Biochimica et Biophysica Acta, 291 (1973) 237–245

BBA 76106

DIFFERENT EFFECTS OF FREE AND CONJUGATED BILE ACIDS AND THEIR KETO DERIVATIVES ON (Na^+,K^+)-STIMULATED AND Mg^{2+}-ATPase OF RAT INTESTINAL MUCOSA

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(Received March 28th, 1972)
(Revised manuscript received August 21st, 1972)

SUMMARY

1. Rat intestinal Mg^{2+}-ATPase present in a semipurified brush border preparation or membrane fraction of a mucosal homogenate was stimulated by the free dihydroxy bile acids, chenodeoxycholic acid and deoxycholic acid; the keto derivatives of cholic acid, a trihydroxy bile acid, caused an even greater stimulation.

2. The glycine or taurine N-conjugates of these dihydroxy bile acids inhibited Mg^{2+}- and (Na^+,K^+)-stimulated ATPase.

3. Lauric and oleic acid stimulated Mg^{2+}-ATPase but the glycine and taurine N-conjugates of lauric acid inhibited both Mg^{2+}- and (Na^+,K^+)-ATPases.

4. Bile acids influenced the rate of phosphate release at a concentration below the critical micellar concentration (CMC). Effects were linearly related to both concentration and time.

5. The data suggest that activation of Mg^{2+}-ATPase by surface-active agents such as dihydroxy bile acids and fatty acids is related to their pK_a, since conjugates with a lower pK_a were inhibitory.

INTRODUCTION

Since Skou[1] first described (Na^+,K^+)-stimulated ATPase in crab nerve, there has been increasing evidence that this enzyme is involved in active transport across biologic membranes. Post *et al.*[2] and Dunham and Glynn[3] showed a close correspondence between several enzyme and transport properties in the erythrocyte cell membrane and concluded that (Na^+,K^+)-ATPase was directly associated with the active transport of Na^+ and K^+ across the membrane.

Forth *et al.*[4] showed that the dihydroxy bile acids, deoxycholic acid and chenodeoxycholic acid, inhibit salt and water absorption in the rat intestine. Mekhjian *et al.*[5] found that these acids, both free and conjugated, caused secretion of salt and water by the perfused human colon. Parkinson and Olson[6], and Faust and Wu[7]

Abbreviation: CMC, critical micellar concentration.

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found that the trihydroxy conjugated bile acids, cholylglycine and cholyltaurine, inhibited active transport of the perfused rat jejunum *in vitro*; Gracey *et al.*[8] showed that free cholic and deoxycholic acids inhibited glucose transport in rat jejunum but that cholyltaurine was without effect. In hamster[9] or human[10] jejunum, only dihydroxy bile acids are inhibitory and inhibition occurs with both free and conjugated dihydroxy acids. Using an intestinal mucosal homogenate, Faust and Wu[11] observed an increase in both (Na^+,K^+)- and (Mg^{2+})-ATPase activities in the presence of cholylglycine and cholyltaurine, but Parkinson and Olson[6] found that cholylglycine inhibited rat small intestinal ATPase. Skou[12] found that low concentrations of deoxycholic acid inhibited (Na^+,K^+)-ATPase. This was confirmed by Pope *et al.*[13] who also found that deoxycholic acid inhibited jejunal transport. In their study, 8 mM cholylglycine and cholyltaurine also inhibited ATPase of rat mucosal homogenates and brush border preparations but had no effect on jejunal transport.

The detergent dodecyl sulfate has been shown to cause a reversible stimulation of human erythrocyte membrane ATPase[14]. Similar effects on brain ATPase were observed with several detergents, including deoxycholic acid[15]. Stimulation of (Na^+,K^+)-ATPase, from a microsomal fraction derived from the outer medulla of the rabbit kidney, by incubation with deoxycholic acid was described by Jørgensen and Skou[16].

We report here the effect of various free and conjugated bile acids and fatty acids, as well as keto derivatives of bile acids (which are not known to have any detergent effect), on rat brush border and membrane-bound ATPase. The aim of these experiments was to determine whether there was any correlation between the secretory properties of bile acids and their effect on intestinal ATPase.

