Failure of Bile Acids to Control Hepatic Cholesterogenesis: Evidence for Endogenous Cholesterol Feedback

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ABSTRACT Studies were undertaken to define the role of bile acids in the control of hepatic cholesterogenesis from acetate. Both biliary diversion and biliary obstruction increase the rate of sterol synthesis by the liver 2.5- to 3-fold. After biliary diversion, however, the bile acid content of the liver is decreased, whereas after biliary obstruction, it is markedly increased. Thus, there is no relationship between the tissue content of bile acid and the rate of hepatic cholesterol synthesis. Furthermore, restoration of the enterohepatic circulation of bile acid in animals with biliary diversion fails to prevent the rise in synthetic activity seen after this manipulation. These data indicate that bile acid plays no direct inhibitory role in the regulation of cholesterol synthesis by the liver.

Other experiments were therefore undertaken to evaluate the possibility that changes in cholesterogenic activity observed after manipulation of the enterohepatic circulation of bile acid actually are the result of changes in the enterolymphatic circulation of cholesterol. In support of this thesis it was found that intestinal lymphatic diversion causes the same specific enhancement of cholesterol synthetic activity as biliary diversion and that both of these operative procedures increase enzymatic activity at the step mediated by β-hydroxy-β-methyl glutaryl reductase. Furthermore, the increase in the rate of sterol synthesis by the liver seen in animals with biliary diversion can be prevented by the infusion of approximately 7 mg of cholesterol/24 hr in the form of chylomicrons. This is an amount of cholesterol circulating normally in the enterolymphatic circulation of the intact rat.

These results indicate that bile acid plays no direct role in the control of hepatic cholesterogenesis, but rather, it is the enterohepatic circulation of endogenous cholesterol that determines directly the rate at which cholesterol is synthesized by the liver.

INTRODUCTION

Liver is one of the two major endogenous sources for circulating serum cholesterol (1–5) and, therefore, considerable investigative work has been undertaken to elucidate the means of physiological control of sterol synthesis in this organ. Numerous publications have dealt with three of these mechanisms. First, cholesterol feeding markedly suppresses hepatic cholesterogenesis (6). This suppression is the result of negative feedback inhibition by exogenous cholesterol of the rate-limiting enzyme in the biosynthetic sequence, i.e., β-hydroxy-β-methyl glutaryl reductase (7–9). Second, fasting also is known to inhibit sterol synthesis in the liver (10). Diminished enzymatic activity at the level of β-hydroxy-β-methyl glutaryl reductase also appears to be responsible for the decreased rate of cholesterogenesis observed after this experimental manipulation (11).

The third major factor influencing the rate of hepatic cholesterogenesis is the state of the enterohepatic circulation of bile acid. More specifically, it has been suggested that bile acid exerts a direct, inhibitory influence on cholesterol synthesis in the hepatic cell. The experimental data that support this concept are of three types. First, it has been reported that the addition of bile acid in vitro to liver slices or to subcellular preparations inhibits cholesterogenesis (12, 13). Second, external di-

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version of the biliary secretions (14–16) or feeding the bile acid sequestrant, cholestyramine, (17) leads to an enhanced rate of hepatic cholesterogenesis. Third, preliminary data suggest that in man expansion of the bile acid pool causes decreased cholesterol synthesis while interruption of the enterohepatic circulation of bile acid results in increased synthesis (18).

While these various studies appear to support the thesis that the quantity of bile acid in the enterohepatic circulation exerts a direct rate-controlling effect upon sterol synthesis in the liver, there are, nevertheless, other data now available that raise considerable doubt as to the validity of this concept. First, in contrast to the results reported above, pure conjugated bile acids do not inhibit sterol synthesis when added to liver slices in vitro (8); similar results have been obtained in this laboratory.[1] While some pure unconjugated bile acids do inhibit various metabolic pathways, including the one responsible for cholesterol synthesis under in vitro conditions, this inhibition is totally nonspecific and probably is secondary to the detergent properties of these compounds[1] (19). Thus, to date there are available no valid experimental data based upon the effects of bile acids added to liver preparations in vitro that support the concept that bile acids exert direct control on the rate of hepatic cholesterogenesis. Second, not only does external biliary diversion and cholestyramine feeding, as outlined above, enhance hepatic sterol synthesis, but ligation of the common bile duct also leads to an elevated rate of cholesterogenesis in the liver (20). Finally, since bile acid functions in a number of other physiological processes, e.g. promoting cholesterol absorption from the intestine, the balance studies in man (18) do not necessarily prove that it is bile acid per se that directly alters the rate of sterol synthesis. Such results could well be the indirect consequence of some other phenomenon related to the size of the bile acid pool.

Thus, it is apparent that the relationship between the rate of cholesterol synthesis in the liver and the amount of bile acid in the enterohepatic circulation is still poorly understood. Yet elucidation of the details of this relationship are obviously important for an understanding of over-all sterol balance in the intact animal and in man. The present experiments were therefore undertaken to evaluate directly the role of bile acids in the control of hepatic sterol synthesis.

METHODS

Animals and operative procedures. All animals in these experiments were 200–250 g female Sprague-Dawley rats[2] maintained on laboratory rat chow[3] ad lib. until used in the experiments. After an overnight fast, each animal was anesthetized with ether and subjected to one or to a combination of the following operative procedures: (*a*) for the purposes of tube feeding, a plastic catheter[4] (O.D., 0.050 in.) was secured by means of a purse string suture into the greater curvature of the stomach and exteriorized through a stab wound in the left flank, (*b*) the common bile duct was cannulated above the level of the pancreas and the catheter (O.D., 0.038 in.) was exteriorized through a stab wound in the lower right flank, (*c*) the intestinal lymphatic duct was cannulated at the base of the mesentery and the catheter (O.D., 0.038 in.) was brought to the outside through a stab wound in the right flank as described by Bollman, Cain, and Grindlay (21), (*d*) for purposes of the intravenous infusion of test solutions a plastic catheter (O.D., 0.024 in.) was passed into the inferior vena cava through a tail vein and taped in place, (*e*) for purposes of infusing the bowel with test solutions, a plastic catheter (O.D., 0.038 in.) was secured into the lumen of the proximal jejunum by means of a purse string suture and was exteriorized through the left flank, and (*f*) after cannulation of the common bile duct in some animals a second catheter (O.D., 0.024 in.) was secured in the proximal portion of the duct above the level of the pancreas so that test solutions could be perfused through the distal common bile duct into the duodenum. The specific combination of these operative procedures performed in each group of animals is described in detail for each experiment. After the abdominal incision was closed, each animal was allowed to recover consciousness in a restraining cage where it remained for the duration of the subsequent 48 hr experimental period.

