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Research Article

Caco-2 Cell Monolayers as a Model for Drug Transport Across the Intestinal Mucosa

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Human colon adenocarcinoma (Caco-2) cells, when grown on semipermeable filters, spontaneously differentiate in culture to form confluent monolayers which both structurally and functionally resemble the small intestinal epithelium. Because of this property they show promise as a simple, *in vitro* model for the study of drug absorption and metabolism during absorption in the intestinal mucosa. In the present study, the transport of several model solutes across Caco-2 cell monolayers grown in the Transwell™ diffusion cell system was examined. Maximum transport rates were found for the actively transported substance glucose and the lipophilic solutes testosterone and salicylic acid. Slower rates were observed for urea, hippurate, and salicylate anions and were correlated with the apparent partition coefficient of the solute. These results are similar to what is found with the same compounds in other, *in vivo* absorption model systems. It is concluded that the Caco-2 cell system may give useful predictions concerning the oral absorption potential of new drug substances.

KEY WORDS: cell culture; transport; intestinal mucosa; permeability; epithelial.

INTRODUCTION

The successful discovery and development of new drug entities have been increasingly aided through the use of appropriate *in vitro* model systems. Among the obvious advantages of such systems relative to whole animal studies is the ability to examine large numbers of samples, decreased quantities of drug required, and clearer interpretation of results. At the present time, most of the *in vitro* studies examining drug uptake and transport in the intestinal epithelium have utilized such models as everted sacs, brush border membrane vesicles, isolated cells, and intestinal rings. In general, these systems suffer from such concerns as tissue viability and polarity, among others (1,2). As an alternative to these models, we and others have been interested in developing monolayers of intestinal epithelial cells as a drug absorption model.

While previous attempts to culture mature intestinal epithelial cells (enterocytes) have met with little success due to problems of dedifferentiation (3,4), recent reports have identified several human colon adenocarcinoma cell lines which appear to undergo enterocyte-like differentiation in culture (5,6). Among these, the Caco-2 line, despite its colonic origin, spontaneously differentiates into polarized, columnar cells which are more representative of the small intestine. Specifically, they exhibit well-developed microvilli (5) and a polarized distribution of brush border enzymes (7–9) and, when grown on plastic, form domes typical of normal, transporting epithelium (10,11). Because of these properties, the Caco-2 cell has been proposed as a model for the study of epithelial cell differentiation (12–14).

More recent work has focused on the Caco-2 cell as a model for the study of transport. When grown on plastic, the monolayers take up and transport ions (10,11), sugars (15,16), and peptides (17,18). Further, when established on porous filters, the cells exhibit polarized transport of such substances as bile acids (19) and vitamin B_{12} (20). However, most of the transport properties studied to date are active carrier- or receptor-mediated processes. In contrast, most drug absorption occurs by passive diffusion from the intestinal lumen. Therefore to be useful as a drug absorption model, the passive as well as active transport properties of the Caco-2 cell monolayers should be similar to what is found *in vivo*.

In order to establish such a comparison, the present study was undertaken. Monolayers of Caco-2 cells were prepared on 3-μm-pore polycarbonate Transwell filters. These monolayers were then used to examine the transport of model solutes varying in lipophilicity and size. Further, since several of the solutes have been employed in similar studies to characterize other, *in vivo* absorption model systems, it was possible to compare the *in vitro* to the *in vivo* results directly.

EXPERIMENTAL

Materials. Caco-2 cells were obtained from the American Type Culture Collection (Rockville, MD) at passage 13. Cells used in this study were passaged fewer than 30 times. Transwell cell culture chambers were obtained from Costar. Culture reagents were from GIBCO. Salicylic acid (7-^{14}C, 58

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mCi/mmol); urea (^{14}C, 56 mCi/mmol); testosterone (4-^{14}C, 50 mCi/mmol), and D-glucose (1-^{14}C, 55 mCi/mmol) were all obtained from New England Nuclear. [^{14}C]Hippuric acid was prepared from glycine (1-^{14}C, 56 mCi/mmol) and benzoyl chloride by standard Schotten-Bauman procedures. The crude product was purified by RP-HPLC (RP-18, 10% CH_3CN, 0.02% CF_3CO_2H) by comparison to an authentic standard.

Cell Culture. Caco-2 cells were grown in T-150 flasks at 37°C in an atmosphere of 5% CO_2 using Dulbecco's modified Eagle medium (D-MEM) at pH 7.2. The medium was supplemented with 4.5 g/liter D-glucose, 584 mg/liter L-glutamine, 1% nonessential amino acids, 1% sodium pyruvate, 1% penicillin–streptomycin, and 10% fetal bovine serum. The medium was changed every other day until the flasks reached 90% confluence. The monolayers were washed three times with Hank's balanced salt solution without calcium or magnesium (HBSS-CMF). The cells were removed from the flasks by incubating the monolayers with 0.25% trypsin in 1 m*M* EDTA solution for 10 min at 37°C. The cells were collected into centrifuge tubes, then spun 10 min at 200 *g*, and the pellet was resuspended in D-MEM. The Caco-2 cells were seeded into 3-μm-pore Transwell inserts at 60,000 cells/cm^2. For the studies examining the influence of attachment factor on cell differentiation, a collagen matrix was applied to the Transwells. The method of Ehrmann and Gey (21) was followed. Briefly, a solution of 0.5 ml of crude rat tail collagen in glacial acetic acid (3 mg/ml) was spread onto the filter, and the solvent removed under high vacuum. All Transwell inserts had a 4.71-cm^2 surface area (Fig. 1).

