Isolation and Provisional Identification of Plasma Membrane Populations from Cultured Human Retinal Pigment Epithelium

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We have attempted to isolate samples of apical and basal-lateral plasma membranes from cultured fetal human RPE. Cells from confluent, dome-forming cultures were disrupted with a Dounce apparatus. Nuclei and melanin granules were sedimented by centrifugation at 2600 g for 10 min. The supernates were layered over gradients of 17.5--65% sorbitol and centrifuged at 122,000 g for 5 hr. Fractions were grouped into “density windows” on the basis of their biochemical marker contents. Na,K-ATPase and alkaline phosphatase overlapped but did not precisely parallel one another, suggesting associations with two partially separated membrane populations; in density window I, alkaline phosphatase was enriched 4.3-fold, and Na,K-ATPase was enriched 1.7-fold, whereas in window II the corresponding enrichment factors were 7.7 and 6.7. These markers were well resolved from a mitochondrial marker, but they were overlapped by endoplasmic reticulum and Golgi markers. Additional density gradient centrifugations, performed after samples had been suspended in 55% sorbitol, further separated alkaline phosphatase- and Na,K-ATPase-containing membranes from endoplasmic reticulum and Golgi membranes, yielding alkaline phosphatase and Na,K-ATPase cumulative enrichment factors of 6.8 and 2.5 for the sample from window I and 9.3 and 10.9 for the sample from window II. Subsequent phase partitioning analysis of the sample from window I further enriched an alkaline-phosphatase-rich membrane population, which is believed to represent the RPE basal-lateral membranes. The sample from density window II contained two membrane populations, both enriched in Na,K-ATPase, alkaline phosphatase, and galactosyltransferase, and both of which appear to be derived from the apical plasma membrane. SDS-PAGE and Western blotting confirmed a correlation between Na,K-ATPase catalytic activity and Na,K-ATPase alpha subunit immunoreactivity. Invest Ophthalmol Vis Sci 31:863--878, 1990

In the vertebrate eye the outer segments of the photoreceptors and the apical membranes of the retinal pigment epithelium (RPE) face each other across an extracellular, or subretinal, space. Transitions between light and dark induce a variety of interactions between the two cells, which have a neuron--glial relationship. For example, the RPE cells phagocytose the rod and cone outer segments and thereby participate in the continual cycle of inner segment photoreceptor synthesis and outer segment disc shedding.1--3 At the same time, there is a considerable traffic of vitamins, amino acids, ions, and water through the RPE.4--10 This transepithelial traffic, which helps modulate the ionic compositions of the cellular milieu and the subretinal and choroidal spaces, is mediated, in part, by a variety of ion pumps, channels, cotransporters, and exchangers present in the RPE apical and basal-lateral plasma membranes.11--13 Many of these transport activities are influenced by small changes in extracellular or subretinal K+ concentration which mimic the light/dark cycle.5,11,12

The recent development of methods for isolating and maintaining RPE in long-term culture14 promises to open new avenues for investigation of mechanisms that underlie the transcellular movement of...
vitamin A and the phagocytosis of photoreceptor outer segments. However, many of the features which complicate studies of electrolyte transport mechanisms in explant preparations will also be present in RPE cultures. For example, all of the ion transporters normally function in concert, so that experimental perturbations of one transport process may indirectly alter the activities of other transporters. Furthermore, voltage changes at one surface of the epithelium are passively shunted to the opposite surface of the epithelium, and voltage clamping of individual cell membranes is impossible because the cells are connected by gap junctions, forming a syncitium in the plane of the epithelial sheet. Finally, although the changes in cytoplasmic ion activities that can be detected with ion-selective microelectrodes can be corrected for changes in cell volume to yield measurements of net ionic fluxes, these measurements do not necessarily provide for accurate estimates of the ionic unidirectional flux rates.

Cell-free preparations should allow one to avoid many of the technical problems that intact epithelial preparations pose for biophysical studies. The electroneutral transporters can be characterized with methods that use radiotracers, pH-sensitive dyes, and ion-sensitive dyes to measure ionic fluxes into isolated membrane vesicles. Purified membrane samples also may be useful in studies of the transport of vitamin A and of photoreceptor outer segment constituents. Therefore, we have devised methods for the analytic isolation of plasma membrane vesicles from cultured human RPE cells. Our strategy has been to survey the distributions of a battery of membrane markers after each step of a sequence of separations. The marker distribution patterns allowed us to delineate membrane populations which were enriched in Na,K-ATPase and alkaline phosphatase, two enzyme activities that have frequently been found in the plasma membranes of epithelial cells. Two similar populations, provisionally designated a' and a", were characterized by the large enrichment factors for Na,K-ATPase and alkaline phosphatase expected for apical plasma membranes. A third population, designated b, was characterized by an excess of alkaline phosphatase over Na,K-ATPase, and therefore, was provisionally identified as basal-lateral membranes.

Materials and Methods

Long-Term Culture of Human RPE

Primary cultures: Cultures of human RPE cells were established from aborted fetuses ranging from 16–22 weeks gestation. Eyes were enucleated, irrigated with sterile saline solution, and immersed in basal tissue culture medium at 4°C until dissection. Under sterile conditions, the eyes were opened approximately 1 mm posterior to the corneal–scleral junction, and the cornea, lens, iris, ciliary body, and vitreous body were removed. The neurosensory retina was cut at its attachment to the optic nerve head and removed. The remaining eyecup then was cut into quadrants which were transferred to a 100-mm culture dish (no. 1007; Falcon Plastics, Oxnard, CA) containing Ca²⁺- and Mg²⁺-free Konigsberg's Hank's balanced salt solution with glucose added, pH 7.3. Pieces of RPE-choroid were separated from the sclera and transferred to another 100-mm culture dish containing balanced salt solution. Fine forceps were used to peel away sheets of RPE from the choroid under a dissecting microscope. Frequently, portions of Bruch's membrane remained with the epithelial sheets. Each quadrant yielded one to several sheets of RPE, which were trimmed to eliminate traces of choroidal tissue.

