Regulation of bile acid synthesis. V. Inhibition of conversion of 7-dehydrocholesterol to cholesterol is associated with down-regulation of cholesterol 7α-hydroxylase activity and inhibition of bile acid synthesis

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Abstract In the chronic bile fistula rat, the administration of a bolus dose of mevinolinic acid, an inhibitor of HMG-CoA reductase, was followed by rapid down-regulation of cholesterol 7α-hydroxylase activity and a decrease in bile acid synthesis. These observations suggested that either newly synthesized cholesterol or some other metabolite of mevalonate may be involved in the regulation of bile acid synthesis. In order to distinguish between these two alternatives, we carried out experiments in which cholesterol synthesis was blocked by AY9944, a compound that inhibits the conversion of 7-dehydrocholesterol to cholesterol, a last step in the cholesterol biosynthesis pathway. Rats underwent biliary diversion for 72 h at which time they were given intravenously either a bolus dose of AY9944 (1 mg/kg) or control vehicle. At 0 (pre-treatment control), 0.5, 1.5, and 3 h post bolus, livers were harvested and specific activities of cholesterol 7α-hydroxylase were determined. At 1.5, 3, and 6 h post bolus, AY9944 inhibited bile acid synthesis by 19 ± 6%, 40 ± 4%, and 41 ± 6%, respectively, as compared to pre-treatment baseline. Cholesterol 7α-hydroxylase activity determined at 0.5, 1.5, and 3 h was decreased by 44 ± 6%, 44 ± 2%, and 36 ± 2%, respectively, as compared to the control value. In in vitro experiments using microsomes from livers of control bile fistula rats, the addition of AY9944 (up to 100 μM) failed to inhibit cholesterol 7α-hydroxylase activity. The results of this study demonstrate that, in the chronic bile fistula rat, acute inhibition of cholesterol synthesis at either early or late steps leads to a rapid down-regulation of cholesterol 7α-hydroxylase activity and decrease in bile acid synthesis. —**Pandak, W. M., Z. R. Vlahcevic, D. M. Heuman, and P. B. Hylemon.** Regulation of bile acid synthesis: V. Inhibition of conversion of 7-dehydrocholesterol is associated with down-regulation of cholesterol 7α-hydroxylase activity and inhibition of bile acid synthesis. *J. Lipid Res.* 1990. **31:** 2149–2158.

Supplementary key words cholesterol • bile acid synthesis • cholesterol 7α-hydroxylase • HMG-CoA reductase • liver • AY9944 • mevinolin

In most experimental situations and under many physiological circumstances, the activities of hepatic HMG-CoA reductase, the rate-limiting enzyme in cholesterol biosynthesis, and cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid biosynthesis, were found to change in the same direction (1–4). These two enzymes are located in the smooth endoplasmic reticulum and the observed synchronous behavior suggests that their regulation may be linked to a common regulatory signal. Moreover, several investigators have reported that newly synthesized cholesterol, rather than preformed plasma cholesterol, appears to be the preferred substrate for cholesterol 7α-hydroxylase (5, 6). The mechanism underlying the observed co-variation between the two enzymes is uncertain.

We have previously reported that administration of mevinolinic acid, a competitive inhibitor of HMG-CoA reductase, to rats with chronic bile fistula caused a prompt down-regulation of cholesterol 7α-hydroxylase activity and inhibition of bile acid synthesis. These effects were prevented by simultaneous administration of mevalonolactone, an intermediate in the cholesterol synthesis pathway (7). However, it was not certain from that study whether down-regulation of cholesterol 7α-hydroxylase caused by mevinolinic acid resulted from a decrease in cholesterol synthesis or from deficiency of mevalonate or some other intermediary in the cholesterol biosynthesis pathway. Nonsterol isoprenoid metabolites of mevalonate have been reported to suppress cholesterol synthesis by decreasing HMG-CoA reductase activity probably at the

Abbreviations: DTT, dithiothreitol; HPLC, high performance liquid chromatography; HMG, 3-hydroxy-3-methylglutaryl.

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post-transcriptional level (8, 9). The intriguing possibility is that mevalonate or its metabolites may also regulate cholesterol 7α-hydroxylase activity (**Fig. 1**).

In order to provide information on these two points, we designed the present study in which conversion of 7-dehydrocholesterol to cholesterol was blocked with AY9944, a late step in the cholesterol biosynthesis pathway. This compound has previously been shown to be a potent inhibitor of cholesterol synthesis in the liver (10, 11). As a result of inhibition of this step in cholesterol biosynthesis, mevalonate and/or metabolic products of mevalonate should be present in excess, which in turn should allow us to determine whether these intermediates had regulatory effects on cholesterol 7α-hydroxylase and bile acid synthesis.

MATERIALS AND METHODS

Chemicals

AY9944 (*trans*-N,N'-bis-[(2-chlorophenyl)methyl]-1,4-cyclohexanedimethanamine dihydrochloride) was obtained through a generous gift from Dr. D. Dvornik (Wyeth-Ayerst Laboratories). [4-^{14}C]Cholesterol (59.4 mCi/mmol), DL-[^{3}H]3-hydroxymethyl-3-glutaryl coenzyme A (57.6 mCi/mmol), [^{14}C]taurocholate (46.7 mCi/mmol), and DL-[^{3}H]mevalonate (30 Ci/mmol) and [^{14}C]mevalonolactone (50.1 Ci/mmol) were obtained from New England Nuclear (Boston, MA). DL-mevalonolactone, dithiothreitol, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 3-hydroxy-3-methylglutaryl coenzyme A, $NADP^+$, and NADPH were obtained from Sigma Chemical Company (St. Louis, MO). All other chemicals were of the highest grade available commercially. Silica gel thin-layer chromatography plates were obtained from Fisher Scientific (Springfield, NJ). Intramedic polyethylene tubing (P-50) and Dow Corning silastic tubing were obtained from American Scientific (Columbia, MD).

