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Research Article

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Regulation of Hepatic Transport of Bile Salts

EFFECT OF PROTEIN SYNTHESIS INHIBITION ON EXCRETION OF BILE SALTS AND THEIR BINDING TO LIVER SURFACE MEMBRANE FRACTIONS

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ABSTRACT The overall transport of bile salts across the hepatocyte is characterized as a carrier-mediated process whose rate-limiting step is biliary secretion. Specific bile salt binding proteins have been identified in liver surface membrane fractions and were postulated to represent the initial interaction in bile salt translocation across both the sinusoidal and canalicular membranes. To test this hypothesis, cycloheximide was administered to rats to inhibit hepatic protein synthesis. 16 h after cycloheximide administration [^{14}C]leucine incorporation into hepatic protein was inhibited by 93% at 1 h and 47% at 12 h. However, values of liver function tests were not increased, although serum albumin, serum alanine aminotransferase, and alkaline phosphatase were significantly decreased. Light and electron microscopy did not demonstrate necrosis or fat accumulation. The latter demonstrated minimal disorganization of rough endoplasmic reticulum and occasional lamellar whorls. 16 h after cycloheximide administration bile salt independent bile flow, basal bile salt excretion, and basal bile flow were unaltered, but the maximum bile salt transport capacity was reduced to 62% of control and 24 h later to 38%. Decreased bile salt transport was reversible, for it returned to control values after 48 h, when hepatic protein synthesis was also normal. Maximum bromosulfophthalein (BSP) transport, on the other hand, was reduced after 16 h to only 85% of control. Both bile salt and BSP maximum transport capacities decreased with time during inhibition of protein synthesis, apparently following first order kinetics. It was estimated that their half-lives are 20 h for bile salt transport and 55 h for BSP transport. These different turnover rates suggest that cycloheximide does not decrease active transport through generalized hepatic dysfunction or alteration of high energy sources possibly required for transport. The maximum number of [^{14}C]cholic acid binding sites in liver surface membrane fractions was determined by an ultrafiltration assay. They were reduced to 68% of control after 16 h of cycloheximide and to 25% after 24 h. This reduction in the number of binding sites is apparently selective, for the activities of the liver surface membrane enzymes (Na^+-K^+)ATPase, Mg^{++}-ATPase, and 5'-nucleotidase were not significantly changed. The associated alterations in bile salt transport and the maximum number of binding sites after cycloheximide administration suggests that these receptors may be the bile salt carriers.

INTRODUCTION

Bile salts are efficiently extracted from the portal blood across sinusoidal membranes by hepatic parenchymal cells (1–3) and after intracellular translocation are transported across the canalicular membrane into bile (4). Hepatic uptake capacity for conjugated bile acids is 5–10 times greater than secretory capacity, and therefore transport across the canalicular membrane is generally assumed to be the rate-limiting step in their hepatic transport (4, 5). The initial rate of bile salt uptake is characterized by saturation kinetics, competitive inhibition, and a requirement for sodium (6–9). In contrast to the convincing evidence supporting a carrier-mediated uptake process, the excretory step is less well characterized. However, a high ratio of biliary to hepatic bile acid concentration, saturation kinetics, and competition with bile acids

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(10–14) also strongly indicate a carrier-mediated process at the canalicular membrane.

Specific bile acid binding sites in liver surface membrane fractions have been identified and characterized by binding techniques, and we have postulated that they represent the initial step in bile acid translocation across both the sinusoidal and canalicular membrane surfaces (15). The hypothesis that these binding sites represent the putative bile acid carrier is based on the following experimental observations: (*a*) specific bile acid binding is detected only in membrane fractions from liver, ileum, and kidney, tissues known to transport bile acids (15, 16), and (*b*) kinetics of the binding reaction and competition studies are consistent with the in vivo uptake process (3, 15).

Inhibition of protein synthesis has been a useful technique to study the physiologic role of rate-limiting steps in transport of a number of substances. This approach has been adopted in the present study to examine the possible role of liver surface membrane bile acid receptors in bile acid transport. This present study indicates that after inhibition of protein synthesis there is a reduction in both the maximum hepatic excretory capacity of bile salt (Tm)[1] and the maximum number of bile acid binding sites.

METHODS

Animals and materials. Male Sprague-Dawley rats (Charles River Breeding Laboratories, Inc., Wilmington, Mass.) weighing 150–250 g were used in all experiments. The animals were maintained in a ventilated room at 24°C with 12 h of light and dark in cages with aspen wood chip bedding (American Excelsior Co., Denver, Colo.). Rats were allowed free access to water and laboratory chow (Ralston Purina Co., St. Louis, Mo.) except 12 h before sacrifice to prevent aspiration of gastric contents. Cycloheximide (Sigma Chemical Co., St. Louis, Mo.) freshly dissolved in 0.9% NaCl to a final concentration of 1.5 mg/ml was injected (150 μg/100 g body weight [BW]) intraperitoneally, whereas control rats received saline. A second injection of cycloheximide (150 μg/100 g BW) was given at 10–12 h after the first injection in rats studied longer than 16 h to maintain protein synthesis inhibition.

