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Weak-Acid Transport in the Small Intestine: Discrimination in the Lamina Propria

Chung-Yui Tai and Michael J. Jackson

Department of Physiology, George Washington University Medical Center, Washington, D.C. 20037

Summary. Studies on the intestinal transport of weak acids suggest that the subepithelial tissues exhibit a modest, but significant, ability to discriminate between the ionized and nonionized forms. This suggestion has been tested directly in experiments using an *in vitro* preparation of rat small intestine from which the epithelial cells were removed, but in which the structural and functional integrity of the subepithelial tissues was maintained. Studies on the effects of potential difference on the fluxes of weak acids in this preparation showed that the ratio of permeabilities for the ionized and nonionized species (P^i/P^{ni}) was indeed less than one, and of a magnitude comparable to the value suggested by analysis of transport in the intact tissue. (P^i/P^{ni}) for the subepithelial tissue decreased as pH was increased, and the discriminatory properties of the tissue were abolished [$(P^i/P^{ni})=1$] on treatment with the cationic macromolecule polyethyleneimine (PEI). These observations suggested that the discriminatory properties of the subepithelial tissues were determined by fixed anionic sites, and morphological studies with PEI indicated that such sites were concentrated in the region of the basement membrane.

The classical studies of Brodie, Hogben, Schanker and their co-workers [1, 5, 14] established the concept that the wall of the small intestine is more readily penetrated by the nonionized forms of weak acids than by the corresponding anions. It is usually accepted that this discriminatory behavior reflects the permeability properties of the epithelial cell layer, and quantitative analyses of weak-acid transport in the small intestine indicate that the permeabilities of the epithelium for the ionized and nonionized moieties may differ by more than four orders of magnitude [7, 10]. A recent study of weak-acid transport across rat small intestine *in vitro* [7] has suggested that the epithelial layer is not the only locus of discriminatory properties in this tissue, and that the subepithelial tissues may exhibit a small but significant level of discrimination. The present study was undertaken to test this proposal using a preparation of rat small intestine from which the epithelium was removed without disruption of subepithelial tissues. Studies on this preparation demonstrated discriminatory properties in good quantitative agreement with the suggestions of previous work, and provided information concerning the morphological basis of the weak-acid transport system in the small intestine.

Materials and Methods

The procedure used to remove the epithelium from the rat small intestine was modified from that described by Nayak and Benet [13]. Under Nembutal® anesthesia the small intestine of male Sprague-Dawley rats was rinsed with 0.9% saline *in situ* and removed by manually stripping from the mesentery. A segment approximately 20 cm in length was cut from the mid-region, everted on a glass rod, and converted into a sac by placing ligatures at each end. The sac was filled with sufficient ice-cold 0.9% saline to give good distension, and placed in 50 ml of ice-cold 0.1 M EDTA (pH 7.5) in a refrigerator for 45 min with intermittent vigorous shaking. The surface of the sac was rinsed twice with 100-ml volumes of iced 0.9% saline, and the sac was placed in a dish of iced 0.9% saline. The surface of the sac was gently abraded by passing the fingertips along its length in alternating directions. After 20 passes the sac was rinsed in 100 ml of fresh iced 0.9% saline before use.

Transmural unidirectional fluxes of weak acids or other solutes were estimated as described previously [7, 10]. Segments of intact or denuded intestine approximately 3 cm in length were opened lengthwise along a line opposite to the mesenteric insertion, and the resulting sheets of tissue were mounted in a Lucite flux chamber system. Because of the narrow form of the rat small intestine the orifice between the two halves of the chamber was made in the form of a slot 2 cm in length and 0.5 cm wide, so that the exposed area of tissue was 1 cm^2. Thirty milliliters of incubation saline were continuously recirculated through each half of the chamber from reservoirs maintained at 37 °C. To determine a flux 2 μCi of an appropriate ^{14}C labeled tracer were added to

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one reservoir, a period of 20 min was allowed to elapse for the steady state to develop, and the flux estimated from the rate of increase in the quantity of tracer present in the initially unlabeled reservoir. Usually the flux was calculated from the average increase in tracer during three or five consecutive 10-min intervals. It was noted that the increases observed during each individual period did not vary systematically with time, indicating that the system was in a steady state during the period of flux estimation.

In most experiments the transmural electric potential difference (PD) was clamped at a predetermined value. To this end each chamber was fitted with two pairs of saturated KCl bridges in 2% agar. The tips of one pair of the bridges were arranged to lie approximately 0.5 cm from the surfaces of the tissue. These bridges were connected to matched calomel half-cells the output of which was fed to a high impedance voltmeter. The second pair of bridges was inserted so that the tips rested approximately 5 cm from the surfaces of the tissue. The latter bridges were connected to silver silver-chloride half-cells which were used to pass current from an external source. The formula used to calculate the current required to establish the desired PD provided appropriate correction for the resistance of the incubation saline between the tips of the voltage measuring bridges.

The incubation saline used in preliminary experiments was of the following composition in mEquiv/liter: Na, 138; K, 5; Ca, 2.5; Mg, 2.5; Cl, 122; phosphate, 1; HEPES, 25. However, the electric resistance of the preparation incubated in this saline was so low that very large currents had to be passed to establish a significant transmural PD. For this reason the sodium and chloride concentrations were decreased by iso-osmotic replacement with mannitol in most of the experiments using denuded tissue. All chemicals used in this study were of reagent grade. ^{14}C-labeled tracers were obtained from commercial sources and were used without further purification.