EXPERIMENTAL

Male Sprague–Dawley rats were fed *ad libitum*; they weighed 200 to 300 g when they were killed, by a blow on the head. The small intestine was removed and irrigated with cold 0.9% NaCl–5 mM EDTA (pH 7.4). All further procedures were carried out at 4 °C. The mucosa was removed by scraping, and the scrapings were homogenized as a 5% homogenate in 5 mM EDTA (pH 7.4) for 25 s in a Waring blender. The homogenate was filtered through coarse and fine nylon. Crude brush border was sedimented by centrifugation at 700 × *g* for 10 min. This fraction was washed three times with 2.5 mM EDTA (pH 7.4), and the final pellet was suspended in 50 mM Tris (pH 7.1) without EDTA. A gelatinous, flocculent material appeared and was removed by filtration through glass fiber as suggested by Harrison and Webster[17]. The brush border fraction was stored at −20 °C until used in the assay.

The supernatant solution from the 700 × *g* centrifugation was centrifuged again at 10000 × *g* for 10 min and the pellet was resuspended in 2.5 mM EDTA (pH 7.4). This fraction is equivalent to the Fraction II of Quigley and Gotterer[18] and is referred to in this paper as cytoplasmic ATPase. It was washed twice with 50 mM Tris (pH 7.1) to remove the EDTA. The final pellet was resuspended in 50 mM Tris (pH 7.1) and stored at −20 °C until used in the assay.

Enzyme assay

ATPase was measured under two conditions: in the presence of Mg^{2+}, Na^+,

and K^+ ("total ATPase") and in the presence of Mg^{2+} alone ("Mg^{2+}-ATPase"). The (Na^+,K^+)-ATPase activity was computed as the difference between these. Within experimental error, the Mg^{2+}-ATPase was the same as the ouabain-insensitive ATPase, the ATPase activity in the presence of Mg^{2+}, Na^+, K^+, and ouabain. All assays were performed in duplicate, and the results are expressed as the mean of six assays. For assay of total ATPase, the system was at pH 7.1 and contained 30 mM Tris, 2 mM ATP (Sigma Chemical Co.), 7.5 mM $MgCl_2$, 120 mM NaCl, and 20 mM KCl. For the measurement of the ouabain-insensitive ATPase, 1 mM ouabain was added to this system. The Mg^{2+}-ATPase was measured in a system at pH 7.1 and containing 30 mM Tris, 2 mM ATP, and 7.5 mM $MgCl_2$. Bile acids, fatty acids, and keto derivatives of bile acids were neutralized with Tris and added to the assay in concentrations of 0.01 to 10 mM. The agents had no effect on the phosphate determination. The total volume was 1.0 ml and the incubation, unless otherwise specified, was for 30 min at 37 °C. The reaction was terminated by adding 0.25 ml of 1.0 M $HClO_4$ and immersing the tubes in an ice bath for several min. Perchlorate and protein were then co-precipitated by the addition of 0.25 ml of 1.0 M KCl. The tubes were centrifuged for 10 min in a clinical centrifuge, and an aliquot of the supernate was assayed for phosphate by the method of Chen *et al.*[19].

The optimal pH and concentrations of ions were determined in preliminary experiments. ATP free of Na^+ and neutralized with Tris (Sigma Chemical Co.) was used in all experiments. In experiments in which CTPase, GTPase, and ITPase were assayed, the experimental conditions were the same as for ATPase except that cytosine 5′-triphosphate, guanosine 5′-triphosphate, and inosine 5′-triphosphate (Sigma Chemical Co.) were used as the substrate in 2 mM concentration instead of ATP.

Alkaline phosphatase was measured with β-glycerophosphate as substrate. The assay system contained 30 mM glycine buffer (pH 9.1), 10 mM β-glycerophosphate, 5 mM $MgCl_2$, and 1 mM $ZnCl_2$, in a total volume of 1.0 ml. Incubation was for 5 min at 37 °C. The reaction was terminated and phosphate analysis was performed in the same manner as for the ATPase assay.

Protein determination was performed by the method of Lowry *et al.*[20] with bovine serum albumin as a standard.

Agents tested

The free bile acids tested were the Tris salts of cholic acid, deoxycholic acid, and chenodeoxycholic acid. The conjugated bile acids tested were cholylglycine (glycocholic acid), deoxycholylglycine (glycodeoxycholic acid), deoxycholyltaurine (taurodeoxycholic acid), and chenodeoxycholylglycine (glycochenodeoxycholic acid). Cholic acid was obtained from Matheson, Coleman and Bell, East Rutherford, N.J., deoxycholic acid was obtained from Sigma Chemical Co., St. Louis, Mo., and chenodeoxycholic acid was obtained from Weddell Pharmaceuticals, London. They were 98% pure when examined by thin-layer chromatography. These bile acids were conjugated with glycine or taurine by the method of Norman[21].

