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Isolation of plasma membranes from the bovine retinal pigment epithelium

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Retinal pigment epithelium plasma membranes have been isolated by differential and density gradient centrifugation of glass-bead-bound, collagenase-treated cells. Electron microscopic evidence indicates that the glass-bead-bound cells were devoid of red blood cells, rod outer segments and other ocular cell contaminants. The plasma membranes were recovered in 4–6 μg/eye yields and purified 10-fold by 5′-nucleotidase and alkaline phosphodiesterase I, and 6.5-fold by ($Na^+ + K^+$)-ATPase. Plasma membrane purity as measured by covalent labeling of the epithelial cell plasma membrane proteins with *p*-(diazonium) benzene[^{32}S]sulfonic acid was 8–19-fold. In purified plasma membranes contamination by mitochondria was undetectable and lysosomal contamination reduced 100-fold, while endoplasmic reticulum was 2-fold enriched. SDS-polyacrylamide gel electrophoresis of the plasma membrane proteins revealed 23–26 major bands by Coomassie blue staining and 12–16 major bands by radioactive labeling. The plasma membranes exhibited a 3-fold lower concentration of docosahexaenoic acid, a 3-fold higher cholesterol/phosphate ratio, and were 10-fold enriched in cholesterol per μg protein when compared to the whole cell fraction. Retinal epithelial plasma membranes contain an average of 1 mol cholesterol per mol of lipid phosphorus, a high palmitic acid concentration (39 mol%) and a low concentration of docosahexaenoic acid (2 mol%). The lipid profile of the retinal pigment epithelial plasma membranes indicates that they are typical of plasma membranes from many other cell types and that they appear to be less fluid than total rod outer segment membranes.

Introduction

The retinal pigment epithelium is a single cell layer of cuboidal epithelial cells which is interposed between the choriocapillaris and the neural retina. This monolayer of cells serves multiple functions in maintaining photoreceptor cell viability, including the transport of nutrients, oxygen diffusion [1], storage, transport and esterification of retinol taken up from the blood [2–4], or from photopigment bleaching during light [5]. The retinal pigment epithelium also serves to maintain rod outer segment length by the process of phagocytosis of the shed tips of the photoreceptor cell outer segments [6]. Different functions are associated with the apical and basal membranes of these cells. The apical membrane, which is in intimate contact with the photoreceptor cell rod and cone outer segments, must contain the proteins responsible for recognition and initiation of endocytosis of the shed tips of these organelles. Recently, Ostwald and Steinberg [7] reported that ($Na^+ + K^+$)-ATPase was found in an apical-membrane-rich fraction microdissected from retinal pigment epithelium. The retinal epithelial cell basal membrane is known to contain the receptor for the uptake of

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retinol from plasma retinol-binding protein in the circulation [8–10].

Knowledge of retinal epithelium cell composition is of great interest not only because of the vital role of these cells within the eye, but also because they exhibit characteristics similar to those of other epithelial cells [11]. Bovine retinal pigment epithelial cells have been isolated and characterized with respect to enzyme activities [12–15] and lipid composition [16]. Siakotos, Aguirre and Schuster [17] employed glass beads to bind and isolate retinal epithelial cells from bovine and rat eyes in high yields, but in the preceding studies contamination by rod outer segments, red blood cells or choroid could not be excluded. Ottonello and Maraini [18] have isolated retinal epithelial plasma membranes and characterized them by electron microscopy, but information regarding their composition is lacking.

The use of charged beads in isolating plasma membranes from red blood cells was reported by Jacobson and Branton [19]. They completely coated polylysine beads using excess red cells and achieved higher plasma membrane yields than with previous methods. This technique, or variations thereof, has since been applied to many different cell types [20,21]. Using the approach of Jacobson and Branton [19] and the findings of Siakotos et al. [17], we describe a useful technique for the isolation of retinal epithelial plasma membranes. The isolated plasma membranes have been characterized by analysis of marker enzyme activities, SDS-polyacrylamide gel electrophoresis and lipid composition. This study also shows that, compared to total rod outer segment membranes [22], the retinal epithelium plasma membranes contains a higher cholesterol/phosphate ratio and a higher content of saturated fatty acids.

A preliminary report of this work was presented at the ARVO Meeting in Sarasota, FL, April, 1983.

Materials and Methods

Isolation of bovine retinal pigment epithelium plasma membrane

Fresh bovine eyes were obtained from a local abattoir and transported on ice in the dark. Under dim red light, the eyes were freed of adhering tissue and cut along the ora serrata. The cornea, lens and vitreous were removed, leaving the eyecup intact with the neural retina in place. The dissected eyecups were then soaked for 30 min at 4°C in phosphate buffered saline (pH 7.2)/2 mM PMSF/2.5 mM iodoacetamide/0.02% EDTA (buffer A) to inhibit proteolytic activity. At the end of this period the retina was gently teased away from the epithelial cell layer and the eyecups soaked for an additional 2 h at 4°C in buffer A to remove adhering rod outer segments. The eyecups were washed in fresh buffer A and an even layer of glass microbeads (Ferro Micro Beads class IV-A, 13–44 μm diameter, Cataphote, Inc., Jackson, MI), previously treated with Tris buffer according to Siakotos et al. [17], was carefully applied to the interior of the eyecup. Excess beads were immediately removed with several volumes of buffer A. Next a 0.1% silane solution, made in buffer A was added to the eyecups to coat the unbound bead surfaces. (Prosil-28, PCR Research Chemicals Gainesville, FL was modified by the addition of 10% water.) After 4 min the eyecups were washed, cut, inverted, and the beads with adhering retinal epithelial cells, gently brushed with a sable brush into fresh buffer A. The beads with adherent cells were allowed to settle at $1 \times g$, and the turbid supernatant aspirated and discarded. The preparation was rewashed with buffer A treated for 30 min with 3.5 units of collagenase (Sigma Type V, 0.1%) containing 0.02% *N*-α-*p*-tosyl-L-lysine chloromethyl ketone HCl (trypsin inhibitor; Sigma) in 0.9% saline/0.02% $CaCl_2$ (pH 7.0). The retinal epithelium cell-bead preparation was then washed and the cells lysed in 5 ml of ice-cold water. To remove cytoplasmic materials the lysed retinal epithelium sample was washed once with 0.9% saline and twice with 0.9% saline adjusted to pH 5.0. The bead-plasma membrane preparation was centrifuged at $120 \times g$ between these washes and melanin which settled on top of the beads was carefully removed by aspiration. The beads were suspended in 1 ml of 40% sucrose and then vortexed for at least 1 min. A discontinuous gradient was prepared by layering the sucrose solution with 1 additional volume of 40% sucrose, 1 ml 32% sucrose and water (final volume 5 ml). All sucrose solutions were made in 0.9% saline. The preparation was centrifuged at $100\,000 \times g$ for 60 min at 4°C

