Some Properties and Mechanism of Action of the β-Hydroxy-β-methylglutaryl Coenzyme A Reductase of Yeast*

Mary E. Kirtley† and Harry Rudney‡

ABSTRACT: 3-Hydroxy-β-methylgutaryl coenzyme A (HMG-CoA) reductase has been purified 200-fold from baker's yeast. Gel filtration using Sephadex G-200 and ultracentrifugation of the partially purified enzyme indicate an approximate molecular weight of 150,000–200,000. The enzyme is unstable in buffer solutions but is partially stabilized in the presence of mercaptoethanol. Incubation with the substrate HMG-CoA, the product CoASH, or *o*-phenanthroline results in progressive loss of enzymatic activity. The same site of inactivation appears to be involved in each instance. Several other CoA esters as well as pantetheine inhibit the enzyme. [3-^{14}C]HMG-CoA is not stably bound to the enzyme in the absence of reduced triphosphopyridine nucleotide (TPNH) as judged by passage of the enzyme and substrate through Sephadex G-200. Initial rate and product inhibition studies are in agreement with a "ping pong" mechanism in which no ternary complexes are formed, *i.e.*, a product must be eliminated before a second substrate can be combined with the enzyme. Product inhibition studies indicate that oxidized triphosphopyridine nucleotide (TPN^+) is a competitive inhibitor with TPNH, TPN^+ is an uncompetitive inhibitor with HMG-CoA, and mevalonate is an uncompetitive inhibitor with TPNH. Two possible mechanisms for the reaction are discussed. One involves formation of an acyl enzyme intermediate followed by sequential reduction. The second involves reduction by TPNH of an enzyme-bound group other than substrate.

The reduction of 3-hydroxy-3-methylglutaryl coenzyme A to mevalonic acid (reaction 1) is an early reaction in the biosynthesis of isoprenoid compounds such as steroids, carotenoids, and coenzyme Q. Partial purification of the yeast enzyme which catalyzes this reaction, HMG-CoA[1] reductase,[2] has been reported by Durr and Rudney (1960) and by Knappe *et al.* (1959). Experiments reported by these groups have shown that the possible intermediate aldehyde, mevaldic acid, does not occur as a free intermediate in the course of the reaction. These groups also showed that the enzyme is inhibited by sulfhydryl reagents. It has been suggested that the reaction occurs through the intermediate acylation of a protein sulfhydryl group by HMG-CoA (Popjak and Cornforth, 1960). In this paper we report the further purification of the enzyme from yeast, kinetic studies of the reaction, and some of the properties of the enzyme.

$$\text{HMG-CoA} + 2\text{TPNH} + 2\text{H}^+ \longrightarrow \text{MVA} + 2\text{TPN}^+ + \text{CoASH} \quad (1)$$

Assay of HMG-CoA Reductase. The reaction mixture contained 45 mM phosphate buffer, 0.4 mM HMG-CoA, 8.0 mM 2-mercaptoethanol, and 0.2 mM TPN^+, and enzyme at pH 6.5 an a final volume of 1.7 ml. The reaction was followed spectrophotometrically by measuring the decrease in optical density at 340 mμ at room temperature in a Zeiss spectrophotometer. Readings were taken at 10- or 30-sec intervals and the initial rate was determined.

Purification of HMG-CoA Reductase. Fresh Red Star baker's yeast (12 lb) was crumbled and frozen rapidly in liquid nitrogen. After partial thawing at room temperature for 6 hr the crude yeast was suspended in 2 l. of 0.3 M K_2HPO_4 and stirred 12 hr at 4°. The suspension was centrifuged 15 min at 10,000*g* and the supernatant solution was discarded. The precipitate was resuspended in 2 l. of 0.3 M K_2HPO_4 and was stirred 2 days at 4°. Centrifugation as before gave a supernatant suspension which contained up to 60 mg of protein/ml, but rarely contained appreciable HMG-CoA reductase activity. Resuspension of the gummy precipitate as before and continued autolysis

* From the Department of Biochemistry, Western Reserve University School of Medicine, Cleveland, Ohio 44106. *Received September 19, 1966.* Supported by grants from the National Institutes of Health (National Heart Institute, grant no. HE 06304-04 and research training grant 2 T I GM 35-06) and the Life Insurance Medical Resarch Fund.

† Parts of this work are from a doctoral dissertation submitted by M. E. Kirtley to the Graduate School of Western Reserve University, January 1964. Present address: Department of Biochemistry, University of Maryland School of Medicine, Baltimore, Md., 21201.

‡ Research Career Awardee GM-K6-993 of the U. S. Public Health Service.

[1] Abbreviations used: HMG-CoA, 3-hydroxy-3-methylglutaryl coenzyme A; MVA, mevalonic acid; MAL, mevalonylaldehyde; TPN^+ and TPNH, oxidized and reduced triphosphopyridine nucleotides; ADH, alcohol dehydrogenase; NADP, nicotinamide–adenine dinucleotide phosphate; AMP, adenosine monophosphate; ADP and ATP, adenosine di- and triphosphates.

[2] The trivial name for mevalonate: NADP-oxidoreductase (acylating-CoA) EC 1.1.1.34.

at 4° for an additional day resulted in solubilization of the enzyme activity.

After centrifugation at 10,000*g* for 20 min the supernatant solution containing HMG-CoA reductase was treated at 4° with an equal volume of acetone that had been cooled to −40°. After 10 min at 4° the precipitate was collected at 10,000*g* for 5 min, suspended in 1 l. of 0.015 M phosphate, pH 7.6, and stirred at 4° for 1–2 hr. Insoluble material was removed by centrifugation at 15,000*g* for 10 min and the brown supernatant solution was adjusted to pH 4.6 by slow addition of 1 N acetic acid at 4° over a period of 10 min. After the precipitate had settled for 20 min it was collected at 16,000*g* for 20 min. The precipitate was suspended in 200 ml of 0.015 M phosphate, the pH was adjusted to 7.6 by dropwise addition of 1 N NaOH, and 1 ml of 2-mercaptoethanol was added. At this step and thereafter the enzyme exhibited marked instability unless the thiol was present.

