prominent was a major protein band located at 33,000 daltons. In a number of membrane preparations the relative amount of the 33,000-mol wt species approximately equaled the amount of 62,000-mol wt species. Other bands

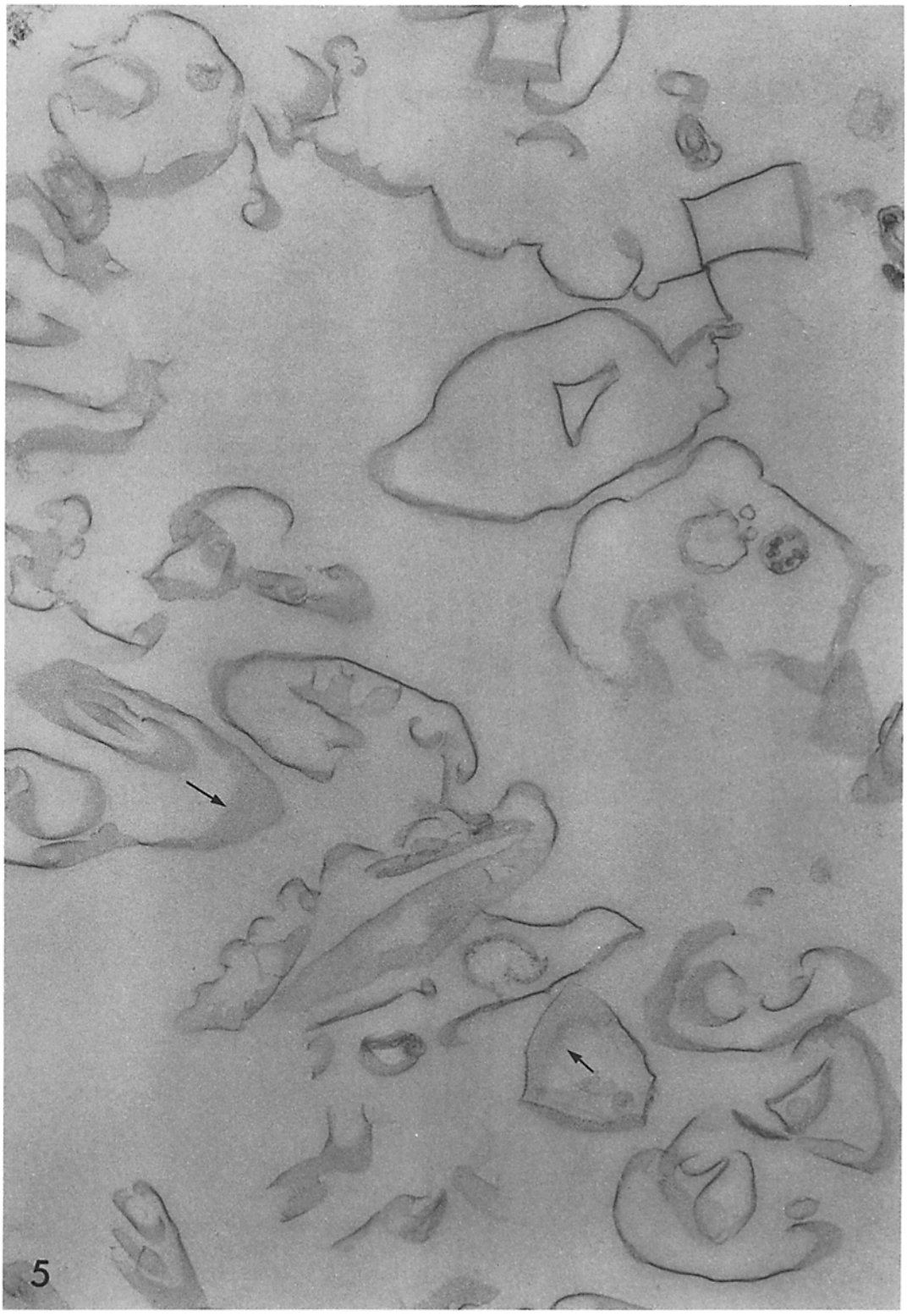

FIGURE 5 Section through pellet of lumenal membranes collected from 1.1 M interface and treated for 15 min in 1% DOC (DOC pellet). Electron micrograph shows typical membrane pieces derived chiefly from plaque regions. Most of the interplaque areas of the membrane are missing. Pieces are often short, tend to curl on end into C-shaped figures, and ~90° angles in the membranes are common. In tangential sections the presence of arrays of particles is revealed (arrows). Note again the absence of attached short filaments or associated long cytoplasmic filaments. × 36,500.

