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ILEAL TRANSPORT OF BILE ACIDS CONJUGATED WITH NORLEUCINE AND LYSINE

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SUMMARY

Cholic and deoxycholic acid were conjugated to the α-amino group of L-lysine to form bile salt derivatives possessing both positive and negative charges on the side chain. These amphoteric bile salts were tested for transport by the bile salt transport system. The everted guinea pig gut sac preparation was used. The introduction of a positive charge on the side chain of a derivative already possessing a negative charge resulted in a marked inhibition, or possible elimination, of its ability to be pumped against a chemical gradient by the intestinal cells. In the case of the deoxycholyl conjugate of lysine an interaction with the bile salt transport system was suggested as evidenced by its apparent ability to enter the ileal mucosal cells, but not the mucosal cells from proximal tissue, and by its ability to inhibit the transport of taurocholate. These observations agree with the idea that the active site of the transport carrier carries a negative and positive charge in proximity to each other which function in the normal uphill transport process of anionic bile salts. When this active site is occupied by a substrate bearing both types of charges, transport against a concentration gradient would not be expected to take place.

INTRODUCTION

Previous structure activity studies of the ileal bile salt transport system have implicated the charged state on the side chain of the bile salt substrate as a critical structural factor in active transport. It was suggested that a single negative charge on the side chain is an absolute requirement for transport[1-3]. In order to test this idea further we have studied the interaction of amphoteric bile salt derivatives (bearing a positive and a negative charge on the side chain) with the ileal bile salt transport system.

METHODS

Materials

Cholic acid and deoxycholic acid used for the preparation of conjugated bile salts were recrystallized before use as previously described[1]. ε-*N*-Carbobenzoxy-L-lysine and L-norleucine were obtained from Nutritional Biochemicals Corp. and

were used without additional purification. [24-^{14}C]Deoxycholic and [24-^{14}C]cholic acid were obtained from Mallinckrodt Nuclear and New England Nuclear Corp., respectively.

Synthesis of α-*N*-cholyl-L-lysine and α-*N*-deoxycholyl-L-lysine: In separate reactions cholic acid and deoxycholic acids were conjugated with ε-*N*-carbobenzoxy-L-lysine by means of the general mixed anhydride method of Norman[4]. The product consisted of the bile acid conjugated to the α-amino group of lysine with the protecting carbobenzoxy group still attached to the ε-amino group. Removal of the carbobenzoxy groups was accomplished by dissolving the materials in 95% acetic acid, adding palladium black, and bubbling H_2 through the solution until CO_2 evolution ceased. The reaction mixtures were filtered to remove the catalyst, and the impure products were recovered by evaporation of the solvent *in vacuo*. Final purification was effected by the reverse phase column chromatographic procedure of Norman[5]; Solvent system C was found to be effective[5]. The eluant fractions were monitored by thin-layer chromatography employing solvent system 2 of Hofmann[6]. In this manner, materials were obtained which were chromatographically pure. The products were crystallized out of ethanol–ethyl acetate. Both amphoteric substances proved to be hygroscopic and apparently retained one molecule of water of hydration even after prolonged drying at 120° *in vacuo*. The theoretical values for elemental analysis include this water of hydration.

α-*N*-Cholyl-L-lysine: m.p. 164. Theory: C, 65.10; H, 9.65; N, 5.06. Found: C, 65.44; H, 9.61; N, 5.11%.

α-*N*-Deoxycholyl-L-lysine: m.p. 229. Theory: C, 67.10; H, 9.93; N, 5.21. Found: C, 67.43; H, 10.16; N, 5.92%.