Morphological Studies

For structural investigations small blocks of tissue (approx. 1 × 3 mm) were fixed in 2% glutaraldehyde, post-fixed in 1% osmium at 4 °C for 2 hr, dehydrated, and embedded in Araldite. Thick sections (0.5–1 μm) from these blocks were examined in the light microscope after staining with Toluidine Blue, and thin sections were stained with uranyl acetate and lead citrate for examination in the electron microscope.

In some experiments samples of intact intestine were treated with polethyleneimine (PEI; Eastman Kodak Co.) prior to processing for ultrastructural examination using the procedure described by Schurer et al. [15]. In these experiments segments of tissue approximately 2 cm in length were incubated in 0.5% PEI in phosphate-free incubation saline for 30 min at room temperature. The tissue was then given three 10-min washes in 0.2 M cacodylate buffer (pH = 7.5) before processing for electron-microscopic examination as described above.

Results

Properties of Denuded Intestine

Figure 1 compares the structure of the intact rat small intestine with that of tissue treated with ice-cold EDTA. The figure shows that the EDTA treatment resulted in complete removal of epithelial cells from both villus and crypt areas, but the structure of the subepithelial tissues was remarkably well-preserved and the villiform organization was maintained. Fig. 2 is an electron micrograph of the denuded tissue taken at the edge of a villus core. Characteristically the edge of the tissue was marked by a continuous dark band suggesting that the basement membrane remained intact when the epithelium was removed.

Table 1 compares some of the functional properties of the intact and denuded preparations. The table shows that removal of the epithelium abolished the spontaneous transmural PD and decreased the electric resistance of the tissue. The Table also shows that removal of the epithelium markedly modified the transport of the weak acids. In the intact tissue the mucosal to serosal (*m* to *s*) flux of a weak acid was always significantly larger than the corresponding *s* to *m* flux, and the flux ratio exhibited a biphasic relation with pK_a, achieving a maximum with the heterocyclic acid, 5:5-dimethyloxazolidene, 2:4-dione (DMO, $pK_a = 6.1$). In contrast the flux ratios of

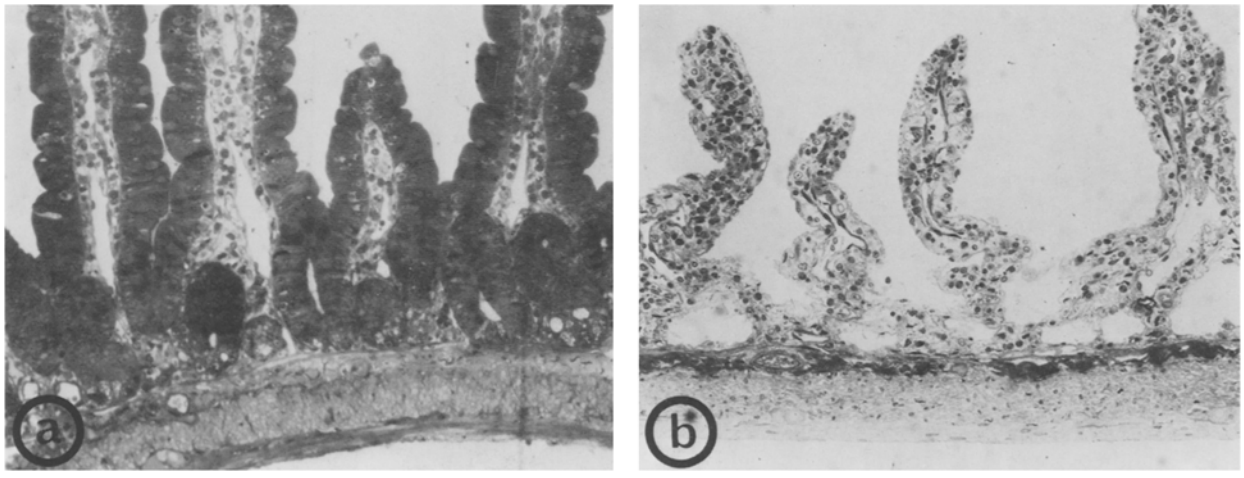

Fig. 1. Comparison of structure in intact (*a*) and denuded (*b*) preparations of rat small intestine (400 ×)

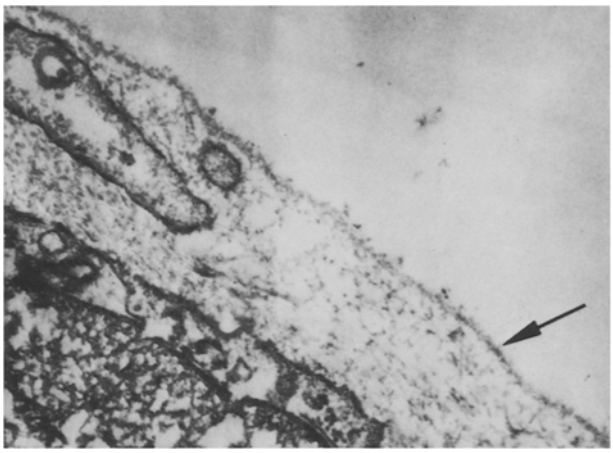

Fig. 2. Electron micrograph of denuded preparation taken at edge of villus core. The arrow indicates the basement membrane (30,000×)

all of the acids tested in the denuded tissue were not significantly different than unity.