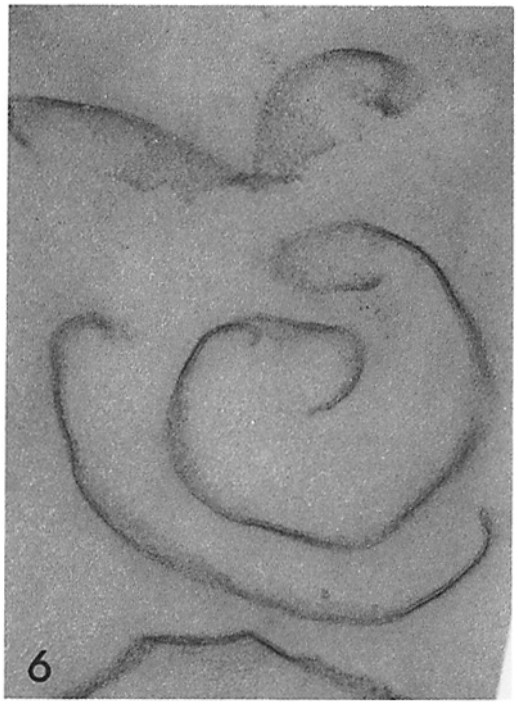

FIGURE 6 Section through isolated DOC pellet (plaque regions). Higher magnification of C-shaped membrane pieces. As in intact cells the asymmetrical unit membrane consists of a relatively thick outer lamella and a thinner inner lamella. × 106,500.

were located at approximately 190,000, 112,000, 88,000, 73,000, 51,000, 40,000, and 15,000 daltons. When a duplicate gel was stained with PAS (Fig. 8), positive reactions were obtained for the 190,000-, 62,000-, 33,000-, and 15,000-mol wt proteins, but the interactions were so weak that bands could be detected only about 50% of the time and with the aid of a light box. However, with the PAS reagent, there appeared an intensely stained band having an R_f of approximately 1.22 and representing, presumably, reaction with glycolipid.

As shown in Fig. 7*b* the DOC pellet (which predominantly comprises the plaque regions of the plasma membrane) exhibited a more resolved SDS-polyacrylamide gel pattern. The plaque region appeared to be characterized by one dominant protein species having an approximate mol wt of 33,000. Bands corresponding to approximately 62,000, 50,000, 41,000, and 15,000 daltons were also present as minor components. After PAS staining, weak positive reactions were obtained with the 62,000-, 33,000-, and 15,000-dalton species, and a very intense stain appeared near the anode (R_f = 1.22), again representing, presumably, interaction between PAS and glycolipid.

Lipid Composition of the Plasma Membrane Fractions

Table II summarizes the lipid composition of the isolated plasma membrane fractions. A lipid-to-protein ratio greater than one was obtained from the plasma membrane 1.1 M interface fraction. There were approximately 0.93 mg of phospholipid and 0.27 mg of cholesterol for each milligram of protein, giving a lipid-to-protein ratio of 1.26. This gives a value of 55% lipids and 45% proteins for the composition of lumenal plasma membrane. The high ratio of lipid-to-protein is in agreement with the low density exhibited by this plasma membrane on the sucrose density gradient.

The molar ratio of cholesterol to phospholipids in the plasma membrane fraction was found to be 0.58; this ratio appears to be in general agreement with published values for other kinds of plasma membranes (22). For the DOC pellet (or fraction enriched in membrane plaques), the cholesterol values were sustained; however, the phospholipid content appeared to be greatly decreased. This decreased value could reflect an exchange between DOC and membrane phospholipid.

Sialic Acid Content of the Isolated Membrane Proteins

The lumenal plasma membrane, isolated as in the 1.1 M interface fraction, was found to have a total sialic acid content of approximately 0.0375 ± 0.009 (n = 8) μmol/mg protein. The plaque region of the plasma membrane showed an elevated level of total sialic acid, having 0.0756 ± 0.031 (n = 9) μmol/mg protein. These values appear to be in general agreement with the value (73.5 ± 1.8 nmol/mg protein) reported by Ibañez et al. (18) for the plasma membrane from sheep urinary bladder.

Measure of Contamination in Isolated Membrane Fractions

Analysis of the isolated plasma membrane fraction has indicated a relatively low RNA content. As shown in Table III, a value of approximately 5.6 μg ribose/mg protein was obtained for the amount of RNA present in the isolated membrane preparation.

