Cyclic AMP-sensitive activation of hepatic sterol synthesis and 3-hydroxy-3-methylglutaryl coenzyme A reductase

Charles D. Goodwin[1] and Simeon Margolis[2]

Departments of Physiological Chemistry and Medicine, The Johns Hopkins School of Medicine, Baltimore, MD 21205

Abstract We previously showed that preincubation of a 10,000 *g* supernatant (S_{10}) from rat liver for 20 min at 37°C dramatically increased the subsequent incorporation of [^{14}C]acetate into sterols. No activation was seen with [^{14}C]mevalonate as substrate. In the present studies we have examined the effect of preincubation on HMG CoA reductase. When microsomes were isolated from S_{10} by calcium precipitation, preincubation of S_{10} increased the specific activity of HMG CoA reductase threefold. No activation of HMG CoA reductase was observed in microsomes isolated by ultracentrifugation. Activation was cyclic AMP-sensitive. When cyclic AMP (0.001–1.0 mM) and MgATP (1 mM) were present during the preincubation period, there was little or no activation of HMG CoA reductase activity or of sterol synthesis from acetate. MgATP alone did not prevent activation. Neither cyclic AMP nor MgATP was inhibitory when present only during the assay of sterol synthesis. We propose that the in vitro activation represents the reversal of a physiologic cyclic AMP-mediated mechanism for the control of hepatic HMG CoA reductase. That a phosphoprotein phosphatase may catalyze the activation was supported by the observation that sodium fluoride, an inhibitor of phosphoprotein phosphatases, inhibited the activation. These results suggest that hormone-induced changes in the cellular level of cyclic AMP may regulate the activity of HMG CoA reductase and the rate of hepatic cholesterol synthesis.

Supplementary key words phosphoprotein phosphatase · protein phosphorylation

Hepatic cholesterol synthesis is regulated by the combined effects of hormones and diet on tissue levels of HMG CoA reductase, the rate-limiting enzyme of the pathway (1). Higgins and Rudney (2) have now provided evidence that the catalytic activity of HMG CoA reductase can also be regulated. In rats fed cholesterol at the beginning of the dark phase of a light–dark cycle, the catalytic activity of HMG CoA reductase was quickly inactivated while the synthesis of new enzyme continued normally for several hours.

Although no further modes of rapid control for cholesterol synthesis have yet been demonstrated, a possible mechanism is the covalent modification of HMG CoA reductase by phosphorylation. In liver, the cyclic AMP-dependent protein kinases participate in the hormonal regulation of hepatic glycogenolysis and glycogen synthesis (3). There is also evidence that cyclic AMP may mediate the hormonal control of hepatic cholesterol synthesis.

Several workers have observed that incubation of rat liver slices with glucagon, epinephrine, or cyclic AMP (2.5 μM–5 mM) inhibited cholesterol synthesis from [^{14}C]acetate, [^{14}C]octanoate, or $^{3}H_2O$ by 40–80% (4–9). In experiments with isolated rat liver cells, the addition of 1–3 mM dibutyryl cyclic AMP to the incubation medium suppressed the incorporation of acetate into cholesterol by up to 80% (10, 11). Misbin, Capuzzi, and Margolis (10) observed that 20 μM epinephrine inhibited cholesterol synthesis in hepatocytes by 30%. In contrast, Edwards (11) observed that 6–60 μM epinephrine or norepinephrine stimulated HMG CoA reductase activity by 50–200% and increased nonsaponifiable lipid synthesis by 20%, apparently by a mechanism that did not involve cyclic AMP. In cell-free preparations of rat liver, cyclic AMP (2.5 mM) inhibited cholesterol synthesis from acetate by up to 80% (12, 13). Perfusion of rat liver with glucagon had variable effects on cholesterol synthesis (4, 14, 15), perhaps because the studies differed in a number of experimental parameters.

During previous studies of the role of cyclic AMP in the control of hepatic cholesterol synthesis, we observed that preincubation of a 10,000 *g* supernatant from rat liver for 20 min at 37°C dramatically in-

Abbreviations: HMG CoA, 3-hydroxy-3-methylglutaryl coenzyme A; S_{10}, 10,000 *g* supernatant of rat liver; cyclic AMP, cyclic adenosine 3′:5′-monophosphate.

[1] Present address: Department of Pharmacology, University of Washington School of Medicine, Seattle, WA 98195.

[2] Investigator of the Howard Hughes Medical Institute. Author to whom reprint requests should be sent.

creased the subsequent incorporation of [^{14}C]acetate into digitonin-precipitable sterols. This activation affected the group of enzymes of cholesterol synthesis that converts acetyl CoA to mevalonate. Activation required both microsomal and cytosolic components (16). This communication further describes the site of activation and proposes a mechanism for this effect. Preliminary accounts of this work have been published (17, 18).

MATERIALS AND METHODS

Chemicals

Cyclic AMP, cycloheximide, digitonin, dithiothreitol, coenzyme A (lithium salt), glutathione, mevalonic acid (dibenzylethylene diamine salt), nicotinamide, theophylline, and the sodium salts of ATP, NADP, and glucose-6-phosphate were obtained from Calbiochem, La Jolla, CA. Rhodamine B, sodium butyrate, sodium fluoride, sodium sulfite, EDTA, and all solvents (reagent grade) were from J. T. Baker Chemical Co., Phillipsburg, NJ. Glucose-6-phosphate dehydrogenase was from Boehringer Mannheim, Indianapolis, IN. Cholesterol and Chromagram TLC sheets (silica gel G) were purchased from Eastman Organic Chemicals, Rochester, NY. Sucrose was from Fisher Scientific Co., Pittsburgh, PA. Mevalonolactone was from Nutritional Biochemicals Corp., Cleveland, OH. HMG CoA was from P-L Biochemicals, Milwaukee, WI. N^6,$O^{2'}$-Dibutyryl cyclic AMP was from Plenum Scientific Research, Inc., Hackensack, NJ. EGTA, 5′-AMP (sodium salt), N^6-monobutyryl cyclic AMP, and Tris (hydroxymethyl) amino methane (Trizma Base) were purchased from Sigma Chemical Co., St. Louis, MO. Instabray and LSC Complete were from Yorktown Research, New Hyde Park, NY.

Sodium [1-^{14}C]acetate, [3-^{14}C]HMG CoA, DL-[2-^{14}C]mevalonic acid (dibenzylethylene diamine salt), and [4-^{14}C]cholesterol were purchased from New England Nuclear, Boston, MA.

