Everted Rat Intestinal Sacs as an *In Vitro* Model for Assessing Absorptivity of New Drugs

Z. T. CHOWHAN ˣ and A. A. AMARO

Abstract □ An *in vitro* model that utilizes everted rat intestinal sacs was evaluated for assessing the absorptivity of several analogs of potential drug substances prior to formulation work and clinical trials. This model not only is a useful qualitative tool for assessing absorptivity of structurally related compounds but also yields some insight into the process involved in drug absorption. Notwithstanding the complexities involved in the absorption processes, the data support the hypothesis that the absorption of organic electrolytes mainly takes place by the partitioning of the unionized species into the lipoidal membranes and then diffusion.

Keyphrases □ Absorptivity, GI—assessed for analogs of potential drug substances using everted rat intestinal sacs □ GI absorptivity—assessed for analogs of potential drug substances using everted rat intestinal sacs □ Models, absorption—everted rat intestinal sacs, GI absorptivity assessed for analogs of potential drug substances

In the development of dosage forms and dosage regimens for new drug entities prior to formulation work and clinical trials, it is important to evaluate the absorptivity of several analogs of the potential drug substances. For this assessment, several simple, relatively rapid, inexpensive, and reproducible screening methods have been used. Since the drug must dissolve and then permeate through GI membranes, both these parameters should be evaluated.

BACKGROUND

For permeability studies, an *in vitro* procedure (1) utilizing everted rat intestinal sacs was valuable (2) in predicting the permeability characteristics of various drug classes. An interesting application of this technique was the study of dosage form variables and the effects of excipients and additives on drug permeation (3–5). This model was used to study the effect of complex formation on drug absorption (6). The effects of physiological (7) and nonphysiological (8) surfactants on drug transport also were investigated using this *in vitro* model. With this same method, the existence of several drug metabolizing enzyme systems in the intestine, which play an important role in drug absorption, was established (9–12). The transfer of salicylic acid across everted rat intestinal sacs was decreased by the presence of potassium and several electrolytes and sugars, such as glucose (13, 14).

The everted rat intestinal sac method has been used for the mechanistic understanding of absorption. The introduction of the pH–partition hypothesis (15–19) stimulated interest in the mechanism of drug absorption. Several studies considered the question of active and passive transport (6, 20–22). The interrelationships among the drug absorption rate, the dissociation constant (pKa), and the absorption site pH is known as the pH–partition hypothesis (15). This hypothesis states that the passage of drugs across the GI barrier is restricted to the undissociated drug form; therefore, the absorption rate depends on the proportion of drug present in this form. In other words, the absorption rate depends not only on the lipid solubility of the drug but on its degree of ionization; the latter, in turn, is a function of the pKa and pH of the absorbing surface.

The validity of the pH–partition hypothesis was tested using the everted rat intestinal sac method. The absorption of flufenamic acid through the everted rat intestine (21) was in agreement with the pH–partition hypothesis. Partial agreement with the pH–partition hypothesis was reported in studies using barbituric acid derivatives and the *in situ* rat small intestine (23). With the pH higher than the pKa, absorption was decreased according to the fraction of unionized form computed from the pKa, indicating that the unionized form was preferentially absorbed. But, at the acidic range, where no significant difference is considered in the fraction of the unionized moiety, the absorption rate of all derivatives studied decreased gradually with the decrease in the perfusion fluid pH.

In vitro and *in situ* absorption experiments using nalidixic acid (24) supported the pH–partition hypothesis; nalidixic acid was absorbed more slowly at a pH yielding a low partition coefficient where the drug was ionized considerably. Phenylbutazone permeation through the everted rat intestine did not obey the pH–partition hypothesis. The noncompliance with the pH–partition theory was described in terms of the general theory of pH effects (25).

This investigation analyzed the usefulness and limitations of the everted rat intestinal sac method as a model for the assessment of absorptivity of new drug analogs. Several complicating factors such as metabolism before and during absorption, accumulation in the intestinal membranes, and the influence of the pH of the perfusing solution on drug permeation, metabolism, and accumulation were considered. The results indicate that this model is a useful qualitative tool for the assessment of absorptivity across GI membranes during early phases of the drug development program.