[24-*carboxyl*-^{14}C]Cholic acid (45 mCi/mmol, >99% radiochemically pure) was obtained from New England Nuclear (Boston, Mass.). Taurocholate was obtained from Maybridge Research Chemicals, Cornwall, England. Purity (>95%) of bile acids was confirmed by thin-layer chromatography (17), AMP, ATP, ouabain octahydrate, bovine albumin fraction V (96–99% purity), EGTA, Tris, D-glucose-6-phosphate (monosodium salt), β-NADH (from yeast grade III), and hydroxy steroid dehydrogenase type I were obtained from Sigma Chemical Co. Azide (sodium salt), TCA, chloroform, ethanol, acetone, methanol (scintillation grade), and ammonium molybdate were obtained from Fisher Scientific Co. (Pittsburgh, Pa.). Hydrazine monohydrate (99–100% purity) was obtained from J. T. Baker Chemical Co. (Phillipsburg, N. J.).

Hepatic bile salt and bromosulfophthalein (BSP) maximal transport capacities. Maximal transport capacity for bile salts was determined as previously described.[2] Taurocholate was dissolved at a concentration of 45 mM in 0.9% NaCl containing 3% albumin and adjusted to pH 7.4 with 1 N NaOH. Bile salts were measured by using β-steroid dehydrogenase (18). Bile salt Tm was determined as the mean of the two highest consecutive values of bile salt secretion obtained during a steady-state period observed usually between 30 and 60 min after the beginning of the infusion of taurocholate and is expressed as micromoles per minute per 100 g BW.

Rats were anesthesized with pentobarbital (Nembutal, 4 mg/100 g BW, i.p.; Abbott Laboratories, North Chicago, Ill.), and bile immediately collected for 30 min during the infusion of only normal saline (basal period). Taurocholate was infused immediately after the basal period at a rate of 1.2 μmol/min per 100 g BW, with a Harvard pump (Harvard Apparatus Co., Inc., Millis, Mass.) through a femoral vein catheter (PE-50 polyethylene catheter, Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, N. J.). Greater infusion rates of taurocholate decreased both bile flow and bile salt secretion, and failed to raise Tm.

Bile was collected in preweighed tubes in 10-min periods for 90 min through a PE-10 polyethylene catheter located just proximal to the bifurcation of the common bile duct. During the experiment, animals were kept at 37 ± 0.5°C with a heating lamp, and temperature was monitored with a rectal thermometer. Bile salt independent bile flow (BSIBF), basal bile flow, basal bile salt secretion, and bile salt Tm were determined in controls and 10, 16, 20, and 24 h after the administration of cycloheximide. In additional experiments, bile salt Tm was determined 2 d after a single injection of cycloheximide to determine reversibility of the drug effect.

Immediately after cannulation of the common bile duct, bile was collected for 30 min while only saline was infused (basal). To determine BSP Tm, BSP (20 mg/ml dissolved in 0.9% NaCl) (Hynson, Westcott & Dunning, Inc., Baltimore, Md.) was infused immediately after this basal period at 0.30 μmol/min per 100 g BW through a femoral vein. A simultaneous infusion of bile salts to replace those lost by bile drainage was not performed. Bile was collected in 10-min periods and BSP was determined by addition of 0.1 N NaOH to aliquots and read at 580 Å wavelength. BSP Tm was expressed as micromoles per minute per 100 g BW from the mean of the two highest consecutive values during a steady-state period of exretion between 30 and 60 min after the beginning of the infusion. During this steady-state period, no further increase in BSP excretion was obtained with increased BSP infusion rates. Blood samples were taken at the end of the experiments, and the serum concentration of BSP and the percent conjugation determined. Conjugated and nonconjugated BSP were separated by thin-layer chromatography in silica gel G (New England Nuclear) by using acetone:H_2O:ammonium hyroxide (82:14:4 vol/vol) (19).

BSIBF determination. BSIBF is that volume of bile which theoretically is secreted in the absence of bile salt secretion. BSIBF was determined indirectly for each group of animals by extrapolating the calculated linear regression of bile flow (*y*-axis) vs. bile salt secretion (*x*-axis) to zero bile salt secretion (21). Values of slope, *y*-intercept (BSIBF), and correlation coefficient were calculated by using all the experimental values of bile flow and bile salt excretion obtained for each group of rats.

Liver surface membranes preparation. Liver surface

[1] *Abbreviations used in this paper*: BSIBF, bile salt independent bile flow; BSP, bromosulfophthalein; BW, body weight; Tm, maximum hepatic excretory capacity.

[2] Simon, F. R., M. C. Gonzalez, E. Sutherland, R. Davis, and L. Accatino. Reversal of ethinyl estradiol-induced bile secretory failure with Triton WR-1339. Submitted for publication.

membranes were prepared as previously described (15) according to the procedure of Neville through step 12 as described by Pohl et al. (21, 22). Then liver surface membrane fractions were washed once each with ice-cold 1 mM $NaHCO_3$ and 0.15 M NaCl to remove sucrose and loosely bound proteins. Liver surface membrane fractions were then resuspended in 1 mM $NaCHO_3$ and stored at −70°C for bile acid binding assays.

Enzyme and chemical assay. (Na^+-K^+)ATPase (ATP phosphohydrolase, EC 3.6.1.3) was determined in liver surface membrane fractions as previously described (23). Total ATPase was determined at pH 7.4 in a final volume of 2.0 ml and contained in millimolars: ATP, 5.0; Mg^{++}, 5.0; Na^+, 120; K^+, 12.5; Tris, 125; Cl^-, 137.5; azide, 5.0; and EGTA, 1.0. (Na^+-K^+)ATPase activity was determined by the difference between total ATPase and the activity inhibited by 1 mM ouabain. Mg^{++}-ATPase (EC 3.6.3.5) was that activity remaining after ouabain inhibition. Correction was made for the nonenzymatic breakdown of ATP, measured as inorganic phosphate. 5′-Nucleotidase activity (EC 3.1.3.5) was measured by the method of Song and Bodansky (24). Enzyme activities were determined by the initial rate of release of phosphorous (25) from appropriate substrates at 37°C and expressed as micromoles of phosphorous released per milligram of protein per hour. Protein was measured by the method of Lowry et al. (26), with bovine serum albumin as a standard.