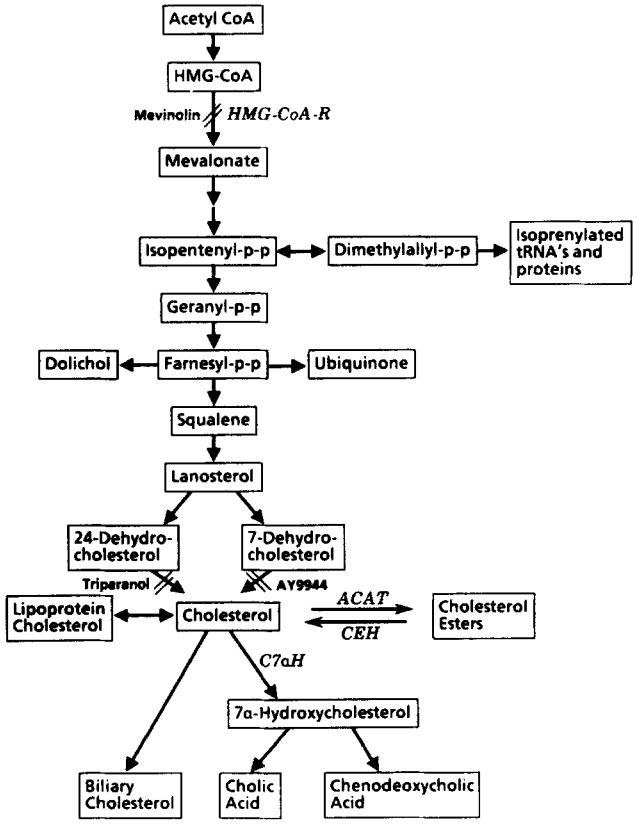

Fig. 1. Schematic of cholesterol biosynthesis pathway. HMG-CoA reductase is the rate-limiting enzyme of cholesterol biosynthesis, and cholesterol 7α-hydroxylase is the rate-limiting enzyme in bile acid biosynthesis. AY9944 inhibits conversion of 7α-dehydrocholesterol to cholesterol, a late step in cholesterol biosynthesis.

Animals

Male Sprague-Dawley rats (Charles River, Cambridge, MA) weighing between 250 and 400 g were housed under controlled lighting conditions (0600–1800 h light phase).

Preparation of animals and experimental protocol

Groups of age- and weight-matched animals were used. With animals under brief methoxyflurane anesthesia, biliary fistula and internal jugular cannulas were inserted. After surgery, animals were placed in individual metabolic cages with free access to water and laboratory chow (Prolab RMH 3000 Agway Corp.). A continuous intravenous infusion of glucose–electrolyte solution was administered at a fixed rate of 1.07 ml/h and was delivered via a syringe pump (Harvard Biosciences, Boston, MA). Each liter of fluid–electrolyte solution contained 100 meq sodium chloride, 30 meq sodium acetate, 6 meq potassium chloride, and 50 g of glucose. In addition, drinking water was supplemented with 50 meq sodium chloride, 15 meq sodium bicarbonate, 3 meq potassium chloride, and 50 g of sucrose per liter. This was done to replace water and electrolyte losses in the bile and , as well, to maintain patency of the IV line for AY9944, [^{14}C]taurocholate, and mevalonolactone infusions. Diverted bile was collected continuously in time increments with a programmed fraction collector. Throughout the experiments, animals were closely monitored. Chow and water consumption, and urine, bile, and stool outputs were determined daily. Rats consuming less than 10 g of chow, drinking less than 15 ml of water, or producing less than 5 ml of urine per 24 h, or exhibiting inconsistent bile flow were eliminated from further study.

In the morning on the fourth postoperative day, after 72 h of chronic biliary diversion, a 1 mg/kg IV bolus of AY9944 or a control vehicle was administered. Bile was collected in timed increments for 2 h before and up to 6 h after the bolus injection of AY9944. Bile flow was monitored throughout the experiment and bile acid synthesis

(equated with biliary bile salt secretion in this model) was determined at 30-min intervals. In some experiments, a constant infusion of mevalonolactone (180 μmol/h) was administered together with the electrolyte solution beginning 0.5 h before administration of AY9944 infusion. In order to assess clearance of bile acids in some experiments, a trace dose of [^{14}C]taurocholate (0.04 μCi; sp act 50 μCi/nmol) was administered intravenously 2 h before the AY9944 IV bolus (internal control period), and again 1 h after the AY9944 administration. At 0.5, 1.5, and 3 h post AY9944 administration, groups of rats were killed and their livers were harvested to be used for the determination of activities of HMG-CoA reductase and cholesterol 7α-hydroxylase, and microsomal cholesterol. In an additional set of experiments, [^{14}C]mevalonolactone (1 μCi; sp act 50 μCi/mmol) was administered intravenously to a group of rats that were killed 3 h after administration of AY9944. Livers were harvested and changes in hepatic microsomal cholesterol were determined.

