chlorides[11] having a purity of 99.5%. The palmitic acid[12] used was recrystallized twice from chloroform and had a melting point of 63.2°. The stearic acid was also recrystallized twice from chloroform and had a melting point of 70°.

The purity of all the samples was determined by vapor phase chromatography.

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[12] Eastman Organic Chemicals, Rochester, N. Y.

Physiologic Surface-Active Agents and Drug Absorption II: Comparison of the Effect of Sodium Taurodeoxycholate and Ethylenediaminetetraacetic Acid on Salicylamide and Salicylate Transfer Across the Everted Rat Small Intestine

STUART FELDMAN* and MILO GIBALDI

Abstract □ The effect of sodium taurodeoxycholate (STDC) and ethylenediaminetetraacetic acid (EDTA) on the transfer of salicylamide and salicylate across the everted rat intestine was studied. STDC produces a small but statistically significant increase (11–21%) in the intestinal transfer rate of salicylamide over a concentration range of 5–100 m*M* but has no measurable effect at a 1 m*M* concentration. There is a small but statistically significant increase (about 20%) in the intestinal transfer rate of salicylate after the intestine has been exposed to 25 m*M* EDTA but the chelating agent has no significant effect on salicylamide transfer. The results indicate that both EDTA and STDC alter membrane structure and thereby affect permeability but by two different mechanisms.

Keyphrases □ Surfactants—drug absorption □ Salicylate, salicylamide transfer—EDTA, sodium taurodeoxycholate effect □ Everted intestine—transfer rates □ Colorimetric analysis—spectrophotometer

The previous report in this series concerned the effects of sodium taurodeoxycholate (STDC) on the transfer rate of salicylate ion across the everted rat intestine (1). The bile salt was found to produce a change in the permeability of the intestine to salicylate; a small increase in transfer rate was noted at concentrations of STDC near or about the CMC and a much larger increase in membrane permeability was observed at STDC concentrations above the CMC. These findings were consistent with other reports on the effects of bile salts on the canine gastric mucosa (2) and goldfish membranes (3).

The purpose of the present study was to contrast the effects of STDC on the membrane transfer of unionized and ionized molecules and to explore the possible commonality of effects of disodium ethylenediaminetetraacetic acid (EDTA) and bile salts on drug transfer across the everted rat small intestine. Salicylate and salicylamide served as model drugs for this purpose.

EXPERIMENTAL

Intestinal Transfer Rate Measurements—The cannulated everted intestine method developed by Crane and Wilson (4) was used with the modifications described previously (1). Male Sprague-Dawley rats (Blue Spruce Farms, Altamont, N.Y) weighing 200–265 g. were fasted 20–24 hr. prior to each experiment. Water was allowed *ad libitum*. Each everted intestinal segment, 10 cm. in length, was suspended in 80 ml. of mucosal solution at 37° and oxygenated with a mixture of 95%:5% oxygen-carbon dioxide. The mucosal solution was Krebs-phosphate buffer (KPB) without calcium or magnesium, at either pH 6.0 or 7.4, and contained varying concentrations of sodium taurodeoxycholate (STDC) or disodium ethyl-

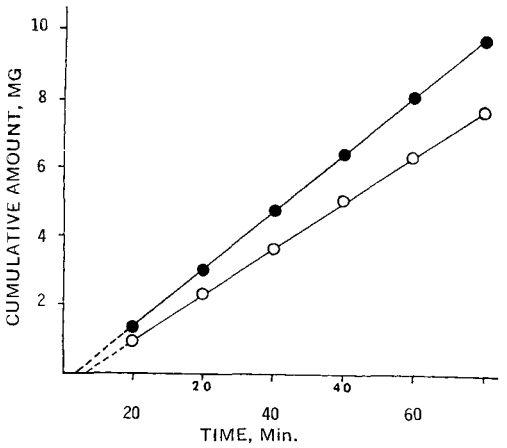

Figure 1—*Effect of 10 m*M *sodium taurodeoxycholate on salicylamide transfer across the everted rat small intestine. Representative plot shows cumulative amount transferred as a function of time after a 1 hr. incubation in 10 m*M *STDC in pH 6.0 buffer solution* (●) *or in pH 6.0 buffer solution* (○).

enediamine tetraacetic acid (EDTA), 25 m*M*. Two milliliters of serosal solution (KPB)[1] at pH 6.0 or 7.4 were introduced into the everted segment. The entire everted intestine was incubated for a period of 1 hr. unless otherwise specified.