Bile acid binding. The binding of [^{14}C]cholic acid (45 mCi/mmol) to surface membrane fractions was measured in an incubation medium containing 66 mM sodium phosphate buffer, pH 6.0, and 1.8 mM cholic acid as previously described (15). After incubating the tissue sample at 4°C for 20 min, the binding reaction was terminated by vacuum filtration of triplicate 0.05-ml aliquots through glass fiber disks (Whatman grade GF/C, W&R Balston Ltd., Maidstone, Kent, England). Filters retaining membrane [^{14}C]cholic acid complexes were placed in scintillation vials, digested with 0.3 ml of Protosol (New England Nuclear) for 12 h, and Toluene-Omnifluor (New England Nuclear) containing [98% 2,5-diphenyloxazole and 2% *p*-bis-(*O*-methyl styryl)-benzene] 4 g/liter of Toluene (New England Nuclear) was added to each vial. Radioactivity of the samples was determined in a Packard 2425 Tri-carb liquid scintillation spectrometer (Packard Instrument Co. Inc., Downers Grove, Ill.). Quench was determined by automatic external standardization. Results are reported as disintegration per minute per milligram protein.

Nonspecific binding of bile acid to membranes was determined after preincubation of the tissue fractions at 37°C for 3 h to denature specific bile acid binding sites. We have previously shown that this method gives values identical to those for nonreversible binding after addition of a 200-fold excess of unlabeled cholic acid to untreated surface membrane fractions (15). Specific bile acid binding is total binding measured in a given experimental condition corrected for the nonspecific value determined under the same condition.

Hepatic morphology. Morphologic studies were obtained from control and cycloheximide-treated rats. Animals were anesthesized with light ether anesthesia, the liver was rapidly removed and washed in ice-cold saline. Small pieces of liver were fixed in 10% formaldehyde buffer phosphate pH 6.0, and sections stained with hematoxylin and eosin and with reticulin. Additional liver samples were quickly frozen, sectioned, and stained for fat with Oil Red O solution.

Tissue for electron microscope examination was obtained from experimental and control animals within 30 s of death. The tissues were finely minced, fixed in phosphate-buffered 4% glutaraldehyde, postfixed in phosphate-buffered 2% osmium tetroxide, and embedded in epon-Araldite (Ciba-Geigy Corp., Ardsley, N. Y.). Ultrathin sections were cut on an LKB ultramicrotome (LKB Instruments, Inc., Rockville, Md.), stained with uranyl acetate and lead citrate, and examined in a JEM 100B electron microscope.

Liver function tests and serum lipids. Serum samples from fasted control and cycloheximide-treated rats were drawn, and bilirubin (27), alkaline phosphatase (by using *p*-nitrophenyl phosphate buffered with AMP) (28), aspartate aminotransferase (2.6.1.1) (29), alanine aminotransferase (2.6.1.2) (30), gamma-glutamyl transpeptidase (31), albumin (32), cholesterol (33), and triglycerides (34) determined in the pediatric microchemistry laboratory of the University of Colorado Medical Center.

Measurement of protein synthesis. To determine whether cycloheximide inhibited protein synthesis, L-[U-^{14}C]leucine 5 μCi (324 mCi/mmol; Amersham Corp., Arlington Heights, Ill.) was administered i.p., and incorporation into liver protein was determined 30 min later. The effect of cycloheximide (150 μg/100 g BW, i.p.) was examined in sets of three rats each, 1 and 12 h later and compared with controls (three rats). After sacrifice, livers were perfused, homogenized in 1 mM $NaHCO_3$; and approximately 1.5 mg of protein was precipitated with an equal volume of ice-cold 10% TCA and collected on glass fiber disks. Samples were serially washed with 5 ml each of ethanol, ether, acetone, and TCA. The disks were transferred to scintillation vials, and protein was digested with Protosol. After 12 h, Toluene-Omnifluor was added to each vial, and the radioactivity determined as previously described.

Statistics. Regression lines, slopes, and correlation coefficients were determined according to the least squares method. Student's *t* test and analysis of variance were used for statistical analysis of data (35). *P* values $\leqq 0.05$ were considered significant. Values are expressed as mean±SEM.

RESULTS

Effect of cycloheximide on body and liver weight. Animals treated with cycloheximide (193±6 g) were of comparable size to controls (206±7 g), and the ratio of liver weight/BW was not significantly different from control values (3.9±0.1 vs. 3.8±0.1 mg/100 g, respectively).

Protein synthesis. Although protein synthesis was 93% inhibited at 1 h, it was only 47% reduced at 12 h. Therefore, a second injection of cycloheximide was administered between 10 and 12 h later if study periods >16 h were performed.

Effect of cycloheximide on liver function tests and serum lipids (Fig. 1). Serum bilirubin, aspartate aminotransferase, and gammaglutamyl transpeptidase values were not changed from control values with cycloheximide administration (Fig. 1), whereas the mean values for serum albumin, alanine aminotransferase, and alkaline phosphatase were significantly lower than normal. Because inhibitors of protein synthesis are known to alter intestinal lipid absorption (36–39), serum cholesterol and triglycerides were also determined. Serum cholesterol was reduced from 52 ±3 mg/dl in controls to 34 ±10 in treated rats ($P < 0.05$), but triglycerides were not significantly changed (90 ±15 vs. 80±18 mg/dl, controls vs. cycloheximide, respectively).