Test agent preparation and administration

AY9944 was dissolved in distilled water, making a solution concentration of 1 mg/ml. A volume containing 1 mg/kg was infused as an IV bolus infusion through the internal jugular venous cannula. Mevalonolactone (100 mg/ml of water) was administered in the intravenous electrolyte solution at a rate of 180 μmol/h (calculated replacement dose accounts for approximate biliary cholesterol secretion, degradation to bile acids, lipoprotein synthesis, and hormonal synthesis). Distilled water in equivalent volumes was infused as the control vehicle solution. [^{14}C]Taurocholate (2 μCi/ml) in a tracer dose of 0.04 μCi was administered through an internal jugular cannula. [^{14}C]Mevalonolactone (1 μCi/ml) in a dose of 1 μCi was also administered intravenously.

Chemical analysis

Bile was extracted according to Folch, Lees, and Sloane Stanley (12). Phospholipid phosphorus was determined using the method of Bartlett (13), and total cholesterol by a modified cholesterol oxidase method (7, 14). Conjugated bile acids were analyzed by reverse phase HPLC using a modification of the method of Nakayama et al. (15). In rats with complete biliary fistulae, bile acid synthesis is equivalent to biliary bile acid secretion. Microsomal proteins were determined by the method of Bradford (16).

Preparation of microsomes

Animals were killed by decapitation, and the livers were removed immediately, rinsed in iced normal saline, and homogenized in buffer A (sucrose, 100 mM; KCl, 50 mM; EDTA, 30 mM; potassium phosphate, 100 mM (pH 7.4); DTT, 3 mM. Homogenates were then centrifuged at 12,000 *g* at 4°C to sediment nuclei, mitochondria, and cellular debris. The resultant supernatant fluid was centrifuged further at 105,000 *g* for 90 min at 4°C. The microsomal pellet was resuspended in buffer A. The resulting microsomes, in a final concentration of approximately 20 mg protein/ml, were frozen in aliquots until analysis.

Enzyme assays

Microsomal HMG-CoA reductase activity was assayed by the method of Whitehead et al. (17). Cholesterol 7α-hydroxylase activity of all microsomes was determined using endogenous cholesterol as substrate by a reverse phase HPLC method described by Hylemon et al. (18). This method allows us to determine simultaneously cholesterol 7α-hydroxylase activity using exogenous [^{14}C]cholesterol as substrate. In some assays, exogenous NADPH cytochrome P-450 reductase was added to the liver microsomes in order to ensure that the reaction was not rate-limiting with respect to this essential cofactor. Purified cytochrome P-450 reductase was a generous gift obtained from Dr. J. Y. L. Chiang. Microsomal free cholesterol was extracted with acetone–ethanol 1:1, and quantified by the method of Abell et al. (19).

Determination of mevalonate sterol metabolites

Animals were killed by decapitation, and livers were removed immediately, rinsed in iced saline, and 5 g was homogenized in buffer A. Cholesterol was extracted according to Folch et al. (12). A 30-ml volume of the chloroform phase was counted for radioactivity. The same volume was dried under N_2, resuspended in chloroform, and analyzed by reverse phase HPLC. Elution peaks were individually collected and radioactivity was counted; the identity of peaks was determined by comparison with retention time and mass of synthetic standards.

RESULTS

Fig. 2 compares the effect of an intravenously administered bolus dose of AY9944 and mevinolinic acid on bile acid synthesis over a period of 6 h. After a 1 mg/kg intravenous bolus of AY9944, bile acid synthesis (or biliary bile salt excretion) was reduced from the infusion baseline by 19 ± 6%, 40 ± 4%, and 41 ± 6% at 1.5, 3, and 6 h, respectively ($P<0.01$). Reduction of bile acid synthesis after the administration of AY9944 was more rapid, but less profound that in experiments in which a bolus dose of mevinolinic acid (10 mg/kg) was administered. Bile flow remained constant for the duration of experiments in both groups of animals (data not shown).

Fig. 3 demonstrates clearance of an IV bolus of [^{14}C]taurocholic acid infused before and after administration of AY9944. Recoveries of the IV bolus trace dose of [^{14}C]taurocholic acid (0.04 μCi) administered 2 h before AY9944 and 1 h after AY9944 were essentially identical.

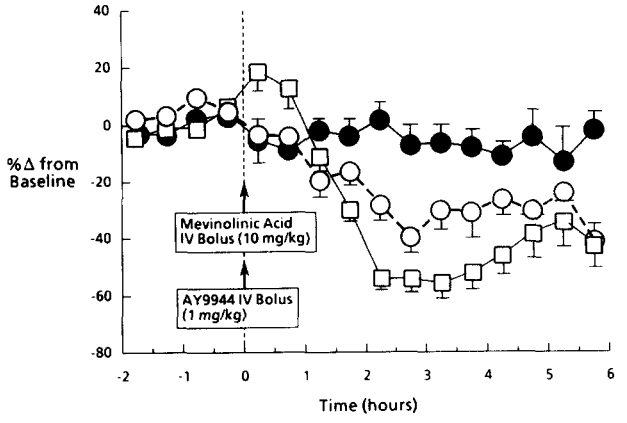

Fig. 2. Effect of AY9944 (1 mg/kg IV bolus) versus mevinolinic acid (10 mg/kg IV bolus) on bile acid synthesis; (●), bile acid synthesis after control vehicle infusion; (○), bile acid synthesis after AY9944; (□), bile acid synthesis after mevinolinic acid. Data are expressed as percent change from pre-drug infusion baseline mean. Each point represents the mean ± SE of four determinations. Bile flow remained unchanged from baseline in both experiments.

