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Biosynthesis of 3-Hydroxy-3-methylglutarate and Mevalonate by Rat Liver Homogenates *in Vitro**

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ABSTRACT: Methods have been developed for studying regulation of 3-hydroxy-3-methylglutaryl–coenzyme A (HMG–CoA) and mevalonate biosynthesis in fractionated rat liver homogenates. Evaluation of individual steps or combinations of steps between acetate and mevalonate, including HMG–CoA condensing enzyme and HMG–CoA reductase, are described. The requirements for each reaction, including optimal concentrations of substrate and cofactors, have been defined, and simplified methods for separating and purifying HMG and mevalonate have been developed. A method for determining the rate of synthesis of HMG and mevalonate, as contrasted with ^{14}C incorporation from labeled precursors, is described. Very little of the HMG formed is bound to protein, and data supporting the role of HMG–CoA as an intermediate in mevalonate synthesis are presented. Using methods described here, accurate delineation of early regulatory points in the cholesterol biosynthetic pathway may be performed. The validity of previous studies, the precautions required, and the usefulness and importance of these methods are discussed.

Biosynthesis of cholesterol from 2-carbon fragments proceeds by well-known pathways. Recently, interest has been shown in control of these pathways and attempts have been made to localize a single rate-limiting step at which regulation occurs. Based on theoretical considerations as well as experimental observations, emphasis has been placed on the early steps in cholesterol formation involving mevalonate synthesis (Rudney, 1963), and it has been suggested that control is exerted on the step catalyzed by 3-hydroxy-3-methylglutaryl–CoA[1] reductase (mevalonate:NADP oxidoreductase, E.C. 1.1.1.34) (Bucher *et al.*, 1960; Siperstein and Fagan, 1964). This reaction, involving TPNH-dependent reduction of HMG–CoA to form mevalonic acid, is considered of importance in cholesterol synthesis because quantitatively significant alternative pathways exist prior to, but not beyond, mevalonic acid formation. By analogy to bacterial systems, its position beyond a branch point on the direct pathway to

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[1] The following abbreviations are used throughout the text: HMG and HMG–CoA for 3-hydroxy-3-methylglutaric acid and its CoA ester; Ac and Ac–CoA for acetate and its CoA ester; AcAc and AcAc–CoA for acetoacetate and its CoA ester; ACP for acyl carrier protein.

cholesterol (Lynen, 1958) and its irreversibility (Rudney, 1963) make it a logical regulatory site (Siperstein and Fagan, 1964).

A variety of manipulations, including radiation, Triton administration, fasting, and cholesterol feeding, lead to altered rates of incorporation of [^{14}C]Ac into cholesterol in cell-free liver systems. Parallel studies have shown that incorporation of [^{14}C]mevalonic acid into cholesterol is not similarly affected (Bucher *et al.*, 1959; Gould and Popjak, 1957) suggesting that the regulatory site lies between Ac and mevalonic acid. Also, in situations that alter incorporation of [^{14}C]Ac into cholesterol, formation of AcAc or incorporation of [^{14}C]Ac into AcAc is not affected by changes in the opposite direction (Bucher *et al.*, 1960; Siperstein and Fagan, 1964). This suggests that the regulatory site lies beyond HMG–CoA, if one assumes that HMG–CoA is the source of AcAc (Lynen, 1958). More recently, dietary-induced alterations in incorporation of [^{14}C]Ac into mevalonic acid with no changes in HMG formation have been reported, and direct inhibition of incorporation of HMG–CoA into mevalonic acid by fasting or dietary cholesterol has been described (Regan *et al.*, Linn, 1967a).

However, changes in HMG–CoA reductase in the same direction as changes in incorporation of [^{14}C]Ac into cholesterol, occurring with various manipulations, does not ensure that this is the only regulatory enzyme in the pathway. In the experiments cited above, definitive assays of the various possible sites of action between Ac and MVA were not performed. In particular, insufficient consideration was given to possible regulation at the step catalyzed by the HMG–CoA condensing enzyme (3-hydroxy-3-methylglutaryl–CoA acetoacetyl–CoA lyase, E.C. 4.1.3.5.), involving condensation of Ac–CoA and AcAc–CoA to form HMG–CoA. Furthermore, it has been assumed that altered rates of incorporation of isotopically labeled precursor can be equated with altered rates of synthesis. To resolve these problems and to assess the validity of assumptions in much of the earlier work, appropriate systems for studing regulation of mevalonic acid formation were developed.