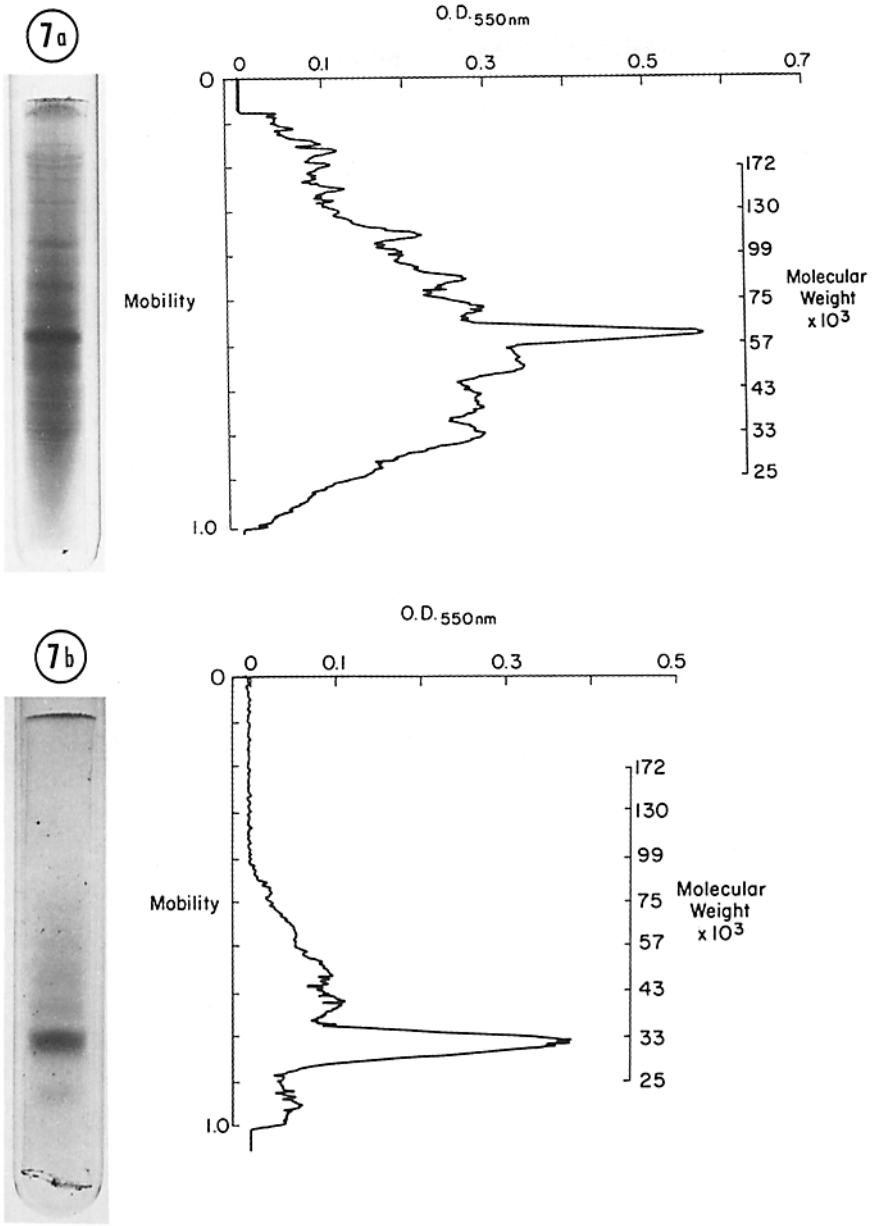

FIGURE 7 (*a*) SDS-polyacrylamide gel electrophoretic pattern and scan of the isolated lumenal plasma membrane (1.1 M interface fraction). (*b*) SDS-polyacrylamide gel electrophoretic pattern and scan of the membrane plaques (DOC pellet fraction).

The extent of contamination by endoplasmic reticulum was estimated by measuring a marker enzyme, NADPH-cytochrome *c* reductase. In general, the amount of this enzymatic activity measured per milligram of protein was extremely low. As shown in Table III, there was no increase in specific activity for NADPH-cytochrome *c* reductase in the plasma membrane (1.1 M interface) fraction above that in the crude homogenate (first low-speed pellet fraction). However, the first supernatant fraction did show an increase in activity, reflecting a separation of microsomes from low-speed membranes into the supernate.

Mitochondrial contamination was assessed by

measuring succinate-cytochrome *c* reductase activity in the isolated membrane fractions. The specific activity for the 1.1 M interface (plasma membrane) fraction was found to be approximately twofold higher than for the first low-speed pellet fraction, but again the overall enzymatic activity was very low. This low rate of mitochondrial activity would be in keeping with the histological observation (15) that very few mitochondria are present in the superficial cytoplasm of the urothelial cells. The first supernatant fraction, as in the case for NADPH-cytochrome *c* reductase, showed an increased specific activity.