Transmission Electron Microscopy. At 4, 7, 10, 14, 21, and 28 days, two monolayers on Transwell inserts with and without collagen were washed three times with Hank's balanced salt solution (HBSS) and fixed with 2.5% glutaraldehyde and 5% sucrose in 0.1 *M* cacodylate buffer, pH 7.4, for 1 hr at 4°C. The monolayers were washed three times with 5% sucrose in 0.1 *M* cacodylate buffer at 4°C, pH 7.4, for 10 min each wash. The cells were postfixed with 1% osmium tetroxide for 45 min at room temperature, followed by washing five times with glass-distilled water, 10 min each wash. The samples were dehydrated in 25, 40, 50, and 60% ethanol dilutions for 2 min in each solution.

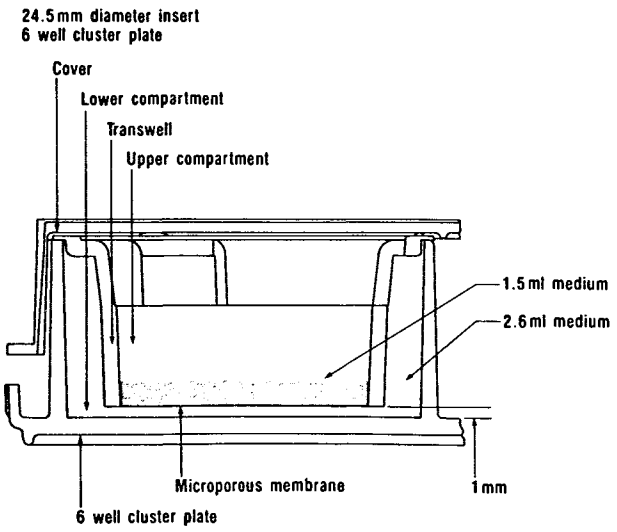

Fig. 1. Costar Transwell System with microporous polycarbonate filter.

The monolayers were stained en bloc with 1% uranyl acetate in 70% ethanol for 30 min and continued dehydration in 75, 85, and 95% ethanol, followed by three changes of 100% ethanol for 2 min in each solution. Freshly prepared Poly/Bed 812 (Polysciences) containing 50 g Epon, 29.4 g DDSA, 29.5 g NMA, and 1.65 ml DMP-30 was diluted 1:1 with 100% ethanol and allowed to infiltrate the monolayer. After 30 min, the diluted epon was removed and replaced with pure Poly/Bed 812 mixture. The epon was changed after 1 hr and replaced with another pure mixture of Poly/Bed 812. One hour after the second change of Epon a final change of PolyBed 812 was made and the samples were left overnight at 25°C before polymerizing in an oven at 60°C for 48 hr.

Cross sections of the monolayer on the polycarbonate filter were made by trimming a 1-mm^2 block face with the monolayer in the middle and sectioned with a diamond knife on a Sorvall MT6000 microtome. Silver sections were collected and poststained with 3% aqueous uranyl acetate for 15 min, followed by 1 min with lead citrate (22). The sections were examined with a JOEL 1200EX electron microscope operated at 60 kV.

Alkaline Phosphatase Cytochemistry. In order to study the functional polarization of the Caco-2 cells, the presence of alkaline phosphatase, a brush border enzyme marker, was followed. The procedure was that of Robinson and Karnovsky (23). Briefly, monolayers grown on the Transwell filters with and without collagen were rinsed three times with HBSS and fixed for 30 min at 4°C with 2.5% glutaraldehyde and 5% sucrose in 0.1 *M* cacodylate buffer, pH 7.4. The monolayers were washed once with 5% sucrose in 0.1 *M* cacodylate buffer, pH 7.4, followed by three 5-min washes of enzyme buffer (2 m*M* $CeCl_3$, 5% sucrose in 0.1 *M* Tris–maleate buffer, pH 8, containing 0.0002% Triton-X). The monolayers were then incubated for 60 min with enzyme buffer containing 2 m*M* β-glycerophosphate as the substrate. Control incubations were carried out at pH 5 (23). After the incubation, the monolayers were washed once with 0.1 *M* Tris–maleate buffer at 25°C, pH 7.4. This was followed by three washes of 5% sucrose in 0.1 *M* cacodylate buffer, pH 7.4, at 25°C. The monolayers were postfixed with 1% osmium tetroxide and embedded in Epon as described above. The cerium phosphate reaction product was visualized by electron microscopy.

Transport Studies. Caco-2 cells grown for 21 days on the Transwell filters with or without rat tail collagen were washed free of medium and allowed to incubate at 37°C in 1.5 ml HBSS in the insert (donor compartment) and 2.5 ml HBSS in the receiver compartment (Fig. 1). In the standard protocol, to initiate the experiment the donor solution was suctioned off and replaced with 1.5 ml fresh HBSS containing [^{14}C]PEG-4000 with a specific activity of 6.5–16.6 × 10^5 dpm/ml. At 30, 60, 90, 120, 180, and 240 min the insert was transferred to fresh receiver solutions of marker-free HBSS. The incubations were maintained at 37°C in a 5% CO_2 atmosphere. The percentage amounts of radionuclide appearing after each sampling interval were added to obtain the cumulative transport.