and the plasma membrane fraction was collected from a narrow band at the 32% sucrose/water interface. The plasma membranes were then precipitated by mixing with fresh glass beads, diluted to 5 vol. with water (less than 10% sucrose) and recentrifuged at 100000 × *g* for 40 min at 4°C. This step was necessary to permit quantitive recovery of the isolated plasma membranes from the ultracentrifuge tube. The precipitated plasma membranes were then collected from the glass-bead pellet with a loss of less than 5% protein. Because of the affinity of these membranes for glass, siliconized glassware was used throughout the study.

In some experiments *p*-(diazonium)benzene[^{35}S]sulfonic acid ([^{35}S]DABS) was used to covalently label the apical plasma membrane proteins of the retinal epithelium. When the label was used, it was added in phosphate-buffered saline to the intact eyecup after the neural retina had been removed and before the addition of glass beads. Radioactive DABS was synthesized from [^{35}S]sulfonic acid according to the method of Dilley et al. [23]. Immediately before use the concentrated [^{35}S]DABS (1 mM, 5.5 mCi) was diluted with phosphate-buffered saline to a final concentration of 0.01 mM. The [^{35}S]DABS was allowed to incubate in the eyecup for 10 min at 4°C after which time the eyecups were washed three times with fresh phosphate-buffered saline to remove excess radioactivity. For tissue analysis, samples were precipitated with 25% trichloroacetic acid, washed with 0.09 M HCl made in acetone and rewashed with acetone. The ^{35}S was counted in the washed sample precipitates by liquid scintillation counting procedures.

Enzyme assays

To determine plasma membrane purity the following enzymatic markers were used: 5′-Nucleotidase, alkaline phosphodiesterase I and ($Na^+ + K^+$)-ATPase. Lysosomal contamination was measured by *N*-acetyl-β-D-glucosaminidase activity. Endoplasmic reticulum and mitochondrial contamination was measured by sulfatase C and cytochrome oxidase activities respectively.

5′-Nucleotidase activity was measured according to Chang et al. [24] with the following modifications. The final concentration of $MgCl_2$ was 80 mM (pH 8.5) and the reaction was stopped with the addition of 0.1 ml of 50% trichloroacetic acid. 0.1 ml of the reagent mixture was utilized for inorganic phosphate determination according to Organisciak and Noell [25].

Alkaline phosphodiesterase I activity was measured as modified from Beaufay et al. [26]. The liberation of *p*-nitrophenol from *p*-nitrophenyl 5′-thymidylate (Sigma) was measured at 400 nm. Incubation was carried out for 60 min at 37°C in 0.5 ml (final volume) containing 1.5 mM substrate/0.1 M glycine (pH 9.6)/0.1% Triton X-100. The reaction was stopped by the addition of 2 ml of 0.1 M NaOH.

Oubain-sensitive ($Na^+ + K^+$)-ATPase activity was measured as modified from Ostwald and Steinberg [7] and Winkler [27]. The reagent mixture comprised 25 mM Tris-HCl (pH 7.4 at 37°C)/1 mM EDTA/2 mM $MgCl_2$/110 mM NaCl/10 mM KCl/2 mM ATP. A reagent blank was run with 0.1 mM oubain. The reaction was allowed to proceed for 30 min at 37°C and was stopped with the addition of 0.1 ml of cold 10% trichloroacetic acid. Inorganic phosphate was determined from 0.1 ml aliquots of the reagent mixture [25].

N-Acetyl-β-D-glucosaminidase activity was measured by the liberation of *p*-nitrophenol from *p*-nitrophenyl-*N*-acetyl-β-D-glucopyranoside (Sigma) at 400 nm. Incubation was carried out for 60 min at 37°C in a 0.5 ml final volume of 6 mM substrate, 0.2 M acetate buffer (pH 5.0) and 0.1% Triton X-100. The reaction was stopped with 2 ml stopping buffer (133 mM glycine/ 83 mM Na_2CO_3/67 mM NaCl (pH 10.7)). This assay was adapted from Beaufay [26].

Sulfatase C was assayed according to Canonico et al. [28].

Cytochrome oxidase was assayed according to Smith and Camerino [29].

DNA in the isolated plasma membranes was measured according to LaBarca and Paigen [30].