N-Cholyl-L-norleucine and *N*-deoxycholyl-L-norleucine: Conjugation of the bile acids with norleucine was effected by the above cited method of Norman. However, the solubilities of the reaction products were sufficiently similar to the starting materials so that final purification by partition chromatography was not possible. Accordingly, the compounds were purified by large batch thin-layer chromatography. Effective separation of the products from organic impurities was accomplished with solvent system S II of Eneroth[7]. The material was eluted from the silica with ethanol and neutralized with NaOH. Recrystallization of the sodium salt was accomplished with ethanol, ethyl acetate. These compounds were used in all biological experiments. In order to obtain adequate amounts of material sufficiently free of traces of silica for elemental analysis, another synthetic approach was necessary. A general method for the preparation of conjugated bile salts was developed using the reagent of Belleau and Malek[8], *N*-ethoxycarbonyl-2-ethoxy-1,2-dihydroquinoline. The experimental conditions for this procedure, which allows one to start with the ethyl ester of amino acids, permit the ultimate separation of the conjugated bile acid ester from traces of starting materials. This procedure will be reported in detail in a separate communication. After hydrolysis and neutralization, the products were found to be identical with the material prepared by the method of Norman.

N-Cholyl-L-norleucine, sodium salt: m.p. 189–194. Theory: C, 66.27; H, 9.20; N, 2.57; Na, 4.23. Found: C, 66.41; H, 9.49; N, 2.21; Na, 4.13%.

N-Deoxycholyl-L-norleucine, sodium salt: m.p. 181–190. Theory: C, 68.28; H, 9.55; N, 2.63; Na, 4.37. Found: C, 68.20; H, 9.71; N, 2.53; Na, 4.25%.

Transport studies

The everted gut sac preparation of Wilson and Wiseman[9] was used in studying bile salt transport or interaction of the modified bile salts with the ileal bile salt transport system. These experiments employed the small intestine of fasted guinea pigs. Gut sacs made from the most distal, quarter of the small intestine have been shown previously to possess this transport system[1,10]. In certain experiments (*e.g.* Fig. 3) gut sacs were also made from regions representing the entire length of the small intestine since the proximal regions, which do not actively transport bile salts, can be utilized for specific controls. All bile salts and their derivatives were also prepared with ^{14}C and used as such when being tested as substrates in these gut sac incubations. Their concentrations in the mucosal and serosal compartments at the end of the incubations were determined by assay by liquid scintillation. Aliquots, 0.2 ml, were measured for radioactivity in a Beckman liquid-scintillation counter Model LS 150 equipped with an external standard. When the amphoteric bile salts (lysine conjugates) were tested for their ability to inhibit the transport of taurocholate, the latter compound was present with its ^{14}C label. The other substances were, of course, not radioactive.

In this type of preparation, everted gut sac, evidence for ability to transport solute against a concentration is the generation of final serosal to mucosal concentration ratios in excess of unity. In addition, the ability of solute to leave the mucosal fluid may be ascertained in evaluating transport phenomena. Such measurements must consider possible changes in the volume of fluid remaining on the mucosal side. When such measurements were made[10] in comparable incubations, the changes in water were found to be sufficiently low to allow the assumption of constancy (10 ml) for all calculations.

RESULTS

Fig. 1 depicts the results of experiments comparing the transport of glycocholate with *N*-cholyl-L-norleucine and α-*N*-cholyl-L-lysine by everted gut sacs prepared from guinea pig ileum. The cholylnorleucine is actually transported by everted gut sacs, as evidenced by the establishment of final serosal to mucosal concentration ratios in excess of 1. Furthermore, the amount of cholylnorleucine leaving the mucosal compartment, 0.71 μmole, is comparable to the amount of glycocholate transported, 0.72 μmole. In contrast, the lysine conjugate is apparently not moved in the direction of the serosal compartment as evidenced by the fact that the final serosal to mucosal concentration ratio is less than one, and in this instance virtually no material left the mucosal compartment.

