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THE ISOLATION AND ANALYSIS OF THE LUMINAL PLASMA MEMBRANE OF CALF URINARY BLADDER EPITHELIUM

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Summary

The luminal plasma membrane of calf urinary bladder epithelium (urothelium) has been isolated by a method designed to preserve enzymic activity as well as structural integrity. The yield was about 80 μg per calf bladder. Low levels of 5′ nucleotidase, Mg^{2+}-ATPase and ($Na^+ + K^+$)-ATPase activities were found in the luminal membrane fraction. Cerebroside was the major lipid present and dodecyl sulphate gel electrophoresis revealed a complex protein and glycoprotein composition in the whole membrane. A membrane fraction consisting of only the plaque areas was shown to have a simpler protein composition with major polypeptides of apparent M_r 12 000 and 22 000. These may associate to form a 30 000 apparent M_r complex which could represent the individual 'particles' of the dodecameric subunits seen by electron microscopy in the plaque regions.

Introduction

The luminal plasma membrane of the mammalian urinary bladder epithelium (urothelium) has an unusual structure and function not found in other cells. When the epithelium is viewed in cross section, the luminal plasma membrane is seen to have a scalloped angular appearance, with an asymmetric thickening in the outer luminal face [1]. This appearance is due to concave plaques separated from each other by thinner symmetrical membrane or interplaque areas [2—4]. When observed by a variety of electron microscopical techniques these plaques are seen to consist of a hexagonal array of subunits [3—8] which project from the luminal face and pass through the membrane bilayer as far as the cytoplasmic face. The hexagonal lattice has also been seen by X-ray diffrac-

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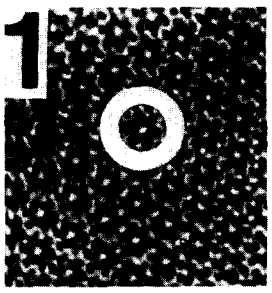


Fig. 1. Luminal plasma membrane negatively stained with potassium phosphotungstate. The electron micrograph was optically processed to remove background noise. The circle shows one 'subunit' consisting of a stellate arrangement of 'particles' (×400 000, taken by Dr. R.C. Warren).

tion of hydrated specimens (Blaurock, A.E. and Stubbs, C.D., unpublished observations). Each of the subunit consists of 12 'particles', approx. 3 nm in diameter arranged in a stellate configuration [8], see Fig. 1. It has been suggested that the main function of the luminal plasma membrane is to form part of the barrier which limits the free flow of ions and water down their concentration gradients across the urothelium, as reviewed by Hicks [9]. In order to determine the chemical basis of its structure and function, luminal plasma membrane has been isolated and analysed from the rat [10,11], rabbit [7], pig [12], sheep [13,14] and cow [4]. In one species, the rat, the low permeability of the membrane was attributed to the presence of the glycosphingolipid, cerebroside [11]. Protein analyses of isolated luminal plasma membranes, by dodecyl sulphate gel electrophoresis, have revealed a complex composition with no single predominant species [11—14]. Studies with an isolated plaque fraction, however, indicated that the subunits in the plaque areas may have a simple protein composition [14].

In this study, luminal plasma membrane was isolated from calf bladders, without recourse to chemical agents such as fluorescein mercuric acetate or thioglycolate which have been used by most previous authors to aid removal of the membrane from the cytoplasm but which might affect enzyme activity. Lipid and protein analyses were performed on whole luminal plasma membrane and a plaque fraction was isolated and analysed for polypeptides. A high level of cerebroside was found in the luminal plasma membrane fraction and this is discussed in connection with its possible role of conferring a low permeability on the membrane. Protein and glycoprotein analysis revealed a complex composition as in previous studies; however there is evidence that the subunits may contain two major peptides.

Materials and Methods

Tissues. Urinary bladders were obtained from 4—6 week old calves from Frank Farge Ltd., Chertsey, Surrey (U.K.) and were transported to the laboratory on ice.

Chemicals. Phospholipids and cerebroside were obtained from Lipid Products, South Nutfield, Redhill, Surrey, (U.K.), acrylamide and bisacrylamide

from Eastman Kodak, Liverpool, (U.K.), proteins of known molecular weight for use as standards from BCL, Lewes, Sussex (U.K.), Schiff reagent from Raymond Lamb Ltd., London (U.K.), Spurr resin from EMscope, London (U.K.), enzyme substrates and other lipids from Sigma, Poole, Dorset (U.K.) and the remaining reagents from B.D.H., Poole, Dorset (U.K.).