Animals

Male Sprague-Dawley rats (Flow Laboratories, Dublin, VA.) had continuous access to water and rat chow from the Country Food Division of Agway, Inc., (Syracuse, NY.). For most of the experiments on activation of sterol synthesis, rats were exposed to a normal lighting pattern. For experiments on activation of HMG CoA reductase, rats were housed in a light-tight chamber with forced-air ventilation and were exposed to a reversed lighting pattern (light from 4 PM to 4 AM and dark from 4 AM to 4 PM) for at least 7 days before use.

Preparation of 10,000 *g* supernatant (S_{10}) and endoplasmic reticulum

Rats weighing 150–300 g were killed by cervical dislocation between 9 and 10 AM. The liver was immediately excised and chilled in cold buffer (0–4°C). For the experiments on sterol synthesis this buffer (pH 7.8) contained 100 mM Tris, 10 mM potassium phosphate, 30 mM nicotinamide, 5 mM glutathione, 0.1 or 0.4 mM EDTA, and 0.6 or 1.2 mM $MgCl_2$ (Tris homogenization buffer). For most of the experiments on HMG CoA reductase, the buffer (pH 7.5) contained 225 mM sucrose, 25 mM Tris-HCl, and 5 mM glutathione (sucrose–Tris homogenization buffer). After clotted blood and connective tissue were trimmed off, the liver was weighed, minced, and homogenized with two strokes of a loose-fitting, motor-driven Teflon pestle in 2.5 ml of buffer per g of liver. The homogenate was centrifuged in an International refrigerated centrifuge (Model B-20) at 10,000 *g* for 20 min at 4°C.

Endoplasmic reticulum was isolated from the 10,000 *g* supernatant (minus the floating fat layer) by either of two methods: centrifugation at 105,000 *g* for 60 min at 4°C in a Beckman model L2-50 ultracentrifuge or calcium precipitation using the previously reported modification (19) of the method of Kamath et al. (20, 21). When S_{10} was prepared in sucrose–Tris homogenization buffer, microsomes were precipitated by dilution of S_{10} in 12.5 mM sucrose containing 8 mM $CaCl_2$, pH 7.5 (19). When S_{10} was prepared in Tris homogenization buffer, microsomes were precipitated by dilution of S_{10} in 15 mM Tris, 8 mM $CaCl_2$, 5 mM glutathione, pH 7.5. Except where otherwise noted, all microsomal pellets were resuspended in buffer (pH 7.2) containing 100 mM sucrose, 50 mM KCl, 40 mM potassium phosphate, 30 mM EDTA, and 20 mM dithiothreitol (sucrose–KCl resuspension buffer) (22).

Measurement of the synthesis of fatty acids and sterols

Lipid synthesis was measured by the incubation of liver supernatant with [^{14}C]acetate and cofactors (23, 24). Aliquots (0.8 ml) of S_{10} from rat liver were added to 40-ml screw-cap tubes. Each assay was initiated by the addition of 0.2 ml of a cofactor–substrate mixture to give the following final concentrations: 1.8 mM sodium [1-^{14}C]acetate (0.4 mC_i/mmol), 50 μM CoA, 1.0 mM ATP, 0.5 mM NADP, and 3.0 mM glucose-6-phosphate. Exceptions to these conditions are noted in the figure and table legends. Incubations, carried out at pH 7.3 and 37°C in a shaking water bath (120 oscillations/min), were stopped after 20 min by the addition of 2 ml of 95% ethanol and 0.5 ml of 60% (w/v)

KOH. The resulting mixtures were saponified at 80°C for 90 min. Nonsaponifiable lipids were extracted into heptane (25) and backwashed once with water.

The incorporation of [^{14}C]acetate into digitonin-precipitable sterols was measured by a modification of the method described by Sperry and Webb (26) and by Crawford (27). An aliquot of each heptane extract was transferred to a 12-ml centrifuge tube, mixed with 0.8 mg of carrier cholesterol in ethanol, and dried under air. Each residue was dissolved in 4 ml of acetone–ethanol 1:1 and mixed with 2 ml of 0.5% digitonin in 50% ethanol. Samples were allowed to stand for at least 3 hr at room temperature. Sterol digitonides were sedimented by centrifugation at 1400 *g* for 10 min, washed with 3 ml of diethylether–acetone 1:1, and then washed with 3 ml of diethyl ether. Dried precipitates were dissolved by gentle heating in 1 ml of chloroform–methanol 2:1 and transferred to scintillation vials. The tubes were rinsed once with 1 ml of chloroform–methanol 2:1 and transferred to scinvent, 1 ml of methanol and 10 ml of LSC Complete were added to each vial for counting.

Fatty acid synthesis was measured in the same incubations as sterol synthesis. Following extraction of nonsaponifiable lipids, the aqueous phase was washed once with 5 ml of heptane and acidified (pH < 2) with 12 N HCl. Fatty acids were then extracted into 10 ml of heptane. An aliquot was dried and counted in LSC scintillation fluid.

In all experiments radioactive samples were counted in a Beckman liquid scintillation spectrometer (LS-133) with a counting efficiency of 58% for ^{14}C. Data were corrected for quenching with the external standard-channels ratio.

HMG CoA reductase assay

HMG CoA reductase was assayed by the procedure of Goodwin and Margolis (19) or by the previously published modification (19) of the method of Shapiro, Imblum, and Rodwell (28).

Definition of activation

Activation was calculated as the amount of labeled substrate incorporated into a product by a preincubated preparation divided by the amount incorporated by a nonpreincubated preparation. Activation data given in the text are averages ± standard errors of the mean.

Protein determination

Protein concentrations were determined by a modification of the biuret method (29, 30). Since dithiothreitol interferes with protein determination by this method, microsomes suspended in buffer containing dithiothreitol were precipitated with 5% trichloroacetic acid. After centrifugation at 1,000 *g* for 10 min, the pellet was resuspended in water and reprecipitated with 5% trichloroacetic acid. Before mixing with the biuret reagent, the protein pellet was dissolved by the addition of 1 ml of H_2O, 0.3 ml of 10% NaOH, and 0.2 ml of 5% deoxycholate.