Dehydrocholic acid (3,7,12-triketo-5β-cholanoic acid) was prepared by chromic acid oxidation of cholic acid: 3α,7α-dihydroxy-12-keto-5β-cholanoic acid and 3-hydroxy-7,12-diketocholanoic acid were prepared by conventional methods[22] and were 98% pure when examined by thin-layer chromatography. The glycine conjugate of dehydrocholic acid was made by the method of Norman[21].

Lauric acid and oleic acid were obtained from Fisher Chemical Co. and the glycine and taurine conjugates of lauric acid were made by the method of Norman[21]. Dehydrocholylglycine, laurylglycine, and lauryltaurine all were more than 95% pure when examined by thin-layer chromatography.

RESULTS

Brush border ATPase: effect of different agents

Mg^{2+}-ATPase activity was stimulated 2-fold by the dihydroxy bile acids, deoxycholic and chenodeoxycholic acids, but only slightly by cholic acid. As a result, the specific activity after addition of those agents became higher than the control total ATPase (Table I). The keto derivatives of the free bile acids were extremely potent stimulators of brush border Mg^{2+}-ATPase, causing more than 5-fold stimulation above control Mg^{2+}-ATPase activity. Conjugated dihydroxy bile acids inhibited both Mg^{2+}-ATPase and (Na^+,K^+)-ATPase, and total ATPase was equal to Mg^{2+}-ATPase. The free bile acids and their keto derivatives had no effect on (Na^+,K^+)-ATPase, the increase in total ATPase activity being entirely reflected in the increase in Mg^{2+}-ATPase.

TABLE I

BRUSH BORDER ATPase

Results shown as μmoles P_i/mg protein per h. Brush border fraction, containing 0.05 to 0.25 mg of protein, was incubated for 15 min (see text for media). The final concentration of each agent was 5 mM, except for lauric and oleic acids it was 0.5 mM.

Agent	*Mg^{2+}-ATPase*	*(Na^+,K^+)-ATPase*	*Total ATPase*
Control (Tris)	14	12	26
Deoxycholic acid	34	12	46
Chenodeoxycholic acid	30	12	42
Cholic acid	18	6	24
3α,7α-Dihydroxy-12-keto-5β-cholanoic acid	96	18	114
3α-Hydroxy-7,12-diketo-5β-cholanoic acid	96	18	114
Deoxycholylglycine	4	0	4
Deoxycholyltaurine	4	0	4
Chenodeoxycholylglycine	4	0	4
Cholylglycine	14	12	26
Cholyltaurine	18	12	30
Lauric acid	32	18	50
Laurylglycine	4	0	4
Lauryltaurine	4	0	4
Oleic acid	36	14	50
Dehydrocholic acid	68	8	76
Dehydrocholylglycine	26	12	38

Lauric and oleic acids at 0.5 mM stimulated Mg^{2+}-ATPase like the free dihydroxy bile acids. The amino acid conjugates of lauric acid inhibited total ATPase like the conjugated dihydroxy acids at the concentration used (10 mM).

Cholyltaurine slightly stimulated Mg^{2+}-ATPase, like free cholic acid, but cholylglycine was without effect. Dehydrocholylglycine stimulated Mg^{2+}-ATPase,

but this stimulation was much less marked than the stimulation by free dehydrocholic acid.

Cytoplasmic ATPase: effect of different agents

The free dihydroxy bile acids stimulated Mg^{2+}-ATPase (Table II), but the stimulation was much less marked than with brush border ATPase. Free cholic acid inhibited cytoplasmic Mg^{2+}-ATPase without affecting (Na^+,K^+)-ATPase. The conjugated dihydroxy bile acids inhibited total cytoplasmic ATPase. The keto derivatives of the free bile acids were more potent stimulators of cytoplasmic Mg^{2+}-ATPase than the free dihydroxy bile acids, but this stimulation was less marked than their stimulation of brush border Mg^{2+}-ATPase. Free lauric and oleic acids stimulated cytoplasmic ATPase, like the free dihydroxy bile acids, and the amino acid conjugates of lauric acid inhibited total ATPase, like the conjugates of the dihydroxy acids.