EXPERIMENTAL

Materials—The following compounds were used as tracers containing tritium: sodium 7-methylsulfinyl-2-xanthonecarboxylate (I), sodium 7-methylthio-2-xanthonecarboxylate (II), sodium 7-isopropoxy-2-xanthonecarboxylate (III), 5-isopropoxy-7-methylthio-2-xanthonecarboxylic acid (IV), methyl 5-isopropoxy-7-methylthio-2-xanthonecarboxylate (V), and *d*-2-(6′-methoxy-2′-naphthyl)propionic acid (VI). The radiochemical purity of these compounds was at least 95% and the chemical purity was 99%.

Sodium chloride[1], potassium chloride[1], calcium chloride[2], anhydrous dextrose[1], anhydrous dibasic sodium phosphate[3], monobasic sodium phosphate[3], citric acid[3], acetic acid[4], and sodium acetate[1] were analytical reagent grade. 1,4-Bis[2-(4-methyl-5-phenyloxazolyl)]benzene[5], 2,5-diphenyloxazole[5], naphthalene[1], 2,6-bis(*tert*-butyl)-4-methylphenol[6], reagent grade methanol, spectrograde dioxane[3], and scintillation grade toluene[2] were used in the scintillation cocktail.

Everted Intestinal Sac Preparation—Young male Sprague–Dawley rats[7], 200 ± 35 g, were used. Food was removed 18 hr prior to use, but water was allowed *ad libitum*. When the rats were sacrificed, the large and small intestines were immediately removed *via* a midline incision of the abdomen and placed in a normal saline solution. Any one of the four intestinal sections was examined from each rat.

For each section, 9-cm segments were cut, sleeved onto a glass rod, everted, and washed in normal saline. The segment was attached at the proximal end of the hose adapter with a silk thread (000), trimmed to 7.5 cm, and ligated at the distal end so that 6.5 cm of the everted sac would be exposed to the mucosal solution. An 8-g stainless steel weight was attached to the ligated end of the sac to maintain it in a vertical position during the experiment.

Permeation Studies—The permeation apparatus (Fig. 1) was a modification of an apparatus described previously (2). It consisted of a 50-ml culture tube with a plastic cap. The tube, containing 40 ml of the buffer solution with the drug, was maintained at 37 ± 1° in a constant-temperature water bath. A mixture of oxygen–carbon dioxide (95:5) was bubbled continuously through an 18-gauge blunt needle. At zero time, the everted sac and assembly were placed in the culture tube.

The serosal side of the sac was filled with the same buffer solution (approximately 2 ml) without the drug, with a fluid level always visible in the vinyl polymer tubing[8]. At 10-min intervals, the solution from the

[1] J. T. Baker Chemical Co., Phillipsburg, NJ 08865.
[2] Mallinckrodt Chemical Works, St. Louis, MO 63147.
[3] Matheson, Coleman & Bell, Norwood, OH 45212.
[4] E. I. du Pont de Nemours & Co., Wilmington, Del.
[5] Arapahoe Chemical, Boulder, Colo.
[6] Aldrich Chemical Co., Milwaukee, WI 53210.
[7] Simonsen Laboratories, Gilroy, CA 95020.
[8] Tygon.

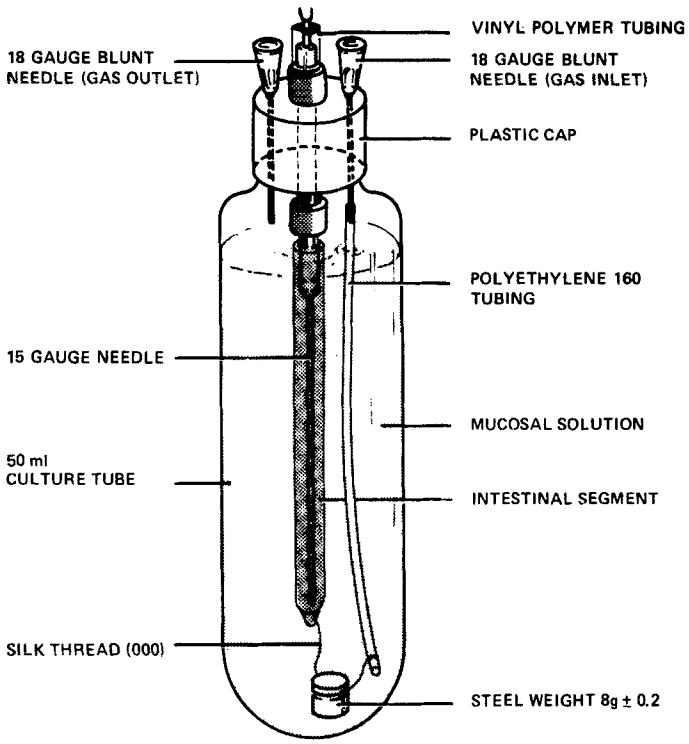

Figure 1—*Schematic representation of the permeation apparatus used in everted rat intestinal sac studies.*

intestinal sac was withdrawn using a hypodermic syringe attached to a long 15-gauge needle. The serosal side of the sac was rinsed by adding 1 ml of fresh buffer solution.