All enzymatic determinations were performed immediately after the preparation of the plasma membranes and all assays were run at protein concentrations for which a linear response was determined. Protein determinations were by the method of Lowry et al. [31] with bovine serum albumin as the standard. Generally, 5′-nucleoti-

dase and one or two other enzymes were measured for each plasma membrane preparation. Note that the bead fraction in these studies contains residual material after removal of plasma membrane by density-gradient centrifugation.

SDS-gel electrophoresis

The basic technique of Laemmli [32] was utilized for SDS-polyacrylamide gel electrophoresis. A 4% stacking gel was used over a 10–15% gradient gel, Dialyzed retinal epithelial membranes were incubated in 3% SDS/1% β-mercaptoethanol/0.05 M Tris-HCl (pH 6.8)/15% glycerol for 30 min at 36°C. Following electrophoresis the proteins were fixed and stained with 0.2% Coomassie blue. Radioactive gels were treated for 90 min in EN^{3}HANCE (New England Nuclear, Boston, MA), washed for 30 min with water, dried under vacuum and then exposed to X-ray film at −80°C.

Lipid analysis

Lipid extracts of the whole cell, bead adherent material and isolated plasma membrane fractions were prepared and analyzed according to previously published methods [22,25]. For the determination of total lipid fatty acid composition, three or four separate plasma membrane preparations were combined and analyzed. Purity of these preparations was checked by 5′-nucleotidase and found to be comparable to those characterized by enzymatic analysis.

Scanning electron microscopy

Samples were prepared for scanning electron microscopy by using Millonig [33] phosphate-buffered osmium fixative. The samples were critical-point-dried, plated with gold and viewed in a Philips-500 scanning electron microscope.

Results

To test the efficacy of the glass-bead plasma membrane isolation procedure, control experiments were performed with beads which were treated or not with silane reagent. As shown in Fig. 1A, treatment of glass beads with silane reagent before their placement into and recovery from a previously dissected eyecup resulted in beads which were devoid of cells. Likewise, when pre-treated beads were mixed with red blood cells, rod outer segments, isolated bovine heart mitochondria or serum albumin, no binding of cells, subcellular organelles or protein could be determined. On the other hand, when non-silane treated beads were utilized, retinal pigment epithelial cells were bound to the beads (Fig. 1B). As can also be seen in Fig. 1B, long strands of fibrous material were also bound to the beads on most of the exposed surfaces. This indicates that during the isolation procedure, extracellular material, subcellular organelles or proteins may bind to the untreated surfaces of glass beads. To minimize extraneous binding, the exposed surfaces of the beads, previously bound to the intact epithelial cell layer, were treated with silane reagent before being brushed from the eyecups. Compared to Fig. 1B, Fig. 1C shows that this treatment appears to be effective in reducing the binding of contaminants (arrows) on exposed bead surfaces. The isolated epithelial cells are found in small sheets adhering to one another, and with good cell morphology. Silane treatment of beads previously mixed with isolated mitochondria or rod outer segments also reduced the binding of these potential contaminants an average of 10 and 25%, respectively. Thus, silane treatment not only coats exposed bead surfaces and prevents extraneous binding, but also displaces a fraction of two potential cellular organelle contaminants. Red blood cells do not bind to the silane-treated or untreated glass beads.

Brushing the glass-bead-bound retinal pigment epithelial cells from the eye frequently results in the simultaneous removal of fragments of Bruch's membrane and choroid cells (Fig. 1C). Fig. 1D shows a small sheet of epithelial cells bound to a glass bead after collagenase treatment. This section and others examined reveal good cell morphology with undetectable red blood cells, rod outer segments or choroidal contamination. A fortuitious section (inset) shows a single retinal pigment epithelial cell with apical processes (asterisk) bound to a glass bead. A single long fiber still bound to the glass bead can also be seen (arrow). Fig. 1E presents a higher magnification of a glass bead with an adherent cell membrane fragment after cell lysis. The plasma membranes bind to the beads with their cytoplasmic surfaces exposed. This membrane orientation may aid in the removal of

