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# PROTEIN-LIPID INTERACTIONS IN ANTIPODAL PLASMA MEMBRANES OF RAT COLONOCYTES *

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**The isolation of apical membranes from rat proximal colonic epithelial cells is described. Differential centrifugation yielded a 'crude' membrane fraction which was further purified using sucrose density centrifugation. The final membrane fraction was enriched 20–28-fold over homogenate in alkaline phosphatase and cysteine-sensitive alkaline phosphatase specific activities. Lipid-protein interactions and lipid dynamics examined in apical and basolateral membranes prepared from colonocytes demonstrated: (1) apical membrane, as assessed by steady-state fluorescence polarization studies have a low lipid fluidity; (2) colonic basolateral membranes possess a greater lipid fluidity than apical membranes; (3) compositional differences in these antipodal membranes appear to explain these differences in lipid fluidity; (4) fluorescence polarization studies using diphenylhexatriene detect a thermotropic transition at 21–23°C in apical membranes and liposomes prepared from lipid extracts of these membranes; (5) alkaline phosphatase and L-cysteine-sensitive alkaline phosphatase activities appear to be functionally dependent on the physical state of the apical membrane's lipid.**

## Introduction

Recently, there has been an increased recognition of the importance of lipid-protein interactions in influencing protein activities of biological membranes [1–3]. In *Escherichia coli* and *Mycoplasma* membranes with well-defined lipid thermotropic transitions §, temperature-dependent alterations in the physical state of the lipid have been shown to effect certain membrane activities dependent on proteins [7–10]. Studies of mammalian plasma membranes have also demonstrated lipid thermotropic transitions and their influence on protein activities [6,11–16]. In these latter studies dif-

* Supplementary data to this article are deposited with, and can be obtained from, Elsevier Science Publishers B.V., BBA Data Deposition, P.O. Box 1345, 1000 BH Amsterdam, The Netherlands. References should be made to BBA/DD/283/72122/773(1984)290. The supplementary information includes: Two tables which show the distribution of marker enzyme activities for each fraction obtained during the fractionation procedure.

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Abbreviations: 2-AS, DL-2-(9-anthroyl)stearic acid; 12-AS, DL-12-(9-anthroyl)stearic acid.

§ The term 'lipid thermotropic transition' is used in a general sense to denote a thermally-induced change in the physical state of the lipid. This change might involve order-disorder transitions of the liquid-crystalline to gel type, lateral phase separations, lipid clusters or other mechanisms [4–6].

ferences in 'lipid fluidity' [§§] appeared to exist between antipodal membranes, i.e., the contralumenal membranes possessed a greater lipid fluidity than their lumenal counterparts [6,11–16]. Compositional differences between these membranes appeared to explain these alterations in lipid dynamics [13,14].

Our laboratory has recently isolated contralumenal (basolateral) membranes from rat colonocytes and examined the lipid dynamics and lipid-protein interactions of these membranes [24,25]. Basic similarities in composition and lipid dynamics between these membranes and contralumenal membranes isolated from rat enterocytes were shown [24,25].

In this present study, using L-cysteine-sensitive alkaline phosphatase as purification marker, we now describe a technique for the isolation of apical membranes from rat colonocytes. This has permitted examination of the lipid dynamics and lipid-protein interactions of these membranes and allowed comparison with colonic basolateral membranes. The results described below demonstrate: (1) apical membranes from rat colon possess a relatively low lipid fluidity, as assessed by steady-state fluorescence polarization studies; (2) colonic basolateral membranes have greater lipid fluidity than apical membranes; (3) the differences in lipid dynamics of these antipodal membranes can be related to compositional differences; (4) a lipid thermotropic transition, as detected by fluorescence polarization using the fluorophore 1,6-diphenyl-1,3,5-hexatriene is present in apical membranes; 95) Arrhenius plots of alkaline phosphatase and L-cysteine-sensitive alkaline phosphatase (activities which are probably mediated by the same enzyme complex) demonstrate breakpoints corresponding to the transition temperature detected by fluorescence polarization.

[§§] The term 'lipid fluidity' as applied to natural membranes is used to express the relative motional freedom of the lipid molecules or substituents thereof. It bears emphasis, however, that this term is broad and includes different types of motion, e.g., rotational or lateral diffusion of a molecule in an array, movements of substituent groups of a molecule, and a flow of molecules under a pressure gradient in accord with a fluidity which is 1/(viscosity of the molecular array) [17–19]. In this report 'lipid fluidity' was assessed by steady-state fluorescence polarization of lipid soluble probes. The anisotropy parameters so obtained are probe dependent and reflect the overall freedom of these molecules without distinguishing the specific mechanisms affecting its motion such as viscous drag of the environment, anisotropic rotations and hindered motions due to structural factors [14,20–23].

## Methods and Materials

*Isolation of colonic epithelial cell apical membranes.* Male albino rats of the Sherman strain weighing 250–300 g were fasted 18 h with water ad libitum before being killed. The animals were killed rapidly by cervical dislocation and their colons excised. The cecum was discarded, the remaining large intestine divided equally into two parts: 'proximal' and 'distal' [26]. Epithelial cells, relatively devoid of goblet cells, were then obtained from the proximal segment using a technique which combined chelation of divalent cations with mild mechanical dissociation as described [24,25].