The sample from the serosal side and the rinse were combined. The mucosal side was sampled using a 50-μl disposable pipet. The samples were assayed for radioactivity using a liquid scintillation counter[9]. The composition of the scintillation cocktail was 1 liter of toluene, 1 liter of dioxane, 600 ml of methanol, 208 g of naphthalene, 13 g of 2,5-diphenyloxazole, 260 mg of 1,4-bis[2-(4-methyl-5-phenyloxazolyl)]benzene, and 250 mg of 2,6-bis(*tert*-butyl)-4-methylphenol.

Drug Solutions—The drug solutions were made in 0.008 *M* phosphate (pH 8, 7, and 6), citrate–phosphate (pH 5), or acetate (pH 3) buffer. The buffer solution also contained 0.134 *M* NaCl, 0.011 *M* KCl, 0.0001 *M* $CaCl_2$, and 0.02 *M* glucose. The concentration of drug varied depending on the experiment.

TLC—Precoated silica gel F-254 plates[10] were utilized with a developing solvent system of 80 ml of ethyl acetate[2], 80 ml of hexane[2], 20 ml of methanol[3], and 4 ml of acetic acid[1]. This system allowed distinct separation of I (R_f 0.302) from II (R_f 0.671). Aliquots of 50 μl of both the serosal and mucosal solutions were spotted along with the standards of I and II. Following development, the plates were scraped and the samples were assayed for radioactivity.

RESULTS AND DISCUSSION

Plots of the cumulative amount of I transported from the mucosal to the serosal side of the everted rat intestinal sacs prepared from duodenum and jejunum *versus* time are given in Fig. 2. After the initial lag time, these plots were linear, indicating that the transport of drug to the serosal side followed zero-order kinetics. This plotting procedure has been widely used (2–7, 13, 26, 27). The rate of drug transport or drug clearance is calculated from the slope of the linear portion. The lag time is determined by extrapolation of the linear segment to the abscissa.

The flux at steady state and the lag time for I obtained in this fashion are summarized in Table I. These data indicate that in spite of the anatomical and physiological differences in various parts of intestine, the flux is similar. However, the lag time showed a decrease in the order duodenum > jejunum > ileum > colon. A similar flux of I through various parts of the everted rat intestine is in agreement with the absorption of salicylic acid and aniline in the upper, middle, and lower one-third of the *in situ* rat small intestine (17).

[9] Nuclear Chicago, Des Plaines, IL 60018.
[10] Brinkmann.

Table I—Steady-State Flux and Lag Time Determined from the *In Vitro* Transport of I across Everted Rat Gut Segments

Segment	Flux, μg/min	Mean Flux, μg/min	Lag Time, min	Mean Lag Time, min
Duodenum	0.075	0.074	13	12.7
	0.073		12.5	
	0.073		12.5	
Jejunum	0.076	0.079	14	10.5
	0.072		9	
	0.079		8	
	0.090		11	
Ileum	0.082	0.078	3	5.7
	0.080		7	
	0.071		7	
Colon	0.103	0.088	1	5
	0.082		5	
	0.079		9	

To determine if the xanthone analogs might be absorbed by specific processes, the transport of I and II was measured at various concentrations. The flux *versus* initial concentration plots were linear over a wide concentration range (Fig. 3). This failure to find evidence of saturation suggests that the xanthone analogs are absorbed by a passive process.

The comparison of the flux data for five analogs of xanthone (I–V) is given in Table II. Because of the low solubility of V in buffer, the flux was extrapolated to the same initial concentration (10 mg/ml) for comparison. In view of the linear relationship between the flux of I and II and the initial concentration on the mucosal side, the extrapolation appeared reasonable. From the flux data given in Table II, qualitative judgment about the absorptivity of these compounds could be made. In agreement with these qualitative observations, the absorption studies in animals and humans showed that II and III were well absorbed from the GI tract while I was not well absorbed orally. Large variations were seen in the average lag time (Table II), and no relationship between lag time and flux was observed.

