Report

In Vitro Measurement of Gastrointestinal Tissue Permeability Using a New Diffusion Cell

George M. Grass[1,2] and Stephanie A. Sweetana[1]

Received July 29, 1987; accepted January 12, 1988

A new diffusion cell, derived from the Ussing chamber, was developed for the measurement of tissue permeability. This cell incorporates the attributes of using a single material and laminar flow across the tissue surface. In addition, the design allows the cell to be manufactured in a wide range of sizes to allow optimization of surface area to volume for a variety of tissues. The apparatus is applicable to the evaluation of transport of compounds through mucosal/epithelial barriers, i.e., gastrointestinal tissue. Active transport, permeability enhancers, enzymatic degradation, and absorption in various tissue sections can be explored. Preliminary data are consistent with the expected effects of molecular size and partition coefficient of a transported molecule on permeability in epithelial tissue. In addition, active transport of D-glucose and inhibition by phloridzin and ouabain can be demonstrated.

KEY WORDS: *In vitro* apparatus; diffusion cell; intestinal permeability.

INTRODUCTION

Prediction of the ability of a drug molecule to traverse an epithelial barrier is difficult to make from simple physicochemical measurements, such as pK_a, molecular size (or weight), and partition coefficient, which tend to oversimplify the driving forces for transport. Direct measurement of tissue permeability would be advantageous in ascertaining whether problems in drug absorption are the result of low solubility or poor tissue permeability.

For the study of ion transport across frog skin, Ussing and Zerahn (1) introduced the use of an *in vitro* short-circuit current technique, i.e., voltage clamping, to distinguish between active and passive ion movement. Since its introduction this technique has been adapted for the investigation of ion transport in various other tissues (2–4), and its validity has been previously reviewed (5). While commonly used in the examination of gastrointestinal pharmacology, it has been little utilized in the examination of intestinal transport of drug substances. Several notable exceptions, such as flux studies using horseradish peroxidase or xylose in intestinal tissue of rabbit (6,7) and piglet (8) and absorption studies in rat (9), do exist; however, routine examination of existing and potential therapeutic agents has not been conducted.

We have attempted to investigate the intestinal permeability of various drug species using the method of Ussing and Zerahn. Several shortcomings of the application of their apparatus for our purposes have led to the development of a new diffusion cell system that has several distinct advantages.

[1] Syntex Research Department of Pharmaceutical Sciences, 3401 Hillview Avenue, Palo Alto, California 94303.

[2] To whom correspondence should be addressed.

EXPERIMENTAL

Materials

Radiolabeled mannitol, L-glucose, D-glucose, methoxyinulin, naproxen, and progesterone were purified by vacuum distillation immediately prior to use. Labeled methanol was used as received. Supplier, specific activity, and type of radiolabel are indicated in Table I for each compound. All other chemicals were either reagent or analytical grade and were used as received. Male albino New Zealand rabbits weighing between 2.5 and 3.5 kg were used throughout the studies. Animals were fasted overnight prior to use in an experiment.

Methods

Preparation of Drug Solutions

Drug solutions were prepared by the addition of tracer amounts of radiolabeled compound to oxygenated (O_2/CO_2, 95/5) Kreb's ringer bicarbonate buffer (pH 7.4), which was prepared daily. The actual concentrations of radiolabeled species applied in the donor chamber are given in Table I. For all compounds the drug concentration is that of the radiolabeled material with two exceptions; unlabeled drug was added for mannitol (40 m*M*) and D-glucose (10 m*M*). To help maintain tissue viability, D-glucose (40 m*M*) was added to the serosal medium. Since even small amounts of glucose on the mucosal side markedly stimulate sodium and water absorption to the serosal side (10), for all experiments except D-glucose permeability measurements, mannitol (40 m*M*) was added to the mucosal solution to provide an equivalent osmotic load. For D-glucose experiments, 30 m*M* mannitol and 10 m*M* D-glucose were placed on the mucosal side.