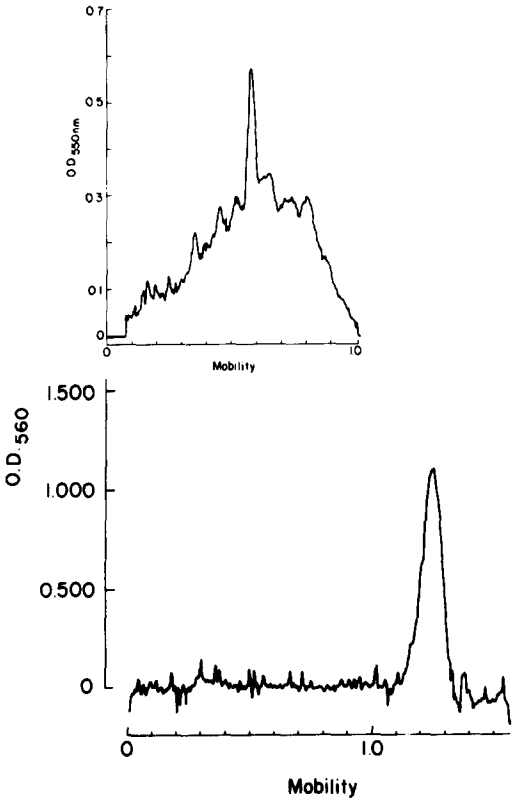

FIGURE 8 SDS-gel electrophoretic scan of plasma membrane 1.1 M interface fraction. Upper scan stained with Coomassie Blue and lower scan stained with PAS.

Measurement of *N*-acetyl-β-D-glucosaminidase activity in the isolated membrane fractions indicated no increase in specific activity of this lysosomal activity in the plasma membrane fraction when compared with the homogenate. A decrease in specific activity was observed for the DOC pellet fraction for which a specific activity of 1.7 nmol/mg per min was measured.

Enzymatic Activities Associated with Plasma Membrane Fractions

The enzymatic profile of the isolated membrane fractions is shown in Table IV. Both 5′-nucleotidase and phosphodiesterase I activities were enhanced in the isolated plasma membrane fraction by a factor of 23 for 5′-nucleotidase and 13-fold for phosphodiesterase I activity when compared to the activity in the first low-speed pellet fraction. Although the specific activity for 5′-nucleotidase is further increased in the DOC pellet, there is no corresponding increase in specific activity for phosphodiesterase I in the DOC pellet. The increase in specific activity of 5′-nucleotidase in the DOC pellet fraction was not caused simply by a detergent activation of the enzymatic activity, for,

TABLE II

Lipid Composition of Isolated Plasma Membrane Fractions

	1.1 M Interface	DOC Pellet
Phospholipid phosphorus		
μg/mg protein	19.666 ± 3.86 (*n* = 12)	5.101 ± 2.96 (*n* = 8)
Phospholipid		
mg/mg protein	0.932 ± 0.18 (*n* = 12)	0.242 ± 0.14 (*n* = 8)
μmol/mg protein	1.203 ± 0.23 (*n* = 12)	0.312 ± 0.18 (*n* = 8)
Total cholesterol		
mg/mg protein	0.270 ± 0.06 (*n* = 11)	0.337 ± 0.09 (*n* = 6)
μmol/mg protein	0.701 ± 0.14 (*n* = 11)	0.873 ± 0.23 (*n* = 6)
Cholesterol/phospholipid		
mol/mol	0.583	2.798
Lipid (cholesterol + phospholipid)		
(by weight), %	54.6	36.7

The value 21.1 was used as micrograms lipid phosphorus per milligram phospholipid after Vergara et al. (44). The value 775 was used as an average molecular weight for phospholipids and 386 for cholesterol.

TABLE III

RNA Content and Marker Enzyme Activities in Isolated Membrane Fractions

	Isolated membrane fractions		
	First low-speed pellet	1.1 M Interface	First Supernate
RNA			
μg ribose/mg protein	–	5.60 ± 2.93 (n = 10)	–
NADPH-cytochrome *c* reductase			
nmol/mg/min	0.26 (n = 6)	0.26 (n = 5)	1.01 (n = 6)
Succinate-cytochrome reductase			
nmol/mg/min	0.63 (n = 6)	1.42 (n = 7)	3.85 (n = 3)
N-acetyl-β-D-glycosaminidase			
nmol/mg/min	5.68 (n = 7)	5.54 (n = 8)	

Fractions from four different isolation preparations were tested for enzymatic activities. In general, six or seven measurements were averaged for each determination, using between 0.1 and 2.0 mg of protein per assay.

TABLE IV

Enzymatic Activities in Isolated Membrane Fractions

	First low-speed pellet	1.1 M Interface	DOC Pellet	Enrichment (1.1 M interface/ low-speed pellet)
		μmol/mg/min		
5'-Nucleotidase	0.0098 ± 0.0051 (n = 6)	0.2297 ± 0.0836 (n = 13)	0.4890 ± 0.1692 (n = 9)	23.4
Phosphodiesterase I	0.0051 ± 0.0016 (n = 6)	0.0657 ± 0.0104 (n = 8)	0.0260 ± 0.0089 (n = 5)	12.9
Mg^{++},Na^+,K^+-ATPase	0.0237 ± 0.0111 (n = 5)	0.1248 ± 0.0242 (n = 6)	0.0380 (n = 2)	5.3
ADPase	0.0400 ± 0.0150 (n = 9)	0.0596 ± 0.0214 (n = 9)	0.0499 ± 0.0316 (n = 7)	1.5