Membrane isolation. A method was developed for the isolation of luminal plasma membrane using separation on two sucrose density gradients followed by rate sedimentation. Bladders were cut open and immersed in 0.12 M sucrose, 2 mM $MgCl_2$, 10 mM Tris-HCl pH 7.4, the same buffer being used throughout the preparation. The urothelium from 25 bladders was removed by scraping, then pooled and concentrated to a small volume by centrifugation at 5000 × *g* for 10 min in an 8 × 40 ml MSE fixed angle rotor using an MSE Superspeed 50 ultracentrifuge. The resulting pellet was homogenised in 15 ml of buffer with a Quickfit ground glass homogeniser (Scientific Supplies, London, U.K.), driven at 2000 rev./min with 10 up and down strokes of the pestle. The degree of disruption of the urothelium was assessed by light microscopy before and after homogenisation. The homogenate, consisting mainly of free nuclei and cell debris, was then layered over 42% (w/w) buffered sucrose and was centrifuged in a 3 × 43 ml MSE swing-out rotor for 30 min at 10 000 × *g*. The material was collected from the interface and pelleted by centrifugation at 24 000 × *g* for 15 min in an 8 × 10 ml MSE fixed-angle rotor. The resulting pellet was then taken up in buffer and layered over a discontinuous sucrose density gradient consisting of steps of 40, 35, and 30% (w/w) sucrose. After centrifugation for 4 h at 110 000 × *g* in a 3 × 25 ml MSE swing-out rotor, fractions were removed with a Pasteur pipette. These fractions were: Fraction a, the material passing through the 40% (w/w) sucrose, Fraction b, the material at the 35/40% (w/w) sucrose interface, Fraction c, the material at the 30/35% (w/w) sucrose interface and Fraction d, the material above the 30% (w/w) sucrose interface. These fractions were pelleted at 24 000 × *g* for 15 min as above and either stored as pellets, at −20°C, or, if they were to be used within 24 h, at 0—4°C. Fraction b was further fractionated by centrifuging at 24 000 × *g* for 15 min. The top of the resulting pellet contained sheets of luminal plasma membrane and the bottom mainly a mixture of membranes. The top of the pellet was collected and the remainder was redispersed in buffer by gentle homogenisation in a ground glass hand homogeniser (five up and down strokes) and repelleted. The procedure of repelleting and collecting the top of the pellet was repeated from four to six times. The pooled material from the top of the pellet was the final luminal plasma membrane fraction, Fraction bs.

Electron microscopy. Pellets were fixed with osmium tetroxide for 40 min according to Millonig [15], cut into 1 mm^3 blocks and dehydrated through a series of alcohols 50—100% (w/v) according to Luft [16]. The blocks were embedded in Spurr resin and ultrathin sections were cut on a Porter-Blum MT2 ultramicrotome. Sections were mounted on copper grids, stained with uranyl acetate and lead citrate according to Watson [17] and examined with a Philips EM-200 electron microscope.

Enzyme analysis. 5′ Nucleotidase (EC 3.1.3.5) was determined by the method of Heppel and Hilmoe [18], glucose 6-phosphatase (EC 3.1.3.9) according to Ginsburg and Hers [19], succinate dehydrogenase (EC 1.3.99.1) according

to Green et al. [20], and acid phosphatase (EC 3.1.3.2) according to DiPietro and Zengerle [21]. Mg^{2+} adenosine triphosphatase (EC 3.6.1.3) was determined in a 0.6 ml reaction mixture consisting of 1 mM disodium ATP, 1 mM $MgCl_2$ and 0.2 ml of membrane in 10 mM Tris-HCl (pH 7.4). For the estimation of Na^+ and K^+ dependent adenosine triphosphatase ($(Na^+ + K^+)$-ATPase) 20 mM KCl was included in the incubation mixture; ouabain sensitivity was determined by the inclusion of 0.06 mM ouabain. Protein was determined by the method of Lowry et al. [22].

Lipid analysis. Lipid was extracted from membrane fractions by a procedure based on that of Folch et al. [23]. Membrane pellets were homogenised in chloroform, methanol (2 : 1, by volume) using a ground glass homogeniser with five up and down strokes of the pestle. Water was then added to give two phases and the lower chloroform phase used for studies of lipid composition. Preliminary tests for gangliosides, which separate into the aqueous phase, indicated that they were not present in any significant amount. The qualitative overall lipid composition was determined by the two dimensional thin layer chromatography system devised by Rouser et al. [24]. The first dimension was developed with chloroform, methanol, 28% (w/v) aqueous ammonia (65 : 35 : 5, by volume) and after drying in air for 30 min was developed in the second dimension by chloroform, acetone, acetic acid, water (2 : 1 : 1 : 0.5, by volume). Lipids were detected by charring, on a hotplate after spraying the thin layer chromatography plate with conc. H_2SO_4. Cerebroside was revealed by spraying with 0.4% (w/v) α-naphthol in conc. H_2SO_4, which gives a purple colour on gentle heating [25]. Individual phospholipids were separated according to Skipski et al. [26] and identified by specific spray reagents [27]. Neutral lipids were resolved by the system of Marzo et al. [28] and cholesterol was identified on the chromatogram by the method of Richter et al. [29]. Total phospholipid was determined by the method of Rouser et al. [30] and individual phospholipids similarly determined after separation by thin layer chromatography. Cholesterol was determined by the method of Webster [31] and cerebroside either by the determination of sphingosine released according to Coles and Gray [32], estimated according to Lauter and Trams [33] or after separation by thin layer chromatography, by the method of Devor [25].

Protein analysis. The protein composition of luminal plasma membrane was investigated by first solubilising the membrane with dodecyl sulphate. This was followed by dodecyl sulphate gel electrophoresis, which separates components according to their polypeptide molecular weight. Relatively mild conditions for solubilising membranes give rise to protein components which are capable of further dissociation into smaller components if stronger conditions of solubilisation are used. Increased dissociation of protein results from increased detergent concentration and increased time and temperature of solubilisation. The dodecyl sulphate concentration and time and temperature of dissociation were varied between 0.1 and 5% (w/v), 37 and 100°C, and 30 and 180 min respectively.

Electrophoresis was brought about in 5% (w/v) polyacrylamide gels containing 0.1% (w/v) dodecyl sulphate, 8 M urea, 10 mM dithiothreitol and 0.01 M Tris-HCl, pH 7.4 [34,35]. Proteins were detected with naphthalene black according to Tasserson et al. [35]. Glycoproteins were revealed by the

periodic acid-Schiff stain (PAS) according to Kapitany and Zebrowski [36], or by ^{131}I-labelled concanavalin A binding. The identification of glycoproteins in the gels by concanavalin A was performed by P.J. Robinson [37].