RESULTS

Activation of in vitro sterol biosynthesis

In a preliminary communication we reported that preincubation of S_{10} from rat liver for 20 min at 37°C increased the subsequent incorporation of [^{14}C]acetate into sterols 8- to 20-fold (16). In a subsequent series of 24 experiments conducted under the same conditions, 9.7 ± 0.8-fold (mean ± SEM) activation of sterol synthesis was observed. The rate of sterol synthesis in nonpreincubated S_{10} was 0.16–2.9 nmol sterol/g liver per hr. Activation was observed in S_{10} prepared at either the peak or at the low point of the diurnal variation of hepatic cholesterol synthesis. Fatty acid synthesis from acetate was activated 3.5 ± 0.3-fold (mean from 26 experiments). The rate of acetate incorporation into fatty acids was 9.1–46 nmol acetate/g liver per hr. Sterol synthesis from mevalonate and acetoacetate synthesis from acetate were not activated by preincubation (16).

The degree of activation observed for sterol synthesis varied with the assay conditions. Sterol synthesis was activated 9.7-fold in the experiments just described where 1.0 mM ATP was present in the assay and the homogenization buffer contained 0.6 mM $MgCl_2$. Later experiments showed that optimal sterol synthesis required 3 mM MgATP. Under the latter conditions sterol synthesis in *nonpreincubated* S_{10} was *increased*; thus the observed activation was *decreased*. In a series of 21 experiments in which 3 mM MgATP was included in the assay, the activation of sterol synthesis averaged 3.2 ± 0.2-fold (range 2–5). In all subsequent studies of sterol synthesis, 3 mM MgATP was present in the assay.

Activation of HMG CoA reductase

The aim of the following experiments was to determine whether HMG CoA reductase, the primary regulatory enzyme of cholesterol synthesis, is activated by preincubation.

An initial series of four experiments examined the effect of preincubation of S_{10} on the subsequent activity of HMG CoA reductase. Aliquots (0.9 ml) of S_{10} prepared in Tris homogenization buffer were prein-

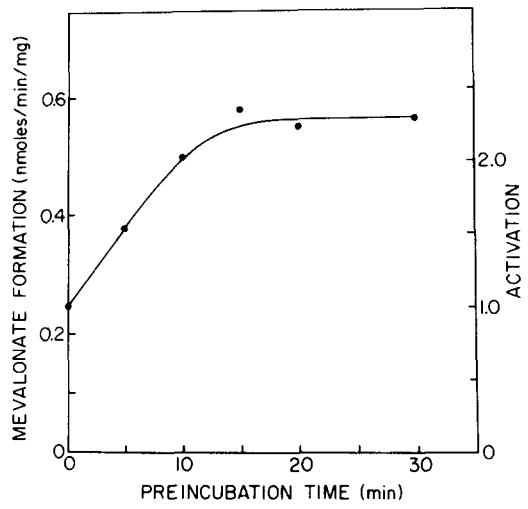

Fig. 1. Effect of preincubation time on the activation of HMG CoA reductase. S_{10} was prepared in buffer containing 225 mM sucrose, 25 mM Tris, and 5 mM glutathione (pH 7.5). Microsomes were isolated by calcium precipitation from S_{10} that was either untreated or preincubated at 37°C for 5–30 min. The methods used for precipitation and resuspension of microsomes and for the subsequent assay of HMG CoA reductase are described in Materials and Methods. The data plotted are averages from three experiments.

cubated for 20 min at 37°C. Aliquots of untreated S_{10} and of preincubated S_{10} were then used for the assay of sterol synthesis from [^{14}C]acetate or for the isolation of microsomes by ultracentrifugation. Microsomes were resuspended in Tris homogenization buffer. Although preincubation activated sterol synthesis 2.4 ± 0.3-fold, there was only 1.3 ± 0.2-fold stimulation of HMG CoA reductase activity as assayed by the method of Shapiro and co-workers (28). This result suggested either *a*) that HMG CoA reductase was not activated or *b*) that the activated form of the reductase was not stable under the conditions of ultracentrifugation. This study was repeated in a series of six experiments in which microsomes were isolated by rapid precipitation with calcium rather than by ultracentrifugation (19). These microsomes were resuspended in Tris homogenization buffer supplemented with 10 mM EDTA. EDTA was essential to recover maximal HMG CoA reductase activity from nonpreincubated preparations, i.e., activity equal to that observed in microsomes prepared by ultracentrifugation (19). Under these conditions HMG CoA reductase was activated 2.7 ± 0.4-fold, thus approaching the 3.2 ± 0.2-fold activation seen for sterol synthesis from [^{14}C]acetate (average from 21 experiments). Although the experiments described here were conducted with liver preparations from male rats, similar 3-fold activation of HMG CoA reductase was observed with female rats.

It should be emphasized that the microsomes isolated by ultracentrifugation were resuspended in Tris homogenization buffer while the calcium-precipitated microsomes were resuspended in this buffer supplemented with 10 mM EDTA. It is possible that either the Ca^{2+} in the precipitation buffer or the EDTA in the resuspension buffer stabilized the activated state of the reductase. Thus, the length of time required for the isolation of microsomes may not have been critical to the observation of an activated reductase.

Fig. 1 shows the kinetics of the activation of HMG CoA reductase as assayed in calcium-precipitated microsomes. Maximal activation (2.5-fold) was attained after 15 min of preincubation, a time course similar to that observed for the activation of sterol synthesis from [^{14}C]acetate. The range of activation for HMG CoA reductase was 2- to 4-fold. The HMG CoA reductase assays were linear for a least 30 min in both preincubated and nonpreincubated preparations, suggesting that no activation occurred under the assay conditions. Some component of the assay mixture may have prevented activation. Alternatively, activation may have required a cytosolic component that was absent during the reductase assay. The second possibility is supported by our observation that activation of sterol synthesis required preincubation of microsomes with cytosol (19).

Activation of HMG CoA reductase was not affected by the presence of any of the following compounds during the preincubation: $CaCl_2$ (8 mM), nicotinamide (30 mM), potassium phosphate (10 mM), NADP (2 mM), or MgATP (1 mM). Also, the presence of 0.1 mM cycloheximide during the preincubation had no effect, suggesting that activation did not require de novo protein synthesis. This observation was not surprising since activation was complete within 15 min (Fig. 1).

Several further observations suggested that the factor that activated HMG CoA reductase was a protein. In these studies intact S_{10} was subjected to various treatments, none of which altered the basal reductase activity of the microsomes. S_{10} was then preincubated at 37°C for 20 min. The extent of activation of HMG CoA reductase by the preincubation was then assessed as in the preceding experiments.