To the cloudy suspension saturated ammonium sulfate (saturated at 25°) was added to 33% saturation at room temperature. The supernatant solution after centrifugation at 16,000*g* for 10 min was adjusted to 66% saturation with saturated ammonium sulfate and centrifuged as before. The ammonium sulfate precipitate readily dissolved in 100 ml of 0.015 M phosphate buffer, pH 7.6, to which was added 0.5 ml of 2-mercaptoethanol. The yellow solution was warmed in a 70° water bath to 60° and maintained thus for 5 min, then cooled rapidly in ice, and centrifuged at 20,000*g* for 20 min.

To the clear yellow supernatant solution 30 ml of 0.33 M zinc acetate was added. After 10 min at 10° the precipitate was collected at 24,000*g* for 10 min. HMG-CoA reductase activity was eluted by batchwise suspension of this precipitate in 25-ml portions of increasing concentrations of phosphate buffer. First the precipitate was suspended in 0.015 M phosphate, pH 7.6, and centrifuged as before. The resuspension procedure was repeated once with 0.15 M phosphate and twice with 0.3 M phosphate, pH 7.6. 2-Mercaptoethanol (0.5 ml) was added to each supernatant solution. Approximately 80% of the HMG-CoA reductase activity appeared in the first 0.3 M phosphate fraction with about 10% in each of the 0.15 M phosphate and the second 0.3 M phosphate fractions. Usually a yellow protein was solubilized in the 0.15 M phosphate or the first 0.3 M phosphate fractions.

Many of the experiments reported here were carried out with the zinc eluate, which is relatively stable. It is known to be contaminated with a small percentage of alcohol dehydrogenase and a variable amount of the yellow protein mentioned above. Both of these contaminating proteins can be separated from the HMG-CoA reductase by chromatography on DEAE-cellulose. The first 0.3 M phosphate eluate was concentrated to 3 ml by filtration through Collodion tubes (Schleicher and Schuell) under vacuum against 0.015 M phosphate, pH 7.6, containing 1.3 mM 2-mercaptoethanol. The concentrated protein was diluted with 5 ml water and put on a DEAE-cellulose column (29 × 2 cm) which had been equilibrated with 0.015 M phosphate, pH 7.6, containing 1.3 mM 2-mercaptoethanol. The column was washed with 230 ml of the same buffer and 9-ml fractions were collected every 4 min. The enzyme was eluted by a gradient of 0.15 M phosphate, pH 7.6, containing 1.3 mM 2-mercaptoethanol into 60 ml of 0.015 M phosphate, pH 7.6, containing 1.3 mM 2-mercaptoethanol. The HMG-CoA reductase activity was preceded off the column by the alcohol dehydrogenase and the yellow protein. The purification is shown in Table I.

TABLE I: Purification of HMG-CoA Reductase from Yeast.

Fraction	Total Protein (mg)	Sp Act. (μ-moles of TPNH/ min mg)	Yield (%)[a]
Acetone precipitate	17,500	0.012	100
Acid precipitate	2,540	0.052	61
Ammonium sulfate precipitate	480	0.257	57
60° supernatant	400	0.281	52
Zinc eluate (first 0.3 M phosphate fraction)	75	0.833	29
DEAE eluate	30	1.40	14[b]

[a] Yield is calculated on the basis of the activity present after acetone precipitation. The HMG-CoA reductase activity present in the crude yeast preparation is too low to be measured spectrophotometrically. Durr and Rudney (1960) have estimated the specific activity of the crude yeast extract as varying between 0.001 and 0.14 in different preparations as measured isotopically. [b] The yield of enzymatic activity is based on the total recovery while the specific activity is that of the peak tubes representing 75% of the total recovered activity.

Materials. TPNH and TPN^+ were obtained from Sigma Chemical Co. CoASH was obtained from Pabst Laboratories. HMG-CoA was synthesized by the procedure of Hilz *et al.* (1958) and contains an equimolar mixture of the two diastereoisomers Kirtley *et al.* (1962).

Separation of [3-^{14}C]HMG-CoA and HMG-CoA Reductase on Sephadex G-200. Zinc eluate enzyme (1 ml) containing 3.7 mg of protein was incubated at room temperature for 70 min with 2.64 [3-^{14}C]HMG-CoA containing 290,000 cpm. This mixture was placed on a 2 × 10 cm column of Sephadex G-200, which had previously been equilibrated with 0.015 M phosphate, pH 7.6, containing 0.1 ml of 2-mercaptoethanol/100 ml. Fractions were collected at 10-min

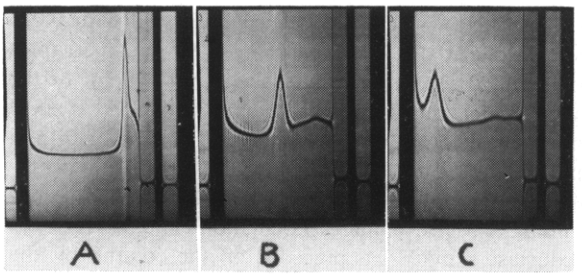

FIGURE 1: Schlieren patterns of HMG-CoA reductase sedimented at 59,780 rpm at 5.1° for (A) 16 min, phase plate angle 55°; (B) 72 min, phase plate angle 45°; and (C) 120 min, phase plate angle 40°. The protein concentration was 5 mg/ml in 0.01 M potassium phosphate, pH 7.6, 0.6 mM EDTA, and 0.04 M 2-mercaptoethanol.

intervals and aliquots were tested for HMG-CoA reductase activity, absorption at 280 and 260 mμ, and total radioactivity. Radioactivity was measured with a Nuclear-Chicago gas-flow counter on aliquots evaporated to dryness on steel planchets.