The purpose of this work was to (1) devise methods for evaluating individual steps or combinations of steps between Ac and mevalonic acid in order to permit delineation of regulatory points in the pathway; (2) determine whether altered rates of isotope incorporation directly reflect similar alterations in synthetic rate, *i.e.*, to develop reliable methods for measuring rates of synthesis; (3) develop a convenient method for isolating and separating HMG and mevalonic acid, and for their purification free of other ^{14}C-containing compounds; (4) determine optimal conditions with respect to substrate and cofactors for obtaining incorporation into products so that only enzyme content and activity determine reaction rate; (5) determine whether biosynthesized HMG exists as a CoA ester or in a protein-bound form. The system that seemed best suited for these studies was the soluble-microsomal system from rat liver which has been shown (Siperstein and Fagan, 1966) to be the most active of all cellular fractions with respect to conversion of acetate into mevalonate.

Methods

Preparation of Tissues. Sprague–Dawley rats weighing 100–250 g and maintained on Purina rat pellets and water *ad libitum* were used throughout the study. The animals were stunned by a blow on the head and killed by exsanguination; livers were immediately removed, chilled, and finely minced with scissors and razor blades fixed 6 mm apart in an aluminum block. All operations were carried out at 4°. The mince was added to 2 volumes of 0.1 M potassium phosphate, buffer pH 7.0, containing 0.03 M nicotinamide and was ground for 45 sec in a Potter-Elvehjem type homogenizer with a loose fitting Teflon pestle. The homogenate was centrifuged at 600*g* for 10 min to remove nuclei, cell debris, and unbroken cells, and the mitochondrial fraction was sedimented by centrifugation at 10,000*g* for 10 min. The supernatant fluid was then divided into microsomal and soluble cell components by centrifugation for 60 min at 105,000*g* in a Spinco preparative ultracentrifuge, and the microsomes were suspended in a small volume of 0.1 M potassium phosphate buffer, pH 7.0, containing 0.03 M nicotinamide and 10^{-3} M dithiothreitol. In most cases, microsomal and soluble fractions were used without further purification. In some experiments, an EDTA extract of microsomes was prepared (Rudney, 1957).

Tissue fractions, substrates, and cofactors were added to 25-ml flasks and incubated in a metabolic shaker at 37° for 1 or 2 hr under air. The contents of the flasks varied for each of six systems as outlined below.

Separation and Purification of Intermediates. Following incubation, the reaction was stopped by addition of 8 N KOH to bring the flask contents to pH 11. Unlabeled HMG (200 μmoles) and mevalonic acid (100 μmoles) were added as carrier, and the flask contents were kept at room temperature for 1 hr to hydrolyze thio ester bonds. The contents were then acidified to pH 2 with concentrated H_2SO_4 and the precipitated protein was removed by centrifugation. The supernatant solution was evaporated by a stream of air, and the dried residue was extracted three times with 5 ml of ethanol. The combined ethanol extracts were evaporated, and the dried material was dissolved in water and treated with Dowex 50W, hydrogen form, to remove cations and to convert mevalonate into mevalonolactone.

For acetoacetate determination, contents of the incubation flask were treated with 1 ml of 25% trichloracetic acid, and aliquots were removed and assayed by the method of Walker (1954).

Separation of HMG from mevalonic acid was achieved by passing the acidified solution through a Dowex-1-formate column, 13 × 1 cm (Rudney, 1957). Mevalonolactone was not retained by the anion-exchange column. The column was eluted with 0.1 N formic acid, and fractions of 25 ml were collected with HMG appearing in fractions between 150 and 350 ml. [^{14}C]Ac was completely removed from both the HMG and mevalonic acid fractions by these procedures. The formic acid fractions were evaporated on a steam bath and the location and amount of HMG were determined by titration with 0.01 N KOH. In early experiments, where preliminary ethanol extraction had not been performed, contamination of HMG with other ^{14}C-containing compounds was present at this stage. In later experiments that included the ethanol extraction step, most of the contamination was eliminated. Fractions containing HMG were combined and rechromatographed on Dowex-1-formate resulting in removal of all contamination. Purity of HMG was verified by (a) constant specific activity in all HMG-containing fractions, and (b) constant specific activity after HMG recrystallization. For

routine HMG analyses, at least three fractions were counted to ensure constant specific activity.