After incubation, the segments were thoroughly rinsed with KPB (prewarmed to 37°) and placed in fresh KPB at pH 6.0 containing either sodium salicylate 2.0 mg./ml. or salicylamide 2.0 mg./ml. Two milliliters of KPB, pH 6.0, were placed in the serosal side of the intestinal segment. Serosal samples were withdrawn at appropriate intervals during the experiment and the serosa was rinsed with 2 ml. of KPB. The rinse was combined with the initial sample and retained for analysis. Another 2 ml. of KPB was then placed in the intestinal segment to serve as serosal solution for the next time interval. The serosal fluid was sampled over a 1-hr period.

The drug solutions were maintained at pH 6.0 since at that pH salicylic acid with a pKa of 3 would be essentially 100% ionized and the transfer of salicylate ion could be studied. At a pH of 6.0, salicylamide with a pKa of 8.3 would be virtually unionized and transfer of the unionized, lipid-soluble species could be studied. The pH of the solutions were checked before and after each experiment and did not vary by more than 0.1 pH unit. All solutions were adjusted to 150 m*M* Na^+ concentration by the addition of sodium chloride.

The experimental approach outlined above, using incubation techniques, was particularly desirable to minimize drug-bile salt interactions which could affect the drug transfer rate (5).

Reversibility or Blocking of EDTA and Taurodeoxycholate Effects—It was of interest to investigate the possibility that the effects of EDTA and STDC on the permeability of the everted intestinal membrane could be reversed by incubation of the intestinal segments in a solution of divalent cations. After incubation of the intestinal segment in KPB containing 25 mmole EDTA at pH 7.4 for 0.5 hr.[2] or in KPB containing 1 mmole STDC at pH 6.0 for 1 hr., the segments were rinsed with KPB (pH 6.0) and placed for 15 min. in a solution containing either 154 mmole sodium chloride (control) or various concentrations of magnesium chloride or calcium chloride plus sufficient Na^+ to yield a total cation concentration of 154 mmole. The segments were then rinsed, placed in 2 mg./ml. salicylate solution at pH 6.0, and the transfer rates were determined as discussed above.

To investigate whether a partially chelated EDTA would "block" or reduce the increase in membrane permeability observed with EDTA, the intestinal segments were incubated in 25 m*M* disodium magnesium ethylenediaminetetraacetic acid (Mg-EDTA) and disodium calcium ethylenediaminetetraacetic acid (Ca-EDTA) at pH 7.4 for 1 hr. In each case a paired segment was exposed to 25 m*M* EDTA under comparable experimental conditions. Transfer rates of salicylate were then determined in the usual manner.

Assay Procedure—The serosal samples were acidified to pH 1 by the addition of several drops of 6 *N* HCl. Sufficient 0.1 *N* HCl was added to bring the total serosal fluid volume up to 5 ml. After appropriate dilutions 5 ml. of Trinder's reagent (6) was added to an aliquot of the serosal fluid and the solution filtered (Millipore filter, 0.45μ). This solution was analyzed in a spectrophotometer (Hitachi-Perkin-Elmer model 139) at a wavelength of 540 mμ. The amount of salicylate or salicylamide in each sample was calculated from standard curves. Neither STDC nor "blank" serosal fluid interfered with the assay procedure. Using the incubation procedures outlined above, EDTA was also found not to interfere with the assay procedure. This fact was ascertained by adding known quantities of salicylate to "blank" serosal fluid from intestinal segments previously incubated with EDTA as described.