Kinetic Measurements. Kinetic studies in the absence of products were made in a Cary recording spectrophotometer Model 14 using a slide wire with full-scale deflection of 0.1 absorbancy. The reactions were carried out at room temperature in 3-ml cuvets with a light path of 1.0 cm. For each measurement substrates, phosphate buffer, 2-mercaptoethanol, and water in a final volume of 2.5 ml were mixed in the cuvet and the pen was adjusted to read 0.9–1.00 at 340 mμ using a blank containing the same concentrations of all components except HMG-CoA. Reaction was initiated by addition of 0.01 mg of enzyme in 10 μl of the same buffer on a polyethylene mixer and the decrease in absorbance at 340 mμ was followed for 1–2 min using a blank containing the same concentration of all components except HMG-CoA. Initial rates were determined from the slope of the tracings obtained in the first phase of the reaction. Product inhibition studies were carried out using a Zeiss spectrophotometer. In the studies in the absence of products each concentration of both substrates was run in triplicate except the lowest concentration each of which was run in quadruplicate. The product inhibition studies were carried out primarily in triplicate with a few points in duplicate.

Results

Partial Purification. The partial purification of HMG-CoA reductase has extended the procedure reported earlier by Durr and Rudney (1960), but has exceeded the extent of purification obtained by these authors. In Table I are shown the results of a typical procedure with 12 lb of commercial baker's yeast. The enrichment achieved is greater than 100-fold. In some instances it has exceeded this figure by a factor of 10. Considerable variation has been found among different batches of yeast with respect to both total extractable activity and stability of the HMG-CoA reductase. The prolonged autolysis necessary to obtain the enzyme in a soluble form suggests that it is bound firmly to particles within the cells. It has been shown by Bucher and McGarrahan (1956) that cholesterol synthesis is present primarily in the microsomal fraction isolated from liver. Unfortunately, the long autolysis may also result in loss of HMG-CoA reductase activity due to proteolysis or inactivation of the enzyme. Thus, the total activity in Table I may represent only a fraction of the HMG-CoA reductase present in the yeast cells.

The enzyme obtained after zinc precipitation can be stored for periods of up to 2 weeks in the presence of 2-mercaptoethanol with gradual loss of activity amounting to approximately 5%/day. Additional stabilization of enzymatic activity is often obtained in the presence of 20% ethylene glycol or 0.3 M sucrose. These preparations may be stored at room temperature or 4° but are rapidly inactivated by freezing. On the other hand, the most purified fractions, eluates from the DEAE-cellulose column, have proved to be quite unstable even in the presence of 2-mercaptoethanol cr ethylene glycol and are generally inactive within 12 hr. For this reason most of the following experiments were carried out with the eluate following zinc precipitation.

Sedimentation and Chromatography. If the enzyme is passed through Sephadex G-200 HMG-CoA reductase activity corresponds to the first protein peak collected from the column and is closely followed by contaminating alcohol dehydrogenase which has a molecular weight of 151,000 (Hayes and Velick, 1954). Schlieren patterns obtained on ultracentrifugation of pooled fractions containing HMG-CoA reductase activity are shown in Figure 1. The calculated $s_{20,w}$ for the major peak is 7.7. It seems reasonable to ascribe the minor peak to alcohol dehydrogenase $s_{20,w}$ 6.7 (Hayes and Velick, 1954). Alcohol dehydrogenase can best be removed by chromatography on DEAE-cellulose. However, because of the instability of this preparation it has not been possible to obtain an ultracentrifuge pattern of active HMG-CoA reductase. Even in the case of the comparatively stable zinc eluate shown in Figure 1 the enzyme had lost 88% of its activity during the ultracentrifugation, while an uncentrifuged aliquot, which was maintained at the same temperature, had lost only 12% of its initial activity. In one experiment, using a movable partition cell where the run was stopped after the major peak had entered the lower half of the cell, all residual activity was found in the lower half of the cell. The results of the behavior of the enzyme on Sephadex G-200 and the sedimentation of the enzyme in the ultracentrifuge are consistent with a molecular weight for the enzyme in the range 150,000–200,000.

The Effect of Inhibitors. As reported in the earlier work (Knappe *et al.*, 1959; Durr and Rudney, 1960), HMG-CoA reductase is inhibited by low concentrations of iodoacetamide or *p*-hydroxymercuribenzoate and enzymatic activity is either protected or stimulated

by the presence of thiol compounds. In the present purification it was found that in all stages following the acid precipitation enzymatic activity was rapidly lost unless a thiol, usually 2-mercaptoethanol, was added. These observations suggest that exposed sulfhydryl groups on the protein are necessary for enzymatic activity.

In addition to its sensitivity to sulfhydryl reagents the enzyme is also inhibited by *o*-phenanthroline. As shown in Table II there is no decrease in activity at the initial exposure of the enzyme to the chelator. However, after preincubation with *o*-phenanthroline the enzyme becomes progressively inactivated even in the presence of 2-mercaptoethanol and phosphate buffer. Complete inactivation due to the presence of *o*-phenanthroline has not been observed even in the presence of high concentrations of the inhibitor. The progressive inactivation of HMG-CoA reductase by *o*-phenanthroline is similar to that observed by Vallee (1960) with yeast alcohol dehydrogenase, a zinc enzyme, and suggests the presence of a tightly bound metal cation which is chelated only slowly by *o*-phenanthroline. Other chelating agents are without effect. EDTA at 0.018 M, bipyridyl, or 8-hydroxyquinoline at 0.053 M do not inactivate the enzyme initially or after 15-min preincubation with the enzyme. The following cations in concentrations up to 2.9×10^{-4} M have no effect on enzymatic activity or stability: Mg^{2+}, Cu^{2+}, Fe^{3+}, Ca^{2+}. In the course of purification the enzyme is precipitated in the presence of zinc and is unaffected by this step even in the absence of EDTA. Thus, there is no requirement for added metal cations nor is their presence inhibitory. It has not been possible to obtain a meaningful pattern in metal analyses performed on the purified active enzyme because of the known contamination by yeast ADH and the instability of the enzyme in the purified state.