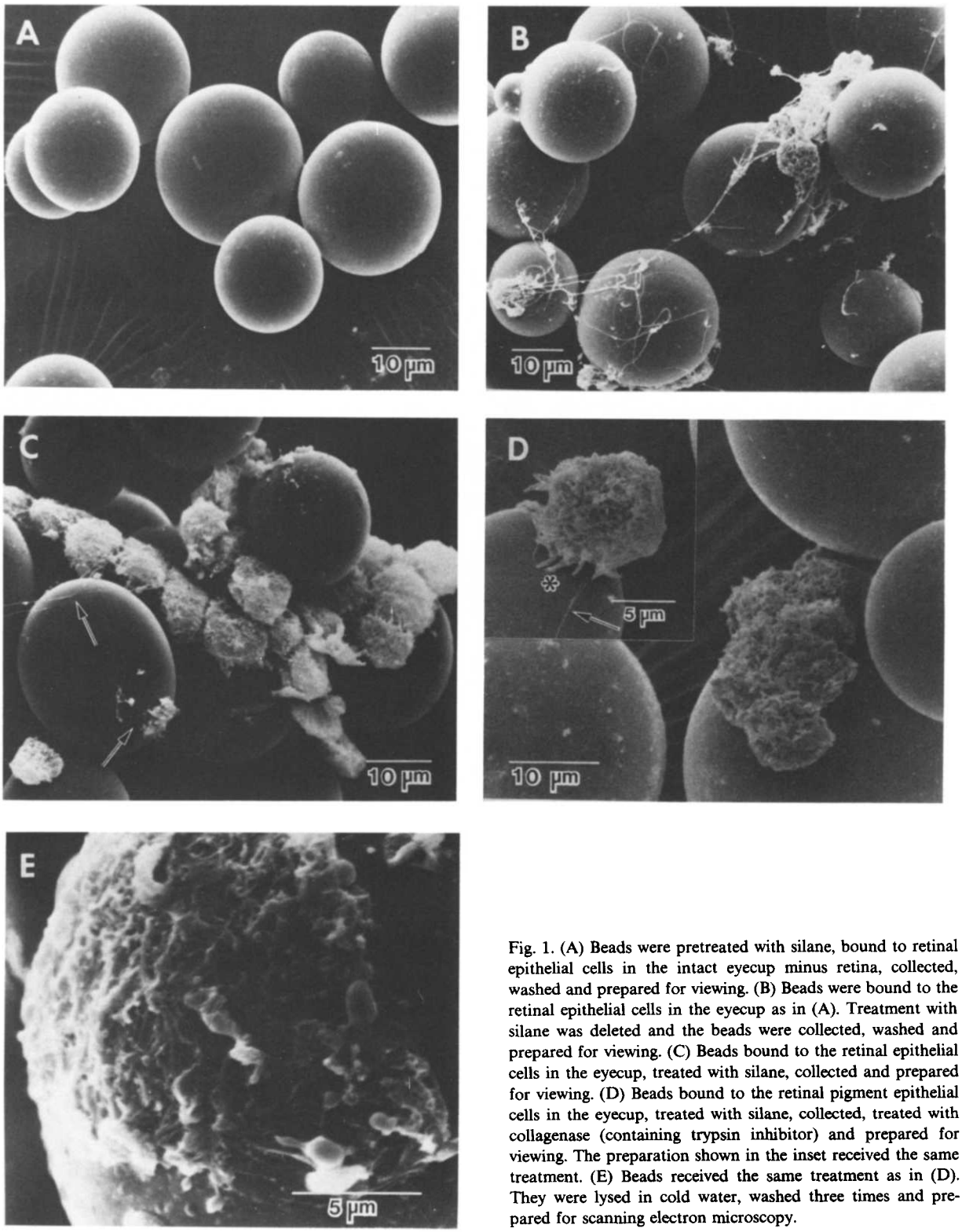

Fig. 1. (A) Beads were pretreated with silane, bound to retinal epithelial cells in the intact eyecup minus retina, collected, washed and prepared for viewing. (B) Beads were bound to the retinal epithelial cells in the eyecup as in (A). Treatment with silane was deleted and the beads were collected, washed and prepared for viewing. (C) Beads bound to the retinal epithelial cells in the eyecup, treated with silane, collected and prepared for viewing. (D) Beads bound to the retinal pigment epithelial cells in the eyecup, treated with silane, collected, treated with collagenase (containing trypsin inhibitor) and prepared for viewing. The preparation shown in the inset received the same treatment. (E) Beads received the same treatment as in (D). They were lysed in cold water, washed three times and prepared for scanning electron microscopy.

intracellular organelles during subsequent wash steps. However, membrane fragments not bound directly to the beads may also be lost by shearing forces resulting in a lower membrane yield.

The enzymatic profiles of the whole cell, bead and plasma membrane fractions are given in Table I. As shown in row 1, 5′-nucleotidase activity in the isolated plasma membrane fraction is 12-fold purified by specific activity and 10-fold purified by the corresponding relative specific activity. These membranes contained an average of 2–3% of the whole-cell 5′-nucleotidase activity. The bead fraction retained an average of 12% of the whole-cell 5′-nucleotidase activity and were 3.6- and 4.2-fold enriched by specific and relative specific activity criteria. Alkaline phosphodiesterase I (row 2) in the plasma membranes is 6.8-fold purified by specific activity and 9.4-fold purified by relative specific activity. Compared to the whole cell, ($Na^+ + K^+$)-ATPase is 6.4–6.5 fold purified in the isolated plasma membranes (row 3). In these plasma membranes, alkaline phosphodiesterase and ($Na^+ + K^+$)-ATPase activities were 1.7–2% of the whole cell activity. Summing activities in various fractions for the plasma membrane marker enzymes, 5′-nucleotidase, ($Na^+ + K^+$)-ATPase and alkaline phosphodiesterase were recovered in 98%, 93% and 70% yields, respectively. In these studies, less than 0.05% of whole cell protein and 0.02% of the nucleotidase activity was present in the 40% sucrose

TABLE I

PURIFICATION OF PLASMA MEMBRANES FROM BOVINE RETINAL PIGMENT EPITHELIUM

Results represent the mean ± S.D. for the number of analyses (n) in parentheses. RSA: Total amount of activity in fraction/total amount of protein in fraction. n.d., not detectable (assay sensitive to 2 μg mitochondrial protein.)

Marker enzyme	Whole cell	Bead		Plasma membrane	
	Spec. act. (RSA)	Spec. act. (RSA)	fold purity	Spec. act. (RSA)	fold purity
5′-Nucleotidase [a] ($n = 6$)	0.52 ± 0.17 (1)	1.87 ± 1.02 (4.18 ± 2.47)	3.6 (4.2)	6.41 ± 2.0 (10.00 ± 3.72)	12.3 (10.0)
Alkaline phosphodiesterase [b] ($n = 3$)	0.12 ± 0.02 (1)	0.06 ± 0.05 (0.18 ± 0.16)	0.5 (0.2)	0.82 ± 0.58 (9.40 ± 6.30)	6.8 (9.4)
($Na^+ + K^+$)-ATPase [a] ($n = 5$)	0.38 ± 0.13 (1)	0.20 ± 0.04 (0.63 ± 0.41)	0.5 (0.6)	2.48 ± 0.85 (6.63 ± 1.12)	6.4 (6.5)
[^{35}S]DABS [c]					
Expt. 1	143	584	4.1	2779	19.0
Expt. 2	602	3174	5.3	4894	8.1
Sulfatase C [d] ($n = 6$)	35.32 ± 8.98 (1)	57.33 ± 34.21 (1.64 ± 0.83)	1.6 (1.6)	62.63 ± 21.56 (1.98 ± 1.01)	1.8 (2.0)
N-Acetyl-β-D-glucosaminidase [b] ($n = 2$)	0.06 [0.05–0.07] (1)	0.04 [0.03–0.05] (0.65) [0.60–0.70]	0.7 (0.7)	0.001 [0.001–0.001] (0.015) [0.01–0.02]	0.02 (0.01)
Cytochrome oxidase [e] ($n = 7$)	0.03 ± 0.03 (1)	0.03 ± 0.03 (1.82 ± 2.56)	1.0 (1.8)	n.d.	
Protein [f] ($n = 18$)	1730 ± 740	42.81 ± 21.2		4.0 ± 2.0	