Analysis of stained gels was carried out by densitometric scanning of photographs of gels or autoradiographs using a Joyce Loebl microdensitometer or of gels using an SP1800 spectrophotometer with a gel scanning attachment. Apparent M_r values were estimated by reference to the mobilities of proteins of known molecular weight.

The effect of treatment with the mild detergent sodium deoxycholate followed by dodecyl sulphate was also investigated. The aim was to preserve any complexes of non-covalently interacting polypeptides present by using relatively mild conditions of dissociation. A pellet of luminal plasma membrane was treated with 1 ml of 2% (w/v) sodium deoxycholate (0.25 mg membrane protein/ml) for 1 h at 25°C. An equal volume of 0.2% (w/v) dodecyl sulphate with 10 mM dithiothreitol was then added and, after 10 min at 25°C, electrophoresis was performed as above.

The possibility that mild detergents might bring about a differential solubilisation of plaque and interplaque regions was also investigated. A membrane pellet was dispersed in 1 ml of a 1% (w/v) solution of either Triton X-100 or sodium deoxycholate. After standing for 1 h at room temperature the volume was made up to 8 ml and the solution centrifuged at 24 000 × *g* for 15 min. A residue was obtained which primarily contained plaque regions. The protein content of this fraction was analysed by gel electrophoresis after solubilisation in 1% (w/v) dodecyl sulphate, 8 M urea, 10 mM dithiothreitol and 0.1 M Tris-HCl pH 7.4, for 180 min at 37°C as above.

Results

Membrane isolation

The method of isolation described here gave a high yield of luminal plasma membrane with minimal contamination by other membranes. Luminal plasma membrane forms only a small percentage of the total urothelial membranes and it was found necessary first to prepare a crude preparation by collecting membranes over 42% (w/v) sucrose. The majority of cell debris, intact nuclei, mitochondria and lysosomes sedimented into the denser sucrose leaving mostly membranes at the interface. After a further separation on the three step sucrose density gradient, luminal plasma membrane was found to collect mainly at the 35—40% sucrose interface (Fraction b) but some small vesicles of non-luminal plasma membrane were also present. When Fraction b was pelleted the larger sheets of luminal plasma membrane tended to collect towards the top of the pellet providing the basis for the final purification step. The final yield of luminal plasma membrane was about 80 μg per calf bladder.

Characterisation of the fractions from the sucrose gradient

(i) Electron microscopy. The subcellular organelles contained in the various fractions were identified by electron microscopy as:

Fraction a; Non-luminal plasma membrane, mitochondria, lysosomes, rough endoplasmic reticulum and some luminal plasma membrane.

Fraction b; Most of the luminal plasma membrane plus large amounts of other membranes.

Fraction c; Small membrane vesicles and some luminal plasma membrane.

Fraction d; Fraction d resembled Fraction c but contained no luminal plasma membrane (Fig. 3).

Fraction bs; Large sheets of luminal plasma membrane with minimal contamination by other organelles (Fig. 2).

(ii) Enzyme analysis. Fraction a, b, c and d contained 5′ nucleotidase, glucose 6-phosphatase, Mg^{2+}-ATPase, and $(Na^+ + K^+)$-ATPase activities, indicating the presence of both plasma membrane and endoplasmic reticulum. Only Fraction a contained the mitochondrial marker succinate dehydrogenase, and then with a very low activity. Fraction bs, the final luminal plasma membrane, contained few other membranes as judged by electron microscopy (Fig. 2) and had no glucose 6-phosphatase activity indicating the absence of endoplasmic reticulum. Mg^{2+}-ATPase, $(Na^+ + K^+)$-ATPase and 5′ nucleotidase activities were found in this fraction (Table I).

(iii) Lipid analysis. The major lipid component of the luminal plasma membrane Fraction bs was cerebroside. Cerebroside sulphate, diglycosyl ceramide and gangliosides, which have characteristic R_F values on the thin layer chromatography system used [24], were not detected. Although cerebroside was more abundant in the luminal plasma membrane fraction it was also found in Fraction d which contained no luminal plasma membrane, whereas relatively more phospholipid was found in Fraction d than in the luminal plasma membrane fraction. Analysis of phospholipid classes revealed the presence of phos-

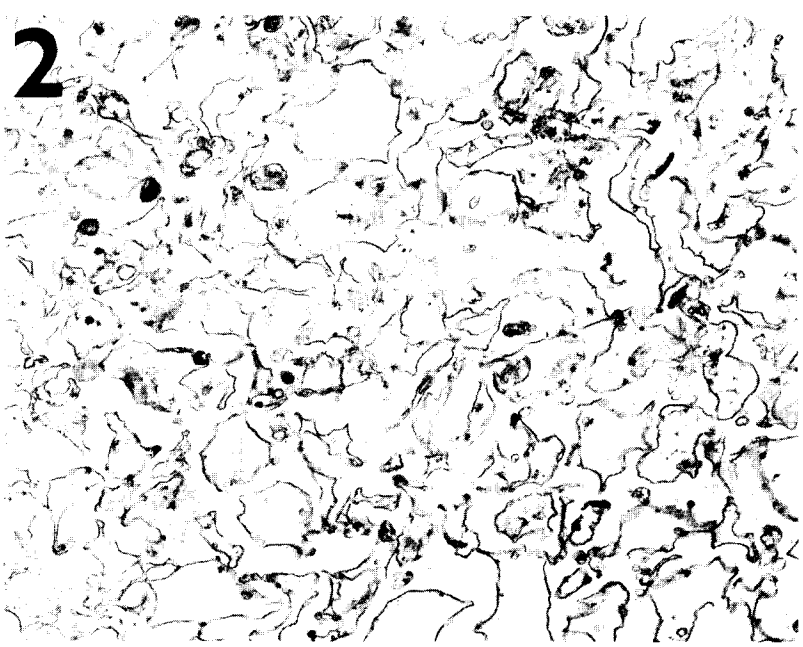


Fig. 2. Electron micrograph of the luminal plasma membrane Fraction bs (×8300).

