Characterization of the Unstirred Water Layer in Caco-2 Cell Monolayers Using a Novel Diffusion Apparatus

Ismael J. Hidalgo,[1] Kathleen M. Hillgren,[1] George M. Grass,[2] and Ronald T. Borchardt[1,3]

Received February 9, 1990; accepted July 30, 1990

Caco-2 monolayers grown on Transwell polycarbonate membranes have been characterized as a valuable tool in drug transport studies. Despite the clear advantages of this system, the lack of stirring may create an unstirred water layer (UWL) whose resistance may limit the transcellular transport of lipophilic molecules. The objective of this study was to evaluate a novel diffusion cell where the transport buffer is mixed by gas lift and to determine the mixing flow rate needed to reduce the thickness (h) of the UWL adjacent to cell monolayers. The transport of the leakage marker, mannitol, remained at least 15-fold lower than the flux of testosterone, indicating that the stirring flow rates used did not affect the integrity of the monolayers. The permeability (P) of testosterone (log PC 3.13) across monolayers mounted on this diffusion cell was 4.07, 10.90, and 14.18×10^{-5} cm/sec at flow rates of 0, 15, and 40 ml/min, respectively, and the apparent UWLs were calculated to be 1966, 733, and 564 μm. P and h in the stagnant Transwell were 3.08×10^{-5} cm/sec and 2597 μm, respectively. On the other hand, h was significantly smaller in the unstirred, cell-free membranes than in their cell-containing counterparts. P was correlated with lipophilicity and, in the case of the more lipophilic compounds, with the mixing flow rate.

KEY WORDS: Caco-2; unstirred water layer; intestinal permeability; steroids; cell culture.

INTRODUCTION

The increasing availability of cell and tissue culture systems has created an interest in using these systems as *in vitro* models to investigate drug transport at the cellular level (1). In particular, the culture of epithelial cells on microporous membranes allows the uptake of nutrients (or drugs) from and the release of waste products (or metabolites) into the media through both the apical (luminal) and the basolateral (serosal) membranes (2). This contrasts with cells grown onto plastic surfaces, where such exchange of material is possible only through the apical membrane (3).

The lack of success in culturing intestinal epithelial cells (4,5) together with the fact that some human colon carcinoma cell lines are known to undergo enterocytic differentiation in culture (3,6) have prompted the consideration of colon carcinoma-derived cell lines as potential *in vitro* cell culture model systems of the small intestinal epithelium (7,8). The Caco-2 cell line has received the greatest attention because, in addition to undergoing a high degree of differentitation under typical culture conditions, this cell line performs ion transport (3).

Our laboratory has characterized Caco-2 cells grown on Transwell polycarbonate membranes as a model transport system of polarized intestinal epithelium (7). After 15 days in culture the cells develop morphological characteristics of the small intestine (microvilli, desmosomes, occluding junctions, and cell polarity). After confluency, alkaline phosphatase, a small intestine brush border marker enzyme, is expressed by the apical but not the basolateral membrane of Caco-2 cell monolayers, indicating the achievement of functional polarity. The integrity of the tight junctions was shown by the fact that the permeability of the membrane-insoluble markers, lucifer yellow, inulin, polyethylene glycol (MW 4000), and dextran (MW 70,000), was less than 0.25%/hr in 10-day-old monolayers (7). Caco-2 cells have been used also as model systems for studying passive drug diffusion (8).

However, before using the Caco-2 cell monolayer system to study transcellular diffusion, the effect of the potentially large unstirred water layer (UWL) adjacent to the cell monolayer on the permeability of lipophilic compounds should be determined. Since the UWL has also been shown to distort the kinetics of active transport (9), the characterization of the relationship between UWL and permeability would further improve the utility of the Caco-2 model system in drug transport studies. Thus, we have developed a side-by-side diffusion cell in which stirring flow rates can be controlled accurately (Fig. 1). This diffusion cell is related to one previously developed for use with intestinal mucosal tissue (10). To test whether changes in stirring flow rates were associated with a reduction of the UWL thickness, the transport of a highly lipophilic steroid, testosterone, was investigated at different flow rates and compared with its diffusion in the stagnant Transwell system. Moreover, a series of steroids of differing lipophilicities was used to determine whether a relationship could be established between permeability and lipophilicity. The minimal manipulation required in mounting the monolayers in this diffusion cell virtually eliminates monolayer damage due to handling. However, to determine whether the exposure of the monolayers to this type of mixing had any effect on the barrier properties of the monolayer, we used the membrane-impermeant mannitol as an internal leakage marker.

