Influence of Various Anions on Intestinal Disappearance of Hexamethonium Chloride and Pralidoxime Chloride in Rats

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Abstract □ The intestinal transfer of two poorly absorbed quaternary ammonium drugs, hexamethonium chloride (I) and pralidoxime chloride (II), in the presence of various organic and inorganic anions was investigated in the rat using a modified *in situ* gut technique. The results were in agreement with those of the conventional *in situ* loop and plasma drug level techniques. Of the anions investigated, cholate, desoxycholate, taurocholate, phoscholate, dehydrocholate, and hyodesoxycholate had the greatest effect on increasing the amount and rate of disappearance of I. Similarly, the amount and rate of disappearance of II were enhanced markedly in the presence of phoscholate and trichloroacetate. The effect of cholate and phoscholate was investigated in detail. The membrane permeability and histological studies indicated that these anions may compromise the structural integrity of the membrane tissue, thus enhancing drug transfer.

Keyphrases □ Hexamethonium chloride—influence of various anions on intestinal disappearance in rats □ Pralidoxime chloride—influence of various anions on intestinal disappearance in rats □ Quaternary ammonium drugs—hexamethonium chloride and pralidoxime chloride, influence of various anions on intestinal disappearance in rats □ Absorption—hexamethonium chloride and pralidoxime chloride, influence of various anions on intestinal disappearance in rats

Quaternary ammonium drugs may have limited therapeutic usefulness following oral administration since they are poorly and erratically absorbed. These compounds are ionized completely at all physiological pH values and thus tend to have high aqueous and low lipid solubilities. According to the pH–partition theory of passive absorption, these ionized moieties should be only insignificantly absorbed since they are transported slowly across lipid membranes.

The poor GI absorption of quaternary ammonium drugs can be illustrated by noting the difference in therapeutically effective oral and parenteral doses. For example, much larger doses of the antihypertensive drug hexamethonium and the cholinesterase reactivator pralidoxime chloride must be given by mouth as compared to parenteral administration to achieve a pharmacological response (1–3).

Various attempts have been made to improve the GI absorption of quaternary ammonium drugs. Isolated studies have shown that, in some cases, the amount and rate of absorption of hexamethonium (4), benzomethamine (5), and isopropamide (6) are increased by the addition of anionic substances. For example, Harington (4) showed that the various salts of hexamethonium are absorbed to different extents. These studies imply that the anionic portion of the salt plays a part in the absorption process since the other moiety always was the positively charged quaternary ion.

Although the existence of ion-pair complexes has been speculated, no conclusive evidence has been found. Furthermore, no data are available showing the effect of these anions on *in vivo* membrane permeability. Further work in this area is warranted to elucidate the mechanism of the enhancing action effected by anions.

The purposes of this study were to investigate techniques for increasing GI transfer of two model quaternary ammonium drugs, hexamethonium chloride (I) and pralidoxime chloride (II), and to study the mechanisms involved in this enhancement.

EXPERIMENTAL

Test Animals—Male Sprague-Dawley rats[1], ~140 g, were housed two per cage and had access to food and water until they attained a weight of 180–200 g. Animals were fasted for 24 hr prior to use but had free access to water.

Chemicals—Hexamethonium chloride[2], hexamethonium iodide[2], pralidoxime chloride[3], and pralidoxime iodide[3] were used as purchased. Chemicals used as anions were purchased from various sources.

The concentration of hexamethonium chloride (I) was 2.6×10^{-3} *M*, while that of pralidoxime chloride (II) was 8.7×10^{-3} *M*. The anions were administered as their sodium salts. When a sodium salt was not available, the desired acid was dissolved in deionized, distilled water with an equivalent quantity of sodium hydroxide. All solutions were prepared fresh on the day of the experiment.

Anesthesia—Each rat was anesthetized with urethan[4] (ethyl carbamate), 1 mg/g ip.

Experimental Design—To determine the number of experimental animals required to evaluate each drug–anion combination, preliminary studies were conducted with I and II in four animals on 4 consecutive days. An analysis of variance indicated that there was no statistical difference between the results from the different animals. This finding suggested that each drug–anion combination studied in four animals would give satisfactory results that could differentiate the effects of different anions on drug disappearance. Therefore, each I–anion and II–anion combination to be studied on any particular day was selected randomly.

The results were analyzed statistically using Duncan's new multiple range procedure (7). This procedure groups the ranked means that do not differ statistically ($p = 0.05$) and is suitable for the interpretation of the results since it distinguishes between a single anion and a group of anions that have similar effects on drug absorption.

Methods of Analysis—A slight modification (4% instead of 1.5% washed isopentanol) of the ion-pair technique described by Auerbach (8) and Mitchell and Clark (9) was used for the determination of hexamethonium in aqueous solutions and plasma. The pralidoxime content in biological fluids was analyzed utilizing the method of May *et al.* (10) and Ellin and coworkers (11, 12). The analytical procedure of Roe *et al.* (13) as modified by Schreiner (14) was employed for determining the inulin content in the aqueous solution. In all cases, the standard curves obeyed the Beer–Lambert law.