The decrease in the tissue resistance associated with removal of the epithelial layer was of some interest because in subsequent experiments it was necessary to clamp the PD across the tissue at values significantly different than zero. The low value of resistance of the preparation incubated in the unmodified saline indicated that very large electric currents would be required to establish a useful range of values for transmural PD. For this reason a second series of comparative experiments was carried out using an incubation saline in which part of the sodium chloride was replaced by iso-osmotic substitution with mannitol. The concentrations of sodium and chloride in this modified saline were 50 and 34 mEquiv/liter, respectively. The results of experiments in this saline are shown in the lower part of Table 1. The decreased ionic strength of the mannitol-substituted saline was associated with a marked increase in the electric resistance of the preparation such that a useful range of values for transmural PD could be established by application of currents in an acceptable range of values. It was noted that the modification to the composition of the saline was associated with some change in the fluxes of the weak acids. In particular, the flux ratios observed in the intact tissue were usually smaller when the tissue was incubated in the mannitol-substituted saline than those seen using the unmodified saline. However, the general pattern of transport was not altered when the concentrations of sodium and chloride were decreased, and the flux ratio continued to exhibit a maximum with DMO. It will be shown subsequently that these changes in the flux ratios do not reflect alterations in the permeability properties of the tissue. In the denuded preparation the decrease in the concentrations of sodium and chloride was associated with statistically significant changes in the fluxes of only one acid, phenobarbital. The reason for the increases in the fluxes of this acid is not known, but the results of these experiments indicate that the modification to the composition of the saline was not associated with a general increase in the permeability of the preparation for weak acids. Because the mannitol-substituted saline effected useful changes in the electrical properties of the tissue without markedly modifying its weak-acid transport properties, the modified saline was used in all subsequent experiments.

Table 1. Functional properties of intact and denuded preparations of rat small intestine

	Intact tissue					Denuded tissue				
	PD	R	J_{ms}	J_{sm}	J_{ms}/J_{sm}	PD	R	J_{ms}	J_{sm}	J_{ms}/J_{sm}
I. Unmodified saline (Na = 138 mEquiv/liter										
Salicylic			53± 6	20±7	2.65			119±12	144±13	0.83
Benzoic			210±13	46±8	4.57			147±27	151±14	0.97
DMO	5.3±0.4	57± 4	132± 3	33±2	4.00	0.0 ±0.01	13±1	139±11	133± 6	1.05
Phenobarbital			98±11	35±3	2.80			127± 9	125±13	1.02
Pentobarbital			101± 9	75±5	1.35			218±20	221±17	0.99
II. Mannitol-substituted saline (Na = 50 mEquiv/liter)										
Salicylic			68± 6	31±3	2.19			151±12	150±16	1.00
Benzoic			136±10	40±3	3.40			139±10	152± 7	0.91
DMO	2.2±0.4	140±11	102± 4	28±2	3.64	0.04±0.02	45±3	160±11	157± 9	1.02
Phenobarbital			64± 8	36±7	1.78			184±11	175±10	1.05
Pentobarbital			105±12	71±6	1.48			212±13	196±11	1.08

Data are means of five experiments ±SEM.
Units as follows: PD, mV; R, $\Omega\,cm^2$; fluxes, nmoles $cm^{-2}\,hr^{-1}$.

Discriminatory Properties of Denuded Tissue

It is shown in the Appendix that the influence of PD on the flux of a weak acid in a simple barrier may be described by an expression of the following form:

$$\frac{J^{\psi}}{J^{o}}=\frac{(P^{i}/P^{ni})\,10^{\alpha}\,\xi}{1+(P^{i}/P^{ni})\,10^{\alpha}}+\frac{1}{1+(P^{i}/P^{ni})\,10^{\alpha}}. \quad (1)$$

In this expression J^{ψ} is the flux of a weak acid observed at a value of PD $=\psi$, and J^{o} is the corresponding flux observed in the absence of a PD; P^{i} and P^{ni} are the permeabilities of the barrier for the ionized and nonionized forms of the acid, and the ratio (P^{i}/P^{ni}) provides a quantitative estimate of the ability of the barrier to discriminate between the ionized and nonionized species; $\alpha=\mathrm{pH}-\mathrm{pK}_{a}$ and reflects the degree of ionization of the acid in the conditions of the system; and $\xi=[zF\psi/RT(1-\exp\{-zF\psi/RT\})]$ in which z, F, R and T have the usual electrochemical significance.

Eq. (1) suggests that a plot of (J^{ψ}/J^{o}) *vs.* ξ should give a straight line with a characteristic slope and intercept which are determined by the discriminatory properties of the barrier and the degree of ionization of the acid. Examination of the equation shows that the sum of the slope and the intercept should be equal to unity and this, together with the linearity of the relation, provides a test of the suitability of the model upon which the analysis is based.