The RPE sheets were transferred to conical centrifuge tubes containing 13 ml fresh balanced salt solution. The sheets were washed and sedimented at 100 g three times. Finally, the balanced salt solution was replaced with low-calcium CEM 2000 growth medium (Scott Laboratories, Fiskeville, RI). Basal culture medium lacking calcium was adjusted to 0.05 mM with CaCl₂, and trace elements and hormones were added as follows: selenous acid, Na⁺ salt (Collaborative Research, Cambridge, MA), 1.75 μg/ml; hydrocortisone (Sigma, St. Louis, MO), 10.0 μg/ml; heat-inactivated calf serum (Hazelton Biologies, Lenexa, KS), 1% by volume; linoleic acid complexed with albumin (Sigma), 84 μg/ml; transferrin (Sigma), 5.0 mg/ml; bovine insulin (Sigma), 5.0 mg/ml; putrescine-HCl (Collaborative Research), 0.3 mg/ml; L-glutamine (Sigma), 292 mg/ml; and triiodothyronine (Sigma), 6.5 ng/ml. Additionally, 1% bovine retinal extract was added to the growth medium. Bovine retinal extract was prepared by briefly sonicating 12 freshly dissected bovine retinas (50 W, Branson Tech 1510) in 100 ml Ca²⁺- and Mg²⁺-free Konigsberg's balanced salt solution at 4°C until no large retinal fragments were visible. The suspensions then were stirred at 4°C for 2 hr in the dark. The mixture was centrifuged at 17,000 × g for 20 min, and aliquots of the supernatant were frozen at −75°C for future use.

Sedimented RPE sheets were transferred from the centrifuge tube to 100-mm-diameter tissue culture dishes (no. 25020; Corning, Corning, NY) containing 7 ml low-Ca²⁺ growth medium. Cultures were maintained in an incubator at 37°C with an atmosphere of 5% CO₂ and 95% air.

Secondary cultures: After approximately 2 weeks in low-Ca²⁺ medium, many individual, rounded cells lifted from the culture dishes and floated in the me-
Subcultures from these floating cells were established by centrifuging them at 100 g for 2.5 min and seeding them at a density of $1.2 \times 10^5$ cells/cm$^2$ on 100-mm plastic dishes containing growth medium with normal Ca$^{2+}$ (1.5 mM). The basal medium consisted of a 1:1 mixture of CEM 2000 and Eagles's Minimal Essential Medium (Scott Laboratories). Trace elements and hormones were added as before. ZnSO$_4 \cdot 7$ H$_2$O, 72 $\mu$g/l; CuSO$_4 \cdot 5$ H$_2$O, 125 ng/l; MnCl$_2 \cdot 4$ H$_2$O, 50 ng/l; and L-ascorbic acid (Sigma), 22.5 mg/l also were added, as was 0.5% bovine retinal extract. For the initial medium change, the serum concentration was raised to 10% to promote attachment. Subsequent medium changes had a serum concentration of 1%. The cell cultures were maintained for 3-7 months prior to analysis. An example of a secondary culture of human RPE is shown in Figures 1 and 2.

Cell Disruption and Differential Centrifugation

For each experiment (of a total of four), cells were collected from 5 of the 100-mm culture dishes. The total number of cells per experiment, estimated by counting selected areas on each culture dish, ranged from $7.7 \times 10^7$ to $1.6 \times 10^8$. The culture medium was removed, and each dish was rinsed with 1 ml isolation buffer, which contained 5% sorbitol (analyzed reagent; J. T. Baker, Phillipsburg, NJ), 0.5 mM NaEDTA, and 5 mM histidine-imidazole, pH 7.5). For the initial harvesting and cell disruption, isolation buffer was supplemented with 0.1 mM phenylmethylsulfonylfluoride, 5 $\mu$g/ml pepstatin, 5 $\mu$g/ml aprotinin, 5 $\mu$g/ml leupeptin, and 5 $\mu$g/ml chymostatin (Sigma) as protease inhibitors. The cells were scraped from the dish with a Teflon$^\circledR$ policeman and pipetted into a 15-ml Dounce homogenizer (Wheaton Scientific, Millville, NJ). Each plate was rinsed with 0.5 ml buffer, which then was combined with the harvested cells. The cells were homogenized by 50-100 passes with pestle A (clearance 25-76 $\mu$m) until 95-99% of the cells were disrupted while nuclei remained intact. Verification of cell disruption was made by phase microscopy.

The initial homogenate was centrifuged at 2600 g for 10 min. After removal of the supernatant, the pellet was resuspended in 4 ml isolation buffer, subjected to 30 gentle strokes of the Dounce apparatus, and again centrifuged at 2600 g for 10 min. The pellet, designated P$_5$, was resuspended in 4 ml isolation buffer.
buffer and saved for biochemical marker determinations. The two low-speed supernatants, together designated $S_0$, were pooled and further analyzed by a sequence of separations that involved two different centrifugations on preformed sorbitol density gradients and partitioning in an aqueous polymer two-phase system; these procedures are summarized by the flow diagram in Figure 3.