Storing S_{10} overnight at 4°C or −20°C completely abolished any subsequent activation of HMG CoA reductase. Treatment of S_{10} at −40°C for 20 min or at 4°C for 3 hr decreased activation by 50–60%. Heating S_{10} at 50°C for 3–5 min completely prevented activation. Activation was not restored by adding 5 mM glutathione or 2 mM NADP to heated S_{10}. These observations suggested that the activator was a labile

macromolecule, probably a protein, and was either in the cytosol or associated with the endoplasmic reticulum.

Further attempts to characterize the activator of HMG CoA reductase involved recombining cytosol with microsomes isolated by ultracentrifugation. Activation of HMG CoA reductase was observed when microsomes were resuspended directly in cytosol and the mixture was preincubated for 20 min at 37°C. The extent of this activation was extremely sensitive to the length of time that elapsed between the preparation of S_{10} and the preincubation of a reconstituted mixture. No activation was observed when microsomes were resuspended in sucrose–Tris homogenization buffer before mixing with cytosol.

Effect of cyclic AMP on the activation of sterol synthesis

The next series of experiments examined the possibility that activation reflected the reversal of a cyclic AMP-mediated inhibition of cholesterol synthesis and HMG CoA reductase. **Fig. 2** shows that the addition of 1 mM cyclic AMP and 1 mM MgATP to S_{10} at the start of the preincubation period prevented activation of sterol synthesis, even within the first 5 min. The control for this experiment was preincubation of S_{10} with 1 mM MgATP alone, which decreased activation by only 22% (average from 10 experiments). It is not known how much of this added MgATP remained at the end of the preincubation period. Cyclic AMP prevented activation at concentrations as low as 1 μM (**Table 1**); lower concentrations were not examined. The addition of 1 mM MgATP or 1 mM MgATP plus 1 mM cyclic AMP to nonpreincubated S_{10} increased sterol synthesis by 20%. Thus, cyclic AMP had little effect on the assay of sterol synthesis.

Prevention of the activation of sterol synthesis by cyclic AMP was absolutely dependent on the concentration of added MgATP (**Fig. 3**). Cyclic AMP (1 mM) alone decreased activation by only 20%, whereas cyclic

TABLE 1. Effect of cyclic AMP concentration on the activation of sterol synthesis

Added Cyclic AMP	Sterol Synthesis: Basal	Sterol Synthesis: Preincubated	Activation	
mM	*nmol/g/hr*		*-fold*	
None	3.5	12.0	3.8 ± 0.4	(9)
0.001	3.7	5.2	1.2 ± 0.6	(3)
0.1	2.1	2.5	1.3	(2)
1.0	4.0	4.4	1.3 ± 0.2[a]	(9)

[a] Inhibition of activation by 1 mM cyclic AMP was significant at $P < 0.001$.

S_{10} was prepared in Tris homogenization buffer and preincubated for 20 min at 37°C with 1 mM MgATP and the indicated concentration of cyclic AMP prior to the assay of sterol synthesis from [^{14}C]acetate. Experiments were conducted as in Fig. 2. The number of experiments with each level of cyclic AMP is indicated in parentheses. The data are averages and, where given, standard errors of the mean.

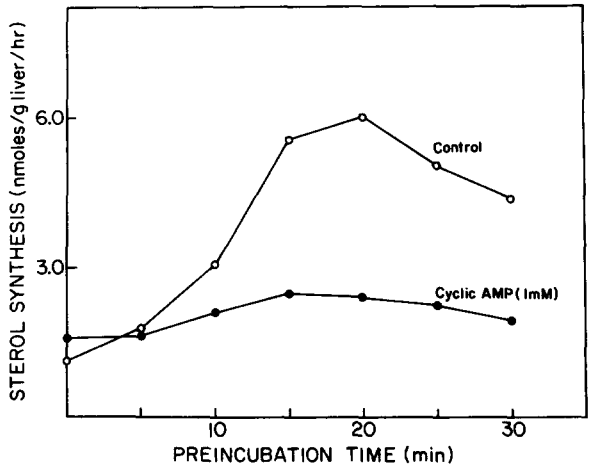

Fig. 2. Effect of 1 mM cyclic AMP on the activation of sterol synthesis from [^{14}C]acetate. S_{10}, prepared in Tris homogenization buffer, was preincubated at 37°C for various periods of time. Sterol synthesis was then measured as described in Materials and Methods, except that 3 mM MgATP replaced 1 mM ATP in the assay. Control samples were preincubated with 1 mM MgATP. Representative data are shown from a single experiment.

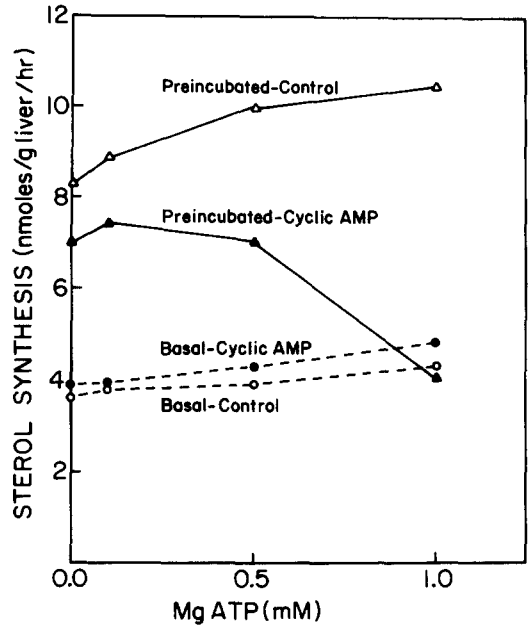

Fig. 3. Effect of MgATP concentration on the activation of sterol synthesis and on its inhibition by cyclic AMP. S_{10}, prepared in Tris homogenization buffer, was preincubated for 20 min with MgATP at the indicated concentration in the presence or absence of 1 mM cyclic AMP. Sterol synthesis from [^{14}C]acetate was assayed as in Fig. 2. The data plotted are averages from two experiments.

