

Redistribution of Membrane Proteins in Isolated Mouse Intestinal Epithelial Cells

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ABSTRACT Single mouse intestinal epithelial cells (IEC) may be isolated by the use of a combination of methods used for the isolation of IEC from other species. Isolated cells remain viable for several hours. The membrane integral enzymes alkaline phosphatase and leucine aminopeptidase of isolated IEC are localized to the brush borders of IEC in tissue and in most newly isolated IEC. With time, both enzymes are found distributed over the entire cell surface. Redistribution appears to occur by diffusion in the plane of the membrane. It is slowed, but not blocked, if cells are maintained at 0°C instead of at 37°C, and it is not blocked by fixation in 0.5–3% paraformaldehyde. Drugs that alter cell membrane potential or that affect cell levels of ATP enhance the rate of redistribution of the enzymes.

Diffusion of integral membrane proteins in the plane of the plasma membrane has been demonstrated in many cells and by several different techniques (for review, see reference 7). The diffusion constants measured are often smaller than predicted for free diffusion. This suggests that lateral diffusion of membrane proteins is somewhat restricted, even in cultured cells with little surface differentiation.

Extremes of spatial localization of proteins in the plasma membrane and, by implication, extremes of restriction on lateral diffusion of these proteins are seen in cells of differentiated tissues such as muscle (2, 18, 19, 20, 41), liver (16, 46, 47), and intestine (10, 15, 21, 22, 23, 24, 25, 28). Cells in the intact tissues are functionally and morphologically polarized, with hydrolytic, secretory, or transport functions being restricted to a particular area of the cell membrane, which in turn is often morphologically differentiated.

In this paper we describe the distribution of two membrane integral enzymes, alkaline phosphatase (AP) and leucine aminopeptidase (LAP) in the membranes of isolated mouse intestinal epithelial cells (IEC). The enzymes are restricted to the brush border of IEC in tissue and in single cells immediately after isolation. Within minutes of preparation, cells can be found that bear both enzymes over the entire surface. The proportion of these cells increases with time until, ~60 min after isolation, almost 100% have delocalized LAP and AP. Experiments with fixed cells and with a variety of drugs indicate that redistribution is not metabolically driven. Though restraints to redistribution may require cell metabolism, redistribution of both enzymes appears to be entirely caused by lateral diffusion in the plane of the membrane.

MATERIALS AND METHODS

Isolation of Intestinal Epithelial Cells

The method is based on published procedures for isolation of intestinal epithelial cells of other species. No one of these procedures alone yielded significant numbers of viable single cells from mouse intestine.

B10.A mice (Jackson Laboratory, Bar Harbor, Maine) were starved overnight, then killed by cervical dislocation. Their small intestines were removed and flushed with cold Dulbecco's phosphate-buffered saline (PBS) to remove fecal contents. An intestine, everted over a plastic-coated wire (8), ligated at one end, distended with cold PBS and tied off (31), was placed in a wide-mouth plastic bottle in 35 ml of hypertonic (360–410 mosM) isolation medium that had been aerated for 1 h before use. The isolation medium was Krebs-Ringer's phosphate-buffered saline, pH 7.3, containing 27 mM sodium citrate (36, 42), 3 mg/ml hyaluronidase 300 (Nutritional Biochemical Corp., Cleveland, Ohio) (8, 23, 25, 28, 31, 36), 2% Dextran 500 (Pharmacia Fine Chemicals, Div. of Pharmacia, Piscataway, N. J.) (42), and 2 mM adenosine 5'-diphosphate, sodium salt (ADP) (Sigma Chemical Co., St. Louis, Mo., Grade IV, from equine muscle) (5). After 10 min, the isolation medium was removed and replaced with another 35 ml of aerated isolation medium for an additional 10 min of shaking and aeration at 37°C. This step was repeated twice. Each decanted solution containing dissociated epithelial cells was centrifuged at room temperature for 5 s at the top speed of an International clinical centrifuge (International Equipment Co., Needham Heights, Mass.) to pellet epithelial sheets and whole villi. The resulting single-cell supernates were further centrifuged at 200 g for 5 min in the cold. The pellets were gently resuspended in 1–2 ml of cold, aerated resuspension medium (345–375 mosM), Krebs-Ringer's phosphate-buffered saline (pH 7.3) containing 2% Dextran 500 and 2 mM ADP (KRP-DA). Cell viability was checked in terms of Nigrosin dye exclusion. Plastic vessels, test tubes, and coverslips, as well as siliconized glass Pasteur pipettes and siliconized glass conical test tubes, were used at all times because the single cells are damaged when exposed to untreated glass vessels and pipettes (24, 42).

Some preparations were made with media containing 0.5 mM dithiothreitol (35). The viability of isolated cells was somewhat (10%) higher than that of cells isolated in the absence of dithiothreitol, but no difference was seen in the rate of redistribution of markers in these cells.

Histochemical Stains

AP was visualized by the sodium- α -naphthyl phosphate-Fast Blue RR (30) or by the naphthol AS-MX phosphate-Fast Blue RR (1) technique. We stained the cytocentrifuged preparations of cells for from 20 s to 2 min. With this brief exposure, reaction product was found only in the brush border of cells and not in the cytoplasm in the Golgi apparatus (29). LAP was visualized by a dye coupling method with L-leucyl-4-methoxy-2-naphthylamide HCl as substrate and Fast Blue B as coupling reagent (1). Specificity of both reactions was checked on cryostat sections of intact intestine. Only villus epithelial cells stained on these preparations. No reaction product appeared in the absence of substrate.

Purification of Immunogens

AP was prepared from 100 g of mouse small intestine following the procedure of Moog et al. (27). The intestines were extracted with butanol, and the enzyme activity was purified from a butanol-water supernate by chromatography on ion exchange cellulose, DEAE-52 (Whatman, Inc., Clifton, N. J.). Enzymatic activity was monitored by the procedure of Fujita et al. (21), in terms of hydrolysis of *p*-nitrophenyl phosphate (Sigma Chemical Co.). The final product was 40-fold purified. One major protein band was seen, which corresponded to the AP activity when examined by electrophoresis in 5% polyacrylamide or 0.6% agarose tube gels. Five other faint protein bands, which did not react histochemically for AP, could be detected when 200 μ g of protein was loaded on a single gel. The use of this material for immunization is described in a later section.

