Regulation of rat liver microsomal cholesterol 7α-hydroxylase: presence of a cytosolic activator

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Abstract Microsomal cholesterol 7α-hydroxylase (EC 1.14.13.7) in rat liver was assayed by a single-isotope-incorporation method, and factors influencing its activity were studied. Crude cytosol contained a non-catalytic activator which was heat-stable and non-dialyzable. This activator enhanced cholesterol 7α-hydroxylase catalytic activity. The stimulatory property of this cytosolic activator was not altered by cholestyramine feeding, and was retained after fractionation by ammonium sulfate of saturation up to 65%. 3-Hydroxy-3-methylglutaryl-coenzyme A (HMG-CoA) stimulated hydroxylase activity at concentrations up to 90 μM but it became inhibitory above 150 μM. ATP at concentrations greater than 1.2 mM inhibited hydroxylase activity. NaF was stimulatory at concentrations up to 50 mM with a maximum stimulatory effect at 10 mM, and was antagonistic in effect to ATP. HMG-CoA and ATP at the above inhibitory concentrations and higher abolished the activating effect of the cytosolic factor.—**Kwok, C. T., W. Burnett, and I. R. Hardie.** Regulation of rat liver microsomal cholesterol 7α-hydroxylase: presence of a cytosolic activator. *J. Lipid Res.* 1981. **22:** 570–579.

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The initial step in the conversion of cholesterol to bile acids is catalyzed by cholesterol 7α-hydroxylase (EC 1.14.13.7) which is rate-limiting (1, 2). This enzyme plays a crucial role in the catabolism of cholesterol to bile acids and the maintenance of the overall pool size of bile salts. Reduction in the amount of bile salts in hepatic bile has been observed in several studies of patients with gallstones (3–5), and a significant reduction in cholesterol 7α-hydroxylase activity has been reported in patients with gallstones (6). The understanding of the regulation of cholesterol 7α-hydroxylase activity under various experimental conditions is thus of great importance in the elucidation of the pathogenesis of gallstones.

Regulation of hydroxylase activity is mediated via active turnover of the enzyme and via modulation of its catalytic efficiency (7), through a feedback mechanism which has been demonstrated in experimental animals and in human subjects (8–10). Cholesterol feeding, cholestyramine treatment, and glucose administration to rats enhance hepatic cholesterol 7α-hydroxylase activity (10, 11), while bile salts administered in the diet or by intraduodenal infusion (12, 13) inhibit activity.

Modulation of hydroxylase catalytic activity at molecular level, however, is not understood. There have been conflicting reports on the effect of the high speed supernatant obtained from the post-microsomal fraction on the catalytic activity of cholesterol 7α-hydroxylase. A stimulating effect of this supernatant on cholesterol 7α-hydroxylase was noted by Spence and Gaylor (14) in contrast to earlier studies by Shefer, Hauser, and Mosbach (15) where this was found to be negative. In this study, we provide evidence for the presence of a noncatalytic, heat-stable cytosolic factor, and data on molecular modulation of cholesterol 7α-hydroxylase by HMG-CoA, ATP, and NaF.

EXPERIMENTAL

Chemicals

[4-^{14}C]Cholesterol (57.8 mCi/mmol) was obtained from Radiochemical Centre, Amersham, Bucks, United Kingdom. ATP, NADP$^+$, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, 3-hydroxy-3-methylglutaryl-CoA, dithiothreitol, and butylated hydroxytoluene were purchased from Sigma Chemical Co., St. Louis, MO. Cholesterol (>99% pure), nicotinamide, and silica gel 60-coated aluminium plates (0.20 mm thickness) were obtained from E. Merck, Darmstadt, Germany. Liquid scintillants, ACS and PCS, were products of Amersham, Arlington Heights, IL. Questran (44.4% cholestyramine) was obtained from Mead Johnson, Crows Nest, N.S.W., Australia. Moni-Trol II from Dade Division, American Hospital

Abbreviation: HMG-CoA, 3-hydroxy-3-methylglutaryl-coenzyme A.

Supply Corporation, Miami, FL, was used for protein standard. 7α-Hydroxycholesterol standard was a kind gift from Dr. H. Danielsson, Karolinska Institutet, Stockholm. 7α- and 7β-hydroxycholesterol were prepared by sodium borohydride reduction of 7-keto cholesteryl acetate according to the method of Fieser et al. (16). All solvents used were of A.R. grade and were used without further distillation.

Treatment of rats

Male Wistar albino rats of body weight 200–250 g were used in all experiments. Animals were fed ad libitum with a stock diet supplemented by 10% Questran, and were conditioned to 12-hr reversed light-dark cycle from 1:30 PM–1:30 AM for at least 1 week. This was done to maximize enzyme activity. Unless otherwise specified, the microsomal fraction obtained from Questran-treated rat liver was used in all experiments.

Preparation of microsomal fraction

In each experiment, rats were killed by cervical dislocation at 7:30 AM ('mid-night' of the reversed lighting cycle). Livers were quickly removed and chilled in ice-cold buffer containing potassium phosphate buffer (50 mM, pH 7.4), NaCl (250 mM), EDTA (30 mM), and dithiothreitol (1 mM). The livers were finely minced with scissors and homogenized in four volumes of the above buffer. The homogenates were spun for 20 min at 4°C at 20,000 *g*. The supernatant from this centrifugation was centrifuged for 2 hr at 4°C at 100,000 *g* to give a supernatant fraction (S_{100}) and a microsomal pellet. The pellet was resuspended in 6.0 ml of the homogenizing buffer. Washed microsomes were obtained by centrifuging the microsomal fraction for a further 1 hr at 100,000 *g*.