TABLE 2. Effect of cyclic AMP concentration on the activation of HMG CoA reductase

Added Cyclic AMP	HMG CoA Reductase Activity		Activation
	Basal	Preincubated	
mM	*nmol/min/mg*		*-fold*
None	0.41	0.70	2.3 (3)
0.1	0.41	0.73	1.7 (2)
1.0	0.57	0.55	1.0 (2)

S_{10} was prepared in Tris homogenization buffer and preincubated for 20 min at 37°C with 1 mM MgATP and the indicated concentration of cyclic AMP. Microsomes were then isolated by calcium precipitation and resuspended in Tris homogenization buffer supplemented with 10 mM EDTA. HMG CoA reductase was assayed as described in Materials and Methods. The data shown are the average specific activities and the average activations from the number of experiments indicated in parentheses.

AMP plus 1 mM MgATP decreased activation by 90%. Basal sterol synthesis (in nonpreincubated S_{10}) was only slightly affected by MgATP concentration, with or without 1 mM cyclic AMP.

Other experiments showed that N^6,$O^{2'}$-dibutyryl and N^6-monobutyryl cyclic AMP (1 mM) were respectively, 60% and 80%, as effective as cyclic AMP in preventing activation. Sodium butyrate (1 mM) did not affect activation. Theophylline (2 mM) did not enhance the effectiveness of 1 mM cyclic AMP, nor did it affect activation by itself. 5'-AMP (1 mM) did not inhibit activation.

Effect of cyclic AMP on the activation of HMG CoA reductase

Subsequent experiments tested the cyclic AMP-sensitivity of the activation of HMG CoA reductase. Activation of HMG CoA reductase was completely prevented by the presence of 1 mM cyclic AMP plus 1 mM MgATP during the preincubation period (**Table 2**); 0.1 mM cyclic AMP decreased activation by 50%. These results were similar to the effects of cyclic AMP on the activation of overall sterol synthesis (Table 1 and Fig. 2). However, in those studies cyclic AMP was effective at concentrations as low as 1 μM.

The following mechanism of activation is proposed. Preincubation may activate HMG CoA reductase through the action of a phosphoprotein phosphatase on an inactive, phosphorylated form of the enzyme. This proposal was examined with the use of sodium fluoride, an inhibitor of phosphoprotein phosphatases (31–33). Following preincubation of S_{10} with or without 50 mM NaF, microsomes were isolated by calcium precipitation, washed once, and HMG CoA reductase activity was assayed. Fluoride prevented the activation of HMG CoA reductase almost completely, but had little effect on HMG CoA reductase in nonpreincubated preparations (**Table 3**). Sodium chloride (50 mM) had little effect on the activation. In other experiments, the addition of fluoride after 5 min of preincubation prevented any further activation during the remaining 15 min of preincubation. These studies support the proposal that a phosphoprotein phosphatase participates in the activation of HMG CoA reductase.

DISCUSSION

We have described two major observations. *1*) Preincubation of S_{10} for 20 min at 37°C dramatically increased the subsequent rate of sterol synthesis from [^{14}C]acetate by directly stimulating HMG CoA reductase activity. *2*) Cyclic AMP accompanied by MgATP prevented this activation; sodium fluoride was also inhibitory.

There are no other reports of in vitro activation of cholesterol synthesis by preincubation of liver homogenates. However, Fang and Lowenstein (34) observed that preincubation of rat liver cytosol for 20 min at 37°C stimulated fatty acid synthesis 11-fold. In that study, activation required either citrate or magnesium at a concentration greater than 15 mM. Similarly, Allred and Roehrig (35) noted that preincubation of a 700 *g* supernatant of rat liver for 30 min at 37°C resulted in a 3.5-fold stimulation of acetyl CoA carboxylase activity.

Other workers have described in vitro conditions that enhance the activity of partially purified HMG CoA reductase. Tormanen et al. (36) noted that preincubation of solubilized HMG CoA reductase for 20 min at 37°C stimulated enzyme activity 1.5-fold. In the presence of NADPH, 3-fold stimulation was obtained.

TABLE 3. Effect of sodium fluoride on the activation of HMG CoA reductase

Additions	HMG CoA Reductase Activity		Activation
	Basal	Preincubated	
	nmol/min/mg		*-fold*
None	0.21	0.56	2.9 (3)
50 mM NaF	0.24	0.32	1.5 (3)
50 mM NaCl	0.20	0.47	2.4 (2)

S_{10} was prepared in buffer (pH 7.5) containing 225 mM sucrose, 25 mM Tris, and 5 mM glutathione. Aliquots of S_{10} (0.9 ml) were preincubated for 20 min at 37°C with the additions indicated above. Microsomes were then isolated by calcium precipitation. Microsomes were also isolated from nonpreincubated S_{10} in the presence of the above additions. Microsomal pellets were washed once with 4.5 ml of 12.5 mM sucrose, 8 mM $CaCl_2$ (pH 7.5) and were resuspended in the sucrose–KCl buffer with 20 mM dithiothreitol. The data shown are the average specific activities and the average activations from the number of experiments indicated in parentheses.

Similarly, Heller and Gould (37, 38) observed that preincubation of solubilized HMG CoA reductase for 20 min at 37°C stimulated enzyme activity 1.5- to 2.5-fold. Microsomal HMG CoA reductase was not activated. The solubilized HMG CoA reductase showed kinetics of activation that were similar to those seen in the present work. Heller and Gould (37, 38) observed an activation half-time (time required to activate to 50% of the maximal activity) of about 7 min. Goodwin and Margolis (16) observed activation half-times of 8 min for sterol synthesis from acetate (16) and of 6 min for HMG CoA reductase activity (Fig. 1). Since Heller and Gould studied solubilized HMG CoA reductase, their observations may reflect a different phenomenon than that described here.

In experiments similar to ours, Berndt and Gaumert (39) observed that incubation of a crude (uncentrifuged) mouse liver homogenate for 1 hr at 37°C stimulated HMG CoA reductase activity 3.5-fold as subsequently measured in microsomes isolated by ultracentrifugation. These workers noted an activation half-time of 15–20 min, somewhat longer than that seen in the present studies. In agreement with our work, Berndt and Gaumert (39) observed that both cytosol and microsomes were required for activation and that no activation occurred when microsomes were preincubated alone. Furthermore, the cytosolic component required for activation was trypsin-sensitive, strongly suggesting that it was a protein (39). The activation observed by these workers appears to be similar to that described here, except with regard to the stability of the activated enzyme during ultracentrifugation. Unlike Berndt and Gaumert, (39) we were not able to demonstrate activation of HMG CoA reductase when microsomes were isolated from preincubated S_{10} by ultracentrifugation. These two studies differed with respect to several experimental parameters: source of liver (mouse vs. rat); composition and pH of the buffers in which livers were homogenized and in which microsomes were resuspended; and centrifugation conditions. One or more of these differences may have led to the observed difference in the stability of the activated reductase.