0724-8741/88/0600-0372$06.00/0 © 1988 Plenum Publishing Corporation

Table I. Radiolabel, Supplier, Specific Activity, and Concentration Applied to the Tissue for Compounds Used in *in Vitro* Permeability Determinations

Drug name	Label	Supplier	Sp act (Ci/mmol)	Actual applied concentration ($\times 10^{-5}$ *M*)
Methanol	^{14}C	NEN[a]	0.058	0.15
Mannitol	^{14}C	Amersham[b]	0.055	4000
L-Glucose	^{3}H	NEN	10.70	0.003
D-Glucose	^{3}H	Amersham	6.44	1000
Methoxy-inulin	^{3}H	NEN	0.93	0.04
Naproxen	^{3}H	Syntex[c]	18.0	0.002
Progesterone	^{3}H	Amersham	56.0	0.0008

[a] New England Nuclear, Boston, Mass.
[b] Amersham, Arlington Heights, Ill.
[c] Syntex Research, Palo Alto, Calif.

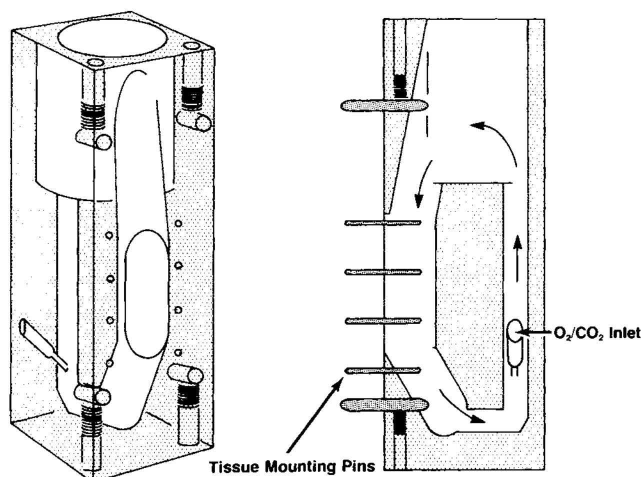

Fig. 1. Sweetana/Grass diffusion cell. Tissue is mounted between acrylic half-cells. Buffer is circulated by gas lift (O_2/CO_2) at inlet and flows in the direction of arrows, parallel to the tissue surface. Temperature control is maintained by heating block (Fig. 2).

In Vitro *Experiments*

Rabbits were sacrificed by rapid injection of sodium pentobarbital through a marginal ear vein. Following a midline incision, 40 cm of small intestine, extending from the apex of the ascending loop of the duodenum, was removed and placed in ice-cold oxygenated Kreb's ringer bicarbonate buffer (pH 7.4). Single jejunal segments were cut, beginning in the region 12 cm from the duodenal end of the excised tissue segment. Peyers patches could be easily identified visually, and sections containing them were not used in these studies. The individual segments were opened along the mesenteric border to expose the epithelial surface.

For Ussing chambers, tissues were mounted by a previously described procedure (11); however, the permeability studies were conducted in an unclamped state. Briefly, the section was placed on an acrylic half-cell (Jim's Instrument Manufacturing, Iowa City, Iowa), the longitudinal muscle fibers were carefully stripped from the serosal side of the tissue, and the matching half-cell was joined to seal the diffusion apparatus. Immediately following tissue mounting, the cell was joined to its reservoir and circulation of the mucosal and serosal solutions was initiated. The exposed tissue surface area was 1.13 cm^2 and the fluid volume in each half cell was 10 ml.

An alternate diffusion cell, designed in our laboratory, was manufactured (Precision Instrument Design, Los Altos, Calif.) and is described in Fig. 1. Tissues were mounted as with the Ussing chamber, however, the cells were preheated to 37°C. After tissue mounting, the assembled cells were placed in an aluminum block heater (Fig. 2). The heating unit was capable of holding six cells and maintained a temperature of 37°C throughout the studies. The reservoirs were filled with warmed oxygenated Kreb's buffer which was circulated by gas lift (O_2/CO_2), controlled by needle valves. The exposed surface area was 2.06 cm^2 and the volume of each half cell was 7 ml.