Ammonium sulfate fractionation

The crude 100,000 *g* supernatant was first boiled at 100°C for 1 min, and the precipitated protein was separated by centrifugation at 100,000 *g* for 1 hr. The clear supernatant was then fractionated at various saturations with ammonium sulfate solution. A 65% saturation fraction with ammonium sulfate was prepared by adding saturated ammonium sulfate to the supernatant (1.86 ml/ml) and that of 40% and 40–65% fractions were obtained by adding 0.67 ml and 1.19 ml, respectively, of saturated ammonium sulfate per ml of the supernatant. The mixture was stirred for 10 min, then centrifuged at 20,000 *g* for 20 min. The precipitated protein and salt were redissolved in the homogenizing buffer (5 ml/60 g liver) and dialyzed overnight against 200 volumes of the same homogenizing buffer.

Incubation and isolation of radioactive products

Incubation was carried out in duplicate for all assays. The standard incubation medium contained potassium phosphate buffer pH 7.4 (80 mM), nicotinamide (3 mM), EDTA (1 mM), dithiothreitol (5 mM), [4-^{14}C]cholesterol (0.05 μCi, 0.1 mM), microsomal fraction (0.5–1 mg protein), and NADPH regeneration system in a final volume of 0.5 ml. [4-^{14}C]Cholesterol was diluted to the required specific activity with non-labeled cholesterol solubilized in Tween 80 (1 mg per assay). After a preincubation of 10 min, the reaction was commenced by adding a NADPH-regenerating system consisting of glucose-6-phosphate (2.5 mM), $NADP^+$ (1.25 mM), $MgCl_2$ (4.5 mM), and glucose-6-phosphate dehydrogenase (1 I.U.). The reaction had an absolute requirement for NADPH.

Supply of endogenous NADPH can only support less than 4% of hydroxylase activity. A boiled control was incorporated in each experiment, and this value was subtracted from the test results. All incubations were carried out in mini-vials (15 × 55 mm) in a constant shaking metabolic water bath at 37°C for 20 min with air as the gas phase. Light was kept at minimal levels by having the lid on the bath. The reaction was terminated by adding 3 ml of chloroform–methanol (2:1 (v/v), containing 0.005% butylated hydroxytoluene).

The radioactive products were extracted with 10 ml of chloroform–methanol (2:1 (v/v) containing 0.005% butylated hydroxytoluene) by the procedure of Folch, Lees, and Sloane Stanley (17). The tubes were vigorously shaken for 1 min and the volume of aqueous phase was made up to 1 ml by adding water; the aqueous layer was removed after separation of the phases by centrifugation. The extracts were then washed with 0.2 volume of 0.88% KCl (w/v). The chloroform layer was carefully withdrawn and dried down to a volume of 20 μl. The radioactive products were co-chromatographed with authentic standards on thin-layer plates developed in diethyl ether at 5°C.

The bands were visualized and marked by exposure to iodine vapor. The band corresponding in R_f to the standard compounds was scraped into counting vials. The purified radioactive product was measured by counting in PCS liquid scintillant (10 ml). This was later changed to the cheaper ACS as the efficiency of counting remained the same. Radioactivity was assayed in a Searle Delta 300 liquid scintillation spectrometer. Counting rates were cor-

rected for quenching by the external standard channels-ratio method. Protein was measured by the method of Lowry et al. (18) by using Moni-Trol II as standard. To avoid interference from dithiothreitol in the buffer, protein was precipitated with 10% trichloroacetic acid.

RESULTS

Incubation conditions and cholesterol 7α-hydroxylase activity

The effect of preincubation on the oxidation of cholesterol was studied (**Fig. 1**). The hydroxylase activity was linear up to 20 min incubation under the present conditions. Optimal conditions for the 7α-hydroxylation of cholesterol required preincubation of at least 10 min before adding the NADPH-regenerating system to the incubation (Fig. 1a). A 10-min preincubation stimulated hydroxylase activity twofold and this stimulation rose to 2.6-fold after a 20-min preincubation. Identification of reaction products according to Fig. 1b showed that the length of preincubation time had no apparent effect on oxidation of cholesterol to 7-ketocholesterol nor any effect on the amount of 7β-hydroxycholesterol formed (Fig. 1c). However, the conversion of cholesterol into cholesteryl esters and cholestane-3β,5α,6β-triol was increased with increasing preincubation time.

Substrate concentration versus cholesterol 7α-hydroxylase activity

The effect of adding cholestyramine to the diet was studied by examining its influence on the kinetic properties of cholesterol 7α-hydroxylase as compared to controls without this addition to the diet. Microsomal fractions prepared from rats at the acrophase of cholesterol 7α-hydroxylase activity (reversed lighting cycle 1:30 PM–1:30 AM) with or without cholestyramine-supplemented diet showed linear activity with respect to cholesterol concentration up to 100 μM cholesterol (**Fig. 2a**). In contrast the microsomal preparation obtained from rats killed 7½ hr after peak cholesterol 7α-hydroxylase activity (normal 12 hr light-dark cycle 6 AM–6 PM) retained linear activity to 160 μM cholesterol. Though the rate of enzymic reaction differed for each group, cholestyramine treatment did not appear to have a marked effect on the hydroxylase affinity for the substrate. An apparent K_m obtained from Lineweaver-Burk plots (Fig. 2b) from three experiments had a mean value of 226 ± 16 μM for the three groups of