MATERIALS AND METHODS

Materials

The Caco-2 cell line was obtained from American Type Culture Collection, Rockville, MD, and used between passage 65 and passage 70. Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), nonessential amino acids (NEAA, ×100), and L-glutamine (200 mM) were obtained from Hazleton Research, Lenexa, KS. Penicillin and streptomycin were obtained as a mixture from Hazleton

[1] Department of Pharmaceutical Chemistry, The University of Kansas, Lawrence, Kansas 66045.

[2] Department of Pharmaceutical Sciences, Syntex Research, Palo Alto, California 94303.

[3] To whom correspondence should be addressed at Department of Pharmaceutical Chemistry, 3006 Malott Hall, The University of Kansas, Lawrence, Kansas, 66045.

0724-8741/91/0200-0222$06.50/0

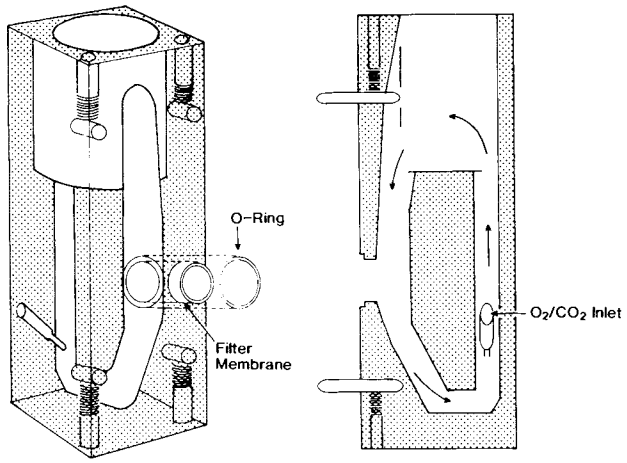

Fig. 1. New side-by-side diffusion cell. Inserts containing cell monolayers are mounted between acrylic half-cells with the aid of an O-ring. Transport buffer is circulated by gas (O_2/CO_2) lift and flows in the direction of the arrows parallel to the surface of the cell monolayer. Temperature is maintained by a heating block connected to a recirculating water bath.

Research. Short (3-mm-high) Transwell inserts with 0.4-μm polycarbonate membranes and 6.5 mm in diameter were kindly provided by Costar, Bedford, MA. Transwell clusters, polyvinylpyrrolidine-free, 24.5-mm diameter, 3.0-μm pore size, were from Costar. The side-by-side diffusion chambers, gas manifold, and block heater were from Precision Instrument Design, Los Altos, CA. Hank's balanced salts, *N*-2-hydroxyethylpiperazine-N'-2-ethanesulfonate (HEPES), paraformaldehyde, and glutaraldehyde were from Sigma Chemical Co., St. Louis, MO. [^{3}H]Testosterone (sp act, 55.2 Ci/mmol) was from NEN Research Products, Boston, MA, [1,2,4,6,7-^{3}H]Dexamethasone (sp act, 84 Ci/mmol), [1,2,6,7-^{3}H]androst-4-ene-3,17-dione (sp act, 91.8 Ci/mmol), and [1,2,6,7-^{3}H]cortisol (sp act, 86 Ci/mmol) were from Amersham, Arlington Heights, IL, and D-[1-^{14}C]-mannitol was from American Radiolabeled Chemicals, St. Louis, MO. All other chemicals were of the highest grade available.

Cell Culture

Caco-2 cells were seeded at a density of 63,000 cells/cm^2 on polycarbonate membranes 6.5 mm in diameter, 0.4-μm pore size, that were part of 3-mm-high inserts provided by Mr. Hank Lane, Costar. These inserts were placed on larger Transwell inserts and bathed in culture medium consisting of DMEM supplemented with 10% heat-inactivated FBS, 1% NEAA, 1% L-glutamine, 100 U/ml penicillin, and 100 μg/ml streptomycin. The cells were fed every other day with culture medium and maintained at 37°C in 90% relative humidity and 5% CO_2. All monolayers were from cells between passage 65 and passage 70 and used 20–30 days after seeding.