The results of the effect of buffer pH on the total flux of I were interesting (Fig. 4). The flux at a pH below the pKa (3.8) was higher; at pH values above the pKa, it was lower. Although these results are consistent with the pH–partition theory, the transport data cannot be explained solely on the basis of the unionized species partitioning into the lipoidal membrane and then permeating.

The effects of lightly shaking the intestinal sacs by means of a wrist-action shaker are also shown in Fig. 4. At lower pH's, the flux increased as a result of shaking. However, at pH 8 the flux was essentially the same,

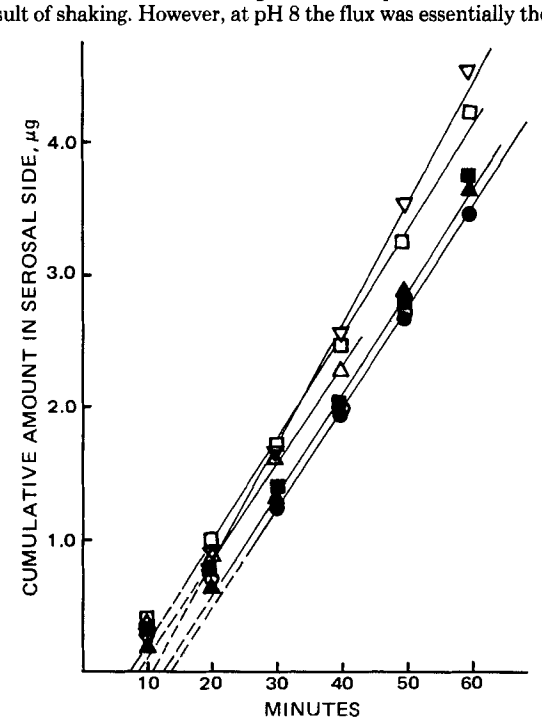

Figure 2—In vitro *transport of I from the mucosal side to the serosal side of everted rat intestinal sacs. Open symbols represent sacs made from jejunum; solid symbols represent sacs made from duodenum. Different symbols represent data points from different rats.*

Table II—Steady-State Flux and Lag Time of Xanthone Analogs at pH 7 and 8 across Everted Rat Intestinal Sacs [a]

Compound	pH 8				pH 7			
	Flux, μg/min	Mean Flux, μg/min	Lag Time, min	Mean Lag Time, min	Flux, μg/min	Mean Flux, μg/min	Lag Time, min	Mean Lag Time, min
I	0.090	0.085	12.5	8.3	0.076	0.079	14	10.5
	0.110		10.5		0.072		9	
	0.055		2.0		0.079		8	
					0.090		11	
II	0.257	0.240	30.0	24.3	0.245	0.233	14.8	14.4
	0.230		23.0		0.230		16.8	
	0.234		20.0		0.225		26.5	
III	0.300	0.290	32.0	25.5	0.300	0.263	24.0	24.3
	0.325		18.5		0.235		28.5	
	0.245		26.0		0.255		30.4	
IV	0.174	0.168	31.5	30.0	0.150	0.131	27.5	31.0
	0.175		25.5		0.135		30.5	
	0.155		33.0		0.108		35.0	
V	—	—	—	—	0.0164	0.020	26.0	22.0
					0.0180		25.0	
					0.0208		20.0	
					0.0256		17.0	

[a] The initial drug concentration was 10 μg/ml.

with or without shaking, suggesting that at this pH there is no effect of shaking on the integrity of the rate-limiting barrier. Since most drug is in the ionized form, there is little effect on diffusion either. This result would at least suggest that the integrity of the rate-limiting barrier is also not being affected at other pH values by the shaking.

The effects of buffer solution pH on the total flux of II and III are illustrated in Fig. 5. These compounds were transported at a faster rate at pH's above the pKa and at a slower rate at pH's close to the pKa. The pKa of the free acid of II, determined by the solubility method at 37° and at 0.1 ionic strength, was 4.4. Shaking the intestinal sacs increased the flux of III but did not alter the general profile. These results are in disagreement with the pH–partition hypothesis.