**Density Gradient Centrifugation**

Density gradients were constructed in 38-ml Ultra-clear® tubes (Beckman, Fullerton, CA) from stock solutions of different sorbitol concentrations, which are expressed as wt/vol; all solutions contained the basic isolation buffer supplemented with 0.2 mM phenylmethylsulfonylfluoride and 9 μg/ml aprotinin. A peristaltic pump was used to transfer the gradient media from a standard two-chamber gradient maker to the centrifuge tube, the end of the delivery being held at the bottom of the centrifuge tube by a Buchler Auto-Densi Flow®. The gradient maker mixing chamber was initially loaded with 16.5 ml 17.5% sorbitol; its volume was held constant with a piston placed at the fluid surface, and it was mixed continuously with a magnetic stir bar. A total of 27.2 ml of a high-density solution was delivered through the mixing chamber. For the first density gradient centrifugation, the high-density solution contained 65% sorbitol, and it was followed by a 3.0 ml cushion of 70% sorbitol. For the second density gradient centrifugation, the high-density solution contained 70% sorbitol. Both gradients were followed by cushions of 3 ml 80% sorbitol.

$S_0$ was layered on to the top of the first density gradient. After 5 hr centrifugation at 122,000 $g$ in an SW 28 rotor, the gradient was collected in 12 equal-volume fractions. Each fraction was diluted to 26 ml with isolation buffer and centrifuged at 250,000 $g$ for 60 min. The high-speed supernatants, collectively designated $Σ S_0$, were pooled, and an aliquot was saved for biochemical marker determinations. Each of the series of high-speed pellets, designated $Σ P_1$, was resuspended in 1 ml isolation buffer. Pellets
No. 5 PLASMA MEMBRANES FROM CULTURED HUMAN RPE / Mircheff et al 867

Homogenate

Centrifuge 2,600 g x 10 min
Resuspend pellet and repeat homogenization and centrifugation

\[ P_e \]

\[ S_p \]

Layer over 17.5% to 65% sorbitol gradient and centrifuge 122,000 g x 5 hr

\[ IF \] (first gradient)

Dilute with isolation buffer and sediment membranes at 250,000 g x 60 min

\[ IS_1 \]

\[ IP_1 \]

Density window I
Density window II
Increase sorbitol concentration to 55%.
Insert into 17.5% to 70% sorbitol gradients, and centrifuge 122,000 g x 5 hr

\[ IS_2 \]

\[ IP_2 \]

Dilute with isolation buffer and sediment membranes at 250,000 g x 60 min

\[ IS_3 \]

\[ IP_3 \]

Fraction 4 from density window I
Fraction 4 from density window II
Fraction 5 from density window II
Analyze by phase partitioning

\[ IF \] (partitioning)

Fig. 3. Summary of the analytic subcellular fractionation procedure.

which formed beneath the 80% sorbitol cushion of the initial density gradient were also resuspended in 1 ml isolation buffer; they were designated fraction 13.

As described under Results, selected pooled fractions from the first density gradient were mixed with concentrated sorbitol (1.3 g 87.4% sorbitol solution per gram sample suspension) and inserted into the 55% sorbitol regions of gradients for the second density gradient centrifugation step. Subsequent centrifugation, fraction collection, and high-speed sedimentation of membranes were as described above.

Phase Partitioning

Selected fractions from the second density gradient centrifugation analysis were analyzed further by phase partitioning in the 120-chamber thin-layer counter-current distribution apparatus described by Albertsson et al.17 This method has been used for subcellular fractionation of a number of different epithelia.18-21 The two-phase systems contained 5% dextran T-500 (Pharmacia Fine Chemicals, Piscataway, NJ), 3.5% polyethylene glycol (Carbowax® 8000; Union Carbide, Danbury, CT), 5% sorbitol, 10 μM EDTA, and 8.33 mM imidazole-HCl, pH 6.8. Phase systems were prepared the day before use and were placed in separatory funnels to equilibrate overnight at 4°C. The circular plates were divided into sectors of 40 chambers each so that three different samples could be analyzed simultaneously. All chambers were loaded with 0.65 ml lower phase. Chambers 5–40 of each sector were loaded with 0.65 ml upper phase, and chambers 1–4 of each sector were loaded with 0.65 ml upper phase in which the sample to be analyzed had been resuspended by low speed homogenization with a Tissumiser® (Tekmar Instruments, Cincinnati, OH). After 36 transfer steps, the contents of each chamber were diluted with 1 ml isolation buffer and emptied into fraction collector tubes. The phase partitioning fractions were pooled into groups of four, and each pooled fraction was further diluted to a total volume of 26 ml. Membranes were harvested by centrifugation at 250,000 g for 60 min, and then resuspended in 1-ml aliquots of isolation buffer.

Analytical Methods

Na,K-ATPase was determined from the K+-dependent p-nitrophenylphosphatase reaction as described by Murer et al22; alkaline phosphatase as described by Mircheff and Wright23; acid phosphatase as described by Michell et al24; NADPH-cytochrome c reductase as described by Mircheff et al25; and succinate dehydrogenase as described by Pennington.26 Galactosyltransferase was measured with the procedure of Rao et al27 as modified by Ahnen et al,28 with UDP-[3H]-galactose (Amersham, Arlington Heights, IL) as donor. Protein was measured with the Bio-Rad (Richmond, CA) dye binding kit. Marker and protein contents of subcellular fractions were expressed as percentages of the total activities recovered after the sequence of separation steps15; the ratio of percent recovered marker activity to percent recovered protein represents the marker cumulative enrichment factor.

Sodium Dodecyl Sulfate–Polyacrylamide Gel Electrophoresis (SDS-PAGE) and Western Blotting

Selected fractions were analyzed by SDS-PAGE according to the method of Laemmli.29 Membrane samples were dissolved in dissociation buffer con-
taining a final concentration of 1% SDS and 5% beta-
mercaptoethanol and were not heated prior to loading
on the gel. Individual gel lanes were loaded with 1
µg protein for silver staining and 2 µg protein for
Western blotting. Ten percent acrylamide gels, 0.5
mm in thickness, were run at constant voltage (200
V). Gels were stained with silver according to Oakley
et al.30 Na,K-ATPase was detected by Western blot-
tting according to the methods of Towbin et al.31
SDS-PAGE-separated proteins were electrophoresed
onto nitrocellulose sheets for 16 hr at a constant cur-
rent of 95 mA. Detection was achieved with a 1:500
dilution of rabbit polyclonal antiserum directed
dilution of goat anti-rabbit IgG conjugated with horseradish peroxidase (Bio-
Rad). Color development was with the Bio-Rad color
development reagent.