TABLE II: Inhibition of HMG-CoA Reductase by *o*-Phenanthroline.[a]

Preincubation Time (min)	% of Initial Activity: No Inhibitor	% of Initial Activity: 0.33 mM *o*-Phenanthroline
0	100	104
5	99	86
15	98	65
25	95	59
60	100	48

[a] Zinc eluate enzyme (0.2 mg) was preincubated at room temperature with 105 μmoles of phosphate, pH 6.5, 14 μmoles of 2-mercaptoethanol, and *o*-phenanthroline as indicated in a final volume of 1.5 ml. At the indicated times 0.45 μmole of TPNH and 1.7 μmoles of HMG-CoA were added in 0.25 ml and the optical density change was recorded at 340 mμ.

The Effect of Substrates on Enzyme Stability. A number of attempts have been made to increase the stability of purified HMG-CoA reductase among which have been the addition of substrates to the enzyme. TPNH has little effect either on stabilization of activity or on rate of inactivation; however, HMG-CoA has a marked destabilizing effect when added to the enzyme in the absence of TPNH (Table III, expt 1). The inactivation by HMG-CoA is dependent on the time of preincubation of the enzyme in the absence

TABLE III: Effect of Substrates on Stability of HMG-CoA Reductase.[a]

Preincubation Time (min)	% Initial Activity: No Substrate	0.29 mM TPNH	0.91 mM HMG-CoA	0.51 mM HMG-CoA	0.69 mM *o*-Phenanthroline	0.51 mM HMG-CoA + 0.69 mM *o*-Phenanthroline
			Experiment 1			
5	100	110	78	—	—	—
15	100	109	41	—	—	—
30	101	106	27	—	—	—
60	98	91	19	—	—	—
			Experiment 2			
15				45	21	43

[a] In expt 1, 0.2 mg of zinc eluate enzyme was preincubated at room temperature for the indicated times with 75 μmoles of phosphate, pH 6.5, 14 μmoles of 2-mercaptoethanol, and the additions in a final volume of 1.5 ml. After preincubation the substrates were added and the optical density change at 340 mμ was measured. In expt 2, zinc eluate enzyme from a different preparation was used and the volume of the preincubation mixture was 1.45 ml.

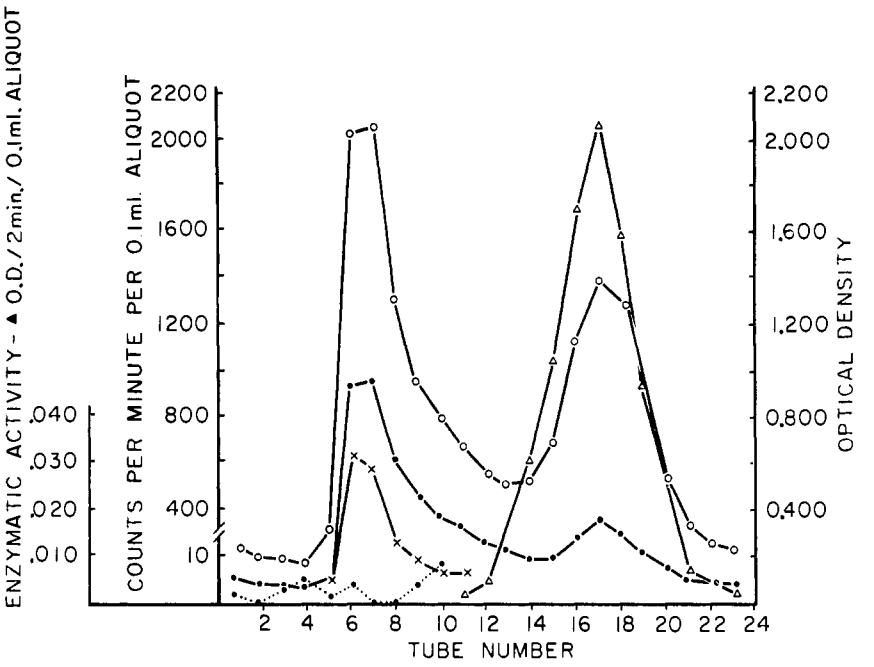

FIGURE 2: Chromatography of HMG-CoA reductase plus [^{14}C]HMG-CoA on Sephadex G-200. Conditions as described in the text. ●····●, radioactivity in tubes 1–10 based on expanded ordinate scale represents one-third of total counts per minute in each tube. Δ——Δ, radioactivity. X——X, enzymatic activity. O——O, optical density at 260 mμ. ●——●, optical density at 280 mμ.

of TPNH. The similarity of this inactivation to that observed with *o*-phenanthroline (Table II) suggested that the two compounds might be acting at the same or related sites. When both inhibitors are preincubated with the enzyme, the inactivation follows that due to HMG-CoA alone. As seen in Table III, expt 2, preincubation of the enzyme with HMG-CoA gave 55% loss in activity while that with *o*-phenanthroline gave 79%. When both substances were present the loss in activity was 57%. The time course of inactivation in the presence of both inactivators was found to follow the course of HMG-CoA inactivation throughout a preincubation period of 1 hr. These results support the possibility that both substances are destabilizing the enzyme in a similar manner and suggest that if the effect of *o*-phenanthroline involves metal chelation, a metal may participate at the active site or binding site for HMG-CoA.

The progressive inactivation seen with HMG-CoA is also exhibited by CoA, acetyl-CoA, and acetoacetyl-CoA when these substances are preincubated with the enzyme Table IV. These compounds are also inhibitory in the over-all reaction. Free HMG is inhibitory in the over-all reaction but produces no inactivation on preincubation with the enzyme. Similar cases of inactivation of an enzyme in the presence of substrates or products have been reviewed by Grisolia (1964). A number of other CoA esters when added to the reaction mixture used for following the over-all reaction showed the following per cent decrease in HMG-CoA reductase activity, *e.g.*, 6.6 × 10^{-3} M propionyl-CoA, 13%; 4.6 × 10^{-4} M butyryl-CoA 7%; 2.1 × 10^{-4} M β-methylcrotonyl-CoA, 17%; 6 × 10^{-4} M dimethylmalonyl-CoA, 63%; and 1.3 × 10^{-3} M succinyl CoA, 23%.