In view of these results, the flux of another organic acid (VI, pKa = 4.45) with known absorption characteristics (28) at different pH's was investigated (Fig. 6). At pH values higher than the pKa, absorption was not decreased according to the fraction of unionized form computed from the pKa, indicating that the unionized form was not preferentially absorbed. These results disagree with the pH–partition hypothesis. As observed earlier for III, the effect of shaking the intestinal sacs was an overall increase in the flux of VI. This increase in flux caused by diffusion layer effects did not change the overall shape of the pH profile.

The results showing the effect of buffer solution pH on the flux of II, III, and VI were similar to the recent observations of the effect of pH on the permeation of phenylbutazone across the everted rat intestine (5). Lovering and Black (5) attributed the disagreement of their data with the pH–partition hypothesis to diffusion layer effects. They claimed that this discrepancy could be best explained by using a modification of Higuchi's general equation relating pH and the permeation rate (25). The shaking and nonshaking results of this study indicate that, although the diffusion layer plays an important role in permeation studies, the major disagreement with the pH–partition hypothesis cannot be explained only on the basis of diffusion layer effects.

The rates of the transport of flufenamic acid (21) and phenylbutazone (5) at different pH's were obtained under the assumption that the drug transport followed zero-order kinetics. The same procedure was used to obtain the pH profiles given in Figs. 4–6. In treating the data, an assumption was made that the drug concentration on the mucosal side of the everted rat intestinal sacs remained constant during the experimental run. Because of differences in the effective partition coefficient values

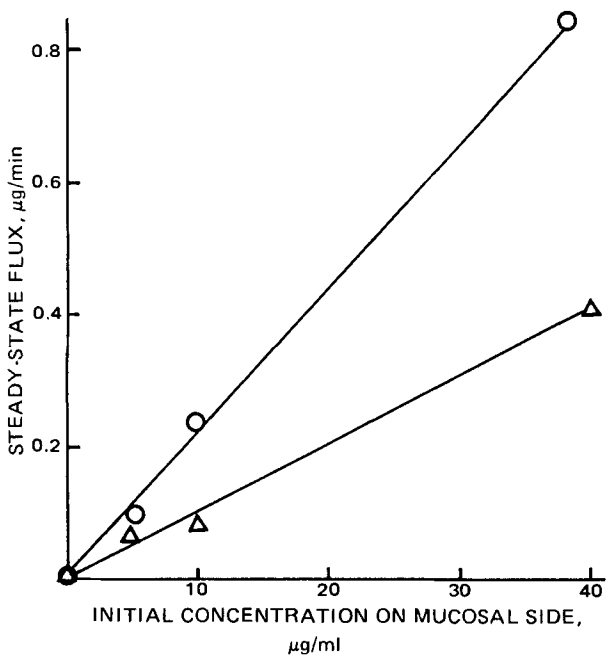

Figure 3—*Steady-state flux across everted rat intestinal sacs as a function of initial concentration. Key: △, I; and ○, II. Each data point represents the mean of at least three rats.*

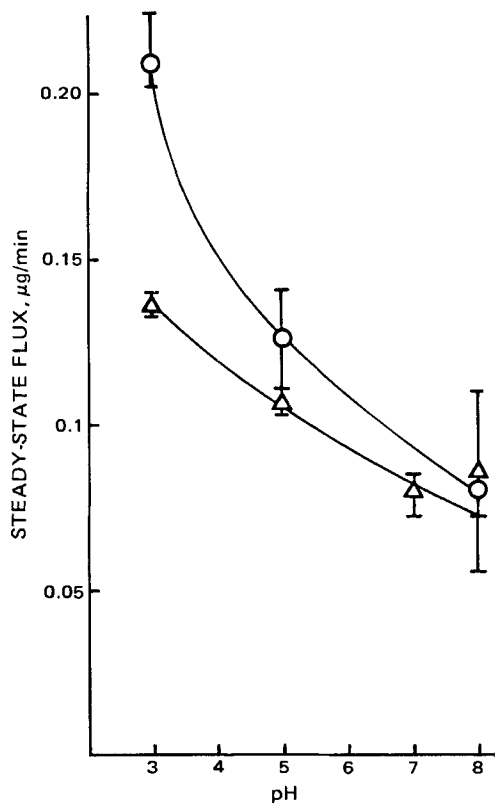

Figure 4—*Effect of pH and shaking on the steady-state flux of I across everted rat intestinal sacs. Each data point represents the mean of at least three rats. Vertical bars represent the range. Key: ○, with shaking, and △, without shaking.*