Data Evaluation—Repeated determination of transfer rate constants under various experimental conditions revealed no significant differences between the first and second intestinal segment. Accordingly, no distinction was made, with respect to segment, in evaluating and reporting the data. All experiments were run in a cross-over fashion with respect to segments.

The apparent zero-order transfer rate constants for the transfer of salicylate or salicylamide were determined from the linear segment of a plot of cumulative amount of drug transferred *versus* time. The steady-state (or linear) region was discerned from rate plots as discussed previously (1).

The data was evaluated statistically by means of Student's *t* test using both unpaired and paired (control *versus* test condition in the two intestinal segments of a single rat) data (7).

Materials—Sodium taurodeoxycholate (STDC), (Maybridge Chemical Co., Ltd., North Cornwall, England) chromatographically pure. Ethylene diamine tetraacetic acid, disodium salt (Fisher certified reagent grade). Disodium calcium ethylenediaminetetraacetic acid and disodium magnesium ethylenediaminetetraacetic acid (K and K Laboratories Inc., Plainview, N. Y.). All other chemicals used in the study were reagent grade and used as such without further purification.

RESULTS

Effect of STDC Concentration on Intestinal Transfer Rate of Salicylamide—Figure 1 shows a typical cumulative plot of the amount of salicylamide transferred (mucosal to serosal) *versus* time across a segment pretreated in mucosal solution containing 10 m*M* STDC in KPB, or KPB alone. The rates of salicylamide transfer were obtained from a least squares fit of the linear region of the cumulative plots. The steady-state transfer rates at various concentrations of STDC are reported in Table I.

The results show a small but significant increase (paired comparison, $p < 0.05$) in the rate of salicylamide transfer after incubation of the intestinal segment in the presence of 5, 10, and 100 m*M* STDC. Statistical comparison of the ratios of the rate of salicylamide transfer after STDC incubation to that after control incubation in paired segments revealed no significant difference between the effects at 5, 10, and 100 m*M* STDC. Transfer rates

Table I—Effect of Sodium Taurodeoxycholate (STDC) on Transfer Rate of Salicylamide Across the Rat Small Intestine at pH 6.0

Incubation Medium	No. of Intestinal Segments	Mean Transfer Rate ± *SD* mcg./min.
Control, buffer solution	17	129 ± 10
STDC, mucosal, m*M*		
100	4	149 ± 16
10	5	156 ± 4
5	3	143 ± 6
1	2	113 (120, 106)
STDC, mucosal-serosal, m*M*		
1	3	131 ± 6

[1] In some experiments the serosal solution also contained a concentration of STDC or EDTA equal to that present in the mucosal solution.

[2] Initial studies showed that incubation of the intestinal segment for up to one hour resulted in identical transfer rates.

Table II—Effect of EDTA on Transfer Rate of Salicylamide and Salicylate Across the Rat Small Intestine[a]

Incubation Medium	No. of Intestinal Segments	Mean Transfer Rate ± *SD* mcg./min.
Salicylamide		
Control, KPB,[b] pH 6.0	6	121 ± 8
EDTA, 25 m*M*, pH 6.0	6	120 ± 11
Control, KPB, pH 7.4	4	125 ± 4
EDTA, KPB, pH 7.4	4	129 ± 8
Salicylate		
Control, KPB, pH 6.0	6	30 ± 3
EDTA, 25 m*M*, pH 6.0	6	34 ± 3
Control, KPB, pH 7.4	4	33 ± 3
EDTA, 25 m*M*, pH 7.4	4	42 ± 6

[a] Data grouped according to paired segments. [b] Krebs phosphate buffer.

obtained after pretreatment with 1 m*M* STDC showed no significant difference from control levels.

Effect of EDTA on Transfer Rate of Salicylamide and Salicylate—Incubation of the everted intestinal segments in 25 m*M* EDTA at either pH 6.0 or 7.4 resulted in no statistical difference in salicylamide transfer rates from control values (Table II).