It was thought that the progressive inactivation by

TABLE IV: Inactivation of HMG-CoA Reductase by CoA and Acyl-CoA Esters.[a]

Preincubation (min)	No Addn	% of Initial Activity		
		0.56 mM CoA	0.74 mM Acetyl-CoA	0.74 mM Acetoacetyl-CoA
0	100	74	100	81
1	—	49	—	—
2	—	39	—	—
5	—	26	—	—
10	100	26	—	—
15	—	—	20	19

[a] Zinc eluate enzyme (0.4 mg) was preincubated at room temperature with 75 μmoles of phosphate, pH 6.5, and the additions in a final volume of 1.35 ml. At the indicated times 0.72 μmole of HMG-CoA and 0.44 μmole of TPNH were added in a volume of 0.35 ml and the optical density change at 340 mμ was measured.

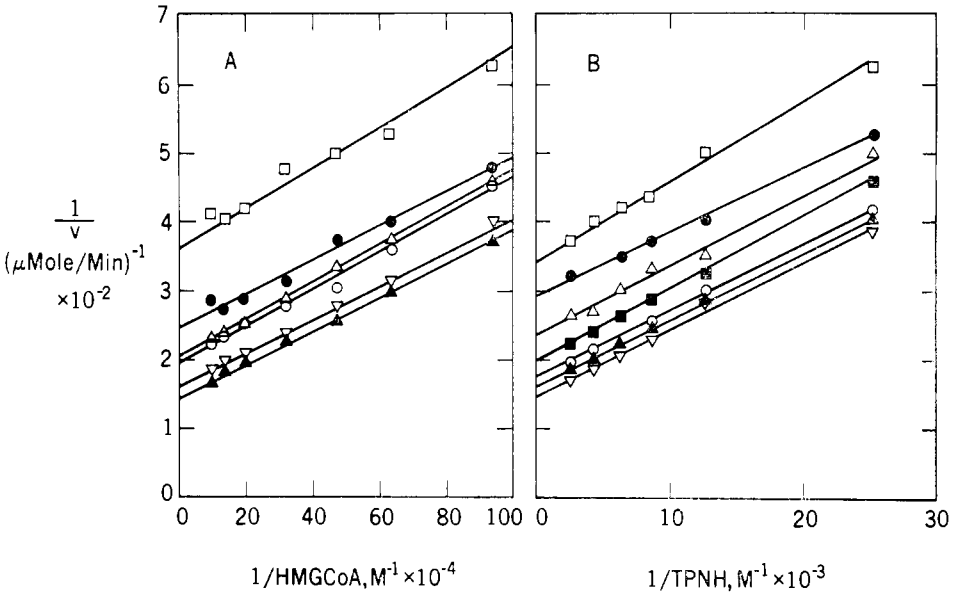

FIGURE 3: Primary double-reciprocal plots of TPNH and HMG-CoA. (A) TPNH held constant at the following concentrations; □, 4.0×10^{-5} M; ●, 7.9×10^{-5} M; Δ, 11.9×10^{-5} M; ○, 15.9×10^{-5} M; ∇, 23.7×10^{-5} M; ▲, 39.6×10^{-5} M. (B) HMG-CoA held constant at the following concentrations; □, 1.1×10^{-6} M; ●, 1.6×10^{-6} M; Δ, 2.1×10^{-6} M; ■, 3.2×10^{-6} M; ○, 5.3×10^{-6} M; ▲, 7.4×10^{-6} M; ∇, 10.6×10^{-6} M. The reaction mixtures contained 0.03 M potassium phosphate and 5.5 mM mercaptoethanol at pH 6.5 in a final volume of 2.36 ml.

the CoA derivatives might be related to the adenine nucleotide portion of these molecules. It was found that 5'-AMP, 3'AMP, ADP, ATP, and adenine were not inhibitory even when preincubated with HMG-CoA reductase. In fact ADP and ATP showed a small activation.

Although the adenine compounds were not inhibitory it was found that pantetheine was a weak inhibitor and also gave a progressive inactivation. At 1.1 mM concentration pantetheine was not inhibitory when added to the enzyme and after 16-min preincubation the activity loss was only 6%; however, with 5.5 mM pantetheine there was a 5% inactivation initially and this increased to 36% following 10-min preincubation with the enzyme. The conditions of this experiment were essentially as outlined in Table IV.

In view of the inactivation of HMG-CoA reductase in the presence of HMG-CoA it was anticipated that binding of HMG-CoA to the enzyme could be demonstrated by incubation with ^{14}C-labeled substrate followed by passage through Sephadex G-200. This treatment would separate the enzyme from the free substrate. The enzyme and [3-^{14}C]HMG-CoA were preincubated until a loss of 30% of the initial activity had occurred. As shown in Figure 2 the HMG-CoA reductase activity was not retarded by the column and appeared with the excluded peak of protein indicated by the absorption at 280 mμ. Suitable aliquots of each tube were plated and counted and it was found that no significant amount of radioactivity was associated with the fractions which contained HMG-CoA reductase activity. All of the radioactivity appeared in a single peak associated with high 260-mμ absorption due to the adenine of HMG-CoA.

Assuming a molecular weight of 150,000, 3.7 mg of protein would represent 0.024 μmole of enzyme. The specific activity of the HMG-CoA added was 110,000 cpm/μmole; therefore, assuming equimolar binding one could expect 2640 cpm bound as a maximal figure. This level of radioactivity would be easily detected under the conditions of the experiment. The failure to demonstrate binding of HMG-CoA to the enzyme by this technique means either that HMG-CoA is not bound at the active site in the absence of the second substrate TPNH, or that binding of HMG-CoA is reversible during passage through the column.