Diets and infusion solutions. The diet fed in most experiments consisted of a mixture of dextrin[5]: casein hydrolysate[6]: water (70 g: 30 g: 100 g) briefly mixed together in a food blender and neutralized to a pH of 7.0. Aliquots of this semipurified diet were fed to each rat via the indwelling gastric feeding tube every 6 hr; the amount of diet fed was calculated to provide 62 kcal to the animal during the 48 hr experimental period and, by direct analysis, contained only 0.6 mg of digitonin-precipitable sterols. In some experiments either a glucose solution or triolein was substituted, isocalorically, for the dextrin-casein diet. All animals, also, were allowed access to drinking water containing 0.25% NaCl.

Solutions of bile acids[7, 8] for infusion intravenously, intrajejunally, or into the common bile duct were prepared in 0.9% NaCl solution and the pH was adjusted with HCl or NaOH to 7.4.

Rat chylomicrons were collected and processed for intravenous infusion in the following manner. Each donor animal was prepared with an indwelling catheter, placed in the intestinal lymphatic trunk at the base of the mesentery (21), and with an indwelling gastric feeding tube. All donor animals were fed raw egg yolk through the feeding tube and lymph was collected continuously for 2–4 days. The lymph from each animal was allowed to drip directly into an Erlenmeyer flask containing 5 ml of 0.5% ethylenedinitrilotetraacetic acid (EDTA) in 0.9% NaCl solution, 15,000 U of penicillin G, and 50 mg of streptomycin; the flask was kept at room temperature and was changed every 24 hr. Lymph so collected from a number of donor animals was pooled,

[1] Unreported observations from this laboratory.
[2] Simonsen Laboratories, Gilroy, Calif.
[3] Ralston Purina Co., St. Louis, Mo.
[4] Clay-Adams, Inc., New York.
[5] Matheson, Coleman, and Bell, Cincinnati, Ohio.
[6] General Biochemicals, Div., Chagrin Falls, Ohio.
[7] Calbiochem, Los Angeles, Calif.
[8] Steraloids, Inc., Pawling, N. Y.

filtered through gauze, and centrifuged at room temperature for 45 min at 20,000 rpm in a SW 25.1 swinging-bucket head. The semisolid lipid layer was resuspended in 0.9% NaCl solution through a 25 gauge needle.

The concentration of bile acid or of cholesterol in chylomicrons in the infusion solutions was adjusted so that the desired amount of these test substances could be delivered to the animals at a standard infusion rate of 1.0 ml/hr by means of a constant rate infusion pump.

Tissue preparation. All experimental manipulations were carried out on appropriate animal preparations over a 48 hr period. At the end of this time the animals were killed and liver and jejunal slices as well as liver homogenates, in some instances, were prepared.

In studies utilizing slices, the liver was immediately excised, washed in cold 0.9% NaCl solution, and cut into cords 3–4 mm thick. Slices, 0.75 mm thick, were then prepared with a McIlwain tissue slicer.[9] Slices were prepared from the midjejunum in a similar manner. 350-mg aliquots of both the liver and the intestinal slices were placed in centerwell incubation flasks containing 5 ml of Krebs' bicarbonate buffer (pH 7.4) previously gassed with 95% O_2: 5% CO_2, 1 μc (0.5 μmoles) of acetate-2-^{14}C, and 10 μmoles of sodium acetate. In some experiments an equal aliquot of slices was also incubated with 0.5 μc (0.1 μmoles) of mevalonate-2-^{14}C in place of the radiolabeled and unlabeled acetate.

In the case of homogenates, the liver was first flushed in situ by injecting 20 ml of cold 0.1 M phosphate buffer into the portal vein. Each liver was then weighed and homogenized in a Dounce[10] homogenizer in cold 0.1 M potassium phosphate buffer (pH 7.8, 1 ml/1.0 g of liver) containing nicotinamide (33 μmoles/1.0 g of liver). Cellular debris, nuclei, and mitochondria were removed by centrifugation at 10,000 rpm for 10 min and the supernatant fluid containing microsomes and soluble enzymes was used without further purification. Each incubation flask contained 2.0 ml of this homogenate, 1 μc (0.5 μmoles) acetate-2-^{14}C, 5 μmoles potassium acetate, 1 mg of reduced nicotinamide adenine dinucleotide phosphate (NADPH), 12.5 mg of glucose-6-phosphate, and 5 μmoles of magnesium chloride. Aliquots of the homogenates also were run using 0.5 μc (0.1 μmoles) of mevalonate-2-^{14}C in place of the radiolabeled and unlabeled acetate.

Both the slices and homogenates were incubated in a metabolic shaker at 37°C for 2 hr at 100 oscillations/min. Under the conditions of these experiments, it has been established in the case of both types of tissue preparations that the rate of incorporation of acetate-2-^{14}C into cholesterol becomes linear with respect to time within the first 5–10 min of the incubation, that the rate of incorporation remains constant throughout the remainder of the 2 hr incubation, that the concentration of substrate is at least five times the apparent K_m value (0.2–0.4 μmoles/ml) for the rate-limiting step in the cholesterogenic pathway so that zero-order kinetics essentially are achieved, and finally, that all of these conditions are obtained in each of the experimental situations described in the results section. The apparent K_m value for the incorporation of mevalonate into cholesterol is approximately 2.5 μmoles/ml in both control and bile-fistula animals. At concentrations of mevalonate high enough to approach V_{max}, there was clearly nonspecific inhibition of the system. Therefore, incorporation of mevalonate into cholesterol was measured at a concentration of substrate that does not yield maximal rates. However, since the apparent values of K_m for the incorporation of mevalonate into cholesterol in control and bile-fistula animals are the same, the comparison of rates measured under these conditions is valid.