***In Situ* Gut Technique**—The experimental technique used to study drug transfer kinetics was a modification of a method described previously (15). The earlier technique did not account for intestinal volume changes. To overcome this difficulty, the ilial syringe was replaced with

[1] Huntingdon Farms, West Conshohocken, Pa.
[2] K & K Laboratories, New York, N.Y.
[3] Aldrich Chemical Co., Milwaukee, Wis.
[4] Matheson, Coleman and Bell, East Rutherford, N.J.

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Table I—Intestinal Disappearance of Hexamethonium Chloride (I) in the Presence of Different Anions for the *In Situ* Gut Technique [a]

Anion	Molar Ratio of I to Anion	$k^* \times 10^3$, min^{-1}
Cholate	1:5	8.33 ± 0.13
Desoxycholate	1:5	8.10 ± 0.17
Taurocholate	1:5	6.10 ± 0.40
Phoscholate	1:5	5.35 ± 0.18
Dehydrocholate	1:5	5.14 ± 0.26
Hyodesoxycholate	1:5	4.85 ± 0.11
Bromide	1:4	4.03 ± 0.05
Glycocholate	1:5	3.85 ± 0.12
Phosphate	1:5	3.53 ± 0.09
Iodide	1:5	3.40 ± 0.11
Chloride	1:3	3.05 ± 0.07
Trichloroacetate	1:5	3.00 ± 0.32
Glycotaurocholate	1:4	2.98 ± 0.25
Tartrate	1:5	2.81 ± 0.15
Propionate	1:5	2.76 ± 0.15
Lactate	1:5	2.62 ± 0.11
Acetate	1:5	2.28 ± 0.13
Citrate	1:5	1.95 ± 0.05
Sulfide	1:5	0.99 ± 0.14

[a] Statistical results of Duncan's new multiple range procedure.

a 10.0-ml graduated pipet. At various times, the drug solution from the intestine was emptied gently into the pipet, the volume was measured accurately, a 0.1-ml aliquot was withdrawn for analysis, and the solution was returned to the intestine.

***In Situ* Loop Technique**—This method was an adaptation of the *in situ* gut technique. The experimental procedure and setup were identical up to the point where the drug solution was to be introduced into the washed intestine. At this stage, the syringe was disconnected from the duodenal cannula and replaced with a 5.0-ml pipet containing exactly 5.0 ml of drug solution. This solution was allowed to flow into the intestinal lumen, and the intestine was ligated at the tip of the duodenal cannula. Similarly, the intestine at the ilial cannula was ligated, with care taken to ensure that no drug solution was in the receiving pipet. The pipet and cannulas then were removed.

After 3 hr, the entire section of small intestine was removed and placed in a beaker containing ~50 ml of distilled water. After the intestine was cut into thin rings, the contents in the beaker were stirred vigorously for 30 min. The mixture was filtered, and the solution was diluted to a desired volume. An aliquot of this solution was analyzed for drug content as described previously. The difference between the amount of drug introduced and the amount remaining at the completion of the experiment yielded the drug lost from the intestinal segment.

At the completion of the experiment, a blood sample was obtained *via* heart puncture from the same animal in which the loop technique was studied. The drug concentration in the plasma was determined as described. The blood volume of the rat was estimated from its body weight using the chart published previously for blood volume *versus* the body weight of male rats (16). The product of the plasma drug concentration and the plasma volume yielded a measure of the amount of drug in the plasma of the rat.

Membrane Permeability Study—The experimental setup was the same as that used in the *in situ* gut technique. The drug solution was introduced into the intestine; simultaneously, a sterile solution of 20 mg of inulin was injected into the femoral vein. At the end of 3 hr, the volume of the intestinal solution was measured accurately, and an aliquot was taken for inulin analysis.

The amount of inulin determined in the sample aliquot and the volume of the intestinal contents yielded the amount of inulin transferred from the blood to the intestinal lumen.

Histological Study—The *in situ* gut technique was utilized to obtain the intestinal tissue samples. The drug solution was introduced into the intestine; after 3 hr, segments from different intestinal areas were removed, gently flushed out with distilled water, and then fixed for at least 24 hr in a 10% formalin and 1% $CaCl_2$ solution. These sections were fixed permanently for later use.

RESULTS AND DISCUSSION

Intestinal Disappearance—The kinetics of intestinal disappearance of the two quaternary ammonium drugs, hexamethonium chloride (I) and pralidoxime chloride (II), in the presence of various anions were observed for 3 hr. The kinetics between 30 and 180 min followed apparent first-order disappearance and, therefore, could be represented mathematically by:

$$m = m_0 e^{-kt} \quad \text{(Eq. 1)}$$

where m is the amount of drug in the intestine at any time t, m_0 is the initial amount of drug in the intestine, and k is the disappearance rate constant.