Results

Cell Culture

After 1 month, the RPE secondary cell cultures
were heavily pigmented (Fig. 1) and exhibited a pro-
nounced apical–basal polarity with dense apical mi-
crovilli and well-developed basal infoldings as deter-
mined by electron microscopy (data not shown). The cultures formed numerous fluid-filled domes (Fig. 2), a presumed indication of apical-to-basal fluid trans-
port.

Differential Sedimentation

The combination of differential sedimentation steps, ie, low-speed centrifugation of the initial homoge-

nate followed by high-speed centrifugation of the density gradient fractions, separated subcellular components into 3 “windows” encompassing three ranges of sedimentation coefficients. Po contained the most massive particles; Pj, particles of intermediate sedimentation coefficient; and SI, soluble constituents. Melanin obviously was concentrated in Po. As indicated in Table 1, Po also contained 52% of the

succinate dehydrogenase and 64% of the acid phos-
phatase. The Na,K-ATPase and alkaline phosphatase
contents of P0 varied markedly among experiments, sug-

gesting that there might have been a large diversity
in the sizes of plasma membrane fragments generated
during cell disruption. In three of four experiments, Po contained large excesses of Na,K-ATPase over alka-
line phosphatase; in the fourth, it contained a 3-fold excess of alkaline phosphatase over Na,K-ATPase.

The discrepancies between the yields of Na,K-
ATPase and alkaline phosphatase in Po are consistent
with the suggestion that the RPE cell plasma mem-
brane contains distinct Na,K-ATPase-rich and alka-
line-phosphatase-rich domains which tend to frag-
ment differently during cell disruption. However, the
relatively low and variable yields of Na,K-ATPase in Po made this fraction unsuitable as a starting point for plasma membrane isolation. On the other hand, the
existence of separate Na,K-ATPase-rich and alkaline-phosphatase-rich membrane populations was
confirmed by fractionation analyses of SI.

First Density Gradient Centrifugation

Figure 4 summarizes the density distributions of
biochemical markers from a typical experiment. Membrane populations which accounted for most of the alkaline phosphatase and Na,K-ATPase activities

<table>
<thead>
<tr>
<th>Marker</th>
<th>Initial</th>
<th>Po (mm)</th>
<th>Σ Pj (mm)</th>
<th>Σ SI (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na,K-ATPase (4)</td>
<td>7.0 ± 1.2</td>
<td>35.9 ± 20.0</td>
<td>59.5 ± 19.5</td>
<td>4.7 ± 5.6</td>
</tr>
<tr>
<td>Alkaline phosphatase (4)</td>
<td>2.6 ± 2.4</td>
<td>13.0 ± 14.4</td>
<td>76.5 ± 8.1</td>
<td>10.4 ± 9.6</td>
</tr>
<tr>
<td>Acid phosphatase (4)</td>
<td>187.9 ± 33.7</td>
<td>63.8 ± 15.3</td>
<td>22.9 ± 8.3</td>
<td>13.3 ± 10.9</td>
</tr>
<tr>
<td>Succinate dehydrogenase (3)</td>
<td>1.4 ± 0.1</td>
<td>51.6 ± 9.8</td>
<td>41.7 ± 11.5</td>
<td>6.7 ± 4.4</td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase (2)</td>
<td>161.5 ± 19.1</td>
<td>6.6 ± 9.3</td>
<td>55.2 ± 8.5</td>
<td>38.3 ± 0.8</td>
</tr>
<tr>
<td>Galactosyltransferase (2)</td>
<td>135.9 ± 91.5</td>
<td>9.3 ± 0.0</td>
<td>84.7 ± 1.5</td>
<td>6.0 ± 1.5</td>
</tr>
<tr>
<td>Protein (4)</td>
<td>58.5 ± 6.0</td>
<td>45.4 ± 9.7</td>
<td>21.0 ± 3.7</td>
<td>33.6 ± 6.1</td>
</tr>
</tbody>
</table>

Values for marker contents in the initial low-speed pellet, the series of high-speed pellets, and the series of high-speed supernatants are mean percentages of total recovered activities ± standard deviations, or for NADPH-
cytochrome c reductase and galactosyltransferase, ± ranges. Numbers in parentheses are numbers of preparations in which markers were determined. Initial amounts are in µmoles/hr for Na,K-ATPase, alkaline phosphatase,
Fig. 4. First density gradient centrifugation analysis. S<sub>p</sub>, was layered over gradients of 17–65% sorbitol and centrifuged for 5 hr at 122,000 g in an SW 28 rotor. Fractions were collected and diluted, and membranes were harvested by high-speed centrifugation.

Na,K-ATPase densities in S<sub>p</sub>, were centered between fractions 3 and 7. They were well resolved from a mitochondrial population, marked by succinate dehydrogenase, and from acid-phosphatase-rich particle populations which equilibrated in fractions 11 and 12 or which sedimented through the 80% sorbitol cushion. A third peak in the acid phosphatase density distribution overlapped the major peaks of alkaline phosphatase and Na,K-ATPase. The endoplasmic reticulum marker, NADPH-cytochrome c reductase, and the trans Golgi marker, galactosyltransferase, had broad, multimodal density distributions, portions of which overlapped the distributions of the major alkaline-phosphatase-rich and Na,K-ATPase-rich membrane populations.

