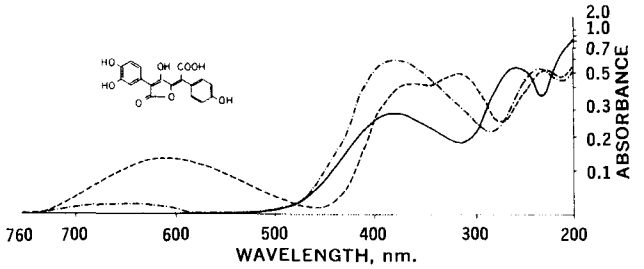

Figure 5—*Xerocomic acid (10.17 mcg./ml.) spectra for pH 2.1 through 5.2 (—), pH 8.5 through 9.3 (- - -), and pH 11.9 (·-·). Analytical wavelength 316 nm.; pKa 6.25 ± 0.05.*

carpophores of both species, and atromentic and xerocomic acids were isolated and identified from the culture mixtures. The tetronic acids were produced equally well when the fungi were grown on a cherry medium and an acidified malt extract medium; the utility of the latter medium was particularly significant for the fermentative production of these compounds, since it offered the advantage of circumventing the isolation problems caused by the anthocyanin pigments in the cherry decoction. Xerocomic acid was the major tetronic acid found in the cultures of both species; the ratios of xerocomic acid to atromentic acid were greater than 200 and approximately 36 for cultures of *P. atrotomentosus* and *P. panuoides*, respectively. An unidentified purple pigment was present in carpophores of both species, and the culture mixtures contained an unidentified red pigment. The observed distribution of pigments in the two *Paxillus* species further confirmed the distinctive metabolic capabilities of carpophores and vegetative mycelium which had been detected initially with *P. atrotomentosus* (3), but any contribution of the two unidentified pigments toward clarifying these metabolic capabilities must await further characterization of the pigments.

The quantitative spectrophotometric studies revealed feasible procedures for determination of atromentic and xerocomic acids in low concentrations (2–24 mcg./ml.) and in mixtures of certain compositions. Application of such quantitative methods, in conjunction with observations on UV and fluorescent properties and on molecular response to electron bombardment, may be useful in future studies to clarify the biologic involvement of tetronic acids and to establish the interrelationship between terphenylquinones and diphenyl-substituted tetronic acids.

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ACKNOWLEDGMENTS AND ADDRESSES

Received April 26, 1971, from the *Drug Plant Laboratory, College of Pharmacy, University of Washington, Seattle, WA 98105*

Accepted for publication June 9, 1971.

Presented to the Pharmacognosy and Natural Products Section, APhA Academy of Pharmaceutical Sciences, San Francisco meeting, March 1971.

Supported in part by National Institutes of Health Research Grant GM 07515-10.

The authors thank David L. Duewer and William N. Howald, Department of Chemistry, University of Washington, for consultation and assistance in preparation of the computerized data for the mass spectral fragmentation patterns. They also extend appreciation to Mrs. Marcella F. San Martini, formerly College of Pharmacy, University of Washington, for technical assistance with the many spectrophotometric analyses required for the determination of ionization constants.

Drug Transfer across Rat Intestinal Musculature after Edetic Acid Treatment

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Abstract □ The columnar epithelium of the *in vitro* rat intestine was separated from the intestinal musculature by treatment with ice-cold edetic acid solution. The *in vitro* absorption kinetics through the denuded intestinal musculature for salicylate ion and acetanilide were followed in pH 7.4 sodium phosphate and potassium phosphate buffers, as well as in an isotonic sodium chloride solution where the effect of rinsing with Mg^{+2} and Ca^{+2} was also studied. Differences in directional transfer rates, as noted in previous studies with the untreated intestinal membrane, were not found. The transfer rates through the muscle layer were found to be at least twice as high as those found in the untreated membrane. As noted previously, nonionized acetanilide transfer rates were still approximately twice those seen for salicylate ion.

Keyphrases □ Drug-transfer rates, *in vitro*—effect of intestinal musculature, rat □ Membrane permeability coefficients—effect of edetic acid treatment □ Absorption kinetics, *in vitro*—salicylate and acetanilide through denuded intestinal musculature, rat □ Edetic acid—treatment of rat intestinal musculature, effect on drug transfer

Recent studies in this laboratory pointed out that drug ions exhibit directional permeability coefficients for passage across the rat intestine *in vitro* (1, 2), with mucosal to serosal transfer occurring at faster rates than serosal to mucosal transfer. It was proposed that the difference in directional permeability coefficients

might be related to a sodium-ion coupled transport, since when sodium ion was completely replaced by potassium in phosphate buffer solutions, the difference in permeability coefficients tended to disappear (1). However, more recently, Benet *et al.* (2) pointed out that salicylate ion also exhibited different directional flux rates in a tromethamine buffer; they suggested that the results seen previously (1) with sodium and potassium phosphate buffers might be better explained in terms of the effects of phosphate on the intestinal mucosa. Nonionized drugs did not show a difference in directional permeability coefficients in either sodium or potassium phosphate buffers.