Preliminary studies indicated that the intestinal disappearance rate of the drugs and their combinations with various anions for the initial 30 min did not follow simple first-order kinetics. For this reason, the constant was redefined as an apparent disappearance rate constant and designated as k^*. The behavior during the initial period was studied in detail and will be discussed later. The k^* values for I and II were estimated using linear regression to be $3.05 \pm 0.07 \times 10^{-3}$ and $4.58 \pm 0.11 \times 10^{-3}$ min^{-1}, respectively.

The influence of an excess of chloride ions while utilizing hexamethonium iodide and pralidoxime iodide also was investigated. The disappearance rates of these iodide salts in the presence of an excess of chloride anions were identical to those of the chloride salts of the drugs. Similarly, another study showed that the apparent rates of I and II in the presence of an excess of iodide ions were not different from the rates for the iodide salts of these drugs. The results indicate strongly that the intestinal disappearance kinetics of I and II are affected by the presence of foreign anions and that the drugs behave as the salt of the excess anion present. A possible reason for such behavior is that the quaternary ammonium salts are ionized completely at all physiological pH values to their corresponding cations and anions, and an excess of foreign anion will swamp the anion of the salt.

All anions, except bromide, when used in the molar ratios of drug to anion of 1:2 to 1:5 had the same effect on I and II. With bromide, a progressive increase in the anion concentration caused a corresponding increase in the disappearance rate of I but not of II. The molar ratio of drug to anion was maintained within the range of 1:2 to 1:5; in a few instances, it was necessary to change (±10%) the anion concentration to achieve an isotonic solution to prevent drastic intestinal volume changes. The pH of the solutions during this investigation ranged from 7.5 to 8.3; only the desoxycholate and taurocholate systems gave slightly lower values.

The results for hexamethonium chloride and pralidoxime chloride in the presence of various anions are presented in Tables I and II, respectively. The brackets enclosing the anion or anions in the first column indicate the results of the statistical analysis by Duncan's new multiple range procedure. The effects on the apparent disappearance rate constant of the drug for the anions included in the same bracket do not differ statistically at $p = 0.05$; however, the effects of the anions grouped in different brackets are significantly different at $p = 0.05$.

The simple ion, bromide, increased the disappearance of I but had no effect on the rate constant of II. Iodide increased the value of II and had no significant effect on the value of I. The results of the hexamethonium studies agree with Harington's findings (4); however, the results of the

Table II—Intestinal Disappearance of Pralidoxime Chloride (II) in the Presence of Different Anions for the *In Situ* Gut Technique [a]

Anion	Molar Ratio of II to Anion	$k^* \times 10^3$, min^{-1}
Phoscholate	1:3	7.37 ± 0.30
Trichloroacetate	1:2	6.56 ± 0.14
Taurocholate	1:2	5.64 ± 0.10
Glycotaurocholate	1:1	5.49 ± 0.21
Hyodesoxycholate	1:2	5.42 ± 0.15
Iodide	1:2	5.25 ± 0.12
Cholate	1:2	5.23 ± 0.27
Dehydrocholate	1:1	5.18 ± 0.51
Glycocholate	1:1	5.19 ± 0.14
Bromide	1:2	4.75 ± 0.71
Chloride	1:2	4.58 ± 0.11
Acetate	1:2	3.73 ± 0.72
Desoxycholate	1:2	3.51 ± 0.13
Tartrate	1:2	3.32 ± 0.12
Propionate	1:2	3.29 ± 0.33
Lactate	1:2	3.24 ± 0.06
Sulfate	1:2	3.16 ± 0.15
Phosphate	1:2	3.07 ± 0.21
Citrate	1:2	2.57 ± 0.38

[a] Statistical results of Duncan's new multiple range procedure.

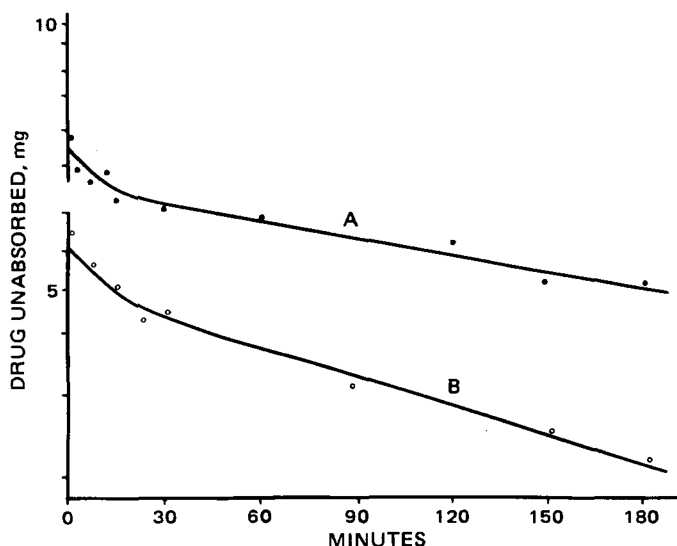


Figure 1—*Intestinal disappearance of hexamethonium chloride (A, ●) and pralidoxime chloride (B, ○) in rats.*

pralidoxime experiments differ from those of Levine and Steinberg (17).