Chemical methods. As previously outlined (7) in the case of tissue slices, at the end of the incubation the contents of the flasks were acidified with 1 N H_2SO_4 and the CO_2 that evolved was trapped in 1 ml of 1 N NaOH previously placed in the center well of each flask. An aliquot of this alkali solution was counted in the scintillation fluid described by Bray (22). The flasks' contents were then saponified, made up to a 50% ethanolic solution, and extracted with petroleum ether to remove nonsaponifiable lipids. After acidification, the residue was next extracted with petroleum ether to remove acidic lipids. These latter petroleum ether extracts were backwashed with water and a sample was counted in a scintillation fluid containing 0.3% 2,5-diphenyloxazole and 0.015% *p*-bis[2-(5-phenyloxazolyl)]benzene in toluene (PPO-POPOP solution). Sterols were isolated from the first petroleum ether extract as the digitonides. The precipitates were then washed with acetone and diethyl ether and dissolved in methanol. A sample of this solution was placed in PPO-POPOP scintillation fluid for ^{14}C assay. Homogenates were handled in a similar manner except that the collection of labeled CO_2 was omitted.

In a few experiments direct assay of β-hydroxy-β-methyl glutaryl reductase activity was undertaken using the gas-liquid chromatography (GLC) method previously described (23).

Chemical determination of cholesterol in liver tissue and in the chylomicron infusions was undertaken using the method of Sperry (24) after saponification and precipitation of the sterol digitonides. Egg phospholipid was prepared as described in reference 25 and by direct determination contained 9.4 mg of cholesterol/g of phospholipid.

The concentration of cholic acid in liver tissue was determined in the following manner. At the time the animals were killed, 2-g aliquots of liver were minced, placed in beakers, and an internal standard of taurocholate-^{14}C was added. The tissue was extracted three times in boiling ethanol for 15 min. The alcohol extracts were combined, dried, redissolved in 5 ml of 1.25 M NaOH, and autoclaved at 120°C for 5 hr. After acidification, the unconjugated bile acids were extracted in diethyl ether and then separated by thin-layer chromatography using Silica Gel H (Merck) in the developing system of Hofmann (26). The area corresponding to cholic acid was scraped from the plate, and the bile acid was eluted from the silica gel in chloroform: methanol (1:1). Equal aliquots of this eluate were then taken for assay of radioactivity and for determination of mass by the photofluorometric method of Levin, Irvin, and Johnston (27).

RESULTS

Role of bile acid in the control of hepatic cholesterogenesis. Initial experiments, shown in Fig. 1, illustrate the results obtained in rats with external biliary diversion and biliary obstruction. The rate of acetate incorporation into cholesterol by slices of liver from control animals (A) equaled 261 ±21 mμmoles/g per 2 hr. This rate increased approximately 2.5 fold to 657 ±57 mμmoles/g per 2 hr and 563 ±44 mμmoles/g per 2 hr in animals with biliary diversion (B) and biliary obstruction (F), respectively.

[9] H. Mickle, Gomshall, Surrey, England.
[10] Blaessig Glass Company, Rochester, N. Y.

The effect of these two operative manipulations was specific for cholesterogenesis because neither the rate of acetate incorporation into fatty acid nor into CO_2 was significantly altered in either of these experimental situations. Also illustrated in Fig. 1 are the concentrations of cholic acid, the major bile acid in the rat, in liver tissue in each of these three experiments. After biliary diversion, hepatic cholic acid content decreased by approximately 50%, whereas after biliary obstruction, it increased over 3-fold. Yet in both situations, the rate of cholesterogenesis was elevated to approximately the same degree.

In order to explore the influence of bile acid on hepatic cholesterogenesis more definitively, the enterohepatic circulation of bile acid was reestablished in animals with biliary diversion by the continuous infusion of taurocholic acid either intravenously, intrajejunally, or into the common bile duct as shown in panels C, D, and E, respectively, of Fig. 1. To assure that the bile acid infused into the intestine was absorbed, the output of bile acid through the biliary cannula was measured and absorption was found to be consistently greater than 90%. As is apparent, in each of these three experimental situ-