The permeability of the blank filters was examined by a

slightly different method. We were unable to transfer the non-Caco-2 cell-containing non-collagen-coated filter in this manner without considerable leakage of the donor solution into the receiver as the cup was lifted from the solution. Therefore, the collagen- and non-collagen- coated blank filters were mounted in a nonstirred side-by-side diffusion cell obtained from Crown Glass. A 3.5-ml solution of the [^{14}C]PEG-4000 in HBSS was added to one compartment, and solute-free HBSS to the other. At 5-min intervals 50-μl aliquots were removed from the receiver and replaced with fresh buffer. The cumulative appearance of radioactivity was determined as above.

For the transport studies with the rest of the model solutes, the standard protocol was followed. Stock concentrations ranged from 1 to 4 μ*M* in HBSS. All these studies were conducted with monolayers of between 14 and 21 days in culture on non-collagen-coated Transwell filters. For the disappearance studies, 10-μl aliquots were removed from the donor compartment at the specified intervals. For the appearance studies, samples of the receiver compartments were analyzed for solute.

Analytical Methods. Radioactivity was determined by liquid scintillation counting in a Beckman LS 3801 Scintillation counter. For the disappearance studies, effective permeability coefficients, P_{eff}, were calculated from the following relationships:

$$ln\left(\frac{C_t}{C_o}\right) = -kt \quad (1)$$

where C_t is the concentration of solute in the donor at time t, C_0 is the initial concentration, and k is the first-order rate constant; and

$$P_{eff} = \frac{kV_D}{A} \quad (2)$$

where V_D is the volume of the donor compartment and A is the surface area of the monolayer, 4.71 cm^2.

Similarly, effective permeability coefficients were calculated from the receiver compartment concentrations and the following relationship:

$$P_{eff} = \frac{V_R}{AC_o} dC/dt \quad (3)$$

where V_R is the volume of the receiver compartment, A is the membrane surface area, C_0 is the initial donor concentration of solute, and dC/dt is the initial slope of a plot of the cumulative receiver concentration with time.

RESULTS

Electron Microscopy of Caco-2 Cells on Collagen-Coated Transwells. The Caco-2 cells readily attached to the matrix and began to spread. After 4 days in culture, tight junction formation was observed between adjacent cells although the monolayers had not reached confluence. The cells contained large amounts of rough endoplasmic reticulum, mitochondria, and small amounts of glycogen. As shown in Fig. 2, the cells appeared squamous in shape, with a sparse distribution of immature microvilli on the apical surface. Confluence was evident by day 7 and the cells also appeared to undergo a change from squamous to cuboidal. Cytoplasmic extensions into the 3-μm filter pores were frequently observed. By day 10, cytoplasm protruded through the filter pore and was beginning to spread along the underside of the filter. Many large vacuoles containing unknown material were present in the cultures at this time but tended to decrease in number as the cultures got older. Despite the large size of the vacuoles, the cultures remained confluent.

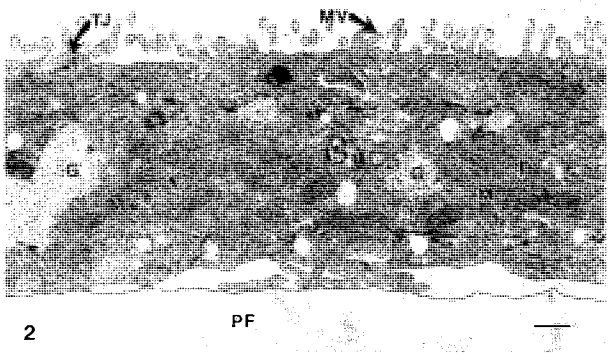

Fig. 2. Day 4 culture grown on polycarbonate filter (PF) coated with rat tail collagen type I. Note the poorly developed microvilli (MV), tight junction (TJ), mitochondria (M), glycogen (G), and rough endoplasmic reticulum (arrows). ×23,800 (bar = 0.5 μm).

At 14 days, the cells had changed from cuboidal to columnar in shape with an eccentrically located nucleus along the basolateral border. The cytoplasm between the nucleus and the brush border membrane contained predominantly glycogen. At 21 days, the cells remained morphologically polar, with a greater number of cytoplasmic processes extending into the receiver chamber (Fig. 3). By day 28, as can be seen in Fig. 4, a substantial spreading of cellular cytoplasm was evident on the underside of the filter, with what appeared to be immature microvilli on the surface projecting into the receiver compartment. While the extent of coverage of the underside of the filter by these cells was not determined, every pore in the sections we did examine showed this cellular invasion.

Electron Microscopy of the Caco-2 Cells on Non-Collagen-Coated Transwells. Similar to the case with the collagen-coated filters, by the fourth day after seeding, patchy clusters of actively dividing cells were observed with evidence of tight junctions between adjacent cells. The squamous-shaped cells contained a large amount of rough endoplasmic reticulum and numerous mitochondria. However, in contrast to the cells on collagen, no glycogen and very few immature microvilli were observed. By day 7 the cells had reached confluence, changed in shape from squamous to cuboidal, and established desmosomes. At 10 days, mature microvilli had developed and the cells appeared as morphologically differentiated as those grown in the presence of collagen. By the 14th day, the cells were columnar in shape, with the nucleus eccentrically located in the basolateral border of the cell. Glycogen was the major component of the cytoplasm between the nucleus and the apical brush border (Fig. 5). Intercellular lumen, containing microvilli, were ob-