There are no previous descriptions of the effect of cyclic AMP on an in vitro activation of sterol synthesis. However, Bloxham and Beg, and their co-workers (12, 13, 40), observed that cyclic AMP (2.5 mM) inhibited cholesterol synthesis from acetate or acetyl CoA by as much as 80% in rat liver supernatants. Our studies differed from these earlier studies in that we were unable to show a direct effect of cyclic AMP on the *assay* of sterol synthesis from [^{14}C]acetate. The *activation* of sterol synthesis was markedly inhibited by 1 μM cyclic AMP, but only if MgATP was also present. This inhibition was observed even in the absence of inhibitors of cyclic AMP phosphodiesterase (Table 1). Thus, cyclic AMP is a potent inactivator of sterol synthesis when studied during a preincubation period, i.e., in the absence of the cofactors required to assay sterol synthesis. This effect of cyclic AMP requires the presence both of cytosolic components and of MgATP. Therefore, cyclic AMP does not act as a direct inhibitor of one of the enzymes of sterol synthesis. Rather cyclic AMP appears to act through a mediating factor such as a protein kinase.

Several laboratories have studied the effect of cyclic AMP and/or MgATP on the activity of hepatic HMG CoA reductase (13, 40–44). Beg et al. (13, 40) observed that HMG CoA reductase activity was decreased by 40–60% when rat liver slices, cells, or 5,000 *g* supernatants were exposed to 5 mM cyclic AMP for 30–60 min. HMG CoA reductase was reversibly inhibited when soluble or microsomal enzyme was preincubated for 10–20 min at 37°C with 1–5 mM MgATP and cytosolic protein fractions. Cyclic AMP enhanced this inactivation of HMG CoA reductase. Although the inhibitory cytosolic fractions contained cyclic AMP-stimulated protein kinase activity, Beg et al. (13) did not try to demonstrate phosphorylation of HMG CoA reductase.

Several workers have confirmed Beg's observation of a MgATP-dependent inactivation of HMG CoA reductase, but have differed in the reported effect of cyclic AMP (41–44). Nordstrom, Mitschelen, and Rodwell (41) observed that microsomal HMG CoA reductase was inhibited by Mg^{2+} and ATP or ADP; inactivation was enhanced by a cytosolic protein fraction termed M^-. Soluble reductase was also inhibited by Mg^{2+} and ATP or ADP, but only in the presence of M^-. A second cytosolic protein, termed M^+, completely reactivated the inhibited forms of both soluble and microsomal reductase (41).

Chow, Higgins, and Rudney (42) observed that the inhibition of HMG CoA reductase by MgATP was extremely variable and did not require cytosol; cyclic AMP also inhibited the enzyme. Furthermore, they were unable to show phosphorylation or adenylation of HMG CoA reductase by either microsomal or cytosolic fractions (42). In related studies, Brown, Brunschede, and Goldstein (43) showed that extracts of human fibroblasts contained a factor that inactivated HMG CoA reductase, but only if ATP or ADP and Mg^{2+} or Mn^{2+} were present. In that case inactivation was unaffected by cyclic AMP. Shapiro et al. (44) observed that MgATP inactivated HMG CoA reductase from L-cells as well as from rat liver. Tormanen et al. determined that cytosol partially inhibited microsomal HMG CoA reductase; inhibition was virtually com-

plete when 4 mM MgATP was present. In the absence of cytosol MgATP had little effect (45).

Our studies of the in vitro inactivation of HMG CoA reductase differed from other work in several important ways. Cytosol was always present during the exposure of microsomal HMG CoA reductase to cyclic AMP and MgATP. In our work MgATP alone had little effect on the activation of HMG CoA reductase. Prevention of activation, however, was dependent on both MgATP and cyclic AMP. Furthermore, all components of S_{10} were diluted considerably during the calcium precipitation and subsequent resuspension of microsomes. Since little of the added cyclic AMP and MgATP remained at the beginning of the HMG CoA reductase assay, the observed effect of these agents was on activation alone.

As a result of the studies described here and those performed by other laboratories, we propose the following mechanism for the in vitro modulation of HMG CoA reductase activity. Preincubation may activate HMG CoA reductase through the action of a phosphoprotein phosphatase on an inactive, phosphorylated form of the enzyme. A cyclic AMP-dependent protein kinase could reverse the reaction, converting the reductase to a phosphorylated, less active form. To date, there is no firm evidence that hepatic HMG CoA reductase can be phosphorylated, either in vitro or in vivo. However, it is known that hepatic microsomes contain endogenous protein kinases, phosphoprotein phosphatases, and substrates for both classes of enzymes (46). Furthermore, Carlson and Kim (47) and Lee and Kim (48) have presented considerable evidence that acetyl CoA carboxylase, the rate-limiting enzyme of fatty acid synthesis, is regulated by phosphorylation–dephosphorylation. Qureshi and his coworkers (49) have proposed similar control for fatty acid synthetase.

In the present work we never observed complete inhibition of HMG CoA reductase. This suggests that there may be two populations of enzyme, only one of which can be inactivated. Alternatively, the mechanism of inhibition may be inherently incapable of eliminating all catalytic activity. In this regard,phosphorylation causes only partial (75–95%) inactivation of acetyl CoA carboxylase (48), fatty acid synthetase (49), and glycogen synthase (50).

Of the many reports of inactivation of HMG CoA reductase by MgATP and/or cyclic AMP, the observations described here constitute the best available evidence for the involvement of a cyclic AMP-dependent protein kinase; in particular the lack of effect of MgATP or cyclic AMP alone on the activation and the absolute dependence of the inactivation on both MgATP and cyclic AMP. Our observation that sodium fluoride inhibits the activation of HMG CoA reductase supports the proposed role of a phosphoprotein phosphatase in the activation process. Berndt et al. (51) observed a similar effect of fluoride.