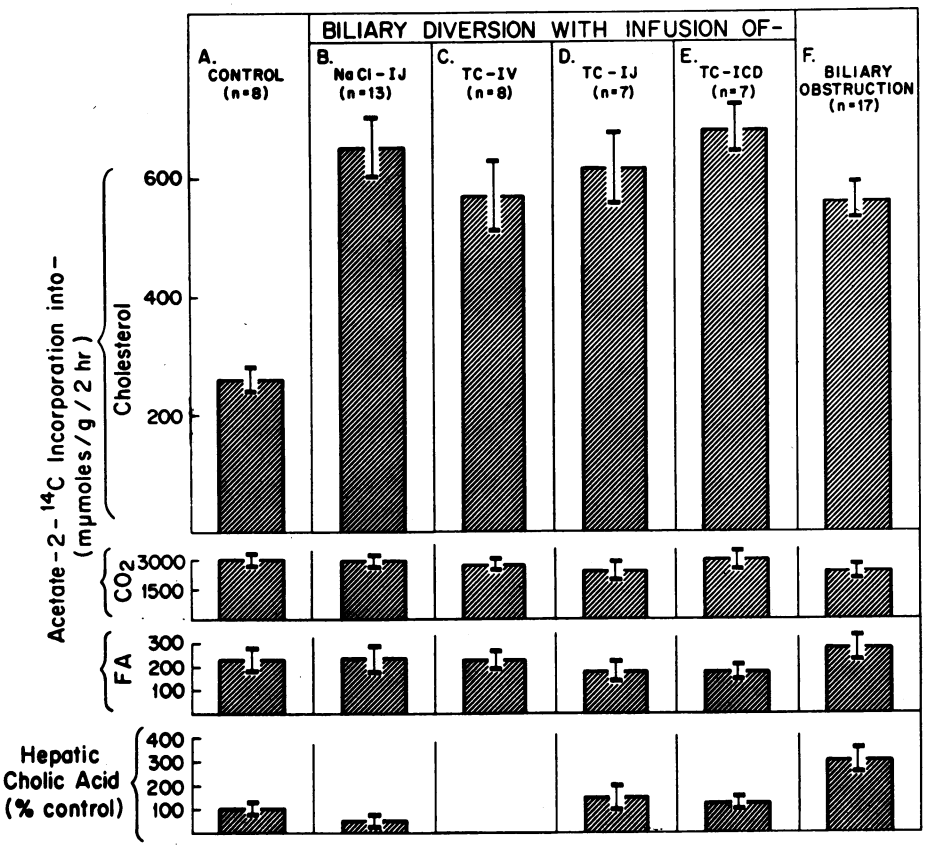

FIGURE 1 Hepatic cholesterogenesis in control rats and in animals with biliary diversion and biliary obstruction. All groups of animals had indwelling stomach tubes and were fed dextrin-casein diet in amounts calculated to equal a caloric intake of 62 kcal/48 hr. The experimental groups were treated as follows. (A) Control animals each had an intact biliary system and an indwelling catheter in the proximal jejunum through which 0.9% NaCl solution was infused. Groups B–E had biliary diversion as well as an indwelling catheter either in the proximal jejunum (IJ), intravenously in a tail vein (IV), or in the proximal common bile duct (ICD). Group B was infused intrajejunally with 0.9% NaCl. Group C was infused intravenously with a solution of taurocholic acid (TC) at a rate of 35 μmoles/hr. Taurocholic acid was infused at a rate of 40 μmoles/hr either intrajejunally or into the proximal common bile duct in groups D and E, respectively. The common bile duct was ligated above the level of the pancreas in group F. After 48 hr, the animals were killed and the ability of liver slices to incorporate acetate-2-^{14}C into cholesterol, CO_2, and fatty acids (FA) was determined. In addition, in all groups except group C the amount of cholic acid extractable from liver tissue at the end of the experiment was quantified. Mean values ±1 SE are shown.

TABLE I

Effect of the Intrajejunal Infusion of Various Bile Acids on Hepatic Cholesterogenesis in Animals with Biliary Diversion

Bile acid and infusion rate	Acetate-2-^{14}C incorporation into		
	Cholesterol	Fatty acid	CO_2
μmoles/hr	*mμmoles/g per 2 hr*		
1. Control	346 ±51	208 ±31	3250 ±450
2. Saline	890 ±72	290 ±51	3901 ±200
3. Taurocholic (40)	965 ±101	195 ±105	2950 ±150
4. Glycocholic (40)	876 ±51	265 ±40	3450 ±351
5. Taurocholic (20) + taurodeoxycholic (20)	1010 ±79	240 ±85	3001 ±250
6. Taurocholic (35) + cholic (5)	1121 ±160	309 ±42	3541 ±350
7. Taurocholic (35) + deoxycholic (5)	910 ±65	301 ±65	2960 ±159

Control animals (1) had intact biliary systems while all other groups of animals (2–7) had external biliary diversion. All groups of animals had indwelling gastric tubes, through which they were fed dextrin-casein diet, and indwelling jejunal tubes through which either 0.9% NaCl or bile acid solutions were infused throughout the 48 hr experimental period. At the end of this time, the animals were killed and the rates of incorporation of acetate-2-^{14}C into cholesterol, fatty acid, and CO_2 by liver slices were determined. There are four or five animals in each group. Mean values ±1 SE are shown.

ations it was impossible to prevent the rise in cholesterogenic activity observed after biliary diversion.

In previous studies reported from this laboratory (28, 29), it was shown that the rate of cholesterol synthesis in the small intestine also increases after biliary diversion and that this increase can be prevented by the infusion of bile acid into the bowel lumen (29). These previous observations were incidentally confirmed in the present study. The rate of cholesterol synthesis in the jejunum of control animals (A) equaled 53 ±6 mμmoles/g per 2 hr and increased markedly in the groups of animals with biliary diversion (B, 331 ±48 mμmoles/g per 2 hr) and biliary obstruction (F, 278 ±27 mμmoles/g per 2 hr). Infusion of taurocholate intrajejunally, but not intravenously, suppressed this high rate of synthesis to control levels. Thus, the specific inhibitory effect of taurocholic acid on intestinal sterol synthesis is in contrast to the lack of effect of bile acid on hepatic cholesterol synthesis.

In order to evaluate the possibility that bile acids other than taurocholic acid might affect the rate of hepatic cholesterogenesis, the studies illustrated in Table I were performed. In this set of experiments acetate was incorporated into cholesterol by the livers of control rats at a rate of 346 ±51 mμmoles/g per 2 hr. This rate increased to 890 ±72 mμmoles/g per 2 hr in animals with biliary diversion infused with saline. As is apparent, the continuous intrajejunal infusion of taurocholic acid, glycocholic acid, or of combinations of taurocholic acid with taurodeoxycholic, cholic, or deoxycholic acid failed to suppress the rate of hepatic cholesterol synthesis to control levels.