One difficulty in extrapolating *in vitro* intestinal flux data to the *in vivo* transport of drugs is the fact that the *in vitro* fluxes are measured through the columnar epithelium *plus* the underlying connective and muscle tissue. Therefore, the rate-limiting step for *in vitro* transport might well be the intestinal musculature, a barrier that would not be encountered in *in vivo* transport. The present study was undertaken to determine the effect of the intestinal musculature on the *in vitro* transmural flux of drugs.

Two drugs, salicylate ion and nonionized acetanilide, were studied with respect to their transfer rates across the *in vitro* rat intestine in both directions. The intestine was pretreated with ethylenediaminetetraacetic acid, edetic acid, by the method of Berlin and Hawkins (3), to slough off the mucosal epithelium, leaving the underlying muscle layers intact for absorption studies.

EXPERIMENTAL

Materials—The following USP, NF, or reagent grade chemicals were used: salicylic acid, acetanilide, monobasic sodium phosphate anhydrous, monobasic potassium phosphate anhydrous, dibasic potassium phosphate anhydrous, sodium chloride, ethylenediaminetetraacetic acid, sodium hydroxide, potassium hydroxide, magnesium chloride hexahydrate, calcium chloride dihydrate, and a 100% oxygen gas.

Procedure—The pH 7.4 sodium phosphate buffers and the potassium phosphate buffers were prepared as previously described (1). Isotonic sodium chloride solution at pH 6.6 was prepared in the usual manner. The pH values of the solutions used were routinely checked at the beginning and end of each experiment on a research pH meter[1].

Male Sprague-Dawley rats, weighing 220–250 g., were fasted for 24 hr. prior to the experiment, but water was allowed *ad libitum*. The rats were rendered unconscious with carbon dioxide in an airtight chamber and were then sacrificed by a sharp blow at the base of the skull. The small intestine (from the pyloric junction to about the middle of the jejunum) was removed at once *via* a midline abdominal incision and flushed with 40 ml. of the buffer (warmed) to be used in the absorption study. The first 15-cm. segment was discarded and the next 2–12.5-cm. segments (stretched length using a 20-g. weight) were everted.

The segments of the gut were kept for 45 min. in a refrigerator in an ice-cold 0.1 *M* edetic acid solution of the appropriate buffer. At the end of this period, the segments of the gut were taken out and rinsed with buffer. The disrupted epithelium was then removed by gently moving a finger back and forth over the length of the everted intestinal segment. In alternating studies, the proximal or distal segments were reeverted. The denuded gut segments were then used to study the *in vitro* absorption kinetics using perfusion devices and techniques previously described (1, 2), where each intestinal segment was used for four consecutive experiments with the solutions changed after each run. At appropriate intervals, 0.1-ml. samples were removed from the inner chamber for assay. In the experiments to study the possibility of Mg^{+2} and Ca^{+2} ions reversing the effects of edetic acid treatment, the intestinal segments were rinsed in $MgCl_2$ and $CaCl_2$ solutions (both 0.1 *M* and at pH 6.6) for 5 min. each after the usual treatment with edetic acid.

For salicylate-ion transfer, the time for the experiment was 2 hr. and 20 min. (four 35-min. transport studies with each segment). For acetanilide transfer, however, absorption measurements were made during the following time periods: initial rate, 0–15 min.; rate 2, 35–50 min.; rate 3, 70–85 min.; and rate 4, 125–140 min. Fifteen-minute transfer experiments were necessary in the acetanilide studies to correspond with requirements described previously (1) so that transfer would appear to follow unidirectional zero-order kinetics.

A histological examination was also made to compare an untreated everted intestinal segment with the: (*a*) one kept in ice-cold isotonic NaCl solution (pH 6.6) for 45 min., (*b*) one kept in ice-cold 0.1 *M* edetic acid solution (pH 6.6) for 45 min., and (*c*) one kept in $MgCl_2$ and $CaCl_2$ (both 0.1 *M* and at pH 6.6) solutions after edetic acid treatment. The tissue was fixed in formalin solution, and the sections were stained with hematoxylin and eosin.