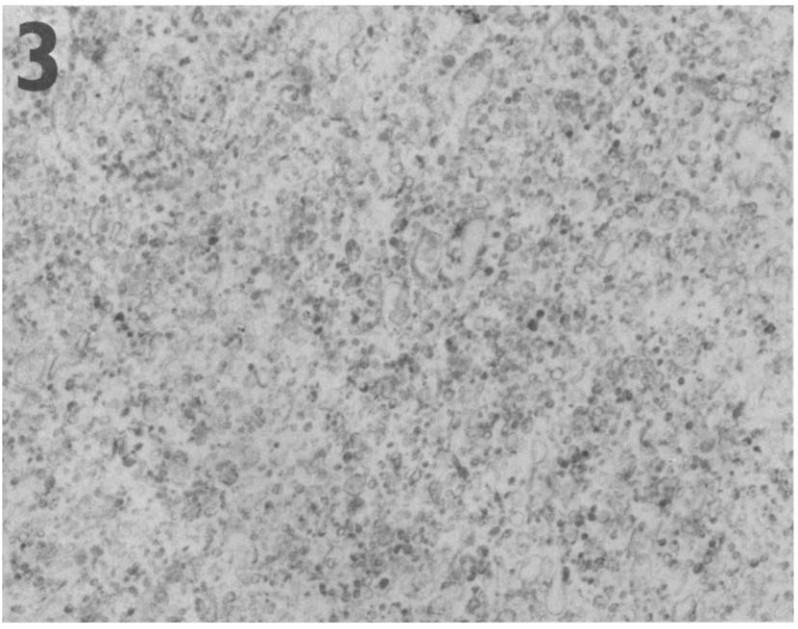


Fig. 3. Electron micrograph of membrane Fraction d (×8300).

TABLE I

COMPOSITION OF LUMINAL PLASMA MEMBRANE (FRACTION bs) AND NON-LUMINAL PLASMA MEMBRANE (FRACTION d)

Results ± S.E.M., number of determinations in brackets.

(a) Enzyme activities	Enzyme activity (μg P_i/h per mg protein)		
	Homogenate	Fraction bs	Fraction d
5′ Nucleotidase	0.22 (1)	6.5 (2) recovery 12.9%	9.2 (2) recovery 11.0%
Glucose 6-phosphatase	0.2 (1)	0 (1)	1.4 (1) recovery 2.3%
Total ATPase (+Mg^{2+}, K^+, Na^+)	0.13 (1)	0.78 ± 0.19 (4) recovery 12.3%	0.51 (1) recovery 4.8%
Mg^{2+}-ATPase	—	0.57 ± 0.17 (4)	0.48 (1)
(Na^+ + K^+)-ATPase ouabain sensitive	—	0.21 ± 0.11 (4)	0.04 (1)

(b) Chemical position	Fraction bs	Fraction d
Protein (%)	41.0	50.0
Lipid (%)	59.0	50.0
Cholesterol:phospholipid (molar ratio)	1.89 ± 0.15 (4)	0.76 ± 0.14 (4)
Cerebroside:phospholipid (molar ratio)	1.81 ± 0.26 (4)	0.61 ± 0.19 (4)
Cholesterol (mol%)	40.0	40.0
Cerebroside (mol%)	38.0	22.0
Phospholipid (mol%)	22.0	38.0
Phosphatidylcholine (%P) *	43.6 ± 2.3 (3)	43.7 ± 1.6 (3)
Phosphatidylethanolamine (%P) *	26.3 ± 1.8 (3)	33.2 ± 4.0 (3)
Phosphatidylserine (%P) *	20.4 ± 1.4 (3)	17.7 ± 2.7 (3)
Lysophosphatidylcholine (%P) *	1.2 ± 1.0 (3)	1.6 ± 1.1 (3)
Sphingomyelin (%P) *	8.5 ± 1.9 (3)	3.8 ± 1.3 (3)

* (%P), percentage of total lipid phosphorus.

phatidylcholine, phosphatidylethanolamine, phosphatidylserine, sphingomyelin and lysophosphatidylcholine. No differences in the quantitative phospholipid class analyses were found between the luminal Fraction bs and the membrane Fraction d (see Table I).

(iv) Protein and glycoprotein analysis. The relative amounts of the lower molecular weight species of polypeptides varied according to the conditions of dissociation. For example, increasing the temperature from 37°C to 100°C caused a relative increase in band 3 (cf. Figs. 5 and 6). Similarly, increasing the dodecyl sulphate concentration and/or time of solubilisation increase the relative amounts of the lower molecular weight species.

A marked relative increase in band 4 could be obtained by pretreatment of the membrane fraction with 1% sodium deoxycholate for 1 h at 25°C before further dissociating with 0.1% dodecyl sulphate for 10 min at 25°C (Fig. 7).