Density gradient fractions 3 and 4, which were designated density window I, were characterized consistently by an excess of alkaline phosphatase over Na,K-ATPase. As indicated in Table 2, density window I contained 15.6 ± 4.9% of the recovered alkaline phosphatase and 6.0 ± 1.7% of the Na,K-ATPase. Window II (fractions 5 and 6) contained only a slight excess of alkaline phosphatase over Na,K-ATPase, and window III (fractions 7 and 8) contained equal percentages of the two markers. The marker density distribution patterns suggested that windows I and II together contained elements of at least four different membrane populations: an alkaline-phosphatase-rich population, centered in window I; an Na,K-ATPase-rich population, centered in window II; and separate NADPH-cytochrome c reductase-rich and galactosyltransferase-rich populations represented in both windows.

Additional analyses were performed to resolve the Na,K-ATPase- and alkaline-phosphatase-rich popu-
lutions from windows I and II. First, the sorbitol concentrations of membrane samples from density windows I and II were increased to approximately 55% by addition of concentrated sorbitol, and the samples were centrifuged after loading onto 17.5–70% sorbitol gradients. Second, selected fractions from these second density gradient analyses were analyzed by phase partitioning.

**Alkaline-Phosphatase-Rich Membranes from Window I**

As depicted in Figure 5, the second density gradient analysis of window I revealed the presence of elements of at least three different membrane populations. To facilitate discussion, these have been given arbitrary letter designations. Population c was apparent from the peak of NADPH-cytochrome c reductase at fraction 3. Population b accounted for the peak of alkaline phosphatase at fraction 4. Population c' accounted for the overlapping peaks of galactosyltransferase and NADPH-cytochrome c reductase spanning fractions 6–8. According to the marker cumulative enrichment factors summarized in Table 3, this separation increased the alkaline phosphatase and Na,K-ATPase activities in the sample of population b an additional 1.5- to 1.6-fold over their values in density window I (Fig. 4, Table 2).

Phase partitioning analysis (Fig. 6) indicated that the sample of population b in pooled fractions 4 and 5 from Figure 5 was still somewhat heterogeneous, ie, that it contained elements of several different membrane populations in addition to population b. Population b itself appeared as a major, broadly distributed peak of alkaline phosphatase centered at partitioning fractions 5 and 6. Most of the Na,K-ATPase and galactosyltransferase activities in the sample appeared to be associated with minor populations, centered at partitioning fractions 4, 6, 7, and 9. Partitioning fraction 5 contained the sample of population b least contaminated by elements of the minor membrane populations. The alkaline phosphatase and Na,K-ATPase enrichment factors in this sample were 15.2 and 3.0.

The NADPH-cytochrome c reductase distribution pattern in Figure 5 suggests that population e might have spilled over into the sample of population b obtained from the second density gradient analysis of window I. Unfortunately, the NADPH-cytochrome c reductase activity was too low to be measured after the phase partitioning analysis depicted in Figure 6. For this reason, it is necessary to recognize the possibility that even the best sample of population b might have contained contaminating elements of population e. On the other hand, since less than half of the cell’s alkaline phosphatase is associated with population b, the remainder being associated primarily with populations a' and a", the observed 15-fold enrichment of alkaline phosphatase indicates that the sample of population b in partitioning fraction 5 has been purified at least 30-fold.

In a separate experiment, density window I was analyzed by phase partitioning without having first been subjected to a second density centrifugation analysis (data not shown). The best sample of population b had alkaline phosphatase and Na,K-ATPase enrichment factors of 10.2 and 2.4. Thus, the separation of minor, Na,K-ATPase-rich and galactosyltransferase-rich populations by phase partitioning analyses nearly doubled the alkaline phosphatase:Na,K-ATPase ratio over its value in the partially purified samples obtained by density gradient centrifugation analyses. Furthermore, it appears that the sequence of two density gradient centrifugation analyses and analysis by phase partitioning is necessary for optimal purification of population b.

### Table 2. Distributions of markers among density windows

<table>
<thead>
<tr>
<th>Marker</th>
<th>Window I</th>
<th>Window II</th>
<th>Window III</th>
<th>Window IV</th>
<th>Window V</th>
<th>Window VI</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na,K-ATPase</td>
<td>6.0 ± 2.2</td>
<td>35.9 ± 15.7</td>
<td>10.2 ± 2.8</td>
<td>4.1 ± 0.9</td>
<td>2.3 ± 0.5</td>
<td>0.9 ± 1.1</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>15.6 ± 4.9</td>
<td>41.0 ± 5.3</td>
<td>10.5 ± 1.5</td>
<td>4.1 ± 1.0</td>
<td>3.3 ± 0.7</td>
<td>3.6 ± 2.5</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>1.9 ± 0.6</td>
<td>6.7 ± 3.5</td>
<td>2.7 ± 0.9</td>
<td>1.8 ± 1.1</td>
<td>1.8 ± 1.3</td>
<td>1.8 ± 1.3</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>0.8 ± 0.4</td>
<td>2.3 ± 1.0</td>
<td>7.0 ± 2.8</td>
<td>2.2 ± 0.4</td>
<td>7.6 ± 2.9</td>
<td>1.1 ± 0.2</td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase</td>
<td>10.5 ± 4.3</td>
<td>13.5 ± 1.1</td>
<td>10.0 ± 2.4</td>
<td>7.4 ± 3.5</td>
<td>6.4 ± 2.0</td>
<td>4.3 ± 1.7</td>
</tr>
<tr>
<td>Galactosyltransferase</td>
<td>14.8 ± 1.3</td>
<td>29.6 ± 3.2</td>
<td>22.0 ± 0.1</td>
<td>10.9 ± 1.7</td>
<td>6.9 ± 0.9</td>
<td>0.7 ± 0.1</td>
</tr>
<tr>
<td>Protein</td>
<td>3.6 ± 1.6</td>
<td>5.4 ± 1.4</td>
<td>3.4 ± 0.7</td>
<td>3.2 ± 0.4</td>
<td>2.8 ± 0.7</td>
<td>2.5 ± 2.2</td>
</tr>
</tbody>
</table>

Values given are mean percentages of total recovered activities present in each density window, ± either standard deviations or, for NADPH-cytochrome c reductase and galactosyltransferase, ranges. Values in parentheses are cumulative enrichment factors. Number of preparations in which markers were determined are as in Table 1.
Fig. 5. Second density gradient centrifugation analysis of window I from Figure 4. The sample sorbitol concentration was brought to 55%, and the sample was inserted into 15.5-70% sorbitol gradients. After centrifugation as in Figure 4, fractions 3-8 and pooled fractions 1-2 and 9-12 were diluted, and membranes were harvested by high-speed centrifugation. Letters indicate the modes of major membrane populations delineated on the basis of the marker distribution patterns.