TABLE II

CYTOPLASMIC ATPase

Experimental conditions as in Table I.

Agent	*Mg^{2+}-ATPase*	*(Na^+,K^+)-ATPase*	*Total ATPase*
Control (Tris)	15	13	28
Deoxycholic acid	21	7	28
Deoxycholylglycine	2	0	2
Deoxycholyltaurine	2	0	2
Chenodeoxycholic acid	23	5	28
Chenodeoxycholylglycine	2	0	2
Cholic acid	8	13	21
Cholylglycine	12	11	23
Cholyltaurine	12	12	24
Lauric acid	22	12	34
Laurylglycine	2	0	2
Lauryltaurine	2	0	2
Oleic acid	25	14	39
Dehydrocholic acid	28	12	40
3α,7α-Dihydroxy-12-keto-5β-cholanoic acid	48	12	60
3α-Hydroxy-7,12-diketo-5β-cholanoic acid	48	14	62
Dehydrocholylglycine	18	12	30

Effect of agent concentration

There was a linear relationship between the effect of the agent and its concentration, but the concentration at which maximal and minimal effects were shown varied from agent to agent (Figs 1 and 2). The agents that inhibited Mg^{2+}-ATPase, the conjugated dihydroxy bile acids, were maximally effective at about 5 mM and had little effect below 1.0 mM. The inhibition of (Na^+,K^+)-stimulated ATPase by conjugated dihydroxy bile acids and lauric acid paralleled the inhibition of Mg^{2+}-ATPase and appeared at a concentration in the ranges of the critical micellar concentration (CMC)[23]. On the other hand, the stimulatory agents, free dihydroxy bile acids, were maximally effective at levels above the CMC and also were effective below the CMC. The detergent properties of the keto bile acid derivatives have not been well characterized, but their ability to solubilize lecithin is considerably

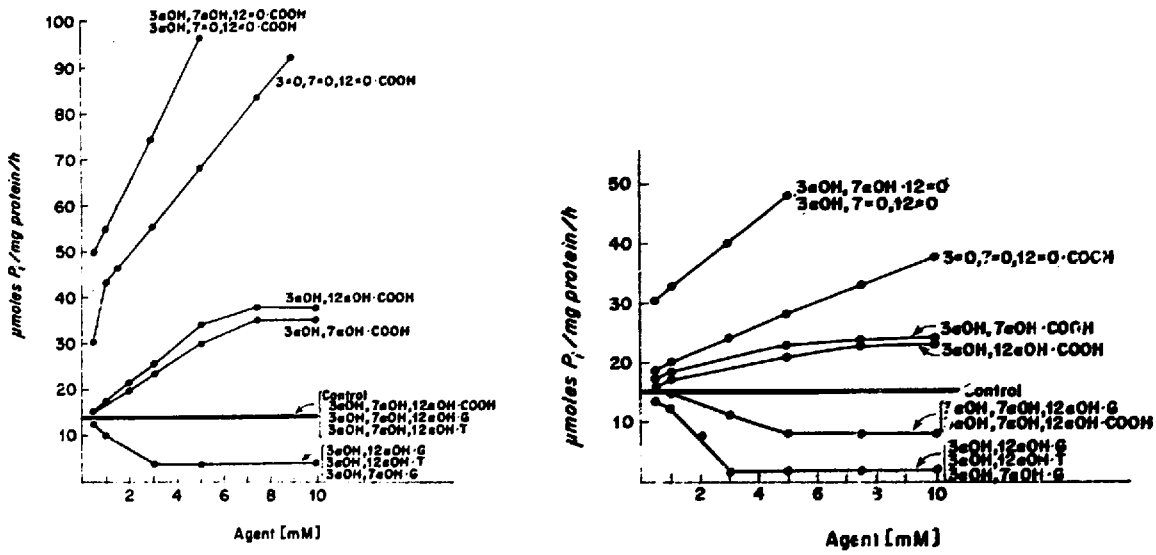

Fig. 1. Effect of different concentrations of agents on brush border Mg^{2+}-ATPase. The compounds tested, in order from top to bottom, were: 3α,7α-dihydroxy-12-ketocholanoic acid; 3α-hydroxy-7,12-diketocholanoic acid; 3α, 12α-dihydroxycholanoic acid (deoxycholic acid); 3α,7α-dihydroxycholanoic acid (chenodeoxycholic acid); 3α,7α,12α-trihydroxycholanoic acid (cholic acid); 3α,12α-dihydroxycholanoyl glycine (deoxycholylglycine); 3α,12α-dihydroxycholanoyl taurine (deoxycholyltaurine); 3α,7α-dihydroxycholanoyl glycine (chenodeoxycholylglycine).