It was previously proposed by several groups (Popjak and Cornforth, 1960; Rudney, 1963; Knappe *et al.*, 1959) that the mechanism of the reaction might involve the formation of an acylated enzyme as shown in reaction 2.

$$\text{HMG-S-CoA} + \text{enzyme-SH} \longrightarrow \text{HMG-S-enzyme} + \text{CoASH} \quad (2)$$

$$\text{HMG-S-enzyme} + H_2O \longrightarrow \text{enzyme-SH} + \text{HMG} \quad (3)$$

It is conceivable that such an acyl protein would be hydrolyzed in the absence of TPNH as shown in reaction 3, thereby implying the HMG-CoA reductase acts as a deacylase in the absence of TPNH. Such deacylase activity measured by the formation of free sulfhydryl groups (Kirtley *et al.*, 1962) has not been detected in the more purified enzyme preparations. In view of the inactivation in the presence of HMG-CoA, it is possible

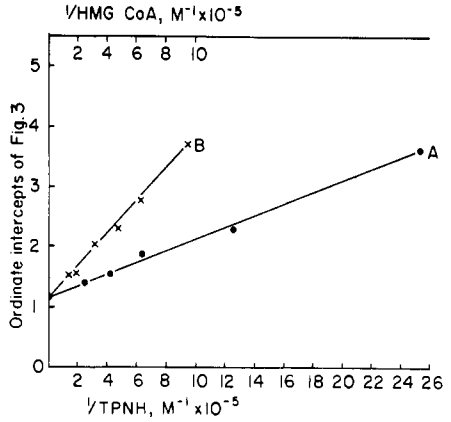

FIGURE 4: Secondary plots of the ordinate intercepts in Figure 3 *vs.* the reciprocal of the concentration of the second substrate. (A) Ordinate intercepts of Figure 3A *vs.* 1/TPNH. (B) Ordinate intercepts of Figure 3B *vs.* 1/HMG-CoA.

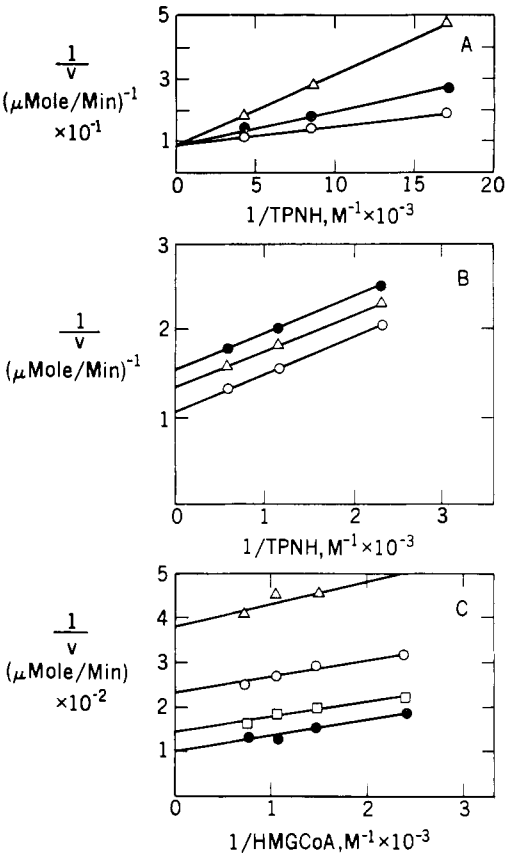

FIGURE 5: Product inhibition studies on HMG-CoA reductase. (A) TPN *vs.* TPNH, HMG-CoA 3.4 × 10^{-4} M; potassium phosphate 0.04 M; pH 6.5 mercaptoethanol 8 × 10^{-3} M; O, TPN^+ none; ●, TPN^+ 1.4 × 10^{-4} M; Δ, TPN^+ 6.7 × 10^{-4} M. (B) Mevalonate *vs.* TPNH. HMG-CoA 3.4 × 10^{-4} M; potassium phosphate 0.04 M, pH 6.5; mercaptoethanol 8 × 10^{-3} M; O, mevalonate none and 5.3 × 10^{-4} M; Δ, mevalonate 27 × 10^{-4} M; ●, mevalonate 54 × 10^{-4} M. (C) TPN^+ *vs.* HMG-CoA, TPNH 1.7 × 10^{-4} M; potassium phosphate 0.06 M, pH 6.5; mercaptoethanol 5 × 10^{-3} M; ●, TPN^+ none; □, TPN^+ 1.8 × 10^{-4} M; O, TPN^+ 4.5 × 10^{-4} M; Δ, TPN^+ 8.9 × 10^{-4} M.

that the enzyme is inactivated during the first turnover along this abortive pathway. The methods used for detection of HMG-CoA deacylation are not sufficiently sensitive to detect a single turnover of the enzyme under usual assay conditions.

Kinetics

It has been possible to study the kinetics of the overall reaction with fresh preparations of the enzyme purified through the zinc precipitation step. The experiments were carried out with very short time periods to obtain initial rates and controls were included to ensure that no loss of activity had occurred during the course of the experiment. The results of these experiments are shown in Figures 3–5.

In Figure 3 (A and B) the double-reciprocal plots of initial velocity at several concentrations of each substrate give a series of parallel lines. The secondary plot of the ordinate intercepts of Figure 3 *vs.* the reciprocal of the second substrate concentration are shown in Figure 4. The slope of line A obtained from the intercepts of Figure 3A is the same as the slopes of the lines in Figure 3B. Similarly the slope of line B obtained from the intercepts of Figure 3B is the same as the slopes of the lines in Figure 3A. This pattern in a two substrate system fits eq 4 as described by Dalziel (1957)

$$\frac{E}{v} = \varphi_0 + \frac{\varphi_A}{A} + \frac{\varphi_B}{B} \qquad (4)$$

where the φ terms include several rate constants and A and B are the substrate concentrations. The intercept of the secondary plot shown in Figure 4 gives $1/V_{max}$ for the reaction at saturation levels of both substrates. From this value and the slopes of the lines the K_m for each substrate at saturation levels of the other substrate is obtained HMG-CoA, 2.4 × 10^{-6} M; TPNH, 8.9 × 10^{-5} M.