Another anion that had opposing effects on the drug transfer rate was phosphate. It increased the disappearance constant of I while it decreased the value of II appreciably.

Citrate, sulfate, lactate, propionate, tartrate, and acetate had similar effects; they all decreased or had no effect on the disappearance rate constants of I and II. These findings parallel those in the literature, although one investigator (18) reported increased intestinal absorption of the quaternary ammonium compound benzomethamine in the presence of acetate and propionate anions. However, this effect occurred only at high acid concentrations (0.2 *M*) and at relatively low pH values (pH 3–4).

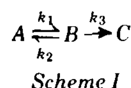
Trichloroacetate had no influence on the results of I while it increased the corresponding value of II. This anion increased the pharmacological response of isopropamide iodide (6).

The most dramatic changes were caused by the bile salt anions. For example, cholate caused an approximately threefold increase in the rate constant for I but only a slight increase for II. Of the bile anions studied, cholate, taurocholate, dehydrocholate, hyodesoxycholate, and glycocholate increased the disappearance rates of I and II. Desoxycholate increased the rate constant of I but was ineffective with II, while glycotaurocholate had an opposite effect.

The most promising of the anions studied was phoscholate. This anion, which is the conjugate base of phoscholic acid (2,2′-phosphinicodilactic acid), significantly increased the disappearance rate constants of both I and II. For example, the rate constant for I in the presence of chloride ions was 3.05×10^{-3} min^{-1}, while its value in the presence of phoscholate was 5.35×10^{-3} min^{-1}. Similarly, the k^* value of II in the presence of chloride ions was 4.58×10^{-3} min^{-1}, which increased to 7.37×10^{-3} min^{-1} when the drug was present with phoscholate.

It was stated previously that the intestinal disappearance of I and II and their combinations with various anions followed a definite pattern; their initial disappearance rate was rapid and was followed by a slower phase. Similar results for other quaternary ammonium drugs (5, 19–21) and other drugs such as phenothiazines (22), haloperidol (22), and steroids (23) also were reported. This pattern may be indicative of the mechanism by which these quaternary ammonium drugs are absorbed through the intestinal barrier.

Figure 1 illustrates the typical kinetic patterns of the intestinal disappearance of I and II. The rate was rapid in the initial period and was followed by a slower phase between 30 and 180 min. An attempt was made to describe mathematically the kinetics of I and II. Of the kinetic models considered, the model shown in Scheme I gave the best fit to the experimental data.



$$A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} B \overset{k_3}{\rightarrow} C$$

Scheme I

In Scheme I, A is the drug in the intestinal lumen, k_1 and k_2 are the constants that are part of the equilibrium step, and k_3 is the constant

Table III—Intestinal Disappearance Rate Constants of Hexamethonium Chloride (I) in the Presence of Different Anions

Anion	Molar Ratio of I to Anion	$k_1 \times 10^2$, min^{-1}	$k_2 \times 10^2$, min^{-1}	$k_3 \times 10^2$, min^{-1}
Chloride	1:3	3.3	11.9	1.1 (0.305)[a]
Cholate	1:5	4.4	12.8	4.1 (0.833)[a]
Phoscholate	1:5	3.0	6.5	1.7 (0.535)[a]

[a] Apparent disappearance rate constant, k^*, in the *in situ* gut technique.

responsible for the disappearance of the drug from the lumen. Assuming that k_1, k_2, and k_3 are first-order rate constants and solving the differential equation for A yield (22, 24, 25):

$$A = A_0 \left[\frac{(k_2 + k_3 - \alpha)e^{-\alpha t} - (k_2 + k_3 - \beta)e^{-\beta t}}{\beta - \alpha}\right] \quad \text{(Eq. 2)}$$

where α and β are given by:

$$\alpha = \tfrac{1}{2}[(k_1 + k_2 + k_3) + \sqrt{(k_1 + k_2 + k_3)^2 - 4k_1k_3}] \quad \text{(Eq. 3)}$$

and:

$$\beta = \tfrac{1}{2}[(k_1 + k_2 + k_3) - \sqrt{(k_1 + k_2 + k_3)^2 - 4k_1k_3}] \quad \text{(Eq. 4)}$$

This derivation gives a mathematical relationship between the defined microconstants k_1, k_2, and k_3 and the disappearance of the drug from the intestine. The β value obtained from the 0–180-min data is identical to the k^* constant determined previously.