Fig. 2 represents a similar experiment except that this series of analogues is based on the dihydroxy bile acid, deoxycholic acid. It would appear that *N*-deoxycholyl-L-norleucine is capable of being actively transported but possibly not as effectively as *N*-cholyl-L-norleucine (Fig. 1). Thus, less material, 0.57 μmole, left the mucosal space when compared with glycodeoxycholate, 0.65 μmole. The final serosal–mucosal concentration ratios for *N*-deoxycholyl-L-norleucine while in excess of one, is less than that observed with the norleucine conjugate of cholic acid. The dipolar derivative, α-*N*-deoxycholyl-L-lysine evidently cannot be transported against its concentration. However, in contrast to the result found for α-*N*-cholyl-L-lysine (Fig. 1), a significant amount of substance did leave the mucosal space.

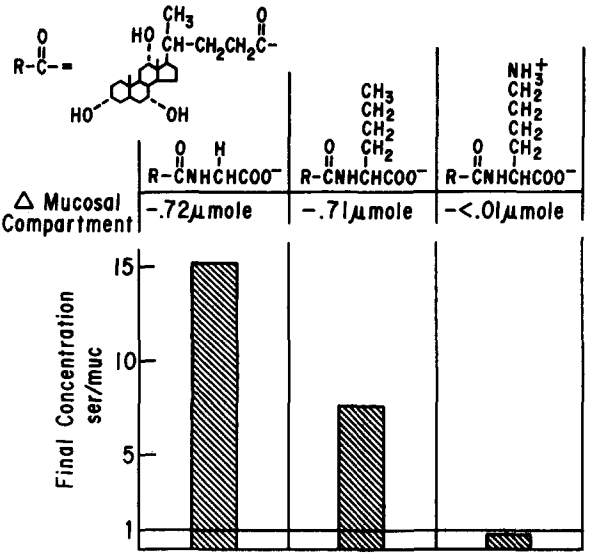

Fig. 1. Comparison of glycocholate transport with that of cholyl-L-norleucine and α-*N*-cholyl-L-lysine. Gut sacs were prepared from the distal ileum, as described[1]. Four animals were used and staggered to obviate differences between animals[13]. Each bar (—) represents the average of 4 gut sacs. Time of incubation 90 min, temp. 37°. Also shown is the amount of material removed from the mucosal compartment. Initial concentration of substrate 50 μg/ml in both serosal and mucosal compartments. Volume of mucosal fluid 10.0 ml. Volume of serosal fluid 1.5 ml.

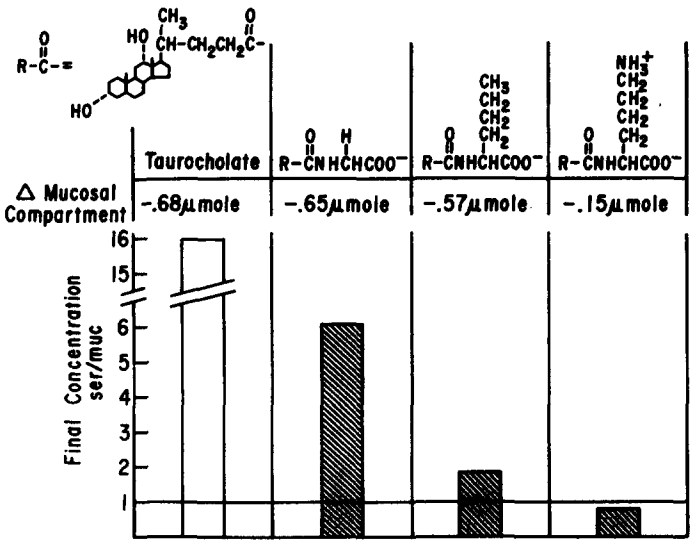

Fig. 2. Comparison of the transport of taurocholate with that of glycodeoxycholate, *N*-deoxycholyl-L-norleucine, and α-*N*-deoxycholyl-L-lysine. Conditions are the same as those described for Fig. 1.