LAP was purified from 100 g of mouse small intestine following the procedure of Maroux et al. for the enzyme from hog (26). A Triton X-100 extract of intestine was purified by ammonium sulfate precipitation and fractionation of the supernate (which contained the bulk of the activity) on DEAE-52, followed by purification on Biogel A 1.5 (Bio-Rad Laboratories, Richmond, Calif.) in 1% Triton X-100. Enzyme activity was followed using L-leucyl-naphthylamide substrate (3). The resulting product was purified 350-fold. However, it gave multiple bands on 5% polyacrylamide gels. Four faint protein bands were LAP-negative; a single prominent band corresponded to all of the enzyme activity determined histochemically on a parallel gel. A single enzyme-positive band was also obtained on 0.6% agarose tube gels.

Rabbit Antibodies to Intact Intestinal Epithelial Cells

Single IEC were prepared as described above. 1.5×10^7 cells were mixed with 1 ml of reconstituted live *Bacillus Calmette-Guerin* (BCG) (Trudeau Institute, Sarnac Lake, N. Y.) and injected into multiple subcutaneous sites and into the hind footpads of a ~4-kg rabbit. The animal was bled 3 wk later and was reimmunized with 1.5×10^7 cells intravenously at week four. 7 d later (week five) the rabbit was bled again, and then every 3 d for the next 2 wk. The sera obtained are designated anti-IEC.

Rabbit Antibodies to Alkaline Phosphatase

Rabbit anti-IEC reacted with purified AP in Ouchterlony double-diffusion gels to give two precipitin bands, only one of which contained AP activity. Further gels were prepared from 35 μ l of antiserum and ~11 μ g of purified AP. The AP-positive precipitin bands were cut out of the washed gels, homogenized in Freund's complete adjuvant, and injected into a rabbit at multiple subcutaneous sites. The animal was further immunized 6 wk later by intraperitoneal injection of another set of precipitin bands prepared from 70 μ l of antiserum and 22 μ g of antigen protein. Bleeding commenced 1 wk after the booster injection and continued for 10 d. The antiserum produced gave a single enzyme-positive band in Ouchterlony gels when tested against purified AP. It did not react with isolated IEC in indirect immunofluorescence. Accordingly, we immunized yet another rabbit with the AP-positive precipitin band produced by the previous anti-AP. This animal was boosted twice by intraperitoneal injection and bled eight times over a period of 3 wk after its third immunization. The antisera gave a single precipitin band, which was AP positive, in double diffusion against purified AP. The band fused with those formed by the other antisera described in this section. The serum also reacted with IEC, as described in Results, but did not react with mouse L cells.

Rabbit Antibodies to Leucine Aminopeptidase

Rabbit anti-LAP was prepared by homogenizing the LAP-positive band from a polyacrylamide gel in Freund's complete adjuvant and injecting the mixture

into two rabbits as described for AP. The animals were reimmunized intraperitoneally 3 wk after the first injection. They were bled during the following 3 wk. The sera were tested in double-diffusion gels against both purified leucine aminopeptidase and crude intestine homogenate. Both antigens gave only two precipitin bands, and both bands were positive for LAP. Serum absorbed with intact IEC no longer gave LAP-positive bands in double-diffusion gels.

Monovalent Antibody Fragments

Fab fragments of anti-AP and anti-LAP were prepared by papain digestion of intact antibody followed by isolation from CM-cellulose (45). Intact IgG and Fab fragments were conjugated with fluorescein isothiocyanate (Baltimore Biological Laboratories, Baltimore, Md.) following published procedures (6). Free fluorescein was separated from conjugated protein on Sephadex G-25. Fluorescein to protein ratio was 0.7 for both Fab conjugates.

Immunofluorescence

Cells were exposed to antibody for 15 min at 0°C, washed by centrifugation through a layer of fetal calf serum and resuspended in HEPES-buffered Hanks' balanced salt solution (BSS) or KRP-DA. Directly labeled cells were then examined without further treatment. In indirect labeling experiments, cells coated with specific antibodies were further treated with a fluorescein-conjugated Fab fragment of goat anti-rabbit Fab and then washed as described. When fluorescence was weak, cells were pelleted onto slides in a cytocentrifuge and examined in a solution of 5×10^{-3} M *o*-phenylenediamine (Matheson, Coleman, & Bell, East Rutherford, N. J.) in 50% ethanol, 50% PBS. This enhanced the fluorescence and retarded its fading. In experiments in which antibodies were used, to follow the rate of redistribution of enzymes with time, cells were fixed after the first antibody layer in 4% paraformaldehyde in PBS for 15 min at 0°C before being washed and before fluorescent antiglobulin reagents were added. This inhibited further redistribution of enzymes (see Results) but did not affect the specificity of staining with antiglobulin.

Electron Microscopy

Immediately after isolation, single IEC were fixed for 30 min in cacodylate-buffered 6.25% glutaraldehyde on ice, and then washed with cold 0.1 M cacodylate buffer, pH 7.2, and postfixed in OsO₄. Fixed cell pellets were dehydrated, embedded in Epon, sectioned, stained with uranyl acetate and lead citrate, and examined in a JEM-100 transmission electron microscope. For scanning electron microscopy, the glutaraldehyde-fixed cells were attached to poly-L-lysine-coated coverslips, postfixed in cacodylate-buffered 2% OsO₄, dehydrated, dried from liquid CO₂ at the critical point, gold coated, and examined in a JEOL JSM-35 scanning electron microscope.

Some cell suspensions were stained to visualize alkaline phosphatase by the method of Hugon and Borgers (22). This lead phosphate method was applied before postfixation in OsO₄. Stained samples were washed in 0.1 M cacodylate buffer before fixation and embedding were continued.

Scoring of Alkaline Phosphatase and Leucine Aminopeptidase Location on Isolated Cells

Only epithelial cells stained for either enzyme. A cell was scored as showing restricted or localized marker if only the brush border or extreme apical portion of the cell was stained. A cell was scored as showing redistributed marker if at least one-half to two-thirds of the cell surface was stained. 100–200 cells were evaluated for the location of enzyme stain for each time sample. In some instances, replicate counts were made on the same sample. These counts were within 20% of one another. The range of variation in counts from experiment to experiment may be judged from Figs. 7 and 8.