Assay Procedures—The 0.1-ml. samples were diluted and assayed as follows. Acetanilide was diluted with 2.0 ml. of the appropriate buffer, and the concentration was determined by UV measurement at 239 nm. on a spectrophotometer[2]; salicylic acid was diluted with 5.0 ml. of 0.1 *N* sodium hydroxide or potassium hydroxide, and the drug concentration was determined on a spectrophotofluorometer[3] at an excitation wavelength of 300 nm. and an emission wavelength of 408 nm. (uncorrected). The choice of diluent depended upon the presence or absence of sodium ion in the environment. Standard curves for each drug were made using five known concentrations of the drug in the appropriate diluent. Blank values were sufficiently low at all times during the sampling period and could be ignored. As in previous experiments (1, 2), no significant difference in transfer rates was observed between the first and second segments of the intestine.

Plots were made of the concentration in the inner chamber *versus* time. Rates of transfer, intercepts, and ratios were obtained by the use of an unweighted least-squares program executed on a desk calculator[4]. By using the everted and noneverted data, the following calculations were made: A, the average everted and noneverted rates and the everted/noneverted ratios for all determinations on a particular drug in a particular buffer; B, the average initial rates (everted and noneverted) and ratios (everted/noneverted), *i.e.*, the first rate measured on each intestinal segment; C, the average increase in rates over the time of the experiment as a percent of the initial rate, *i.e.*, (4th rate/1st rate) × 100; D, the average *X*-intercept or lag time observed with initial rate measurements; and E, the average total amount (milligrams) of a drug transferred across the gut during the time for the experiment (four runs). Differences between everted and noneverted values were tested at 95% or greater confidence levels using Student's test for paired data.

RESULTS AND DISCUSSION

The summary of the data is presented in Table I for salicylate ion and in Table II for nonionized acetanilide according to the format used previously (2). The level of significance for each of the five averages (A–E) used to compare transfer through everted and noneverted segments is also reported in Tables I and II. No significant differences between everted and noneverted transfer rates (at 95% or greater confidence levels) were observed for acetanilide in all three isotonic solutions or for salicylate ion in the isotonic solutions containing sodium. Previous studies (1, 2) showed that significant differences in directional permeability coefficients existed for ionized drugs in the presence of sodium ion.

The values of directional permeability coefficients reported in Tables I and II for both the salicylate ion and the nonionized acetanilide are at least twice as high as those obtained earlier with the untreated gut (2). Previous studies (3–14) reported changes in membrane permeability after treatment with, or in the presence of, edetic acid. In all of these studies, the transfer rates for different

[1] Beckman.

[2] Beckman DB-G.

[3] Aminco-Bowman.

[4] Hewlett-Packard model 9100A.

Table I—Summary of Transfer Data ± Standard Deviation for Salicylate Ion in Three Isotonic Solutions through Rat Intestinal Tissue from Which the Mucosa Was Removed by Treatment with Edetic Acid

	Number	Everted	Noneverted	Ratio	Level of Significance, Everted *versus* Noneverted
		Sodium Phosphate pH 7.4 Buffer			
A[a]	4	4.27 ± 0.74	4.47 ± 0.40	0.96 ± 0.14	N.S.[b]
B	4	3.93 ± 0.50	4.35 ± 0.54	0.92 ± 0.17	N.S.
C	4	117 ± 8	108 ± 19		N.S.
D	4	1.18 ± 0.87	1.21 ± 0.61		N.S.
E	4	8.96 ± 1.61	9.41 ± 1.01	0.95 ± 0.15	N.S.
		Potassium Phosphate pH 7.4 Buffer			
A	4	4.01 ± 0.43	5.33 ± 0.77	0.76 ± 0.12	$p < 0.05$
B	4	3.94 ± 0.42	5.45 ± 0.74	0.74 ± 0.15	$p < 0.05$
C	4	105 ± 8	97 ± 7		N.S.
D	4	1.65 ± 1.06	1.80 ± 1.25		N.S.
E	4	8.35 ± 0.94	11.00 ± 1.51	0.77 ± 0.10	$p < 0.05$
		Isotonic Sodium Chloride pH 6.6			
A	4	4.13 ± 0.78	4.76 ± 1.01	0.88 ± 0.12	N.S.
B	4	3.26 ± 0.80	4.15 ± 1.12	0.81 ± 0.18	N.S.
C	4	153 ± 21	134 ± 16		N.S.
D	4	2.89 ± 0.84	2.16 ± 1.48		N.S.
E	4	8.38 ± 1.51	9.92 ± 2.06	0.86 ± 0.13	N.S.
		Isotonic Sodium Chloride pH 6.6[c]			
A	4	4.92 ± 0.67	5.62 ± 0.63	0.88 ± 0.09	N.S.
B	4	4.58 ± 0.56	5.45 ± 0.94	0.85 ± 0.15	N.S.
C	4	107 ± 28	104 ± 32		N.S.
D	4	6.08 ± 2.46	4.70 ± 1.40		N.S.
E	4	9.34 ± 1.08	11.01 ± 0.65	0.85 ± 0.07	$p < 0.05$