The pattern observed in Figures 3 and 4 and described by eq 4 is that of the ping pong mechanism in the terminology of Cleland (1963). The parallel lines obtained in the double-reciprocal plots of initial rates (Figures 3 and 4) and the absence of terms containing both A and B in the initial rate equation imply that the binding of each substrate to the enzyme is followed by the release of a product from the enzyme before a second substrate can react. In this reaction there are three substrates: HMG-CoA and two TPNH molecules. The possibility arises that a term φ_{AB}/AB could occur in which both A and B are TPNH. The occurrence of such a term would result in an upward curvature of the lines plotted in Figure 3. Since the data do not

suggest such a curvature it is concluded that this term as well as the possible term φ_{ABC}/ABC does not occur.

In order to determine the order of addition of substrates and release of products, the effect of some of the products on initial rates were determined and the data are shown in Figure 5A–C. The significance of these results is considered in the discussion of the general mechanism of the HMG-CoA reductase reaction.

Discussion

Substrate Effects. The significance of the inhibitory interactions with CoA, pantetheine, HMG-CoA, and other acyl-CoA derivatives is still speculative. The effects may indicate a change of conformation of the enzyme on the addition of substrate or product but further work is necessary to examine this point. Similarly, it is premature to ascribe to these reactions a physiological role in control of the biosynthesis of cholesterol. In this connection no inhibition by ergosterol and mevalonate 5-phosphate could be detected. As yet it has not been possible to reactivate the enzyme once it has been inactivated either by incubation with HMG-CoA, CoA, *o*-phenanthroline, or simply by storage. Among the techniques which have been tried are: reduction with 2-mercaptoethanol, sodium borohydride, or sodium hydrosulfite; addition of metal cations; dialysis; precipitation with ammonium sulfate and solution in phosphate or Tris buffers; concentration or dilution; incubation with combinations of substrates and products; warming to 37 or 50°; cooling; addition of ethylene glycol or sucrose. TPNH does not affect the stability of the enzyme under any of the conditions studied.

General Mechanism of the HMG-CoA Reductase Reaction

The general form of the reaction is shown in reactions 5–8

$$E + A \underset{k_2}{\overset{k_1}{\rightleftharpoons}} EA \underset{k_4}{\overset{k_3}{\rightleftharpoons}} E' + P \qquad (5)$$

$$E' + B \underset{k_6}{\overset{k_5}{\rightleftharpoons}} E'B \underset{k_8}{\overset{k_7}{\rightleftharpoons}} E'' + Q \qquad (6)$$

$$E'' + C \underset{10}{\overset{k_9}{\rightleftharpoons}} E''C \underset{k_{12}}{\overset{k_{11}}{\rightleftharpoons}} E''' + R \qquad (7)$$

$$E''' \underset{k_{14}}{\overset{k_{13}}{\rightleftharpoons}} E + S \qquad (8)$$

The three substrates are HMG-CoA and two TPNH molecules and the four products are mevalonic acid, CoASH, and two TPN^+ molecules. In the absence of products the initial rate of the reaction is described by eq 9.

$$E/v_f = \varphi_0 + \varphi_A/A + \varphi_B/B + \varphi_C/C \qquad (9)$$

Derivation of the steady-state rate equation by the method of King and Altman (1956) gives the following values of the constants: $\varphi_0 = 1/k_3 + 1/k_7 + 1/k_{11} + 1/k_{13}$; $\varphi_A = (1/k_1)(1 + k_2/k_3)$; $\varphi_B = (1/k_5)(1 + k_6/k_7)$; and $\varphi_C = (1/k_9)(1 + k_{10}/k_{11})$. Product inhibition studies on the initial rate give the following results. TPN^+ is competitive with TPNH (Figure 5A) and gives uncompetitive inhibition with respect to HMG-CoA (Figure 5C). Mevalonic acid is uncompetitive with respect to TPNH (Figure 5B). Uncompetitive inhibition by a product in this type of mechanism occurs when the product binds to a form of the enzyme which differs from that form which binds the substrate in question. For example, uncompetitive inhibition would result if mevalonic acid were product S and TPNH were substrates B and C or if mevalonic acid were product R and TPNH were substrate A + B.

The ping pong mechanism is characterized by the cyclic conversion of the protein through different forms. In the case of transaminases which behave according to this mechanism (Velick and Vavra, 1962; Henson and Cleland, 1964). the enzyme is interconverted between two species in which the bound cofactor is alternately pyridoxal and pyridoxamine (Hughes *et al.*, 1962; Metzler *et al.*, 1954). D-Amino acid oxidase (Massey *et al.*, 1961), lipoyl dehydrogenase (Massey *et al.*, 1960), xanthine oxidase (Massey and Veeger, 1963), glutathione reductase (Mapson and Isherwood, 1963), as well as l-lactate cytochrome-c oxidoreductase (Morton and Sturtevant, 1964; Morton *et al.*, 1961; Hinkson and Mahler, 1963), also follow a ping pong mechanism in which the bound cofactor is alternately oxidized and reduced. The initial reaction in these mechanisms depends on which form of the enzyme is initially present. The sequence of reactions shown in eq 5–8 could be written in four equivalent ways starting with each of the reactions. The mechanism is consistent with that proposed by Popjak and Cornforth (1960) as written in the form of reactions 10–13 (mechanism 1).