Fig. 3 demonstrates that only gut sacs made from the distal small bowel can affect the disappearance of α-*N*-deoxycholyl-L-lysine from the mucosal fluid. The average final serosal to mucosal ratios for 4 gut sacs from the most distal regions was 0.84 (range 0.78–0.89). Simultaneous control gut sac incubations were performed with sodium taurocholate in order to demonstrate that the particular tissue employed in these experiments was capable of active transport. These control sacs were taken from ileal sections of intestine immediately adjacent to the experimental sacs. It should be noted that in order to ensure that maximum serosal to mucosal ratios be attained, all the incubation periods were run for 90 min and the incubation fluids were prepared with 5 μmoles sodium succinate per ml. These are conditions which enhance the final ratios[1,10,13].

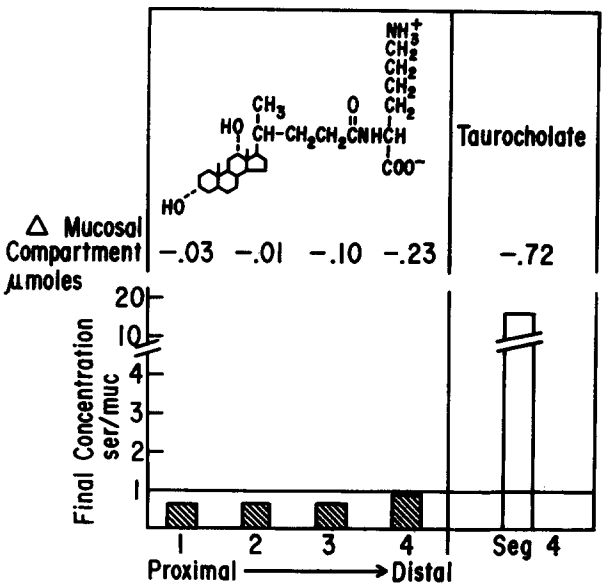

Fig. 3. The interactions of α-*N*-deoxycholyl-L-lysine with gut sacs from various regions of the guinea pig small bowel. 9-cm gut sacs were made from the center of each quarter of intestine[1]. The incubations with taurocholate employed similar sacs taken from the most distal quarter adjacent to the experimental sacs. Concentrations of substrates 0.50 μg/ml in both serosal and mucosal compartments. Initial volume mucosal fluid 10.0 ml, serosal 1.5 ml, time 90 min, temp. 37°.

The results presented in Table I show that the presence of α-*N*-cholyl-L-lysine does not interfere with the transport of taurocholate, while α-*N*-deoxycholyl-L-lysine at equivalent concentrations lowered the transport of the natural substrate, taurocholate.

DISCUSSION

Previous *in vitro*[1,2] and *in vivo*[3] studies have demonstrated that bile salts bearing two potential negative charges on the side chain are transported by the intestinal (ileal) bile salt transport system less readily than their natural analogues which have single negative charges in this region of the molecule. The small amount

TABLE I

INTESTINAL TRANSPORT OF TAUROCHOLATE IN THE PRESENCE OF ZWITTERION DERIVATIVES OF BILE SALTS

Incubation was 60 min at 37°. Each value represents the average of 4 gut sacs. Ileal gut sacs were used as described[1].

Taurocholate (*μmole/ml*)	*Inhibitor* (*μmole/ml*)	*Formula*	*Serosal/ mucosal ratio*	*Taurocholate transported* (*μmoles*)	% *Control*
0.37	0	—	6.3	2.26	
0.37	0.64	NH_3^+ CH_2 CH_2 CH_2 CH_2 Cholyl–NHCHCOO⁻	6.6	2.33	103
0.37	0.66	NH_3^+ CH_2 CH_2 CH_2 CH_2 Deoxycholyl–NHCHCOO⁻	2.3	1.11	49

of transport observed with these unnatural compounds could represent transport of those molecules present in solution at a particular pH in their singly charged state.