**Na,K-ATPase- and Alkaline Phosphatase-Rich Membrane from Window II**

The second density gradient analysis of density window II, depicted in Figure 7, indicated the presence of samples of three different membrane populations. Population c" was relatively enriched in NADPH-cytochrome c reductase and galactosyltransferase, and it was relatively depleted of alkaline phosphatase and Na,K-ATPase. Populations a' and a" were relatively enriched in Na,K-ATPase, alkaline phosphatase, and galactosyltransferase. Populations a' and a" were only poorly resolved from each other, and they resembled each other with respect to their marker contents. The distinction between them was initially based on the differences between their Na,K-ATPase:alkaline phosphatase and Na,K-ATPase:galactosyltransferase ratios, which were relatively lower in a' and higher in a". As documented in Table 3, the alkaline phosphatase cumulative enrichment factors in the samples of populations a' and a" were similar to the alkaline phosphatase cumulative enrichment factor in the partially purified sample of population b obtained by the second density gradient analysis of window I. In contrast, the Na,K-ATPase cumulative enrichment factors in the samples of populations a' and a" were 3.6-fold higher than in the sample of population b.

**Table 3. Marker cumulative enrichment factors in samples obtained from second density gradient analyses**

<table>
<thead>
<tr>
<th>Marker</th>
<th>b (window)</th>
<th>a' (fraction)</th>
<th>a&quot; (fraction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Na,K-ATPase</td>
<td>2.5 ± 0.0</td>
<td>9.3 ± 2.0</td>
<td>8.2 ± 0.1</td>
</tr>
<tr>
<td>Alkaline phosphatase</td>
<td>6.8 ± 0.2</td>
<td>10.9 ± 0.3</td>
<td>8.0 ± 2.2</td>
</tr>
<tr>
<td>Acid phosphatase</td>
<td>0.8 ± 0.4</td>
<td>1.4 ± 0.4</td>
<td>1.8 ± 0.6</td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase</td>
<td>1.7</td>
<td>1.6</td>
<td>2.7</td>
</tr>
<tr>
<td>Galactosyltransferase</td>
<td>2.9</td>
<td>6.7</td>
<td>4.3</td>
</tr>
</tbody>
</table>

Values given are mean cumulative enrichment factors ± range for markers determined in two separate experiments. NADPH-cytochrome c reductase and galactosyltransferase were determined in one experiment. Samples of the populations were obtained as follows:

<table>
<thead>
<tr>
<th>Population</th>
<th>First gradient</th>
<th>Second gradient</th>
</tr>
</thead>
<tbody>
<tr>
<td>b</td>
<td>I</td>
<td>4</td>
</tr>
<tr>
<td>a'</td>
<td>II</td>
<td>4</td>
</tr>
<tr>
<td>a&quot;</td>
<td>II</td>
<td>5</td>
</tr>
</tbody>
</table>
Fig. 6. Phase partitioning analysis of the sample of population b obtained by the second density gradient analysis of window I (Fig. 5, fraction 4). Fraction 1 contains material nearest the origin, or sample loading zone, and fraction 10 contains material furthest from the origin. Thus, the extent of its rightward movement reflects the tendency of a membrane population to partition into the mobile, polyethyleneglycol-rich phase. The sample of population b (indicated by letter) least contaminated by elements of the minor, Na,K-ATPase-rich and galactosyltransferase-rich populations is in partitioning fraction 5.

Fig. 7. Second density gradient centrifugation analysis of window II from Figure 4. Samples were treated as in Figure 5. Letters indicate the fractions at which the major membrane populations were sampled.
The samples of populations a' and a" from Figure 7 were subjected to separate phase partitioning analyses. Analysis of the sample of population a', depicted in Figure 8, indicated that it was dominated by a single major membrane population which accounted for most of the recovered Na,K-ATPase, alkaline phosphatase, and galactosyltransferase activities. However, the sample also contained a minor membrane population, which could be discerned from a peak of acid phosphatase activity centered at partitioning fraction 9. Analysis of the sample of population a", depicted in Figure 9, indicated that it, too, was dominated by a single major membrane population which accounted for most of the recovered Na,K-ATPase, alkaline phosphatase, and galactosyltransferase activities. As was the case for a', the analysis of a" also revealed the presence of a minor, acid-phosphatase-rich population; it was centered at partitioning fraction 7. NADPH-cytochrome c reductase activities were too low to be measured reliably in the phase partitioning fractions. Therefore, it was not clear whether the NADPH-cytochrome c reductase activities apparent in the samples of a' and a" from the second density gradient analysis (Figure 7, Table 3) were associated with these populations or with elements of some other membrane population.

Population a' peaked in partitioning fraction 6 (Fig. 8), whereas population a" peaked in partitioning fraction 4 (Fig. 9). This difference in partitioning behavior indicated that the two populations differed from each other with respect to the surface properties detected by the phase partitioning analysis. Samples of the two populations were separated from each other on the basis of this surface difference in an experiment in which density window II was analyzed by phase partitioning without an intervening second density gradient analysis (data not shown).