Figure 3 shows the relation between the *m* to *s* fluxes of two representative weak acids and PD in the denuded tissue plotted in the format suggested by Eq. (1). The statistical analysis of these data and those obtained in similar studies on other weak acids is given in Table 2. Examination of these data indicate that the relations between (J^{ψ}/J^{o}) and ξ was well approximated by straight lines for all of the acids tested, and statistically significant correlations were observed ($P<0.001$ in all cases). It was also observed that the sum of the slope and the intercept was close to unity in each case, although the values of these variables changed from one acid to another. In the cases of the strongest acids tested, salicylic and benzoic acids (pK_{a} values 3.0 and 4.2, respectively) the intercept was not significantly different than zero, but significant positive intercepts were observed for the other three acids.

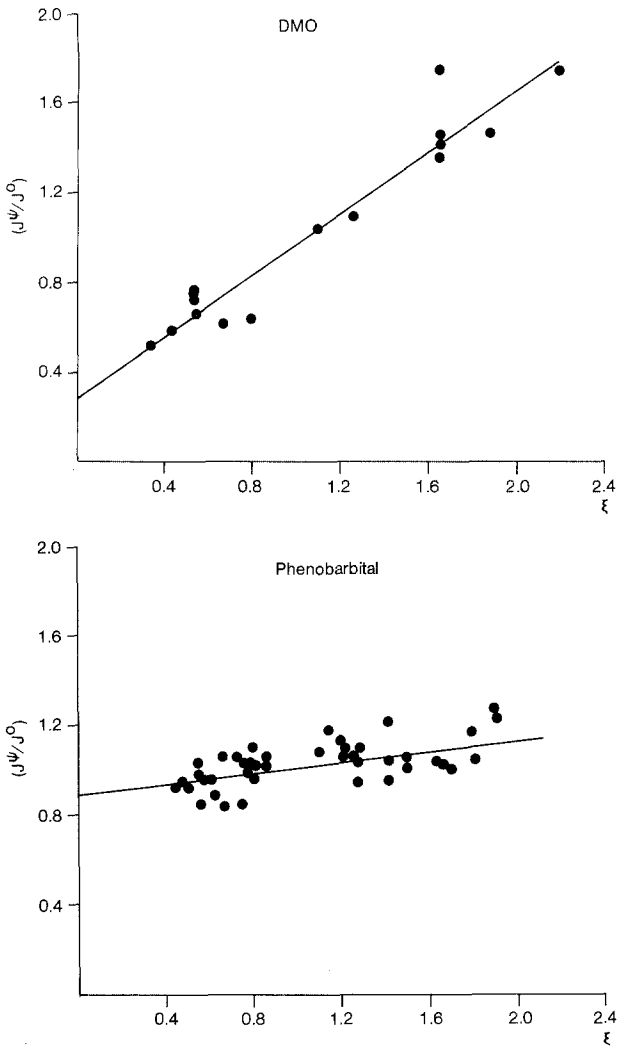

Fig. 3. Influence of PD on fluxes of representative weak acids in denuded rat intestine. The rationale for the format used in these plots is described in the text

It is shown in the Appendix that estimates of the ratio (P^{i}/P^{ni}) may be calculated from data of the form shown in Fig. 3 using the formula:

$$(P^{i}/P^{ni})=\frac{\text{Slope}}{\text{Intercept}}\times 10^{-\alpha}.$$

Table 2. Regression analysis for relation of (J^{ψ}/J^{o}) *vs.* ξ for weak-acid fluxes in denuded rat intestine

Acid	pK_{a}	Slope	Intercept	n	(Slope+Intercept)	(P^{i}/P^{ni})
Salicylic	3.0	0.98±0.03	0.04±0.05	18	1.02	–
Benzoic	4.2	1.04±0.12	0.00±0.15	18	1.04	–
DMO	6.1	0.69±0.05	0.28±0.06	16	0.97	9.8×10^{-2}
Phenobarbital	7.3	0.12±0.02	0.89±0.03	43	1.01	8.5×10^{-2}
Pentobarbital	8.0	0.07±0.03	0.93±0.03	36	1.00	2.3×10^{-1}

Values of slopes and intercepts are means ±SEM for the number of observations listed under *n*.

Table 3. Effect of pH on estimates of (P^i/P^{ni}) in denuded rat intestine

Acid	pK_a	pH	Slope	Intercept	(P^i/P^{ni})
DMO	6.1	6.5	0.63 ± 0.06	0.42 ± 0.03	5.8×10^{-1}
		7.0	0.67 ± 0.04	0.41 ± 0.04	2.1×10^{-1}
		7.5	0.74 ± 0.04	0.30 ± 0.03	9.8×10^{-2}
		8.5	0.88 ± 0.06	0.09 ± 0.07	3.9×10^{-2}
Phenobarbital	7.3	6.5	0.03 ± 0.10	1.04 ± 0.09	–
		7.5	0.15 ± 0.05	0.81 ± 0.03	1.2×10^{-1}
		8.0	0.30 ± 0.04	0.68 ± 0.05	8.8×10^{-2}
		8.5	0.46 ± 0.05	0.45 ± 0.02	6.4×10^{-2}
Pentobarbital	8.0	6.5	0.07 ± 0.08	0.97 ± 0.10	–
		7.5	0.09 ± 0.03	0.90 ± 0.04	3.2 ± 10^{-1}
		8.5	0.26 ± 0.05	0.74 ± 0.07	1.1×10^{-1}

Clearly when either the slope or the intercept is not significantly different than zero, the formula cannot be used to yield realistic estimates of (P^i/P^{ni}). For this reason the data derived from the studies with salicylic and benzoic acids were not analyzed further, and attention was focused on the three less well ionized acids. Table 2 includes the estimates of (P^i/P^{ni}) calculated from the regression analyses of the plots of J^ψ/J^o *vs.* ξ for these three acids. It was noted that the resulting estimates were always less than unity and indicated that the permeabilities for the nonionized species were usually approximately one order of magnitude greater than that of the corresponding anions.