RESULTS

The supernate, obtained after a 10-min incubation of intestine in isolation medium at 37°C, contained few cells, but a large amount of mucus and intestinal parasites and flora, and was always discarded. The supernates obtained after the 20- and 30-min incubations contained little mucus and large numbers of single epithelial cells, as well as single cells from the lamina propria, cell clumps, epithelial sheets, whole villi, and free nuclei. Large clumps, sheets, and villi were sedimented for 5 s at high speed in a clinical centrifuge. The supernates contained

suspensions of single cells slightly contaminated with two-, three-, and four-cell epithelial strips, free nuclei, and some nonepithelial cells. From 3×10^6 to 1×10^7 isolated IEC were obtained from a single animal, depending on the length and the diameter of the intestine used. 80–98% of the isolated cells kept at 37°C excluded Nigrosin dye for at least 1 h after isolation.

The dextran 500, ADP, and Ca^{++} and Mg^{++} ions in the isolation and collection media, as well as prior aeration of both media, minimized the cell swelling that commonly occurs in isolated intestinal epithelial cell preparations. Cells isolated in the absence of any of these five components were extremely swollen within 1–1.5 h after isolation.

Isolated IEC examined in the transmission electron microscope (TEM) were columnar in shape, although often rounder and shorter than those in the intact intestine. The cells retained their apical brush borders. Cells were always vacuolated to some extent. Vacuoles were concentrated toward the basal ends of most cells; the degree of vacuolization varied from preparation to preparation and from cell to cell within a given preparation. Sections through three cells, indicating the degree of organization at the cytoplasm of isolated IEC, are shown in Fig. 1.

Intact isolated IEC examined in the scanning electron microscope (SEM) were largely columnar in shape (Fig. 2) and averaged about 4–6 μm wide and 10–12 μm long. The pits seen in the surface of the cell in Fig. 2 are a common feature of the preparation. The cells, in all cases, had a rough surface, which was shaggy in cells kept alive for 45–60 min after cell isolation. The morphology of the IEC in strips appeared identical to that of the single cells.

Histochemical Localization of Alkaline Phosphatase and Leucine Aminopeptidase by Light Microscopy

Both enzymes were localized to the brush borders of the villar epithelial cells in paraffin-and-cryostat sections of mouse small intestine. The lateral and basal plasma membranes of these cells were not stained. Villus core and crypt cells were also negative. With the staining procedure used, we found no reaction for alkaline phosphatase that could be specifically associated with the Golgi apparatus (29).

Immediately after isolation, the majority (75–90%) of the single IEC and all of the small cell strips had AP stain only on their brush borders, although some (10–25%) single IEC had stain over the entire cell (six experiments) (Fig. 3). Cells lacking a brush border were not stained. With increasing time after isolation, a larger proportion of the single IEC had enzyme reaction product over the entire cell surface. Though the unstained basolateral positions of some cells appear as faint dark images in the photograph, these were readily distinguished in practice. Similar results were found for LAP; 92–99% of all cells reacting for LAP showed reaction product restricted to the brush border immediately after isolation (three experiments). Most cells retained a concentration of enzyme on the brush border, though brush borders were greatly reduced in extent after 40-min incubation at all temperatures. The proportion of cells that were basolaterally stained for alkaline phosphatase by the electron microscope cytochemical method of Hugon and Borgers (22) also increased with time after isolation. An example of such a uniformly stained cell is shown in Fig. 4. We could not find sufficient numbers of well-oriented cells that

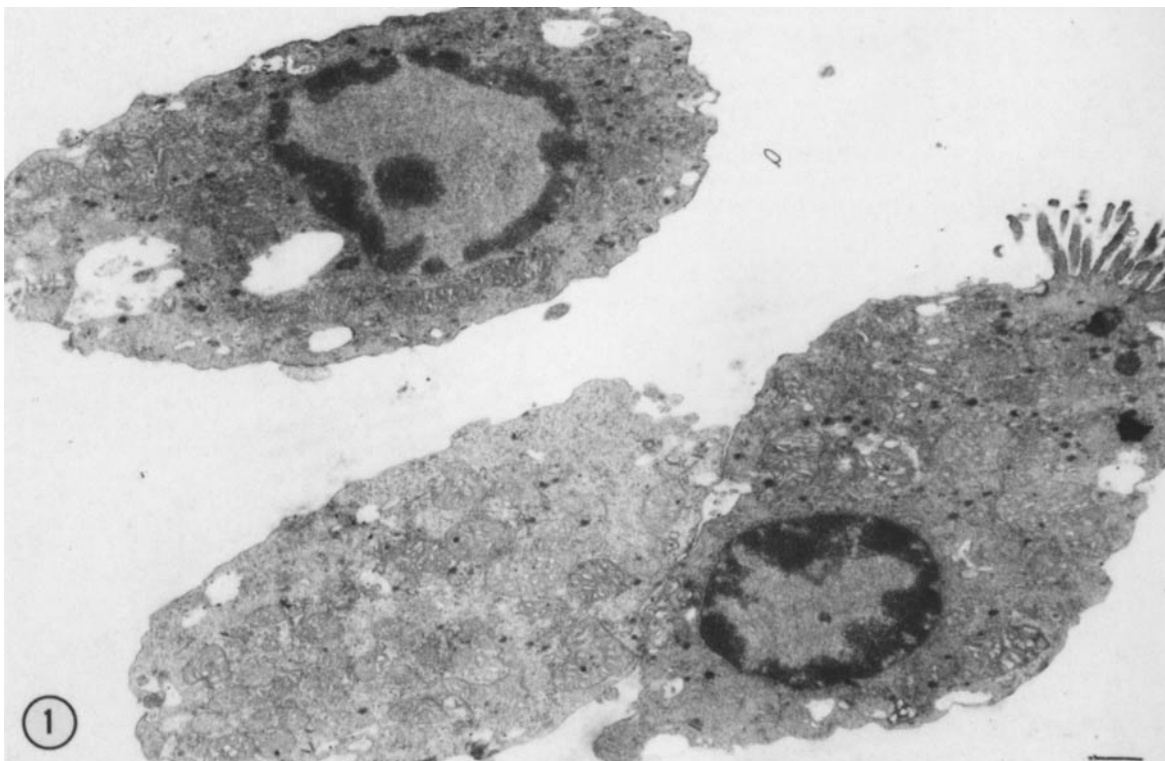


FIGURE 1 Freshly isolated IEC. Some microvilli are evident in one cell, but the glancing section does not allow an estimate of their full extent. Some vacuoles are present in the cytoplasm of all three cells. These cells are from a control (no substrate) sample in a series stained for alkaline phosphatase. The small dark spots in the cytoplasm are nonspecific lead deposits (cf. Fig. 4). Bar, 1 μm . $\times 7,600$.