**SDS-PAGE and Western Blotting**

Samples of each density window from the first sorbitol gradient analysis and selected samples of populations resolved by the second gradient analyses were subjected to SDS-PAGE and Western blot analysis (Fig. 10). All six of the samples from the first gradient had discernible silver-stained bands (Fig. 10a) at an apparent molecular mass of 94 kD, although the 94-kD band from density window VI was faint. Western blot analysis with a rabbit polyclonal antiserum directed against the alpha subunit of bovine brain Na,K-ATPase (Fig. 10b) also revealed bands at this molecular mass, with the exception of window VI. The most prominent bands were observed in the lanes containing samples from windows I–III, with the most intense band in window II. This is consistent with the observations (Table 2) that the percentage of recovered Na,K-ATPase activity was highest in windows I–III; that it peaked in window II; and that it had its lowest level in window VI.

A 94-kD band was visible in the partially purified...
sample of population b, in pooled samples of populations a’ and a”, and in a sample of population c”; the sample of population c” was negative. The most intense 94-kD silver-stained (Fig. 10a) band was observed in pooled samples of populations a’ and a”. Similarly, the Western blot (Fig. 10b) had antibody-stained bands at 94 kD corresponding to their silver-stained counterparts. The most intense band was observed in the pooled sample of populations a’ and a”, and no staining was observed in the sample of population c”. Again, these results are consistent with the marker cumulative enrichment factors (Table 3) and with the marker distributions (Figs. 5, 7), which indicated that Na,K-ATPase had its highest specific activities in the samples of populations a’ and a”; that it was present in the sample of population b; that it was present at lower levels in the sample of population c”; and that it was present only at very low levels in the sample of population c”.

Discussion

Analyses of the low-speed supernatant fraction from lysates of cultured human retinal pigment epithelium have revealed the presence of three different membrane populations—populations a’, a”, and b—in which a general plasma membrane marker, alkaline phosphatase, was enriched 7-fold or more.

Populations a’ and a” were characterized by similar enrichment factors for alkaline phosphatase and for Na,K-ATPase, and by 1.7- to 1.9-fold excesses of their plasma membrane marker enrichments over their galactosyltransferase enrichments. Both populations equilibrated in density window II after having been loaded onto sorbitol gradients in a 5% sorbitol medium, but they were separated from each other by equilibrium centrifugation after having been loaded onto gradients in a 55% sorbitol medium. The physical basis for this separation is not known, but we speculate that it may be related to differences in the extent to which the two membranes reswell and allow sorbitol to permeate after osmotic shrinkage in the hypertonic medium. In addition to the distinction revealed by their behavior during the second density gradient analysis, the two Na,K-ATPase- and alkaline-phosphatase-rich populations also differed from each other with respect to the surface property which determines their partitioning behavior in an aqueous polymer two-phase system. Partitioning analyses in this phase system yielded evidence of only minor contamination by elements of additional membrane populations.

Given the evidence that Na,K-ATPase is concentrated in the RPE cell apical plasma membrane, the observations that populations a’ and a” together accounted for most of the Na,K-ATPase catalytic activity in the low-speed supernatant fraction (Fig. 6, Table 2) and that they were the loci of the greatest
Fig. 10. Silver-stained SDS-PAGE (a) and Western blot analysis (b) of selected membrane fractions obtained by density gradient centrifugation. Lane S in (a) contains phosphorylase B, 94 kD; bovine serum albumin, 68 kD; carbonic anhydrase, 30 kD; soybean trypsin inhibitor, 22 kD; and lysozyme, 14 kD. Lanes 1–6 contain aliquots of density windows I–VI. Samples analyzed in the remaining numbered lanes are as follows:

<table>
<thead>
<tr>
<th>Lane</th>
<th>Population</th>
<th>First gradient (window)</th>
<th>Second gradient (fraction)</th>
</tr>
</thead>
<tbody>
<tr>
<td>7</td>
<td>b</td>
<td>I</td>
<td>4–5</td>
</tr>
<tr>
<td>8</td>
<td>c'</td>
<td>I</td>
<td>7</td>
</tr>
<tr>
<td>9</td>
<td>a' + a''</td>
<td>II</td>
<td>4–6</td>
</tr>
<tr>
<td>10</td>
<td>c''</td>
<td>II</td>
<td>7</td>
</tr>
</tbody>
</table>

Lanes 1–3 indicate that windows I–III from the first density gradient analysis have the highest levels of alpha subunit immunoreactivity. Lanes 8 and 10 indicate relatively low levels of alpha subunit immunoreactivity in the presumed Golgi membrane samples resolved by the second density gradient analyses, whereas lanes 7 and 9 indicate relatively high levels of alpha subunit immunoreactivity in the plasma membrane samples obtained with these analyses. As illustrated in Figure 6, much of the Na,K-ATPase in the partially purified sample of population b was associated with minor membrane populations that could be resolved by phase partitioning.

observed enrichments of Na,K-ATPase catalytic activity (Table 3) and of Na,K-ATPase alpha subunit immunoreactivity (Fig. 10) lead to the conclusion that they were derived from the apical surface of the RPE. We can suggest several possible explanations for the observed physical differences between these populations. They might have represented different microdomains of the apical surface of one homogeneous cell type; distinct populations of inside-out and right-side-out vesicles formed from a single apical membrane domain; or apical membranes from two different subtypes of cell in the cultures.