Table 3 shows the effect of varying pH on the estimates of (P^i/P^{ni}) obtained in the denuded tissue from observations on the fluxes of DMO, phenobarbital, and pentobarbital. In all cases the slopes and intercepts of the lines relating (J^ψ/J^o) and ξ were markedly changed when the pH of the incubation saline was varied in the range 6.5 through 8.5, and in some cases the variations were such as to preclude reliable estimates of (P^i/P^{ni}). For example, at pH 6.5 the slopes of the lines for phenobarbital and pentobarbital became insignificant. However, it was possible to obtain estimates of (P^i/P^{ni}) for at least two values of pH in the case of each of the acids tested, and the resulting data consistently showed that (P^i/P^{ni}) decreased as pH was increased.

Effects of Polyethyleneimine (PEI)

PEI has been shown to bind to fixed anionic sites in biological tissues, and two series of experiments were conducted using this agent. In one series of experiments pieces of intact intestine were incubated in 0.5% PEI in normal saline for 30 min at room temperature. The tissue was then washed and processed for ultrastructural examination. Figure 4 is an electronmicrograph taken from tissue processed in this way. Characteristically, small darkly stained deposits were observed to be scattered through the subepithelial connective tissue, and in many cases the deposits were organized as a regular, evenly spaced array apparently associated with a collagen fiber. Such deposits were observed to be distributed throughout the lamina propria suggesting that the PEI had penetrated the tissue uniformly. However, the deposits were most abundant in the amorphous basement membrane, suggesting that the PEI binding sites were most prevalent in this region.

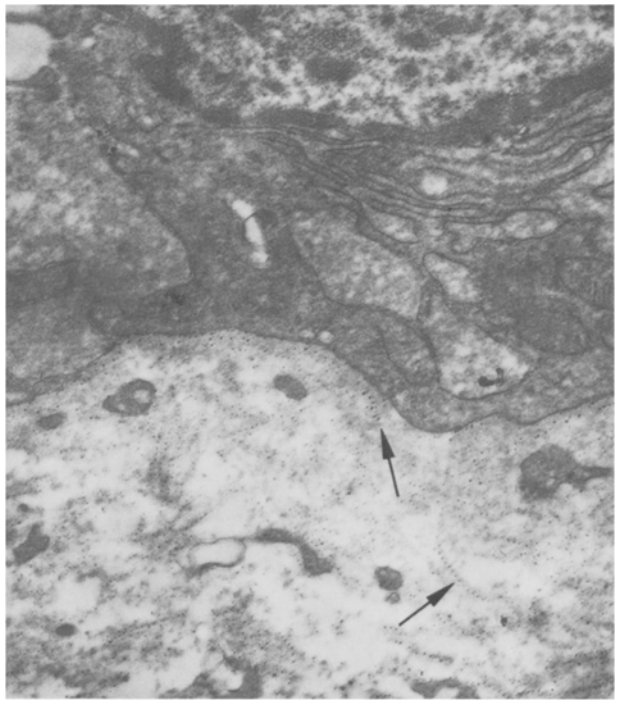

Fig. 4. Electron micrograph of rat intestine treated with PEI *in vitro*. Arrows indicate the small darkly stained deposits referred to in the text (33,000×)

In the second series of experiments 0.5% PEI was added to the incubation saline used in studies on the effects of PD on the fluxes of weak acids through the denuded tissue. The results of these experiments are shown in Table 4. It was found that the presence of PEI increased the slope, and decreased the intercept of the relation between (J^ψ/J^o) and ξ for each of the acids studied. In the case of DMO the changes in the parameters of the relation were so marked as to preclude estimation of a value for (P^i/P^{ni}) in the presence of PEI, but reliable estimates could be made with the other two acids in this condition, and in both cases it was found that PEI increased (P^i/P^{ni}) to a value close to unity.