The presence of excess chloride did not affect the rate constants of I and II. This finding is in agreement with the results of earlier studies. The presence of cholate and phoscholate increased the disappearance rate constant, k_3, of I and II. They also caused a corresponding increase in the apparent disappearance rate constants of I and II in the *in situ* gut study. For comparison purposes, the k^* values are listed in Tables III and IV in parentheses.

Therefore, it is possible that the quaternary ammonium drugs I and II may be absorbed in the manner described by the proposed mathematical model. Nakamura *et al.* (26–28) investigated the mechanism of intestinal absorption of four highly ionized, poorly lipid-soluble dyes and noted that these compounds must bind to mucus as the first step in absorption. Mucus, which coats the epithelial surface, is secreted by the goblet cells of the small intestine.

Martin *et al.* (29) found that the bile salts sodium deoxycholate, sodium taurodeoxycholate, and sodium glycocholate cause breakdown of the mucus structure. In addition to possibly causing membrane changes, the anions also may elicit their effect in this manner.

The investigation so far indicated that the intestinal disappearance of quaternary ammonium drugs can be influenced by the presence of a suitable anion and also that the *in situ* gut technique can be used to study such enhancement. Since this method determines only the disappearance of drugs from the intestinal lumen, several possibilities could produce misleading results. These possibilities are drug degradation, binding of the drug to the membrane or intestinal contents, intramembrane storage, and mechanical error during sampling. Therefore, before any definite conclusion could be drawn from the study, further investigation of the method was warranted.

To determine the stability of I and II in solution, different drug–anion solutions were prepared and kept at room temperature for 1 week. Almost 100% recovery of each drug from these solutions indicated that the drugs were stable in aqueous solutions containing the different anions. Similar experiments were conducted using the membrane and intestinal contents. Again, almost 100% recovery indicated that the drugs were stable over the experimental period. These results agree with previous reports (4, 17).

Table IV—Intestinal Disappearance Rate Constants of Pralidoxime Chloride (II) in the Presence of Different Anions

Anion	Molar Ratio of II to Anion	$k_1 \times 10^2$, min^{-1}	$k_2 \times 10^2$, min^{-1}	$k_3 \times 10^2$, min^{-1}
Chloride	1:2	4.2	9.5	1.7 (0.458)[a]
Cholate	1:2	3.1	5.5	1.7 (0.523)[a]
Phoscholate	1:2	1.9	3.6	2.9 (0.730)[a]

[a] Apparent disappearance rate constant, k^*, in the *in situ* gut technique.

Table V—Intestinal Disappearance and Appearance in Plasma of Hexamethonium Chloride (I) and Pralidoxime Chloride (II) in the Presence of Different Anions for the *In Situ* Loop Technique

	I		II	
Anion	Intestinal Loss after 3 hr, mg	Amount in Plasma at 3 hr, mg	Intestinal Loss after 3 hr, mg	Amount in Plasma at 3 hr, mg
Chloride	1.6 ± 0.1	0.46 ± 0.03	3.6 ± 0.1	0.038 ± 0.006
Cholate	2.4 ± 0.2	0.74 ± 0.10	5.4 ± 0.4	0.068 ± 0.007
Phoscholate	2.3 ± 0.1	0.81 ± 0.07	6.3 ± 0.3	0.110 ± 0.004

To determine whether the loss of drug from the intestine was a result of absorption, two experiments were conducted. The first experiment was a study of the effects of selected anions on the intestinal absorption of I and II using a conventional *in situ* loop technique; the second experiment was a study of the amount of drug transferred from the intestine into the blood at the end of 3 hr. Chloride, cholate, and phoscholate were the three anions studied in combination with I and II. The chloride anion was selected because it is the most commonly used anion in the synthesis of quaternary ammonium drugs. Furthermore, the chloride salts of hexamethonium and pralidoxime frequently are used as therapeutically active agents. Cholate was representative of the bile salts, while phoscholate was the most promising anion, and both enhanced the disappearance rates of I and II.

The results for I and II in the presence of cholate and phoscholate as determined by the loop technique are presented in Table V. Both cholate and phoscholate increased the amount of I and II absorbed as compared to the standard, chloride. These findings are in agreement with those of the *in situ* gut technique.

The plasma samples obtained at the completion of the loop experiments indicated the presence of drug, thereby qualitatively substantiating that both I and II were absorbed to some degree. As seen in Table V, both cholate and phoscholate caused increased intestinal loss of I and II (as compared to chloride), and this loss, in turn, increased the quantity of drug in plasma.

The amount of drug lost could be calculated from the *in situ* gut technique data. This value was the difference between the amount of drug known to be present in the intestine at time zero and the amount remaining in the intestine after 180 min. The amount of drug lost was converted to a percentage, and this value then was compared to the results of the *in situ* loop technique. The gut and loop techniques yielded similar values for the percent of drug lost (Table VI). Both cholate and phoscholate enhanced the disappearance of I and II.