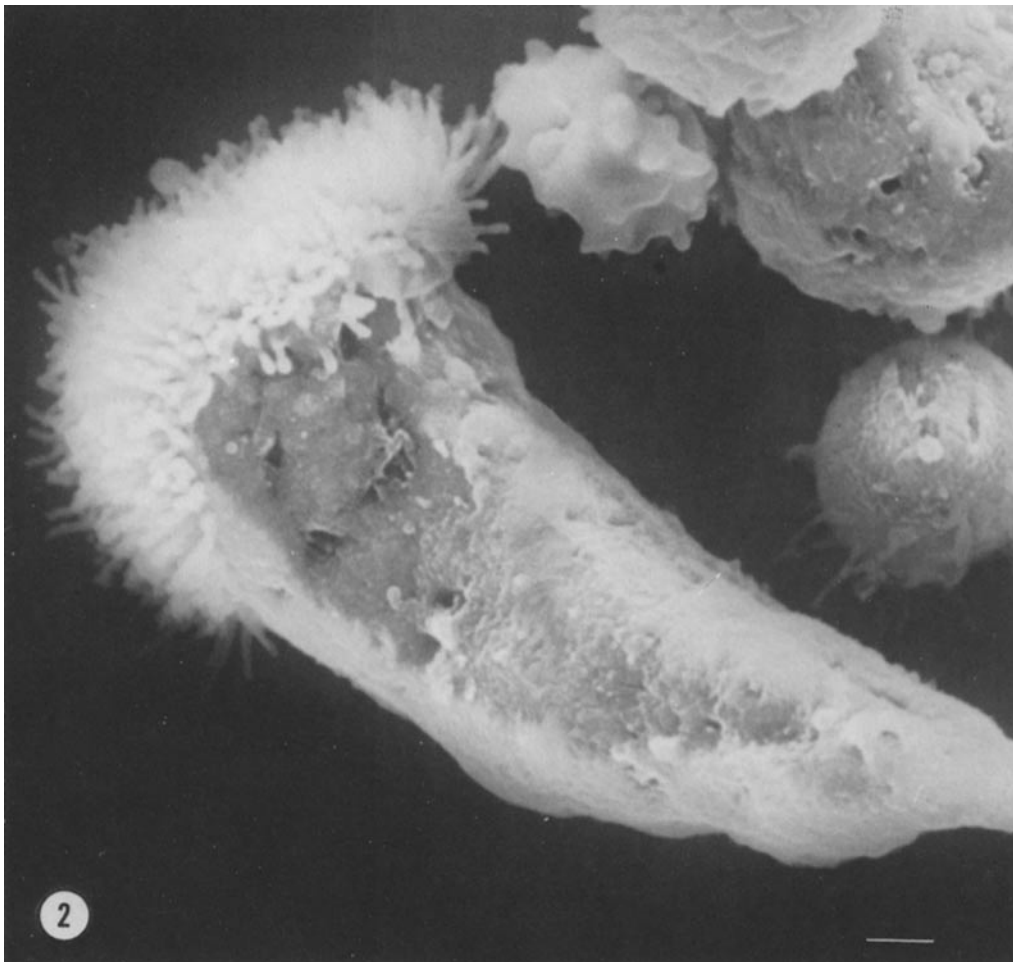


FIGURE 2 Scanning electron micrograph of an isolated intestinal epithelial cell. Note the luxuriant brush border and rough, pitted basolateral membrane. An erythrocyte and a cell of the lamina propria are also seen. Bar, 1 μm . $\times 8,858$.

were cut to show both the brush border and the nucleus to allow us to quantitate our results by electron microscopy.

Time-Course of Alkaline Phosphatase and Leucine Aminopeptidase Redistribution Determined Cytochemically

Redistribution of AP over the cell surface (detected by light microscope cytochemistry) occurred in 50% of the single IEC at 0°C in 40 min ($t_{1/2}$). The redistribution was faster at 37°C, the $t_{1/2}$ being ~12 min. In a few experiments, cell morphology deteriorated after long periods at 37°C; the brush borders of the cells were no longer clearly visible and cells appeared very irregular in shape. However, cell viabilities measured by dye exclusion were excellent (>80%) at all the time points. Intermediate $t_{1/2}$ s were obtained at intermediate temperatures. The $t_{1/2}$ values are summarized in Table I, together with the $t_{1/2}$ values for LAP redistribution as a function of temperature. The latter, determined cytochemically, are greater than those of AP.

Reaction of Intestinal Epithelial Cells with Antibodies and Antibody Fragments

To ensure that IEC of adults did not bind immunoglobulins nonspecifically, we examined a number of control sera in addition to the anti-IEC and antienzyme sera and antibody

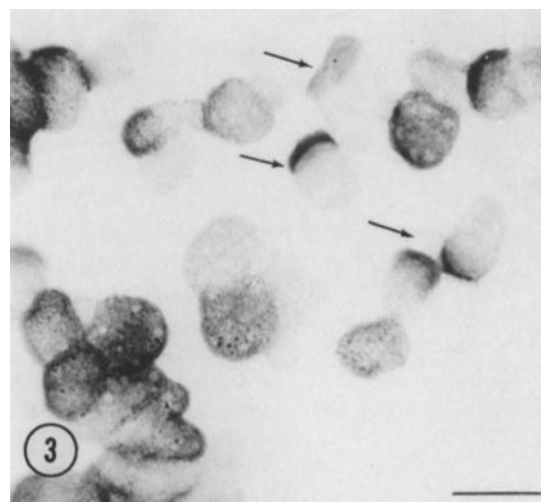


FIGURE 3 Cytocentrifuge slide of IEC stained for alkaline phosphatase by the sodium- α -naphthyl phosphate-Fast Blue RR technique. Cells with reaction product restricted to the brush border (arrows) and those with stain on basolateral surfaces as well may be seen.

fragments. The results of these controls, the staining patterns given by the specific antisera, and data on absorption of these antisera by purified antigen are given in Table II. Staining is specific; a number of normal sera or irrelevant antisera do not

TABLE I
Half-Times and Temperature Coefficients, Q_{10} , for Appearance of Isolated IEC Bearing Alkaline Phosphatase or Leucine Aminopeptidase Over the Entire Cell Surface

Enzyme	Detected	$t_{1/2}$ (min) at degrees centigrade					Q_{10}
		0	15	22	30	37	
Alkaline phosphatase	Cytochemically*	40	40	26	20	12	1.64
	Immunochemically‡	40	—	20	—	9	1.46
Leucine aminopeptidase	Cytochemically§	80	—	56	—	42	1.18
	Immunochemically	48	—	24	—	16	1.35

* Five experiments at 0°, 22°, 37°C; standard deviation was 10%; one experiment at 15° and 30°C.

