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Research Article

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The Role of Bile Salts in Controlling the Rate of Intestinal Cholesterogenesis

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ABSTRACT According to current concepts, the liver and gastrointestinal tract are considered to be the major, if not the sole, sources of circulating serum cholesterol. While several mechanisms have been described which control the rate of hepatic cholesterogenesis, only biliary diversion is known to alter the rate of sterol synthesis in the intestine. The present study was designed to identify the inhibitory constituent of bile and to define its anatomic and biochemical sites of action.

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This feedback inhibition has been localized in the biosynthetic sequence to a step between acetyl CoA and mevalonic acid and, presumably, is at the enzymatic step mediated by hydroxymethylglutaryl reductase.

These studies emphasize the close interrelationship which exists between the mechanisms of control of cholesterogenesis in the liver and small intestine. Sterol synthesis in the liver is regulated by exogenous cholesterol intake, whereas the rate of intestinal sterol synthesis is controlled by bile salt, the major end product of the hepatic catabolism of cholesterol.

INTRODUCTION

Nearly every tissue in the mammal has been shown to be capable of cholesterogenesis (1-3). In both the rat and monkey the highest rates of synthesis per unit weight are found in liver and terminal small intestine, whereas at the other end of the spectrum, tissues which manifest the least active rates of sterol synthesis include muscle and central nervous system (2, 3). The variation in rates of cholesterogenic activity among the various tissues of the body becomes even more impressive when organ weight is taken into consideration. Thus, in a recent publication from this laboratory it was demonstrated that the amount of cholesterol synthesis calculated to occur in the liver and gastrointestinal tract alone accounts for 92% of the total demonstrable cholesterogenic activity found in all tissues of the squirrel monkey (3).

While such in vitro studies emphasize the potential importance of liver and intestine to over-all

sterol metabolism in the intact animal, of still greater physiologic importance are the observations which indicate that these same two organs represent the principal if not the sole endogenous sources for circulating serum cholesterol. Thus, early observations from several laboratories provided convincing evidence that liver made an important contribution to the circulating sterol pool (4-7). More recent studies have, in addition, clearly demonstrated that cholesterol synthesized de novo from acetate in the intestinal wall reaches the circulation by way of the intestinal lymphatics (8, 9). When the synthesis of cholesterol by the liver is blocked and intestinal lymph is diverted from the bloodstream, as recently shown by Wilson, virtually no labeled cholesterol appears in the circulating pool of the monkey injected with radio-labeled acetate (9).

The early recognition of the importance of the liver to sterol metabolism led to the elucidation of several mechanisms that alter the rate of cholesterologenesis in this organ and so, presumably, are important in controlling the hepatic contribution to the circulating sterol pool. For example, feeding cholesterol and fasting both result in marked suppression of sterol synthesis by the liver (2-4, 10-14). Biliary diversion, on the other hand, enhances hepatic cholesterologenesis two to threefold (15). In contrast to these findings in the liver, exogenous cholesterol and fasting were shown to have essentially no effect upon sterol synthesis in the intestine (2, 3, 12, 14, 16, 17) and it has therefore been generally assumed that intestinal cholesterologenesis is devoid of metabolic control. However, in a recent publication from this laboratory, biliary diversion was demonstrated to result in a marked enhancement of sterol synthesis by the small intestine while the infusion of bile caused a striking inhibition of sterol production (14). Since the intestine is now known to contribute significantly to the circulating cholesterol pool, elucidation of this unique control mechanism should lead to a further understanding of the over-all control of sterol metabolism in the intact animal. The present investigation, therefore, was undertaken to identify the constituent of bile responsible for inhibition of intestinal cholesterologenesis, to determine its biochemical and anatomic sites of action, and to define the kinetics of this inhibition.

METHODS

Animal preparations and diets. Female Sprague-Dawley¹ rats weighing 200-225 g were used in these investigations; in preliminary experiments, however, identical results were obtained with male animals. All animals were allowed water and Purina Rat Chow² ad lib. before they were placed on an experimental diet or operated upon. In various experiments four different diets were used: (a) low cholesterol control diet consisted of 5 g of triolein added to each 100 g of ground Purina Rat Chow;² (b) high cholesterol diet was prepared by dissolving 1.5 g of cholesterol in 5 g of hot triolein and mixing this with 100 g of ground rat chow; (c) 3 or 5% cholestyramine diet was prepared by thoroughly mixing 3 or 5 g, respectively, of cholestyramine resin³ with 100 g of ground rat chow; and (d) liquid diets for intragastric feeding consisted either of a solution of sucrose and casein (500 g of sucrose plus 200 g of casein hydrolysate made up to 1 liter with water and adjusted to a pH of 7.4) or of a powdered milk formula (100 g of Lactum powder³ plus 100 ml of water homogenized together in a food blender).

In many of these experiments an *in vivo* assay animal preparation was utilized in which indwelling catheters were placed in the stomach, small intestine, and common bile duct. With the animal under light ether anesthesia, the abdominal cavity was entered through a mid-line incision. A polyethylene feeding tube (o.d. 0.050 inches) was inserted into the stomach through a puncture wound in the greater curvature; it was then secured with a purse-string suture and exteriorized through a stab wound in the left flank. A second catheter (o.d. 0.038 inches) was similarly inserted through the antimesenteric border of the mid-jejunum, secured with a purse-string suture and exteriorized. The common duct was catheterized (o.d. 0.038 inches) just below the bifurcation of the right and left hepatic ducts and above the level of the pancreas, and the catheter was brought to the outside through a stab wound in the right flank of the animal. The abdominal incision was then closed in two layers, and the animal was placed in a restraining cage. All animals were awake and were allowed water ad lib. throughout the subsequent 48-hr experimental period. Bile was collected from the common duct catheter directly into test tubes. The small intestine of each animal was perfused continuously with saline or with various test solutions by means of the indwelling small bowel catheter using a constant rate infusion pump⁴ at rates of 1.0 or 1.5 ml/hr. In addition, each animal was fed 2.0 ml of the sucrose-casein diet through the indwelling stomach tube at 6-hr intervals (approximately 28 kcal/24 hr). In some experiments the powdered milk diet was substituted for the sucrose-casein diet and was fed at the rate of 1.0 ml every 6 hr.

¹ Simonsen Laboratories, Gilroy, Calif.

² Ralston Purina Co., St. Louis, Mo.

³ Mead Johnson & Co., Evansville, Ind.

⁴ B. Braun, Melsungen, West Germany.