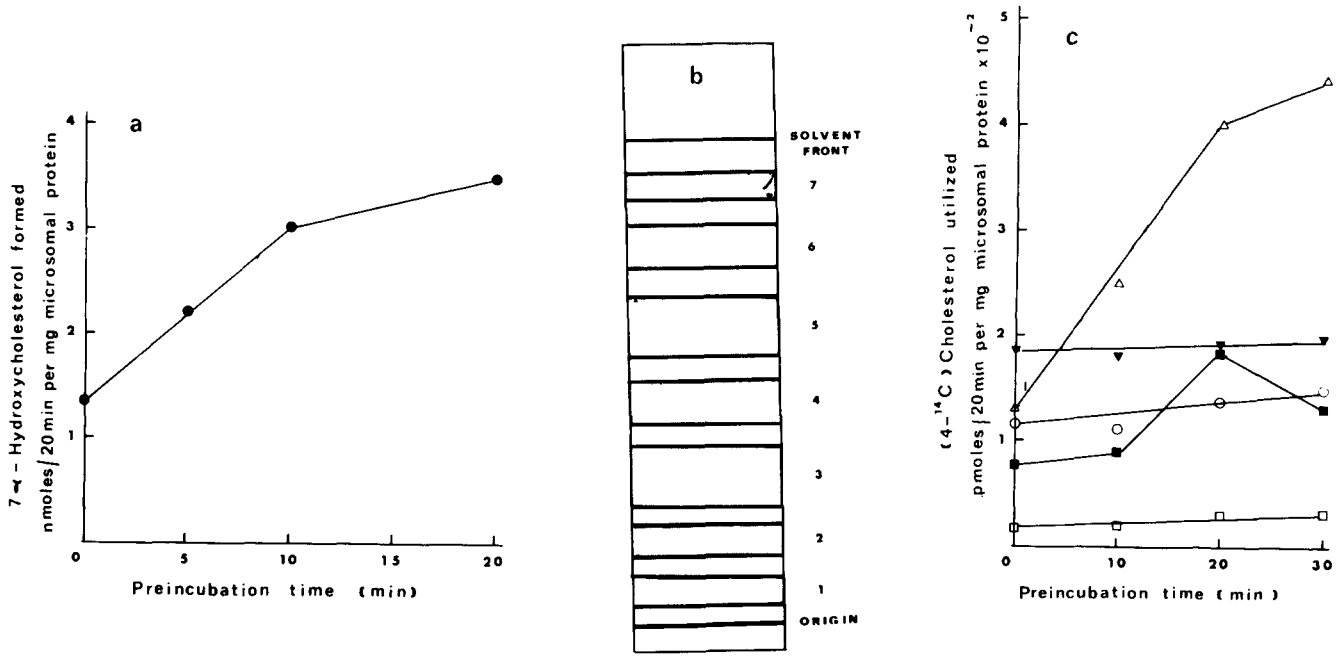

Fig. 1. Effect of preincubation. Cholesterol 7α-hydroxylase activity was assayed as described in the Experimental section. Reaction was initiated at various intervals of preincubation by adding the NADPH-regenerating system. Radioactive products were extracted and separated on thin-layer plates with diethyl ether as developing solvent at 5°C. The following bands, visualized by exposure to iodine vapor and shown in diagram b), were identified as illustrated by Mitropoulos et al. (22): 1), cholestane-3β,5α,6β-triol (■); 2), 7α,12α-dihydroxycholest-4-en-3-one (□); 3), 7α-hydroxycholesterol (●); 4), 7β-hydroxycholesterol (▲); 5), 7-ketocholesterol (○); 6), cholesterol and 7), cholesteryl esters (△). Effect of preincubation on cholesterol 7α-hydroxylase activity is shown in a) and on the oxidation of [4-^{14}C]cholesterol in c).

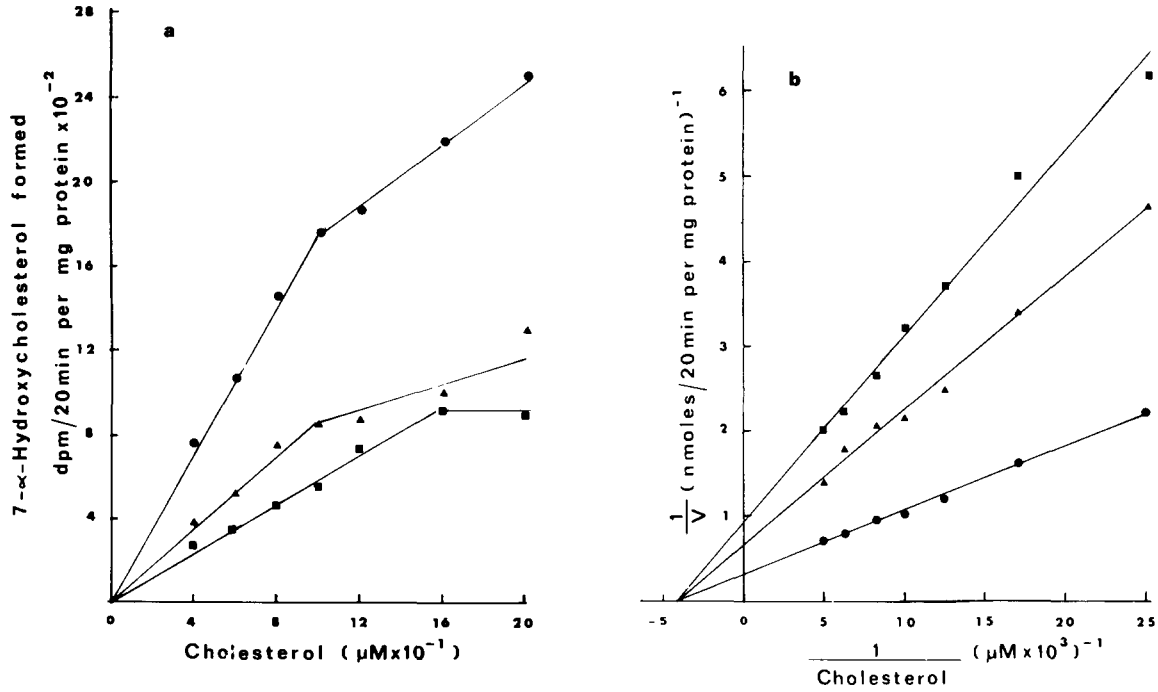

Fig. 2. Effect of cholesterol concentration on cholesterol 7α-hydroxylase activity. Cholesterol 7α-hydroxylase activity was measured with increasing amounts of [4-^{14}C]cholesterol with microsomes prepared from rats exposed to a normal 12-hr light-dark cycle (6:00 PM–6:00 AM) (■), and from rats on a reversed lighting cycle (1:30 PM–1:30 AM) with (●) or without (▲) cholestyramine added to the diet as shown in a). K_m and V_{max} were obtained from a Lineweaver-Burk plot as shown in b). The figures are representative of three independent experiments.

rats. The effect of cholestyramine treatment appears to be an increase in the V_{max} of cholesterol 7α-hydroxylase. The V_{max} for the catalytic activity of cholesterol 7α-hydroxylase obtained from three experiments had the mean values (±S.D.) of 1.42 ± 0.35; 2.15 ± 0.60; 3.31 ± 0.43 nmol/20 min per mg protein for rats kept at normal lighting cycle, reversed lighting cycle without and with cholestyramine-supplemented diet, respectively.