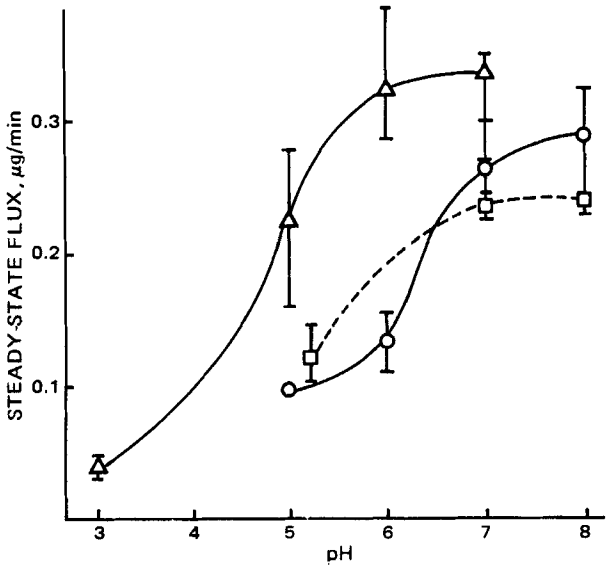

Figure 5—*Effect of pH on the steady-state flux of II and III. Key: △, III with shaking; ○, III without shaking; and □, II without shaking. Each data point represents the mean of at least three rats. Vertical bars represent the range.*

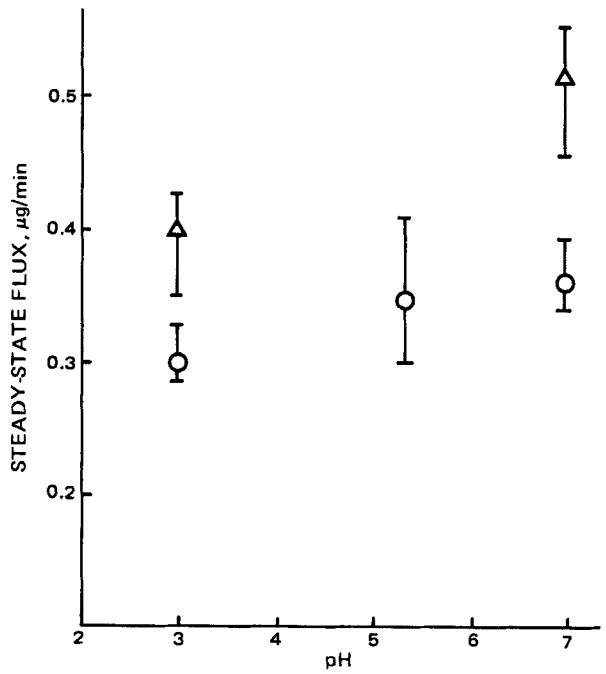

Figure 6—*Effect of pH on the steady-state flux of VI. Key: △, with shaking; and ○, without shaking. Each data point represents the mean of at least three rats. Vertical bars represent the range.*

between the lipoidal membrane and the buffer solutions resulting from the intrinsic partition coefficient difference of structurally related compounds and different degrees of ionization as a result of different pH's of the buffer solutions, drug saturation levels in the membranes could vary from compound to compound and from pH to pH. Under these circumstances, it is conceivable that the drug concentration on the mucosal side may constantly decrease or remain constant after the apparent lag time.

An examination of the amount of I and III accumulated in the everted rat intestinal sac *versus* time at different pH's confirmed that the drug accumulation in the membranes was a function of the effective partition coefficient of the drug, which, in turn, was determined by the degree of ionization. The results in Fig. 7 were obtained by subtracting the mucosal plus serosal amounts in solution from the total drug added. The accumulation of I at pH 8 in the intestinal sacs was very small. The result of decreasing the buffer solution pH was a severalfold increase in the drug accumulation (Fig. 7). At pH 3, approximately 5% drug was accumulated in the membrane during the first 20 min. An additional 4% drug accumulation resulted between 20 and 80 min. The total accumulation of drug at pH 8 was only about 2.5% at the end of 80 min.