Transport Studies

Mixing in the diffusion cell was achieved by a 5% CO_2/O_2 airlift. After adjustment of the mixing rate to 0, 15, or 40 ml/min, the cell inserts were placed in the diffusion cell (Fig. 1), which was maintained at a constant temperature of 37°C by a water-heated jacket and 2.5 ml of transport medium (Hank's balanced salt solution containing 25 m*M* glucose and 10 m*M* Hepes, pH 7.35) was placed on each compartment (i.e., donor and receiver). To prevent potential adsorption of the steroids to the walls of the diffusion cell, the donor (apical) side had 10^{-4} M of the respective steroid and the receiver (basolateral) side had 10^{-4} M of the steroid plus 0.01% bovine serum albumin. After 15 min of preincubation the solution in the receiver side was replaced with steroid-free transport medium and the donor side was spiked with the radioactive steroids (10^6 dpm/ml). Samples were taken from both the donor (50 μl) and the receiver (750 μl) sides at selected times. The samples taken from the receiver side were replaced with an equal volume of transport medium. The radionuclides were measured on a Beckman LS-5801 liquid scintillation counter (Beckman Instruments, Palo Alto, CA).

Data Treatment

Permeability coefficients (P) were calculated using the following equation:

$$P = \frac{V \cdot dC}{A \cdot C_0 \cdot dt} \quad (1)$$

where dC/dt is the flux across the monolayer (dpm/ml/sec), V the volume of the receiver chamber (i.e., 2.5 ml), A the diffusion area (i.e., 0.33 cm^2), and C_0 the initial concentration (dpm/ml) in the donor compartment. The transport of testosterone across cell-free membranes slowed down considerably after 30 min, presumably because of back diffusion. Thus, to avoid underestimating the apparent permeability across cell-free membranes, the permeability was calculated from those data obtained during the first 15 min. On the other hand, the permeability across cell monolayers was calculated from the slope of the line describing the amount transported versus time (which was linear through 60 min).

The apparent UWL thickness (h) was determined by using the equation:

$$h = D/P \quad (2)$$

where D is the diffusion coefficient, and P the effective permeability coefficient. The diffusion coefficient was assumed to be equal to 8×10^{-6} cm^2/sec (11).

Statistical differences between two means were evaluated using the one-sided Student's t test and differences among more than two means using one-way analysis of variance followed by the Student–Neumann–Keuls multiple-range test (12). All statistical differences were evaluated at a significance level of 5%.

RESULTS AND DISCUSSION

Diffusion Across Cell-Free Membranes

The objective of this study was to evaluate the usefulness of a new diffusion apparatus (Fig. 1) which can be used

with cultured cell monolayers grown on microporous membranes. This diffusion cell provides stirring (by gas lift) in order to minimize the potential influence that the UWL adjacent to the monolayer could have in the permeability of lipophilic compounds. The Caco-2 cell monolayer was chosen because the system had been extensively characterized as a model system for intestinal epithelial permeability (7,8).

To determine the potential barrier properties of the polycarbonate membrane alone, we studied first the diffusion of mannitol and testosterone across cell-free membranes. We found that in the absence of stirring, both mannitol and testosterone diffused readily and in comparable amounts (Table I, Fig. 2), indicating that the polycarbonate membrane was not a diffusion barrier. When the flow rate increased from 0 to 40 ml/min the permeability of testosterone across cell-free membranes increased by 38.2%, while the corresponding increase in mannitol permeability was only 10.2% (Table I). These results suggest the presence of a relatively small UWL adjacent to the cell-free polycarbonate filters mounted on the diffusion apparatus. The diffusion of mannitol and testosterone across cell-free polycarbonate membranes in the unstirred Transwell inserts was virtually identical to those values obtained in the unstirred diffusion cell (Table I, Fig. 2), suggesting that the apparent UWL present in the large cell-free Transwell insert is comparable to that found in the small insert mounted in the unstirred diffusion cell. A recent study showed that while the rate of adsorption of 10^{-4} *M* concentrations of propranolol (log PC 1.93) onto polycarbonate filters without cells was only 0.9%/hr, the corresponding adsorption onto nitrocellulose filters was 41.3%/hr (8). In this study, the amounts of these marker compounds that were adsorbed to the polycarbonate membrane were negligible (not shown) and thus not likely to interfere with their diffusion.