Peak secretion of ^{14}C radioactivity in bile was observed 60 ± 5 min after each bolus with >90% of the total dose secreted within 2 h. This 60-min delay represents dead space in the biliary collecting system. Prompt secretion of [^{14}C]taurocholate after infusion of AY9944 suggests that administration of AY9944 did not interfere with uptake and/or secretion of bile salts. Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) levels in animals infused with AY9944 and/or mevalonolactone were not elevated over controls (data not shown). Also, bile flow was not affected under any of the experimental conditions used. These data exclude the possibility that changes occurring after administration of AY9944 resulted from cholestasis or liver injury.

The specific activities of cholesterol 7α-hydroxylase and HMG-CoA reductase in rats killed 0.5, 1.5, and 3 h after

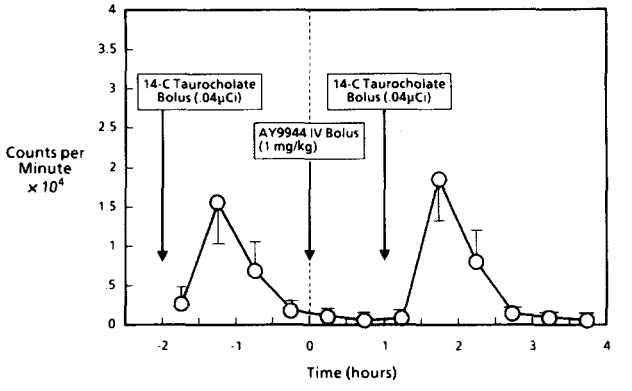

Fig. 3. Effect of AY9944 on biliary excretion of [^{14}C]taurocholate. An IV bolus of [^{14}C]taurocholate (0.04 μCi) given 2 h prior to and 1 h after AY9944 administration. In each case peak secretion of [^{14}C]taurocholate in bile was observed 60 ± 5 min after infusion, with greater than 90% of the total dose secreted into the bile within 2 h. Each point is the mean ± the SE of four determinations. Each animal served as his own control.

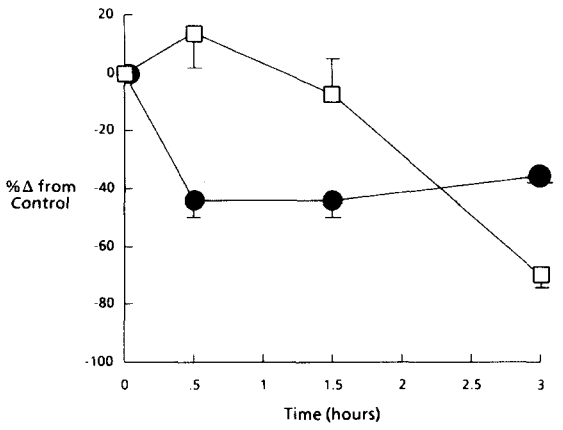

Fig. 4. Effect of AY9944 on the activities of HMG-CoA reductase and cholesterol 7α-hydroxylase. A profound decrease in cholesterol 7α-hydroxylase activity (●) occurs by 30 min post administration of AY9944. In contrast, onset of down-regulation of HMG-CoA reductase activity (□) lagged behind that of cholesterol 7α-hydroxylase. Data are expressed as percent change from controls. Each point represents the mean ± SE of four determinations. Control enzyme specific activities for HMG-CoA reductase and cholesterol 7α-hydroxylase expressed in nmol/h · mg^{-1} of microsomal protein are 7.2 ± 0.76 and 4.36 ± 0.29, respectively.

administration of a bolus dose of AY9944 are shown in **Fig. 4**. Cholesterol 7α-hydroxylase activities were decreased by 44 ± 6%, 44 ± 2%, and 36 ± 2%, respectively. Cholesterol 7α-hydroxylase activity was measured as conversion of endogenous microsomal cholesterol to 7α-hydroxycholesterol (18). In some assays measuring cholesterol 7α-hydroxylase specific activities of control and AY9944-treated rats, purified NADPH cytochrome P-450 reductase was added to the assay system in excess in order to ensure that the reaction was not rate-limiting with regard to this essential cofactor. No significant changes in enzyme specific activities were noted after addition of exogenous cytochrome P-450 reductase (data not shown). HMG-CoA reductase activities were unchanged at 0.5 and 1.5 h. By contrast, at 3 h there was a marked down-regulation of HMG-CoA reductase. We hypothesize that this change resulted from accumulation of mevalonolactone-derived cholesterol precursors which have been previously shown to down-regulate HMG-CoA reductase (8, 9). However, these findings only provide indirect evidence that the down-regulation of HMG-CoA reductase activity observed is related to accumulation of sterol or nonsterol precursors of cholesterol.