During the *in situ* gut technique experiments, if the drug had adhered to or been bound to the intestinal membrane or contents, then the sample aliquot obtained at any time would not have truly represented the actual amount of drug in the lumen. This possibility cannot be fully discounted since no recovery experiments were conducted. However, the results of the two methods were in close agreement. In the *in situ* loop technique, the entire intestine and contents are assayed, so the drug not accounted for must be located in areas other than the intestine. Finally, since no samples are removed until the conclusion of the experiment, mechanical errors such as frequent sampling cannot account for the different kinetic results.

Membrane Permeability—Quaternary ammonium drugs are completely ionized at all physiological pH values, and these drug solutions always contained their corresponding anions. It seemed reasonable that these anions may have affected membrane permeability. This assumption was investigated in a study with a sodium chloride solution as the control. The molar ratios of drug to anion were the same as those of the *in situ* gut technique for all test solutions. Again, the anions selected were chloride, cholate, and phoscholate.

Table VI—Comparison of *In Situ* and Loop Techniques for the Intestinal Disappearance of Hexamethonium Chloride (I) and Pralidoxime Chloride (II) in the Presence of Different Anions

	Drug Lost, %			
	I		II	
Anion	Gut Technique	Loop Technique after 3 hr	Gut Technique	Loop Technique after 3 hr
Chloride	44	46	54	48
Cholate	68	69	67	72
Phoscholate	60	66	79	84

Table VII—Effect of Different Drug-Anion Combinations on Membrane Permeability in the Rat Intestine in the Membrane Permeability Study

Drug-Anion Combination	Molar Ratio of Drug to Anion	Inulin in Intestine after 3 hr, mg × 10^2	Percentage Change[a]
Chloride	—	61 ± 8	Control
I + chloride	1:3	62 ± 5	No change
I + cholate	1:5	84 ± 10	35
I + phoscholate	1:5	90 ± 12	38
I + sulfate	1:5	48 ± 9	−26[b]
II + chloride	1:2	64 ± 8	No change
II + cholate	1:2	86 ± 12	38
II + phoscholate	1:2	83 ± 10	35
II + citrate	1:2	38 ± 5	−39

[a] Percentage change as compared to control. [b] Negative number indicates less than control.

The drug-anion combinations tested for their possible effects on membrane permeability are listed in Table VII. Table VII also shows the percentages of inulin in the intestine after an intravenous dose of 20 mg. The percentages of inulin reported in Table VII are in terms of the control.

The drug-chloride combinations and the chloride alone (control) gave similar results. However, the presence of cholate and phoscholate in the drug solutions increased the amount of inulin in the intestine by ~35%. These results indicate that there were changes in the intestinal membrane permeability when the drug solutions containing cholate and phoscholate were in the intestine for 3 hr.

The control solution containing chloride ion apparently affected the membrane permeability because ~3% of the inulin was found in the intestine when this solution was tested. However, this anion in combination with the drugs gave the same effect as the chloride ion alone. Furthermore, the changes in permeability with phoscholate and cholate in the presence of both drugs were similar. These findings seem to indicate that the anionic components of the solutions were responsible for the permeability changes of the intestinal membrane and not the drug molecules.

Feldman and coworkers (30–34) studied the enhancement of drug absorption by bile salts; in all cases, the increases were attributed to permeability changes. Other investigators (35, 36) demonstrated a similar effect by the bile salts, resulting in better drug absorption. These results suggest that the enhancement of the transfer of I and II caused by cholate and phoscholate is the result of permeability changes in the rat intestine. Also, it is suggested that the anionic components and not the drug molecules are responsible for these effects.

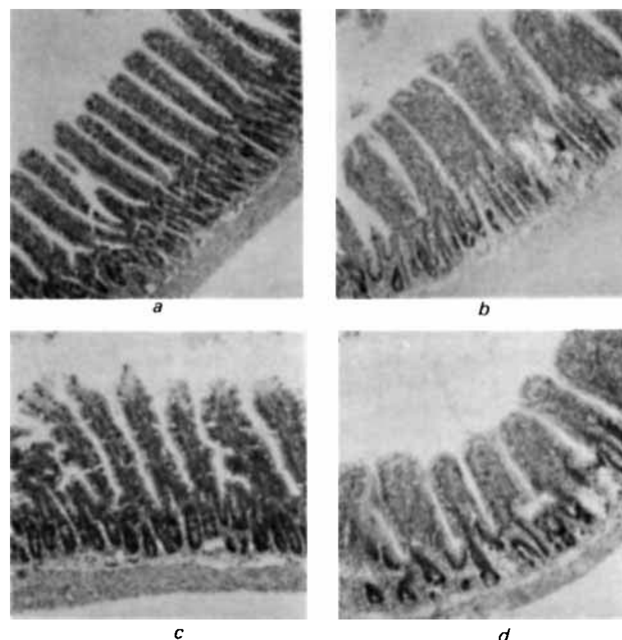


Figure 2—*Examples of intestinal morphological changes. Key:* a, *control;* b, *fusion of villi;* c, *loss of epithelial cells; and* d, *edema of the lamina propria.*