Effect of 100,000 *g* supernatant (S_{100}) on cholesterol 7α-hydroxylase

This series of experiments showed considerable variation in the absolute level of hydroxylase activity from rat to rat and from season to season, despite maintenance of the basic experimental conditions as constant as possible. These variations were overcome by expressing the activity in the presence of an added factor as a ratio of that without any added factor, using each experiment as its own internal control. The specific activity of various factors would then be compared.

The post-microsomal 100,000 *g* supernatant was found to contain a factor(s) which stimulated cholesterol 7α-hydroxylase activity (**Table 1**). The origin of S_{100}, from rats under different experimental conditions, had no apparent effect on its stimulatory activity: S_{100} prepared from rats kept at a normal lighting cycle and from rats kept at a reversed lighting cycle on cholestyramine-supplemented diet enhanced

TABLE 1. Effect of 100,000 *g* supernatant cholesterol 7α hydroxylase activity

	7α-Hydroxycholesterol Formed	
Microsomes from normal rat (M_n)	0.97 ± 0.09	1[a]
M_n + S_{100N}	1.04 ± 0.18	1.17 ± 0.04
M_n + S_{100C}	1.09 ± 0.13	1.19 ± 0.01
Microsomes from cholestyramine-rat (M_C)	2.85 ± 0.77	1[a]
M_c + S_{100N}	3.58 ± 0.86	1.23 ± 0.12
M_c + S_{100c}	3.49 ± 0.89	1.30 ± 0.06
M_c + Boiled S_{100c}	3.62 ± 0.75	1.26 ± 0.08
S_{100C}	0	0
Boiled M_c + S_{100C}	0	0

[a] Ratio of specific activity relative to microsomes without added S_{100}.

Enzyme activities were determined as described in the Experimental section. Equal amounts of 100,000 *g* supernatant (S_{100}) (300 μg) protein from rats at normal lighting cycle (S_{100N}) and at reversed lighting cycle with dietary supplement of 4.4% cholestyramine (S_{100C}) were added to each assay. Data are expressed as nmoles of 7α-hydroxycholesterol formed/20 min per mg microsomal protein. The mean of four independent experiments ± S.D. is presented.

Abbreviations in parentheses denote source of microsomal preparation.

hydroxylase activity to the same extent. S_{100} had a comparatively less stimulatory effect on hydroxylase from normal rats that already had very low hydroxylase activity in comparison to cholestyramine-treated rats. These results suggest that S_{100} activity is not modulated by cholestyramine treatment.

S_{100} by itself had no catalytic activity, as shown by incubation with S_{100} alone and incubation with S_{100} added to a heat-inactivated microsomal fraction (Table 1). However, S_{100} was not heat labile: when it was boiled for 1 min and assayed, the activating effect was not destroyed. Addition of graded amounts of boiled cytosolic protein produced a steady increase in hydroxylase activity (**Fig. 3**), from 0.15 nmol at 135 μg protein to 0.44 nmol above the basal activity at 675 μg of cytosolic protein. When the boiled S_{100} was separated into a supernatant and a pellet fraction by centrifugation for 1 hr at 100,000 *g*, the activator was located predominantly in the supernatant, which produced a sharp increment of hydroxylase activity in the order of twofold, from 0.26 nmol at 10 μg protein to 0.57 nmol at 40 μg of cytosolic protein added in the assay. In comparison, the pellet retained some activity that was significantly less marked then that of the supernatant. This supernatant was fractionated by 65% saturated ammonium sulfate solution, which retained the activator activity (**Fig. 4**). Subsequent fractionation into 0–40% and 40–65% saturated ammonium sulfate solutions showed that the latter contained the active fraction. The 0–40% saturated ammonium sulfate fraction was as active at low concentrations but this activity decreased at higher concentrations.

Table 2 shows further results obtained in a preliminary characterization of S_{100}. S_{100} was non-dialyzable and had negligible supporting activity for cholesterol 7α-hydroxylase in the absence of added NADPH-regenerating system. It contained only trace amounts of NADPH that were completely removed by dialysis. The catalytic activity of cholesterol 7α-hydroxylase was enhanced by washing the microsomal preparation, the washed microsomal fraction having on the