Fig. 5 compares the specific activities of cholesterol 7α-hydroxylase in groups of rats that received AY9944 and rats receiving mevinolinic acid over a period of 3 h. After administration of AY9944 cholesterol, 7α-hydroxylase activity was down-regulated at 0.5 h, while there was no change with mevinolinic acid at the same time period. In contrast, specific activity of cholesterol 7α-hydroxylase after mevinolinic acid administration became reduced at

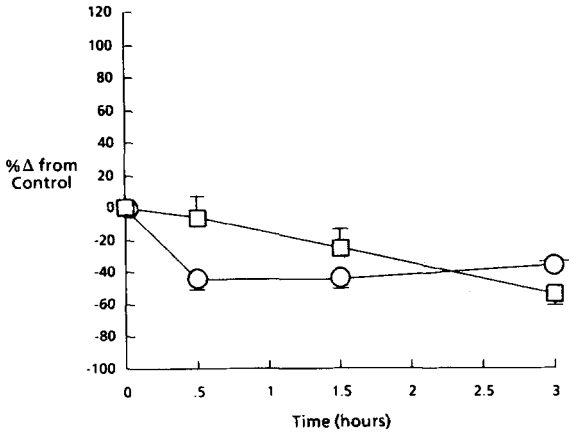

Fig. 5. Effect of AY9944 versus mevinolinic acid on the activity of cholesterol 7α-hydroxylase. An immediate decrease in cholesterol 7α-hydroxylase activity was noted after administration of AY9944 at time zero (○); by contrast, after administration of mevinolinic acid (□), the down-regulation of cholesterol 7α-hydroxylase activity was delayed.

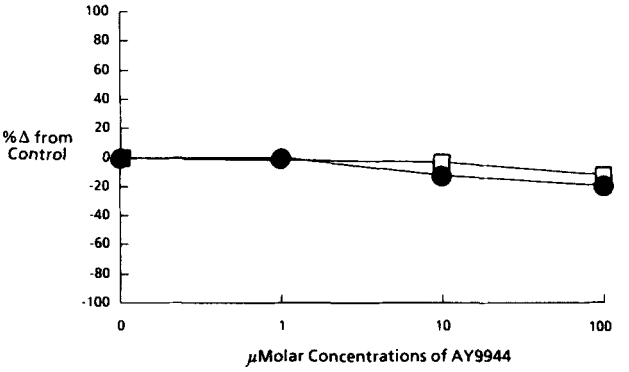

Fig. 6. Effect of AY9944 in vitro on the activity of HMG-CoA reductase (●) and cholesterol 7α-hydroxylase activity (□). Data are expressed as percent change from control. Each point is the mean of two determinations.

1.5 h, and was 54% of control at 3 h. The specific activities of cholesterol 7α-hydroxylase after AY9944 or mevinolinic acid administration were about equal at 3 h.

In one group of rats killed 3 h post AY9944 administration, cholesterol 7α-hydroxylase activity in microsomes was measured simultaneously by reverse phase HPLC using both endogenous and exogenous [4-^{14}C]cholesterol as substrate. Cholesterol 7α-hydroxylase activity measured by conversion of endogenous microsomal cholesterol to 7α-hydroxycholesterol was decreased 35% from control values after AY9944 administration. Similarly, isotope incorporation, defined as the ratio of radioactivity collected in the 7α-hydroxycholesterol peak to that in the cholesterol peak, decreased 51% from that found in control bile fistula microsomes (data not shown).

When the data on bile acid synthesis and cholesterol 7α-hydroxylase acitivity were normalized, i.e., expressed as percent of control, there was a very close correlation between the two measurements. This demonstrates that measurements of cholesterol 7α-hydroxylase acutely reflect the rates of bile acid synthesis and vice versa.

Fig. 6 shows that in vitro addition of AY9944 at concentrations of 1, 10, and 100 μM to microsomes of bile fistula rats did not result in a significant direct inhibition of cholesterol 7α-hydroxylase or HMG-CoA reductase activities.

Continuous intravenous infusion of mevalonolactone (180 μmol/h) to rats receiving AY9944 failed to normalize bile acid synthesis rates (**Fig. 7**). This finding is different from that of our previous studies using mevinolinic acid as a cholesterol biosynthetic inhibitor in which normalization of bile acid synthesis was observed after continuous infusion of mevalonolactone (7). Also, mevalonolactone infusion did not potentiate the inhibitory effect of AY9944.

Fig. 8 shows the specific activities of cholesterol 7α-hydroxylase 3 h after three different experimental manipulations. AY9944 significantly ($P<0.001$) inhibited (40%) cholesterol 7α-hydroxylase activity as compared to control. Administration of mevalonolactone alone caused no significant change in cholesterol 7α-hydroxylase activity when compared to control values. Administration of mevalonolactone plus AY9944 did not alter the activity of cholesterol 7α-hydroxylase when compared to AY9944 alone. HMG-CoA reductase activity under all three experimental conditions (mevalonolactone, AY9944, mevalonolactone plus AY9944) was down-regulated.