[a] nmol P_i/μg protein per h.
[b] nmol PNP/μg protein per h [range].
[c] Acid precipitated cpm/μg protein.
[d] Fluorescence units/μg protein per h.
[e] μmol O_2/μg protein per min.
[f] μg protein/eye.

layer above the beads. No plasma enzyme activities were detected in the supernatant from the precipitated plasma membranes.

Since contaminating plasma membranes from other cell types (e.g., choroid) probably contain the same enzymes, retinal pigment epithelial cell plasma membranes were labeled with [^{35}S]DABS in situ. In two such experiments (row 4; Table I), [^{35}S]DABS labeled plasma membranes were 8- and 19-fold purified, while the residual bead-bound radioactivity was 4–5-fold greater than in whole cells. The similarity of the fold purification of plasma membranes by 5′-nucleotidase and [^{35}S]DABS labeling suggests that these plasma membranes are not contaminated by plasma membranes from other ocular cells.

Contamination of retinal pigment epithelial plasma membranes by subcellular organelles was also determined by enzyme markers. Sulfatase C, (row 5) an endoplasmic reticulum specific enzyme in some cells [28], was 1.8–2.0-fold purified in the plasma membrane fraction, but it contained only 0.3% of the whole cell activity, whereas the bead fraction was 1.6-fold enriched in sulfatase C and contained 6% of the whole cell enzyme activity. In these experiments total recovery for sulfatase C activity averaged 90%. *N*-Acetyl-β-D-glucosaminidase activity was 50–100-fold decreased in the plasma membranes, with less than 0.01% of the lysosomal activity of whole cell (row 6). The mitochondrial marker, cytochrome oxidase (row 7), was undetectable in seven plasma membrane preparations (lower limit of detectability is 2 μg mitochondrial protein). In one preparation, however, cytochrome oxidase activity was 0.1 and relative specific activity 0.4 (data not shown). In the bead fraction, cytochrome oxidase was 1.8-fold enriched and it contained 3% of the whole cell activity. The plasma membranes also contained less than 0.3 μg DNA per 40 μg protein (data not shown).

The yield of plasma membrane protein (row 8; Table I) was 0.2% of the whole cell protein (4.0 μg/eye), while on the average, 43 μg of protein (2.5% of whole cell) remained bound to the glass beads. Several approaches to increase membrane yield were utilized, but in each instance increased protein yield resulted in decreased membrane purity. For example, 4% poly(ethylene glycol) treatment [17] of glass beads increased the yield of plasma membranes 10-fold. However, these membranes were only 4.5-fold purified by 5′-nucleotidase, while sulfatase C activity increased 5-fold (data not shown). A 5-fold increase in plasma membrane protein was achieved with smaller diameter glass beads (3–30 μm), but ($Na^+ + K^+$)-ATPase and 5′-nucleotidase were only 1.4- and 2.5-fold purified, respectively. Therefore, subsequent membrane characterization studies were performed with the higher purity plasma membranes.

Fig. 2 shows the SDS-polyacrylamide gel electrophoresis profile of proteins from the whole cell, bead and plasma membrane fractions stained with Coomassie blue (left) and the X-ray film (right) from a [^{35}S]DABS experiment. In this experiment the plasma membranes were 8.1-fold purified from whole cell. A comparison of the stained gel and X-ray film for the whole cell lanes reveals numerous protein bands of various molecular weights ranging from less than 14.4 kDa to greater than 94.0 kDa. Additional high-molecular-weight protein material did not enter the gel. The bead lane of the gel reveals a great reduction of stainable protein from whole cell, while the film shows a similarity in the radioactive bands between the two fractions. The plasma membrane lane of the Coomassie gel shows a loss of both high- (over 94 kDa) and low- (under 20 kDa) molecular-weight protein bands when compared to either bead or whole cell. In these plasma membranes, 23–26 protein bands were stained, while 12–16 bands were labeled. Two highly radioactive bands (15–18.0 kDa) are seen in the X-ray film. These proteins, however, did not stain intensely with dye and may be present in low concentration in the plasma membranes.