In order to exclude the possibility that the semisynthetic dextrin-casein diet altered or abolished an inhibitory effect of bile acid on the cholesterol biosynthetic pathway in the liver, experiments were performed using other dietary regimens. In column A of Table II are

TABLE II

Effect of Taurocholic Acid Infusion on Hepatic Cholesterogenesis in Animals with Biliary Diversion Fed Various Diets

Experimental group	Acetate-2-^{14}C incorporation into—	A. Dextrin-casein (62 kcal/48 hr)	B. Glucose (62 kcal/48 hr)	C. Triolein (62 kcal/48 hr)	D. Fasting
		mμmoles/g/2 hr			
1. Control	Cholesterol	408 ±28	379 ±63	256 ±34	77 ±13
	Fatty acids	348 ±101	299 ±66	50 ±10	14 ±2
	CO_2	3505 ±376	3206 ±162	1985 ±581	2000 ±286
2. Biliary diversion (intrajejunal NaCl infusion)	Cholesterol	1117 ±18	987 ±153	179 ±50	235 ±37
	Fatty acids	291 ±10	265 ±13	36 ±15	24 ±5
	CO_2	3066 ±246	2638 ±214	2499 ±603	1731 ±225
3. Biliary diversion (intrajejunal TC infusion)	Cholesterol	1232 ±138	947 ±179	1000 ±308	236 ±48
	Fatty acids	467 ±51	208 ±52	50 ±8	25 ±6
	CO_2	3895 ±290	2654 ±352	1445 ±226	1245 ±137

Three experimental groups of animals were utilized in this study: 1) control animals with an intact biliary system, 2) animals with biliary diversion infused intrajejunally with 0.9% NaCl solution, and 3) animals with biliary diversion infused intrajejunally with taurocholic acid (TC) (40 μmoles/hr). From four to eight of these animal preparations were then fed, isocalorically, one of three diet regimens through indwelling gastric tubes. A fourth set of animals was fasted. After 48 hr, liver slices were prepared and assayed for their ability to incorporate acetate-2-^{14}C into cholesterol, fatty acids, and CO_2. Mean values ±1 SE are shown.

again shown the results obtained in a group of animals fed the dextrin-casein diet in an amount equal to 62 kcal/48 hr. Biliary diversion increased the rate of cholesterol synthesis by the liver from a control value of 408 ±28 mμmoles/g per 2 hr to 1117 ±18 mμmoles/g per 2 hr and restoration of the enterohepatic circulation of taurocholic acid did not alter this enhanced rate of synthesis (1232 ±138 mμmoles/g per 2 hr). Substitution of glucose, isocaloric for the dextrin-casein diet, yielded similar results (column B). Isocaloric substitution of triolein was poorly tolerated and a number of animals, particularly those with biliary diversion, developed diarrhea. As shown in column C, biliary diversion did not enhance cholesterogenesis in this group of animals. This result very likely is due to the fact that these animals had significant malabsorption and therefore were partially starved relative to the intact control animals. The infusion of bile acids corrected the malabsorptive defect; and as shown in the third part of column C, the rate of hepatic cholesterogenesis rose to the same levels seen in animals fed either the dextrin-casein diet or the glucose diet. Finally as shown in column D, the effects of these three experimental situations were evaluated in fasted animals. As expected, the rate of cholesterogenesis was suppressed to approximately 20% of the value found in control animals fed a dextrin-casein diet. However, after biliary diversion, the same relative 3-fold increase in synthetic activity occurred as the rate of acetate incorporation into cholesterol increased from 77 ±13 mμmoles/g per 2 hr to 235 ±37 mμmoles/g per 2 hr and infusion of taurocholic acid did not suppress the enhanced rate of cholesterol synthesis to control levels.

TABLE III

Effect of Phospholipid on Hepatic Cholesterogenesis in Animals with Biliary Diversion

Experimental group	Dietary regimen	Calculated caloric intake	Acetate-2-^{14}C incorporated into cholesterol
		kcal/48 hr	*mμmoles/g/2 hr*
1. Control	Dextrin-casein	62	408 ±27
2. Biliary diversion	Dextrin-casein	62	1117 ±18
3. Biliary diversion	Dextrin-casein + taurocholic acid	62	1232 ±138
4. Biliary diversion	Dextrin-casein + taurocholic acid + egg phospholipid	62	946 ±89

Control animals had an intact biliary system while the other groups of animals had biliary diversion. Both groups were fed the various diets by continuous infusion into the stomach through indwelling gastric cannulas Animals in groups 3 and 4 received taurocholic acid at the rate of 40 umoles/hr; group 4 received 4.5 mg of egg phospholipid/hr. The amount of dextrin-casein diet administered to group 4 was decreased so that the total calculated caloric intake in these animals was the same as in the first three groups despite the addition of phospholipid to their diet. There were 4–10 animals in each group. Mean values ±1 SE are shown.

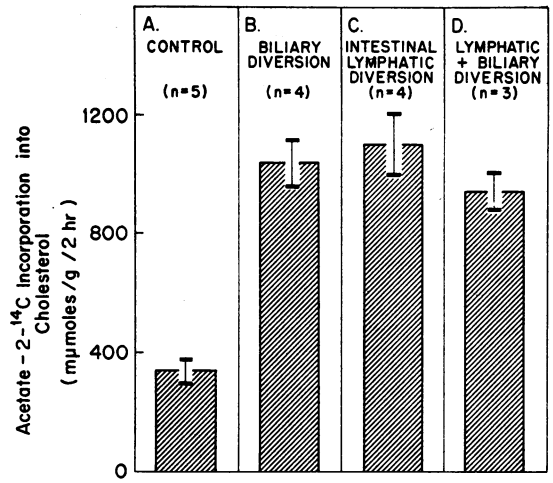

FIGURE 2 Comparison of hepatic cholesterogenesis in animals with biliary and intestinal lymphatic diversion. All animals were fed the dextrin-casein diet (62 kcal/48 hr) through indwelling gastric tubes. Animals in group B had external biliary diversion while those in group C had cannulation of the intestinal lymphatic outflow with external diversion of intestinal lymph. Group D had both biliary and intestinal lymphatic diversion performed. After 48 hr, the animals were killed and liver slices were assayed for their ability to incorporate acetate-2-^{14}C into cholesterol. Mean values ±1 SE are shown.

Thus, it is apparent that under each of these dietary regimens bile acid failed to alter the rate of sterol synthesis by the liver. One final possibility that might serve to explain these results was investigated. In whole bile it is known that bile acids are associated with phospholipids in the form of mixed micelles and that phospholipids, like bile acids, participate in an enterohepatic circulation. The effect of the addition of phospholipids to the infusate of bile acid was therefore tested. As shown in Table III, biliary diversion increased the rate of incorporation of acetate into cholesterol by nearly 3-fold and the infusion of taurocholic acid alone did not suppress this enhanced synthetic activity. Furthermore, the addition of phospholipid to the infused bile acid solution did not cause significant suppression.