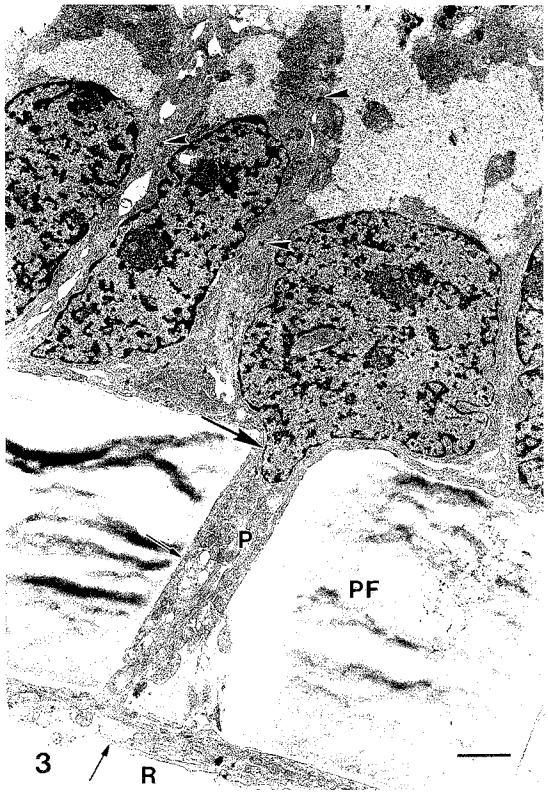

Fig. 3. Day 21 culture grown on polycarbonate filter (PF) coated with rat tail collagen type I. Note the cytoplasm (small arrows) extending through the 3.0-μm filter pore (P) into the receiver chamber (R) and part of the nucleus that protrudes into the filter pore (large arrow). Desmosomes are indicated by arrowheads. ×6000 (bar = 2.0 μm).

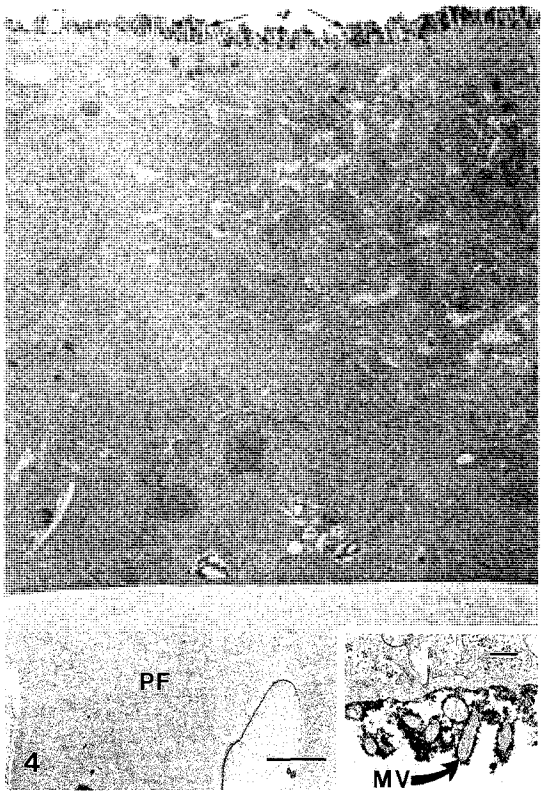

Fig. 4. Day 28 culture grown on polycarbonate filter (PF) coated with rat tail collagen type I. Brush border membranes on both sides of the polycarbonate filter stain positive for alkaline phosphatase (arrows). ×6500 (bar = 2.0 μm). Inset: High-power view of cytoplasm extending through the 3.0-μm pore with well-developed microvili (MV) and positive staining for alkaline phosphatase. ×30,000 (bar = 2.0 μm).

served running parallel to the filter, but in contrast to the collagen-attached cells, no cytoplasm was seen invading the filter pores. However, after 21 days small cytoplasmic extensions into the filter were found which increased in number and content up through 28 days. No microvilli were observed in the receiver chamber in these cells (Fig. 6).

Alkaline Phosphatase Cytochemistry. The appearance of what appeared to be microvilli on the surface of the cytoplasm extending through the collagen-coated polycarbonate filter suggested that the cells were losing polarity at this time in culture. In order to ascertain if this was happening, the localization of alkaline phosphatase on these cells was examined. In normal intestinal epithelial cells, this enzyme is highly polarized and located exclusively on the apical, brush border membrane. As can be seen in the inset to Fig. 4, there is a heavy staining of the cell surface on the underside of the filter when the cells were grown on the collagen matrix, consistent with a loss of polarity of the cell. However, in several of the sections, we found nuclei in the space between the microvilli and the filter surface, suggesting that these were cells which had actually squeezed through the filter pore into the receiver rather than depolarizing. In contrast, no phosphatase staining or nuclei were seen on the underside of the filter for the 28-day cells grown in the absence of added collagen. However, extensive staining was seen on the apical surface, consistent with normal cell polarization (inset, Fig. 6).

Permeability of the Monolayers to PEG-4000. Very rapid flux of [^{14}C]PEG-4000 was observed through the blank filters. In 25 min, 10% crossed the collagen-coated Transwell membranes, while 8% crossed the untreated filters. In marked contrast, the filters containing 21-day-old Caco-2 cells were essentially impermeable to the label. For the collagen-coated matrix, 0.33% of the PEG-4000 crossed the monolayer in 4 hr, while 0.26% of the label crossed the monolayers grown on non-collagen-coated filters in the same time period. Considering that the relative surface areas of the filter available for diffusion across the non Caco-2 cell-containing filters was 0.66 cm^2, compared to 4.71 cm^2 for the monolayers, it is clear that the major barrier to diffusion of PEG-4000 is the Caco-2 cells.