Fig. 2. Effect of different concentrations of agents on cytoplasmic Mg^{2+}-ATPase. For agents, see Fig. 1.

inferior to that of the common (nonketo) bile acids[24] and, based on solubilization of azobenzene[25], the 3α,7α-dihydroxy-12-keto compound has a CMC greater than 100 mM (A. F. Hofmann, unpublished data).

Time course of effect of agents on Mg^{2+}-ATPase

The inhibition caused by the conjugated dihydroxy bile acids (Figs 3 and 4) and amino acid conjugates of lauric acid persisted during a 60-min incubation. The

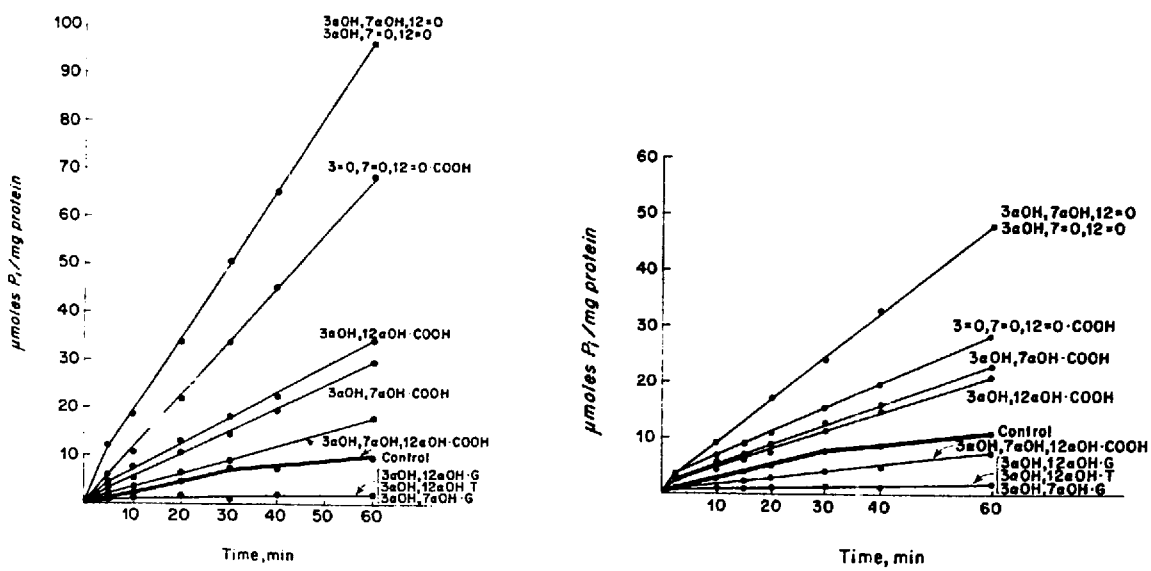

Fig. 3. Time course of effect of agents on brush border Mg^{2+}-ATPase. For agents, see Fig. 1.

Fig. 4. Time course of effect of agents on cytoplasmic Mg^{2+}-ATPase. For agents, see Fig. 1.

stimulated activity caused by the free dihydroxy bile acids and the keto derivatives of the bile acids, like the basal ATPase activity, was linear from 5 to 60 min.

Effect of agents on other substrates

Alkaline phosphatase was unaffected by any of the agents tested (Table III). CTPase and GTPase were affected similarly to ATPase, with stimulation by the free dihydroxy bile acids and the keto derivatives of the bile acids and inhibition by the dihydroxy bile acid conjugates. ITPase, on the other hand, was inhibited by both free and conjugated bile acids, whether these were dihydroxy or trihydroxy. Slight stimulation of ITPase occurred when the keto derivatives of the bile acids were tested.