For both cell types, 0.1-ml samples of the donor phase (mucosal) were taken immediately prior to addition to the diffusion cell and from the donor reservoir at the conclusion of the experiment. Receptor-phase (serosal) samples (1 ml) were taken at indicated time points, with replacement of the sampled volume by blank (non-drug-containing) buffer. Samples were placed in scintillation vials, 15 ml of scintillation cocktail (Aquasol, New England Nuclear, Boston, Mass.) added, and counted on a scintillation counter (Beckman LS 8100, Beckman Instruments, Fullerton, Calif.) using an external standardization method. All experiments continued for 2 hr with the exception of L-glucose and D-glucose studies, which continued for 4 hr. In separate experiments to examine active transport, inhibitors were added to either donor (1 m*M* phlorizin) or receptor (0.2 m*M* ouabain) solutions. The number of animals used and experiments conducted were as indicated in the tables.

After each experiment the acrylic cells of both types were sonicated in cleaning solution (Count-off, New England Nuclear, Boston, Mass.) and thoroughly rinsed with distilled water. For the Ussing chamber, the circulating reservoir was cleaned by flushing with ethanol followed with distilled water.

RESULTS

Figure 3 gives representative data for a series of permeability experiments conducted in rabbit jejunum with methanol. Permeabilities were calculated from the slope of similar plots for all compounds studied. For more water-soluble compounds, slopes were linear throughout a 120-min time period. However, for more lipophilic compounds, e.g., progesterone, a lag time of up to 30 min was incorporated, with the determined slope taken from the remaining points. Unless noted, permeabilities were determined over a 120-min time period only. For glucose determinations (Fig. 4), slopes were calculated before and after 120 min to determine differences in permeability over 240 min. *In vitro* permeability coefficients were calculated from the following equation:

$$P = \frac{V \cdot dC}{A \cdot C_0 \cdot dt} \tag{1}$$

where dC/dt is the change in concentration as dpm per milliliter sample per unit time and V is the volume of the receptor phase as milliliters. Therefore the quantity $V \cdot dC/dt$ is the

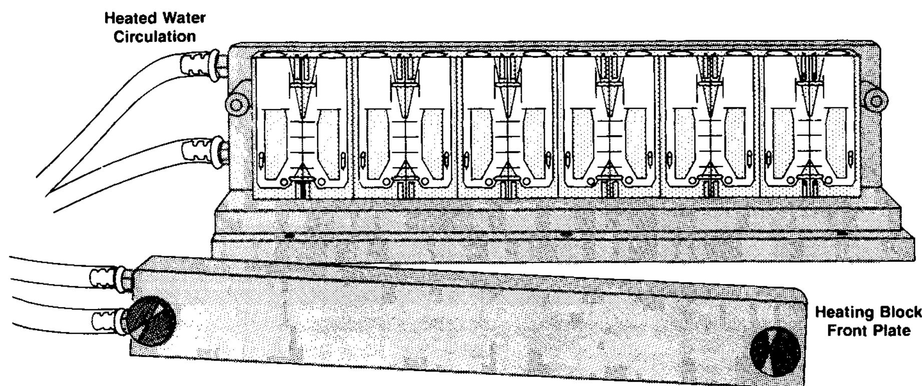

Fig. 2. Diffusion-cell heating block—maintains six assembled diffusion cells at 37°C. Water from heated reservoir is circulated through aluminum block. Front plate fits over upper half of diffusion cell from surface, allowing access to air inlet ports.

steady-state flux as dpm per unit time. A is the surface area of the intestinal segment and C_0 is the initial concentration of the diffusing drug.

Table II gives the combined permeability measurements from both systems along with their molecular weight and partition coefficient values. No statistical differences in permeability measurements were found between the two systems, however, variability was much greater with the Ussing chamber system. The permeability of progesterone could not be accurately determined in the Ussing chamber apparatus since a large percentage of the drug was absorbed by the connecting tubing of the system.

Figure 4 and Table III demonstrate that glucose permeability was nearly constant up to 120 min but decreased to about 65% of this value between 120 and 240 min. In contrast, L-glucose transport (Table II) appears nearly constant over the full 4-hr range and was 20% of the D-glucose permeability.

In the presence of the inhibitor phlorizin, the magnitude of D-glucose transport decreased and was slightly less than that measured for D-glucose from 120 to 240 min. Similarly, 0.2 m*M* ouabain was added in some experiments to the serosal medium to examine the effect of this potent inhibitor of sodium transport on active transport of glucose. The result was comparable to that of phlorizin although slightly decreased in effect. For both inhibitors, permeability was nearly constant over the 4-hr time period, similar to L-glucose transport.