Light and electron microscopy (Figs. 2 and 3). Ex-

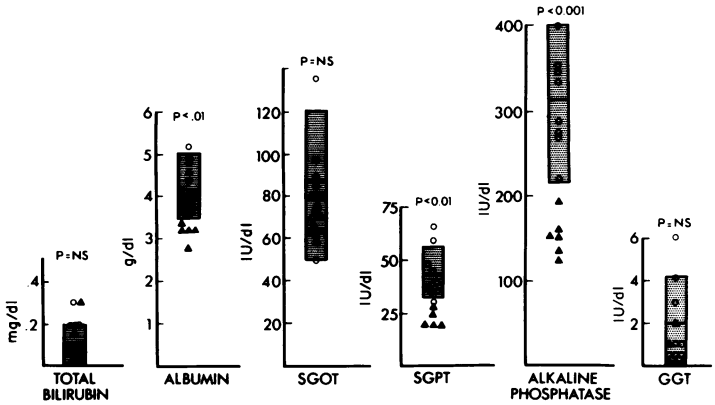

FIGURE 1 Effect on liver function studies 16 h after cycloheximide administration (150 μg/100 g BW). Individual control values (○) are shown, and the stippled columns ▩ indicate the mean ±1 SD. Individual cycloheximide determinations are shown by ▲. NS, not significant.

amination of liver tissue by light microscopy from rats 16 h after cycloheximide appeared unchanged from controls. There was no evidence of cell necrosis or accumulation of fat. Minimal changes seen by electron microscopy were characterized by: loss of bound ribosomes, partial disorganization of the parallel arrays of rough endoplasmic reticulum, and occasional lamellar whorls (Fig. 2). On the other hand, no accumulation

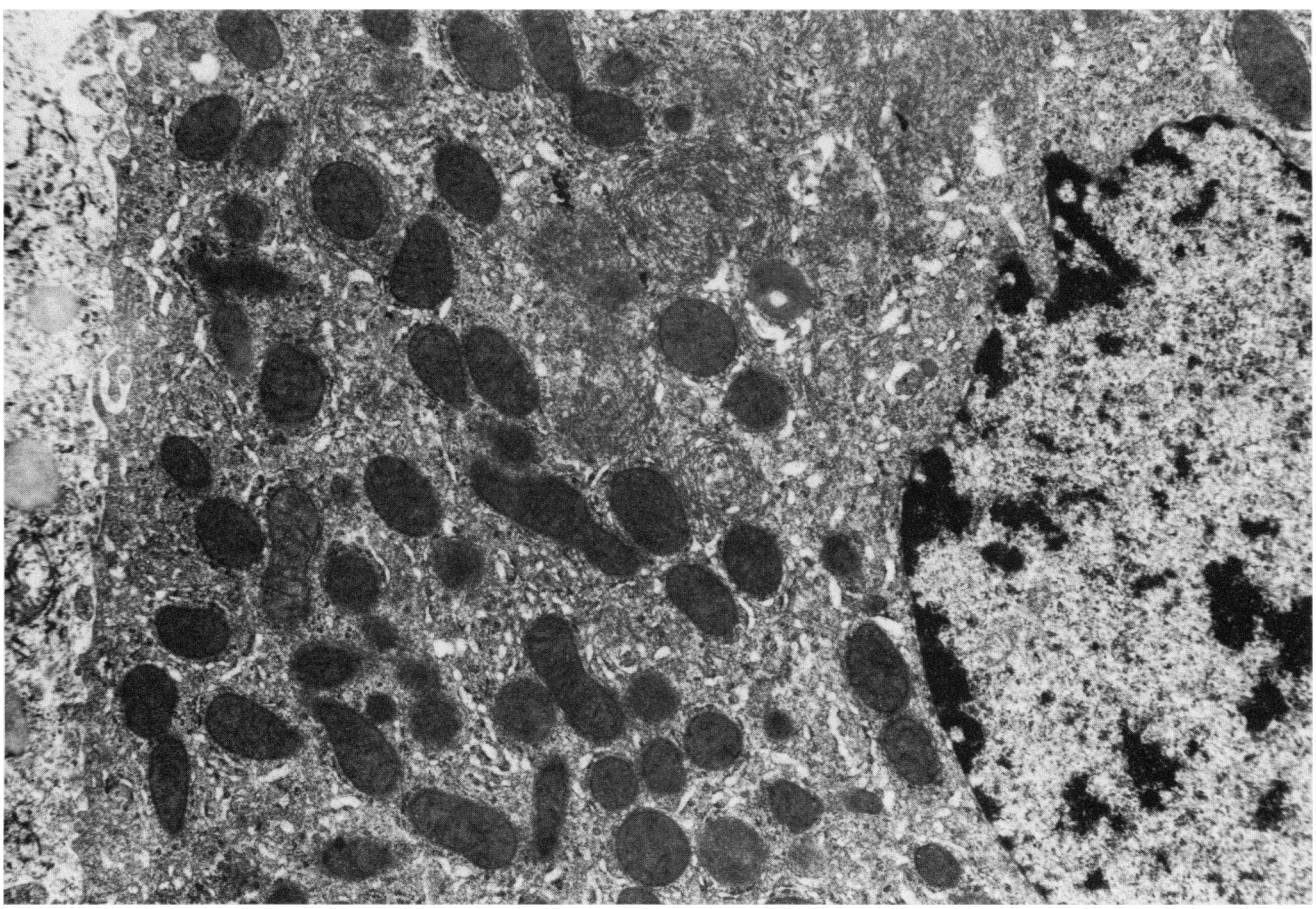

FIGURE 2 (A) The ultrastructural changes observed in liver cells 16 h after cycloheximide administration (150 μg/100 g BW). Minimal loss of bound ribosomes, partial disorganization of rough endoplasmic reticulum (RER) and occasional lamellar whorls are seen (×6,000).