In summary, the infusion of a variety of bile acids into groups of animals with biliary diversion maintained on several different dietary regimens consistently failed to prevent a rise in the rate of hepatic cholesterogenesis after external diversion of biliary secretions. These results, along with those described above in the case of biliary obstruction, provide strong evidence that bile acid plays no direct role in the regulation of cholesterol synthesis by the liver.

Role of endogenous cholesterol in the control of hepatic cholesterogenesis. Since the studies reported thus far have demonstrated that interruption of the entero-

hepatic circulation of bile acids cannot account for the enhanced hepatic sterol synthetic rates seen in bile-diverted animals, the next set of experiments was designed to explore the possibility that this effect was, in fact, due to interruption of the absorption of endogenous cholesterol. If the effect of biliary diversion or obstruction on hepatic sterol synthesis is the consequence of a secondary interruption of the enterolymphatic circulation of endogenous cholesterol, then intestinal lymphatic diversion should cause an elevation in the rate of hepatic sterol synthesis that is quantitatively identical with that observed after interruption of the biliary outflow. Such experiments are shown in Fig. 2. After biliary diversion for 48 hr, the rate of acetate incorporation into cholesterol increased 3-fold (panel B). However, as shown in panel C, in animals with an intact enterohepatic circulation of bile acids, interruption of the intestinal lymphatic outflow resulted in an identical rise in hepatic cholesterogenic activity. Finally, interruption of both the biliary and intestinal lymphatic outflow produced no greater rise in synthetic activity than seen with either operative procedure alone. Intestinal lymphatic diversion, like biliary diversion, enhanced the rate of incorporation of acetate into cholesterol but did not alter the rate of incorporation of acetate into fatty acid or CO_2.

While biliary and intestinal lymphatic diversion produced similar effects on hepatic cholesterogenesis, the effect of these two operative procedures on intestinal cholesterogenesis was quite different. The rate of cholesterol synthesis in the small bowel increased 6- to 8-fold after biliary diversion but remained at control levels in animals with intestinal lymphatic diversion.

The enzymatic site in the biosynthetic sequence responsible for the increased rate of acetate incorporation into cholesterol was next elucidated. As shown in Table IV, the rate of acetate-2-^{14}C incorporation into cholesterol increased by 4- to 5-fold in homogenates of livers prepared from animals with biliary diversion, biliary obstruction, and intestinal lymphatic diversion. Similar enhanced synthetic activity was present in homogenates prepared from livers of animals with biliary diversion that were infused with taurocholic acid. As shown in column B, however, when aliquots of these same homogenates were incubated with mevalonate-2-^{14}C, no significant differences were found in the rates of incorporation of this precursor into cholesterol. This finding indicates that the biochemical site of release of inhibition in the cholesterogenic pathway brought about by either biliary diversion, biliary obstruction, or intestinal lymphatic diversion occurs before the formation of mevalonic acid. Finally, this site was localized specifically by direct assay of the incorporation rate of acetate-2-^{14}C into mevalonic acid. Homogenates from control,

TABLE IV

Localization of the Biochemical Site of Release of Inhibition in Rats with Biliary Diversion, Biliary Obstruction, and Intestinal Lymphatic Diversion

Experimental group	A. Acetate-2-^{14}C → cholesterol	B. Mevalonate-2-^{14}C → cholesterol
	mμmoles/2 hr	
1. Control (intrajejunal NaCl infusion)	15.9 ±1.1	19.8 ±0.7
2. Biliary diversion (intrajejunal NaCl infusion)	86.9 ±11.0	17.2 ±0.9
3. Biliary diversion (intrajejunal TC infusion)	64.9 ±16.5	16.6 ±0.3
4. Biliary obstruction (intrajejunal NaCl infusion)	80.3 ±11.5	18.3 ±0.9
5. Intestinal lymphatic diversion (intrajejunal NcCl infusion)	81.8 ±11.0	20.2 ±1.4

Each animal was fed the dextrin-casein diet through an indwelling stomach tube. In addition, each animal had an indwelling catheter in the proximal jejunum through which either a 0.9% NaCl solution or a solution of taurocholic acid (TC) (40 μmoles/hr) was infused continuously. At the end of 48 hr, liver homogenates were prepared and 2.0-ml aliquots were incubated with acetate-2-^{14}C and mevalonate-2-^{14}C. As stressed in the methods section, acetate incorporation was measured under conditions that yield maximal rates, whereas mevalonate incorporation was measured at substrate concentrations less than those necessary to achieve maximal rates. Homogenates were prepared from four animals in each group. Mean values ±1 SE are shown.

bile-diverted, and intestinal lymphatic-diverted animals had incorporation rates of 10,810 ±919 cpm/2 hr, 38,316 ±4100 cpm/2 hr, and 38,617 ±3310 cpm/2 hr, respectively. Under the conditions of this assay, only the reduction of β-hydroxy-β-methyl glutarate is rate limiting (7, 9). Therefore, these data, along with those in Table IV, clearly establish that interruption of the biliary system and the intestinal lymphatic system increases hepatic sterol synthesis by enhancing enzymatic activity at the same site in the biosynthetic sequence, i.e., the conversion of β-hydroxy-β-methyl glutarate to mevalonic acid by the enzyme β-hydroxy-β-methyl glutaryl reductase.

Another prediction that would follow from the thesis that biliary diversion or obstruction augments hepatic cholesterogenesis by interruption of the delivery of endogenous cholesterol into the circulation is that the enhanced rate of sterol synthesis seen under these experimental conditions should be suppressed by the infusion of cholesterol in the form of chylomicrons. Such experiments are shown in Fig. 3. In these studies in which varying amounts of cholesterol in the form of chylomicrons were infused continuously into the rats throughout the 48 hr period of biliary diversion, it is apparent that the rate of hepatic cholesterogenesis is related in a first-order manner to the dose of cholesterol administered intravenously. Despite the inhibition of synthetic activity, the cholesterol content of the livers of control rats and animals receiving chylomicrons was

not significantly different. The concentration of cholesterol equaled 1.82 ±0.09 mg/g in control animals infused with saline and 1.91 ±0.06 mg/g in those animals receiving 10–16 mg of cholesterol/24 hr in chylomicrons.