TABLE V

Effect of DOC on 5'-Nucleotidase and Phosphodiesterase I Activities

DOC Added mg/mg protein	5'-Nucleotidase	Phosphodiesterase I
	μmol/mg/min	
0	0.328	0.220
0.1	0.303	
0.2	0.224	0.225
0.5	0.188	
1.0		0.234
4.0	0.207	
10.0	0.199	0.221

Enzyme activities were tested with the 1.1 M interface fraction.

as shown in Table V, increasing the concentrations of DOC from 0.1 to 10.0 mg DOC/mg protein had no stimulatory effect but rather a 40% maximal inhibitory effect on the 5'-nucleotidase activity of the 1.1 M interface fraction. Furthermore, no effect of DOC could be shown on the phosphodiesterase I activity. It would appear, therefore, that the 5'-nucleotidase activity is being concentrated in the membrane subfraction corresponding to the plaque regions.

ADPase activity was measured in the isolated membrane fractions, but, as shown in Table IV, very little or no enrichment in specific activity was observed in the plasma membrane fraction when compared to the first low-speed pellet fraction. A low rate of Mg^{++}, Na^+, K^+-dependent ATPase activity was found consistently in our membrane preparations. This activity was about five times greater in the plasma membrane (1.1 M interface) fraction than in the first low-speed pellet fraction. Between 15 and 30% of the measured Mg^{++}, Na^+, K^+-stimulated ATPase activity was inhibited by ouabain.

The decrease in specific activity for ATPase in the DOC pellet probably finds explanation in the inhibition study presented in Fig. 9. Increasing concentrations of DOC were added to the assay tubes for the determination of the inhibition profile for ATPase activity. At a DOC concentration of 4.0 mg/mg protein, there was total inhibition of ATPase activity. Since this detergent is used during the isolation procedure to fragment the plasma membrane and to isolate the plaque regions at a level of at least 4 mg DOC/mg protein, it is possible that the specific activity measured for the plaque region fraction is underestimated.

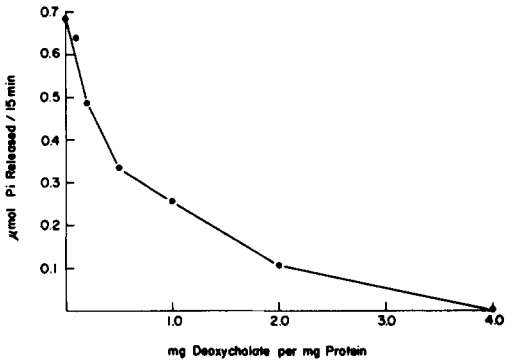

FIGURE 9 Effect of DOC on the Mg^{++}, Na^{+}, K^{+}-dependent ATPase activity of the low-speed pellet fraction isolated in 0.25 M sucrose 0.01 M Tris-HCl, pH 7.4.

The possibility that the thioglycolate which is used in the isolation procedure might inhibit ATPase activity was also tested. As shown in Table VI, there was no significant effect of thioglycolate on the ATPase activity for two of the membrane fractions, the low-speed pellet and the 1.1 M interface, isolated in the absence of thioglycolate.

Amino Acid Composition of the Plaque Regions

The amino acid composition of the DOC pellet fraction is presented in Table VII. Two different preparations of the DOC pellet were hydrolyzed in HCl and analyzed for amino acids, and an additional preparation was oxidized with performic acid before acid hydrolysis. SDS-gel electrophoresis showed the DOC pellet fraction to have one protein species in much greater proportion than the other four relatively minor protein subunits (Fig. 7*b*). The polarity index was calculated by summing the mole percentages of Asp, Thr, Ser, Glu, Lys, His, and Arg (4). The plaque proteins appear to be hydrophobic in nature with a polarity of 37.1%.

DISCUSSION

The desire to know the molecular structure and function of the bladder lumenal plasma membrane, and of plasma membranes in general, no doubt served as a simulus for the isolation of the lumenal membranes, and these membranes have been isolated in several laboratories (16, 7, 44, 18, 5). All the methods so far devised have taken advantage of specific chemical reagents that interact with the epithelium. Normally, subjection of the bladder lumenal membrane to water at room temperature for 1 h does little more than distend the extracellular spaces between epithelial cells (14). However, pretreatment of the bladder epithelium with fluorescein mercuric acetate (16) al-

TABLE VI

Effect of Increasing Concentrations of Thioglycolate on Mg^{++}, Na^{+}, K^{+}-ATPase Activity

Fraction	Concentration of thioglycolate	Mg^{++}, Na^{+}, K^{+}-ATPase
	mM	*μmol Pi/15 min*
Low-speed pellet isolated in ST*	0	0.342
	0.1	0.368
	0.5	0.368
	1.0	0.402
	5.0	0.385
	10.0	0.335
	20.0	0.312
	50.0	0.361
1.1 M Interface isolated in KCl‡	0	0.545
	5.0	0.545
	10.0	0.561
	20.0	0.545
	50.0	0.561

* The isolation was carried out under standard experimental conditions as described earlier except that inverted bladders were not pretreated with thioglycolate but were scraped immediately into a 0.25 M solution of sucrose and 0.01 M Tris-HCl pH 7.4 (ST).