The effluent from the acidified ethanol extract passed through a Dowex-1-formate column contained mevalonic acid free of HMG, but contained other ^{14}C-labeled nonionic substances. These contaminants, which represented up to 40% of the initial radioactivity in the effluent, cochromatographed with mevalonic acid in an ascending paper chromatography system using propanol–NH_4OH–H_2O (8:1:1) and Whatman No. 1 paper. Ether extraction of a slurry formed by adding anhydrous Na_2SO_4 to the acidified mevalonate removed 90–95% of the contaminating material. However, complete purification was obtained by means of Celite chromatography (Swim and Utter, 1957). The water effluent was evaporated to dryness, the residue dissolved in 0.6 ml of 3 N H_2SO_4, and 1.2 g of Celite was added. This was packed on a 10-g Celite column and mevalonic acid was eluted with chloroform. The bulk of the mevalonic acid appeared from 25 to 125 ml. In several early experiments, purity of mevalonic acid was verified by determining specific activity of mevalonic acid in individual chloroform eluate fractions between 25 and 125 ml. The mevalonic acid content was determined by titration or hydroxamate determination as described below. ^{14}C content corresponded to mevalonic acid content, indicating the absence of ^{14}C-containing nonmevalonate material. In later studies, only the 50–75 ml fraction, containing the mevalonic acid peak, was used. The chloroform eluate containing mevalonic acid was evaporated to dryness and the residue dissolved in water.

Analytical Methods. The formic acid fractions containing HMG from the second Dowex-1-formate column were evaporated to dryness, and HMG was titrated with 0.01 N KOH. Aliquots containing 5 μmoles of the dipotassium salt were plated on stainless steel planchets, evaporated, and counted on a Nuclear-Chicago end-window gas flow counter. For each sample, aliquots of three different fractions were counted.

Incorporation of ^{14}C into mevalonic acid was determined by adding an aliquot of the purified aqueous solution to 15 ml of a *p*-dioxane solution containing 125 g of naphthalene, 7.5 g of 2,5-diphenyloxazole and 375 mg of 1,4-bis[2-(5-phenyloxazolyl)]benzene per liter, and counting in a liquid scintillation spectrometer. To determine mevalonic acid concentration, another aliquot was acidified to form the lactone and treated with neutral hydroxylamine hydrochloride, and the hydroxamate formed was assayed spectrophotometrically as the ferric chloride color complex at a wavelength of 500 mμ (Lynen and Grassl, 1959).

Specific Activity of Acetoacetic Acid. When incorporation of ^{14}C-labeled substrate into products is measured, the specific activity of the product or its immediate precursor pool must be determined to assess the rate of product synthesis (see below). This was done indirectly be measuring specific activity of AcAc. To an aliquot of the ethyl acetate extract of the formazan derivative prepared by the method of Walker (1954) 2 mg of unlabeled formazan derivative was added as carrier and the solution was evaporated to dryness. The residue was dissolved in 0.2 ml of ethyl acetate and 0.8 ml of ethanol and crystallization occurred upon the addition of water. Recrystallization was repeated to constant specific activity, and the purified derivative dissolved in ethyl acetate. Aliquots were plated on stainless steel planchets, evaporated, and counted on an end-window gas flow counter. Suitable dilutions of other aliquots were assayed colorimetrically at 450 mμ and compared with samples of initial unlabeled formazan derivative to correct for losses during purification and to enable calculation of total incorporation of ^{14}C into acetoacetate. Specific activity of acetoacetate could then be calculated [sp act. (cpm/μmole) = total incorporation (cmp)/acetoacetate content (μmole)].[2]