‡ Two experiments.

§ Three experiments.

|| Three experiments.

Q_{10} are calculated from linear least squares fits to regressions of $\log t_1$ on temperature in degrees centigrade.

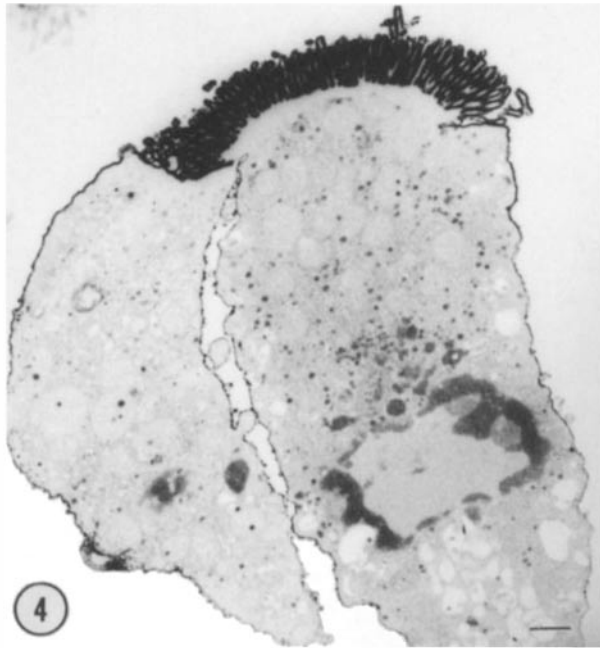


FIGURE 4 Reaction of lateral portions of an IEC for alkaline phosphatase after 20 min at room temperature. A definite outline stain of reaction product may be seen along the membrane. It remains heaviest in the brush border. The control cells, for comparison, are in Fig. 1. Bar, 1 μ m. \times 6,500.

stain the cells. The sera that are expected to react either stain the entire epithelial cell surface, in the case of anti-IEC and anti-mouse cell, or stain only the brush border of freshly isolated cells (anti-AP and anti-LAP). The antisera stained epithelial cells in cryostat sections of intact intestine with the pattern seen on isolated epithelial cells. Anti-AP and anti-LAP sera did not react with the cells of the lamina propria or cells of the cultured L-cell subline, cl 1d. One set of reagents, detecting the major histocompatibility antigens, H-2, stained only the basolateral surface of isolated cells. In all cases, damaged cells stained uniformly in the cytoplasm. These were not counted in any of the work with antibodies or fragments.

Our observations with histochemical stains for surface enzymes show that redistribution occurs even at 0°C. Because at least 15 min are required for labeling of cells with antibodies, even using the quick washing procedure described in Methods, the initial samples of stained cells showed higher proportions of cells labeled on cell surfaces than were found in histochemical preparations. 22–43% of cells were labeled with antibody

TABLE II
Reaction of Single Intestinal Epithelial Cells With Various Antisera and Antibody Fragments by Direct and Indirect Immunofluorescence

Serum or antibody fraction	Fluorescence intensity	Fluorescence pattern
None*	—‡	—
Preimmunization rabbit serum*	—	—
Rabbit anti-fish cell	—	—
Rabbit anti-mouse spleen cells	+++‡	Ring stain
Rabbit anti-IEC	+++	Ring stain
Rabbit anti-IEC, absorbed on mouse lymphoid cells and L cells	+++	Ring stain
Mouse anti H-2§	++	Basolateral membranes only
Rabbit anti-AP	+++	Brush border only
Rabbit anti-AP absorbed with purified AP	—	—
Rabbit anti-AP absorbed with AP-positive band eluted from agarose gel electrophoresis	—	—
FI-Fab rabbit anti-AP	+++	Brush border only
Rabbit anti-LAP	+++	Brush border only
Rabbit anti-LAP absorbed with LAP positive band eluted from agarose gel electrophoresis	—	—

* All rabbit antisera were detected with a fluorescein-conjugated Fab fragment of goat anti-rabbit Ig_{FAB}.

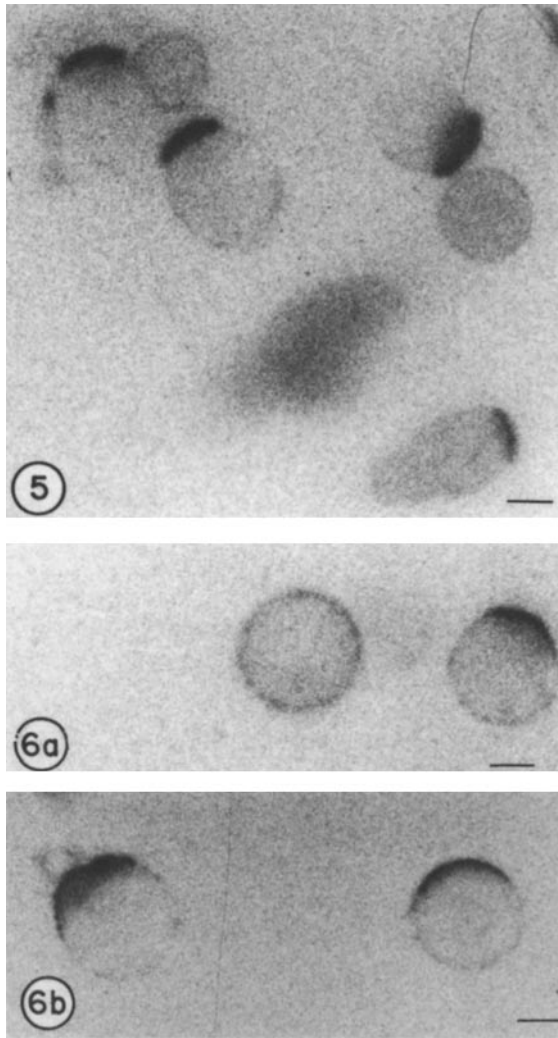
‡ Fluorescence was scored on a scale of brightness from — (no fluorescence) to ++++ (bright fluorescence).