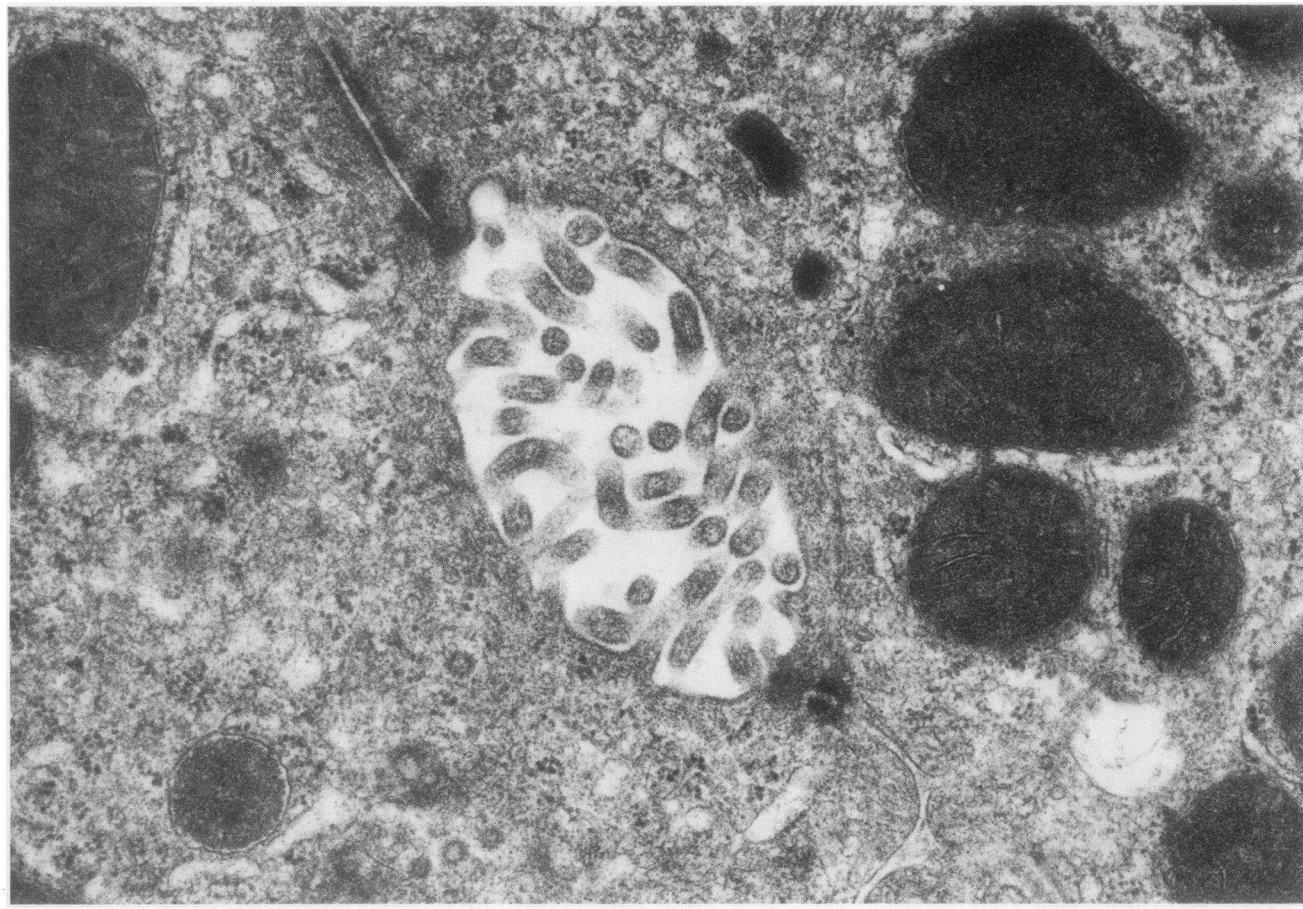

FIGURE 3 Intact bile canaliculi are seen 16 h after cycloheximide administration (×20,000).

of fat was noted and the bile canaliculi were intact (Fig. 3).

Effect of cycloheximide on bile salt excretion and bile flow (Table I). Cycloheximide significantly reduced bile salt Tm to 62% of control values 16 h after administration. Decreased bile salt Tm was associated with a significant reduction in both the maximum bile salt concentration and maximum bile flow. In contrast, basal bile salt excretion and basal bile flow were not significantly changed. BSIBF was also not significantly changed suggesting that cycloheximide may selectively alter biliary secretory pathways.