Table II presents the lipid composition of the retinal pigment epithelial whole cell, bead and plasma membrane fractions. All three fractions are characterized by high concentrations of phosphatidylcholine (PC) (40–54%) and phosphatidylethanolamine (PE) (22–26%). However, plasma membrane contains a higher concentration of PC and a slightly lower PE concentration than in whole cell. Moderate levels of sphingomyelin (9–11.8%) and phosphatidylinositol (PI) (7.5–8.6%) are contained in these fractions. Phosphati-

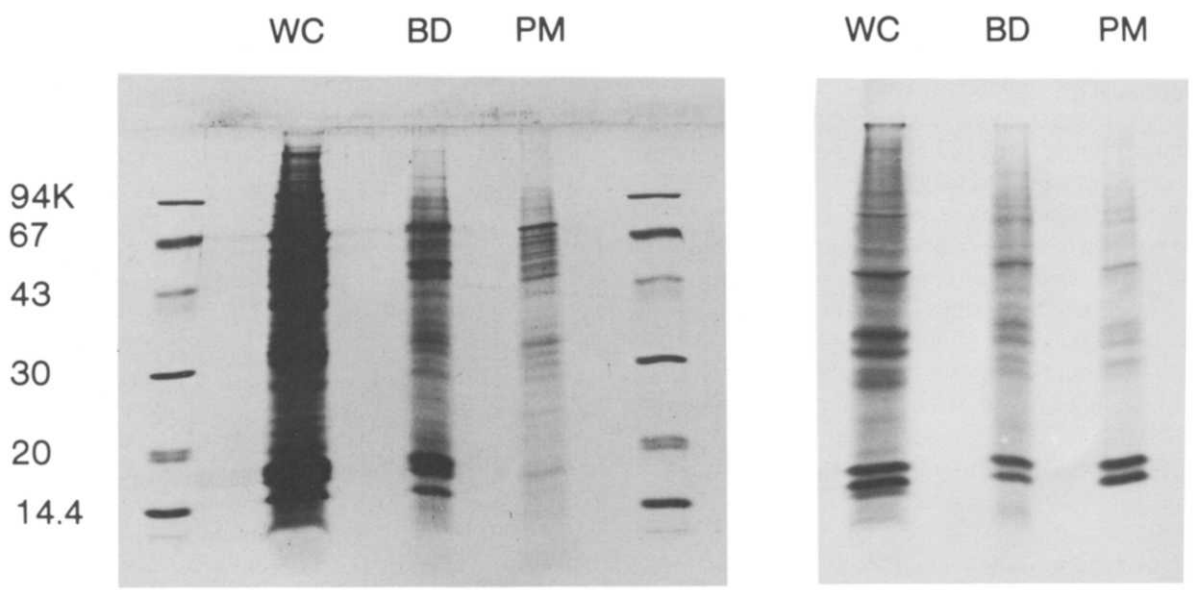

Fig. 2. Intact eyecups (minus retina) were incubated with [^{35}S]DABS for 10 min and the plasma membranes were then purified. The gel was first stained with Coomassie blue (left) and then treated with EN^{3}HANCE for 30 min, dried and exposed to X-ray film (right). WC, whole cell sample; BD, remaining bead sample and PM, plasma membranes.

TABLE II

LIPID COMPOSITION OF BOVINE RETINAL PIGMENT EPITHELIUM FRACTIONS

Results (% lipid phosphorus) are the mean ± S.D. for the number of determinations in parentheses. Abbreviations: DPG, diphosphatidylglycerol; PE, phosphatidylethanolamine; PA, phosphatidic acid; PS, phosphatidylserine; PC, phosphatidylcholine; PI, phosphatidylinositol; Sph, sphingomyelin; LPC, lysophosphatidylcholine.

Lipid	Whole cell (4)	Bead (5)	Plasma membrane (5)
DPG	2.6 ±0.2	1.5 ±0.7	1.3 ±0.7
PE	25.8 ±2.6	24.6 ±3.7	21.6 ±3.8
PA	0.6 ±0.6	0.7 ±0.5	1.1 ±1.1
PS	5.4 ±0.6	3.5 ±0.7	3.6 ±1.7
PC	39.9 ±2.4	48.7 ±2.8	54.0 ±4.9
PI	8.6 ±1.3	8.5 ±1.7	7.5 ±1.7
Sph	11.8 ±2.6	9.0 ±2.3	10.3 ±2.4
LPC	3.8 ±2.0	2.4 ±0.6	0.8 ±0.4
Chol/PO_4 [a]	0.33 ±0.03	0.52 ±0.06	1.1 ±0.2
Chol/μg protein	0.034 ±0.013		0.34 ±0.11

[a] Molar ratio cholesterol : lipid phosphorus.

dylserine (PS) and diphosphatidylglycerol (DPG) are found in lower and fairly constant amounts in all fractions. Lysophosphatidylcholine (lyso PC) ranged from less than 1% in plasma membranes to 3.8% of the whole cell lipids. The cholesterol : organic phosphate ratio of the whole cell lipid extracts averaged 0.33 mol/mol, while in beads it was 0.52. The plasma membranes, however, had a molar ratio of 1.1 cholesterol/phospholipid, which is 3.3-fold higher than in whole cell and 2-fold higher than the ratio in the beads. The fold enrichment of cholesterol (Chol/μg protein) in the plasma membranes fraction was 10.

The fatty acid profiles of total lipid extracts from the various fractions are shown in Table III. Palmitic acid (16 : 0) and stearic acid (18 : 0) are the major saturated fatty acids, which together represent 50 mol% of the total in whole cell and beads and nearly 60% of the plasma membrane fatty acids. These saturated fatty acids are slightly higher in plasma membrane compared to whole cell (16 : 0; 40–34% respectively; 18 : 0; 18–15%). Oleic acid (18 : 1) and arachidonic acid (20 : 4) are the major unsaturates, together representing nearly

TABLE III

FATTY ACIDS OF BOVINE RETINAL PIGMENT EPITHELIUM MEMBRANE LIPIDS

Results (mol%) represent the mean ± S.D. for the number of determinations in parentheses. Abbreviations stand for chain length: number of double bonds; 22:5$^{w6 \text{ or } w3}$ identify the first double bond from the methyl end for this pair of equal chain length fatty acids.