In contrast, incubation of the everted intestine preparation in 25 m*M* EDTA at pH 6.0 resulted in a small (14%) but significant ($p < 0.05$) increase in the rate of salicylate transfer based on paired comparisons. Incubation of the intestinal segments in 25 m*M* EDTA at pH 7.4 resulted in a larger (29%) increase in the transfer rate of salicylate when compared to control values. Comparison of the transfer rates of salicylate after incubation of the sement at pH 6.0 and 7.4 in the presence of EDTA resulted in a significant difference ($p < 0.05$) between the two experimental conditions.

Influence of Divalent Cation Rinse on the Reversibility of the EDTA and Taurodeoxycholate Effects on Salicylate Transfer Rate—The data in Table III show that rinsing with 100 m*M* Mg^{++} after exposure to EDTA had little effect in reversing the change in membrane permeability induced by EDTA. In more limited studies as to the effects of a Ca^{++} rinse after exposure of the intestinal segment to 1 m*M* STDC there was again no indication of reversibility. In fact rinsing with 154 m*M* Ca^{++} yielded transfer rates in two experiments which were somewhat higher than observed in paired control segments which were treated with bile salt and then rinsed with 154 m*M* Na^{+}.

Effect of Divalent Substituted EDTA on Transfer Rate of Salicylate—Incubating the intestinal segments in EDTA, calcium EDTA, or magnesium EDTA results in an increase in salicylate transfer rates compared to control levels. The data shown in Table III indicate no discernible difference however between the three salts of EDTA with respect to their effect on salicylate transfer.

A reduction in the EDTA effect on membrane permeability to salicylate was observed only when the intestinal segment was incubated in 25 m*M* magnesium EDTA to which was added an additional amount of calcium chloride to make the solution 25 m*M* with respect to Ca^{++}. Statistical comparison of the salicylate transfer rates in intestinal segments exposed to 25 m*M* magnesium EDTA plus 25 m*M* Ca^{++} or 25 m*M* EDTA indicated that the former treatment resulted in significantly decreased rates, though the rates were still somewhat higher than control levels. The addition of excess calcium chloride to 25 m*M* calcium EDTA resulted in precipitation which was due to an interaction between excess Ca^{++} and the phosphate components of the buffer.

DISCUSSION

The effect of various concentrations of STDC on the rate of transfer of salicylate across the everted rat intestine has been discussed in a previous report (1). There appears to be two levels of bile salt effect on the transfer rate of salicylate; a relatively small effect at concentrations of STDC below or about the CMC and a more pronounced effect at concentrations of STDC above the CMC. The results of the experiments with salicylamide at various concentrations of STDC did not show the two levels of effects which were observed with salicylate. The increase in salicylamide transfer rate was small (11–21%) and apparently independent of STDC concentration from 5–100 m*M* STDC (see Table I).

The marked qualitative and quantitative differences in the STDC effects on salicylamide and salicylate transfer leads one to suspect that the two molecules traverse the membranes in a markedly different manner. The experimental conditions were such that salicylate existed as the ionized species while salicylamide existed in the unionized form. Hence, although the two species have essentially the same molecular weight, a major difference exists in their respective lipid solubilities. The same STDC-induced membrane alteration results in a greater than two-fold increase in the transfer rate of the ionized species (salicylate) (1) but less than a 25% increase in the transfer rate of the unionized drug (salicylamide). Based on these findings the authors are presently investigating the use of bile salts as a probe for differentiating absorption mechanisms.