The rapid regulation of HMG CoA reductase by a cyclic AMP-dependent protein kinase, functioning in addition to known determinants of the level of the enzyme, would increase the sensitivity of the physiologic control of hepatic cholesterol synthesis. This mechanism would also contribute to the sensitive, overall modulation of hepatic metabolism by hormones such as glucagon. In this regard Bloxham and Akhtar proposed that cyclic AMP serves an anti-anabolic role in liver, such that lipid and protein synthesis are coordinately inhibited under conditions in which gluconeogenesis and glycogenolysis are active (9). The present work supports that proposal.

This work was supported by Grants HL 15930 and GM 00184 from the National Institutes of Health, United States Public Health Service.

Manuscript received 8 June 1977; accepted 12 January 1978.

REFERENCES

1. Rodwell, V. W., J. L. Nordstrom, and J. J. Mitschelen. 1976. Regulation of HMG CoA reductase. *Adv. Lipid Res.* **14:** 2–74.
2. Higgins, M., and H. Rudney. 1973. Regulation of rat liver β-hydroxy-β-methylglutaryl-CoA reductase activity by cholesterol. *Nature (London), New Biol.* **246:** 60–61.
3. Krebs, E. G. 1972. Protein kinases. *Curr. Top. Cell. Regul.* **5:** 99–133.
4. Raskin, P., J. D. McGarry, and D. W. Foster. 1974. Independence of cholesterol and fatty acid biosynthesis from cyclic adenosine monophosphate concentration in the perfused rat liver. *J. Biol. Chem.* **249:** 6029–6032.
5. Berthet, J. 1960. Action du glucagon et de l'adrenaline ser le métabolisme des lipides dans le tissu hépatique. *Proc. 4th Int. Congr. Biochem.* **17:** 107.
6. Akhtar, M., and D. P. Bloxham. 1970. The co-ordinated inhibition of protein and lipid biosynthesis by adenosine 3′:5′ cyclic monophosphate. *Biochem. J.* **120:** 11P.
7. Bricker, L. A., P. L. Kozlovskis, and G. S. Levey. 1976. Adenosine 3′:5′-monophosphate and the regulation of rat hepatic sterol synthesis: a reexamination based on Sutherland criteria. *Metabolism.* **25:** 477–481.
8. Calandra, S., and M. Montaguti. 1973. Effect of prostaglandin E_1 on cholesterol biosynthesis in rat liver. *Experientia.* **29:** 1361–1362.
9. Bloxham, D. P., and M. Akhtar. 1972. An anti-anabolic role of adenosine 3′:5′-cyclic monophosphate in the control of lipid metabolism. A hypothetical mechanism for gluconeogenesis. *Int. J. Biochem.* **3:** 294–308.
10. Misbin, R. I., D. M. Capuzzi, and S. Margolis. 1972. Inhibition of lipid synthesis by epinephrine in isolated rat liver cells. *Horm. Metab. Res.* **4:** 176–178.