Other animals were prepared with "self-emptying" intestinal blind loops at the level of the mid-jejunum as previously described (18). Briefly, these consisted of a blind pouch of jejunum 10 cm in length which communicated with the remainder of the jejunum through a Roux-en-Y anastomosis; the loop was prepared surgically in such a way that peristalsis continuously emptied the isolated segment.

Infusion solutions. In various experiments the small intestines of assay animals were perfused with solutions of either saline, bile salts dissolved in saline, or whole rat bile. In some experiments whole rat bile was first depleted of its bile salt content by passing it through a chromatographic column packed with cholestyramine (1.0 g of resin/10 ml of whole bile). Micellar solutions of cholesterol were prepared by shaking a mixture of 500 ml of 15 mM taurocholate in isotonic saline, 1.0 g of egg phospholipid, and an excess of cholesterol for 36 hr, after which the undissolved cholesterol was removed by centrifugation. All infusion solutions were adjusted, if necessary, to an osmolality of 285–295 mOsm/kg and a pH of 7.4–7.6.

Bile salts⁵ used in these studies were tested for purity by thin-layer chromatography using several different solvent systems (19–21). Particular attention was directed toward the amount of unconjugated bile salt contaminating preparations of conjugated bile salt. If this was greater than approximately 0.5%, the bile salt was dissolved in water, acidified to pH 3.0, extracted four times with diethyl ether, neutralized, and finally lyophilized. In addition, most preparations of taurocholate were reprecipitated from ethanol by the addition of diethyl ether or were recrystallized from an aqueous alcohol solution (22). In a few experiments highly purified taurocholate free of any detectable extraneous conjugated or unconjugated bile salts was isolated by preparative thin-layer chromatography.

Tissue preparations. At the termination of the experimental periods each animal was killed by decapitation and the gastrointestinal tract was immediately excised, flushed with cold saline, and placed in cold Krebs' bicarbonate buffer. The entire small bowel was divided into 10 segments of equal length and numbered 1 through 10, for purposes of identification, proximal to distal. Slices 0.75 mm thick were then prepared from each segment level with a McIlwain tissue slicer;⁶ these were thoroughly rinsed in cold buffer solution and blotted on filter paper. In most experiments 400 mg of slices were used, but in the studies on the kinetics of sterol synthesis only 200 mg were utilized; these were placed in incubation flasks (fitted with center wells) containing 5.0 ml of Krebs' bicarbonate buffer (pH 7.4), 1.0 μ C of acetate-2-¹⁴C, and 10 μ moles of sodium acetate. The flasks were gassed with 95% O₂:5% CO₂, capped, and placed in a metabolic shaker (100 oscillations/min) at 37°C for 2 hr. In order to localize the anatomic site of feedback control, we used a scraping technique, as described previ-

ously (14), to obtain preparations of jejunal villi and crypts which we then incubated as above. On histologic examination the crypt preparations were relatively pure while those of intestinal villi contained fragments of crypt; this was particularly true of the villi prepared from the intestines of animals with biliary diversion where contamination with crypts equalled approximately 5% of the tissue as judged from histologic sections.

Chemical methods. Methods for determining the incorporation of acetate-2-¹⁴C into cholesterol, long-chain fatty acids, and CO₂ have been described previously in detail (17). Briefly, these procedures may be outlined as follows: after incubation, the contents of the flasks were acidified with 1 N H₂SO₄ and the CO₂ 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 (23). 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 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 ¹⁴C assay. All samples were counted in a Packard liquid scintillation counter, series 314E. The data are expressed as the μ moles of acetate-2-¹⁴C incorporated into cholesterol, fatty acids, and carbon dioxide per gram wet weight of tissue per 2-hr incubation.

In order to determine the concentration of bile salts within the intestinal lumen, we gently expressed the intestinal contents of each segment into cold centrifuge tubes. These were immediately capped and centrifuged. Measured aliquots of the supernatant fluid (usually 50 or 100 μ l) were transferred to a second set of tubes and lyophilized. 100 μ l of chloroform:methanol (1:1) was then added and the tubes were tightly capped, vigorously agitated on a vortex mixer, and again centrifuged. 25 μ l of the supernatant fluid was spotted in duplicate on thin-layer plates coated with Silica Gel H⁷ (made from a 50% slurry) which were then developed in the system of Hofmann (19). After drying, we sprayed the plates with water to identify individual spots, and scraped those spots corresponding to taurocholate, taurodeoxycholate, taurochenodeoxycholate, and glycocholate from the plate into separate centrifuge tubes. 2.0 ml of chloroform:methanol (1:1) was added, and the tubes were capped and vigorously agitated on a vortex mixer. After centrifugation, 100 and 500 μ l-aliquots were transferred to test tubes and taken to dryness under nitrogen. 5.0 ml of concentrated sulfuric acid was added to each test tube and bile salts were then quantitated by the spectrofluorometric

⁵ Calbiochem, Los Angeles, Calif.

⁶ H. Mickle, Gomshall, Surrey, England.

⁷ Brinkmann Instruments, Inc., Westbury, N. Y.

method of Levin, Irvin, and Johnston (24). Recoveries were consistently >85% when checked by means of internal standardization with ^{14}C -labeled bile salts or by the systematic addition of known amounts of bile salt to intestinal contents and rat bile.

RESULTS

Effects of biliary diversion and cholestyramine feeding on intestinal cholesterogenesis. In panel A of Fig. 1 is shown the variation in the relative rates of incorporation of acetate into cholesterol, fatty acids, and CO_2 at different levels of the small intestine in animals fed a low cholesterol control diet. The rate of cholesterol synthesis declines slightly down the length of the proximal small bowel reaching a minimum value approximately at the junction of the jejunum and ileum; distally the

rate increases so that maximal cholesterogenic activity is found in the terminal 20% of the small bowel. Fatty acid synthesis is more uniform along the length of the intestine although a modest peak of activity is found in the proximal ileum. Finally, the rate of acetate oxidation to CO_2 gradually increases down the length of the small bowel so that slices taken from the ileum are nearly twice as active in this regard as those taken from the jejunum.