To determine whether decreased bile salt Tm is the result of mechanisms other than inhibition of protein synthesis, measurements were also examined at a time when protein synthesis is known to return to normal

TABLE I
Effect of Cycloheximide on Bile Acid Excretion and Bile Flow

	Bile acid Tm	Basal bile acid excretion	Basal bile flow	Maximal bile acid concentration	Maximal bile flow	BSIBF
	μmol/min/100 g	*μmol/min/100 g*	*μl/min/100 g*	*μmol/ml*	*μl/min/100 g*	*μl/min/100 g*
Controls (9)	1.0±0.05	0.26±0.02	7.4±0.4	82.3±4.9	13.0±0.8	5.2±0.1
Cycloheximide, 16 h (5)	0.6±0.06	0.24±0.02	7.7±0.5	64±6.5	10.6±0.4	6.2±0.6
Percent difference	−38	−8	+4	−22	−19	+19
P value	<0.001	NS	NS	<0.05	<0.01	NS

Cycloheximide (150 μg/100 g BW) was administered and biliary secretory function measured as described in Methods, 16 h later. Values are expressed as mean±SEM. Parentheses represent the number of separate experiments.

TABLE II
Effect of Cycloheximide on BSP Tm, Maximal Bile Flow, and BSP Concentration in Bile and Serum

	BSP Tm	Maximal bile flow	Maximal BSP concentration in bile	Percent conjugated BSP in serum
	μmol/min/100 g BW	*μl/min/100 g BW*	*μmol/ml*	
Controls (3)	0.213±0.003	14.7±0.6	469±28	31±5 (3)
Cycloheximide (4)	0.185±0.003	12.4±0.3	416±17	59±9 (3)
Percent difference	−15	−19	−13	+93
P value	<0.01	<0.01	0.15	<0.025

BSP Tm was determined as described in Methods, 16 h after cycloheximide administration (150 μg/100 g BW). Values are expressed as mean±SEM. Parentheses indicate the number of separate experiments.

(40). 48 h after cycloheximide, reduced bile salt Tm returned to control values (1.0±0.20 vs. 1.0±0.05 μmol/min per 100 g BW; controls vs. cycloheximide) suggesting decreased transport results from altered protein synthesis, rather than generalized cell damage.

Effect of cycloheximide on BSP Tm. To examine further whether the effect of cycloheximide is selective on hepatic transport of bile salts, BSP Tm was also determined. Although the change was not as great as for bile salt Tm, cycloheximide significantly reduced BSP Tm to 85% of control values 16 h after administration (Table II). Reduced BSP Tm was due primarily to a reduction in maximal bile flow and a modest decrease in the maximum concentration of BSP in bile. Although this decrease was not statistically significant at 16 h, there was a significant reduction at 24 h after cycloheximide (385±17 vs. 469±28 μmol/ml, $P < 0.025$).

To determine whether BSP was infused at rates saturating the biliary excretory capacity, the percent of conjugated BSP in serum was determined 16 h after cycloheximide. Conjugated BSP was significantly greater in treated rats (59±8) than controls (31±5) (Table II), thus indicating that the defect in transport was probably at the level of excretion.

Kinetics of degradation of hepatic transport processes (Fig. 4). Bile salts and BSP are transported by different mechanisms through the liver (41). In addition, BSIBF may in part depend on the hydrolysis of ATP for active transport of sodium by (Na^+-K^+)-ATPase (42, 43). Decreased transport capacities for bile salts and BSP were measured at various times after inhibition of protein synthesis (Fig. 4). These changes appear to follow first order kinetics, permitting approximation of their half-lives. Bile salt half-life ($t_{1/2}$ = 20 h) is significantly faster than that observed for BSP ($t_{1/2}$ = 55 h), consistent with a differential effect on transport which is dependent on the turnover of the specific pathways. In contrast to its effect on organic anion transport pathways, cycloheximide did not alter BSIBF.

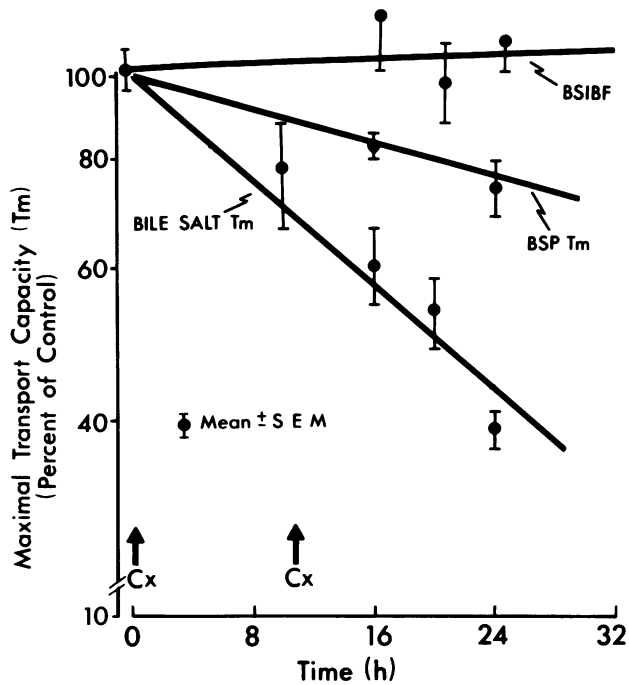

FIGURE 4 Time-course of change in bile salt independent bile flow, BSP Tm, and bile salt Tm after cycloheximide administration (150 μg/100 g BW). Biliary secretory function parameters were measured as described in Methods. Each point represents the mean±SEM for three to nine different experiments at each individual point. Cycloheximide (CX) was administered again when time periods >16 h were studied. BSIBF did not change from 100%. The best fit linear function was determined by least-squares regression. BSP Tm ($y = -0.0249 \times +100$, $r = 0.995$, $P < 0.0025$) and bile salt Tm ($y = -0.0251 \times +101$, $r = 0.995$, $P < 0.0025$) half-lives were estimated to be 55 and 20 h, respectively.