TABLE III

EFFECT OF AGENTS ON BRUSH BORDER ENZYMES

Results shown as μmoles P_i/mg protein per h. Brush border containing 0.05 to 0.25 mg of protein was incubated for 15 min at 37 °C with 30 mM glycine buffer (pH 9.1), 10 mM β-glycerophosphate, 5 mM $MgCl_2$, and 1 mM $ZnCl_2$ for assay of alkaline phosphatase. For assay of CTPase, GTPase, and ITPase, 0.05 to 0.25 mg of protein was incubated for 15 min at 37 °C with 2 mM CTP, GTP, or ITP *plus* 30 mM Tris (pH 7.1), 7.5 mM $MgCl_2$, 120 mM NaCl, and 20 mM KCl. The final concentration of each agent was 5 mM, except for lauric acid it was 0.5 mM.

Agen.	*Alkaline phosphatase*	*CTPase*	*GTPase*	*ITPase*
Control (Tris)	55	12	15	64
Deoxycholic acid	55	31	38	44
Chenodeoxycholic acid	55	31	38	46
Cholic acid	55	14	20	48
Deoxycholylglycine	55	4	4	34
Cholylglycine	55	14	12	22
Lauric acid	55	40	19	84
Laurylglycine	55	3	3	22
Dehydrocholic acid	55	44	45	76
3α,7α-Dihydroxy-12-keto-5β-cholanoic acid	55	51	58	89
3α-Hydroxy-7,12-diketo-5β-cholanoic acid	55	56	60	92

DISCUSSION

The results indicate that, when an intestinal brush border preparation or a membrane fraction of cytoplasm is exposed to bile acids or their keto derivatives, ATPase may be stimulated. The mechanism of this stimulation is unknown but is possibly due to alteration of the type of binding of the enzyme to membranes, permitting greater exposure of enzyme to substrate. Since the stimulation occurs well below the CMC of the bile acids and since the stimulation also occurs when keto derivatives of the bile acids, which have no detergent effect, are used, it would seem that the disruptive effect of these agents is not related to their dispersant properties. Moreover, since conjugation of the stimulatory agents with glycine or taurine abolishes the stimulatory effect but should not alter surface activity greatly for a given degree of conjugation, it seems possible that the lower pK_a of the conjugated bile acids inhibits binding or other interaction with the brush border preparation.

Previous studies have shown that deoxycholate may stimulate (Na^+,K^+)-ATPase from different tissues[12–16], but this is the first study in which an effect on

Mg^{2+}-ATPase has been shown. Furthermore, the concentration of deoxycholic acid that stimulated ATPase in this study was as high as 10 mM, a concentration that is nearly 10 times the concentration used in other studies in which stimulation of ATPase by deoxycholate was shown. Jørgensen and Skou[16], who showed stimulation of kidney (Na^+,K^+)-ATPase by deoxycholate, used a concentration of 0.6 mg/ml (about 1.5 mM) but found that higher concentrations inactivated the enzyme.

This is the first instance in which the effect of bile acids and their keto derivatives on intestinal ATPase has been studied, except for the short studies by Parkinson and Olson[6], and Faust and Wu[7] who studied the effect of only cholylglycine and cholyltaurine. The concentration of bile acids in the lumen of the small intestine is normally about the concentration at which stimulation of ATPase was observed *in vitro* with free bile acids and inhibition was observed with conjugated bile acids. Normally, all bile acids excreted by the liver into bile are conjugated with either glycine or taurine, and they remain conjugated in the small intestine proximal to the ileum except when a stagnant loop is present[26]. Deconjugation of bile acids occurs only as a result of bacterial enzymes, and these bacteria, being fastidious anaerobes[27], occur only in the colon or terminal ileum[28]. It is possible that conjugation of bile acids may prevent their disrupting brush border enzymes such as ATPase. Our data do not support the speculation that inhibition of salt and water absorption by conjugated bile acids[9,10] is related to an effect of these agents on ATPase.

Keto bile acids, which are synthetic analogs of bile acids, are strong secretagogues of bile acids by the liver[29]. Their effect on small intestinal function is unknown but it is possible that their effect on hepatic bile secretion may be related to an effect on hepatic ATPase since our data indicate that they are marked stimulators of intestinal (Na^+,K^+) and Mg^{2+}-ATPase.