Total microsomal free cholesterol was not significantly altered from control values by infusions of AY9944 or mevalonolactone alone (**Table 1**). However, after simul-

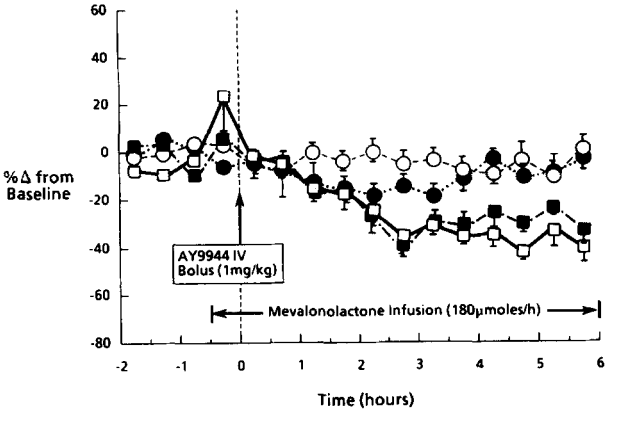

Fig. 7. Effects of mevalonolactone (constant infusion) and/or AY9944 (IV bolus) on bile acid synthesis. Data are expressed as percent change from pre-drug infusion baseline mean. Each point is the mean ± SE of four determinations; (○---○), effects of control vehicle; (●···●), effects of mevalonolactone; (■— ·-■), effects of AY9944 and mevalonolactone; (□——□), effects of AY9944 alone. Actual baseline values (mean ± std) are (*μmol/100 g·h^{-1}*) *control*, 2.10 ± 0.03; *mevalonolactone*, 2.62 ± 0.07; mevinolinic acid, 2.33 ± 0.08; AY9944 + mevalonolactone, 2.19 ± 0.17.

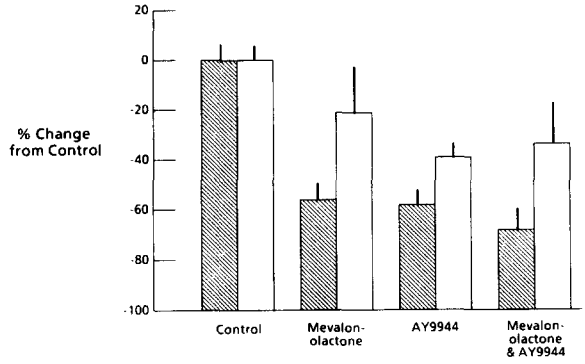

Fig. 8. Effects of AY9944 (1 mg/kg IV bolus) and /or mevalonolactone (180 μmol/h constant IV infusion) on HMG-CoA reductase activity (shaded bars) and on cholesterol 7α-hydroxylase activity (clear bars) 3 h after initiation of the experiment. Data are expressed as % change from time zero controls. Each point represents the mean ± SE of four determinations.

taneous infusion of both AY9944 and mevalonolactone, a significant increase in digitonin-precipitable sterols was seen. We speculate that the increase in digitonin-precipitable sterols, observed when a bolus dose of AY9944 was coupled with continuous infusion of mevalonolactone, reflected free cholesterol plus accumulated sterol precursors of the biosynthesis pathway.

The administration of AY9944 resulted in inhibition of cholesterol synthesis at the last step in the cholesterol biosynthetic pathway as evidenced by accumulation of 7-dehydrocholesterol within the liver, and a decrease in the rate of incorporation of [^{14}C]mevalonolactone into cholesterol (10, 11). The first of these two findings is demonstrated in **Fig. 9**. Fig. 9C shows the appearance of an HPLC retention peak after AY9944 administration which correlates with the retention peak (peak 2) of the cholesterol precursor, 7-dehydrocholesterol (Fig. 9A), a peak not apparent in the control bile fistula rat (Fig. 9B). Gas-liquid chromatographic analysis of microsomes of rats treated with AY9944 confirmed these observations, also demonstrating accumulation of a retention peak correlating with 7-dehydrocholesterol (data not shown). The second of these findings is demonstrated by the following observations. The ratio of [^{14}C]cholesterol/total cholesterol in the liver after administration of an intravenous bolus of [^{14}C]mevalonolactone 1 h prior to killing in the control bile fistula rat was 4.29×10^{-6} dpm/mmol cholesterol. After inhibition of cholesterol synthesis with AY9944, cholesterol specific activity was 2.02×10^{-6} dpm/mmol representing a 53% decrease from the control value. This decrease in specific activity after administration of AY9944 roughly correlates with the reduction in measurements of cholesterol 7α-hydroxylase activity and bile acid synthesis after AY9944 administration.

DISCUSSION

The synchronous behavior of HMG-CoA reductase and cholesterol 7α-hydroxylase activities and the putative role of newly synthesized cholesterol in the regulation of cholesterol 7α-hydroxylase served as a basis for a postulated interrelationship between the cholesterol and bile acid biosynthesis pathways (4, 5, 20). Studies recently performed in our laboratories have shown that in chronic bile fistula rats inhibition of HMG-CoA reductase with mevinolinic acid led to a marked down-regulation of cholesterol 7α-hydroxylase activity and reduction in bile acid synthesis. Continuous infusion of mevalonolactone reversed the effects of mevinolinic acid on cholesterol 7α-hydroxylase and bile acid synthesis. These results provided further evidence for the existence of close interrelationship between the cholesterol and bile acid biosynthetic pathways (7). It was not certain from that study, however, whether down-regulation of cholesterol 7α-hydroxylase was caused solely by a decrease in the supply

TABLE 1. Effect of AY9944 (IV bolus) and/or mevalonolactone (constant infusion) on microsomal cholesterol

Condition	Time Post Bolus AY9944 (or Control Vehicle)	Microsomal Cholesterol	
		n	Mean ± SD
	h		*μmol/g microsomal protein*
Control	0	6	47.8 ± 8.26
AY9944	0.5	5	37.3 ± 4.99
AY9944	1.5	5	49.7 ± 11.8
AY9944	3	6	46.0 ± 15.2
Control	3	4	45.8 ± 5.84
Mevalonolactone	3	4	51.2 ± 8.38
AY9944	3	6	46.0 ± 15.2
Mevalonolactone + AY9944	3	4	79.9 ± 12.7[a]

[a]Significantly different from control value, $P < 0.02$.