‡ The isolation procedure was carried out as described above except that the isolation medium was 0.6 M KCl instead of 0.25 M sucrose, 0.01 M Tris-HCl pH 7.4.

TABLE VII

Amino Acid Composition of DOC Pellet Fraction

Aspartic acid	9.66
Threonine	4.14
Serine	2.41
Proline	5.86
Glutamic acid	8.62
Glycine	10.00
Alanine	10.00
Valine	9.31
Cysteic acid	2.07
Methionine	0.69
Isoleucine	6.21
Leucine	12.76
Phenylalanine	6.21
Lysine	5.86
Histidine	1.72
Arginine	4.83
Polarity index, %	37.1

Results are presented in moles of each amino acid per 10 mol of glycine.

lows modification of the permeability of the membrane to water, permitting hypotonic extraction of the plasma membrane. Also, another procedure for the isolation of the lumenal membrane was developed (7), based on the chemical interaction of the epithelium with sodium thioglycolate. It has been shown (14) that exposure of the bladder membrane to sodium thioglycolate resulted in damage to the permeability barrier to water. Although the exact mechanism of the initial damage to the membranes of the intact epithelium by the hypotonic thioglycolate solutions is not yet clear, it seems probable that, once inside the cells, this reagent, which breaks disulfide bridges, detaches and solubilizes cytoplasmic filaments attached to the membrane (7) and allows the lumenal membranes to float free and be isolated from urinary bladders on a discontinuous sucrose gradient. Modifications of procedures employing thioglycolate now allow isolation of lumenal plasma membranes from pig (44) and sheep in high enough quantity and purity to permit biochemical analysis.

The unique morphology characterizing the lumenal plasma membranes was used as direct identification during isolation procedures. In profile, the "wavy" or scalloped appearance due to alteration of concave plaque regions measuring approximately 120 A in diameter and crested interplaque regions measuring approximately 80 A in diameter precisely identified the lumenal membranes in sectioned materials. In tangential sections a hexagonal pattern can be seen in, specifically, the plaque regions where the lumenal membrane is thickened and asymmetrical, the leaflet adjacent to the lumen being thicker than the cytoplasmic leaflet (40). Interplaque regions, however, appear to be devoid of particles. Thus, morphologically, the DOC pellet could be identified specifically with the plaque regions. It would appear that the action of detergents, DOC or Triton X-100, on the lumenal membranes is foremost at the interplaque regions, presumably disrupting the hydrophobic cohesive forces dominant within the interplaque regions and thereby releasing individual plaque regions.

On the basis of electron microscope observation, the degree of contamination in the lumenal plasma membrane fraction with other cytoplasmic membranes was found to be minimal. A rough estimate of the amount of impurity contained within our plasma membrane fraction was made by using the following marker enzyme activities: succinate-cytochrome *c* reductase activity for mitochondria and NADPH-cytochrome *c* reductase for microsomal and/or mitochondrial outer membrane activities. In both cases, a low level of contamination could be measured. When the specific activity of the plasma membrane fraction was compared directly to the specific activity of the crude homogenate fraction, a ratio of 1.02 was calculated for the NADPH-cytochrome *c* reductase activity, and a ratio of 2.26 was obtained for the mitochondrial enzymatic activity. These ratios closely resemble those obtained by Johnsen et al. (19) during the isolation of plasma membrane from suspensions of HeLa S_3 cells. These authors show a ratio (specific activity in plasma membrane fraction/specific activity in homogenate) of 0.68 for the NADPH-cytochrome *c* reductase and a ratio of 1.51 for the mitochondrial cytochrome *c* oxidase activity.

An RNA content of 5.6 μg/mg protein was measured in our plasma membrane fraction. RNA in isolated plasma membranes has been found to vary from 10 to 72 μg/mg protein (42). Johnsen et al. (19) have suggested that ribosomes present in the isolated plasma membrane fraction are probably trapped within membrane ghosts.

A high lipid-to-protein ratio was obtained for the isolated lumenal plasma membrane from sheep bladders, and the lipid (cholesterol plus phospholipid) content was calculated to be approximately 55% by weight. Lipid-to-protein ratios greater than one have also been reported for lumenal membranes from pig (44), calf (41), and rat (21). In common with bladder lumenal plasma membranes of different species, the sheep lumenal membrane was found to have a relatively high cholesterol content. The molar ratio of cholesterol to phospholipid reported for plasma membranes from various sources has ranged from 0.26 to 0.80 (22, 42). A molar ratio of 0.58 was obtained by us for the sheep lumenal plasma membrane, and this compares favorably with the ratio of 0.6 reported by Ketterer et al. (21) for rat bladder lumenal membrane and 0.47 obtained by Vergara et al. (44) for pig bladder lumenal membrane.