[a] A, average rates—all runs, mcg./ml./min.; B, average initial rates, mcg./ml./min.; C, average rate increase, 4th rate/initial rate as %; D, average lag time for initial runs, min.; and E, average total amounts, mg. [b] Not significant. [c] Intestinal segments rinsed with 0.1 *M* $MgCl_2$ and 0.1 *M* $CaCl_2$, both at pH 6.6.

substances increased except in one case (8), where the uptake of cyanocobalamin was found to be markedly diminished. In a study on the intestinal absorption of pralidoxime ion, 2.5 m*M* edetic acid did not affect the transfer rates of the drug (15).

The effect of edetic acid on intestinal permeability has been related to its removal of bivalent ions from the structure of the membrane (7) and to the loss of adhesiveness of cells (8) due to disruption of the intercellular epithelial bridges. The relationship between membrane permeability and the depletion of bivalent ions from the membrane by chelation with edetic acid is supported by many observations. Feldman and Gibaldi (13) reported a significant increase in the salicylate transfer rate after incubation of the gut in 25 m*M* edetic acid. The effects were greater at pH 7.4 than at pH 6.0, which may be related to increased binding capacity of edetic acid for bivalent ions with increasing pH. The increase in phenol red absorption from the rat intestine observed by Tidball (7), after edetic acid exposure, could be reversed by rinsing the intestine with $CaCl_2$ or $MgCl_2$. It was also pointed out that the calcium salt of edetic acid had no effect on the permeability of the rat intestinal epithelium to phenol red. This was also found to be true in the absorption of heparin (11). These data postulate that edetic acid alters the aqueous permeability of intestinal epithelium by depleting Mg and Ca.

The alteration of permeability after treatment with edetic acid was previously thought to be independent of any mechanical damage to the cell (9) but, more recently, it was shown (10), with the help of electron micrographs, that treatment with edetic acid causes a reversible alteration in the fine structure of the tissue. In this laboratory, the histological examinations of the intestinal segments treated with 0.1 *M* edetic acid revealed that the epithelial layer of the mucosa was completely stripped off, with about 25% of basal cells of the mucosa still remaining. Edema was observed in muscularis mucosa. This edema was absent in the section of the gut rinsed with $MgCl_2$ and $CaCl_2$ solutions after edetic acid treatment. The muscle layer or the serosa was found to be intact after edetic acid treatment when compared with an untreated section.

The present findings, high transfer rates through the muscle layer and no difference in directional transport, are in accord with observations made earlier (4, 6) that the selective and absorptive elements are epithelial in the absorption of various drugs. A more conclusive statement must await further work being carried out in this laboratory where the intestinal musculature will be removed by surgical techniques and transport across the short-circuited epithelial membrane will be followed using radioactive tracers.

The values in Row C of Tables I and II give the average changes in rates observed during each 2-hr. and 20-min. transport experiment. No changes in transport rates were observed, except for the case where salicylate ion was placed in the unbuffered 0.9% sodium chloride solution. Therefore, except for this one unexplained case, the intestinal musculature was able to maintain a relatively constant transfer rate over the time of the experiment, and the transfer rates observed here might be considered the maximum values possible when transport through the *in vitro* intestine is considered.

As was noted in the previous untreated transport studies (2), the nonionized acetanilide yielded lag times (Row D) that were not significantly different than zero (at 95% confidence levels) for all three of the solutions studied. The lag times observed for salicylate ion were significantly different than zero, even though the lag time values found for transport through the intestinal musculature were one-half to one-third those found previously in the intact intestinal segment. As would be expected, from the values reported in Row C, there was little difference between the average initial rates and the average rates for all runs, as reported in Rows B and A. Likewise, since there was no significant difference in directional transport rates, the ratios found using the total amounts transferred (Row E) were almost identical to those found for the average rates (Row A).