Table 4. Effect of PEI on estimates of (P^i/P^{ni}) in denuded rat intestine

Acid	pK_a	Condition	Slope	Intercept	n	(P^i/P^{ni})
DMO	6.1	Control	0.68 ± 0.05	0.28 ± 0.06	16	1.0×10^{-1}
		+ PEI	1.03 ± 0.12	-0.03 ± 0.07	18	–
Phenobarbital	7.3	Control	0.13 ± 0.03	0.86 ± 0.04	22	9.5×10^{-2}
		+ PEI	0.64 ± 0.05	0.43 ± 0.06	18	9.4×10^{-1}
Pentobarbital	8.0	Control	0.07 ± 0.03	0.94 ± 0.04	28	2.4×10^{-1}
		+ PEI	0.24 ± 0.07	0.75 ± 0.03	18	1.01×10^{0}

Discussion

The present studies have confirmed earlier work [7, 10] in demonstrating that the rat small intestine incubated *in vitro* may effect net transport of weak acids in the absence of transmural gradients of electrochemical potential. In the earlier work it was proposed that the mechanism for intestinal transport of weak acids could be described in terms of the model system shown in Fig. 5. The system consists of a series arrangement of three aqueous compartments: the mucosal and serosal bulk phases, *M* and *S*, and an intermediate compartment, *X*. Adjacent compartments are separated by the barriers *a* and *b*. Both barriers are considered to be permeable to both the ionized and nonionized forms of a weak acid, but the barriers may discriminate between the two species, and the discriminatory properties of the two barriers may be different. It has been shown [6, 10] that the ratio of the steady-state transmural fluxes of a weak acid in this system is described by an equation of the following form:

$$\frac{J_{ms}}{J_{sm}} = \frac{\left[1+\left(\frac{P^i}{P^{ni}}\right)_a 10^{(pH_m - pK_a)}\right]}{\left[1+\left(\frac{P^i}{P^{ni}}\right)_a 10^{(pK_x - pK_a)}\right]} \frac{\left[1+\left(\frac{P^i}{P^{ni}}\right)_b 10^{(pH_x - pK_a)}\right]}{\left[1+\left(\frac{P^i}{P^{ni}}\right)_b 10^{(pH_m - pK_a)}\right]} \quad (2)$$

where J_{ms} and J_{sm} are the transmural fluxes of a weak acid in the directions indicated by the subscripts; pH_m and pH_x refer to the pH values of the bulk phases and the intermediate compartment, respectively; and the ratios $(P^i/P^{ni})_a$ and $(P^i/P^{ni})_b$ describe the relative permeabilities of the ionized and nonionized species at the two barriers, and provide a quantitative description of the abilities of the barriers to discriminate between the two forms of the weak acid.

In a previous study on the intestinal transport of weak acids [7] it was shown that Eq. (2) provided an excellent fit to the relation between flux ratio and pK_a when a unique set of values for the unknown

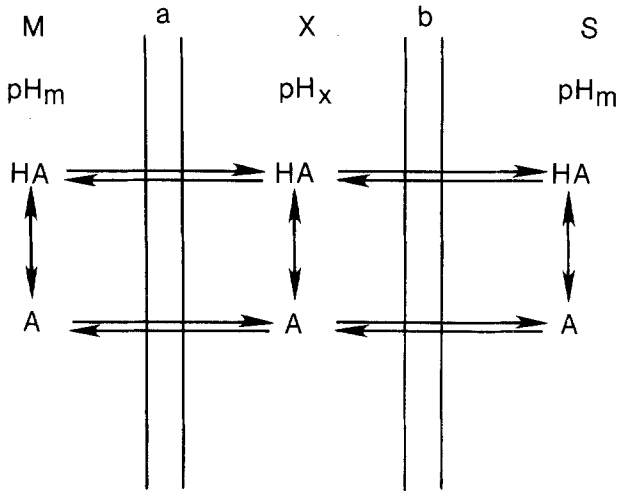

Fig. 5. Three-compartment model for the mechanism of weak-acid transport in rat small intestine

variables were inserted into the equation, and the utility of the equation in this respect was confirmed in the present experiments. Figure 6 is a plot of flux ratio *vs.* pK_a based on the data given in Table 1. The individual data points shown in the figure were taken from Table 1, and the lines connecting these points were calculated from Eq. (2) using an iterative procedure in which the unknown variables were assigned a series of arbitrarily chosen values and examined for fit to the empirical data. As in the previous study [7] it was found that a satisfactory fit to a particular group of data was obtained only when a specific set of values was assigned to the unknown variables. Table 5 compares the critical values for these variables obtained in the previous study [7] with those estimated from the experiments described here. The table shows that the estimates derived from the present study were in good agreement with those obtained previously. The values of pH_x were all within 0.1 unit, and the present study yielded values for the permeability ratios which were within one order of magnitude of the corresponding values obtained in the earlier study. Comparison of the data derived