The effects of biliary diversion for 48 hr and feeding of the bile salt sequestrant cholestyramine for 4 days on these three parameters of intestinal metabolism are shown in panels B and C, respectively. As previously reported from this laboratory (14), biliary diversion results in a marked enhance-

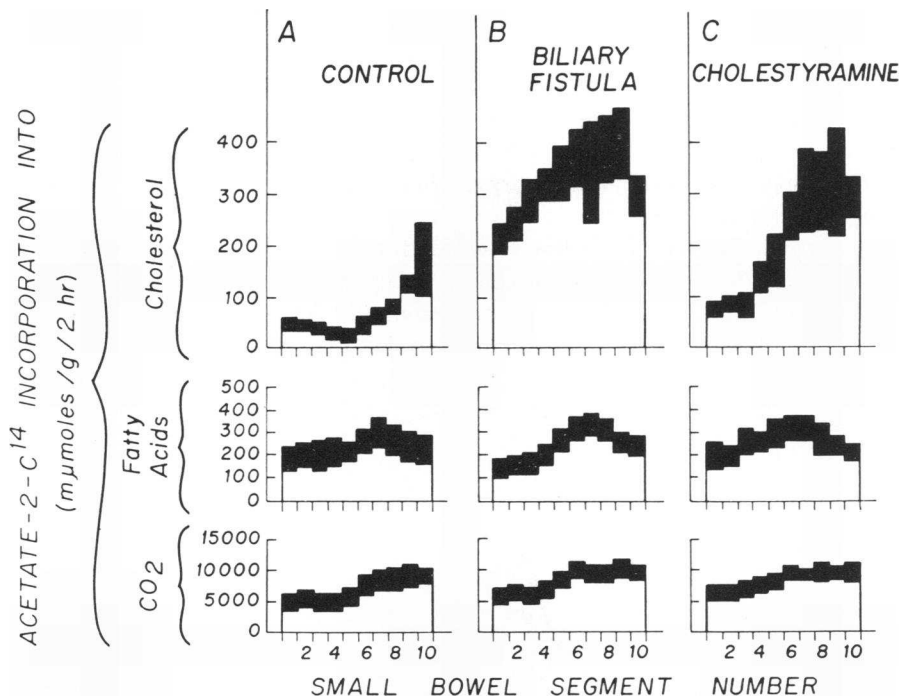


FIGURE 1 Intestinal cholesterogenesis in animals with bile fistula or animals fed cholestyramine. Control animals (A) were sham operated while external biliary diversion was performed in the bile fistula animals (B); both groups of rats were then placed in restraining cages and allowed ground rat chow and water ad lib. for 48 hr. The third group of rats (C) was fed ad lib. 5% cholestyramine diet for 4 days. At the end of the respective experimental periods the animals were killed by decapitation, and the small bowel was divided into 10 segments of equal length numbered from 1 to 10, proximal to distal. 400 mg of slices from each segment were incubated in 5 ml of Krebs' bicarbonate buffer containing $1 \mu\text{C}$ acetate- $2\text{-}^{14}\text{C}$ and $10 \mu\text{moles}$ sodium acetate for 2 hr at 37°C in a metabolic shaker. The data are expressed as the $\text{m}\mu\text{moles}$ of acetate- $2\text{-}^{14}\text{C}$ incorporated into cholesterol, fatty acids, and CO_2 per g wet weight of slices per 2 hr incubation. The shaded area represents the mean $\pm 1 \text{SE}$ for four animals in each group.

ment of the rate of acetate incorporation into sterols by intestinal slices taken from every level of the small bowel; this increase equals nearly ten-fold in the mid-intestinal segments. A similar, although less marked, increase in sterol synthesis can be produced in the intact rat by feeding cholestyramine, as shown in panel C.

It should be emphasized that both of these experimental manipulations alter greatly the rate of cholesterogenesis without affecting the rate of acetate incorporation into fatty acids or CO_2 , as shown in the lower panels of Fig. 1. Furthermore, careful analysis at various times throughout the 2 hr incubation revealed that the rate of incorporation of acetate into cholesterol became linear, i.e. achieved zero order kinetics, within 10 min and remained linear throughout the subsequent 110 min of incubation. This was true for intestinal slices obtained both from control animals and from animals with biliary diversion.

Bile salt concentrations down the length of the small bowel. In order to evaluate the possible relationship between the concentration of bile salt in the intestinal contents and the level of cholesterogenic activity in the adjacent bowel wall, we performed the experiments illustrated by the solid lines in Fig. 2. Animals fed low cholesterol control diet for 1 wk were killed at midnight when their intestines were filled with food. It is apparent that there is a relatively good inverse relationship between the luminal bile salt concentration

and the rate of serol synthesis found at each level of the small intestine. Thus, increasing concentrations of bile salt down the length of the jejunum are associated with declining rates of cholesterol synthesis, while the marked fall in the concentrations of bile salt seen in the distal small bowel occur coincident with an abrupt rise in the rate of acetate incorporation into sterols by slices taken from these same segments.

These initial experiments, therefore, provide inferential evidence along two lines which suggests that bile salt is the constituent of bile responsible for the specific inhibition of intestinal cholesterogenesis in the intact animal. First, cholestyramine, a resin which binds bile salts intraluminally, is nearly as effective as biliary diversion in enhancing intestinal sterol synthesis and, second, an inverse relationship exists down the length of the small bowel between the rates of cholesterogenesis and the intraluminal bile salt concentration.

Identification of bile salt as the inhibitor present in bile. Experiments designed to definitively establish the identification of bile salt as the inhibitor present in bile are shown in Fig. 3. Assay animals were prepared with biliary diversion and indwelling catheters in the stomach and small bowel. Various test solutions were infused continuously for 48 hr into the small bowel at the level of the third intestinal segment, as indicated by the arrows in Fig. 3. Panel A demonstrates the expected low rate of cholesterol synthesis found in

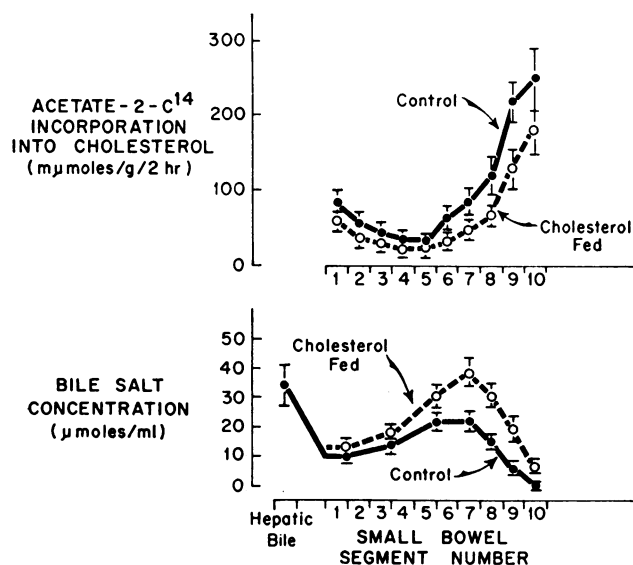


FIGURE 2 Effect of cholesterol feeding on intestinal cholesterogenesis and on conjugated bile salt concentration at all levels of the small bowel. Two groups of six rats were maintained for one wk on either a control or a 1.5% cholesterol diet fed ad lib. The animals were then killed and cholesterol synthetic activity in the small bowel wall and the concentration of conjugated bile salts in the intestinal contents was assayed at each level of the small intestine. Mean values ± 1 SE are shown.