§ Detected with intact fluorescein-conjugated goat anti-mouse Fab.

|| Fluorescein-conjugated Fab fragment of rabbit anti-AP used in direct immunofluorescence.

on the basolateral as well as on the luminal surface, compared with 10–25% labeled cytochemically. Examples of cells labeled on the brush borders only or over the entire surface are shown in Figs. 5 and 6. With time, the proportion of cells stained on all surfaces increased, as a function of temperature. As noted above, stain was usually most intense on the brush border, though some ring-stained cells were found (Fig. 6). The data for AP are shown in Fig. 7 and those for LAP in Fig. 8, plotted as first-order decay processes. The $t_{1/2}$ values are tabulated in Table I. Similar values were obtained when directly labeled Fab fragments were used instead of intact antienzyme antibodies.

The significant redistribution of both enzymes at 0°C, even



FIGURES 5 and 6 Cells labeled with anti-AP antibody and a fluorescent anti-Fab. Three cells in Fig. 5 are labeled in the brush border, whereas those in Fig. 6 a and b are labeled around their entire periphery. The greater concentration of surface in the brush border makes its fluorescence more intense than that over the basolateral portion of the membrane, though one cell (Fig. 6) shows ring stain. The photographs are printed as negative images to enhance contrast. Bar, 10 μm . $\times 600$.

after a layer of antibody was added, suggest that cell metabolism did not play a role in the movement of enzyme from the brush border to the basolateral membrane of isolated cells. Accordingly, we examined redistribution of surface label in cells labeled with antienzyme, fixed for 15 min in various concentrations of paraformaldehyde at 0°C, and then incubated for a further 15 min with fluorescein-conjugated anti-globulin. Under these conditions, redistribution of both enzymes is inhibited only by 4% paraformaldehyde (Fig. 9).

Work in our laboratory, as well as work by others, had indicated that in some cells diffusion of membrane integral proteins does not occur at the maximum rate expected; rather, it appears to be hindered by the cytoskeleton. We tested the effects of a number of drugs on the rate of redistribution of AP and LAP in isolated IEC. These were an inhibitor of serine proteases (21), phenylmethanesulfonyl fluoride (PMSF), drugs directly affecting cytoskeletal elements, colchicine and cytochalasin B, and drugs expected to alter membrane potential,

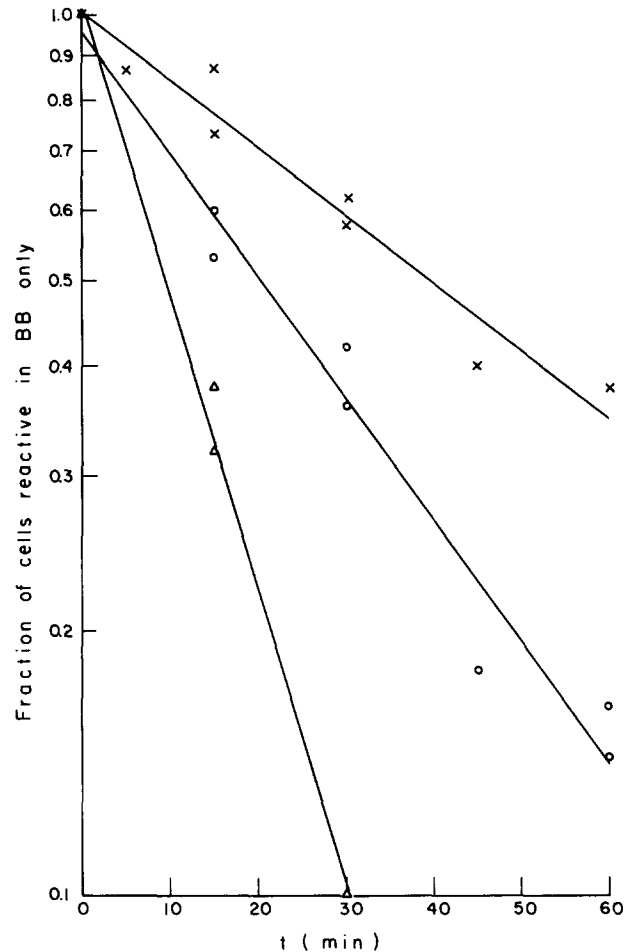


FIGURE 7 The fraction of all AP-stained IEC with fluorescence restricted to the brush border as a function of time after isolation and of temperature. (Δ) 37°C. (\circ) 22°C. (\times) 0°C.

ouabain, 5,5'-diphenylhydantoin (DPH), carbonyl cyanide *m*-chlorophenylhydrazone (CCCP), dinitrophenol (DNP), and 56 mM K^+ medium (17). Cells were exposed to all of these in collection medium (KRP-DA), HEPES-buffered Hanks' BSS, or PBS for 10 min at 37°C. They were then pelleted onto microscope slides in the cyto-centrifuge and stained cytochemically for enzyme. PMSF, colchicine, and cytochalasin B had no effect on the fraction of all stained cells with redistributed enzyme. All of the other treatments enhanced the fraction of cells staining for AP and LAP over the entire cell (Table III). CCCP was most effective at 10^{-4} M but had some effect at 10^{-6} M. DPH was effective in enhancing redistribution of AP only in PBS; it enhanced redistribution of LAP even in KRP-DA.

In contrast to the results in single cells, no redistribution of LAP, detected with antibody, was found in three- or four-cell strips of isolated IEC. Redistribution was observed in half the two-cell pairs examined (though it might be seen for only one of the two cells of a pair).