Glycine dihydroxy bile acids cause secretion of water and electrolytes from the human jejunum[10], and cholyltaurine appears to inhibit the release of pancreozymin from the intestinal mucosa[25]. These effects on jejunal mucosa occur despite the fact that little or no absorption of these bile acids actually occurs in the jejunum[30]; bile acids are reabsorbed almost exclusively in the ileum and colon[30]. Conceivably, bile acids may act on a cell membrane or brush border enzyme without passing into the cell. Such an action would be analogous to that proposed for a number of hormones which are believed to exert an action on surface receptors.

ACKNOWLEDGMENTS

We acknowledge the invaluable technical assistance of Miss Susan B. Coffin.

This investigation was supported in part by Research Grant AM-6908 from the National Institutes of Health, Public Health Service.

REFERENCES

1 J. C. Skou, *Biochim. Biophys. Acta*, 23 (1957) 394.
2 R. L. Post, C. R. Merritt, C. R. Kinsolving and C. D. Albright, *J. Biol. Chem.*, 235 (1960) 1796.
3 E. T. Dunham and I. M. Glynn, *J. Physiol., London*, 156 (1961) 274.
4 W. Forth, W. Rummel and H. Glasner, *Naunyn Schmiedebergs Arch. Pharmakol.*, 254 (1966) 364.

5 H. S. Mekhjian, S. F. Phillips and A. F. Hofmann, *J. Clin. Invest.*, 50 (1971) 1569.
6 T. M. Parkinson and J. A. Olson, *Life Sci.*, 3 (1964) 107.
7 R. G. Faust and S. L. Wu, *J. Cell. Physiol.*, 65 (1965) 435.
8 M. Gracey, V. Burke and A. Oshin, *Scand. J. Gastroenterol.*, 6 (1971) 273.
9 M. V. Teem and S. F. Phillips, *Gastroenterology*, 62 (1972) 261.
10 D. Wingate, S. F. Phillips and A. F. Hofmann, *J. Clin. Invest.*, in the press.
11 R. G. Faust and S. L. Wu, *J. Cell. Physiol.*, 67 (1966) 149.
12 J. C. Skou, *Biochim. Biophys. Acta*, 58 (1962) 314.
13 J. L. Pope, T. M. Parkinson and J. A. Olson, *Biochim. Biophys. Acta*, 130 (1966) 218.
14 P. C. Chan, *Biochim. Biophys. Acta*, 135 (1967) 53.
15 J. Somogyi, M. Budai, L. Nyírő, G. A. Kaluza, W. Nagel and F. Willig, *Acta Biochim. Biophys. Acad. Sci. Hung.*, 4 (1969) 219.
16 P. J. Jørgensen and J. C. Skou, *Biochim. Biophys. Acta*, 233 (1971) 366.
17 D. D. Harrison and H. L. Webster, *Biochim. Biophys. Acta*, 93 (1964) 662.
18 J. P. Quigley and G. S. Gotterer, *Biochim. Biophys. Acta*, 173 (1969) 456.
19 P. S. Chen, Jr, T. Y. Toribara and H. Warner, *Anal. Chem.*, 28 (1956) 1756.
20 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
21 A. Norman, *Ark. Kemi*, 8 (1955) 331.
22 O. Hammarsten, *Ber. Dtsch. Chem. Ges.*, 14 (1881) 71.
23 A. F. Hofmann, *Biochem. J.*, 89 (1963) 57.
24 R. D. Soloway, A. F. Hofmann, P. J. Thomas, L. J. Schoenfield and P. D. Klein, *J Clin. Invest.*, in the press.
25 J. R. Malagelada, V. L. W. Go, E. P. DiMagno and W. H. J. Summerskill, *J. Clin. Invest.*, 51 (1972) 59a.
26 S. Tabaqchali and C. C. Booth, *Lancet*, 2 (1966) 12.
27 K. Shimada, K. S. Bricknell and S. M. Finegold, *J. Infect. Dis.*, 119 (1969) 273.
28 S. L. Gorbach, *Gastroenterology*, 60 (1971) 1110.
29 H. O. Wheeler and K. K. King, *J. Clin. Invest.*, 51 (1972) 1337.
30 I. M. Weiner and L. Lack, in C. F. Code and W. Heidel, *Handbook of Physiology, Section 6: Alimentary Canal*, Vol. 3, Am. Physiol. Soc., Washington, D.C., 1968, p. 1439.