The relative amounts of the molecular weight species of glycoproteins also varied according to the method of dissociation used. When the temperature of dissociation was 37°C and PAS staining was used, three main broad bands of carbohydrates were observed in regions corresponding to band numbers 3, 5 and 9—11 (Fig. 9). At 100°C the amounts of higher molecular weight bands were diminished while the amount of band 3 was relatively increased (Fig. 10). When carbohydrates were revealed by ^{131}I-labelled concanavalin A binding, the patterns were similar to those seen with PAS, but the resolution was much increased (Figs. 11 and 12).

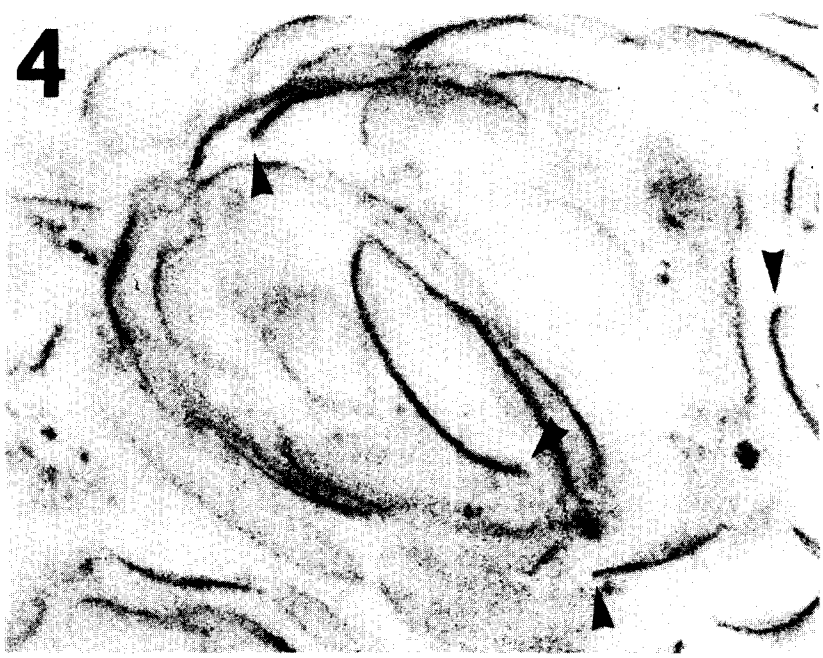
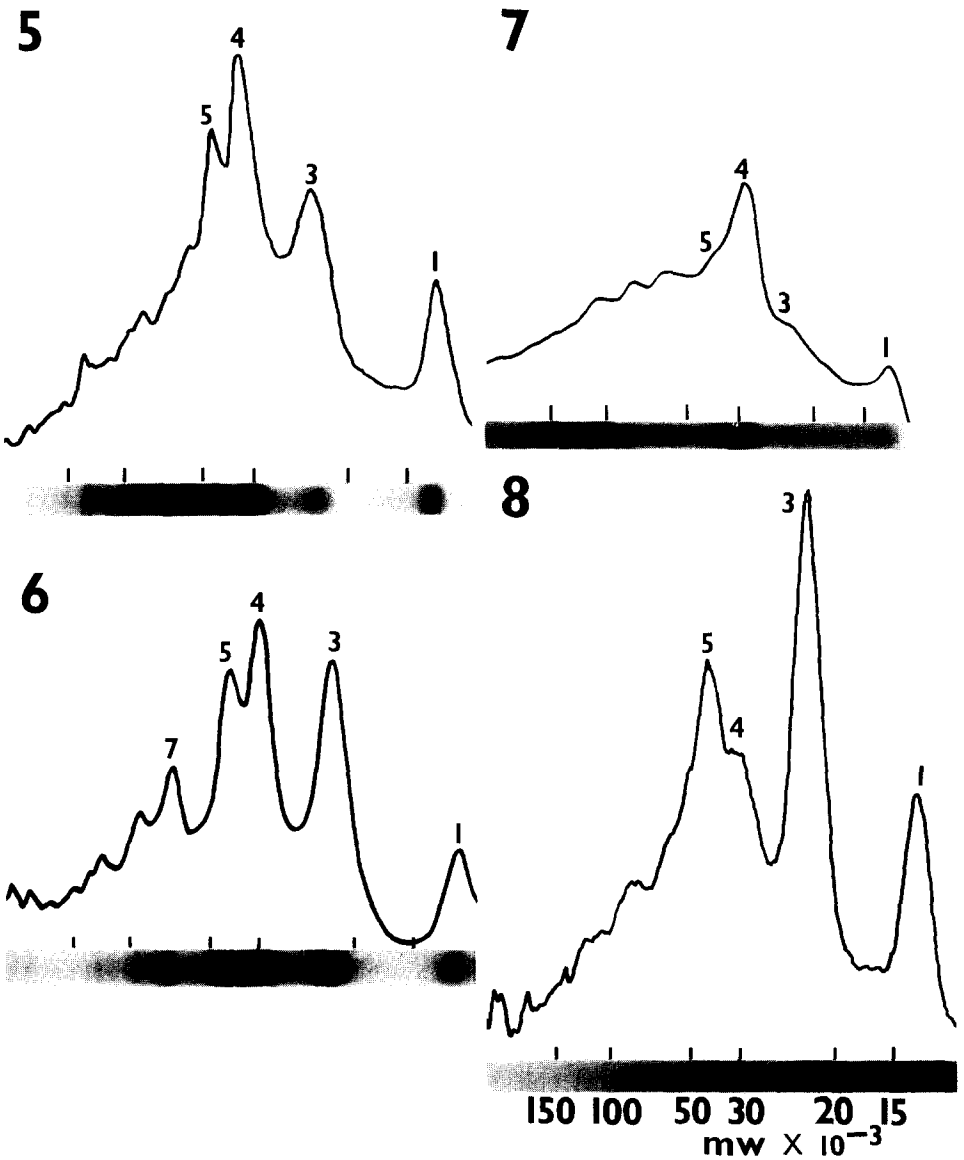