Fatty acid	Whole cell (6)	Bead (6)	Plasma membrane (7)
14:0	1.9 ± 0.6	4.1 ± 1.1	6.5 ± 2.0
16:0	34.4 ± 1.8	35.1 ± 1.6	39.6 ± 2.8
16:1	2.8 ± 1.0	4.5 ± 1.6	6.8 ± 2.0
18:0	15.4 ± 0.5	14.9 ± 1.1	17.8 ± 3.4
18:1	12.5 ± 1.9	14.1 ± 1.9	14.2 ± 0.7
18:2	9.2 ± 1.0	8.9 ± 0.8	5.4 ± 0.4
20:4	12.1 ± 0.9	8.1 ± 1.7	3.0 ± 1.7
20:5	0.7 ± 0.2	1.4 ± 1.0	0.7 ± 0.7
22:5^{w6}	1.0 ± 0.6	1.2 ± 1.0	0.9 ± 0.3
22:5^{w3}	2.2 ± 0.4	1.8 ± 0.8	0.6 ± 0.5
22:6	6.1 ± 0.7	4.4 ± 0.7	2.3 ± 1.1
Satd./unsatd.	1.1	1.2	1.8

25% of the whole cell and 17% of the plasma membrane fatty acids. While the 18:1 content of the various fractions remained fairly constant (12–14%), 20:4 decreased from 12 mol% in whole cell to 3 mol% in the plasma membrane lipids. Linoleic acid (18:2) also decreased from 9% in whole cell lipid to 5% of the plasma membrane fatty acids. The polyunsaturated fatty acid docosahexaenoic acid (22:6) which is 47 mol% of bovine rod outer segment lipid fatty acids [34], is 6% of the retinal pigment epithelial whole cell fatty acids. Plasma membrane lipids contain only 2.3% 22:6, which is nearly 3-times lower than in whole cell and 20-times lower than reported for rod outer segments [34]. The saturated to unsaturated fatty acid ratio of the plasma membrane lipids is 1.8, while the whole cell and bead lipid ratios are 1.1 and 1.2, respectively. Taken together, the data in Tables II and III indicate that the plasma membrane lipids of retinal pigment epithelium contain more cholesterol and saturated fatty acids than the lipids of intact epithelial cells.

Discussion

The location of the retinal pigment epithelial cell layer within the eye and its vital role in maintaining the visual process has stimulated interest in compositional and functional studies. Morphologically intact retinal pigment epithelial cells have previously been isolated and characterized [2,14,15,18]. Recently, Siakotos et al. [17] employed a glass bead column to recover these cells in high yield. In previous studies, however, contamination of retinal pigment epithelium with other ocular cells, rod outer segments or red blood cells could not be quantitated. The use of the glass-bead binding technique has advantages for the isolation and the study of epithelial cell plasma membranes. For example, red blood cells do not bind to glass beads [17], which eliminates one potential contaminant. The affinity of retinal pigment epithelium for glass surfaces also results in a higher effective cell density. Thus, bound cells sediment rapidly, while those contaminants which remain in suspension can be removed. This is especially advantageous after treatment of the bound epithelial cells with collagenase. Following collagenase, choroid cells and fragments of Bruch's membrane are removed from intact cells in subsequent steps.

Two lines of evidence support this contention. First, electron micrographs of the post-collagenase treated cells (Fig. 1D) are largely devoid of cellular debris and fibrous strands which may arise from Bruch's membrane. The size and cell morphology of these cells also suggest that they are retinal pigment epithelial cells and not choroid cells. Similarly, in examination of other micrographs of bound cells, rod outer segments and red blood cells were not observed. Second, the use of [^{35}S]DABS, a membrane labeling agent which does not penetrate intact cells [23], shows 8–19-fold purification which is similar to the purification of plasma membranes by 5′-nucleotidase (Table I). This suggests that the enzyme activity associated with the purified plasma membranes is that of retinal pigment epithelium and not that of other ocular cell plasma membranes. In addition, after collagenase treatment of isolated retinal epithelial cells, the aspirated material (e.g., choroid, Bruch's membrane) contained little radioactivity but considerable protein.

While collagenase treatment removes or dramatically reduces choroidal contamination, it may also loosen pigment epithelial cells that are not directly bound to the beads. This loss could account for the lower whole cell protein recovered with our procedure in comparison to previous studies [2,16,17]. As a result, the yield of plasma membrane is a major drawback of this technique. Another source of plasma membrane loss may occur after cell lysis, therefore, pigment epithelial plasma membranes must be handled carefully to minimize shearing forces. The inability of vortexing to release all of the glass bead bound membranes is a third source of plasma membrane loss (see Table I). In attempts to increase yield (e.g., smaller beads; 4% poly(ethylene glycol)), membrane yield increased 10-fold but purity decreased 3-fold, while contamination by endoplasmic reticulum also increased 5-fold.

The use of a silane solution to coat the unbound surfaces of glass beads is also effective in reducing plasma membrane contamination by extraneous proteins and subcellular organelles. Although the majority of rod outer segments are removed from epithelial cells by soaking the eyecups after removal of the retinas, control studies showed that silane also displaces 25% of bound rod outer segments. Similarly, 10% of bound mitochondria were removed from glass beads by silane treatment. The bead fraction also appears to retain the remaining bound mitochondria during isolation, as cytochrome oxidase was undetectable in seven plasma membrane preparations (Table I). At the concentration of silane used in these studies (0.1%), no effects on the activities of 5′-nucleotidase, alkaline phosphodiesterase or $(Na^+ + K^+)$-ATPase were detected.