These heterogeneous changes in hepatocyte transport mechanisms strongly suggest that the effect of cycloheximide is specific to its effect on protein synthesis and not a generalized nonspecific decrease in transport capacity.

Effect of cycloheximide on bile salt binding and liver surface membrane enzymes (Table III). The possibility that decreased bile salt transport is the result of a reduced number of putative bile acid "carriers" was examined by measuring the maximum number of bile acid binding sites in liver surface membrane fractions isolated from cycloheximide-treated rats. 16 h after cycloheximide, the maximum number of specific bile acid binding sites was reduced 32% from control values (Table III). This effect is apparently not because of direct inhibition, for addition of cycloheximide in vitro at concentrations from 0.01 to 1 μM did not alter binding. Reduction in the number of binding sites is apparently selective, for activities of the liver surface membrane enzymes (Na^+-K^+)ATPase (sinusoidal location), Mg^{++} ATPase (canalicular location), and 5'-nucleotidase (sinusoidal as well as canalicular) were not significantly changed (Table III).

The inference that reduced bile salt binding sites may account for the observed decrease in bile salt Tm was explored further by analysis of the maximum number of binding sites present 24 h after cycloheximide administration. At 24 h cycloheximide significantly reduced the number of specific bile salt binding sites (8.2±1.5 nM/mg protein, $P < 0.005$) as well as bile salt Tm (0.38±0.03 μmol/min per 100 g BW) compared with controls ($P < 0.005$) and when tested against values at 16 h ($P < 0.005$).

DISCUSSION

In the past few years increasing evidence has accumulated from both eukaryotic (44–47) as well as prokaryotic systems supporting the role of carriers in the translocation of substances across the surface membrane (48–50). These studies demonstrate that carriers are symmetrical proteins which span the lipid membrane bilayer and that maximum rates of transport can be regulated by the number of carriers, the lipid environment, and coupled energy (51, 52). Thus, membrane transport processes may be potentially altered at three distinct steps.

Carrier-mediated transport is composed of two distinct steps, recognition (binding) and translocation; because the demonstration of specific bile acid binding permits differentiation between these two processes, it is currently possible to examine directly whether reduced hepatic transport of bile acids is the result of changes in the number of putative bile acid carriers. The use of inhibitors of protein synthesis has underscored the importance of proteins in the transport of sugars (53), amino acids (54–57), and lipids (36–39). However, in these studies it was assumed that these pharmacological agents decreased transport through changes in the number of putative carriers. This study shows that cycloheximide decreases bile acid transport and also the number of bile acid receptors, and strongly

TABLE III

Number of Bile Acid Binding Sites and Enzymatic Activity of Liver Surface Membrane Fractions from Control and Cycloheximide-Treated Rats

	Specific cholic acid binding	Enzyme activity		
		(Na+−K+)ATPase	Mg++−ATPase	5'-Nucleotidase
	nmol/mg protein	*μmol Pi/mg protein/h*		
Control	32.2±2.8 (9)	26.5±1.7 (16)	53.2±3.5 (16)	49.0±3.6 (6)
Cycloheximide	21.9±2.5 (8)	26.2±0.5 (4)	55.0±6.1 (4)	56.8±8.7 (4)
Percent difference	−32	−1	+3	+16
P value	<0.01	NS	NS	NS

Liver surface membrane fractions were prepared through step 12 of Neville (22) and enzymatic activities determined as described in Methods. Surface membrane fractions were incubated with [^{14}C]cholic acid (initial concentration 1.8 mM) in standard buffer, pH 6.0, for 20 min at 4°C. Specific cholic acid binding was determined as described in Methods. Values are mean±SEM. The numbers of separate experiments are shown in parentheses. Rats were sacrificed 16 h after cycloheximide administration (150 μg/100 g BW).

suggests that these proteins are involved in membrane transport.

The use of pharmacologic inhibitors of protein synthesis (e.g. cycloheximide) for experimental models to study hepatic transport processes may be difficult to interpret because of the inherent toxicity of the drugs employed. The effect of these drugs on hepatic morphology, liver function tests, and bile secretory capacity was therefore carefully examined. Although by light microscopy, with a number of stains, the livers of treated rats were not different from controls, electron microscopy demonstrated minimal changes in the rough endoplasmic reticulum, primarily characterized by their partial disorganization and formation of whorls. Similar features have been noted by others (39, 58), and appear to be related to the ability of cycloheximide to selectively inhibit nascent peptide synthesis on membrane-bound polyribosomes (39). Lack of cell necrosis, fat accumulation, and alterations in bile canaliculi permit cycloheximide-treated rats to be excellent models for the study of the molecular steps in bile acid transport.

Although many agents known to inhibit protein synthesis are known to cause cell damage, these changes were not apparent after cycloheximide for serum indicators of cellular necrosis (aspartate aminotransferase, alanine aminotransferase) and cholestasis (alkaline phosphatase, gammaglutamyl transpeptidase) were not elevated. The cause of reduced serum albumin, alanine aminotransferase, and alkaline phosphatase values is unknown, although impaired synthesis or release into the serum may account for these small changes (59).

Further evidence against a generalized toxic effect of cycloheximide on liver function is unaltered basal bile flow, bile salt excretion, and BSIBF. Similar observations have been made by others with even higher doses of cycloheximide (60). Therefore, it is concluded that during at least 16 h of protein synthesis inhibition, cycloheximide produces selective changes in hepatocyte function without causing hepatitis or cholestasis.