Several features of this relationship deserve emphasis. The slope of the best fit line on the semilogarithmic plot in Fig. 3 is equal to $-0.082x$. This figure corresponds to a decrease in cholesterol synthetic activity by a factor of approximately 0.19 for each 1.0 mg of cholesterol infused/24 hr. Furthermore, and of particular importance, is the fact that approximately 7 mg of cholesterol/24 hr is required to maintain hepatic cholesterogenesis at control levels. This effect is specific for cholesterogenesis since acetate incorporation into CO_2 and fatty acid was not altered by chylomicron infusion. Finally, the infusion of cholesterol inhibited hepatic cholesterogenesis by suppressing specifically β-hydroxy-β-methyl glutaryl reductase activity since the rate of incorporation of mevalonate-2-^{14}C was uniform in all animals regardless of the amount of cholesterol infused.

These data along with those reported above provide strong evidence that the enterohepatic circulation of endogenous cholesterol plays a major role in the regulation of hepatic sterol synthesis and furthermore, indicate that it is this mechanism, rather than a direct effect of bile acid, that accounts for the changes in the rate of hepatic cholesterogenesis observed after manipulation of the enterohepatic circulation.

DISCUSSION

It has been suggested that bile acid exerts a direct rate-controlling effect in the liver upon the synthesis both of cholesterol from acetate (12, 13) and of bile acid from cholesterol (30, 31). As outlined in the introduction, however, there are few definitive data to support the first thesis that bile acid participates directly in regulation of hepatic cholesterogenesis. Indeed, data presented in the present study demonstrate that bile acids play no direct role in the control of sterol synthesis by the liver. Rather, evidence has been presented that strongly suggests that alterations in the rate of hepatic cholesterogenesis observed after manipulation of the enterohepatic circulation are, in fact, the result of changes in the enterolymphatic circulation of endogenous cholesterol.

Several important aspects of the interrelationship of bile acid and cholesterol metabolism in the intestine and liver deserve comment. Bile acid, secreted into the proximal small intestine through the common duct, is absorbed across the bowel wall at all levels of the intestine by several passive and active transport mechanisms

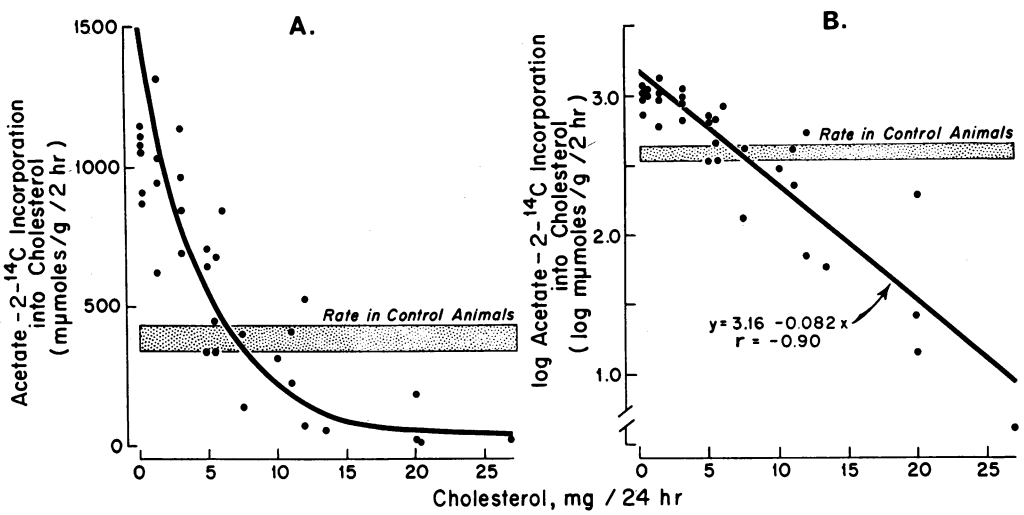

FIGURE 3 The effect of cholesterol administered in the form of chylomicrons on the rate of hepatic cholesterogenesis. Each point represents the result obtained in a single test animal. Each test animal had external biliary diversion and indwelling gastric and intravenous cannulas. The dextrin-casein diet (62 kcal/48 hr) was fed through the indwelling gastric tube. In addition, each animal received various amounts of rat chylomicrons intravenously throughout the 48-hr experimental period. At the end of this period the animals were killed and hepatic cholesterogenic activity was assayed. Panel A shows the rate of hepatic cholesterogenesis plotted against the amount of cholesterol administered in the form of chylomicrons/24 hr. Panel B shows these same data plotted semilogarithmically. The line in panel B is the best fit line by the method of least squares; the line in Panel A is the antilog curve of the line in panel B. The mean rate of hepatic cholesterogenesis ±1 SE for 10 control animals is shown by the shaded area in each panel.

(32–34) and hence, enters the portal circulation and is recycled through the liver. In contrast, cholesterol is absorbed exclusively into the intestinal lymphatic circulation after being incorporated into chylomicrons (35). While the absorption of bile acid is thus anatomically dissociated from the absorption of cholesterol, bile acid, nevertheless, plays several important roles in the absorption of sterols. For example, it is well known that bile acid solubilizes cholesterol in the intestinal contents. Such micellar solubilization is essential for uptake of cholesterol across the mucosal membrane of the intestinal epithelium (36, 37). In addition, recent evidence indicates that bile acid also is required for the ultimate movement of cholesterol, incorporated in chylomicrons, from the intestinal wall into the lymphatic circulation (38). Thus, any experimental manipulation of the enterohepatic circulation of bile acids alters, secondarily, the enterolymphatic absorption of cholesterol. Indeed, in the absence of bile acid, movement of cholesterol into the intestinal lymph is markedly reduced (38).