Materials. [1-^{14}C]Acetyl-CoA was prepared from [1-^{14}C]-acetic anhydride (Simon and Shemin, 1953) and acetoacetyl-CoA from diketene (Lynen, 1958). [3-^{14}C]HMG anhydride was prepared by the method of Hilz *et al.* (1958); the product obtained melted at 101–103°. This was treated with CoA to form [3-^{14}C]HMG-CoA (Hilz *et al.*, 1958). In each case, unreacted free acid was removed by acidification to pH 2 and subsequent ether extraction. Coenzyme A, ATP, and TPN^+ were obtained from P-L Biochemicals, DL-mevalonolactone from Nutritional Biochemicals, HMG from Mann Research Laboratories, dipotassium glucose 6-phosphate and Dowex 50W cation-exchange resin from Sigma Chemical Co., glucose 6-phosphate dehydrogenase from Boehringer, dithiothreitol from Calbiochem, Celite 535 from Johns Manville Co., and [1-^{14}C]acetate, [2-^{14}C]acetate, [1-^{14}C]acetic anhydride and [3-^{14}C]HMG from New England Nuclear Corp. or Tracerlab. Dowex formate was generated from Dowex 1-X10 anion-exchange resin, chloride form, obtained from Bio-Rad Laboratories.

Results

Separation and Purification of Intermediates. Attempts to cleanly separate HMG and MVA have been unsuccessful. Siperstein *et al.* (1966) were unable to separate these intermediates by paper or column chromatography or by derivative formation and recrystallization, but did achieve separation by a gas–liquid chromatographic method. This procedure has been repeated by Kandutsch and Saucier (1969) and precautions with its use have been emphasized. Linn (1967b) separated mevalonic acid from HMG using thin-layer chromatography but did not achieve sufficient purification when [^{14}C]Ac was the substrate.

By utilizing the fact that mevalonic acid forms a lactone at low pH that is not retained by an anion-exchange column, mevalonolactone and HMG were separated with ease. No cross-contamination between mevalonic acid and HMG occurred, although other ^{14}C intermediates contaminated both fractions requiring further purification; this was obtained by Celite chromatography. These methods must be used in conjunction to ensure complete purification.

HMG-CoA Condensing Enzyme. Three systems were developed for assay of HMG-CoA condensing enzyme, and each of them, when optimized, revealed various factors which must be considered in evaluating the extent of synthesis from pools of 2-carbon units.

[2] In early experiments, initial values for AcAc were determined and subtracted from final AcAc so as to calculate net synthesis and to correct for any endogenous AcAc present at the beginning of incubation. This was not done in later experiments, since (1) initial AcAc content was insignificant relative to the amount formed; (2) when [^{14}C]HMG-CoA of known specific activity was incubated, the specific activity of the AcAc formed was the same as that of HMG-CoA, indicating no significant pool dilution by endogenous AcAc.

TABLE I: Effect of Mg^{2+} on Substrate Incorporation into HMG or Mevalonic Acid.[a]

Substrate	Tissue Fraction	Mg^{2+} Added (μmoles)	Incorporation of Substrate (mμmoles)	
			HMG	Mevalonic Acid
[1-^{14}C]Acetate	Soluble fraction		44.0	
		30	13.0	
[1-^{14}C]Acetate	Soluble fraction		61.5	
		30	21.0	
[1-^{14}C]Acetate	Soluble fraction		31.0	
		5	27.5	
		10	22.5	
		30	17.5	
[^{14}C]Ac–CoA	Microsomal fraction		46.6	
		15	33.6	
[^{14}C]Ac–CoA	EDTA microsomal extract		23.2	
		15	9.8	
[^{14}C]Ac–CoA	Soluble fraction		17.5	
		12	12.3	
[1-^{14}C]Acetate	Soluble + microsome		124.0	
		30	70.0	
[1-^{14}C]Acetate	Soluble + microsomes		3.2	51.5
		6	15.0	166.0
		12	17.5	111.0
		30	17.0	66.5

[a] Conditions as described in text.