After detergent emulsification of the interplaque regions of the lumenal plasma membrane, the resultant DOC pellet was found to retain a high cholesterol content (approximately 0.87 μmol/mg protein), but the phospholipid content was greatly diminished. The extent of exchange between phospholipid and detergent molecules was not assessed, but such an exchange could conceivably account for the decrease in phospho-

lipid content within the plaque regions. However, it is equally possible that the plaque regions have an altered membrane composition, that is, an elevated protein-to-lipid ratio. This would be in agreement with electron microscope observations of thin section and freeze-etch preparations, both showing particle associations with the plaque, and not interplaque, regions of the lumenal plasma membrane (40).

The sialic acid content of rat liver plasma membrane has been reported as 33 nmol/mg protein (9) and 48 nmol/mg protein (42). Our value of approximately 38 nmol/mg protein would therefore be in the expected range for superficial membranes. When SDS-polyacrylamide gels of the isolated plasma membrane fraction were stained for carbohydrate with PAS, the dominant species observed was an intensely stained, fast running band located near the anode, presumably a glycolipid. Ibañez et al. (18) also found that the fastest moving band in a gel which stained for carbohydrate had no corresponding band in the gel stained for protein. According to their experimental data (18), roughly 23% of the sialic acid of plasma membranes of transitional epithelium is lipid bound. The presence of carbohydrate might be expected from electron microscope observations showing that the plasma membrane of transitional epithelium of extrarenal urinary passages has a defined glycocalyx (29). Further, in unpublished experiments, we have found that upon exposure to ruthenium red, thin sections of transitional epithelium showed intense staining of the entire lumenal surface of the plasma membrane.

In contrast to the conclusions reached by Hicks and co-workers (13, 41), enzymatic activities were found to be associated with the lumenal plasma membrane fraction of sheep bladders. The specific activities for 5′-nucleotidase, phosphodiesterase I, and Mg^{++}, Na^{+}, K^{+}-dependent ATPase were found to be increased considerably in the plasma membrane fraction. Our observation that the 5′-nucleotidase activity was enhanced to a greater degree than ATPase activity would agree, presumably, with the hypothesis put forth by Widnell and Unkeless (49) and by Johnsen et al. (19) that 5′-nucleotidase does not co-purify exactly with the ouabain-sensitive ATPase. In pig bladder lumenal membranes, Vergara et al. (44) found Mg^{++}, Na^{+}, K^{+}-dependent ATPase activity to be enriched approximately five times over the specific activity in the homogenate; this enrichment factor is similar to that observed by us for sheep lumenal membranes. Moreover, Ibañez et al. (18), working with sheep bladder lumenal membranes, found both Mg^{++}-dependent ATPase activity (approximately 0.16 μmol/min per mg protein) and 5′-nucleotidase activity (approximately 0.088 μmol/min per mg protein), and these values are in rough agreement with those obtained by us.

The suggestion that 5′-nucleotidase is not a good marker enzyme for the plasma membrane fraction because it is also measured in fractions of nuclear membranes (23) and endoplasmic reticulum (49) has largely been dispelled by the studies of Song and Bodansky (38) and by Touster et al. (42). Isolated nuclear membranes were found (38) to contain a negligible amount of 5′-nucleotidase activity, whereas isolated plasma membranes showed increase in specific activity for this enzyme. Also, comparison of marker enzyme activities in different isolated cell membrane fractions by Touster et al. (42) strongly suggested that 5′-nucleotidase was probably associated with neither the nuclear membranes nor the endoplasmic reticulum but was present in plasma membranes.

The protein spectrum for the isolated plasma membrane shows the 62,000- and 33,000-mol wt species to be the major proteins. After detergent treatment of the plasma membrane, a fraction enriched in plaque regions can be separated. Gel electrophoresis of the membrane plaques indicates that the 33,000-mol wt species is dominant. Similarly, Vergara et al. (44) found in pig bladder plasma membrane two major bands located around 27,000 daltons and a less dense one located around 55,000. It would appear possible that the 33,000-mol wt species is a dodecameric subunit of the protein cluster making up the hexagonal pattern seen under electron microscope observations (48, 40). Vergara et al. (44) have suggested that the subunit molecular weight could be about 27,000 and that the 55,000 band could then be interpreted as beng a dimer which resisted denaturation.

Amino acid analysis of the plaque regions protein isolated as the DOC pellet demonstrates the nonpolar nature of this apparently intrinsic protein. The high proline content (8.4 mol %) seen by Hicks and co-workers (21, 17) for lumenal membranes was not observed in our preparations of either lumenal plasma membranes (unpublished observations) or plaque regions.