Diffusion Across Cell Monolayers

In the presence of cell monolayers the permeability of mannitol and testosterone exhibited different characteristics, consistent with their physical chemical properties. Without stirring, the permeability of mannitol across cell monolayers mounted on the diffusion cell was 0.26×10^{-5} cm/sec, compared to 12.1×10^{-5} cm/sec across cell-free membranes, indicating that the cells do represent a formidable barrier to the diffusion of lipophobic compounds. Simi-

Table I. Permeability of Testosterone and Mannitol Across Caco-2 Cell Monolayers

System[a]	Flow rate (ml/min)	Permeability ($\times 10^{-5}$ cm/sec) Testosterone	Mannitol
		Filter alone	
TW	0	7.25 ± 0.7[b]	13.9 ± 1.0
DC	0	8.1 ± 0.7	12.1 ± 8.6
DC	40	11.1 ± 1.4	13.3 ± 0.5
		Filter plus cells	
TW	0	3.1 ± 0.04	0.19 ± 0.06
DC	0	4.1 ± 1.1	0.26 ± 0.07
DC	15	10.9 ± 1.6	0.97 ± 0.10
DC	40	14.2 ± 1.6	0.87 ± 0.47

[a] TW, Transwell system; DC, diffusion cell.
[b] Values are mean ± SD (N = 3–6).

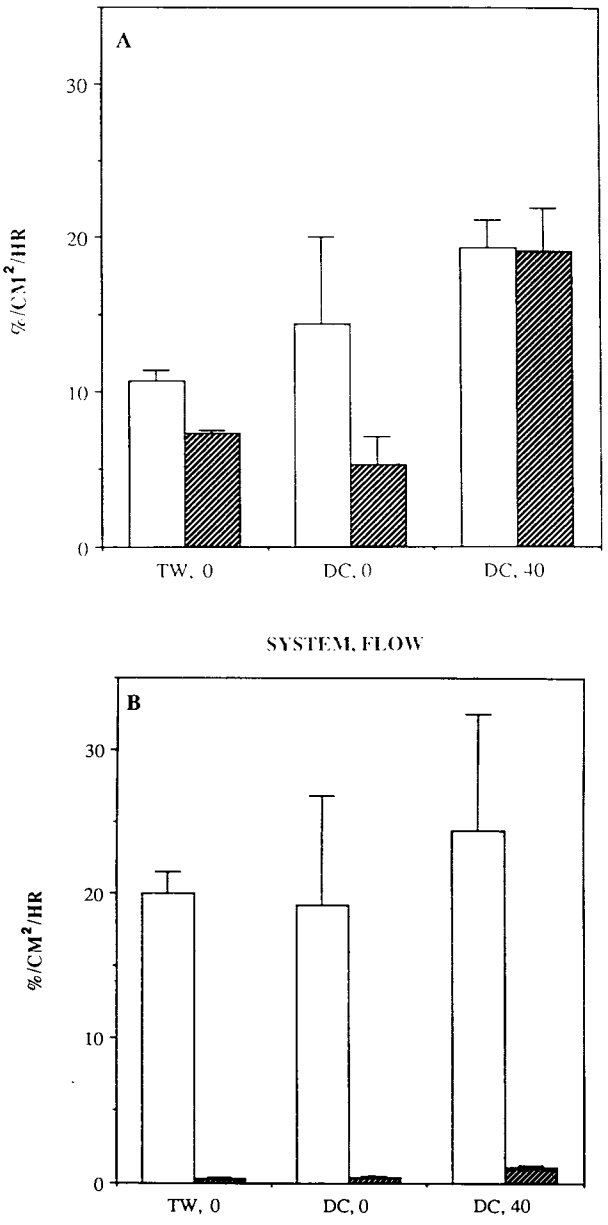

Fig. 2. Testosterone and mannitol flux through Caco-2 cell monolayers. Testosterone (A) and mannitol (B) were applied as a mixture to 24.5-mm-diameter Transwell inserts with or without cells that were positioned on six-well trays and incubated without stirring (TW, 0). The same solution was applied to short, 6.5-mm-diameter inserts with or without cells that were mounted in the new side-by-side diffusion cell (DC) and incubated at 37°C under stirring flow rates equal to 0 or 40 ml/min. DC, diffusion cell. Open bars indicate membrane alone; hatched bars indicate membrane plus cell monolayers; vertical bars represent standard deviation of three to five membranes or monolayers.

larly, in the stagnant Transwell, there was a decrease in mannitol permeability from 13.9×10^{-5} cm/sec across cell-free membranes to 0.2×10^{-5} cm/sec across cell monolayers. This observation is in agreement with a previous study which showed that Caco-2 monolayers 10 days or older restricted dramatically the paracellular diffusion of the membrane-impermeant compounds lucifer yellow CH (MW 453), dextran (MW 70,000), polyethyleneglycol (MW 4000), and horseradish peroxidase (MW 40,000) (7).