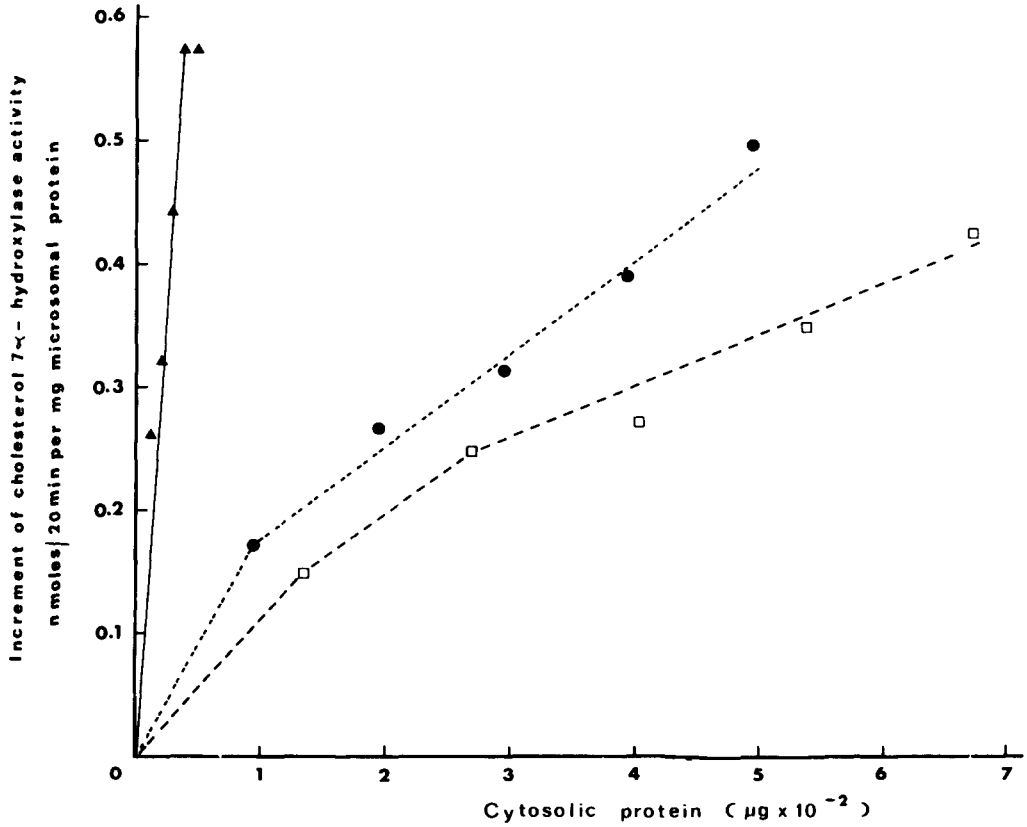

Fig. 3. Effect of boiled 100,000 *g* supernatant on cholesterol 7α-hydroxylase activity. The 100,000 *g* supernatant was boiled in water for 1 min. Precipitated protein was separated by centrifugation for 1 hr at 100,000 *g*. Preincubation was carried out with graded concentrations of boiled 100,000 *g* supernatant (□), boiled and spun 100,000 *g* pellet (●), and boiled and spun 100,000 *g* supernatant (▲). The activities of these fractions were assayed as described in the Experimental section. The activities of these fractions were measured as increment of basal cholesterol 7α-hydroxylase activity (1.47 nmol/20 min per mg microsomal protein) against the amount of these preparations added.

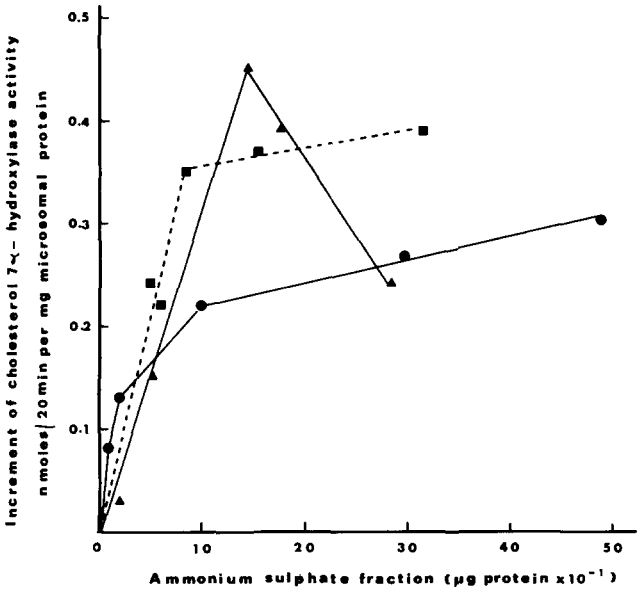

Fig. 4. Activation of cholesterol 7α-hydroxylase by ammonium sulfate-fractionated cytosolic fractions. The supernatant from heat-treated cytosol was adjusted to 40% (▲), 65% (●), or 40–65% (■) saturations with ammonium sulfate solution as described in the Experimental section. The activities of these fractions were measured as increment of basal cholesterol 7α-hydroxylase activity (1.04 nmol/20 min per mg microsomal protein) versus amount of these fractions added.

average 58% higher activity than the unwashed microsomes. Catalytic activity in washed microsomes was stimulated to a greater extent by S_{100} than that in unwashed microsomes, being 28% greater in unwashed and 45% in washed microsomal fractions. The same order of increase in activity was observed with S_{100} that had been dialyzed for 2 hr (16% and 39% for unwashed and washed microsomes, respectively). Organic solvent extraction of S_{100} with diethyl ether and chloroform–methanol 2:1 (v/v) in four experiments showed that the stimulatory effect of S_{100} (activity relative to no S_{100} = 1.30 ± 0.05) was stable to diethyl ether (relative activity 1.28 ± 0.07) but not chloroform–methanol (relative activity 1.03 ± 0.18).

Effect of ATP, fluoride ions, and HMG-CoA on cholesterol 7α-hydroxylase activity

A study was undertaken on modulation of cholesterol 7α-hydroxylase catalytic activity at the molecular level, investigating the possibility of modulation by ATP and fluoride ions parallel to that of HMG-CoA reductase (19), and the relationship of these factors to S_{100}. The effects of ATP, NaF, and HMG-CoA on hydroxylase activity in the absence or presence of S_{100} are shown in **Table 3.** These effectors produced small but consistent changes in hydroxylase activity. In the absence of S_{100}, both fluoride ions at 45 mM and HMG-CoA (85 μM) stimulated the catalytic activity of cholesterol 7α-hydroxylase, but ATP (1.8 mM) was inhibitory. On the other hand, ATP strongly counteracted the activating effect of S_{100}, decreasing hydroxylase activity from 1.32 to 0.98 relative to the activity with no added effectors.