The above cited *in vivo* and *in vitro* experiments have also shown that the relative transport of these dibasic bile salt derivatives increases when the pH of the fluid bathing the mucosal cells is lowered. Since more of the singly charged species will exist in solution at lower pH, these findings support this idea that only singly charged bile salt molecules are transported. It should be noted that transport was not compromised when the second negative charge was introduced into the molecule at a site other than the side chain[11]. Finally, when taurocholate was modified in a manner where its sulfonate radical was replaced by a positively charged quaternary amine, a compound was obtained which was not transported by everted gut sacs and which did not interfere with the transport of natural bile salts[1].

Considerations of reasons why a second negative charge properly placed is so dramatically inhibitory led to our current operational hypothesis based on the idea that, under normal conditions, the bile salts' negative charge interacts with a positive charge on the membrane carrier. It is speculated that closely associated with this positive charge is a negatively charged region or radical, which repels those synthetic substrates which had two negative charges. Furthermore, this negative charge might interact with Na^+ in such a way as to function in the overall concentrating transport process. In order to initiate some preliminary tests of this hypothesis (which at this point is speculative), we synthesized and tested the above described amphoteric bile salts. It should be noted that pH titration curves of both of these lysine conjugates indicated that the first pK was approx. 4.0, while that for the amine group (pK_2) was above 9.0. Thus, at the pH of our incubation medium, 7.3, these compounds carried both positive and negative charges.

Reference to Fig. 1 demonstrates that the introduction of a positively charged group into a bile salt already possessing a negative charge may significantly alter its transport properties. Cholyl-L-norleucine and α-*N*-cholyl-L-lysine while not isosteric, both have the same carbon skeleton. However, it is apparent that cholyl norleucine can be translocated from the mucosal to the serosal compartment against its own concentration while α-*N*-cholyl-L-lysine, which differs by having a potentially positive amine group, is not transported in these preparations. The final serosal–mucosal ratio was not in excess of one, and more to the point, no detectable amount of substrate left the mucosal compartment. Previous mutual inhibition studies[1,12] have shown that dihydroxy bile salts are better inhibitors of the transport of trihydroxy bile salts than the converse, implying that the apparent affinity of dihydroxy bile salts for the transport system is greater than that of the trihydroxy compounds. This fact was utilized in order to design an amphoteric bile salt derivative with sufficiently enhanced attraction to produce some apparent interaction between substrate and transport system.

When ileal gut sacs were incubated with glycodeoxycholate, deoxycholyl-L-norleucine, and α-*N*-deoxycholyl-L-lysine (Fig. 2), a pattern similar to that for the trihydroxy bile salts was observed except that a significant amount of the deoxycholyllysine ampholyte left the mucosal fluid. However, uphill translocation was not observed (average final serosal to mucosal concentration ratio 0.79, range 0.68–0.95).

Before any consideration can be given to the idea that this disappearance of substance from the mucosal space represents a specific interaction of the substrate with the transport system with facilitated penetration into the mucosal cell rather than non specific binding with mucosal tissue, additional facts had to be ascertained. This finding should only take place when gut sacs from distal regions of the small intestine are used since it is only in this region that the transport system is found. Additionally, α-*N*-deoxycholyl-L-lysine should inhibit the transport of natural bile salts, but the trihydroxy compound would be expected to be inactive in this regard. These expectations have been borne out by the experiments summarized in Fig. 3 and Table I (see Results).

The demonstration that a bile salt of this type can interact in a specific manner with the transport system but that this interaction is accompanied by a marked decrease or disappearance of its transport against a concentration gradient suggests that the presence of a positive charge on the substrate was in some manner responsible in bringing this about. These findings are in accordance with our current ideas that the active site of the transport carrier possesses positive and negative areas in proximity to each other which function in the transport process. When the active site is occupied by a bile salt also containing a positive and a negative charge, normal translocation against a concentration gradient would be expected to be disrupted.

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