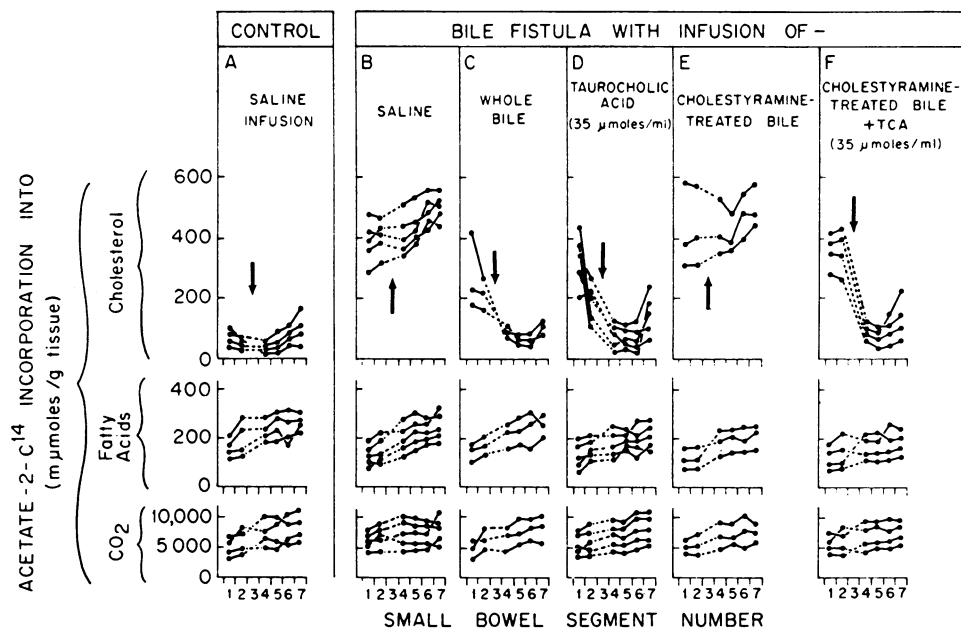


FIGURE 3 Inhibition of intestinal cholesterol synthesis by bile salts infused *in vivo*. Assay animals were prepared with external bile fistula (except for control animals) and with indwelling stomach and small bowel catheters. With the animals in restraining cages, various test solutions were infused into the small intestine at 1 ml/hr for 48 hr. During this period the rats were allowed water *ad lib.* and were fed a sucrose-casein solution, 2 ml every 6 hr, through the indwelling stomach tube. The animals were then killed and intestinal slices were prepared from the first seven segments and were incubated with acetate-2-¹⁴C. The arrows indicate the level of the infusion in the small intestine (segment No. 3). The data from individual animals are shown.

the intestines of control animals with intact biliary systems and with intra-intestinal infusions of saline. For comparison, panel B shows the greatly enhanced rates of synthesis seen in animals with biliary diversion whose proximal small intestines also were perfused with only saline. In contrast, when such animals were perfused with whole rat bile, as shown in panel C, there was inhibition of cholesterol synthesis distal to the infusion point to the low rates seen in control animals. However, of particular importance is the observation demonstrated in panel D that the infusion of taurocholate alone, at the physiologic concentration of 35 nmoles/liter, resulted in as much inhibition of sterol synthesis as the infusion of whole rat bile.

Finally, the importance of bile salt as the inhibitory constituent of whole bile is confirmed by the observations shown in the last two panels of Fig. 3. If whole rat bile is put through a chromatographic column packed with cholestyramine to deplete it of its bile salt content, then essentially all

of the inhibitory activity of the bile is lost, as shown in panel E. When, however, the bile salt content of such cholestyramine-treated bile is restored to 35 nmoles/liter by the addition of pure taurocholate, then there is complete restoration of the inhibitory activity, as shown in panel F. Therefore, these studies provide strong evidence that bile salt is the constituent of bile responsible for inhibition of intestinal cholesterol synthesis in the intact animal. Again, it should be emphasized that this is an effect of bile salt specific for cholesterol synthesis, for as shown in the lower panels of Fig. 3, bile salt exerts no regulatory effects upon fatty acid synthesis or acetate oxidation.

Relationship of inhibition to the intraluminal bile salt concentration. In order to determine the relationship between the steady-state intraluminal bile salt concentration and the rate of cholesterol synthesis in the adjacent wall, we infused assay animals intra-jejunal for 48 hr with solutions containing varying concentrations of taurocholate.

The rate of cholesterogenesis in the jejunum plotted as a function of the steady-state concentration of taurocholate in the lumen of this same area of the bowel is shown in Fig. 4. It is apparent that an inverse relationship exists between these two variables; furthermore, it should be emphasized that the greatest effects upon the rate of intestinal cholesterogenesis occur over a range of intraluminal bile salt concentrations which is encountered physiologically in this area of the small bowel. Thus, variations in the luminal bile salt concentration would be expected to modulate effectively sterol synthesis in the adjacent wall.

Other bile salts in addition to taurocholate were assayed for inhibitory activity in a similar manner. The infusion of glycocholate, taurochenodeoxycholate, taurodeoxycholate, and unconjugated cholate resulted in specific inhibition of intestinal cholesterogenesis to approximately the same degree, at comparable intraluminal concentrations, as that shown for taurocholate in Fig. 4. The infusion of unconjugated deoxycholate, however, resulted in nonspecific inhibition of several parameters of mucosal cell metabolism in addition to sterol synthesis.

Possible role of cholesterol in mediating the inhibitory effect of bile salt. While these data clearly demonstrate that bile salt is the inhibitory constituent of bile, it does not necessarily follow that it is bile salt per se which is directly responsible for the inhibition of cholesterogenesis at the intracellular level. An alternative possibility is that bile salt acts only indirectly in regulating mucosal cell metabolism by promoting the intracellular absorption of a second substance which in turn directly affects the cholesterol biosynthetic sequence. The known role of cholesterol in feedback control of hepatic cholesterogenesis and the absolute requirement of bile salt for the absorption of this 3- β -OH sterol (25) raises the possibility that cholesterol might be the agent directly responsible for the feedback inhibition of intestinal cholesterogenesis. To test this important point, we performed the experiments shown in Fig. 2 and Table I.