The collected cells were combined, pelleted, washed and brought up in 20 ml of mannitol/Tris/$MgCl_2$ buffer (0.05 M mannitol/0.002 M Tris/0.01 M $MgCl_2$ (pH 7.4)) (buffer 1). The suspension of cells was homogenized using a polytron PCU-2-110 (Brinkman Instruments, Westbury, NY) at a power setting of 5 for 15 s × 4 at 4°C. The homogenate was then diluted with an equal volume of mannitol/Tris buffer (0.05 M mannitol/0.002 M Tris (pH 7.4)) (buffer 2), filtered through a single layer of Nitex (Tetko, Inc., Elmsford, NY) with a pore diameter of 100 $\mu$m to remove debris and unbroken cells, and washed with additional 10–20 ml of buffer 2. The homogenate was centrifuged at 6000 × *g* for 30 min at 0–4°C in a Sorvall centrifuge (Dupont Instruments, Model RC-5B) to obtain an initial pellet ($P_1$) and supernatant ($S_1$). $S_1$ was discarded while $P_1$ was brought up in 80 ml of buffer 2 and homogenized ×8 strokes, using a tight fitting Dounce homogenizer (pestle A), and spun at 6000 × *g* for 30 min ($P_2$,$S_2$). $S_2$ was discarded. $P_2$ was brought up in 26 ml of 15% sucrose/0.01 M Tris (pH 7.4) (buffer 3) (w/v) and dounced ×75 strokes. Four discontinuous gradients were then prepared by placing 3 ml of 40% sucrose/buffer 3 at the bottom of '5/8 × 3' cellulose nitrate tubes (Beckman, Palo Alto, CA) and successively applying 4 ml of 33% sucrose/buffer 3 and filling the

remainder of the tubes with the material in the 15% sucrose/buffer (approximately 6.5 ml). The discontinuous gradients were centrifuged at 160 000 × *g* for 75 min at 4.0 °C in a Beckman SW 36 rotor using a Beckman L5-50 ultracentrifuge (Palo Alto, CA). Five fractions were collected (15%, 33%, 40% and two interfaces), diluted with distilled $H_2O$ and pelleted by ultracentrifugation, using a Beckman 40 rotor, at 105 000 × *g* for 60 min. Each pellet was then brought up in 1 ml of buffer 2 and analyzed immediately or quick frozen with acetone and dry ice and stored at −70 °C.

*Isolation of colonic basolateral membranes.* Basolateral membranes were prepared from rat 'proximal' colonocytes as previously described [24]. Purity of basolateral suspensions were assessed by the estimation of ouabain-sensitive sodium-potassium-dependent adenosine triphosphatase (($Na^+ + K^+$)-ATPase) specific activities. Preparations used were purified 15–20-fold as compared to the original homogenates. Membranes were relatively free of contamination by other intracellular membranes or organelles.

*Enzyme assays.* Assay conditions were chosen for each enzyme to assure linear kinetics with respect to time and protein. ($Na^+ + K^+$)-ATPase was quantified by the method of Siegel and Goodman [27]. NADPH-cytochrome *c* reductase, succinate dehydrogenase, magnesium-dependent adenosine triphosphatase ($Mg^{2+}$-ATPase) and alkaline phosphatase (*p*-nitrophenylphosphatase) were assayed as previously described [12–14]. Acid phosphatase, ouabain-sensitive and -insensitive potassium-dependent phosphatase were assayed as described [28], using *p*-nitrophenyl phosphate as substrate. Exogeneous galactosyltransferase activity was measured, using ovalbumin (Sigma Chemical Co.) as exogeneous acceptor, according to the method of Kim et al. [29] as modified by Podolsky and Weiser [30] using 727 μM UDP[$^3$H]galactose (spec. act. 1.14 Ci/mmol) prepared to a final specific activity of 0.076 Ci/mmol by the addition of unlabelled UDPgalactose (Sigma Chemical Co.).

Cysteine-sensitive alkaline phosphatase (*p*-nitrophenylphosphatase) activity was estimated by using as substrate 5 mM *p*-nitrophenyl phosphate (Sigma Chemical Co.) in 34 mM glycine buffer of pH 9.3 containing 3.4 mM $MgCl_2$, 0.34 mM $ZnCl_2$ ± 10 mM L-cysteine (pH 9.3). After adding an aliquot of an appropriate fraction, the tubes were incubated at 37 °C for 15–30 min before addition of 2 ml of 1 M NaOH. The cysteine-sensitive alkaline phosphatase was calculated as the difference in substrate hydrolysis in the presence and absence of cysteine.

Arrhenius plots were determined for alkaline phosphatase and L-cysteine-sensitive alkaline phosphatase as previously described [11–15].

*Iodination of isolated cell surface membrane.* The method of Morrison [31] using glucose oxidase to generate peroxide was used [32] as described by Weiser et al. [33].

*Slab gel electrophoresis of apical membrane.* Gel electrophoresis was performed using gradient gels PAA 4/30 (Pharmacia Fine Chemicals, Piscataway, NJ) following general procedures as previously described [34,35]. At the end of the electrophoresis, gels were stained for protein with Coomassie blue.

*Compositional studies of the antipodal colonic plasma membranes.* Total lipids were extracted from membranes by the method of Folch et al. [36]. The composition of total neutral and phospholipids extracts of these membranes were examined by thin-layer chromatography according to the method of Katz et al. [37]. Fatty acids of the total lipid extract of membranes were derivitized as previously described by Gartner and Vahouny [38]. Fatty acid methyl esters were determined on a Jeol JGC-20K gas chromatograph equipped with a flame ionization detector and interfaced with a Hewlett-Packard 3390A integrator [38]. Authentic fatty acid methyl esters were utilized to identify retention times. Protein was estimated by the method of Lowry et al. [39] using bovine serum albumin as standard. DNA content was measured using the method of Burton [40], as modified by Giles and Myers [41]. Sonicated dispensions of extracted lipid (liposomes) were prepared as described [12].