In all studies of isolated plasma membranes, one concern is that the procedure may result in the purification of a small non-representative fraction of the membranes. For retinal pigment epithelial cells it is known that $(Na^+ + K^+)$-ATPase is an apical plasma membrane marker [7]. Assuming that 5′-nucleotidase and alkaline phosphodiesterase are uniformly distributed in these plasma membranes, then the fold purifications of these enzymes indicate that these membranes contain both basal and apical plasma membranes. Likewise, if 5′-nucleotidase is located on the basal and lateral portions of the plasma membrane, as in intestinal villous cells [11], our membrane preparations are still representative of the epithelial cell plasma membranes. Although we did not measure retinol-binding protein receptor, study of this known retinal epithelial cell basal membrane marker [8–10] will allow a more quantitative estimate of the apical and basal membrane content in these plasma membranes.

Analysis of subcellular organelle marker enzymes indicates that lysosomal and mitochondrial contamination of these plasma membranes is minimal (Table I). As judged by sulfatase C activity, endoplasmic reticulum is 2-fold enriched in these plasma membranes. However, it should be noted that sulfatase C activity accounted for only 0.3% of the whole cell activity. Retinal pigment epithelial cells are known to contain substantial amounts of endoplasmic reticulum [35] which may be continuous with plasma membrane. Thus, endoplasmic reticulum may be co-purified with the plasma membrane fraction to a small extent. Sulfatase C activity was also found in the gradient at a density of 40% sucrose while our plasma membranes were isolated at the 32% sucrose/water interface. It appears therefore, that retinal epithelium endoplasmic reticulum has a slightly higher density than plasma membrane and that a small fraction of endoplasmic reticulum (that continuous with plasma membrane) is isolated with this procedure.

SDS-polyacrylamide gel electrophoresis profiles of the whole cell, bead and plasma membranes indicates that in the plasma membrane fraction a loss of both high- and low-molecular-weight proteins occurred (Fig. 2). Further, a comparison of the Coomassie stained gel and the corresponding X-ray film shows that approximately one-half of the stainable protein bands contain detectable radioactivity. These radioactively labeled proteins may be more accessible to the [^{35}S]DABS reagent or may contain higher concentrations of reactive amino acid residues [23] than the unlabeled proteins. However, the possibility that some of the unlabeled proteins may arise from intracellular membranes cannot be excluded. The two low-molecular-weight protein bands (15–18 kDa) highly labeled by [^{35}S]DABS do not appear to arise from proteolytic activity during the isolation

procedure. Studies with and without the use of proteolytic inhibitors during membrane isolation revealed no detectable differences in molecular weight or concentration of these two proteins. Recently, McLaughlin and Cooper [36] reported the isolation of retinal pigment epithelial membrane microvilli. They also found two low-molecular-weight bands (12.3 and 18.4 kDa) similar to those reported in this study. Although we cannot completely rule out proteolytic degradation as a source of these proteins, they may be subunits of larger membrane proteins.

The lipid class composition of the plasma membranes reveals that despite the close proximity of retinal pigment epithelium and rod outer segments in the eye, the two types of membranes are very different (Table II). In both the retinal epithelium and rod outer segments, PC and PE are the major glycerophospholipids. In pigment epithelial plasma membranes however, these lipids are present at a 2.5 : 1 molar ratio which is in contrast to a ratio of 0.8 PC to PE in membranes of bovine rod outer segments [34]. Likewise, PS is 5-times lower and sphingomyelin approx. 10-times higher in pigment epithelium plasma membrane than in rod outer segments [34]. Retinal pigment epithelial plasma membranes contain an average of 1 mol cholesterol per mol of lipid phosphorus versus a molar ratio of 0.1–0.13 mol/mol in total rod outer segment membranes from rat [22]. This striking difference indicates that the retinal epithelium plasma membrane may be less fluid than the opposed rod outer segment membranes.

The retinal epithelial cell lipid profile in this study (Table II) and that of Anderson et al. [16] are similar, despite the different methodologies employed in these two studies. In this study retinal epithelial cells are judged to be largely devoid of choroid contamination, a factor which may contribute to the differences in lipid composition in this study and the previous work [16]. At this time, we have no way to conclude that phospholipase activity did not occur during our isolation procedure. However, as judged by the lyso PC content of our whole cell lipids this is estimated to be less than 10%.

The plasma membrane fatty acid profiles (Table III) indicate that the epithelial cell membrane is composed largely of saturated fatty acids, which is in contrast to the fatty acid profile of vertebrate rod outer segment membranes [34] (for review, see Daemen [37]). The content of 22 : 6 is only 6% of whole cell lipids and 2.3% of plasma membrane lipids, enforcing the suggestion that these plasma membranes are less fluid than those of total rod outer segments. A comparison of the plasma membrane fatty acid profiles from rod outer segments and the retinal pigment epithelium also indicates that our plasma membranes contain at least a 10-fold lower 22 : 6 content than the plasma membrane of rod outer segments [38].

From a consideration of the lipid profile, it is clear that the retinal epithelial plasma membrane which contains a high cholesterol/phospholipid ratio and low polyunsaturated fatty acid content resembles the plasma membranes of other cell types, including liver [39]; fibroblast [40,41], myoblasts [42] and red blood cells [43] (for review, see Rothfield [44]). The lipid characteristics of these plasma membranes are thought to contribute to a more rigid membrane than for most subcellular organelles [40,43]. Because of the functional similarity between retinal pigment epithelium and other epithelial cells [11] the glass-bead procedure may prove effective in the isolation of plasma membranes from other epithelial cells. How and whether the lipid makeup of the retinal pigment epithelial plasma membranes influences the processes of rod cell metabolism, vitamin A exchange and phagocytosis remains to be resolved.

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