The conclusions drawn from this comparison of Caco-2 cell monolayers grown in both the presence and the absence of collagen suggested that the collagen matrix was unnecessary for establishment of confluent monolayers. Further, the collagen seemed to promote cell migration through the filter at early times in culture, resulting in depolarization of the model. Based on these results, the remainder of the transport studies were all conducted with monolayers grown on Transwells without added collagen. Also, based on the cell migration observations, the monolayers were used within a period of 14–21 days after seeding.

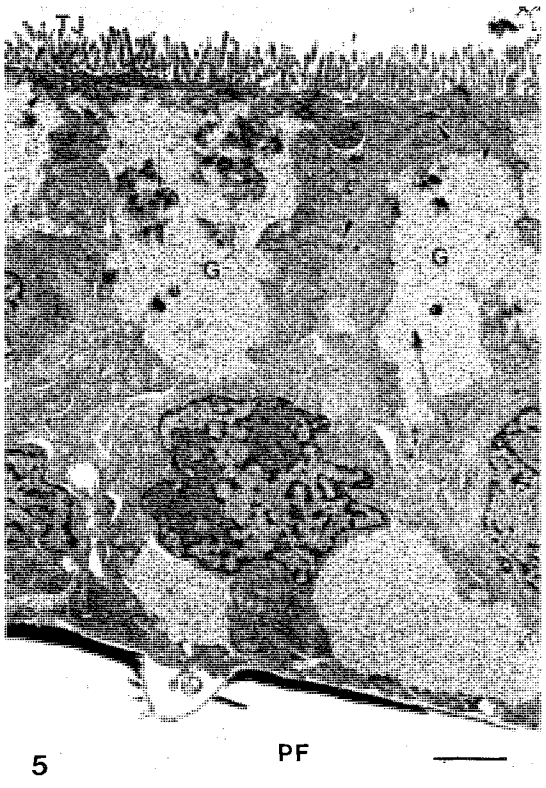

Fig. 5. Day 14 culture grown on non-collagen-coated polycarbonate filter (PF). Oblique section through 3.0-μm pore, tight junction (TJ), desmosomes (arrowheads), glycogen (G), and multivesicular bodies (arrows). ×8000 (bar = 2.0 μm).

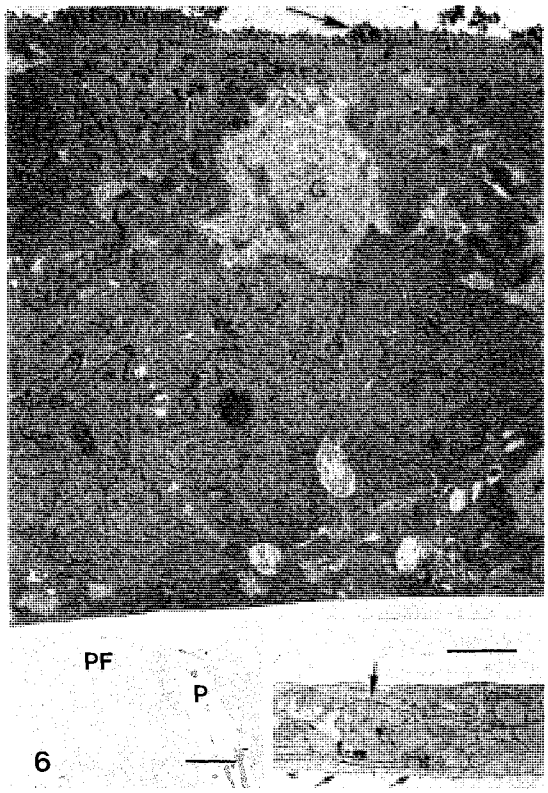

Fig. 6. Day 28 culture grown on non-collagen-coated polycarbonate filter (PF). Glycogen (G), 3.0-μm pore (P), and multivesicular bodies (small arrows). Note the positive alkaline phosphatase stain along the apical brush border membrane (large arrow). ×5500 (bar = 2.0 μm). Inset: Basal cytoplasm extending through the 3.0-μm pore and into the receiver chamber does not stain for alkaline phosphatase along the basal membrane. The experimental groups and controls stained for nonspecific phosphatase along the lateral membrane (large arrow). ×7700 (bar = 2.0 μm).

Solute Disappearance from the Donor Compartment. For all the solutes except hippuric acid, slow to rapid disappearance from the donor compartment was observed. The slowest rate was found for urea and the most rapid, essentially identical disappearance rates were seen with salicylic acid at low pH, testosterone, and dilute glucose (Fig. 7). In the case of hippuric acid, a second experiment was performed in which the incubation intervals were 60 min. However, even with these long intervals, the decrease in concentration in the donor was too small to measure accurately.

Using the observed disappearance rates from Fig. 7 and Eqs. (1) and (2), the permeability coefficients were calculated and are summarized in Table I. Again, no permeability coefficient could be obtained for hippuric acid by the disappearance method.

In order to examine the relationship of permeability across the Caco-2 cells to lipophilicity, the octanol–water partition coefficients for the solutes were obtained from the literature and are included in Table I. For the ionizable solutes salicylic acid and hippuric acid, the apparent partition coefficients (P_{app}) was calculated from the following relationship:

$$P_{app} = \left(\frac{H^+}{K_a + H^+}\right) P_i \qquad (4)$$

where H^+ is the hydrogen ion concentration in the donor solution, K_a is the acid dissociation constant for the solute, and P_i is the intrinsic octanol water partition coefficient for the uncharged solute.