When a comparison was made between the rates of cholesterol synthesis in the small intestines of animals fed either a low cholesterol diet or a high cholesterol diet, it is apparent, as shown in the upper panel of Fig. 2, that cholesterogenesis is lower in the intestines of animals fed cholesterol.

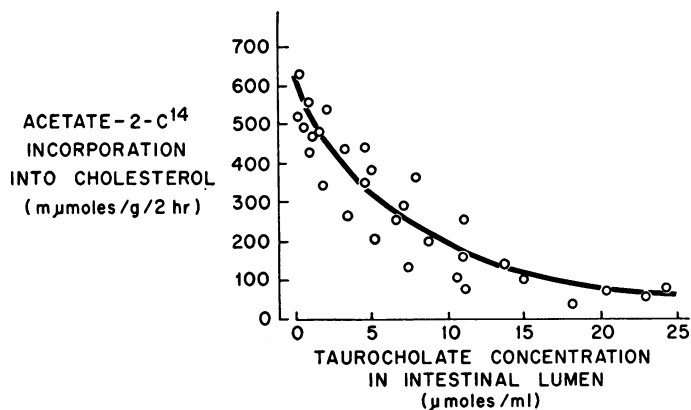


FIGURE 4 Relation of the steady-state concentration of taurocholate in the intestinal lumen to the rate of intestinal cholesterogenesis. Assay animals with biliary diversion and indwelling stomach and small bowel catheters were used in this study. The animals were fed a sucrose-casein solution intragastrically, 2 ml every 6 hr, while their small intestines were perfused continuously with isosmotic taurocholate solutions of varying concentrations (2.5–60 mmoles/liter) at the rate of 1.5 ml/hr. At the end of the 48 hr experimental period, luminal fluid from the mid-jejunum was aspirated for determination of the intraluminal taurocholate concentration and slices from this same area of small bowel were assayed for cholesterogenic activity. Each point represents the results from one animal.

TABLE I
*Effect of Cholesterol Feeding on Intestinal Cholesterogenesis in Rats with Bile Fistula**

Means of administering infused cholesterol	Acetate-2- ¹⁴ C incorporation into cholesterol				
	SB ₂	SB ₄	SB ₆	SB ₈	SB ₁₀
<i>μmoles/g per 2 hr</i>					
A. Dissolved in triolein					
Control	266 ± 8	245 ± 14	313 ± 5	332 ± 17	237 ± 26
+ Cholesterol, 40 mg/day	294 ± 22	324 ± 59	359 ± 25	334 ± 18	222 ± 20
B. Dissolved in powdered milk formula					
Control	316 ± 40	368 ± 76	477 ± 78	419 ± 52	272 ± 27
+ Cholesterol, 40 mg/day	425 ± 56	434 ± 44	428 ± 48	421 ± 21	252 ± 40
	SB ₃	SB ₄	SB ₅	SB ₆	SB ₇
C. Dissolved in micellar solution					
Control	241 ± 27	147 ± 29	64 ± 12	99 ± 26	84 ± 17
+ Cholesterol, 4 mg/day	207 ± 22	127 ± 12	39 ± 10	81 ± 21	80 ± 14

* Rats were prepared with external bile fistula and with indwelling stomach tubes; the animals in Experiment C had, in addition, catheters placed in the third small bowel segment. In Experiment A, either triolein alone or triolein containing dissolved cholesterol (10 mg/ml) was fed intragastrically at a rate of 1 ml every 6 hr. In Experiment B either a powdered milk formula alone or the formula containing dissolved cholesterol (10 mg/ml) was similarly fed intragastrically at a rate of 1 ml every 6 hr. In Experiment C cholesterol was solubilized in a micellar solution of 15 mM taurocholate and egg phospholipid, 2 mg/ml; the data compare the effects of the infusion of the taurocholate-phospholipid solution alone or of this solution containing solubilized cholesterol (0.11 mg/ml) into the small intestine at 1.5 ml/hr. After 48 hr all groups of animals were killed and cholesterogenic activity of slices taken from various levels of the small bowel was assayed. Mean values ± 1 SE of six animals are shown. The designation SB_n indicates the small bowel segment number from which the slices were obtained.

This difference is significant ($P < 0.05$) in intestinal segments six through nine. However, it is also apparent in the lower panel of Fig. 2 that the concentration of bile salt in these same segments is significantly higher in the cholesterol-fed animals ($P < 0.05$). Thus, this experiment provides no definitive evidence for an inhibitory effect of cholesterol on intestinal sterol synthesis since the lower rates of cholesterogenesis found in the cholesterol-fed animals can be attributed to the higher concentrations of bile salt found in the luminal contents of these animals.

In a further attempt to demonstrate such an inhibitory effect, however, large amounts of cholesterol dissolved either in triolein or in a powdered milk formula diet were administered to animals with biliary diversion. As shown in Experiments A and B of Table I, however, the rates of intestinal cholesterol synthesis in such cholesterol-fed animals were not significantly altered from the rates found in appropriate control animals.

In a final attempt to demonstrate a rate-controlling effect of cholesterol on the small bowel, a comparison was made between the amount of

inhibition produced by the intra-intestinal infusion of a micellar solution of taurocholate and phospholipid and the amount of inhibition resulting from the infusion of this micellar solution plus cholesterol. Again, however, as shown in Experiment C of Table I, no greater inhibition of intestinal cholesterogenesis was evident with the infusion of the micellar solution containing cholesterol than with the infusion of the micellar solution containing no sterol.

Thus, in three separate experimental situations it has not been possible to demonstrate an inhibitory effect of cholesterol on intestinal sterol synthesis apart from that directly attributable to bile salt. These data, therefore, do not support the concept that bile salt inhibits intestinal cholesterogenesis only secondarily by promoting the intracellular absorption of cholesterol; rather, they are consistent with the concept that it is bile salt per se which regulates intracellular sterol synthesis.