DISCUSSION

The use of an isolation method, which is a composite of several published procedures, for rat small intestine resulted in preparations of single intestinal epithelial cells from the mouse which, though vacuolated, could be maintained in good mor-

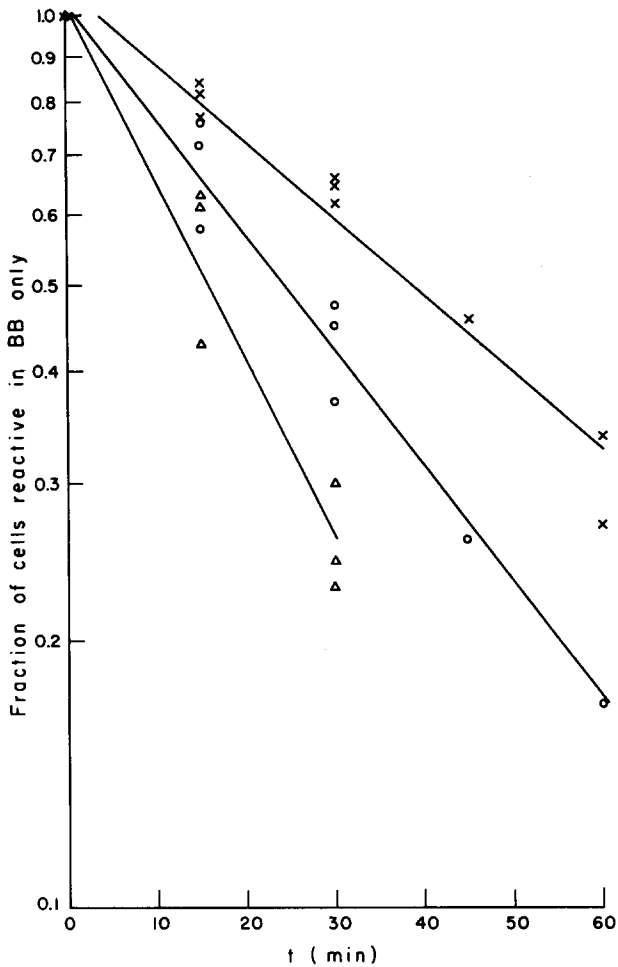


FIGURE 8 The fraction of all LAP-stained IEC with fluorescence restricted to the brush border as a function of time after isolation and of temperature. (Δ) 37°C. (O) 22°C. (x) 0°C.

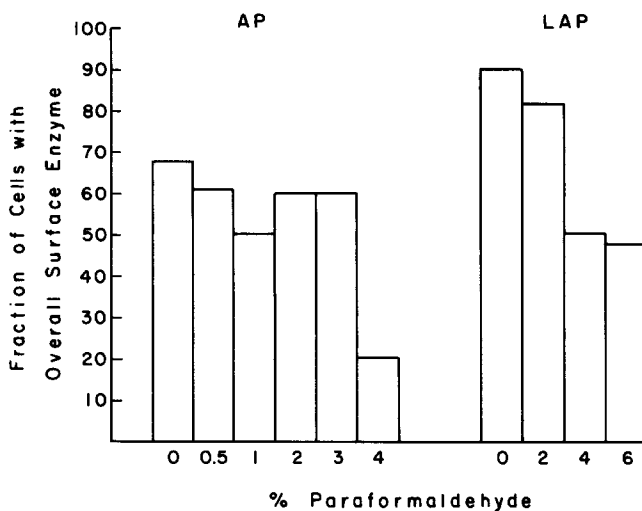


FIGURE 9 The effect of paraformaldehyde fixation on subsequent redistribution of AP and LAP in isolated IEC.

phological condition for at least 1 h. Viability and yield of the cells were not altered by the addition of dithiothreitol to the isolation or collection medium (35).

AP and LAP were localized to the brush border of IEC before and after isolation. This was demonstrable cytochemically and by the use of specific antibodies to the enzymes.

TABLE III
Effect of Drugs on the Fraction of All IEC With Diffuse Enzyme Stain

Drug*	Percent treated cells with redistributed enzyme/percent control cells with redistributed enzyme \ddagger	
	Alkaline phosphatase	Leucine aminopeptidase
CCCP, 10 ⁻⁴ M in PBS	2.1	—
CCCP, 10 ⁻⁵ M	1.4	—
CCCP, 10 ⁻⁶ M	1.1	—
CCCP, 10 ⁻⁴ M in KRP-DA	—	1.7
5,5'-Diphenylhydantoin, 10 ⁻⁴ M in PBS	2.0	—
5,5'-Diphenylhydantoin, 10 ⁻⁴ M in KRPA	1.0	1.5
Ouabain, 10 ⁻² M in PBS	1.9	—
56 mM K ⁺ Hanks' BSS§	2.3	—
Cytochalasin B, 10 ⁻⁵ M in KRP-DA	—	1.0
Cytochalasin B, 10 ⁻⁵ M in PBS	1	—
Colchicine, 10 ⁻⁴ M in KRP-DA	—	0.9
Colchicine, 10 ⁻⁴ in PBS	1	—

* Freshly isolated cells were exposed to drugs for 10 min at 37°C, then cytocentrifuged and histochemically stained for AP or LAP.

\ddagger Controls were held for 10 min at 37°C in the appropriate salt solution containing 1% DMSO in ethanol when these had been used in the drug's stock solution.

§ Exposure time was 40 min at 0°C before transfer to 37°C for 10 min.

|| Mean of three experiments.

Populations of isolated IEC appeared to be mixtures of cells with localized enzymes and of cells with enzymes present over the entire cell surface. The proportion of cells with general surface staining for either enzyme increased with time after isolation. This proportion was also a function of temperature, but significant redistribution occurred even in cells kept at 0°C. Such distribution has been reported in dissociated bladder epithelial cells (42) and is implied by recent observation in isolated gastric mucosal cells (38).

We examined the effect of temperature on redistribution of AP and LAP using cytochemical and immunological methods to detect the enzymes. The half-times measured for appearance of cells bearing redistributed AP were shorter when measured with antienzyme antibodies than with cytochemical stains. This discrepancy was more marked for LAP. We believe that the longer half-times measured cytochemically are artifacts of the method. A cell is scored as bearing diffuse or redistributed enzyme when the low level of stain present on the basolateral portion can be distinguished from the background level of stain always seen. The contrast between background and positive stain is much greater in fluorescence microscopy than in bright-field microscopy. Thus, conservative scoring tends to decrease the number of cells seen as bearing diffuse label, and this skewing will be greatest at times soon after isolation when little redistribution has occurred. We draw support for our view from the calculated values for Q_{10} , the rate of increase in the $t_{1/2}$ with increasing temperature, which are similar for AP and LAP (1.55 and 1.25) and are also similar whether cytochemical or immunological methods are used to detect the enzymes (Table I).