Finally, to confirm that the pH 4.5 buffer used in the transport study with salicylic acid was not damaging the cells and increasing solute flux by this mechanism, two further experiments were performed. In three monolayers, disappearance of salicylic acid at pH 7.1 was followed for 1 hr. The donor solution was then removed and replaced with a salicylic acid solution at pH 4.5. Disappearance was again followed for 1 hr, at which point the pH 4.5 was removed and replaced by the pH 7.1 salicylate solution. Disappearance was followed for another hour. The results of this experiment are shown in Table II. The slower disappearance rate is seen for salicylic acid upon return of the pH from 4.5 to 7.1, suggesting that the 1-hr exposure of the cells to acid does not significantly affect their barrier properties.

We further examined the cells by microscopy for any evidence of physical changes due to the acid pH. After a 1-hr incubation at pH 4.5 a monolayer was fixed and stained as described under Experimental and examined by electron microscopy. No morphological differences were seen between these cells and those which had not been subjected to the acid buffer.

Solute Appearance in the Receiver Compartment. For the experiments with salicylic acid at neutral pH, testoster-

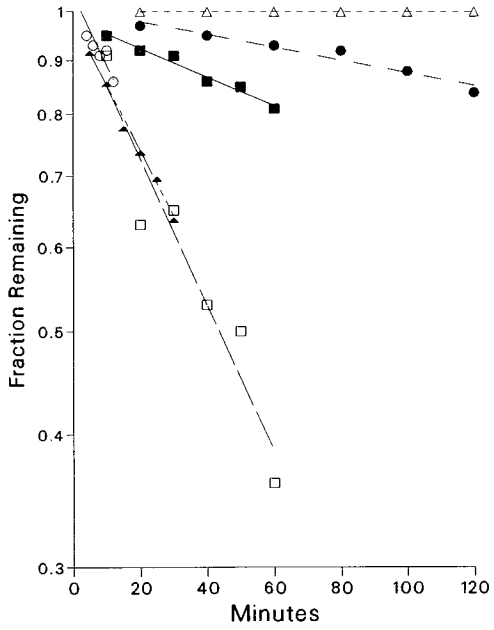

Fig. 7. Transport of the model solutes into Caco-2 cells—disappearance from the donor compartment. Stock solutions of (■) 2.0 μ*M* salicylic acid at pH 7.1, (□) 3.7 μ*M* glucose, (●) 1.7 μ*M* urea, (○) 1.7 μ*M* testosterone, (▲) 2.0 μ*M* salicylic acid at pH 4.5, or (△) 1.5 μ*M* hippuric acid were incubated in the Transwell system at 37°C. Each point represents the mean of at least three determinations.

one, and hippuric acid, the cumulative increase in concentration in the receiver compartment was also measured. These results are shown in Fig. 8. Consistent with the disappearance results, testosterone disappeared fastest from the donor and appeared most rapidly in the receiver. The other solutes showed intermediate rates which reflected their disappearance. In the case of hippuric acid, although no measurable disappearance could be seen, the appearance of the transported acid was easily quantitated. Again, from these results and Eq. (3), effective permeability coefficients were calculated and are included in Table I.

Table I. Permeability Coefficients for the Model Solutes in the Caco-2 Cell System

Solute	Conc. (μ*M*)	Log octanol/water PC (apparent at pH 7.1)	P_{eff} (cm/sec × 10^5)[a] Disappearance	Appearance
D-Glucose	3.7	−3.24	8.5 (0.3)	—
Salicylic acid (pH 4.5)	2.0	1.74[b]	7.8 (1.2)	—
Hippuric acid	1.5	−3.0[c]	0[d]	0.067 (0.002)
Urea	1.7	−1.09	0.75 (0.1)	0.76 (0.01)
Salicylic acid (pH 7.1)	2.0	−0.82[b]	1.8 (0.2)	1.9 (0.2)
Testosterone	1.7	3.31	8.2 (1.0)	6.3 (0.3)

[a] Values are means (±SD) for at least three determinations.
[b] Calculated from log PC intrinsic = 3.28 and pK_a = 3.0.
[c] Calculated from log PC intrinsic = 0.49 and pK_a = 3.64.
[d] Unable to be measured.

Table II. Effect of Low pH on the Permeability Properties of Caco-2 Cell Monolayers

Donor pH	Time (min)	P_{eff} (salicylic acid)[a]
7.1	0–60	1.8 (0.16)
4.5	60–120	7.66 (0.54)
7.1	120–180	1.59 (0.01)

[a] cm/sec × 10^5. Values are the means (±SD) for three samples.

DISCUSSION

Although the Caco-2 cell has received a great deal of attention as a model for epithelial cell differentiation, only recently have attempts to utilize this system for drug transport studies been reported (24,25). The series of experiments described in this work was undertaken to characterize further the growth and transport properties of these cells.