DISCUSSION

Permeability measurements conducted in two *in vitro* apparatuses, the Ussing chamber and a new diffusion cell design from this laboratory (Figs. 1 and 2), showed no statistical difference in values when corrected for surface area. In addition, trends of the permeability data are generally as one would expect. For example, methanol, mannitol, and methoxyinulin demonstrate permeabilities that decrease as the molecular size increases.

To provide some information about the viability of the tissue, electrodes were inserted in each Ussing chamber half-cell in early experiments to measure electrical potential and resistances of the tissue. The implication of this measurement was that in order to maintain an electrical potential, the tissue must be able to transport sodium ions actively (1,2). These measurements (unpublished data) repeatedly afforded nearly constant values for over 4 hr, although by gross examination, most tissue samples appeared severely discolored and hydrated at these later time periods. This finding is consistent with results of Corbett *et al.* (12), who monitored intestinal tissue viability by electrical potential and concluded that the samples were viable for a period of at least 180 min.

Several investigators have questioned the applicability

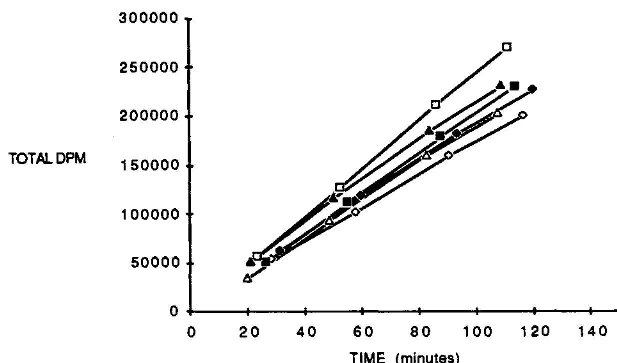

Fig. 3. Disintegrations per minute in receiver solution (7 ml) of receiver solution versus time for methanol permeability in rabbit jejunum. Results from six individual experiments (tissue sections) are shown.

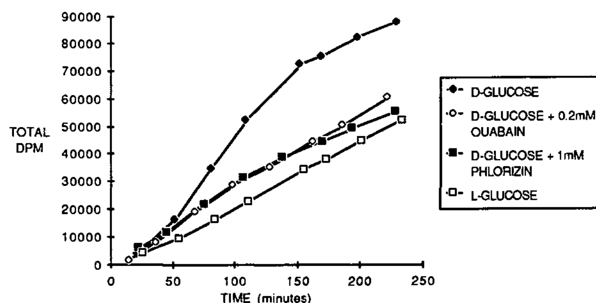

Fig. 4. Disintegrations per minute in receiver solution (7 ml) versus time for various glucose permeability experiments conducted with rabbit jejunum.

Table II. Combined Values for Permeability Determinations from Ussing Chambers and a Novel Diffusion Cell

Drug	MW	Log partition coefficient[a]	Permeability coefficient (cm/sec $\times 10^{-6}$)	*N* (experiments/animals)
Methanol	34	−0.74	44.40 (4.10)[b]	25/5
L-Glucose	180	−3.29	3.03 (0.33)	18/5
Mannitol	184	−3.10	3.59 (0.22)	13/3
Naproxen	253	0.18	8.52 (0.84)	11/3
Progesterone	315	3.87	1.30 (0.18)	22/4
Methoxy-inulin	~5200	−2.90	1.61 (0.14)	25/5

[a] Log octanol/water partition coefficient.
[b] Number in parentheses represents the standard error of the mean.

and validity of the use of short current as a measure of active transport (13,14). Since short circuiting is established when the algebraic sum of the mucosal and serosal potential becomes zero, one may question the status of the membrane diffusion barrier. Most recently, the permeability of a nonapeptide through the intestinal barrier has been evaluated in the unclamped state utilizing an Ussing chamber system (15). Since we believe that it more closely represents the *in vivo* situation, our studies were also conducted in the unclamped state.