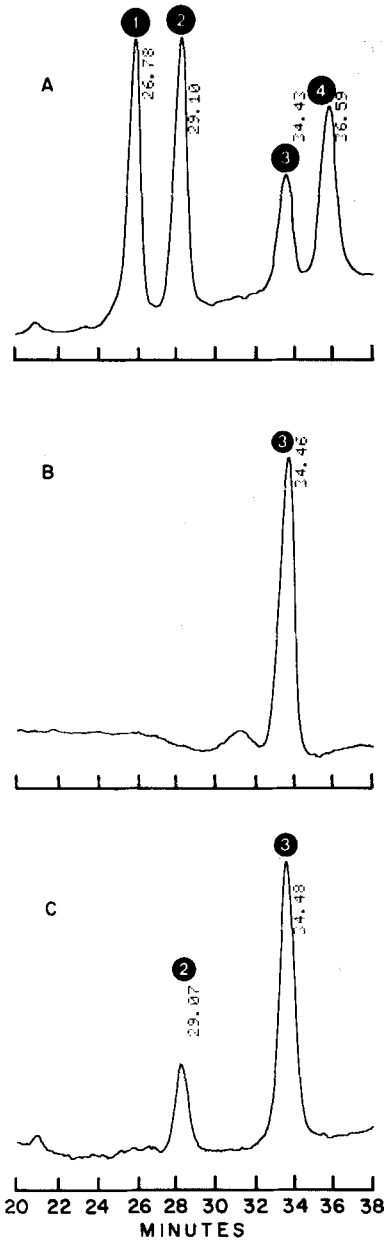

Fig. 9. Effect of AY9944 on the accumulation of three cholesterol precursors. A) Represents the HPLC retention peaks of 1 mM concentration of known standards: 24-dehydrocholesterol (1), 7-dehydrocholesterol (2), cholesterol (3), and lanosterol (4). B) HPLC retention peaks observed in the chronic bile fistula rat. C) Retention peaks in the AY9944-treated rat.

of newly synthesized cholesterol or as a result of reduction of an intermediate (mevalonate or a metabolic product of mevalonate) in the cholesterol biosynthesis pathway. A hypothesis, that mevalonate or a metabolic product of mevalonate may play a role in regulation of cholesterol 7α-hydroxylase, was novel and consistent with the obtained data. The role of newly synthesized cholesterol in regulation of bile acid synthesis was also suggested by the findings of Bilhartz, Spady, and Dietschy (21), who reported that after withdrawal of mevinolin, a marked increase in bile acid synthesis occurred.

The present study was designed to answer two questions: *1*) is down-regulation of cholesterol 7α-hydroxylase specific to inhibition of HMG-CoA reductase, or does it occur in response to inhibition of cholesterol synthesis at any step of the pathway; and *2*) does mevalonate (or metabolic product of mevalonate regulate cholesterol 7α-hydroxylase? Inhibition of a late step in the cholesterol synthesis pathway with AY9944 should result in the accumulation of biosynthetic intermediates. If a putative intermediate in the cholesterol biosynthesis pathway plays a role in regulation of cholesterol 7α-hydroxylase, then its accumulation should up-regulate cholesterol 7α-hydroxylase activity.

Several studies have demonstrated that AY9944 inhibits solely the last step in the cholesterol biosynthetic pathway (10, 11). In contrast, Gibbons and Mitropoulos (22) suggested that at high concentrations of AY9944 (1×10^{-4} M) two additional late steps in cholesterol biosynthesis may be affected, steps involved in the conversion of lanosterol to 7-dehydrocholesterol. We should point out, however, that our conclusions are not dependent on whether, in addition to inhibition of conversion of 7-dehydrocholesterol to cholesterol, other steps in the cholesterol synthesis are involved.

The data obtained in the present study provide additional evidence that, in chronic bile fistula rat, newly synthesized cholesterol plays a role in regulation of cholesterol 7α-hydroxylase. Administration of AY9944 inhibited bile acid synthesis and suppressed cholesterol 7α-hydroxylase activity. These effects were similar to those previously observed with mevinolinic acid, an inhibitor that blocks an early step in cholesterol biosynthesis. Several differences were observed, however. First, inhibition of bile acid synthesis after AY9944 administration was maximal by 30 min, whereas mevinolinic acid maximally inhibited bile acid synthesis at 180 min. This difference probably reflects the time required for complete utilization of cholesterol precursors. Second, the magnitude of inhibition of bile acid synthesis after AY9944 administration was somewhat less than that observed in previous studies using mevinolinic acid (40% vs 54% suppression), possibly because conversion of lanosterol to cholesterol can follow two pathways, only one of which is blocked by AY9944 (Fig. 1). Third, AY9944's inhibitory effects were neither reversed nor potentiated by simultaneous administration of mevalonolactone, a cholesterol precursor. This is in contrast to the experiments in which mevinolinic acid was used (7). This last result can be best explained by the fact that AY9944 prevented the conversion of cholesterol biosynthetic intermediates

into the newly synthesized cholesterol. These findings suggest that newly synthesized cholesterol is necessary to sustain the activity of cholesterol 7α-hydroxylase under conditions of maximal bile acid synthesis.