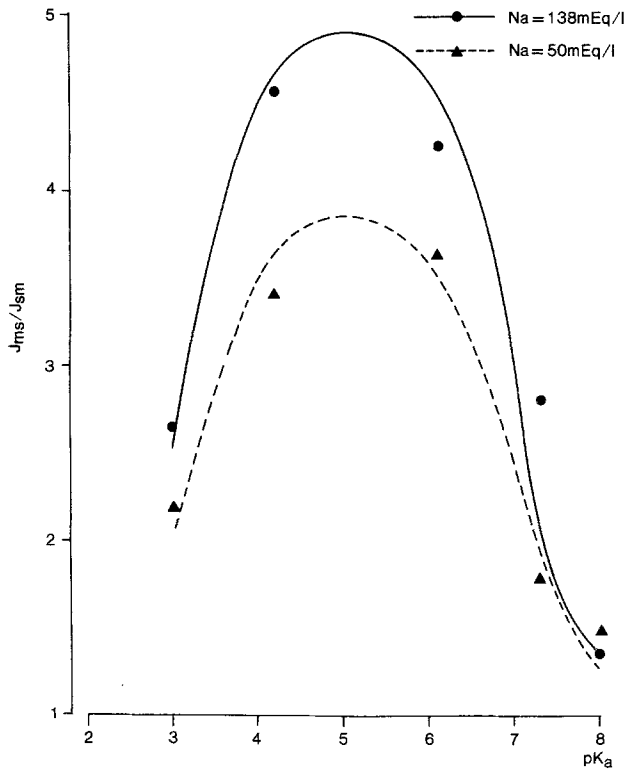

Fig. 6. Variation of flux ratio and pK_a for weak-acid transport in rat small intestine. Individual data points were taken from Table 1 and the continuous lines were calculated from Eq. (2)

from the studies in the unmodified and mannitol-substituted salines in the present experiments indicates that the difference between the flux ratios of weak acids observed in these two conditions may be ascribed in part to a difference in the values of pH_x. The finding that the value of pH_x was decreased in the mannitol-substituted saline is consistent with the previously demonstrated sodium dependence of the serosally directed alkalinization component of intestinal acid-base metabolism [9]. The latter process is considered to be responsible for the maintenance of the high pH of the intermediate compartment which represents the driving force for weak acid transport in this system.

Both the present and earlier studies [7, 10] have shown that barrier *a* of this system discriminates well between the ionized and nonionized forms of weak acids, and it has been suggested that the values of $(P^i/P^{ni})_a$ may reflect the relative permeabilities of the extracellular shunt channels for anions [8], and of the cellular element for nonionized acids [11].

All of the studies listed in Table 5 show that barrier *b* exhibits a modest but significant ability to discriminate between the ionized and nonionized forms of weak acids, and it has been suggested that barrier *b* may be represented by the subepithelial

Table 5. Values for determinants of weak-acid transport in rat small intestine

Source of data	pH_x	$(P^i/P^{ni})_a$	$(P^i/P^{ni})_b$
Previous study [7]	8.1	5.0×10^{-5}	5.0×10^{-1}
Present study: unmodified saline	8.2	1.0×10^{-5}	3.0×10^{-1}
Present study: mannitol-substituted saline	8.1	1.5×10^{-5}	3.0×10^{-1}

connective tissue [7]. The main objective of the present investigation was to examine the discriminatory properties of the subepithelial tissue in the rat small intestine for concurrance with the properties indicated by the analysis of weak acid transport in the intact tissue.

An important question which arises in connection with this study concerns the possibility that the procedure used to remove the epithelial layer may have modified the permeability properties of the subepithelial tissues. Three observations suggest that such modifications were minor or negligible:

(*i*) Only the mucosal surface of the tissue was exposed to EDTA-containing solution during the procedure used to remove the epithelium, and the serosal surface was bathed in a saline solution without EDTA. Observation showed that the EDTA exposure did not itself result in cell loss from the epithelial layer but rather rendered the epithelium susceptible to the subsequent mechanical disruption. Thus the subepithelial tissues were separated from the EDTA solution by the epithelium at all times, and it may be suggested that the exposure of the subepithelial tissues to EDTA was more limited than that of the epithelial layer.

(*ii*) The actions of EDTA on biological tissues are usually considered to be associated with chelation of divalent metal ions, and have been shown to be reversible by replacement of alkaline earth metals [4]. The incubation saline used in the flux studies contained normal concentrations of calcium and magnesium, so that any changes in the properties of the subepithelial tissues associated with exposure to EDTA should have been restored when the tissue was placed in the incubation saline.

(*iii*) Histological and electron-microscopic examination of the denuded tissue did not reveal morphological changes comparable to those described in epithelia, the permeabilities of which have been altered by EDTA treatment [2, 17]. For these reasons we consider that the properties of the denuded preparation used in our experiments reflect those of the subepithelial tissue present in the intact tissue.

The method used in the present experiments for the evaluation of the ratio (P^i/P^{ni}) in the denuded tissue is subject to some important limitations. In particular, in some conditions the flux of one form of a weak acid may be so large as to obscure the flux of the second species and in this situation reliable estimates of (P^i/P^{ni}) cannot be obtained from observations of the effects of PD on the movements of a weak acid. In the case of a poorly discriminatory barrier this condition occurs when the concentrations of the ionized and nonionized species differ by more than one order of magnitude, and for this reason our estimates of (P^i/P^{ni}) in the denuded tissue were confined to a few acids, the pK_a values of which are such that both the ionized and nonionized species contribute significantly to the observed flux in solutions of physiological pH. However, it should be noted that the permeability properties of the subepithelial connective tissues are probably determined chiefly by the diffusive properties of the penetrating species in the aqueous environment included in the fibrous matrix. Such a system may be expected to exhibit a limited degree of selectivity and, in particular, the ratio (P^i/P^{ni}) probably does not vary widely from one weak acid to another [6].