Maximum hepatic bile salt transport capacity was determined in control and experimental rats by the infusion of taurocholate at rates above its Tm. Others have observed that bile flow and bile acid excretion decreased, presumably as a result of a high bile acid infusion rate (61). However, in the present study, there is no evidence of this toxic effect because bile flow and bile salt excretion did not decrease during the maximum infusion rate. Thus, we believe that the reduced Tm value after cycloheximide administration represents the maximum bile salt transport capacity and may reflect a decreased number of hypothetical membrane carriers. We propose that this inhibitory effect of cycloheximide is mediated through blockade of protein synthesis. The finding that bile acid Tm and protein synthesis were the same as in untreated rats 48 h after cycloheximide administration is consistent with this suggestion.

If cycloheximide inhibition of transport is nonspecific, resulting from blocked synthesis of high-energy compounds or necessary membrane components required for transport of all organic anions, the maximum transport capacities will be equally inhibited. On the other hand, if one assumes that specific membrane components mediate active transport of organic anions, and these components differ for different pathways, such as bile salt and BSP, the time-course of protein inhibition should vary for bile acid and BSP transport. This study demonstrates that the rates of transport for both bile salts and BSP decreased progressively, following apparent first order kinetics during the duration of protein inhibition. This decrease suggests that existing transport components may be degraded without replacement. The differential rates of inhibition of transport observed for bile acids and BSP strongly suggest that the effect of cycloheximide on bile salt transport cannot be explained by a decrease in intracellular enzymes involved in the production of high-energy intermediates or membrane lipids possibly necessary for transport. More likely, the effect is the result of inhibition in the synthesis of specific membrane transport components that exhibit different turnover rates.

Inasmuch as recognition (binding) is the initial step in membrane translocation of ligands, and it is possible to measure this step independently of the translocation process, the maximum number of bile acid binding sites were determined in liver surface membrane fractions at 16 and 24 h after cycloheximide administration. The number of [^{14}C]cholic acid binding sites decreased progressively with time (Fig. 5). This observation that [^{14}C]cholic acid binding sites and transport capacity for bile acids are proportionately decreased is in accord with the inference that bile acid receptors may represent the putative membrane carriers.

The reduction of bile acid binding sites after cycloheximide administration is apparently not due to a generalized toxic effect on membrane proteins, for membrane enzyme markers of the sinusoidal [(Na^+-K^+)-ATPase] as well as the canalicular (Mg^{++}-ATPase) surfaces were not significantly changed. In particular, (Na^+-K^+)ATPase activity, which is believed to in part regulate BSIBF, was not changed. Although this is consistent with the lack of significant change in BSIBF, it was a surprising observation. The estimated $t_{1/2}$ of (Na^+-K^+)ATPase from other studies was approximately 2.5 d, and thus one would expect a reduction of approximately 15% at 16 h. Because inhibitors of protein synthesis may decrease degradation rates of

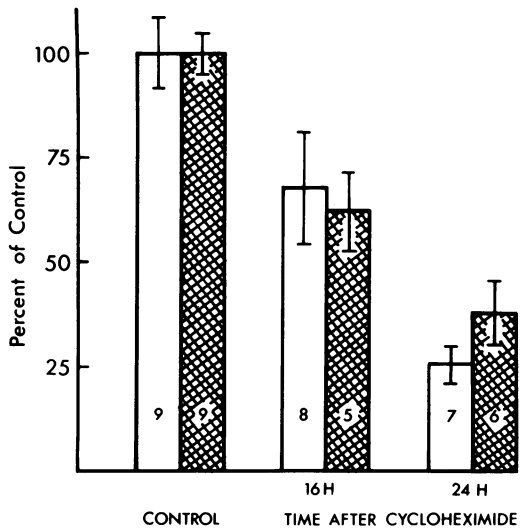

FIGURE 5 Association between the change in percent of bile salt Tm (□) and [^{14}C]cholic acid binding (▩). Liver surface membrane fractions were prepared according to Neville (22) from controls and rats treated 16 and 24 h previously with cycloheximide as described in Methods. Bile salt Tm and [^{14}C]cholic acid binding were determined as described in Methods. Columns represent the percent mean ± SEM for the number of separate experiments indicated. *, significantly different ($P < 0.005$) from respective previous experimental group as determined by analysis of variance.

selective enzymes as well as inhibit protein synthesis (62), a decreased degradation rate may explain this result. Failure of cycloheximide to change hepatic activities of Mg^{++}-ATPase and 5′-nucleotidase is not known, but altered degradation rates or slow turnover rates are possible explanations. Examination of these alternatives will require measurement of isotopic decay of purified protein components.

Proteins of the rat liver surface membrane are known to undergo differential degradation as determined by chemical and detergent fractionation techniques (63, 64). The turnover of (Na^+-K^+)ATPase by pharmacological time-course techniques and NAD glycohydrolase ($t_{1/2}$ = 18 d) measured by isotope incorporation methods are the only surface membrane proteins examined (22, 65). These observations, in addition to the present observations of the bile salt receptor, lend further support to the differential turnover of membrane proteins.

Although the biochemical basis for heterogeneous protein turnover is unknown, Berlin and Schimke (66) have suggested that the levels of rate-limiting proteins will have a rapid rate of turnover. This would permit rapid response (either increase or decrease) to changes in the environment such as substrate or hormonal levels. That a substrate may alter biliary secretory capacity has recently been demonstrated after prolonged bile acid infusions (67).

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