The diffusion of the lipophilic compound, testosterone, did not exhibit as dramatic a decrease as mannitol (Table I, Fig. 2) when cell monolayers were present on the polycarbonate membrane. In fact, both the unstirred diffusion cell system and the stagnant Transwell system showed only about a 50% reduction in diffusion when the cell monolayers were present with respect to the cell-free membranes. This decrease in testosterone permeability may be due partly to the presence of an UWL in the unstirred system and partly to the diffusional resistance of the cell membrane. An increase in flow rate from 15 to 40 ml/min resulted in only a 30% increase in testosterone permeability, indicating that at stirring flow rates equal to or greater than 15 ml/min, the UWL may not necessarily constitute the principal component of the total diffusional resistance to testosterone.

The reason for the selective restriction of lipophobic compounds is that lipophilic compounds such as testosterone can undergo transcellular diffusion due to their ability to traverse the cell membrane, while lipophobic compounds such as mannitol can permeate the monolayer only through paracellular diffusion or fluid-phase transcytosis. Since the extent of transcytosis in intestinal cells is minimal (13) and, further, since the cell surface area is much greater than the junctional area (14), the potential diffusion of testosterone is not expected to be affected substantially by the presene of the monolayer. The paracellular diffusion of mannitol increased roughly by a factor of three when the flow rate was increased from 0 to 40 ml/min. Although the reasons for this increase in mannitol permeability are not clear, one can speculate that it may result from subtle changes in the monolayers such as loosening of defective tight junctions or the possible detachment of dead cells from the filter caused by the stirring used. However, the minimal leakage of inulin across cell monolayers ($0.06\% \cdot cm^{-2} \cdot hr^{-1}$) compared with the diffusion across cell-free filters ($19.1\% \cdot cm^{-2} \cdot hr^{-1}$) and the apparent lack of morphologic changes in tight junction and cell morphology under transmission and scanning electron microscopic examination (Hidalgo *et al.*, unpublished results) do not support this hypothesis. In a related study we also used light microscopy and transmission and scanning electron microscopy to confirm that no significant morphologic changes were induced on the cells by the flow rates used (16). It was found that at the highest stirring flow rate used, 40 ml/min, there was no alteration on the structure or organization of microvilli, nor was the structure of the tight junctions affected. Moreover, the attachment of the cells to the microporous filter remained unchanged after exposure to this flow rate for up to 2 hr. Despite the increase in the permeability of mannitol at 40 ml/min, the permeability of this compound remained more than 15-fold lower than that of testosterone (Table I). The minimal leakage of mannitol at all flow rates used is also indicative of no appreciable breakdown in the integrity of the epithelial barrier even at flow rates as high as 40 ml/min. These data agree with a recent study which determined that opening tight junctions with chelating agents affected substantially the diffusion of lipophobic β-adrenergic blocking agents but not the permeability of hydrophobic ones (15).

Permeation of Steroids Across Cell Monolayers

To examine further the effect of flow rate, hence the UWL, on permeability, a series of steroids of differing lipophilicities was employed. Because most of the reduction in apparent UWL took place at 15 ml/min, the effect of flow rate on permeability was evaluated only at 0- and 15-ml/min flow rates. The apparent increase in permeability with increasing lipophilicity observed in the diffusion experiment employing stirring lacked statistical significance due to the relatively large errors associated with the permeability values (Table II). The permeability of the two compounds with higher partition coefficients (deoxycorticosterone and testosterone) was more sensitive to the changes in flow rate than that of the less lipophilic compounds. In addition, the permeability of those compounds with a lower lipophilicity showed less of a change in permeability as a result of a change in the mixing rate because their permeation is not significantly affected by the presence of an UWL as is the case for lipophilic drugs (11). For example, neither cortisol's (log PC 1.53) nor dexamethasone's (log PC 1.74) permeability was greatly affected by the flow rate (Table II).