The flux data obtained from the cumulative amount *versus* time plots may be quite valid at high pH's. However, no definite conclusion about the validity of the pH–partition hypothesis is possible because of the complications resulting from the degrees of drug saturation in the intestinal membranes and the time to reach a plateau level in the membranes. For III, there was a remarkable difference in the rates and amount of drug accumulation resulting from a relatively small change in pH (Fig. 7). For example, the accumulated amount in the intestinal sacs at the end of 80 min at pH 7 was approximately 16% of the total amount, as compared to 25% accumulation at pH 6. No plateau effect was seen at either pH. At lower pH's, the rate and amount of drug accumulation in the intestinal sacs would be larger because of the higher concentration of unionized species in solution. It should be obvious that the plots of flux *versus* pH given in Figs. 4–6 have very little meaning, and this treatment of the data cannot be explained on the basis of diffusion layer effects as reported previously for phenylbutazone (5).

These data suggest that the cumulative amount of drug transported to the serosal side *versus* time plots only provide qualitative information on the absorptivity of several analogs of potential drug compounds. At constant pH, the rate and amount of drug accumulation in the membranes for several analogs may be completely different because of differences in the effective partition coefficient. Different degrees of drug accumulation in the intestinal sac membranes could also result from differences in the solution concentration of unionized species resulting from changes in the pH.

The experimental design generally used for studying the absorptivity of drugs across the everted rat intestinal sac model is optimally suitable for monitoring the drug transport by sampling the serosal side. The results of this study indicate that, although the transport data obtained by sampling the serosal side appears to follow zero-order kinetics, the concentration in the mucosal solution may vary depending upon the drug and the mucosal solution pH. The data given in Fig. 7 suggest that a nonsteady-state situation in the membranes may exist during the entire experimental run. In view of these findings, it may be important to examine the concentration changes in the mucosal solution.

Figure 8 gives the log of the drug remaining in the mucosal solution *versus* time plots for compounds (II, III, and VI) showing higher drug accumulation in the membrane. These curves suggest that the uptake of drug from the mucosal solution follows a biexponential function. These

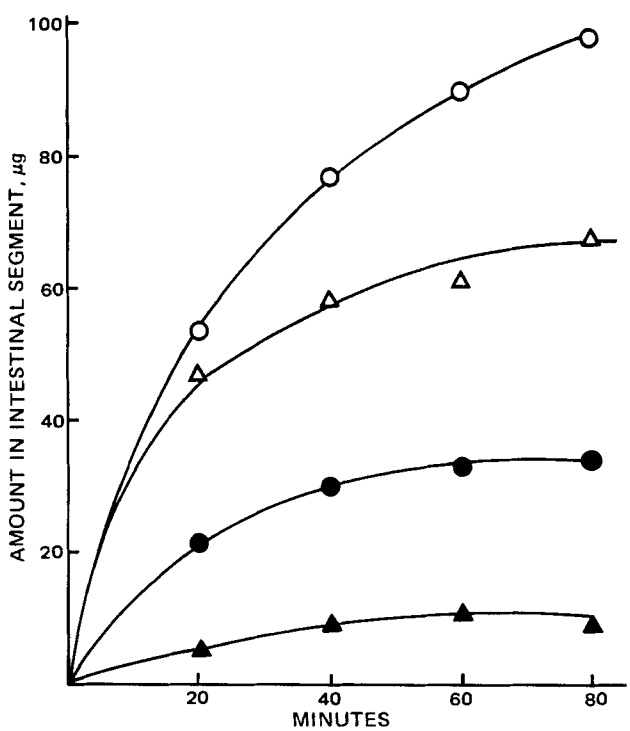

Figure 7—*Accumulation of I and III in the everted intestinal sacs as a function of time at different pH's. Key: ▲, I, pH 8; ●, I, pH 3; △, III, pH 7; and ○, III, pH 6.*