Modulation by these effectors with respect to concentration on the catalytic activity of cholesterol 7α-hydroxylase was further investigated (**Fig. 5**), and the preceding observation (Table 3) was confirmed. The effect of S_{100} decreased in the presence of increasing ATP concentrations. In the absence of S_{100}, ATP had little effect, appearing slightly stimulatory at less than 1 mM, but became inhibitory at concentrations higher than 1.2 mM (Fig. 5a). Fluoride ions, however, were stimulatory (Fig. 5b) with a maximum effect at 10 mM NaF, beyond which concentration the stimulatory effect diminished. In the presence of S_{100}, fluoride ions appeared to have a slight but not significant stimulatory effect on hydroxylase

TABLE 2. Some properties of S_{100}

Addition	7α-Hydroxycholesterol Formed			
	Unwashed Microsomes		Washed Microsomes	
Nil	2.08 ± 0.95 (5)		2.96 ± 1.18 (5)	
S_{100}	2.65 ± 1.02 (5)	1.28 ± 0.07*	3.85 ± 1.09 (4)	1.45 ± 0.18*
S_{100} (No NADPH)	0.04 (1)		0.041 (1)	
S_{100D}	3.87 (1)		5.76 (1)	
S_{100D} (No NADPH)	0.00 (1)		0.00 (1)	
S_{100DO}	2.37 ± 0.95 (5)	1.16 ± 0.17*	3.92 ± 1.49 (4)	1.39 ± 0.15*
S_{100DO} (No NADPH)	0.00 (1)		0.00 (1)	

Details of standard assay conditions are given in the Experimental section. Microsomes, washed and unwashed as indicated, were incubated with 100,000 *g* supernatant (S_{100}) and S_{100} treated by dialysis at 4°C against 100 volumes of homogenizing buffer for 2 hr (S_{100D}) and overnight (S_{100DO}). Enzyme activity is expressed as nmol/20 min per mg microsomal protein. Activity with the addition of untreated or treated S_{100} is shown as both absolute values and as relative values (*) compared to the activity without S_{100}. The number of independent experiments is given in parentheses.

TABLE 3. ATP, fluoride ions, and HMG-CoA effects on cholesterol 7α-hydroxylase activity

Addition	7α-Hydroxycholesterol Formed	
Control	1.89 ± 0.63 (9)	1*
S_{100}	2.42 ± 0.74 (9)[a]	1.32 ± 0.01 (9)
ATP	1.74 ± 0.40 (7)[b]	0.87 ± 0.02 (7)
S_{100}, ATP	2.03 ± 0.90 (7)[d]	0.98 ± 0.02 (7)
NaF	2.18 ± 0.31 (6)[c]	1.10 ± 0.01 (6)
S_{100}, NaF	2.46 ± 1.08 (7)[b]	1.30 ± 0.05 (7)
HMG-CoA	2.51 ± 1.10 (8)[a]	1.30 ± 0.15 (8)
S_{100}, HMG-CoA	2.58 ± 1.48 (6)[c]	1.26 ± 0.02 (6)

[a] $P < 0.01$ (compared to controls).
[b] $P < 0.02$ (compared to controls).
[c] $P < 0.05$ (compared to controls).
[d] $P < 0.02$ (compared to S_{100}).

Washed microsomes were preincubated with ATP (1.8 mM), NaF (45 mM), and HMG-CoA (85 μM) as denoted in the table, with Mg^{2+} ions with or without S_{100} added in the standard incubation mixture. Cholesterol 7α-hydroxylase activity was assayed as described in the Experimental section. Enzyme activity is expressed as nmoles of 7α-hydroxycholesterol formed/20 min per mg microsomal protein. The results are also shown as the mean ± S.D. of specific activity (*) relative to the basic incubation which served as the internal control in each experiment. The number of independent experiments is given in parentheses. The non-parametric Wilcoxon test was applied to determine the significance of difference between controls and the effectors. The statistical significance of S_{100} and S_{100} with effectors added was also compared.

activity. The stimulatory effect of HMG-CoA peaked at 90 μM (Fig. 5c), and then decreased, becoming inhibitory above 150 μM either in the presence or in the absence of S_{100}.

The interaction of NaF on ATP was then studied. Inclusion of NaF (45 mM) in the preincubation with increasing concentrations of ATP abolished the inhibitory effect of ATP (**Fig. 6**). In the presence of the crude cytosol, NaF produced a slight stimulation of hydroxylase activity at 0.9 mM ATP, and reduced the inactivating effect of higher ATP concentrations.

DISCUSSION

Cholesterol 7α-hydroxylase belongs to a group of mixed function oxidases linked to the microsomal cytochrome P-450 oxygenases (20, 21) requiring NADPH and molecular oxygen for activity. For optimal catalytic efficiency, hydroxylase requires the presence of thiol protective agents, EDTA and nicotinamide (20, 22, 23). The presence of these reagents prevents the lipoperoxidative action on cholesterol and hence minimizes the non-enzymic formation of 7-ketocholesterol, 7α- and 7β-hydroxycholesterol, and cholestane-3β,5α,6β-triol. Therefore, it is important to optimize the assay conditions to maximize the formation of 7α-hydroxycholesterol.

The effect of preincubation on catalytic efficiency of cholesterol 7α-hydroxylase has received little attention to date. Our results indicate that preincubation has a marked effect on the catalytic activity of cholesterol 7α-hydroxylase. Maximum enzyme catalytic activity is reached after a 20-min preincubation and this resulted in a 2.6-fold enhancement of enzyme activity. However, a 10-min preincubation was chosen as a compromise, because longer preincubation time would lead to enhanced conversion of cholesterol to cholesteryl esters in addition to the undesirable effect of increased formation of cholestane-3β,5α,6β-triol. Otherwise, preincubation has little effect on the formation of 7β-hydroxycholesterol and 7-ketocholesterol.