Anatomic localization of the site of inhibition by bile salts of intestinal cholesterogenesis. Experiments were next undertaken to identify the anatomic site within the intestinal wall where bile

TABLE II
*Localization of the Anatomic Site of Feedback Control of Intestinal Cholesterologenesis**

	Acetate-2- ¹⁴ C incorporation into					
	Cholesterol		Fatty acids		CO ₂	
	Villi	Crypts	Villi	Crypts	Villi	Crypts
	<i>μmoles/10 cm per 2 hr</i>					
A. Control	3 ± 1	33 ± 5	13 ± 5	73 ± 12	628 ± 50	3392 ± 555
B. Bile fistula	32 ± 9	235 ± 15	20 ± 8	106 ± 15	521 ± 163	3191 ± 117

* Groups of six animals were either sham operated or prepared with external bile fistula; after 48 hr the animals were killed and their jejunums were excised. Segments 10 cm long were cut open along the mesenteric border and scraped to yield preparations of intestinal villi and crypts which were then incubated separately. The data are expressed as the mean ± 1 SE values of the μmoles of acetate-2-¹⁴C incorporated into cholesterol, fatty acids, and CO₂ by the amounts of villi or crypts obtained from each 10 cm long intestinal segment.

salt feeds back to inhibit the rate of sterol synthesis; the results of these experiments are shown in Table II. As previously reported from this laboratory (14), nearly all of the cholesterologenic activity of the intestine was found in the mucosal cell layer; the anatomic site of this activity in the mucosa could be localized further, as shown in line A, to the cells of the intestinal crypt. Similarly, these same cells were far more active than those of the villi in incorporating acetate into fatty acids and CO₂.

After diversion of bile salts from the intestinal lumen, nearly all of the greatly enhanced sterol synthetic activity found in the small bowel could be accounted for by a marked increase in the rate of synthesis in the intestinal crypts, as demonstrated in line B of Table II. The intestinal villi also showed some increased synthetic activity, but this was attributable to contamination of these preparations by small amounts of crypt tissue. Again, the inhibitory effect of bile salt on crypt cell cholesterologenesis was specific, for, as also shown in line B of Table II, biliary diversion did not alter the rates of incorporation of acetate into fatty acid or CO₂ by this tissue.

Biochemical localization of the site of inhibition by bile salt of intestinal cholesterologenesis. In order to localize the site of feedback inhibition by bile salt on the cholesterol biosynthetic sequence, we compared the rates of incorporation of acetate-2-¹⁴C and of mevalonate-2-¹⁴C into cholesterol in intestinal slices obtained from control and from bile-diverted animals. As shown in Table III, the rate of incorporation of acetate-2-¹⁴C into sterol

was enhanced nearly sixfold, from 1350 ± 107 to 7940 ± 317 cpm/2 hr, after biliary diversion; in contrast, the rate of incorporation of mevalonate-2-¹⁴C into cholesterol increased only slightly, from 1524 ± 110 to 1985 ± 189 cpm/2 hr, after elimination of bile salt from the intestinal lumen. These data indicate that bile salt inhibits sterol synthesis primarily at a step before the formation of mevalonate. Since biliary diversion does not alter the rates of incorporation of acetate into fatty acid or CO₂ (Fig. 1), neither the intracellular penetration of acetate nor its activation to acetyl CoA is rate limiting under the conditions of these experiments. Therefore, the point of feedback inhibition by bile salt on intestinal cholesterologenesis can be localized more precisely to one of the few enzymatic steps between the condensation of acetyl

TABLE III
*Localization of the Biochemical Site of Feedback Control of Intestinal Cholesterologenesis**

	Acetate-2- ¹⁴ C	Mevalonate-2- ¹⁴ C
	incorporation into cholesterol	incorporation into cholesterol
	<i>cpm/2 hr</i>	
A. Control	1350 ± 107	1524 ± 110
B. Bile fistula	7940 ± 317	1985 ± 189

* Intestinal slices were prepared from sham-operated animals and from animals which had biliary diversion for 48 hr. 400 mg aliquots of these slices were incubated in 5 ml of Krebs' bicarbonate buffer containing 1 μc of either acetate-2-¹⁴C or mevalonate-2-¹⁴C for 2 hr. The data represent the mean ± 1 SE of 10 determinations in each group.

TABLE IV
Effect of Prolonged Cholestyramine Administration on Intestinal Cholesterogenesis*

	Acetate-2- ¹⁴ C incorporation into cholesterol				
	SB ₂	SB ₄	SB ₆	SB ₈	SB ₁₀
	<i>mμmoles/g per 2 hr</i>				
A. Control	90 ± 11	40 ± 4	46 ± 7	129 ± 49	236 ± 36
B. Cholestyramine, 5 days	100 ± 10	165 ± 20	321 ± 29	363 ± 62	263 ± 51
C. Cholestyramine, 30 days	149 ± 44	170 ± 70	271 ± 68	400 ± 47	237 ± 60

* Control animals (A) were maintained on ground rat chow ad lib. while the animals in Experiments B and C were fed a 3% cholestyramine diet for 5 and 30 days, respectively. At the end of the experimental periods, intestinal slices were prepared from various levels of the small intestine and assayed for cholesterogenic activity. The data are mean ± 1 SE values from four animals. The designation SB_n indicates the small bowel segment number from which the slices were obtained.

CoA to form acetoacetyl CoA and the reduction of hydroxymethylglutaryl-CoA to mevalonate.

Kinetics of bile salt inhibition of intestinal cholesterogenesis. In order to determine the kinetics of bile salt inhibition of intestinal cholesterogenesis, we prepared common pools of slices from the proximal small bowels of control animals and of animals with biliary diversion for 48 hr. Sterol synthetic rates were then assayed in 200 mg aliquots of these slices incubated in buffer containing acetate concentrations which varied from 0.022 to 2.200 μmoles/ml. The data were processed by a computer programmed for analysis of the double reciprocal plot of Lineweaver and Burke. In the absence of bile salt the maximum rate of incorporation (V_{max}) of acetate into cholesterol by the jejunum was calculated to equal 450.8 ± 74.8 mμmoles/g per 2 hr; half maximal velocity (K_m) was achieved at an acetate concentration of 0.11 ± 0.06 μmoles/ml (four animals).

In the presence of physiologic concentrations of bile salt in the proximal small intestine, V_{max} equaled only 59.4 ± 5.4 mμmoles/g per 2 hr, while the value of K_m was calculated to be 0.30 ± 0.11 μmoles/ml (four animals). Thus, it is apparent from these data that inhibition of intestinal cholesterogenesis by bile salt is characterized by a marked decrease in the maximum rate of incorporation of acetate into sterol per unit weight of tissue without any significant change in the substrate concentration at which these maximal velocities are achieved.