As an alternate method to assess tissue viability, the permeability of D-glucose was determined for up to 4 hr. In contrast to passively transported compounds such as L-glucose, which exhibited linearity for up to 4 hr, the permeability of D-glucose decreased to 65% of its initial value after approximately 120 min. It is assumed that this is due to depletion of available ATP within the epithelial cells, and studies are continuing to verify this. The value obtained for 0 to 120 min for D-glucose transport is similar to that reported by Takaori and co-workers for 3-*O*-methylglucose (2.89×10^{-5} cm/sec) for rabbit jejunum using Ussing chambers (15). The magnitude of glucose permeability after 120 min was similar to that found for D-glucose with phlorizin, a known specific inhibitor of Na-dependent glucose transport. Phlorizin appears to bind to the glucose transporter site, located on the mucosal brush border, in a sodium dependent manner (16). Ouabain, a specific Na, K ATPase inhibitor (17), was added in some experiments to the serosal medium and produced a response similar to that of phlorizin, although slightly decreased in effect. For both inhibitors, permeability was nearly constant over the 4-hr time period, similar to L-glucose transport. This behavior of D- and L-glucose is consistent with known differences in binding affinities of the hexose transport system reported by various investigators. The measured affinity, K_m, of glucose in the hamster small intestine to the binding site is much less for L-glucose than for D-glucose, 65 and 1.5 m*M*, respectively (18,19). It has been assumed that similar relative affinities exist in other tissues. The measurements with and without inhibitors suggest that the permeability of D-glucose after 120 min may approach the residual passive tissue transport component, after loss of the active transport capabilities of the cells. Similarly, the permeabilities measured for mannitol and L-glucose appear to represent the passive component of transport available to a molecule of a size, partition coefficient, and hydrogen bonding capability similar to those of glucose (10).

While similar results were obtained with both diffusion cell systems, the diffusion cell designed in our laboratory incorporates several novel attributes useful for permeability screening of drug substances. For example, the Ussing chamber is generally constructed of two or three different materials, including the glass reservoir, acrylic cell, and connecting tubing. In early experiments conducted in this

Table III. Changes in Permeability over Time for an Actively Transported Molecule, D-Glucose, with and Without Inhibitors

Drug	Time (min)	Permeability (cm/sec $\times 10^{-6}$)	*N* (experiments/animals)
D-Glucose*	<120	14.99 (2.02)[a]	32/9
D-Glucose	>120	9.78 (1.64)	26/9
D-Glucose + 1.0 m*M* phloridzin	<120	6.77 (0.95)	12/4
D-Glucose + 1.0 m*M* phloridzin	>120	5.40 (1.11)	12/4
D-Glucose + 0.2 m*M* ouabain	<120	8.20 (0.45)	12/4
D-Glucose + 0.2 m*M* ouabain	>120	6.05 (0.89)	6/2

[a] Number in parentheses represents the standard error of the mean.
* Differences between D-Glucose permeability measurements pre- and post 120 min were significant to $P \leq 0.0005$.

laboratory, HPLC procedures were used as the analytical method for permeability determinations. Unidentified peaks were traced to leachables (presumably monomers) from the connecting tubing used in the Ussing chamber apparatus. Testing of tubing material lead to selection of a suitable medical grade which eliminated this problem. Yet it was still impossible to conduct permeability studies with very lipophilic compounds such as progesterone in that system, due to the large degree of drug adsorption to the tubing. The newer cell design was fabricated to have only one material type (acrylic) in contact with the drug-containing fluids. This also decreases fluid loss from the diffusion cells due to leakage since there are no connections between the tissue compartment and the reservoir.

Another improvement incorporated in this system was a fluid flow parallel to the tissue surfaces, as it would be *in vivo*. In general, very little attention has been devoted to the rate of flow in diffusion chambers with respect to erosion of tissue surfaces, diffusion layers, available oxygen, and mixing. In the newer cell design, the bubble size of the gas lift which circulates the fluid has been decreased in order to provide much finer and reproducible control of this parameter and allows investigation of these variables in a controlled manner. These parameters are currently being investigated in this laboratory. The Ussing chamber design can be arranged so that its fluid flows directly toward the tissue surface or somewhat parallel to it but causes flow with eddy formation from the bottom of the cell. This can be demonstrated with dye circulation studies. The latter configuration was used in our Ussing chamber studies and can be obtained by crossing the connecting tubes.