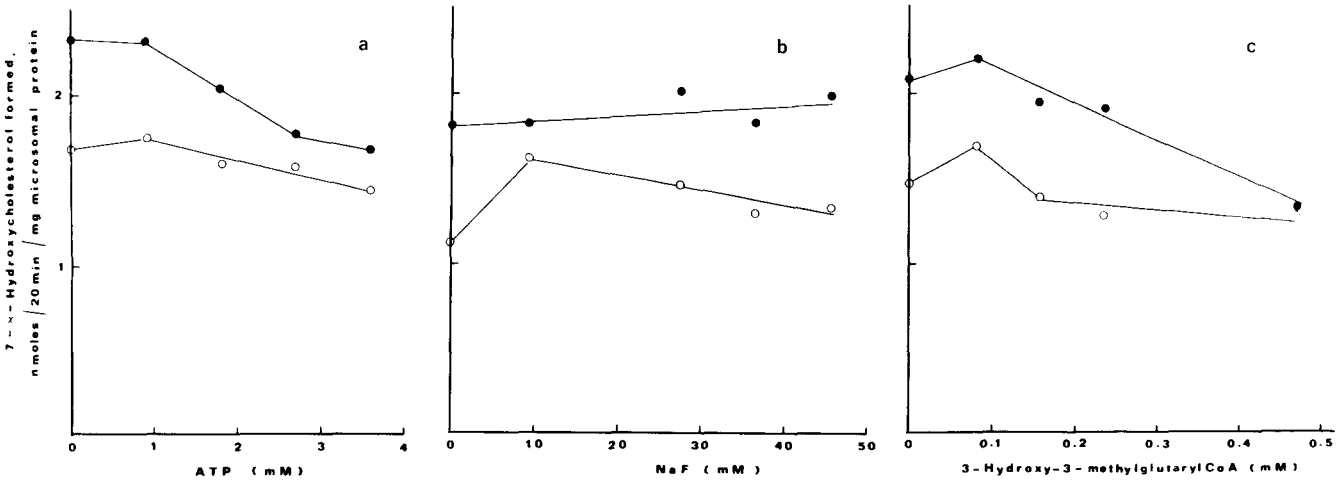

Fig. 5. Interactions of cholesterol 7α-hydroxylase with some small molecular metabolites. Preincubation was carried out with increasing concentrations of ATP, a); NaF, b); and HMG-CoA, c) in three separate experiments. Closed circles (●) and open circles (○) indicate that the reaction was carried out in the presence or absence of 100,000 *g* supernatant, respectively. Cholesterol 7α-hydroxylase activity was assayed as described in the Experimental section.

The single-isotope-incorporation method we have employed can produce inaccurate measurements of the absolute catalytic activity of cholesterol 7α-hydroxylase because of participation of an endogenous pool of cholesterol in the assay. It has been shown that the endogenous pool of cholesterol directly accessible to the enzyme ranges from 33 to 70% (23–25). However, this technique has been compared with the mass fragmentographic technique in assessing the influence of biliary drainage and cholesterol feeding (25) and also with a double-isotope-derivative dilution procedure in assessing the effects of cholestyramine and of phenobarbitone administration (26). Similar results were obtained in both studies with all three techniques. The single-isotope-incorporation technique is therefore valid under the experimental conditions and has been retained because of its greater simplicity.

Results from the substrate enzyme saturation plot support observations by Balasubramaniam, Mitropoulos, and Myant (24) that the supply of cholesterol as substrate for cholesterol 7α-hydroxylase is not saturated under normal conditions. Cholesterol 7α-hydroxylase in rats killed at the beginning of the light phase in a normal 12-hr light-dark cycle required 60% more exogenous cholesterol (160 μM: 100 μM cholesterol) for equilibration than those on a reversed lighting cycle, when the latter were at the acrophase of circadian rhythm at the time of killing. The acrophase corresponds to 'mid-night' of the circadian rhythm at which time active cholesterol 7α-hydroxylase synthesis takes place coupled with an increased synthesis of cholesterol that is a result of increased activity of HMG-CoA reductase (27). These results are consistent with the observations that the marked increase of enzyme catalytic activity during the acrophase is a result of concomitant increased enzyme synthesis and supply of cholesterol (23, 27). Feeding of cholestyramine (Questran), a bile acid anionic binding-resin, did not alter the substrate-enzyme saturation pattern though interruption of the return of bile acids to the liver increases the supply of substrate for cholesterol 7α-hydroxylase by stimulating the activity of HMG-CoA reductase (29). These results are consistent with those of Mitropoulos, Balasubramaniam and Myant (10) who showed that the size of the substrate pool was unchanged in cholestyramine-treated rats. Mitropoulos et al. (10) suggested that the increased rate of formation of 7α-hydroxycholesterol due to cholestyramine treatment was mediated via activation or increased synthesis of cholesterol 7α-hydroxylase, coupled with an increase in the supply of substrate. The present results show that cholestyramine treatment alters the catalytic activity of cholesterol 7α-hydroxylase by increasing the V_{max}. The apparent K_m under the three experimental conditions in the present studies is unchanged at 226 μM. This value agrees remarkably well with that of 0.2 μM obtained by Balasubramaniam et al. (24) using the double-isotope-derivative dilution method.