Redistribution of AP and LAP may have occurred by release of enzyme and its subsequent reabsorption, by diffusion in the plane of the membrane, or by internalization and reinsertion

of enzyme molecules, derived from the brush border, into the basolateral cell surface. The first possibility, release and reabsorption of enzyme activity, is extremely unlikely for three reasons. First, we could observe cells partly labeled on the lateral but not the basal surfaces, though the opposite was never seen. Second, release of soluble enzyme would result in its dilution in a large volume of medium. Third, and most important, both AP and LAP are integral membrane proteins (9, 27). These proteins are not released from membranes in the absence of detergents or proteases, and, in the case of LAP, the water-soluble form produced by protease digestion will not integrate with a lipid bilayer (9). The third possibility is given some force by studies showing the rapid recycling of plasma membrane in a variety of cells (39, 40, 43, 44). However, we believe that the sum of our experiments with temperature, fixation, and drug treatments rule out a recycling mechanism to account for redistribution of AP and LAP in isolated cells. Lowered temperature lowers diffusion rates, and, whereas the effect may not be linear with temperature, it is continuous and does not show threshold effects seen, for example, when attempting to cap surface antigens over a range of temperatures (cf. references 14 and 32).

The low values for Q_{10} also argue against a membrane recycling mechanism for redistribution of enzymes. Measured values of Q_{10} for pinocytosis in L cells, the system best studied for membrane recycling, are 2.7 (44). Our values are far smaller and are comparable to the values for effect of temperature on diffusion of rhodopsin in disk membranes (C. L. Wey, personal communication and reference 34).

A second point arguing against redistribution of the enzymes by recycling is the need to fix antibody-labeled cells with high concentrations of paraformaldehyde to block redistribution of antibody-labeled enzyme. Whereas we could not perform the experiment on intestinal cells, we have found that as little as 0.5% paraformaldehyde suffices to fix L cells so that they will not reattach to glass or plastic. 4% paraformaldehyde was required to inhibit redistribution of antibody-labeled AP and LAP. Again, this parallels the work on diffusion of membrane proteins in single cells in which it is often found that (12, 34) diffusion is readily measured in fixed cells unless high concentrations of, or long periods in, fixative are used.

A third point arguing against redistribution of AP and LAP caused by membrane recycling and in favor of redistribution by diffusion is the effects of drugs on the rate of the process. None of the drug treatments slowed the rate of appearance of cells with uniform enzyme reaction; all of the effective drugs enhanced this rate. Drugs acting on cytoskeletal elements failed to alter the observed rate in any way. Other drugs, notably the uncouplers DNP and CCCP, which are effective inhibitors of pinocytosis, and, presumably, of membrane recycling, enhanced rather than interfered with the redistribution of AP and LAP in isolated cells.

In summary, three different treatments, lowered temperature, fixation, and inhibition of ATP generation, do not affect the rate and extent of redistribution of AP and LAP in the membrane of isolated IEC. At least some of these treatments should affect redistribution as a result of membrane recycling or other processes driven by cell metabolism. Instead, it appears that the redistribution occurs by diffusion of the enzymes in the plane of the cell surface membrane.

A rough estimate of the diffusion constants for AP and LAP may be made by considering the boundary conditions for the freshly isolated IEC, as a band or disk of AP or LAP at one

end of a cylinder. Diffusion is then one-dimensional and, to a first approximation, the diffusion constant, D , for the enzymes is given by $D = (x^2/2t) \cdot \text{cm}^2 \cdot \text{s}^{-1}$, where x is the length of the cylinder and t is the half-time for diffusion. Taking $x = 10 \mu\text{m}$, 10^{-3} cm , and $t_{1/2,37^\circ} = 9 \text{ min}$ (540 S) for AP and $t_{1/2,37^\circ} = 16 \text{ min}$ (960 s) for LAP, we find $D = 9 \times 10^{-10}$ for AP and $D = 5 \times 10^{-10}$ for LAP. Both of these numbers may be underestimates to the extent that our initial conditions assume an infinite sink for the diffusing enzymes, but, in fact, the concentration of enzyme in the basal portions of the cells rapidly becomes large. The calculated numbers approach the values obtained for unrestricted diffusion of membrane integral proteins, rhodopsin (34), $3 \times 10^{-9} \cdot \text{cm}^2 \cdot \text{s}^{-1}$, and histocompatibility antigens in some mouse-human heterokaryons, $2 \times 10^{-9} \cdot \text{cm}^2 \cdot \text{s}^{-1}$ (12).

Some drug treatments enhanced the rate at which cells bearing diffusely distributed enzymes appeared in the population. This enhancement may be caused by an effect of freeing an initially immobile enzyme population for free diffusion, or it may be caused by alterations of twofold to fivefold in the diffusion rate of mobile enzyme, in turn presumably caused by modification of the interaction of the diffusing molecules with the cell cytoplasm. The treatments with high external potassium ion, ouabain, DPH, CCCP and DNP, all affect membrane potential and some, notably the uncouplers CCCP and DNP, should drastically reduce cell levels of ATP; DPH may also decrease cell ATP by stimulating the working of the sodium-potassium ATPase. If DPH, in fact, also alters the level of ATP in cells, this may explain its effects in our system; in other diffusion experiments the effects of DPH and ouabain are an antithetical (13).

The constraints to diffusion of AP, LAP, and other membrane integral proteins do not appear to be the tight junctions of these cells. The time-course of breakdown of tight junctions parallels that of redistribution of the surface enzymes, but, though somewhat unraveled, substantial amounts of tight junctions are found on single cells and in cell pairs as late as 20 min the IEC is isolated (C. Ziomek, J. Sheffield, and M. Edidin, manuscript submitted for publication). Also, LAP is not found redistributed on cells of a small number of strips sampled immediately after isolation, yet these strips must have broken junctional contacts with their neighbors on two sides because they were isolated as linear arrays of cells, not as clumps.

We conjecture that cell metabolism, supplying ATP, is required to keep membrane integral proteins localized to particular regions of the surface. When cell metabolism is seriously disrupted, as it is upon dissociation, the anchorage of integral proteins is lost, and these proteins may diffuse in the membrane. However, the rate of diffusion need not be maximal; weak interactions with cytoplasmic elements, affected in turn by alterations in membrane potential, may slightly restrict diffusion. The first point in the conjecture is supported by recent work showing that uncouplers cause dispersion of acetylcholine receptors on cultured myotubes (4) and by the observation that amphibian embryo cells, which do not appear to be seriously deranged by isolation (46), retain their polarization after isolation. The second is supported by some data on the effects of drugs and calcium isophores on lateral diffusion (M. Edidin and T. Wei, manuscript in preparation). Both points of the conjecture suggest the need for further experiments on isolated cells and cells in tissue, which should elucidate the mechanisms by which functionally polarized cells maintain this polarity on their surfaces.

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