For each of the three acids tested the properties of which allowed reliable estimates to be made, the value of the ratio (P^i/P^{ni}) in the denuded tissue was less than unity and usually close to 1×10^{-1}. Thus these experiments have demonstrated that the subepithelial tissue does indeed exhibit a modest but significant ability to discriminate between the ionized and nonionized forms of weak acids and, in this respect, our experiments have shown that the subepithelial tissue exhibits properties which are quantitatively comparable to those predicted for barrier *b* of the model by the analysis of weak-acid transport in the intact tissue. Accordingly, we consider that the present studies support previous suggestions that barrier *b* of the model is represented by the subepithelial connective tissue in the intestinal system.

It has been suggested that the discriminatory properties of the subepithelial connective tissue may reflect the influence of fixed anionic sites which have been shown to be present on the surface of collagen fibers [3, 15, 16], and which may be expected to retard the diffusion of the ionized forms of weak acids relative to that of their nonionized moieties. Two series of experiments included in the present study support this suggestion. First, the negative site tracer PEI increased the permeability ratios (P^i/P^{ni}) for all of the acids tested in the denuded tissue, and in most experiments the preparation lost its discriminatory character in the presence of this agent. Second, the preparation became more discriminatory [i.e. (P^i/P^{ni}) decreased] as pH was increased. These observations are consistent with the proposal that the discriminatory properties of the denuded tissue are determined by the degree of ionization of acidic sites which are progressively titrated as pH increases. The finding that this titration occurs in the range of pH values 6.5 through 8.5 indicates that the pK_a values of the acidic sites may also lie in this range of values, and suggests that the sites may be phosphate groups.

The ultrastructural study of PEI localization indicated that the anionic sites which determine discrimination in the subepithelial tissue are concentrated in the region of the epithelial basement membrane. Similar localizations for anionic sites have been described in several other tissues [3, 12, 15]. In the context of present studies, this observation identifies the basement membrane as a major locus of the properties characteristic of barrier *b* of the model. Because it is considered probable that barrier *a* of the model reflects the properties of the epithelial layer, the identification of barrier *b* with the basement membrane carries the implication that the intermediate compartment is represented by a space between the epithelium and the basement membrane, and suggests that the lateral intercellular spaces of the epithelium may represent the intermediate compartment in the intestinal system for weak-acid transport.

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Appendix

The objective is to provide a quantitative description for the influence of a PD on the flux of a weak acid. Consider a system consisting of two aqueous compartments, 1 and 2, containing well-stirred solutions of a weak acid, and separated by a barrier which may be penetrated by both the nonionized and anionic forms of the acid. It will be assumed that the movement of the acid through the barrier consists of simple diffusive fluxes of the two forms, and that these fluxes do not interact. Thus the steady-state unidirectional flux of the acid from compartment 1 to compartment 2 can be represented as the sum of the unidirectional fluxes of the ionized (J^i_{12}) and nonionized (J^{ni}_{12}) species:

$$J_{12} = J^{ni}_{12} + J^{i}_{12}.$$

Each of the component fluxes in this expression can be represented as the product of a permeability and a driving force:

$$J^{ni}_{12} = P^{ni}[NI_1]; \quad \text{and} \quad J^{i}_{12} = P^{i}[I_1]\,\xi$$

where P^{ni} and P^i are the permeabilities for the two species; the square brackets denote concentrations; and $\xi = [zF\psi/RT(1 - \exp\{-zF\psi/RT\})]$ in which z is the charge on the anion, ψ is the electric potential difference at the barrier, and F, R and T have the usual electrochemical significance. The concentration terms in these expressions may be substituted using the Henderson-Hasselbalch equation:

$$[NI_1]=\frac{C_1}{1+10^{\alpha_1}}, \quad \text{and} \quad [I_1]=\frac{C_1}{1+10^{-\alpha_1}}$$

in which C_1 refers to the concentration of the weak electrolyte in the compartment of origin of the flux, and $\alpha_1=\mathrm{pH}_1-\mathrm{pK}_a$ and reflects the degree of ionization in that compartment. When these substitutions are made, on simplification and rearrangement we obtain for the flux of the weak acid:

$$J_{12}=\frac{p^{ni}C_1}{1+10^{\alpha_1}}[1+(P^i/P^{ni})10^{\alpha_1}\xi].$$

The form of the electrical term, ξ, is such that $\xi\to 1$ as $\psi\to 0$. Thus the ratio of the unidirectional flux (J^ψ) observed at any particular value of potential difference at the barrier and the unidirectional flux (J^o) observed in the absence of an electric potential difference is given by:

$$\frac{J^\psi}{J^o}=\frac{1+(P^i/P^{ni})10^{\alpha_1}\xi}{1+(P^i/P^{ni})10^{\alpha_1}}.$$

This expression may be expanded to give:

$$\frac{J^\psi}{J^o}=\frac{(P^i/P^{ni})10^{\alpha_1}\xi}{1+(P^i/P^{ni})10^{\alpha_1}}+\frac{1}{1+(P^i/P^{ni})10^{\alpha_1}}. \tag{A1}$$

Equation (A1) shows that the function (J^ψ/J^o) varies with ξ as a straight line. Examination of the equation shows that the slope and intercept of this relation are related, and that the ratio of these parameters is given by

$$\frac{\text{Slope}}{\text{Intercept}}=(P^i/P^{ni})10^{\alpha_1}.$$

Thus when pH, and pK_a are known the ratio (P^i/P^{ni}) can be estimated from the parameters of the line relating (J^ψ/J^o) and ξ.

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