In the previous report (1) it was suggested that the below-CMC effects of STDC on membrane permeability may be due to a depletion of membrane calcium by the bile salt similar to the presumed mechanism of EDTA-mediated membrane effects. The influence of EDTA on both salicylate and salicylamide transfer was therefore studied to explore further the possibility of a common mechanism. These studies also demonstrated, as shown in Table II, that alteration of the membrane produces different effects on the transfer rate of the ionized and unionized drugs. Incubation of the intestinal segments in 25 m*M* EDTA either at pH 6.0 or 7.4 resulted in no change in salicylamide transfer rates compared to control values. On the other hand, EDTA-incubation significantly increased salicylate transfer rates. In addition, the effects of EDTA on salicylate transfer were significantly greater at pH 7.4 than at pH 6.0. This difference may correspond to the increased binding capacity of EDTA for divalent ions with increasing pH. Raising the level of EDTA to 50 m*M* at pH 6.0 produced no further increase in salicylate transfer rates.

Interestingly, all previous reports on the effects of EDTA on the permeability of biologic membranes utilized ionized and/or highly water soluble compounds as the absorption tracer. Windsor and Cronheim (8) observed an increase in the absorption of heparin from the gastrointestinal tract of the rat and of the dog in the presence of EDTA. Schanker and Johnson (9) report similar effects of EDTA on the absorption of mannitol, inulin, decamethonium, sulfanilic acid (at pH 7.4), and edetate ion in the rat. Tidball (10) has reported an increase in the absorption of phenol red from the rat intestine in the presence of EDTA. The lack of effect of EDTA on salicylamide transfer, reported in the present study, suggests the possibility that the membrane alteration mediated by EDTA may not influence the rate-limiting barrier to the transfer of lipid-soluble drugs through the everted intestinal preparation.

Previous reports indicate a reversibility of the EDTA-mediated effect on drug absorption. Tidball (10) was able to reduce the increase in phenol red absorption in the rat intestine after EDTA exposure, by rinsing the intestine with calcium chloride or mag-

Table III—Reversibility or Blocking by Divalent Cations of the Effects of 25 m*M* EDTA and 1 m*M* Sodium Taurodeoxycholate (STDC) on Transfer Rate of Salicylate Across the Rat Small Intestine[a]

Experimental Conditions	No. of Intestinal Segments	Mean Transfer Rate ± *SD* mcg./min.
EDTA, 154 m*M* Na^{+} rinse	6	39 ± 7
EDTA, 100 m*M* Mg^{++} rinse	6	37 ± 5
EDTA	4	41 ± 4
Ca-EDTA	2	41 (40, 42)
Mg-EDTA	2	42 (39, 44)
EDTA	4	43 ± 7
Mg-EDTA + 25 m*M* Ca^{++}	4	38 ± 8
STDC, 154 m*M* Na^{+} rinse	1	46
STDC, 15.4 m*M* Ca^{++} rinse	1	44
STDC, 154 m*M* Na^{+} rinse	2	39, 35
STDC, 154 m*M* Ca^{++} rinse	2	44, 39

[a] Data grouped according to paired segments.

Table IV—Effect of Simultaneous Treatment with Sodium Taurodeoxycholate (STDC) and 25 m*M* EDTA on Transfer Rate of Salicylate Across the Rat Small Intestine

Incubation Medium	No. of Intestinal Segments	Mean Transfer Rate ± *SD* mcg./min.
STDC, 5 m*M*	2	(57, 55)
STDC, 5 m*M* + EDTA	2	(76, 61)
STDC, 50 m*M*	4	83 ± 4
STDC, 50 m*M* + EDTA	4	93 ± 8[a]

[a] Significantly different from results at 50 m*M* STDC ($p < 0.05$), Student's *t* test, method of paired comparisons.

nesium chloride solutions. Magnesium chloride was found to be much more effective in this respect. Tidball (10) also found that "calcium EDTA" had no effect on the permeability of the rat intestinal epithelium to phenol red. Windsor and Cronheim (8) report that substitution of a divalent cation (Mg^{++} or Ca^{++}) for monovalent cations (Na^+, K^+, or NH_4^+) in the EDTA molecule abolishes the effect of EDTA on heparin absorption. Based on these data it has been postulated that EDTA alters membrane permeability by depleting magnesium and calcium loosely bound in the structure of the membrane which regulate the aqueous permeability of the intestinal epithelium (10).