Fig. 4. Electron micrograph of luminal plasma membrane after 1 h treatment with Triton X-100. The plaque areas which are normally joined by interplaque areas have been separated from each other as indicated by the free edges-marked by arrows. The 'unit membrane' structure was difficult to resolve after Triton X-100 treatment. (X120 000).



Figs. 5—8. Dodecyl sulphate gel electrophoresis of luminal plasma membrane-polypeptides, revealed by naphthalene black. 5, solubilisation in 1% (w/v) dodecyl sulphate, 8 M urea, 10 mM dithiothreitol for 3 h at 37°C. 6, solubilisation in 1% (w/v) dodecyl sulphate, 8 M urea, 10 mM dithiothreitol for 30 min at 100°C. 7, treated with 1% (w/v) sodium deoxycholate for 1 h at 25°C followed by 0.1% (w/v) dodecyl sulphate for 10 min at 25°C. 8, the Triton X-100 resistant plaque fraction solubilised in 1% (w/v) dodecyl sulphate, 8 M urea, 10 mM dithiothreitol for 3 h at 37°C.

Dodecyl sulphate electrophoresis of the other membrane Fraction d (Figs. 13 and 14) revealed protein and glycoprotein patterns qualitatively similar to that of the luminal plasma membrane Fraction bs but there were quantitative differences. For example there was relatively more of bands 2, 6 and higher molecular weight species in Fraction d (see Figs. 5 and 13).

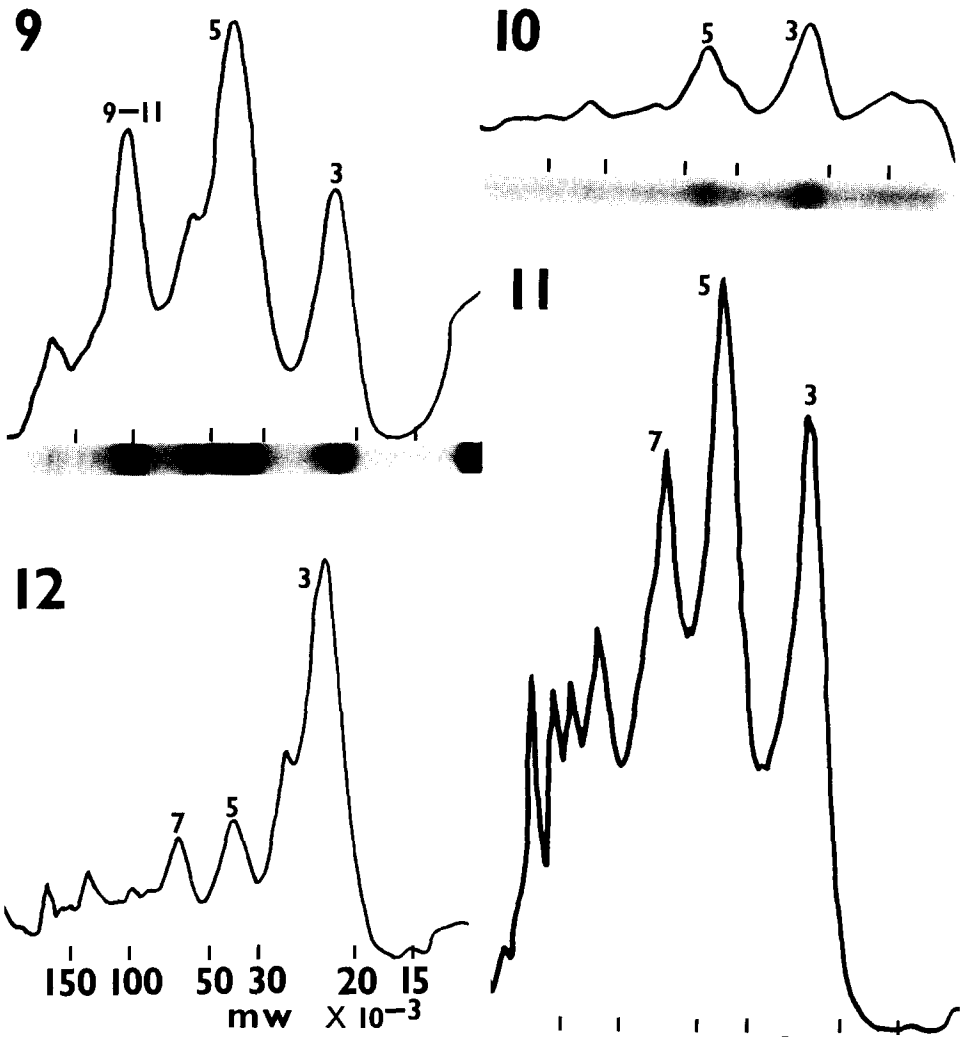
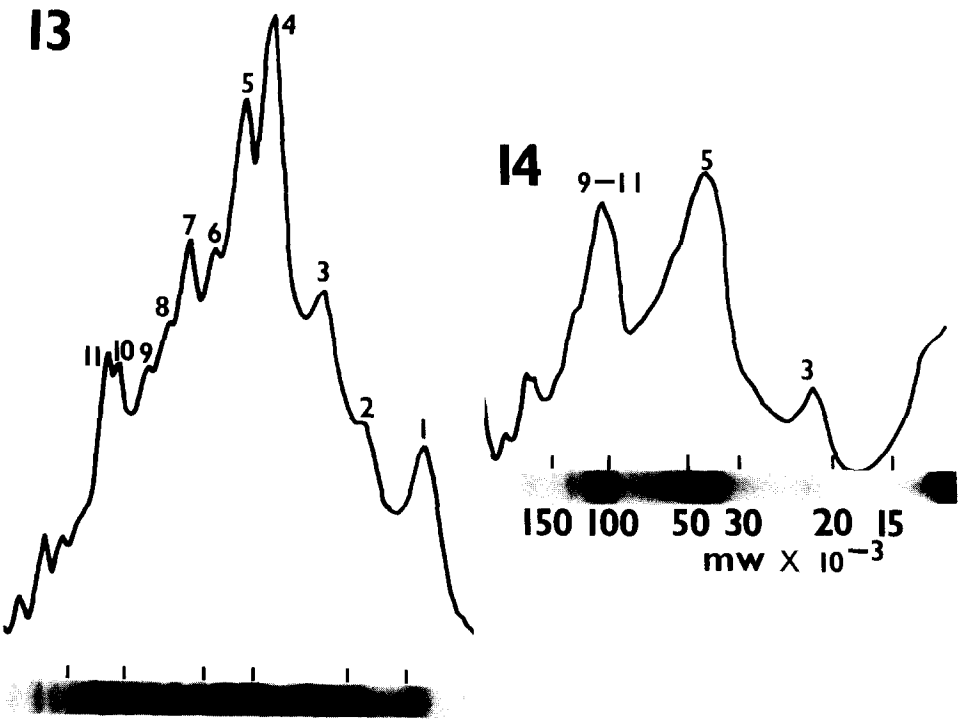