Initial experiments in this study were designed to determine whether bile acid fluxing through the liver during its normal enterohepatic circulation exerts a direct regulatory effect on the rate of hepatic cholesterol biosynthesis. These studies were undertaken in animals fed a semipurified diet of dextrin and casein so that isocaloric intake was assured even in those animals with exclusion of bile acid from the intestinal lumen. Such rigid dietary control is essential since the rate of hepatic cholesterogenesis is markedly influenced by variation of caloric intake.

As shown by the data in Fig. 1 and Tables I–IV, restoration of the enterohepatic circulation of various bile acids in animals with biliary diversion consistently failed to prevent the rise in hepatic cholesterogenic activity observed after interruption of the biliary outflow. Furthermore, a quantitatively similar rise in synthetic activity was shown to occur after biliary obstruction where the content of bile acid was very high in whole liver tissue. While no data are available on the concentration of bile acid at the intracellular site of cholesterol biosynthesis, the fact that the concentration of bile acid is high both in blood and in cannalicular bile after obstruction of the common bile duct makes it very likely that the concentration of bile acid in the hepatocyte is at least normal or, more likely, elevated. This failure of bile acid to directly control hepatic cholesterogenesis was shown not to be influenced by dietary effects (Table III). An interesting aside is that even in the fasting state biliary diversion results in enhanced hepatic cholesterogenesis, an effect which also cannot be abolished by the infusion of bile acid. These various experimental results, therefore, indicate that bile acid exerts no direct regulatory influence on the rate of cholesterol synthesis in the liver.

Interruption of the enterohepatic circulation of bile acid, as discussed above, leads to interruption of the enterolymphatic absorption of cholesterol. Even in these experiments in which a diet essentially free of cholesterol was used, there is, nevertheless, cholesterol always present in the intestinal lumen. This endogenous cholesterol is derived from bile, other gastrointestinal secretions, and from the intestinal wall. It is therefore possible that the rate of hepatic cholesterogenesis is primarily controlled through feedback inhibition by circulating endogenous sterol and that diversion of bile acid from the intestinal lumen affects sterol synthesis in the liver only secondarily insofar as such a maneuver interferes with the delivery of cholesterol into the lymphatic circulation.

The second series of experimental observations presented here suggests strongly that this latter thesis is correct. First, intestinal lymphatic diversion results in enhanced hepatic cholesterol synthesis to a degree quantitatively identical with that observed after biliary diversion. Second, there is no additive effect of diverting both the biliary secretions and the intestinal lymphatic outflow in the same animal. Third, both biliary and intestinal lymphatic diversion increase hepatic cholesterol synthesis by enhancing enzymatic activity at the step mediated by β-hydroxy-β-methyl glutaryl reductase. It should be emphasized that this is the same point of feedback control at which exogenous cholesterol exerts its inhibitory effect (7–9). Fourth, while restoration of the enterohepatic circulation of bile acid consistently failed to prevent the rise of synthetic activity observed after biliary diversion, restoration of the enterohepatic circulation of cholesterol in the form of chylomicrons did prevent this enhanced activity, as shown in Fig. 3. This inhibitory effect was specific for cholesterogenesis and was directed at the enzymatic step, i.e. β-hydroxy-β-methyl glutaryl reductase, at which biliary diversion has been shown to enhance hepatic cholesterogenic activity. Finally, as is also apparent in Fig. 3, it requires the infusion of approximately 7 mg of cholesterol/24 hr (in chylomicrons) to maintain the rate of hepatic cholesterogenesis in animals with biliary diversion at control levels. These data, in turn, imply that in the intact animal receiving no cholesterol in the diet approximately 7 mg of endogenous cholesterol/24 hr are circulating through the enterolymphatic circulation. It is therefore of considerable interest that direct measurement of the cholesterol content of intestinal lymphatic outflow in animals receiving no cholesterol in their diet yields values equal to approximately 6–9 mg/24 hr.[1]

The concept that hepatic cholesterogenesis is controlled in a significant way by endogenous cholesterol

has several implications that deserve comment. It is apparent, for example, that in the intact animal receiving no exogenous cholesterol the rate of cholesterogenesis in the liver equals only approximately 30–40% of the maximal achievable rate, i.e., cholesterogenesis in the liver of the intact animal is always partially suppressed. Maximal rates of synthesis are seen only after interruption of the enterolymphatic circulation of endogenous cholesterol as occurs with either lymphatic or biliary diversion or after the liver cell has undergone malignant degeneration and loses its specific responsiveness to feedback inhibition by cholesterol, a situation recently studied in detail (39, 40). In regard to this latter point, the rate of cholesterol synthesis achieved in slices of the Morris minimal deviation hepatoma, 7787, has been found to be very nearly identical with those rates found in this study of livers of animals with various diversion procedures (41). In the animal with a hepatic tumor, the enterolymphatic circulation of endogenous cholesterol undoubtedly is intact, but the malignant hepatic cells have lost the capacity to respond to feedback control and so synthesize cholesterol at maximal rates (40).

Such an internal feedback system would operate effectively to maintain cholesterol homeostasis under a variety of abnormal states. For example, such diverse conditions as biliary diversion (14–16), biliary obstruction (14, 20), ileal resection or bypass, (42) and the feeding of cholestyramine (17) are all known to enhance the rate of hepatic cholesterol synthesis. Each of these conditions is associated with a decreased amount of bile acid in the intestinal lumen and, hence, interruption of the enterolymphatic circulation of cholesterol. This, in turn, would account for the increased rate of hepatic sterol synthesis seen in these various situations. On the other hand, feeding exogenous bile acid to intact animals enhances cholesterol absorption from the intestine and causes suppression of the rate of cholesterol synthesis in the liver below control levels (7, 43). Finally, adding exogenous cholesterol to the diet increases even more the amount of cholesterol reaching the liver in chylomicrons and so even greater inhibition of sterol synthesis is produced. Thus, control of the entire range of cholesterol synthetic ability in the liver can be explained in terms of a single mechanism, i.e., the amount of cholesterol, either endogenous or exogenous, reaching the liver through the enterolymphatic circulation and suppressing biosynthetic activity via the negative feedback mechanism described by Siperstein and Fagan (8, 9).

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