*Fluorescence polarization studies.* Three fluorescent probes were used: 1,6-diphenyl-1,3,5-hexatriene, DL-2-(9-anthroyl)stearic acid (2-AS) and DL-12-(9-anthroyl)stearic acid (12-AS). The methods used to load the membranes and the quantification of the polarization of fluorescence in an SLM polarization spectrofluorometer have been described [11–15,25]. The polarization of flu-

orescence was expressed as the fluorescence anisotropy parameter, $((r_o/r)-1)^{-1}$, was determined over the range of 0–40 °C and 0–50 °C, respectively, for apical membranes and liposomes using diphenylhexatriene. The logarithm of the anisotropy parameter was plotted against $1/T$ ($K^{-1}$) as described [11,13,42], to detect thermotropic transitions. No changes in the excited-state lifetimes, as assessed by total fluorescence intensity, were demonstrated using all three probes, in apical or basolateral membranes or their respective liposome preparations [25].

*Electron microscopy.* The membrane fractions were prepared for positive staining by fixing in glutaraldehyde (3.0 g/100 ml) in 0.1 M cacodylate buffer, pH 7.5 for 2 h as previously described by Weiser et al. [33] and examined with an electron microscope (Zeiss EMS-9 New York, NY).

*Materials.* Diphenylhexatriene was obtained from Aldrich Chemical Co. 2-AS and 12-AS were purchased from Molecular Probes INc. All radioactive materials were purchased from New England Nuclear Company (Boston, MA). All other reagents, unless indicated, were obtained from Fisher Chemical Co. or Sigma Chemical Co.

*Statistical methods.* All results are expressed as mean values ± S.E. Paired or unpaired *t*-tests were used for all statistical analysis. A $P$ value $< 0.05$ was considered significant.

## Results

### *Cell isolation*

80–90% of the cells were found to exclude 0.1% Trypan blue dye. In agreement with previous studies [24,25], 10–15% were stained by periodic acid-Schiff reagent (i.e., goblet cells).

### *Isolation of apical membranes*

The isolation of apical membranes from rat proximal colonocytes was carried out in two stages. After disruption of the cells by homogenization, the resulting organelles and membrane fragments were fractionated by differential centrifugation. This procedure yielded a 'crude' membrane fraction ($P_2$). While this fraction contained approx. 57% and 65% of the total alkaline phosphatase and cysteine-sensitive alkaline phosphatase present in the homogenate, respectively, the percent total activity for several other markers was also high, indicating that $P_2$ was significantly contaminated by other cellular organelles (Table I). Alkaline phosphatase and cysteine-sensitive alkaline phosphatase showed a 2.5–3.0-fold increase in specific activity compared to homogenate. In general, the specific activities of the other marker enzymes were unchanged or slightly increased, although DNA and succinate dehydrogenase activity were increased 2.0–2.5-fold, respectively (Table I).

The second stage of the purification scheme involving density gradient velocity sedimentation of the $P_2$ fraction using a discontinuous sucrose gradient. The specific activity and percent yield of each of the marker enzymes for the final membrane fraction (15/33) are presented in Table I. The 15/33 fraction appeared maximally enriched in alkaline phosphatase and cysteine-sensitive alkaline phosphatase demonstrating a 18–28-fold increase in specific activity compared to homogenate for these markers. This fraction also contained approx. 15–20% of the total activity of each of these enzymes. Contamination by intracellular material, as assessed by appropriate marker enzymes, was minimal (Table I). $(Na^+ + K^+)$-ATPase and ouabain-sensitive $K^+$-phosphatase were enriched 1.9-fold. Yields of these later enzyme activities, however, averaged only 0.8–1.4% of the total homogenate values in contrast to the 15–20% yield seen with the apical membrane markers *.

Ouabain-insensitive $K^+$ dependent phosphatase activity demonstrated a 5-fold increase in specific activity in the 15/33 fraction compared to homogenate but this fraction only contained 1.7% of the total activity. Additionally, other fractions of the discontinuous sucrose gradient possessed greater total activity *. These data suggest that this enzyme in rat proximal colonic apical membranes, unlike distal apical membranes in the rabbit [28], does not appear to co-purify with alkaline phosphatase and is not suitable as a 'purification' marker for this membrane.

### *$^{125}I$-labeling of proximal colonic cell apical membrane*

As noted previously, the method used for ob-

* BBA Data Deposition, see footnote on p. 290.

TABLE I

DISTRIBUTION OF ENZYME MARKERS DURING FRACTIONATION PROCEDURE

Values are average of four experiments with a S.E. less than 8% for recovery. Specific activities (mean ± S.E.) are given in parenthesis. All enzyme activities are given in nmol per mg protein per min except for the following: succinate dehydrogenase and NADPH-cyto-

| Fraction | Protein | Alkaline phosphatase | Cysteine-sensitive phosphatase | $(Na^+ + K^+)$-ATPase | $Mg^{2+}$-ATPase |
|---|---|---|---|---|---|
| Homogenate | 100% (3.1 ± 0.4) | 100% (16.5 ± 2.9) | 100% (9.0 ± 1.5) | 100% (20.3 ± 1.6) | 100% (54.7 ± 1.9) |
| 'Crude' membrane fraction ($P_2$) | 32.9% (1.5 ± 0.1) | 57.3% (40.7 ± 3.6) | 62.9% (28.0 ± 1.0) | 41.0% (24.9 ± 2.2) | 38.2% (76.9 ± 6.8) |
| Apical membranes (15/33) | 0.8% (0.8 ± 0.1) | 15.5% (299.0 ± 7.9) | 20.1% (255.4 ± 7.1) | 1.4% (38.9 ± 0.9) | 1.6% (155.7 ± 1.3) |

taining isolated proximal colonocytes may produce as many as 20% of cells that cannot exclude vital dyes [24,25]. Therefore, it was important that proper controls be done; (a) no glucose oxidase, and (b) no lactoperoxidase or glucose oxidase. These controls were taken throughout all steps of the isolation procedure and represent 'non-specific' labeling, amounting to approx. 20–25% of the counts incorporated.

The results as shown in Fig. 1, indicate that the 15/33 fraction was most prominently labeled. This data, together with the high specific activities of alkaline phosphatase and cysteine-sensitive alkaline phosphatase strongly supports the contention that this fraction appears to contain apical plasma membranes.