The phase partitioning analyses illustrated in Figures 8 and 9 revealed close parallels between the distributions of Na,K-ATPase, alkaline phosphatase, and galactosyltransferase. These parallels suggest that galactosyltransferase, which is commonly used as a marker for membranes derived from trans elements of the Golgi complex, is also present in populations a' and a''. The analytic fractionation data indicated the presence of additional membrane populations which were very likely derived from the Golgi complex because they were enriched in galactosyltransferase and relatively depleted of Na,K-ATPase and alkaline phosphatase. Examples of the Golgi-derived populations could be seen in density window III after the first density gradient centrifugation analysis (Fig. 4) and centered about fraction 7 after the second density gradient centrifugation analyses of windows I (population c', Figure 5) and II (population c'', Figure 6). Therefore, it appears that galactosyltransferase is associated both with the Golgi complex and with apical membranes of the cultured human RPE. We are not aware of previous evidence for such a galactosyl-
transferase localization in RPE. However, the existence of plasma-membrane-expressed galactosyltransferases has been documented in several other cell types.19,34,35

Several groups have previously isolated apical membrane samples from bovine and rat RPE preparations. Procedures have involved adsorption to glass beads,36,37 adsorption to wheat germ agglutinin-conjugated sepharose beads,38 sucrose gradient centrifugation of cell lysates,39,40 and a sequence of differential centrifugation and Percoll density gradient centrifugation.41 The Na,K-ATPase enrichment factors which have been reported range from 3.537 to 16.6,41 and the alkaline phosphatase enrichment factors range from 5.537 to 12.8.41 The Na,K-ATPase and alkaline phosphatase enrichment factors of populations a' and a'' fall within these ranges (Table 3).

Population b was characterized by a 3- to 6-fold excess of alkaline phosphatase enrichment over Na,K-ATPase enrichment. This population equilibrated in density window I after having been loaded onto sorbitol gradients in a medium containing 5% sorbitol (Fig. 4) and at a similar position after having been loaded onto gradients in a medium containing 55% sorbitol (Fig. 5). The second density gradient analysis yielded a partially purified sample of population b, which also contained elements of several additional membrane populations. The contaminating populations were resolved by phase partitioning analysis, yielding a more highly purified sample of population b (Fig. 7).

To our knowledge, this is the first report of the isolation of a membrane sample with the characteristics described for population b (Figs. 5, 6; Table 3). This population was characterized by a large cumulative enrichment factor for alkaline phosphatase (6.8 after the second density gradient analysis and 15.2 after phase partitioning) and by a 3- to 5-fold excess of alkaline phosphatase enrichment over Na,K-ATPase enrichment. The marked excesses of alkaline phosphatase over Na,K-ATPase (Fig. 6), NADPH-cytochrome c reductase (Fig. 5), galactosyltransferase (Fig. 6), succinate dehydrogenase (Fig. 4), and acid phosphatase (Fig. 6) serve to exclude the basal–lateral plasma membranes, endoplasmic reticulum, Golgi complex, mitochondria, and lysosomes as likely subcellular origins for this population.

We believe that population b most likely originated from the RPE basal–lateral plasma membrane. Alkaline phosphatase is known to be associated with the plasma membranes of a variety of nonepithelial cell types.15 In the small intestinal epithelium, alkaline phosphatase is localized preferentially to the apical plasma membrane, but it is also present, at a some-what lower specific activity, in the basal–lateral membranes, which are the major site of Na,K-ATPase activity.23 A somewhat different relationship between the marker localizations is apparent in acinar cells of the rat exorbital lacrimal gland. Alkaline phosphatase and Na,K-ATPase are both present at both surfaces of the acinar cell, but the alkaline phosphatase:Na,K-ATPase ratio is greatest in the apical membranes, and the Na,K-ATPase:alkaline phosphatase ratio is greatest in the basal–lateral membranes.19

The existence of an alkaline-phosphatase-rich membrane population that is distinct from the apical plasma membrane is not an artifact of the use of cultured RPE cells, since we have also observed such populations in subcellular fractionation analyses of RPE preparations isolated from frog and bovine eyes (Mircheff, Miller, and Farber, unpublished observations). Although our preliminary results suggested that it would be feasible to isolate differentially apical membranelike and basal–lateral membranelike populations from freshly isolated RPE, several technical considerations made the cultured RPE used in the current study a more attractive starting point. The cultures provide a larger amount of material, with 65.8 mg protein obtained from five plates versus 1.9 mg protein from 10 bovine eyes.41 The cultures virtually eliminate the question of potential contamination from photoreceptors or choroidal tissue, which also have substantial Na,K-ATPase activities.42 The RPE cells harvested from tissue culture plates appear to be in better condition than the RPE cells freshly obtained by brushing procedures, since soluble protein accounts for 34% of the protein recovered from the former preparations (Table 1) and 7% of the protein recovered from the latter.41 There is no need for cell purification immediately prior to the subcellular fractionation analysis. Finally, it should be possible to design experiments in which biophysical studies on intact epitheliallike RPE sheets are performed in parallel with biochemical studies of membrane vesicles isolated from these sheets.

In studies designed to characterize constituents of populations a', a'', and b, it will be possible to obtain partially purified samples of the populations with the sequential density gradient analyses depicted in Figures 4, 5, and 7. Phase partitioning analyses are required for resolution of relatively well-purified samples, particularly of population b. These analyses depend on equipment which is not widely available.17 However, the partially purified samples that can be obtained by sequential density gradient centrifugation analyses should be useful for a variety of purposes. For example, it will be possible to determine
the properties of receptors and of ion and substrate transporters which are associated with population b membranes, even though other membrane types are also present in the sample. It also should be possible to determine how these constituents are distributed among the plasma membrane populations and among the likely contaminating populations by evaluating their density distributions relative to those of alkaline phosphatase, Na,K-ATPase, galactosyltransferase, and NADPH-cytochrome c reductase after each of the density gradient analyses.

Key words: apical membranes, basal-lateral membranes, Golgi complex, Na,K-ATPase, phase partitioning

References

35. Roth J, Lentze MJ, and Berger EG: Immunocytochemical