Fig. 9—12. Dodecyl sulphate gel electrophoresis of luminal plasma membrane-glycopolypeptides. 9, solubilisation in 1% (w/v) dodecyl sulphate, 8 M urea, 10 mM dithiothreitol for 3 h at 37°C, revealed by PAS stain. 10, solubilisation in 1% (w/v) dodecyl sulphate 8 M urea, 10 mM dithiothreitol for 30 min at 100°C, revealed by PAS stain. 11, solubilisation in 1% (w/v) dodecyl sulphate, 8 M urea, 10 mM dithiothreitol for 3 h at 37°C, revealed by ^{121}I-labelled concanavalin A binding. 12, solubilisation in 1% (w/v) dodecyl sulphate, 8 M urea, 10 mM dithiothreitol for 30 min at 100°C, revealed by ^{131}I-labelled concanavalin A binding.

(v) Preparation of a plaque fraction from the luminal plasma membrane. When the luminal plasma membrane Fraction bs was treated with 1% Triton X-100 for 1 h, electron microscopy showed the interplaque areas to be preferentially solubilised leaving plaque areas intact and separable by sedimentation (Fig. 4).

When this plaque fraction was solubilised in 1% (w/w) dodecyl sulphate, 8 M urea at 37°C for 3 h, subsequent electrophoresis revealed a much less com-



Figs. 13 and 14. Dodecyl sulphate gel electrophoresis of Fraction d (non-luminal plasma membrane). Solubilisation in 1% (w/v) dodecyl sulphate, 8 M urea 10 mM dithiothreitol for 3 h at 37°C. 13, polypeptides revealed by naphthalene black. 14, glycopolypeptides revealed by PAS stain.

plex pattern than from that found in the whole luminal plasma membrane Fraction bs. Bands 1 and 3 were considerably more abundant and accounted for approx. 50% of the plaque fraction (Fig. 8). Little carbohydrate could be detected in the plaque fraction by PAS staining by comparison with that found in the whole luminal plasma membrane Fraction bs.

Discussion

In this investigation a method was developed in which a very mild procedure was used to remove and isolate luminal plasma membrane. The isolated membrane was shown to be almost entirely free of other cell membranes and organelles by morphological and enzymic markers. In common with the studies of Vergara et al. [12] and Caruthers and Bonneville [14], $(Na^+ + K^+)$-ATPase was found to be associated with the isolated membrane. In theory, this enzyme could help to maintain the large osmotic gradient across the membrane by pumping sodium into the lumen, but evidence suggests that in the adult bladder no significant active transport of sodium occurs [38—41]. It could thus represent a physiologically latent form of the enzyme which is active in the foetal bladder, as demonstrated in the sheep by France et al. [42]. Similarly, 5′ nucleotidase and/or Mg^{2+}/ATPase which were not demonstrated cytochemi-

cally in the membrane in situ [1], may also be physiologically latent in vivo and both these enzymes may be unmasked by the physical conditions of the isolation procedure as has been suggested previously [9].