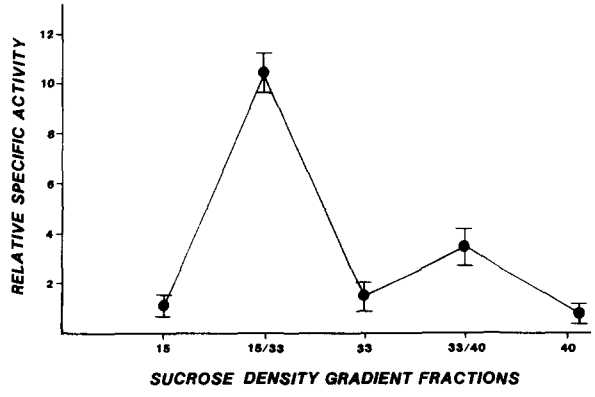


Fig. 1. Sucrose density gradient profile after labeling isolated cells with $^{125}I$ by the method of Morrison [23]; see Material and Methods for further details. Values represent means ± S.E. of $^{125}I$ dpm/mg protein in the fractions relative to that present in the starting homogenate obtained from three separated experiments.

The only other material to show significant labeling was the 33/40 fraction. Since whole cells were labeled and this fraction demonstrated a 3–4-fold increase in specific activities of $(Na^+ + K^+)$-ATPase and ouabain-sensitive $K^+$-dependent phosphatase compared to starting homogenate values *, this fraction appears to contain basolateral plasma membranes.

*Electron microscopy appearance of the 15/33 fraction*

The 15/33 fraction showed fairly uniform membrane vesicles surrounded by well preserved bilaminar membranes. Particles suggestive of other subcellular fractions were not evident (not shown).

*Fluorescence polarization studies*

The effects of temperature on the anisotropy parameter, $((r_0/r)^{-1})^{-1}$, of diphenylhexatriene in apical membranes and liposomes prepared from membrane lipids of proximal colonocytes are illustrated by representative Arrhenius plots in Fig. 2. The plots initially appear reasonably linear until approximately 21–23°C, but then a distinct change in slope occurs with further cooling and the plots became somewhat curvilinear. As shown in Table

* BBA Data Deposition, see footnote on p. 290.

chrome *c* reductase are in $\Delta A$ per mg protein per min; galactosyltransferase is in pmol per mg protein per min; protein is expressed in mg/ml; DNA is expressed in $\mu$g per mg protein.

| NADPH-cytochrome *c* reductase | Succinate dehydrogenase | DNA | Exogenous galactosyltransferase | Acid phosphatase | Ouabain-sensitive $K^+$-phosphatase | Ouabain-insensitive $K^+$-phosphatase |
|---|---|---|---|---|---|---|
| 100% (10.8 ± 1.9) | 100% (276.8 ± 21.9) | 100% (114.5 ± 14.6) | 100% (68.3 ± 5.2) | 100% (45.7 ± 4.7) | 100% (4.4 ± 0.1) | 100% (6.1 ± 0.5) |
| 48.7% (17.8 ± 3.5) | 61.2% (512.3 ± 36.1) | 84.2% (280.3 ± 17.4) | 22.1% (53.0 ± 1.1) | 40.8% (50.5 ± 3.4) | 55.3% (6.2 ± 0.2) | 53.1% (9.2 ± 0.5) |
| 0.7% (16.9 ± 2.7) | 0.8% (355.2 ± 30.5) | 0.6% (127.1 ± 8.1) | 0.6% (84.0 ± 2.4) | 0.6% (63.3 ± 7.8) | 0.8% (8.4 ± 0.7) | 1.7% (30.6 ± 3.4) |

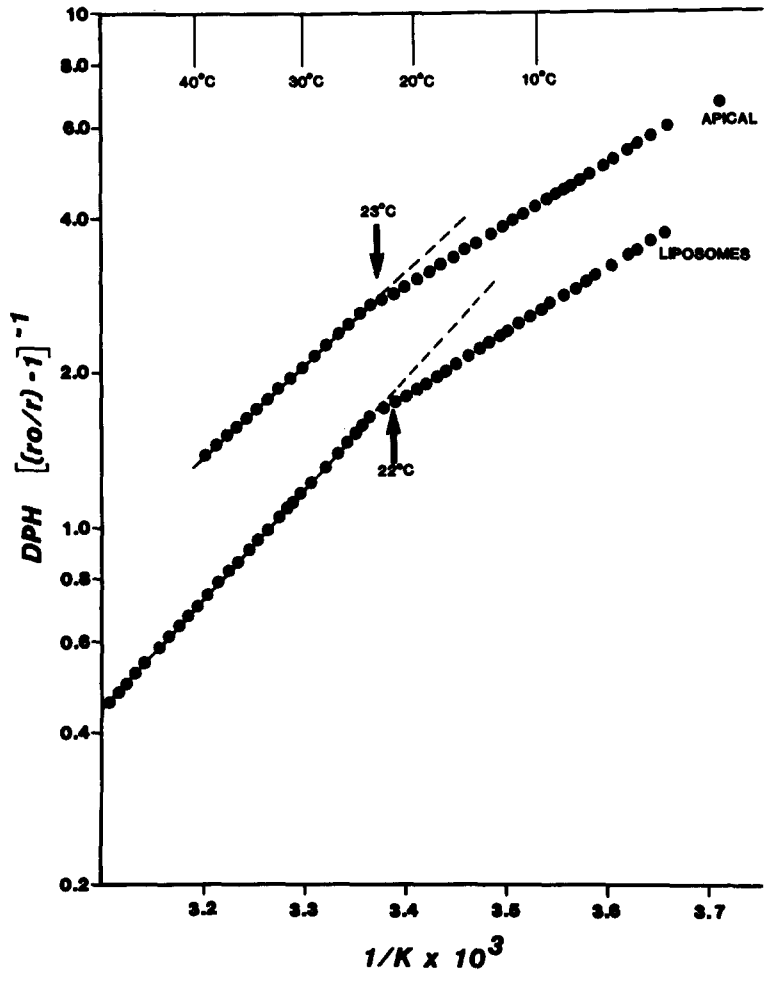


Fig. 2. Arrhenius plot of the anisotropy parameter of diphenylhexatriene (DPH) in a sample of isolated apical membranes (apical, upper curve) and in liposomes prepared from a lipid extract of these membranes (liposomes, lower curve) prepared from proximal rat colonic epithelial cells.