From a cell attachment and differentiation standpoint, it appears that collagen may not be necessary for the establishment of Caco-2 cell monolayers on the polycarbonate Transwells. Comparing the results for the 3-μm filters with and without collagen, we find that the initial rate at which the cells attached and began to differentiate was dependent upon the presence of collagen. After 4 days, cells grown on collagen developed immature microvilli and had a large amount of rough endoplasmic reticulum and mitochondria in the cytoplasm. Small amounts of glycogen were present which increased rapidly as the cells matured. By comparison, differentiation was slower in the absence of collagen at the early times. The cells had few immature microvilli and no glycogen in the cytoplasm. However, by 10 days, these cells ap-

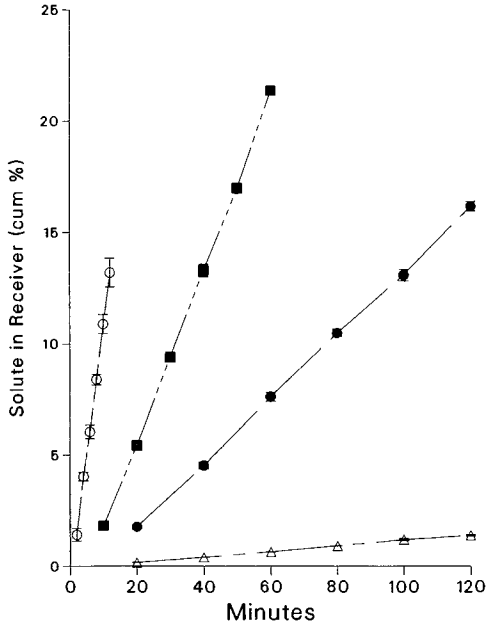

Fig. 8. Transport of the model solutes across Caco-2 cell monolayers—appearance in the receiver compartment. Stock solutions of (○) 1.7 μ*M* testosterone, (■) 2.0 μ*M* salicylic acid at pH 7.1, (●) 1.7 μ*M* urea, or (△) 1.5 μ*M* hippuric acid were incubated in the Transwell system at 37°C. Each point represents the mean and standard error of at least three determinations.

peared to be identical to those in the collagen-containing monolayers.

These results are distinct from earlier work examining the growth of Caco-2 cells on nitrocellulose. In that study, cells seeded onto non-collagen-containing filters showed decreased attachment and failed to form monolayers. However, cells seeded onto collagen-containing nitrocellulose formed confluent monolayers (11).

The general features of the cells in the monolayers after reaching confluence were similar for the collagen-free and collagen-coated filters. The cells were elongated in both cases, with well-developed microvilli, and displayed desmosomes and occluding junctions localized to the apical surface of the cells. These tight junctions appear to be fully functional as a diffusion barrier, substantially restricting the flux of the probe PEG-4000 to the same extent in both the presence and the absence of collagen. In contrast, endothelial cells in culture frequently do not develop such barrier properties (26).

Although it has been shown that such polarization features can be dependent upon the nature of the matrix to which the cells are attached, these results suggest that at least with Caco-2 cells on polycarbonate, the collagen does not influence cell differentiation. The similar accumulation of glycogen in both cases further supports this notion, as it has been shown that the cytoplasmic concentration of glycogen is a unique phenomenon of Caco-2 cells associated with spontaneous differentiation (27).

In only one respect did we find a clear influence of collagen. The presence of collagen seemed to accelerate the extension of cell cytoplasm into the filter pore at an early time in culture. This was much less prominent in the absence of collagen. At 28 days in culture, the cells appeared to have actually crawled through the pore and become established on the opposite side. The clear presence of alkaline phosphatase on the surface of these migrated cells suggested that they too had become polarized. Again, this phenomenon was much less pronounced in the absence of collagen.

Based on these preliminary studies, we concluded that the collagen matrix was not necessary for the establishment of useful Caco-2 cell monolayers. Also, from purely morphological considerations, there appeared to be an optimum "window" for use of the monolayers after seeding. Before day 14 the cells appeared not to have achieved maximum differentiation, while after approximately day 21 the cytoplasmic extensions into and through the pore matrix were resulting in a functional depolarization of the monolayer.

With respect to the study of drug transport across these monolayers, evidence from *in vivo* studies suggest that a combination of aqueous and lipid diffusion pathways is responsible for absorption across the intestinal mucosa. The lipid pathway is most favorable for lipophilic compounds, while highly polar molecules are much less permeable to the cell membrane due to poor partitioning properties. For these molecules an alternate, presumably aqueous "pore" pathway plays a greater role in absorption, resulting in a greater dependence on the size of the solute rather than on lipophilicity (28). Further, it has been shown that a sigmoidal relationship exists between absorption rate and lipophilicity, with an optimal lipophilicity (log octanol–water partition coefficient) of about 2.5 (29).

The results obtained in these studies are consistent with this model. When transport from the donor solution is observed, the permeability coefficients are clearly related to the octanol water partition coefficient of the solute. Thus, when salicylic acid is present in solution in the protonated form at pH 4.5 with an apparent log PC of 1.74, it is absorbed approximately four times faster than at pH 7.1 where the apparent lipophilicity is much less. Moreover, when the permeability coefficients are plotted as a function of partition coefficient as in Fig. 9, the sigmoidal relationship is readily apparent. The polar solutes urea and hippurate ion show very low permeabilities, consistent with the "pore" hypothesis. In principal, it is possible to compare the relative rates of diffusion of a series of polar solutes of different sizes and estimate the effective size of the pore. While not enough data are available from this study, we have made such a correlation with a series of small charged peptides and estimate an effective pore radius of about 0.8 nm in these monolayers (30).

Glucose is an exception to this correspondence, showing absorption much greater than expected based on its partition coefficient. This is consistent with the active transport of glucose similar to that found *in vivo* and recently shown to be present in Caco-2 cells (31).