As can be seen in Table I, significant differences were found between the everted and noneverted transport rates of salicylate ion in the potassium phosphate buffer. In this buffer, the everted transfer rates were similar to those found in the sodium phosphate and sodium chloride solutions. However, there was a marked increase in the rates observed through the noneverted segments. This was in contrast to the results found for the intact intestinal segments (1), where similar transfer rates were observed across the noneverted intestinal segment in both potassium and sodium phosphate buffers and a slight decrease in the everted transfer rate was found in going from the sodium to potassium phosphate buffers. At the present time, we can offer no explanation for this larger noneverted rate found in the edetic acid-treated segments. However, the potassium phosphate buffer is not causing a time-dependent degradation of the membrane, since the values in Row C are very close to 100%.

Table II—Summary of Transfer Data ± Standard Deviation for Acetanilide in Three Isotonic Solutions through Rat Intestinal Tissue from Which the Mucosa Was Removed by Treatment with Edetic Acid

	Number	Everted	Noneverted	Ratio	Level of Significance, Everted *versus* Noneverted
		Sodium Phosphate pH 7.4 Buffer			
A[a]	4	8.94 ± 0.55	9.47 ± 1.27	0.95 ± 0.09	N.S.[b]
B	4	8.94 ± 1.00	9.73 ± 0.89	0.92 ± 0.07	N.S.
C	4	103 ± 8	93 ± 6		N.S.
D	4	0.43 ± 0.27	0.21 ± 0.52		N.S.
E	4	8.31 ± 0.48	8.42 ± 0.69	0.99 ± 0.07	N.S.
		Potassium Phosphate pH 7.4 Buffer			
A	4	8.82 ± 0.64	8.72 ± 0.68	1.02 ± 0.11	N.S.
B	4	8.80 ± 1.74	8.83 ± 0.69	0.99 ± 0.13	N.S.
C	4	101 ± 19	100 ± 14		N.S.
D	4	0.54 ± 0.58	−0.38 ± 0.83		N.S.
E	4	8.01 ± 0.61	7.98 ± 0.57	1.01 ± 0.11	N.S.
		Isotonic Sodium Chloride pH 6.6			
A	4	8.94 ± 1.29	9.19 ± 0.52	0.97 ± 0.14	N.S.
B	4	8.34 ± 1.87	8.70 ± 0.46	0.97 ± 0.25	N.S.
C	4	112 ± 13	113 ± 9		N.S.
D	4	1.67 ± 2.29	1.71 ± 1.96		N.S.
E	4	8.06 ± 1.38	8.21 ± 0.82	0.98 ± 0.09	N.S,
		Isotonic Sodium Chloride pH 6.6[c]			
A	4	8.16 ± 0.22	8.35 ± 1.07	0.98 ± 0.12	N.S.
B	4	7.73 ± 1.25	8.18 ± 0.98	0.96 ± 0.22	N.S.
C	4	111 ± 17	108 ± 16		N.S.
D	4	0.77 ± 0.43	0.57 ± 0.55		N.S.
E	4	6.87 ± 0.96	7.00 ± 0.80	0.98 ± 0.08	N.S.

[a] See *Footnote a*, Table I. [b] Not significant. [c] See *Footnote c*, Table I.

When the segments were rinsed with $MgCl_2$ and $CaCl_2$ following edetic acid treatment, there was no significant change in acetanilide transport through membranes bathed in isotonic sodium chloride solutions. (See Table II, isotonic sodium chloride values with and without divalent-ion rinsing.) However, initial everted transfer rates of salicylate, as well as the lag times for both everted and noneverted transport, increased significantly (95% confidence levels) following the divalent cation rinses. At the same time, there was no significant change in average rates—all runs—and in total amounts transferred. As was noted earlier in the histological preparations, the muscularis mucosa showed edema following edetic acid treatment, but this edema was absent after the divalent cation rinse. Following the reasoning presented in the previous publication (2), the differences in lag time for salicylate might be explained on the basis of a size change in pores or intracellular channels. The size change could be influenced by the presence or absence of edema. These results in the muscle layer are in contrast to those recently reported by Mayersohn *et al.* (16) for intact intestinal sacs, where transport rates decreased as a result of increased water uptake by the segment. However, this difference may be explained in terms of the rate-limiting step being the epithelial membrane rather than the muscle layer. As previously noted, the acetanilide rates and lag times are unaffected by the presence or lack of edema; thus, this drug would appear to be absorbed through a different route.

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ACKNOWLEDGMENTS AND ADDRESSES

Received October 8, 1970, from the *School of Pharmacy, University of California, San Francisco, CA 94122*

Accepted for publication June 18, 1971.

This investigation was supported by Public Health Service Research Grant 17243 from the National Institute of General Medical Sciences and by a grant-in-aid from the research funds of the Academic Senate, San Francisco Division, University of California.

The authors thank Miss Denise Jamin for valuable technical assistance.