Table VIII—Histological Study of Effect of Drugs and Anions on the Structural Integrity of the Intestinal Membrane [a]

Anion, Drug, or Drug–Anion Combination	Loss of Epithelial Cells	Loss of Goblet Cells	Fusion of Villi	Edema of Lamina Propria	Changes in Mucous Membrane
Control	0	0	0	0	0
I	1	1	2	0	1
II	0	1	1	0	0
Chloride	1	3	1	1	1
Bromide	4	4	3	0	4
Cholate	2	3	2	0	3
Phoscholate	2	2	2	0	2
I + chloride	1	2	1	0	1
I + cholate	2	1	1	0	1
I + phoscholate	1	2	2	0	2
II + chloride	0	3	1	0	2
II + bromide	4	3	3	0	4
II + cholate	2	1	2	0	2
II + phoscholate	3	3	2	0	2

[a] Key: 0 = normal, 1 = slight, 2 = moderate, 3 = severe, and 4 = very severe.

Histological Study—This study was undertaken to investigate the effect of I and II, selected anions, and their combinations on the morphology of the intestine. Since the mucous membrane of the intestine comes in direct contact with the drug, the changes in its structure were of concern. The three layers of importance in absorption are the epithelial lining, lamina propria, and muscularis mucosa. The gross morphological changes in these layers were studied by the following observations:

1. Loss of epithelial cells—These cells form the innermost layer of the intestine, lining the villi of the intestinal mucosa. The epithelium consists of columnar cells and the basement membrane. Since absorption occurs through the columnar cells, any damage or loss of these cells could easily affect the transfer process.

2. Loss of goblet cells—Intermingled with the absorptive cells in the mucous membrane are goblet cells. These cells secrete mucus, which protects the epithelial lining from injury by the materials in the intestine.

3. Fusion of villi—The mucosal surface provides a large surface from which absorption occurs. Since the fusion of the villi means a reduction in the surface area, this fusion possibly could affect the absorption of materials from the intestines.

4. Edema of lamina propria—The lamina propria supports the epithelium and connects it to the muscularis mucosa. It carries both the blood and the lymphatic capillaries close to the epithelial surface, and diffusing materials therefore travel only a short distance through the tissue fluid of the lamina propria before gaining entrance into either type of capillary. Damage to the lamina propria could affect the transfer of drugs and their subsequent entrance into the blood.

5. Changes in mucous membrane—These changes did not concern any particular tissue of the mucous membrane but were overall changes in the absorptive surface.

The results of the morphological changes were classified as: 0 = normal, 1 = slight, 2 = moderate, 3 = severe, and 4 = very severe. Examples of these changes are presented in Fig. 2, and the results of this study are shown in Table VIII.

Compound I alone caused only minimal intestinal morphological changes; II caused even milder changes. These results can be correlated with those of the permeability study where the drugs did not affect the permeability of the intestinal membrane.

The responses elicited by the anions were varied. Chloride ions caused considerable loss of goblet cells; however, the overall effect on the mucous membrane was slight. This anion, as well as cholate and phoscholate, earlier was shown to affect membrane permeability. This study demonstrated that these anions had a moderate effect on the morphological structure of the membrane. The most dramatic changes were caused by bromide ions. Bromide was included since it was found previously that increasing the bromide concentration increased the loss of I.

The effects of drugs in combination with anions on the intestinal membrane morphology were similar to the effect of the specific anion alone. This finding supports the view that the drug molecule did not cause any further morphological changes.

None of the drugs or the anions or their combinations caused edema of the lamina propria, while all caused fusion of the villi to varying degrees. Moreover, some goblet cells were lost in all cases. The phoscholate ion and its combination with a drug caused moderate damage to epithelial cells. These findings are in agreement with those of Low-Beer *et al.* (37), who demonstrated a similar morphological change in the presence of foreign materials in the intestine. Cholate ions had a similar effect on the membrane.

The anions that enhanced the absorption of I and II also caused permeability and histological changes in the intestinal membrane. Although these results seem to indicate that the permeability change may have been caused by histological changes, which then led to enhanced drug absorption, they should be viewed cautiously since histological results are not available for the anions that actually decreased drug transfer.

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Separation and Quantitation of Chlortetracycline, 4-Epitetracycline, 4-Epianhydrotetracycline, and Anhydrotetracycline in Tetracycline by High-Performance Liquid Chromatography

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Abstract □ The analysis of tetracycline epimers in tetracycline preparations by high-performance liquid chromatography is described. The method uses a microparticulate phenyl column (3.9 mm i.d. × 30 cm) with a step gradient of 12–22% acetonitrile in 0.2 *M* phosphate buffer at pH 2.2. The analysis takes 22 min. The relative standard deviations of the method (2σ, $n = 6$) for the analysis of chlortetracycline, 4-epitetracycline, 4-epianhydrotetracycline, and anhydrotetracycline in tetracycline were ±3.68, ±4.47, ±7.60, and ±2.77%, respectively.