The effects of EDTA on salicylate transfer across the everted intestinal preparation were not reversible. Rinsing the intestine with magnesium chloride, after initial incubation in EDTA, did not modify the enhanced permeability of the membrane to salicylate transfer under the experimental conditions (see Table III). Furthermore, treatment of the intestinal preparation with magnesium EDTA or calcium EDTA resulted in a permeability increase equal in magnitude to that observed after incubation in unsubstitued EDTA (see Table III). Thus it appears that the permeability effects of EDTA on the everted intestinal preparation cannot be prevented or reversed by the addition of a divalent cation. However, a study by Cassidy and Tidball (11), in which the cellular mechanisms of intestinal alteration produced by chelation depletion were examined, showed that the stripped-mucosa content of magnesium and calcium was approximately 8 meq. divalent ions/kg. in controls and 5 meq./kg. in EDTA-treated animals, a change sufficient to markedly increase permeability to phenol red. Thus, little divalent cation need be removed to attain changes in permeability. It is therefore possible that the monosubstituted divalent EDTA still retained sufficient chelating ability to produce an effect on salicylate transfer. To investigate this possibility, the intestinal segment was incubated in 25 m*M* magnesium EDTA and 25 m*M* Ca^{++}. As shown in Table III, there is a significant decrease in the transfer rate of salicylate under these experimental conditions.

The failure to reverse the EDTA effect on salicylate transfer by a divalent cation rinse prevents a definitive conclusion as to the mechanism of membrane alteration in the isolated intestine. Although apparent saturation of the EDTA molecule with magnesium and calcium did reduce the activity, which suggests a possible role for divalent cations in the overall mechanism, other possibilities must also be considered. For example, edetate ion itself may be responsible for the effects on membrane permeability by some disrupting influence on the organization of the membrane which does not involve chelation. Saturation of the binding sites with divalent cations may then merely reduce the effective concentration of edetate ion. A more attractive hypothesis is that EDTA does function *via* a chelation mechanism but the nature of the intestinal preparation itself precludes the possibility of reversibility, perhaps by not providing an endogeneous source of mobile calcium.

Attempts to study the influence of divalent cations on the membrane permeability effects of STDC were largely unsuccessful. As noted in Table III, rinsing the prepartion with calcium chloride solution, after exposure to STDC, provided no evidence of reversibility. Addition of Ca^{++} or Mg^{++} directly to STDC solutions, to effect a blocking, resulted in precipitation of the divalent salts of the bile acid. Exposure of the intestinal segment to 5 m*M* STDC to which was added Ca^{++} sufficient to just avoid precipitation (4–5 m*M*) yielded salicylate transfer rates identical to those observed after exposure of the segment to 5 m*M* STDC alone.

Since both EDTA and STDC markedly affect the transfer rate of salicylate but have little or no effect on the transfer rate of salicylamide the possibility arises that they share a common mechanism. To resolve this point, intestinal segments were incubated in solutions containing 25 m*M* EDTA and 5 m*M* or 50 m*M* STDC, at pH 6.0. A paired segment was incubated in bile salt alone. After a 1-hr. incubation, salicylate transfer rates were determined in the usual manner. The results are shown in Table IV. In each case the addition of EDTA to a solution of STDC resulted in a further increase in transfer rate of salicylate compared to that observed with STDC alone. If EDTA and STDC acted *via* the same mechanism then one would not anticipate an increase in transfer rate since 25 m*M* EDTA and 10 m*M* STDC represent the minimum concentrations required to attain maximum changes in permeability in the respective systems (1). Based on these findings there probably is a difference in the mechanism by which EDTA and STDC affect membrane permeability.

The results of these investigations suggest that: (*a*) both STDC and EDTA increase the permeability of the everted rat small intestine to ionized drugs but have little or no effect on the transfer of unionized drugs; (*b*) there may be at least two routes for the transfer of drugs across the intestinal membranes; and (*c*) EDTA and STDC alter membrane permeability by different mechanisms.

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