Along with glucose, the most rapidly transported substances in this study are testosterone and salicylic acid, which all disappear at essentially the same rate. In Fig. 9 these solutes represent the maximum obtainable permeability for this model system, which is approximately 8×10^{-5} cm/sec. Mechanistically this occurs when the membrane transport rate is very rapid relative to the diffusion rate of the solute through the unstirred aqueous barrier directly adjacent to the membrane. Such a condition is found for very lipophilic substances and for actively transported molecules when the concentration is much smaller than the K_m for the transporter (32,33). The thickness of this barrier can be estimated from the observed maximum permeability for such a solute through the relationship:

$$P_{eff} = D/L$$

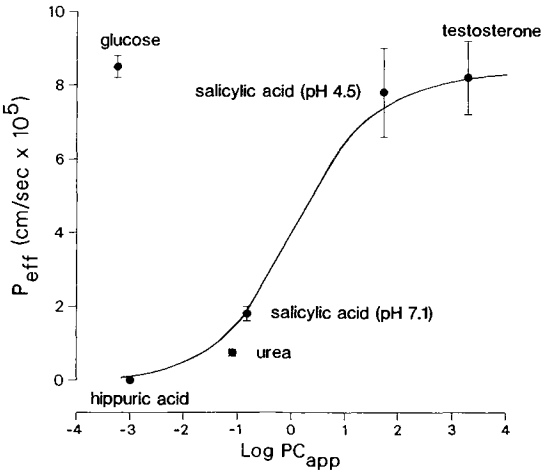

Fig. 9. Relationship of the observed permeability coefficients (P_{eff}) and apparent octanol–water partition coefficients (log PC_{app}) for the model solutes in the Caco-2 cell model system. The permeability coefficients were calculated from the donor disappearance results.

where D is the diffusion coefficient for the solute in water or buffer and L is the thickness of the unstirred barrier. Using the value of P_{eff} determined here and D of 8×10^{-6} cm^2/sec as a reasonable diffusion coefficient for these solutes in neutral buffer at 37°C (32,34), we estimate an unstirred layer of about 1000 μm. This is a rather substantial barrier, much greater than the 100- to 300-μm thickness which is found in other stirred or agitated systems (32). The most serious experimental consequences of the unstirred layer are that it results in an underestimation of permeability coefficients for passively transported molecules and an overestimation of the K_m for actively transported substances. For example, in the extreme case of a significant unstirred layer and a large intrinsic K_m, the overall transport process would appear to be a nonsaturable process over a reasonably large substrate concentration, more representative of passive diffusion, as has been shown from theoretical considerations (33).

Perhaps the relevance of transport data obtained with Caco-2 cell monolayers is best assessed by comparison with data from *in vivo* models. Several of the solutes used in this work have been employed by others to characterize the transport properties of *in vivo* intestinal absorption model systems. A comparison of the Caco-2 results with the results for two of these model systems is shown in Table III. Qualitatively the results are very similar. Quantitatively the differences in the permeabilities in the different models are probably related to the unstirred layer effects, which will be model dependent. However, it does seem clear from this work that the Caco-2 system gives results which are consistent with what is found in these other, more sophisticated models.

While all of the discussion thus far has focused on transport based on substrate disappearance from the donor compartment, one of the principal strengths of this type of model is the ready examination of the receiver compartment. When analyzing disappearance data, the assumption is usually made that what disappears from the luminal compartment is absorbed into the blood (in the *in vivo* situation). Clearly, if the drug is metabolically labile or sequestered in some intracellular organelle during absorption, this assumption is not valid. Thus, the ability to sample the receiver compartment allows for a more thorough examination of the transport process.

As shown in Fig. 8, the appearance rates for the transport of several solutes show essentially the same characteristics as the disappearance rates. In principle, if no metabolism during transport takes place, the disappearance and appearance rates should be equal at steady state for a passive process. Comparing the permeabilities for urea and salicylate calculated from the appearance and disappearance rates, this is indeed what we find. The results for testosterone, on the other hand, show a smaller P_{eff} value calculated from appearance than disappearance. One possible explanation for this may be that the approximations made in the calculation of the permeability coefficients, especially in the value of the membrane surface area, may be different on the receiver and the donor side respectively. Such an argument has previously been made for monolayers prepared from MDCK cells (35).

In any case, it seems clear that the easy access to the receiver solution will be useful when metabolism is a problem or the total amount of absorbed material is very small such that accurate measurement of the slight decrease in the donor concentration is difficult. This is the situation for hippurate transport, where, even with lengthy sampling intervals, no disappearance was detected, but accumulation in the receiver was easily determined. This ability to quantitate readily the transport of relatively impermeable molecules may be one of the principal strengths of the model.

In summary, we feel that the Caco-2 cell grown as monolayers on the Transwell filter system offers an easy, reliable model for the study of drug transport through the intestinal mucosa. Qualitatively, the model gives transport results which are similar to what is found with more complicated models. Quantitatively, the model suffers from a substantial unstirred water layer which will tend to underestimate the rate of rapid passive transport and overestimate K_m values for active transport. With this limitation in mind, the model should prove useful for developing an understanding of how structure influences absorptivity for new drug substances.

Table III. Comparison of Permeability Coefficients Obtained *In Vitro* and *In Vivo*

Solute	P (cm/sec × 10^5)		
	Caco-2	Rat[a]	Rabbit[b]
D-Glucose	8.5	13.7	50.7
Salicylic acid (pH 4.5)	7.8	13.4	47.1
Testosterone	8.2	14.7	—

[a] Data from Ref. 36.
[b] Data from Ref. 37.

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