The relationship between the surface area of the tissue and the volumes of fluid in the bathing medium is critical to quantifying low permeabilities, especially with relatively insoluble substances. Compared to the 10-ml volume of the Ussing chamber, the newer system uses a 7-ml reservoir. In addition, the surface area has been doubled by making the tissue opening an oblong strip of tissue instead of a circular segment. Tissues from several different species (dog, rabbit, monkey, and minipig) have been successfully mounted, including various segments, i.e., duodenum, ileum, jejunum, colon, and stomach. The cell can be manufactured with various size openings, from less than 0.5-cm^2 surface area to a total of over 4 cm^2, without affecting the flow characteristics of the device. This allows the mounting of intestine from other species, such as rat and mouse, as well as variations of tissue surface area and residual volume. In addition, various other tissues, such as buccal and nasal, have also been mounted.

Finally, from a more practical standpoint, the temperature of these units is easier to control, since a group of six cells is heated together, and a greater surface of the device is actually heated when compared to the Ussing chamber, which heats only the fluid reservoir and not the diffusion cell itself. Cleaning of the cells is also more rapid and efficient, since they can easily be removed from the heating block and all of the surfaces which contact the tissue or bathing fluids can be soaked in a sonic bath. This is not practical with the Ussing chamber since it requires separation of the reservoirs from the heating/circulating bath.

REFERENCES

1. H. H. Ussing and K. Zerahn. *Acta Physiol. Scand.* 23:110–127 (1951).
2. G. S. Schultz and R. Zalusky. *J. Gen. Physiol.* 49:567–584 (1964).
3. W. S. Marshall and S. D. Klyce. *J. Membr. Biol.* 54:302–312 (1972).
4. T. E. Machen, D. Erlij, and F. B. P. Wooding. *J. Cell Biol.* 54:302–312 (1972).
5. M. J. Jackson. In L. R. Johnson (ed.), *Physiology of the Gastrointestinal Tract, Vol. 2,* Raven Press, New York, 1987, pp. 1597–1621.
6. M. Heyman, R. Ducrol, J. F. Desjeux, and J. L. Morgat. *Am J. Physiol.* 242:G558–G564 (1982).
7. M. Heyman, A. M. Dumontrier, and J. F. Desjeux. *Am. J. Physiol.* 238:G326–G331 (1980).
8. D. J. Keljo and J. R. Hamilton. *Am. J. Physiol.* 244:G637–G644 (1983).
9. G. Barnett, T. Lockwood, A. Arancbia, and L. Z. Benet. *J. Pharm. Sci.* 67:224 (1978).
10. J. S. Fordtran, F. C. Rector, M. F. Ewton, N. Sorter, and J. Kinney. *J. Clin. Invest.* 44:1935–1944 (1965).
11. M. Field, D. Fromm, and I. McColl. *Am. J. Physiol.* 220:1388–1396 (1971).
12. C. L. Corbett, P. E. T. Isaacs. A. K. Riley, and L. A. Turnberg. *Gut* 18:136–140 (1977).
13. B. Z. Ginzburg and J. Hogg. *J. Theor. Biol.* 14:316–322 (1967).
14. W. S. Rehm. In F. Brommer and A. Kleinzeller (eds.), *Current Topics in Membranes and Transport, Vol. 7,* Academic Press, New York, pp. 217–27017; P. K. Crane. In C. F. Code (ed.), *Handbook of Physiology, Vol. III/6. Alimentary Canal,* American Physiological Society, Washington, D.C., 1968, pp. 1323–1358.
15. K. Takaori, J. Burton, and M. Dinowitz. *Biochem. Biophys Res Comm.* 137(2):682 (1986).
16. D. F. Diedrich. *Arch. Biochem. Biophys.* 117:248–256 (1966).
17. S. G. Schultz and R. Zalusky. *J. Gen. Physiol.* 47:1043–1059 (1964).
18. W. F. Caspary and R. K. Crane. *Biochim. Biophys. Acta* 211:244 (1968).
19. R. K. Crane. *Physiol. Rev.* 40:789 (1960).