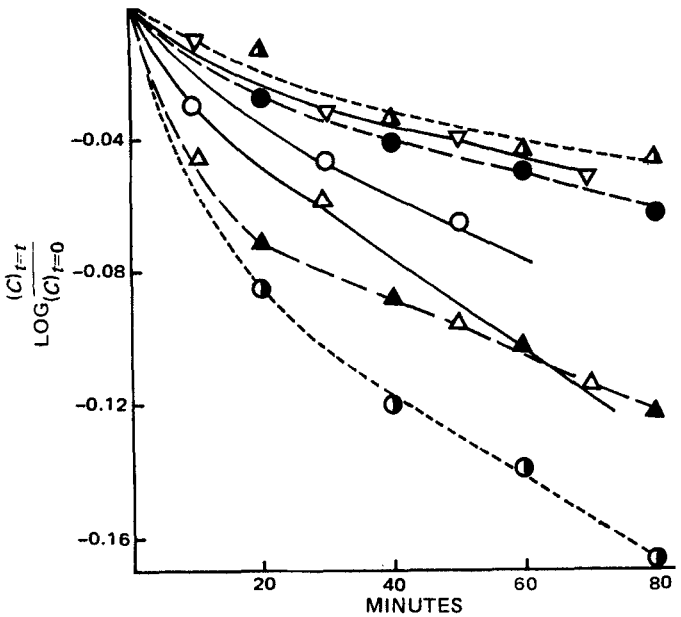

Figure 8—*Semilogarithmic plots of the drug remaining* [$(C)_{t=t}$ = *total drug concentration in the mucosal solution at time* t, *and* $(C)_{t=0}$ = *total drug concentration in the mucosal solution at time zero*] *in the mucosal solution* versus *time at different bulk mucosal solution pH's. Key:* ▽, *III, pH 7;* ○, *III, pH 6;* △, *III, pH 5;* ●, *II, pH 8;* ▲, *II, pH 5.25;* ◭, *VI, pH 7; and* ◑, *VI, pH 3.*

data also indicate higher uptake rates by the intestinal membranes from the mucosal solutions containing a higher concentration of the unionized drug, which is consistent with the pH–partition theory. Since these results cannot be explained solely on the basis of the unionized species partitioning into the lipoidal region of the membranes and diffusing, these compounds may also be transported through the aqueous-filled pores.

The differences seen in the drug transport on the mucosal and serosal sides of the everted sacs cannot be explained on the basis of drug binding to the intestinal tissue. The data given in Fig. 3 suggest that the absorption of these compounds takes place by nonsaturable processes such as passive diffusion. Also, the drug binding in the intestinal tissues would not automatically lead to a zero-order transport on the serosal side. It is possible that the barrier to drug transport in these *in vitro* studies is located in the muscle tissue or in the serosa rather than in the mucosal epithelium.

This idea may not be too surprising if one considers the changes in the functional and structural integrity of the everted intestine. On the basis of histological studies of the everted rat gut preparations, Levine *et al.* (29) reported that, although the intestinal sacs were morphologically intact after eversion, they began to lose structural integrity rapidly after 10–15 min of incubation under conditions normally employed even in the absence of drug. The apparent lack of effect of loss of the structural integrity on everted intestine transfer rates of nonpolar, lipid-soluble compounds was pointed out earlier (13, 30). This suggestion led to the observation (13, 30) that the epithelial border may not be the rate-limiting barrier to transport across the everted rat intestinal sac.

In spite of these complications from the absorption mechanism viewpoint, the everted rat intestinal sac model provides a useful quick screening procedure for assessing the absorptivity of several analogs of potential drugs. It may also provide information related to the rate of drug disappearance from the intestinal lumen, drug accumulation in the intestinal membranes, and metabolism before and during the absorption process. Preliminary studies indicated that I in pH 7 buffer solution, on incubation at 37° for 24 hr with the everted rat intestinal sacs, was completely transformed to II. In light of this observation, it seemed important to investigate the degree of transformation during a permeability run.

When the serosal side samples were analyzed by separating the components by TLC, 82, 81, and 84% of the total drug transported between 0 and 10, 20 and 30, and 50 and 60 min, respectively, was I. A similar analysis of the mucosal side solution at the end of 70 min indicated that 96.8% of the total drug was I. These data indicated that the transformation of I to II was taking place either by the gut microflora or by the intestinal enzymes. Incubation of the everted rat intestinal sacs for 24 hr at 37° with I dissolved in pH 5 buffer solution resulted in no transformation of I to II. A similar TLC analysis of the serosal side solution (pH 7) during the transport of II indicated significant transformation to I by the intestinal lumen. No transformation of III–VI by the everted rat intestine was seen during the *in vitro* transport runs.

In summary, the everted rat intestinal sac model is useful for assessing absorptivity of several analogs of potential new drugs prior to formulation work and clinical trials. This model also provides qualitative information related to the absorption processes including biotransformation before and during absorption through the intestinal membranes. The model does not lend itself to definite conclusions about the mechanism of drug absorption, mainly because of the complicated nature of the processes involved. In spite of the complications involved in treating the transport data, the results of this study suggest that the major species involved in the absorption of organic electrolytes are the unionized species.

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