TABLE II

FLUORESCENCE POLARIZATION STUDIES

Values are means ± S.E. DPH, diphenylhexatriene.

| Probe | Preparation | Number of preparations | Transition temp. (°C) | Mean anisotropy, $r$ at 25°C | Anisotropy parameter $((r_0/r)-1)^{-1}$ at 25°C |
|---|---|---|---|---|---|
| DPH | Apical intact | 10 | 23.1 ± 0.7 | 0.26 ± 0.01 [a,b] | 2.56 ± 0.18 [a,b] |
| | Apical liposomes | 4 | 22.0 ± 1.0 | 0.22 ± 0.01 [b] | 1.50 ± 0.12 [b] |
| | BLM intact | 4 | n.d. | 0.23 ± 0.01 | 1.70 ± 0.09 |
| | BLM liposomes | 4 | n.d. | 0.19 ± 0.02 | 1.03 ± 0.10 |
| 2-AS | Apical intact | 4 | n.d. | 0.13 ± 0.02 [b] | 0.84 ± 0.08 [b] |
| | BLM intact | 4 | n.d. | 0.09 ± 0.01 | 0.46 ± 0.02 |
| 12-AS | Apical intact | 4 | n.d. | 0.10 ± 0.01 [b] | 0.54 ± 0.06 [b] |
| | BLM intact | 4 | n.d. | 0.07 ± 0.01 | 0.30 ± 0.03 |

[a] $P < 0.01$ compared to corresponding liposome value.
[b] $P < 0.05$ compared to corresponding BLM value.

II, the transition temperatures for the apical membranes and their liposomes are similar, i.e., approx. 22–23°C. Although not determined in this series of experiments, previous studies in our laboratory have shown transition temperatures for proximal colonic basolateral membranes and their liposomes at 21–23°C [25].

The anisotropy parameter values of diphenylhexatriene, at all temperatures measured including 25°C (Table II) for both plasma membranes, were approx. 70% greater than for their respective liposome preparation, indicating that the fluidity of the lipids is considerably greater in the liposomes.

As can also be seen in Table II, apical membranes possessed significantly higher diphenylhexatriene anisotropy parameter values than basolateral preparations. Liposomes prepared from these membranes showed similar differences. To examine further whether differences in lipid fluidity existed between these antipodal membranes as well as to overcome certain limitations in the technique as described [14], additional studies were conducted using two other fluorophores 2-AS and 12-AS, which differ in shape and structure and localize in various domains of the bilayer [43–45]. In each case, the anisotropy parameter for the apical membrane was significantly greater than for basolateral membranes at 25°C (Table II), further demonstrating that the lipid molecules in the apical membrane generally experience greater restraint to motional freedom, and in this sense, the apical membranes have a lower 'lipid fluidity' [14].

Similar differences in anisotropy parameter values were seen for both membranes using all three probes at 15°C and 37°C (data not shown).

*Temperature dependence of enzyme activities*

Four membrane preparations were examined for alkaline phosphatase and L-cysteine-sensitive alkaline phosphatase activities to detect breakpoints in their Arrhenius plots and to examine the relationship of such breakpoints to the lipid thermotropic transition temperature. Each preparation and activity tested demonstrated a discontinuity in Arrhenius plot, as shown by plots of the mean values seen in Fig. 3. The apparent breakpoint temperatures and energies of activation, $\Delta E$, for these experiments are summarized in Table III. The breakpoints observed for both activities were similar 22.0–22.5°C (Table III), i.e., close to the lipid thermotropic transition at 22.0–23.1°C observed by fluorescence polarization (Table II).

Inasmuch as an apparent break in the Arrhenius plot of any enzyme activity may result from temperature dependent changes in the half-saturation constant ($K_m$) of the substrate [46], both membrane activities at each temperature were also estimated at five different substrate concentra-

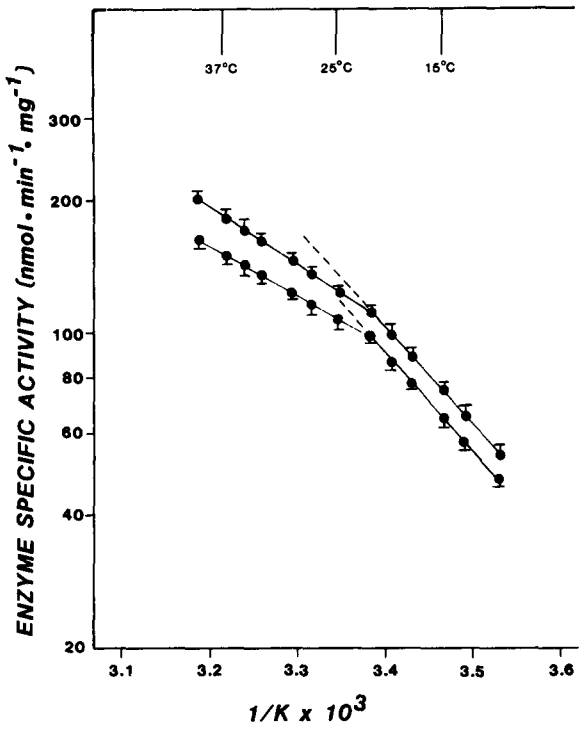


Fig. 3. Arrhenius plots of rat proximal apical membrane alkaline phosphatase (upper curve) and L-cysteine-sensitive alkaline phosphatase (lower curve). Table III lists the number of preparations, the apparent energies of activation, and the temperatures of the breakpoints.

tions. The maximal velocity ($V_{max}$) and $K_m$ parameters were evaluated from double-reciprocal plots [47] and Arrhenius plots were constructed for each parameter. The $V_{max}$ values again demonstrated a break at approx. 22–24°C, whereas the $K_m$ values did not. The $V_{max}$ values were almost identical with the activities in Fig. 3, (data not shown). Hence, the enzyme activities in Fig. 3, assayed at (substrate concentration)/$K_m$ ratios of 3–12 are in fact $V_{max}$ values.