Mechanism 1

$$HMG\text{-}CoA + ESH \rightleftharpoons ES\text{-}HMG + CoASH \qquad (10)$$

$$ES\text{-}HMG + TPNH + H^+ \rightleftharpoons ES\text{-}MAL + TPN^+ \qquad (11)$$

$$ES\text{-}MAL + TPNH + H^+ \rightleftharpoons ES\text{-}MVA + TPN^+ \qquad (12)$$

$$ES\text{-}MVA \rightleftharpoons E\text{-}SH + MVA \qquad (13)$$

The intermediate species of enzyme in this mechanism differ in the state of oxidation of the bound substrate.[3]

An alternate type of mechanism is also consistent

[3] In order to obtain the observed product inhibition patterns the following rate constants must be negligibly small: mechanism 1, k_6 and k_{12}; mechanism 2A, k_2 and k_8; mechanism 2B, k_1 and k_{10} where the constants are those shown in eq 5–8 of the general mechanism.

with the observed kinetics of the reaction. In this case the intermediate species of the enzyme differ in the oxidation state of enzyme-bound groups other than the substrate. Two variations of such a mechanism are shown in reactions 14–17 (mechanism 2A) or reactions 18–21 (mechanism 2B).[3]

Mechanism 2A

$$E + TPNH + H^+ \rightleftarrows E\text{-}H_2 + TPN^+ \quad (14)$$

$$E\text{-}H_2 + TPNH + H^+ \rightleftarrows E\text{-}(H_2)_2 + TPN^+ \quad (15)$$

$$E\text{-}(H_2)_2 + HMG\text{-}CoA \rightleftarrows E\text{-}H(CoA) + MVA \quad (16)$$

$$E\text{-}H(CoA) \rightleftarrows E + CoASH \quad (17)$$

Mechanism 2B

$$E\text{-}H + TPNH + H^+ \rightleftarrows E\text{-}H(H_2) + TPN^+ \quad (18)$$

$$E\text{-}H(H_2) + HMG\text{-}CoA \rightleftarrows E\text{-}MAL + CoASH \quad (19)$$

$$E\text{-}MAL + TPNH + H^+ \rightleftarrows E\text{-}H\text{-}(TPN^+) + MVA \quad (20)$$

$$E\text{-}H\text{-}(TPN^+) \rightleftarrows E\text{-}H + TPN^+ \quad (21)$$

In Mechanism 2A the enyzme contains two different redox groups which are reduced by TPNH with release of TPN^+. The reduced enzyme reacts with HMG-CoA to form an enzyme CoA complex and mevalonate is released. The enzyme–CoA complex is regenerated to the oxidized form with the release of CoASH. In Mechanism 2B, the enzyme contains a group reducible by TPNH. The reduced enzyme reacts with HMG-CoA to form the enzyme–aldehyde complex which is reduced by TPNH to mevalonic acid and an enzyme–TPN^+ complex. Various other combinations can be made which incorporate the basic idea of reducible groups on the enzyme.

Mechanisms 2A and B are consistent with uncompetitive inhibition between TPN^+ and HMG-CoA if reactions 15 and 18 are irreversible. Indeed assumptions of irreversibility are necessary at various points in a detailed analysis of all possible combinations of substrates and products which are in agreement with the product inhibition data.[3] The over-all reaction is practically irreversible (Durr and Rudney 1960; Knappe *et al.*, 1959).

The mechanism requires that there are enzyme-bound groups which can be reduced by TPNH in the absence of HMG-CoA. In this case it might be possible to demonstrate the partial reactions 14 and 15 in the absence of HMG-CoA. Preliminary experiments with the enzyme purified through the DEAE-cellulose step were carried out with randomly tritiated TPN^+. When unlabeled TPNH and tritiated TPN^+ were incubated in the absence of HMG-CoA the label appeared in TPNH in the presence of the enzyme. The demonstration that this activity is due to HMG-CoA reductase must await stabilization of the enzyme and demonstration of its freedom from contamination by other enzymes.

At present it is not possible to choose between the two proposed types of mechanism for this reaction. Further studies are planned to elucidate the details of the mode of action of this interesting enzyme.

References

Bucher, N. L. R., and McGarrahan, K. (1956), *J. Biol. Chem. 222*, 1.

Cleland, W. W. (1963), *Biochim. Biophys. Acta 67*, 104.

Dalziel, K. (1957), *Acta Chem. Scand. 11*, 1706.

Durr, I. F., and Rudney, H. (1960), *J. Biol. Chem. 235*, 2572.

Grisolia, S. (1964), *Physiol. Rev. 44*, 657.

Hayes, J. E., and Velick, S. F. (1954), *J. Biol. Chem. 207*, 225.

Henson, C. P., and Cleland, W. W. (1964), *Biochemistry 3*, 338.

Hilz, H., Knappe, J., Ringelmann, E., and Lynen, F. (1958), *Biochem. Z. 329*, 476.

Hinkson, J. W., and Mahler, H. R. (1963), *Biochemistry 2*, 209.

Hughes, R. C., Jenkins, W. T., and Fischer, E. H. (1962), *Proc. Natl. Acad. Sci. U. S. 48*, 1615.

King, F. L., and Altman, C. (1956), *J. Phys. Chem. 60*, 1375.

Kirtley, M. E., Rudney, H., and Durr, I. F. (1962), *J. Biol. Chem. 237*, 1781.

Knappe, J., Ringelmann, E., and Lynen, F. (1959), *Biochem. Z. 332*, 195.

Mapson, L. W., and Isherwood, F. A. (1963), *Biochem. J. 86*, 173.

Massey, V., Gibson, Q. H., and Veeger, C. (1960), *Biochem. J. 77*, 328, 341.

Massey, V., Palmer, G., and Bennett, R. (1961), *Biochim. Biophys. Acta 48*, 1.

Massey, V., and Veeger, C. (1963), *Ann. Rev. Biochem. 32*, 579.

Metzler, D. E., Ikawa, M., and Snell, E. E. (1954), *J. Am. Chem. Soc. 76*, 648.

Morton, R. K., Armstrong, J. M., and Appleby, C. A. (1961), *in* Haematin Enzymes, Falk, J. H., Lemberg, R., and Morton, R. K., Ed., London, Pergamon, p 501.

Morton, R. E., and Sturtevant, J. M. (1964), *J. Biol. Chem. 239*, 1914.

Popjak, G., and Cornforth, J. W. (1960), *Advan. Enzymol. 22*, 281.

Rudney, H. (1963), *Proc. Intern. Congr. Biochem., 5th, 1961, Moscow, VII*, 254.

Vallee, B. L. (1960), *Enzymes 2*, 225.

Velick, S. F., and Vavra, T. (1962), *Enzymes 6*, 219.