At this point there is morphological similarity between the ATPase isolated from *Streptococcus faecalis* by Schnebli and Abrams (36, 37) and the

particles seen in the urinary bladder. In negatively stained preparations, the isolated ATPase of the streptococcal membrane showed a substructure that suggested a planar, hexagonal array of six globules, each of which is made up of an α- and a β-subunit. The diameter of each globule was about 40 Å and the longest dimension across the hexagon was about 120 Å. The molecular weight of the ATPase was determined to be 385,000, with each of the 12 subunits corresponding to a molecular weight of 33,000. As shown in reconstitution studies by Abrams and Baron (1), the isolated ATPase molecules could be reinserted into specific sites on the membrane, suggesting that the enzyme may fit into a mosaic of hexagonally shaped particles of similar size in the membrane. Particles having the appropriate dimensions and forming hexagonal patterns have been visualized on the surface of bacterial membranes in negatively stained preparations (11).

The similarities in morphological dimensions, subunit structures and molecular weights between the ATPase particles derived from the *S. faecalis* membranes and the particles making up the plaque regions of the urothelial plasma membrane are striking. However, the possibility that the particles in the plaque regions of the urothelial lumenal membrane represent corresponding ATPase molecules seems unconvincing to us. The ATPase from the bacterial membrane is released by low ionic strength, osmotic shock treatments. For isolation of the urothelial plasma membrane, on the other hand, hypoosmotic conditions are not sufficient, and the presence of thioglycolate appears to be a necessary requirement. Further, subfractionation of the lumenal plasma membrane into plaque and interplaque regions is achieved through detergent action, presumably through breakdown of hydrophobic forces holding plaque regions in association with interplaque regions of the lumenal membrane. This is in contrast to the ionic linkages involved in the attachment of ATPase to the plasma membrane of *S. faecalis*. Thus, while the ATPase appears to be a peripheral protein associated with the bacterial membrane, the particles making up the urothelial plaque regions appear to be integral proteins constituting the membrane structure. In support of this notion, amino acid analysis of the bacterial ATPase (37) showed a significant polar content (polarity index = 51.3% when calculated according to Fisher (10) or according to Capaldi and Vanderkooi (4) but with the inclusion of tyrosine in the polar class) in contrast to the dominantly nonpolar character of the urothelial plaque region particles.

The relative impermeability of the lumenal plasma membrane of the bladder to water and ions (14) must depend on its chemical composition. Approximately 75% of the plasma membrane is covered by plaque regions in which hexagonally arranged particles can be seen (40). The interplaque regions appear to be devoid of visible particle structure as judged by both conventional electron and freeze-etch microscope techniques (40). Thus, the two distinct regions of the lumenal membranes (plaque and interplaque) might be expected to have differing chemical compositions. Whatever the difference in chemistry between plaque and interplaque regions, there appears to be no resultant effect upon the passive permeability barrier offered by the entire surface membrane.

Although the lumenal plasma membrane of sheep bladder was found to have a high lipid-to-protein ratio, an inverse relationship has been predicted specifically for the plaque region. From electron microscope data (40) a higher protein-to-lipid ratio might be expected within the plaque regions. Would this change in lipid-to-protein ratio be reflected in a change of permeability characteristics between plaque and interplaque regions of the membrane? Most of the accumulated evidence suggests that even within the plaque regions there exists a hydrophobic interior. The amount of lipid associated with the plaque proteins is uncertain, but a high level of cholesterol can be measured in the DOC pellet. When examined in thin section, the characteristic unit membrane appearance is maintained. Plaque regions are capable of undergoing cleavage during freeze-fracture, indicating a hydrophobic plane within which cleavage occurs (40). The major proteins constituting the units of the membrane plaques show, in amino acid analysis, a predominantly hydrophobic character which is seen for many intrinsic membrane proteins (4). Thus, within the interior of both plaque and interplaque regions of the lumenal plasma membrane, there appears to exist a strongly hydrophobic environment within which lies the passive permeability barrier characteristic of the bladder lumenal membrane.

We are grateful to Judy Krause and Barbara van der Wege for their expert technical assistance, and we wish to thank Karen Anderson for expert electron microscope assistance. We thank heartily Mr. Albert Widmark for

conscientious transportation of sheep bladders to the laboratory, and we acknowledge the generous donation of sheep urinary bladders by Wilson Co., Inc., Denver, Colo. and United Packing Co., Denver, Colo. In addition, we thank Dr. Steve Goodman, Department of Pediatrics, University of Colorado Medical Center, Denver, for the amino acid analyses of our samples. Special appreciation is extended to Dr. L. A. Staehelin for many helpful discussions.

This work was supported in part by a U. S. Public Health Service grant GM18267.

Received for publication 11 June 1976, and in revised form 28 December 1976.

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