The lipid composition of the bladder luminal membrane has proved to be of interest. A previous study of rat luminal plasma membrane [11] demonstrated a high level of the glycosphingolipid cerebroside, and this was also found here in the calf bladder. The effects of cerebrosides on model membrane systems have been investigated by a number of workers. Quinn and Sherman [43] first showed that cerebroside forms relatively more condensed lipid monolayers than do phospholipids. Sharom [44] demonstrated in an NMR study that the addition of cerebroside to a phospholipid bilayer resulted in decreased molecular motion of both lipids resulting in closer packing and lower fluidity. Pascher [45] has provided evidence for the formation of lateral hydrogen bonds between the hydroxyl groups of glycosphingolipids alone, from which it was postulated that the resulting closer packing could lower fluidity and permeability. It has also been suggested that the orientation of the carbohydrate group at the surface of the membrane may act to exclude water and ions [46]. Direct evidence of an effect on permeability comes from a study by Cohen [47] who showed that the permeability of phospholipid liposomes to water was lowered by the addition of cerebroside. It may be concluded that the presence of cerebroside in the epithelial cell membrane will confer a relatively low permeability, especially on the luminal plasma membrane which has the highest cerebroside content.

The protein composition of the bladder luminal membrane was studied by dodecyl sulphate gel electrophoresis. The dissociation of bladder luminal membrane proteins required stronger conditions than were previously used, namely 0.1% dodecyl sulphate at 37°C for 1 h [11,14] where a single major polypeptide of 60—70 000 apparent M_r was observed in both the rat [11] and the sheep [14] together with high M_r material which barely penetrated the gel. A comparable result was obtained in the present study when mild conditions of dissociation were used (see Fig. 7) when a single major species of 30 000 apparent M_r was found. When, however, dodecyl sulphate concentration was increased to 1% and the time of incubation to 3 h at 37°C, all protein entered the gel and a large number of components were separated including components of lower M_r than 30 000 M_r. There were five major species, refered to as band 1, 3, 4, 5 and 7 of apparent M_r 12 000, 22 000, 30 000, 43 000 and 67 000 (see Fig. 5). If the temperature was increased to 100°C, the 22 000 M_r component increased at the expense of the higher M_r species (see Fig. 6), demonstrating that this component is normally part of a higher M_r complex.

The highly ordered morphological substructure of the plaque regions of the bladder luminal membrane might be due to an underlying chemical subunit of relatively simple composition. In previous studies by Chlapowski et al. [48] and Caruthers and Bonneville [14] sodium deoxycholate was used to solubilise the interplaque regions and allow the plaque areas to be isolated. In the present work, Triton X-100 was used to free the plaques from the rest of the membrane, and the isolated plaques appeared to be intact in thin sections when viewed by electron microscopy. In contrast Knutton and Robinson [4] reported that no recognisable membrane remains after 1 h exposure to this

detergent. Warren and Hicks [49] in a negative stain study on rat membrane observed partial degradation of the subunit organisation by Triton X-100, but unaffected plaques still remained (Warren, R.C., personal communication). When the isolated fraction was solubilised in 1% dodecyl sulphate at 37°C for 3 h, gel electrophoresis revealed major polypeptides of 12 000 and 22 000 apparent M_r. The milder conditions of dissociation used by Caruthers and Bonneville on their deoxycholate-produced plaques from the sheep bladder luminal membrane produced a single major polypeptide of 30 000 apparent M_r. In our present study a 30 000 apparent M_r component was produced (see Fig. 7) by mild conditions of dissociation of the whole membrane using deoxycholate followed by 0.1% dodecyl sulphate. These conditions were thus very similar to those used by Caruthers and Bonneville in the preparation and dissociation of their plaque fraction. The 12 000 and 22 000 apparent M_r components reported here were produced by stronger conditions of dissociation of the plaques. It seems likely that these two lower M_r polypeptides arise from a single 30 000 M_r complex. This apparent M_r of 30 000, though it can only be approximate when determined by dodecyl sulphate electrophoresis [50], is consistent with the approximate M_r which can be calculated from measurements of electron micrograph images of single membrane particles.

In the whole membrane fraction, a band of carbohydrate coincided with a polypeptide of 22 000 apparent M_r but no comparable band of carbohydrate was found in the plaque fraction. It is probable that this 22 000 M_r region in the whole membrane contains at least 2 proteins, namely a glycoprotein of the interplaque region and a polypeptide from the plaque. It is suggested above, that the 22 000 M_r component of the plaque region is derived by dissociation of a 30 000 M_r component. This is supported by the fact that the 30 000 M_r component in our whole membrane, like the 22 000 M_r component of the plaque, does not stain for carbohydrate.

When the Fraction d containing other membranes from the urothelium, is compared with the luminal membrane Fraction bs it can be seen that the patterns of glycoproteins are similar in the two fractions. We have established that glycoproteins only occur in the interplaque region of the luminal membrane. Fraction d is more complex than the luminal membrane fraction and contains several additional polypeptide bands, but it should be borne in mind that one-dimensional dodecyl sulphate gel electrophoresis is not sufficiently resolving to completely separate all the proteins which may be present. Fraction d also contains cerebroside but in considerably smaller amounts than the luminal membrane fraction.

This study was undertaken in an attempt to relate structure, chemical composition and function in the bladder luminal membrane. We have demonstrated the membrane to have an unusually high cerebroside content which must contribute to its function as a permeability barrier to water and ions. The permeability barrier presented by the membrane will be further reinforced by the order imposed upon the lipid bilayer by the subunit structure of the plaques, as discussed elsewhere [51]. In the present paper we have further shown the plaques to contain two principle polypeptides of 12 000 and 22 000 apparent M_r which may associate to form a 30 000 apparent M_r complex. Such polypeptide complexes may represent the individual 'particles' of the dodecameric subunits seen by electron microscopy.

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