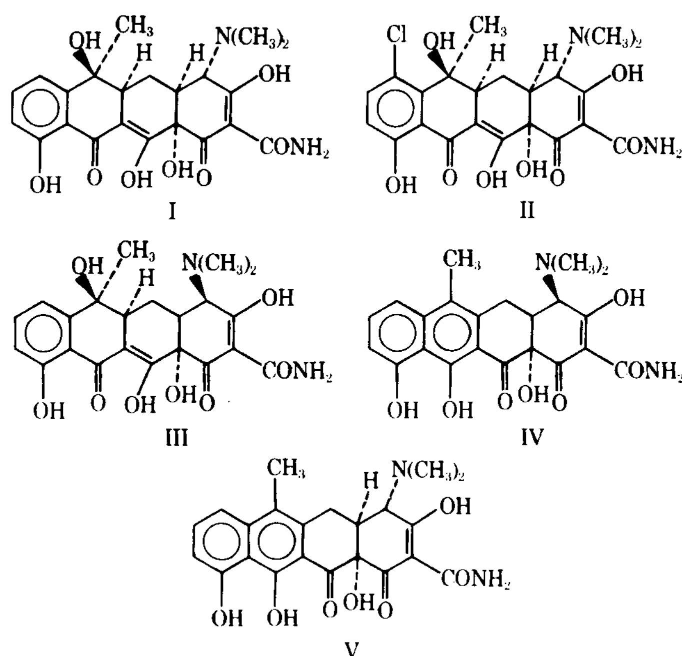
Keyphrases □ Tetracycline—simultaneous high-performance liquid chromatographic analysis of tetracycline epimers in tetracycline preparations □ High-performance liquid chromatography—analysis, tetracycline epimers in tetracycline preparations □ Antibacterials—tetracycline, high-performance liquid chromatographic analysis of tetracycline epimers in tetracycline preparations

Pharmaceutical preparations of tetracycline (I) contain small quantities of related compounds as impurities. The most important compounds are chlortetracycline (II), 4-epitetracycline (quatrimycin, III), 4-epianhydrotetracycline (IV), and anhydrotetracycline (V). Their permitted concentrations are listed in the Federal Register (1) for tetracycline dosage forms marketed in the United States and in the British Pharmacopoeia (2) and European Pharmacopoeia (3) for the European markets. The need for a simple assay prompted this investigation of the conditions under which these compounds could be separated and quantitated by high-performance liquid chromatography (HPLC) without a solvent gradient system.

BACKGROUND

TLC (4, 5), paper chromatography (6), and column chromatography followed by UV spectrophotometry (7, 8) have proved laborious and generally are not sufficiently sensitive or precise. A GLC method (9) requires prior formation of the trimethylsilyl derivative under carefully controlled conditions.

Several HPLC methods have been applied to separate III–V from I using a cation-exchange column with an ethylenediaminetetraacetic acid buffer (10), a C_{18} column (5–7% loading) with phosphate buffer and a linear gradient of 10–60% acetonitrile (11), a C_{18} column (10–18% loading) with a linear gradient of methanol–water–0.2 *M* phosphate buffer at pH 2.5 (30:60:10) and methanol–acetonitrile–water–0.2 *M* phosphate buffer at pH 2.5 (50:30:20:10) (12), and a C_{18} column with water–acetonitrile–perchloric acid in two steps (13).



In the present study, a microparticulate phenyl column with a step gradient of 12–22% acetonitrile in 0.2 *M* phosphate buffer at pH 2.2 (using an automatic switching valve) at a flow rate of 2.6 ml/min was used to separate II–V from I in 22 min. The method did not require a solvent gradient system and needed only one pump. It was tested for the analysis of several different tetracycline formulations.

EXPERIMENTAL

Apparatus—The liquid chromatographic apparatus shown in Fig. 1 was used[1].

Reagents and Materials—Ammonium 4-epitetracycline[2], 4-epianhydrotetracycline hydrochloride[2], anhydrotetracycline hydrochloride[2], chlortetracycline hydrochloride[3], acetonitrile[4], phosphoric acid[5], potassium hydroxide[5], and ammonium hydroxide[5] were used.

[1] A Valcor series SV-72 automatic switching valve, a Waters Associates model 6000 A pump, a Valco loop injector, a Waters Associates μBondapak phenyl column, and a Waters Associates model 440 absorbance detector were used.
[2] Bristol reference standards, Bristol Laboratories, Syracuse, N.Y.
[3] USP reference standard.
[4] Burdick & Jackson Laboratories, Muskegon, Mich.
[5] Fisher Scientific Co., Fair Lawn, N.J.

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