*Membrane composition*

Analysis of the protein pattern of the 15/33 fraction by SDS-polyacrylamide gel electrophoresis demonstrated at least 20 separate bands with molecular weights varying between 16000 and 210000, the majority in the range of 30000 to 100000 (data not shown).

The neutral and phospholipid composition of this membrane was analyzed by thin-layer chromatography. Expressed as percent of weight of

TABLE III

TEMPERATURE DEPENDENCE OF PROXIMAL COLONIC APICAL MEMBRANE ENZYME ACTIVITIES

Values presented are means ± S.E. $N$, number of preparations.

| Enzyme activity | $N$ | $t_b$ (°C) | Apparent energy of activation $\Delta E$ (kcal/mol) | |
|---|---|---|---|---|
| | | | below $t_b$ | above $t_b$ |
| Alkaline phosphatase | 4 | 22.0 ± 1.6 | 12.3 ± 1.0 | 6.4 ± 0.8 |
| L-Cysteine-sensitive alkaline phosphatase | 4 | 22.5 ± 1.5 | 12.0 ± 0.7 | 5.3 ± 0.6 |

TABLE IV

COMPOSITION OF LIPID EXTRACTS OF RAT APICAL MEMBRANES

Values are means ± S.E. for lipid extracts of 33 determinations using seven preparations of apical membranes.

| Component | Neutral and phospholipid composition of apical membrane (% (w/w) of total) |
|---|---|
| Cholesterol | 22.3 ± 1.1 |
| Cholesterol esters | 2.9 ± 0.4 |
| Triacylglycerols | 2.5 ± 0.3 |
| Fatty acids | 13.3 ± 1.2 |
| Phosphatidylcholine | 33.2 ± 1.4 |
| Lysophosphatidylcholine | 1.0 ± 0.1 |
| Sphingomyelin | 13.8 ± 0.9 |
| Phosphatidylethanolamine | 12.3 ± 0.9 |

TABLE V

COMPOSITION OF TOTAL FATTY ACIDS OF RAT PROXIMAL COLONIC APICAL MEMBRANES

Values are means ± S.E. for lipid extracts from seven preparations of proximal colonic apical membranes.

| Fatty acid | % by mass | Fatty acid | % by mass |
|---|---|---|---|
| 12:0 | 0.31 ± 0.38 | 18:2 | 7.70 ± 0.88 |
| 14:0 | 4.50 ± 0.87 | 20:0 | 0.90 ± 0.26 |
| 14:1 | 2.55 ± 0.60 | 20:1 | 1.41 ± 0.30 |
| 16:0 | 25.65 ± 1.12 | 20:2 | 0.39 ± 0.09 |
| 16:1 | 8.08 ± 1.60 | 20:3 | 0.61 ± 0.02 |
| 18:0 | 16.86 ± 1.06 | 20:4 | 4.36 ± 1.32 |
| 18:1 | 27.74 ± 1.51 | | |

TABLE VI

COMPOSITIONAL ANALYSIS OF LIPID EXTRACTS OF RAT PROXIMAL COLONIC APICAL AND BASOLATERAL MEMBRANES

Values are means ± S.E. for lipid extracts of four preparations each of apical and basolateral membranes.

| Parameter | Apical | Basolateral |
|---|---|---|
| Cholesterol/phospholipid (mol/mol) | 0.8 ± 0.1 [b] | 0.4 ± 0.1 |
| Sphingomyelin/phosphatidylcholine (mol/mol) | 0.5 ± 0.1 [b] | 0.4 ± 0.1 |
| Protein/lipid (w/w) | 0.6 ± 0.1 | 0.6 ± 0.1 |
| Saturation index [a] | 0.7 ± 0.1 [b] | 0.5 ± 0.1 |

[a] Calculated by dividing the total saturated acyl chains by the sum of each unsaturated chain multiplied by the number of double bonds.

[b] $P < 0.05$ compared to basolateral membrane values.

total lipid (Table IV), the major neutral lipids were cholesterol and free fatty acids. The major phospholipids were phosphatidylcholine (33.2%), sphingomyelin (13.8%) and phosphatidylethanolamine (12.3%). Analysis of the total fatty acids of this membrane by GLC (Table V) revealed that the major fatty acids present were oleic (18 : 1) (27.7%), palmitic (16 : 0) (25.7%), stearic (18 : 0) (16.9%) and linoleic (18 : 2) (7.7%) acid.

As assessed by steady-state fluorescence polarization, the colonic apical membranes appeared to possess a lower lipid fluidity that basolateral membranes. Prior studies in model bilayers and natural membranes have correlated differences in lipid fluidity with variation in the lipid and protein composition [11,13,48–51].

It was therefore of interest to examine these parameters in both plasma membranes. As can be seen in Table VI, apical membranes possessed significantly greater molar ratios of cholesterol/phospholipid and sphingomyelin/phosphatidylcholine as well as a higher fatty acyl saturation index than basolateral membranes. The ratios of protein/lipid (w/w) in both membranes was similar.