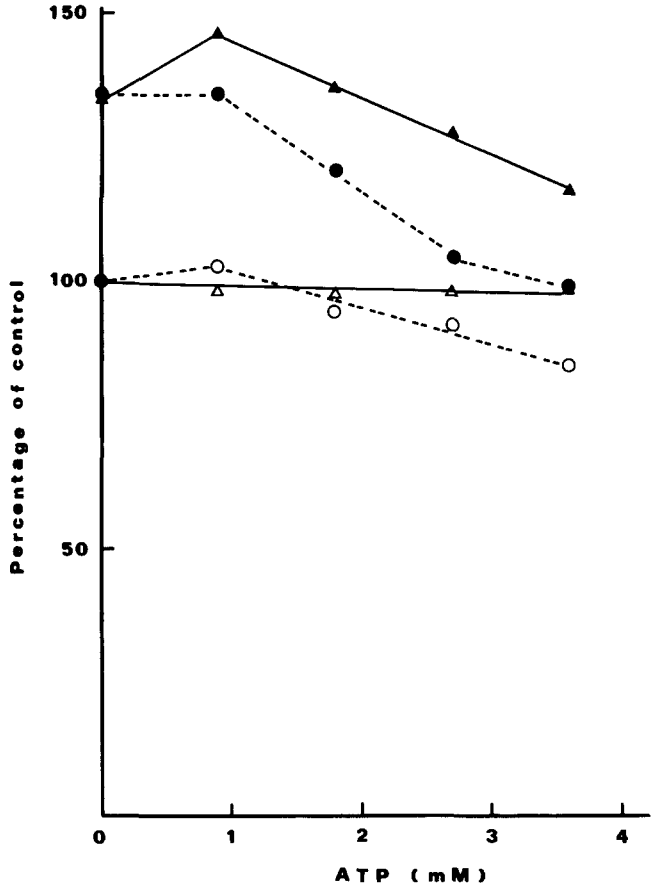

Fig. 6. Effects of NaF on ATP inactivation of cholesterol 7α-hydroxylase. The inactivation of cholesterol 7α-hydroxylase by graded concentrations of ATP, as shown in Fig. 5a (and reproduced here as ○, ●) was compared to the reaction in the presence also of 45 mM NaF (△, ▲) in the preincubation medium. The experiment was carried out either with or without the crude cytosol added, as indicated by the closed and open symbols, respectively. Then enzyme activities were assayed as described in the Experimental section, and were expressed as a percentage relative to control tube without ATP and the crude cytosol added.

The presence of a circadian rhythm running in parallel for cholesterol 7α-hydroxylase and HMG-CoA reductase is well established (27, 28, 30), and homeostasis of these two enzymes is crucial to balance in cholesterol and bile acid metabolism. Changes in physiological conditions and exposure to various biochemicals can exert either an inductive or inhibitory effect on the amount and the catalytic efficiency of these enzymes, but only recently has any significant knowledge on modulation of HMG-CoA reductase

become available, with the implication of a non-catalytic cytosolic protein in the mediation of regulatory control of HMG-CoA reductase (14, 19, 31). Spence and Gaylor (14) also noted that cholesterol 7α-hydroxylase activity may be activated by a non-catalytic cytosolic protein and this is confirmed by our results. However, our results differ from those of Spence and Gaylor (14) with respect to the heat stability of S_{100}. These authors found that S_{100} was labile to heating for 1 min at 80°C after ammonium salt fractionation, whereas the present results show that it is stable to heating for 1 min at 100°C *before* treatment by ammonium sulfate fractionation and subsequently is precipitable by ammonium sulfate solution of saturation up to 65%. Our results also show that the stimulatory effect of S_{100} cannot be due to the presence of NADPH alone as interpreted by Mitropoulos et al. (22), whose results also suggest that the S_{100} factor is heat stable; nor is it due to cholesterol in the supernatant, since the factor(s) is nondialyzable and nonextractable by diethyl ether. The activating effect of S_{100} apparently is not altered by cholesytramine treatment, since S_{100} obtained from normal and cholestyramine-treated rats has a similar activating effect on cholesterol 7α-hydroxylase. Danielsson, Einarsson, and Johansson (1) reported that S_{100} obtained from bile fistula rats stimulated 7α-hydroxylation of cholesterol to the same extent as that from normal rats, and our observations agree that the regulation of cholesterol 7α-hydroxylase activity is mediated via the microsomes.

The washed microsomal fraction enhances the catalytic activity of cholesterol 7α-hydroxylase. Whether the washing procedure removes an inhibitory factor, as in the case of HMG-CoA reductase (19), is yet to be investigated. Washing also enhances the stimulatory effect of S_{100} on hydroxylase activity, with a 28% stimulation in unwashed microsomes and a 32–45% stimulation in washed microsomes. The activating effect of S_{100}, however, can be counteracted by ATP in concentrations above 1.2 mM. The inhibitory effect of ATP on cholesterol 7α-hydroxylase activity found in these preliminary studies is similar to the effect of ATP on HMG-CoA reductase. However, different mechanisms may be involved in view of the different effects of fluoride ions on the two enzymes. Fluoride ions inhibit HMG-CoA reductase but are also stimulatory to cholesterol 7α-hydroxylase. This dissimilarity in the modulation of cholesterol 7α-hydroxylase has been confirmed in our laboratory.[1]

[1] Kwok, C. T., W. Burnett, and I. R. Hardie. Unpublished data.

HMG-CoA, a precursor in the biosynthetic pathway of cholesterol, is strategically located in a committed step catalyzed by HMG-CoA reductase. The present results show that HMG-CoA plays a critical and sensitive role in the modulation of cholesterol 7α-hydroxylase. At concentrations up to 90 μM, HMG-CoA was stimulatory, but above this concentration the effect diminished and it became inhibitory at concentrations greater than 150 μM. Similar effects were also observed in the presence of S_{100}. The results suggest that a build-up of HMG-CoA above physiological concentrations, due either to over-synthesis or blockage of HMG-CoA reductase activity, could result in inhibition of the biosynthesis of bile acids.

The present findings show that regulation of cholesterol 7α-hydroxylase catalytic activity under conditions of active enzyme synthesis and increased supply of cholesterol is mediated via increase in the V_{max}, while the enzyme affinity for cholesterol is unchanged. The hydroxylase activity can be modulated by a cytosolic activator whose properties are not, however, modified by cholestyramine treatment. The stimulatory effect of this cytosolic activator, however, can be counteracted by ATP and HMG-CoA whose inhibitory actions are concentration-dependent. These findings also indicate that short-term regulation of cholesterol 7α-hydroxylase activity can be modulated by more than one effector. Modulation by HMG-CoA possibly involves allosteric interaction and the mechanisms will be investigated further.

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