11. Edwards, P. A. 1975. The influence of catecholamines and cyclic AMP on 3-hydroxy-3-methyl glutaryl coenzyme A reductase activity and lipid biosynthesis in isolated rat hepatocytes. *Arch. Biochem. Biophys.* **170:** 188–203.
12. Bloxham, D. P., and M. Akhtar. 1971. Studies on the control of cholesterol biosynthesis: the adenosine 3′:5′-cyclic monophosphate-dependent accumulation of a steroid carboxylic acid. *Biochem. J.* **123:** 275–278.
13. Beg, Z. H., D. W. Allmann, and D. M. Gibson. 1973. Modulation of 3-hydroxy-3-methylglutaryl coenzyme A reductase activity with cAMP and with protein fractions of rat liver cytosol. *Biochem. Biophys. Res. Commun.* **54:** 1362–1369.
14. Regen, D. M., and E. B. Terrell. 1968. Effects of glucagon and fasting on acetate metabolism in perfused rat liver. *Biochim. Biophys. Acta.* **170:** 95–111.
15. Exton, J. H., J. G. Corbin, and S. C. Harper. 1972. Control of gluconeogenesis in liver. V. Effects of fasting, diabetes, and glucagon on lactate and endogenous metabolism in the perfused rat liver. *J. Biol. Chem.* **247:** 4996–5003.
16. Goodwin, C. D., and S. Margolis. 1973. Specific activation of in vitro cholesterol biosynthesis by preincubation of rat liver homogenates. *J. Biol. Chem.* **248:** 7610–7613.
17. Goodwin, C. D., and S. Margolis. 1974. Activation of hepatic cholesterol synthesis and 3-hydroxy-3-methylglutaryl coenzyme A reductase: inhibition by cyclic AMP. *Circulation.* **50:** III–46.
18. Goodwin, C. D. 1975. Cyclic AMP-sensitive activation of hydroxymethylglutaryl coenzyme A reductase: studies with calcium-precipitated endoplasmic reticulum. *Federation Proc.* **34:** 548.
19. Goodwin, C. D., and S. Margolis. 1976. Improved methods for the study of hepatic HMG CoA reductase: one-step isolation of mevalonolactone and rapid preparation of endoplasmic reticulum. *J. Lipid. Res.* **17:** 297–303.
20. Kamath, S. A., and K. A. Narayan. 1972. Interaction of Ca^{2+} with endoplasmic reticulum of rat liver: A standardized procedure for the isolation of rat liver microsomes. *Anal. Biochem.* **48:** 53–61.
21. Kamath, S. A., and E. Rubin. 1972. Interaction of calcium with microsomes: a modified method for the rapid isolation of rat liver microsomes. *Biochem. Biophys. Res. Commun.* **49:** 52–59.
22. Heller, R. A., and R. G. Gould. 1973. Solubilization and partial purification of hepatic 3-hydroxy-3-methylglutaryl coenzyme A reductase: *Biochem. Biophys. Res. Commun.* **50:** 859–865.
23. Bucher, N. L. R. 1953. The formation of radioactive cholesterol and fatty acids from ^{14}C-labeled acetate by rat liver homogenates. *J. Am. Chem. Soc.* **75:** 498.
24. Bucher, N. L. R., and K. McGarrahan. 1956. The biosynthesis of cholesterol from acetate-1-^{14}C by cellular fractions of rat liver. *J. Biol. Chem.* **222:** 1–15.
25. Entenman, C. 1957. General procedures for separating lipid components of tissue. *Methods Enzymol.* **3:** 299–317.
26. Sperry, W. M., and M. Webb. 1950. A revision of the Schoenheimer-Sperry method for cholesterol determination. *J. Biol. Chem.* **187:** 97–106.
27. Crawford, N. 1958. An improved method for the determination of free and total cholesterol using the ferric chloride reaction. *Clin. Chim. Acta.* **3:** 357–367.
28. Shapiro, D. J., R. L. Imblum, and V. W. Rodwell. 1969. Thin-layer chromatographic assay for HMG-CoA reductase and mevalonic acid. *Anal. Biochem.* **31:** 383–390.
29. Gornall, A. G., C. J. Bardawill, and M. M. David. 1949. Determination of serum proteins by means of the biuret reaction. *J. Biol. Chem.* **177:** 751–766.
30. Jacobs, E. E., M. Jacob, D. R. Sanadi, and L. B. Bradley. 1956. Uncoupling of oxidative phosphorylation by cadmium ion. *J. Biol. Chem.* **223:** 147–156.
31. Téllez De Iñoń, M. T., and H. N. Torres. 1973. Regulation of glycogen phosphorylase *a* phosphatase in *Neurospora crassa*. *Biochim. Biophys. Acta.* **297:** 399–412.
32. Maeno, J., and P. Greengard. 1972. Phosphoprotein phosphatases from rat cerebral cortex. Subcellular distribution and characterization. *J. Biol. Chem.* **247:** 3269–3277.
33. Kato, K., and J. S. Bishop. 1972. Glycogen synthase-D phosphatase. I. Some new properties of the partially purified enzyme from rabbit skeletal muscle. *J. Biol. Chem.* **247:** 7420–7429.
34. Fang, M., and J. M. Lowenstein. 1967. Citrate and the conversion of carbohydrate into fat. The regulation of fatty acid synthesis by rat liver extracts. *Biochem. J.* **105:** 803–811.
35. Allred, J. B., and K. L. Roehrig. 1973. Inhibition of rat liver acetyl coenzyme A carboxylase by N^6,$O^{2'}$-dibutyryl cyclic adenosine 3′:5′-monophosphate in vitro. *J. Biol. Chem.* **248:** 4131–4133.
36. Tormanen, C. D., M. E. Ackerman, W. L. Redd, and T. J. Scallen. 1975. Activation of 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase by preincubation with NADPH. *Federation Proc.* **34:** 560.
37. Heller, R. A., and R. G. Gould. 1974. Reversible cold inactivation of microsomal 3-hydroxy-3-methylglutaryl coenzyme A reductase from rat liver. *J. Biol. Chem.* **249:** 5254–5260.
38. Heller, R. A., and R. G. Gould. 1975. Prevention of cold inactivation of 3-hydroxy-3-methylglutaryl coenzyme A reductase by NADPH. *Biochim. Biophys. Acta.* **388:** 254–259.
39. Berndt, J., and R. Gaumert. 1974. Evidence for an activating–inactivating system of 3-hydroxy-3-methylglutaryl-CoA reductase in mouse liver. *Hoppe-Seyler's Z. Physiol. Chem.* **355:** 905–910.
40. Beg, Z. H., D. W. Allmann, P. J. Anderson, E. Pruden, and D. M. Gibson. 1974. Dampening of rat liver 3-hydroxy-3-methylglutaryl coenzyme A (HMG CoA) reductase with ATP (Mg^{2+}) in vitro *Federation Proc.* **33:** 1428.
41. Nordstrom, J. L., J. J. Mitschelen, and V. W. Rodwell. 1976. Reversible modulation of rat liver 3-hydroxy-3-methyl glutaryl-CoA (HMG CoA) reductase activity. *Federation Proc.* **35:** 1471.
42. Chow, J. C., M. J. P. Higgins, and H. Rudney. 1975. The inhibitory effect of ATP on HMG CoA reductase. *Biochem. Biophys. Res. Commun.* **63:** 1077–1084.
43. Brown, M. S., G. Y. Brunschede, and J. L. Goldstein. 1975. Inactivation of 3-hydroxy-3-methylglutaryl coenzyme A reductase in vitro. An adenine nucleotide-dependent reaction catalyzed by a factor in human fibroblasts. *J. Biol. Chem.* **250:** 2502–2509.

44. Shapiro, D. J., J. L. Nordstrom, J. J. Mitschelen, V. W. Rodwell, and R. T. Schimke. 1974. Micro assay for 3-hydroxy-3-methylglutaryl-CoA reductase in rat liver and in L-cell fibroblasts. *Biochim. Biophys. Acta.* **370:** 369–377.
45. Tormanen, C. D., M. V. Srikantaiah, J. E. Hardgrave, and T. J. Scallen. 1977. Evidence for the presence of a natural inactivating factor of 3-hydroxy-3-methylglutaryl coenzyme A reductase in soluble extracts from rat liver microsomes. *J. Biol. Chem.* **252:** 1561–1565.
46. Jergil, B., and R. Ohlsson. 1974. Phosphorylation of proteins in rat liver. Endogenous phosphorylation and dephosphorylation of proteins from smooth and rough endoplasmic reticulum and free ribosomes. *Eur. J. Biochem.* **46:** 13–25.
47. Carlson, C. A., and K. H. Kim. 1974. Regulation of hepatic acetyl coenzyme A carboxylase by phosphorylation. *Arch. Biochem. Biophys.* **164:** 478–489.
48. Lee, K. H., and K. H. Kim. 1977. Regulation of rat liver acetyl coenzyme A carboxylase. Evidence for interconversion between active and inactive forms of enzyme by phosphorylation and dephosphorylation. *J. Biol. Chem.* **252:** 1748–1751.
49. Qureshi, A. A., R. A. Jenik, M. Kim, F. A. Lornitzo, and J. W. Porter. 1975. Separation of two active forms (holo-*a* and holo-*b*) of pigeon liver fatty acid synthetase and their interconversion by phosphorylation and dephosphorylation. *Biochem. Biophys. Res. Commun.* **6:** 344–351.
50. Huang, T.-S., and E. G. Krebs. 1977. Amino acid sequence of a phosphorylation site in skeletal muscle glycogen synthetase. *Biochem. Biophys. Res. Commun.* **75:** 643–650.
51. Berndt, J., F. G. Hegardt, J. Bove, R. Gaumert, J. Still, and M.-T. Cardo. 1976. Activation of 3-hydroxy-3-methylglutaryl-coenzyme A reductase in vitro. *Hoppe-Seyler's Z. Physiol. Chem.* **357:** 1277–1282.