The effects of chronic bile salt depletion on intestinal cholesterogenesis. While the experiments reported thus far were all relatively short term, it was of interest to determine if the enhanced rate of cholesterogenesis persisted even after a prolonged period of time. The first type of experiment designed to explore this point is shown in Table IV. Three groups of four animals were placed on

TABLE V
Effect of Prolonged Intestinal Isolation on Intestinal Cholesterogenesis*

	Acetate-2- ¹⁴ C incorporation into					
	Cholesterol		Fatty acids		CO ₂	
	SB ₃	BL	SB ₃	BL	SB ₃	BL
	<i>mμmoles/g per 2 hr</i>					
A. Blind loop, 5 days	67 ± 20	256 ± 68	155 ± 19	147 ± 27	3838 ± 580	4222 ± 514
B. Blind loop, 1 yr	56 ± 7	34 ± 4	149 ± 22	131 ± 33	4279 ± 635	4962 ± 526

* Animals were prepared with "self-emptying" blind intestinal loops at the level of the third small bowel segment. After 5 days (A) or 1 yr (B), slices were prepared from the blind loop (BL) and from the third small bowel segment (SB₃) and assayed for their ability to incorporate acetate-2-¹⁴C into cholesterol, fatty acids, and CO₂. Data represent the mean ± 1 SE of four animals in each group.

either a control diet or on a 3% cholestyramine diet for 5 days or for 30 days. It is apparent that the same degree of enhanced cholesterol synthesis was present at the end of one month of cholestyramine feeding as after only 5 days of feeding.

In a second type of experiment, animals were prepared with a "self-emptying" blind pouch of jejunum which was still in continuity with the remainder of the proximal small bowel through a Roux-en-Y anastomosis. Peristalsis continually emptied the isolated loop so that little bile salt was in contact with this area of intestinal mucosa, whereas normal concentrations of bile salt were present in the lumen of the adjacent jejunum. As shown in Table V, 5 days after such an operation, sterol synthesis in the blind loop was selectively enhanced fourfold. However, after the blind loop had been in place 1 yr, cholesterol synthesis markedly decreased to a level which was even lower than that found in the adjacent jejunum. Thus, even though bile salt concentration in this area remained very low, when the mucosa was isolated from the main fecal stream for 1 yr the enhanced rate of sterol synthesis was not maintained. Such data have relevance to sterol balance in humans in which ileal bypass has been performed to promote weight loss or to lower serum cholesterol levels.

DISCUSSION

Even though every tissue which has been tested in the mammal is capable of at least some degree of cholesterogenesis, only two, the liver and gastrointestinal tract, are now considered to contribute significantly to the circulating cholesterol pool (4-9). Knowledge of the mechanisms which control the rates of synthesis in these two organs is therefore essential for an understanding of over-all sterol synthesis in the intact animal.

Hepatic cholesterogenesis is now known to be affected by several dietary and operative manipulations. Since the initial observations of Gould (4), it has been confirmed repeatedly that the rate of cholesterol synthesis is dramatically suppressed by cholesterol feeding (2, 3, 11-14, 17). In a recent publication by Siperstein and Fagan, the point of feedback inhibition by exogenous cholesterol in the biosynthetic sequence has been shown to be at the conversion of hydroxymethylglutarate to mevalonic acid (26). Because of the resemblance of several features of this feedback inhibition to

those previously described in bacterial systems, these authors suggested that exogenous cholesterol primarily suppressed hepatic synthesis by allosteric inhibition of the rate-limiting enzyme in the cholesterol biosynthetic sequence, i.e., hydroxymethylglutaryl reductase. Fasting for periods of 48-72 hr causes a similar suppression of hepatic cholesterogenesis (2, 3, 10, 14, 27, 28). The report of Bucher, Overath, and Lynen suggests that the decrease in synthesis seen after deprivation is also due to depressed enzymatic activity at the level of hydroxymethylglutaryl reductase (28). In contrast to the inhibitory effects of cholesterol feeding and fasting, biliary diversion has been shown to enhance hepatic cholesterogenesis two to threefold (15). The implication has been drawn from these experiments that the bile salt pool, which is depleted after biliary diversion, in some way also exerts a rate-controlling effect upon sterol synthesis from acetate in the liver; however, it is likely that this effect is due to an effect of bile salt on cholesterol absorption (29 and unpublished observations). In summary, the rate of hepatic cholesterol synthesis depends upon the total caloric intake, the cholesterol content of the diet, and the enterohepatic circulation of bile salt.

The situation is quite different with respect to intestinal cholesterogenesis. Several early publications demonstrated that cholesterol feeding did not significantly suppress sterol synthesis in the small intestine (12, 14, 16, 17). This finding was confirmed in both the rat and the monkey in recent publications from this laboratory (2, 3), and even though in the present study lower rates of synthesis were found in segments of the proximal and midileum of cholesterol-fed animals, this difference was attributable to a higher bile salt concentration in these same segments and not directly to the high cholesterol content of the diet. In addition, it was demonstrated in the rat and monkey that fasting for 48-96 hr had little effect upon the rate of intestinal cholesterogenesis, even though the rate of hepatic cholesterogenesis in these animals was suppressed 12- to 15-fold (2, 3). Thus, neither of the dietary manipulations which so dramatically alter cholesterol synthesis in the liver plays an important role in the regulation of intestinal sterol synthesis.

However, diversion of bile from the intestinal lumen results in a five to tenfold increase in the

rate of incorporation of acetate into sterols at all levels of the small intestine both in the rat and in the monkey. Furthermore, in these two species this effect is specific for the small bowel since the rates of acetate incorporation into cholesterol by the esophagus, stomach, and colon are not altered by biliary diversion (2, 3).

It should be stressed that these alterations in the rates of acetate-2-¹⁴C incorporation into digitonin precipitable sterols brought about by biliary diversion represent changes in the actual rates of sterol synthesis. The constancy of the incorporation of acetate into CO₂ and fatty acids, as shown in Fig. 1, make it unlikely that changes in the rate of acetate penetration into the cell, the tissue acetate pool, or the rate of formation of acetyl CoA account for the specific stimulation of the incorporation of acetate-2-¹⁴C into sterols seen after diversion of bile from the gastrointestinal tract. Furthermore, the demonstration that the appearance of labeled acetate in sterols becomes linear with respect to time within 10 min after initiation of the incubation of intestinal slices from both control animals and animals with biliary diversion indicates that in both of these preparations all functionally important intermediate pools between acetyl CoA and the digitonin precipitable sterols have attained isotopic equilibrium. Finally, it also should be emphasized that since the value of K_m for the incorporation of acetate into cholesterol equals approximately 0.30 μ moles/ml, sterol synthesis in these experiments was measured at a substrate concentration approximately seven times the K_m ; thus, in slices from both control rats and rats with biliary diversion rates of cholesterogenesis were measured at essentially zero order kinetics.