We have previously hypothesized that the regulatory linkage between the pathways of cholesterol and bile acid synthesis might be mediated via nonsterol metabolite(s) of mevalonate (7). Precedent for such a regulatory mechanism comes from studies of Brown and associates (8, 9) who investigated the mechanism of regulation of HMG-CoA reductase. These investigators found that suppression of HMG-CoA reductase activity in cultured fibroblasts was submaximal with addition of high concentrations of lipoprotein cholesterol. They also found that addition of mevalonate to the culture medium was required for maximal suppression of HMG-CoA reductase. This observation suggested the existence of a second mechanism for regulation of the HMG-CoA reductase enzyme. The authors proposed that mevalonate or some product of mevalonate metabolism other than cholesterol down-regulates HMG-CoA reductase activity. Subsequent studies also suggested that the regulatory metabolite acted at a post-transcriptional level by enhancing degradation or inhibiting translation of HMG-CoA reductase mRNA (22-24). The data obtained in the present study suggest that cholesterol 7α-hydroxylase is not regulated by mevalonate or a metabolic product of mevalonate. We base this conclusion on two findings: *a*) inhibition of bile acid synthesis and cholesterol 7α-hydroxylase activity with AY9944 was similar to that seen with mevinolinic acid; and *b*) mevalonolactone failed to prevent the inhibitory effects of AY9944 on cholesterol 7α-hydroxylase, despite accumulation of putative precursors of cholesterol synthetic pathway. Although the evidence is indirect, it is plausible to suggest that the observed decrease of HMG-CoA reductase with AY9944 can likely be attributed to suppression of HMG-CoA reductase synthesis by the accumulated mevalonate or a metabolic product of mevalonate. This is the first demonstration of such an effect on HMG-CoA reductase in in vivo experiments.

Based on these observations, we conclude that down-regulation of cholesterol 7α-hydroxylase activity can occur as a result of inhibition of cholesterol biosynthesis at any step of the pathway. This conclusion is appropriate for the conditions in which the bile acid synthesis rate is high; i.e., there is an up-regulation of cholesterol 7α-hydroxylase activity. It is not certain whether cholesterol 7α-hydroxylase activity will be changed under circumstances in which cholesterol and bile acid synthesis are normal such as in animals with intact enterohepatic circulation. Björkhem (25) has shown that administration of mevinolin for 3 days did not result in appreciable changes of cholesterol 7α-hydroxylase in rats, suggesting that newly synthesized cholesterol may have a lesser role in rats with intact enterohepatic circulation. An additional conclusion from our study is that changes in cholesterol synthesis rates, rather than the accumulation of a cholesterol precursor(s), are responsible for the down-regulation of cholesterol 7α-hydroxylase activity and a decrease in bile acid synthesis.

The mechanism by which inhibitors of cholesterol synthesis down-regulate cholesterol 7α-hydroxylase activity is not understood. It is tempting to postulate that reduced availability of newly synthesized cholesterol is the most plausible explanation for the observed findings. This tempting hypothesis cannot fully explain the data. Specifically, two different assays of cholesterol 7α-hydroxylase activity, using either endogenous microsomal cholesterol or exogenous [^{14}C]cholesterol as substrate, showed fractional decreases in the specific activity after administration of AY9944. Similar findings were observed after administration of mevinolinic acid (7). This suggests that the observed reduction in bile acid synthesis resulted from a true decrease in the activity of cholesterol 7α-hydroxylase rather than a decrease in the availability of cholesterol substrate. A variety of other mechanisms could be suggested to account for regulation of activity of cholesterol 7α-hydroxylase by the cholesterol. It is possible that microsomal cholesterol may regulate turnover of cholesterol 7α-hydroxylase by binding to the enzyme active site to form a complex that resists degradation of cholesterol, or a cholesterol metabolite may mediate changes in enzyme synthesis at the transcriptional and/or translational level. Finally, cholesterol-induced changes in cholesterol 7α-hydroxylase activity could reflect altered catalytic efficiency resulting from product accumulation (26), from specific covalent modification such as phosphorylation (27, 28), or from nonspecific changes in the physicochemical environment of the enzyme (29).

Cholesterol 7α-hydroxylase is thought to be regulated by bile acids that circulate in the enterohepatic circulation (30–32). Recent studies from our laboratories have shown that hydrophobic but not hydrophilic bile salts down-regulate HMG-CoA reductase and cholesterol 7α-hydroxylase in roughly similar proportions (33–35). These and previous studies (7) provide additional evidence that cholesterol 7α-hydroxylase may also be regulated by the newly synthesized cholesterol. It therefore appears that regulation of bile acid synthesis may be affected by factors other than bile acids. In spite of recent advances, the mechanism of regulation of cholesterol 7α-hydroxylase is not clearly defined. Further progress in this field should be forthcoming as cholesterol 7α-hydroxylase has been recently purified and cloned (36–39).

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