Determination of the Apparent UWL Thickness

The apparent UWL was not calculated for the more lipophobic compounds since their transport is not diffusion controlled but rather restricted by the cell membrane. The previous results suggest that, in the absence of stirring, a large apparent UWL was present in this cultured cell monolayer model system. Thus, we utilized the most lipophilic steroid, testosterone, whose permeability is thought to be UWL-controlled, to determine the thickness of the apparent UWL adjacent to monolayers in the stagnant Transwell and in the diffusion cell under different stirring conditions. To test this hypothesis the apparent UWL thickness was also

Table II. Effect of Mixing Rates on the Permeability[a] of Compounds of Different Lipophilicities

Compound	log PC[b]	Flow rate (ml/min) 0	15
Cortisol	1.53	2.88 ± 0.34[c]	2.15 ± 0.03
Dexamethasone	1.74	1.16 ± 0.34	1.46 ± 0.27
Corticosterone	1.89	5.39 ± 0.70	6.16 ± 0.64
Androstenedione	2.75	5.99 ± 0.99	6.60 ± 0.77
Deoxycorticosterone	2.88	5.29 ± 0.82	6.88 ± 0.23
Testosterone	3.31	4.07 ± 1.13	10.90 ± 1.56

[a] Permeability values are $\times 10^{-5}$ cm/sec.
[b] Octanol/water (from Ref. 11).
[c] Values are mean ± SD ($N = 3$).

Table III. Relationship Between Stirring Flow Rate and Unstirred Water Layer Thickness

System[a]	Flow rate (ml/min)	h (μm)
	Filter alone	
TW	0	1103 ± 106[b]
DC	0	992 ± 92
DC	40	718 ± 89
	Filter plus cells	
TW	0	2597 ± 34
DC	0	1966 ± 546
DC	15	733 ± 105
DC	40	564 ± 66

[a] TW, Transwell system; DC, diffusion cell.
[b] Values are mean ± SD (N = 3–6).

determined in cell-free membranes both mounted in the diffusion cell and in Transwell inserts. The large apparent UWL adjacent to the monolayers in the diffusion cell in the absence of stirring was reduced by over 60% (i.e., from 1966 to 733 μm) when a stirring flow rate equal to 15 ml/min was applied (Table III). Increasing the flow rate to 40 ml/min caused only an additional 8% decrease, suggesting that the cell membrane may offer some resistance to the diffusion of testosterone. Because of this diffusional resistance of the cell membrane, Eq. (2) may result in an overestimation of the true UWL. Thus our estimate of UWL thickness (i.e., using testosterone) should be considered an apparent UWL. In a related study butanol, a compound whose higher lipophilicity makes it more permeable across the cell membrane, was shown to diffuse faster than testosterone. Therefore, the resulting estimate of the UWL thickness obtained with butanol is expected to be somewhat smaller than that obtained with testosterone.

Our apparent UWL values are similar to those estimated for human small intestine (17,18) and demonstrate that the apparent UWL obtained in the unstirred system mimicks closely those of the small intestine. This was consistent with one of our goals which was to determine whether stirring achieved apparent UWL comparable to those found in the small intestine. While the total elimination of the UWL does not model the physiologic situation, its minimization may make it possible to understand the mechanisms underlying the absorption of drugs and nutrients at the cellular level.

Although we have limited our studies to demonstrating the effect of the apparent UWL on the passive diffusion of lipophilic compounds, there is evidence that the presence of an UWL distorts the kinetics of both passive and active transport (9,19–21). Finally, the need to reduce the UWL will probably result in the development of new diffusion devices. However, in developing diffusion cells for the study of drug transport and metabolism across cultured cell monolayers, the validation of any stirring protocol should include an extensive characterization of the effect of stirring not only on the UWL but also on the integrity of the cell monolayer.

ACKNOWLEDGMENTS

This work was supported by a grant from The Upjohn Company. The authors wish to thank Mr. Hank Lane, Costar, for providing the short Transwell inserts. K. M. Hillgren was supported by National Institutes of Health Predoctoral Training Grant GM-08359.