Thus, it is clear that some component of whole bile maintains cholesterogenesis in the small bowel in a relatively suppressed state in the intact animal. As shown by the data in Fig. 3, this inhibitory constituent is demonstrated by the present studies to be conjugated bile salt. Infusion of taurocholate, for example, causes as much specific inhibition of intestinal cholesterogenesis as the infusion of whole bile. Furthermore, removal of bile salt from whole bile results in loss of inhibitory activity whereas restoration of the bile salt content of this depleted bile with pure taurocholate restores all of the suppressive activity on intestinal sterol synthesis.

Finally, the degree of inhibition is a function of the steady-state bile salt concentration in the intestinal lumen, as shown in Fig. 4, so that half maximal suppression of cholesterogenesis in the intestine is attained at a luminal bile salt concentration of approximately 5 μ moles/ml.

Conjugated bile salt has been reported to suppress cholesterol synthesis when added in vitro to intestinal slices (30). However, as pointed out by Pope, Parkinson, and Olson, it is now apparent that many of the inhibitory effects ascribed to conjugated bile salt in in vitro experiments are artifacts due to contamination of these preparations with small amounts of unconjugated bile salts such as deoxycholate (31). As demonstrated by these authors as well as in this laboratory, pure conjugated bile salts do not inhibit intestinal cholesterogenesis when added to intestinal slices in vitro (31-33). Such results are not at variance with the bile salt feedback inhibition described in the present study, for it also has been shown that no inhibition of intestinal sterol synthesis becomes manifest under in vivo conditions until after 6 hr of intestinal perfusion with whole bile (14). Therefore, no inhibition would be expected in the short-term in vitro experiments.

While these studies clearly establish bile salt as the inhibitory constituent of whole bile, it does not necessarily follow that it is bile salt per se which acts at the cellular level to inhibit intestinal cholesterogenesis. An alternative possibility is that bile salt acts in this inhibitory system only by facilitating the entrance of a second substance into the mucosal cell; the known role of cholesterol in feedback inhibition of the liver (26) and the absolute requirement of bile salt for cholesterol absorption across the intestinal wall (25) make this sterol a possible candidate for this hypothetical intracellular inhibitor. In addition, even though cholesterol was not present in the infusates in these studies, approximately 2-4 mg is sloughed into the intestinal lumen during the 48 hr duration of these experiments and so would be available for absorption. On the other hand, a strong argument against cholesterol being the actual inhibitor of intestinal cholesterogenesis is presented by the data in Table I where no direct inhibitory effect of cholesterol on cholesterol synthesis by the intestine was demonstrable. Even when 8 mg of cholesterol was

infused in a micellar solution of bile salt, no greater inhibition resulted than could be attributed to the infusion of bile salt alone.

There is ample evidence that bile salt is able to penetrate the intestinal mucosa at every level; proximally, this occurs by way of passive ionic diffusion, whereas distally, both passive ionic and nonionic diffusion as well as active transport act in concert to promote the transmural movement of bile salts (34). In the jejunum, the rate of intracellular movement due to passive diffusion is related to the concentration of bile salt in the intestinal lumen; the rate of penetration, in turn, should determine the steady state intracellular concentration and, hence, the degree of inhibition of cholesterogenesis. This hypothetical scheme correlates well with the experimental findings that the steady-state concentration of bile salt in the jejunal lumen, both in control and in experimental animals, was always inversely related to the rate of cholesterol synthesis in the adjacent wall.

All of these various findings, then, support the conclusion not only that bile salt is the constituent of bile responsible for inhibition of intestinal cholesterogenesis, but, in addition, that it is very likely that it is bile salt per se which acts intracellularly to specifically inhibit the conversion of acetate to cholesterol.

As shown in Table II, feedback inhibition by bile salt is directed at synthesis in the cells of the intestinal crypt, the major site for cholesterogenesis in the intestine. In addition, the point of inhibition in the cholesterol biosynthetic sequence is before the formation of mevalonic acid as demonstrated by the data in Table III. If the enzyme relationships in the mucosal cell are the same as those which have been described for liver (17, 26), then the only rate-limiting step between acetyl CoA and mevalonate is at the reduction of hydroxymethylglutarate; these data, therefore, further imply that the site of feedback inhibition by bile salt is at the enzymatic step mediated by hydroxymethylglutaryl reductase. Finally, the kinetics of this inhibition indicate that bile salts act by suppressing the amount of effective enzyme per unit weight of tissue at the rate-limiting step without altering the substrate binding characteristics of this enzyme.

As pointed out previously, Siperstein and Fagan

have suggested that the cholesterol negative feedback manifested in liver may involve allosteric inhibition of the rate-limiting enzyme, hydroxymethylglutaryl reductase (26). While precise definition of the mode of inhibition involved in the bile salt negative feedback in intestine described in the present investigation must await studies using purified, isolated enzyme systems, several characteristics of this inhibition, nevertheless, allow speculation as to the mechanism of metabolic control. Thus, the latent period between the initiation of bile salt infusion into the bowel of the intact animal and the appearance of inhibition, the inability to inhibit sterol synthesis in intestinal slices by the addition of bile salt *in vitro*, and the kinetics of this inhibition which indicate a marked change in V_{max} without a significant alteration in K_m are all consistent with enzyme repression at the genetic level rather than with allosteric inhibition or a negative effector action of bile salt on the rate-limiting enzyme.

These studies emphasize the close interrelationship which exists between the control of cholesterol synthesis in the liver and in the intestine. The endogenous synthesis of cholesterol by the liver is sensitive to the amount of exogenous cholesterol reaching this organ from the diet. The endogenous synthesis of cholesterol by the intestine, on the other hand, is controlled by bile salt, the catabolic end product of hepatic degradation of cholesterol. Under conditions of low cholesterol intake, the rate of hepatic cholesterogenesis is higher than that found in intestine since the bile salt pool normally present in the small bowel maintains intestinal synthesis at a relatively suppressed level. After the administration of a high cholesterol diet, cholesterol synthesis in the liver essentially stops, but, as demonstrated by Wilson in the rat, the production of bile acids increases (35). As a consequence, bile acid concentration in the small bowel increases, as shown in Fig. 2, and this, in turn, is associated with slight additional suppression of cholesterogenesis in the ileum. Nevertheless, exogenous cholesterol most dramatically inhibits hepatic cholesterogenesis so that in the cholesterol-fed animal the intestine becomes the most active site of sterol synthesis. Finally, interruption of the enterohepatic circulation of bile salt increases sterol synthesis both in the liver, probably by decreasing chole-

terol absorption, and in the small intestine by the direct effect demonstrated in the present study. Since cholesterogenesis is enhanced to a greater degree in intestine (five to tenfold) than in liver (two to threefold), the rate of intestinal synthesis may equal or even exceed the rate of hepatic sterol synthesis in such animals.

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