## Discussion

The procedure described for the isolation of apical plasma membranes from rat proximal colonocytes is simple, reproducible, and rapid (requiring 6–7 h for completion). An approximate yield of 15–20% of this membrane is obtained, based on total and cysteine-sensitive alkaline phosphatase recovery with a 20–28-fold purification over homogenate.

The use of these enzymes as markers for this membrane appear valid since: (1) a cysteine-inhibitable alkaline phosphatase has recently been shown to be preferentially localized to the brush border of rat and rabbit colonocytes [52]; (2) alkaline phosphatase activity has previously been used as a purification marker for lumenal plasma membrane prepared from distal rabbit colonocytes [28] and mammalian enterocytes [53–55]. The minimal enrichment of the basolateral membrane markers $(Na^+ + K^+)$-ATPase and ouabain-sensitive $K^+$-phosphatase in the 15/33 fraction together with the results of the surface radio-iodination studies further suggest that the material present in this fraction originates from the apical plasma membrane region of the colonocyte.

A number of procedures for isolation of this membrane from rat enterocytes have used scraped material as starting material. Since colonic mucosa contains a marked heterogeneity of cell types [56,57], in the present procedure this was specifically avoided. To date, the only other published method for isolation of apical plasma membranes from rat colon used scraped mucosa as starting material [58]. The membranes obtained in that study, therefore, most likely represented a mixture of membranes from goblet and epithelial colonic cells.

The present results demonstrate that a lipid thermotropic transition can be detected in apical membranes, by steady-state fluorescence polarization using the fluorophore diphenylhexatriene, at 22–23°C. As previously discussed [13,25], although these temperatures are well below 37°C, this does not preclude a physiological role for this alteration in the physical state of the lipid. Earlier studies have shown that this technique detected only the lower critical temperature of broad transitions observed by differential scanning calorimetry in rat enterocyte [13] and hepatic plasma membranes [6]. In the present study the transition temperatures detected in rat apical membranes therefore probably represents the lower critical

temperature of the transition.

These studies also demonstrate that the membrane lipid may influence certain protein-dependent activities, i.e., alkaline phosphatase and L-cysteine-sensitive alkaline phosphatase. Arrhenius plots of these activities showed breakpoints in the vicinity of the lipid thermotropic transition temperatures. Although temperature-sensitive changes in protein conformation may be independent of membrane lipids [46,59], all studies were performed at constant pH and under maximal velocity conditions to preclude artificial breaks in slope. Studies [12] in our laboratory have furthermore shown delipidation removed the breakpoint seen in Arrhenius plots of the rat enterocyte microvillus membrane's *p*-nitrophenylphosphatase and relipidation restored the breakpoint to its original temperature. This suggests that this (these) activity(ies) are 'intrinsic membrane activities', i.e., are mediated by proteins which are influenced by the physical state of their membrane lipid [12–15].

Prior studies [14] revealed that the motional freedom of lipid molecules was considerably less in microvillus membranes than in basolateral membranes prepared from rat enterocytes. Gray et al. [60], using an electron spin resonance technique, have confirmed this finding. Lumenal membranes of dog kidney cells [16] and canilicular membranes of rat liver cells [61] also appear to possess a lower lipid fluidity than their contralumenal counterparts, suggesting that this gradient of fluidity may be a general phenomenon.

The present data demonstrate that proximal colonic apical membranes, like other lumenal plasma membranes, possess a relatively low lipid fluidity, although its anisotropy parameter values are not as high as those previously documented in microvillus membranes of rat enterocytes [12–15,62].

In this regard, a low lipid fluidity has been correlated with high molar ratio of cholesterol/phospholipid and sphingomyelin/phosphatidylcholine, high ratios of protein/lipid (w/w) [11,13] as well as greater saturated or longer acyl chains in phospholipid [50,51]. The relatively low fluidity of rat colonic apical membranes appears to result from high values for these parameters in this membrane (Table VI). The luminal (apical) membranes of rat proximal colonocytes also possess a lower lipid fluidity than contralumenal (basolateral) membranes at all temperatures examined, as assessed by fluorescence polarization studies using three different probes (Table II). Differences in lipid dynamics of these antipodal membranes appear, at least in part, to be due to differences in membrane composition (Table VI).

Further studies of these antipodal colonic membranes should clarify questions concerned with the functional significance of this gradient of fluidity and mechanisms for the maintenance of these differences in composition.

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## References

1 Razin, S. (1975) Prog. Surf. Membrane Sci. 9, 257–342
2 Fox, C.F. (1975) in Biochemistry of Cell Walls and Membranes (Fox, C.F., ed.), Vol. 2, pp. 279–306, Park Press, Baltimore, MD
3 Tada, M., Yamamoto, T. and Tonomura, Y. (1978) Physiol. Rev. 58, 1–79
4 Lee, A.G. (1977) Biochim. Biophys. Acta 472, 237–281
5 Lee, A.G. (1977) Biochim. Biophys. Acta 472, 285–344
6 Livingstone, C.J. and Schachter, D. (1980) J. Biol. Chem. 255, 10902–10908
7 Overath, P., Schairer, H.V. and Stoffel, W. (1970) Proc. Natl. Acad. Sci. U.S.A. 67, 606–612
8 Rosen, B.P. and Hackette, S.L. (1972) J. Bacteriol. 110, 1181–1189
9 Linden, C.D. and Fox, C.F. (1973) J. Supramol. Struct. 1, 535–544
10 Schechter, E., Letellier, L. and Gulik-Krzywicki, T. (1974) Eur. J. Biochem. 49, 61–76
11 Schachter, D. and Shinitzky, M. (1977) J. Clin. Invest. 59, 536–548
12 Brasitus, T.A., Schachter, D. and Mammouneas, T.G. (1979) Biochemistry 18, 4136–4144
13 Brasitus, T.A., Tall, A.R. and Schachter, D. (1980) Biochemistry 19, 1256–1261
14 Brasitus, T.A. and Schachter, D. (1980) Biochemistry 19, 2763–2769
15 Brasitus, T.A. and Schachter, D. (1980) Biochim. Biophys. Acta 630, 152–156