REFERENCES

1. K. L. Audus, R. L. Bartel, I. J. Hidalgo, and R. T. Borchardt. The use of cultured epithelial and endothelial cells for drug transport and metabolism studies. *Pharm. Res.* 7:435–451 (1990).
2. R. T. Borchardt, I. J. Hidalgo, K. M. Hillgren, and M. Hu. Pharmaceutical applications of cell culture. An overview. In G. Wilson, L. Illum, and S. S. Davies (eds.), *Pharmaceutical Applications of Cell and Tissue Culture,* Plenum, New York (in press).
3. M. Pinto, S. Robine-Leon, M.-D. Appay, M. Kedinger, N. Triadou, E. Dussaulx, B. Lacroix, P. Simon-Assmann, K. Haffen, J. Fogh, and A. Zweibaum. Enterocytic differentiation and polarization of the human colon carcinoma cell line Caco-2 in culture. *Biol. Cell* 47:323–330 (1983).
4. F. Raul, M. Kedinger, P. Simon, J. Grenier, and K. Haffen. Behaviour of isolated rat intestinal cells maintained in suspension or monolayer cultures. *Biol. Cell* 33:163–168 (1978).
5. A. Quaroni, J. Wands, R. L. Trelstad, and K. J. Isselbacher. Epithelioid cell cultures from rat small intestine. Characterization by morphologic and immunologic criteria. *J. Cell Biol.* 80:248–265 (1979).
6. M. Pinto, M.-D. Appay, P. Simon-Assman, G. Chevalier, N. Dracopoli, J. Fogh, and A. Zweibaum. Enterocytic differentiation of cultured human colon cancer cells by replacement of glucose by galactose in the medium. *Biol. Cell* 44:193–196 (1982).
7. I. J. Hidalgo, T. J. Raub, and R. T. Borchardt. Characterization of the human colon carcinoma cell line (Caco-2) as a model system for intestinal epithelial permeability. *Gastroenterology* 96:736–749 (1989).
8. P. Artursson. Epithelial transport of drugs in cell culture. I. A model for studying the passive diffusion of drugs over intestinal absorptive (Caco-2) cells. *J. Pharm. Sci.* 79:476–482 (1990).
9. J. M. Dietschy and H. Westergaard. The effect of unstirred water layers on various transport processes in the intestine. In T. Z. Csaky (ed), *Intestinal Absorption and Malabsorption,* Raven, New York, 1975, pp. 197–206.
10. G. M. Grass and S. A. Sweetana. In vitro measurement of gastrointestinal tissue permeability using a new diffusion cell. *Pharm. Res.* 5:372–376 (1988).
11. I. Komiya, J. Y. Park, A. Kamani, N. F. Ho, and W. I. Higuchi. Quantitative mechanistic studies in simultaneous fluid flow and intestinal absorption using steroids as model solutes. *Int. J. Pharm.* 4:249–262 (1980).
12. W. C. Schefler. *Statistics for the Biological Sciences,* Addison, Menlo-Park, 1979.
13. M. Heyman, R. Ducroc, J.-F. Desjeux, and J. L. Morgat. Horseradish peroxidase transport across adult rabbit jejunum in vitro. *Am. J. Physiol.* 242:G558–G564 (1982).
14. P. F. Curran and A. K. Solomon. Ion and water fluxes in the ileum of rats. *J. Gen. Physiol.* 4:143–168 (1957).
15. P. Artursson and C. Magnusson. Epithelial transport of drugs in cell culture. II. Effect of extracellular calcium (Ca^{2+}) concentration on the paracellular transport of drugs of different lipophilicities across monolayers of intestinal epithelial (Caco-2) cells. *J. Pharm. Sci.* 79:595–600 (1990).
16. I. J. Hidalgo, K. M. Hillgren, G. M. Grass, and R. T. Bor-

chardt. A new side-by-side diffusion cell for studying transport across epithelial cell monolayers (submitted for publication).
17. F. A. Wilson and J. M. Dietschy. The intestinal unstirred layer: Its surface area and effect on active transport kinetics. *Biochim. Biophys. Acta* **363**:112–126 (1974).
18. T. Levitt, T. Aufderheide, C. A. Fetzer, J. H. Bond, and D. G. Levitt. Use of carbon monoxide to measure luminal stirring in the rat gut. *J. Clin. Invest.* 74:2056–2064 (1984).
19. D. Winne. Unstirred layer, source of biased Michaelis constant in membrane transport. *Biochim. Biophys. Acta* **298**:27–31 (1973).
20. A. B. R. Thomson. Limitations of the Eadie-Hofstee plot to estimate kinetic parameters of intestinal transport in the presence of an unstirred water layer. *J. Membr. Biol.* 47:39–57 (1979).
21. A. B. R. Thomson. Limitations of Michaelis-Menten kinetics in presence of intestinal unstirred layers. *Am. J. Physiol.* **236**:E701–E709 (1979).