16 LeGrimmellec, C., Giocondi, M.C., Carriere, B., Carriere, S. and Cardinal, J. (1982) Am. J. Physiol. 242, F246–F253
17 Blank, M. (1976) in Membranes and Diseases (Bolis, L., Hoffman, J.F. and Leaf, A., eds.), pp. 81–88, Raven Press, New York
18 Lakowicz, J.R., Prendergast, F.G. and Hogen, D. (1979) Biochemistry 18, 508–519
19 Lakowicz, J.R., Prendergast, F.G. and Hogen, D. (1979) Biochemistry 18, 520–527
20 Chen, L.A., Dale, R.E., Roth, S. and Brand, L. (1977) J. Biol. Chem. 252, 2163–2169
21 Dale, R.E., Chen, L.A. and Brand, L. (1977) J. Biol. Chem. 252, 7500–7510
22 Kawato, S., Kinosita, K., Jr. and Ikegami, A. (1977) Biochemistry 16, 2319–2324
23 Kinosita, K., Kawato, S. and Ikegami, A. (1977) Biophys. J. 20, 289–305
24 Brasitus, T.A. and Keresztes, R.S. (1983) Biochim. Biophys. Acta 728, 11–19
25 Brasitus, T.A. (1983) Biochim. Biophys. Acta 728, 20–30
26 Shamsuddin, A.K.M. and Trump, B.F. (1981) J. Natl. Cancer Inst. tt, 375–379
27 Siegel, G.J. and Goodwin, B.G. (1972) J. Biol. Chem. 247, 3630–3637
28 Gustin, M.C. and Goodman, B.P. ((181) J. Biol. Chem. 256, 10651–10656
29 Kim, Y.S., Perdomo, J. and Whitehead, J.S. (1972) J. Clin. INvest. 51, 2024–2032
30 Podolsky, D.K. and Weiser, M.W. (1975) Biochem. Biophys. Res. Commun. 65, 545–551
31 Morrison, M. (1974) in Methods Enzymol. 32, 103–109
32 Hubbard, A.L. and Cohn, Z.A. (1972) J. Cell Biol. 55, 390–405
33 Weiser, M.M., Neumeier, M.M., Quaroni, A. and Kirsch, K. (1978) J. Cell Biol. 77, 722–734
34 Fairbanks, G., Steck, T.L. and Wallach, D.F.H. (1971) Biochemistry 10, 2606–2617
35 Steck, T.L. and Yu, J. (1973) J. Supramol. Stuct. 1, 220–232
36 Folch, J., Lees, M. and Sloane-Stanley, G.A. (1957) J. Biol. Chem. 226, 497–509
37 Katz, S.S., Shippley, G.G. and Small, D.M. (1976) J. Clin. Invest. 57, 200–211
38 Gartner, J.L. and Vahouny, G. (1972) Am. J. Physiol. 22, 1121
39 Lowry, O.H., Rosebrough, N.J., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
40 Burton, K. (1956) Biochem. J. 62, 315–323
41 Giles, K.W. and Myers, A. (1965) Nature (London) 93, 4979
42 Shinitzky, M. and Barenholz, Y. (1974) J. Biol. Chem. 249, 2652–2657
43 Thulborn, K.R., Treloar, E. and Sawyer, W.H. (1978) Biochem. Biophys. Res. Commun. 81, 42–49
44 Thulborn, K.R. and Sawyer, W.H. (1978) Biochim. Biophys. Acta 611, 126–140
45 Cadenhead, D.A., Kellner, B.M.J., Jacobson, K. and Papahadjopoulous, D. (1977) Biochemistry 16, 5386–5392
46 Silvius, J.R., Read, B.D. and McElhaney, R.N. (1978) Science 299, 903—905
47 Lineweaver, H. and Burk, D. (1934) J. Am. Chem. Soc. 56, 658–666
48 Chapman, D. and Penkett, S.A. (1966) Nature (London) 211, 1304–1305
49 Hubbell, W.L. and McConnell, H.M. (1971) J. Am. Chem. Soc. 93, 314–326
50 Shinitzky, M. and Inbar, M. (1976) Biochim. Biophys. Acta 433, 133–149
51 Shinitzky, M. and Berenholz, Y. (1978) Biochim. Biophys. Acta 515, 367–394
52 Vengesa, P.B. and Hopfer, U. (1979) J. Histochem. Cytochem. 27, 1231–1235
53 Murer, H., Ammann, E., Biber, J. and Hopfer, U. (1976) Biochim. Biophys. Acta 433, 509–519
54 Eicholz, A. and Crane, R.K. (1965) J. Cell Biol. 6m 687
55 Hopfer, U., Sigrist-Nelson, E., Ammann, E. and Murer, H. (1976) J. Cell Physiol. 89, 805–810
56 Kaye, C.I., Fenoglio, C.M., Pascal, R.P. and Lane, N.L. (1973) Gastroenterology 65, 926–945
57 Fenoglio, C.M., Richart, R.M. and Kaye, C.I. (1975) Gastroenterology 69, 100–109
58 Jackson, R.J., Stewart, H.B. and Sachs, G. (1977) Cancer 40, 2487–2496
59 Fourcans, B. and Jain, K. (1974) Adv. Lipid. Res. 12, 147–226
60 Gray, J.P., Henderson, G.I., Dunn, G.D., Swift, L.L., Wilson, F.A. and Hoyumpa, A.M. (1981) Gastroenterology 80, 1162 (Abstr)
61 Storch, J., Schachter, D., Inoue, M. and Wolkoff, A.W. (1983) Biochim. Biophys. Acta 727, 209–212
62 Brasitus, T.A. and Schachter, D. (1982) Biochemistry 21, 2241–2246