A. [1-^{14}C]ACETATE → HMG. The soluble fraction was fully competent with respect to HMG formation; addition of microsomes, EDTA extract of microsomes, or microsomal membranes after EDTA extraction produced no further incorporation into HMG. In the presence of microsomes alone, no incorporation into HMG occurred. Both CoA and ATP were required for optimal activity. The dependence on CoA could be demonstrated by removing endogenous CoA by partial purification of the soluble fraction by ammonium sulfate precipitation (0–60%) followed by dialysis of the resuspended precipitate. High concentrations of added CoA were inhibitory (*e.g.*, 2×10^{-4} M CoA inhibited incorporation by 83%); optimal CoA concentration was 10^{-5} M.

An [1-^{14}C]Ac concentration of 7×10^{-4} M gave maximum incorporation into HMG. The rate of incorporation was directly proportional to the amount of soluble supernatant added. Mercaptoethanol or dithiothreitol, 10^{-3} M, did not influence incorporation of [^{14}C]Ac into HMG. Addition of Mg^{2+} (Table I) or omission of EDTA (Table II) led to decreased activity. The possibility was considered that omission of Mg^{2+}, required for Ac–CoA carboxylase, would result in decreased activity of Ac–CoA carboxylase, leading to increased substrate availability for HMG formation. However, inhibition of Ac–CoA carboxylase with avidin, in the presence of Mg^{2+}, had no effect on HMG formation, indicating that this was not the mechanism by which magnesium inhibited incorporation into HMG.

Effects of Mg^{2+} and EDTA on AcAc formation were opposite to those on incorporation into HMG. Addition of Mg^{2+} increased, while EDTA decreased or abolished AcAc formation from Ac or Ac–CoA. Mg^{2+} and EDTA did not influence AcAc formation when AcAc–CoA or HMG–CoA were substrates.

Addition of mevalonic acid or a TPNH generating system, used routinely for studies of HMG–CoA reductase (see below), had no influence on incorporation of [^{14}C]Ac into HMG. When microsomes were present, however, decreased incorporation of [^{14}C]Ac into HMG was often observed in association with increased mevalonic acid formation.

Optimal conditions were determined to be as follows. The soluble fraction of supernatant derived from 160 mg of tissue was incubated for 1 hr with 5 μmoles of [1-^{14}C]Ac, 0.1 μmole of CoA, 10 μmoles of ATP, 2 μmoles of EDTA, and 300 μmoles of potassium phosphate buffer, pH 7.0, in a volume of 7 ml. If determination of AcAc specific activity was required (see below), AcAc formation was optimized by incubating the soluble fraction derived from 320 mg of tissue with 10 μmoles of [2-^{14}C]Ac, 0.2 μmole of CoA, 10 μmoles of

TABLE II: Effect of EDTA on Substrate Incorporation into HMG or Mevalonic Acid.[a]

Substrate	Tissue Fraction	EDTA (μmoles)	Incorporation of Substrate (mμmoles) HMG	Mevalonic Acid
[1-^{14}C]Acetate	Soluble fraction		11.8	
		2	32.8	
[1-^{14}C]Acetate	Soluble fraction		14.1	
		2	69.2	
[^{14}C]Ac–CoA	Microsomal fraction		21.7	
		2	33.6	
[^{14}C]Ac–CoA	Microsomal fraction		25.8	
		2	46.6	
[^{14}C]Ac–CoA	Soluble fraction		3.4	
		2	17.5	
[^{14}C]Ac–CoA	Soluble fraction	2	79.0	
		10	78.0	
[1-^{14}C]Acetate	Soluble + microsomes		2.5	5.7
		10	8.4	27.0
[1-^{14}C]Acetate	Soluble + microsomes		5.3	6.4
		10	13.4	15.2

[a] Conditions as described in text.

ATP, 50 μmoles of $MgCl_2$, and 300 μmoles of potassium phosphate buffer, pH 7.0, in a volume of 7.0 ml.

B. [^{14}C]ACETYL–CoA → HMG (MICROSOMAL SYSTEM). When [^{14}C]Ac–CoA was the substrate, incorporation into HMG occurred readily with whole microsomes or an EDTA microsomal extract as previously described (Rudney, 1957). ATP was not required. The addition of 10^{-3} M dithiothreitol resulted in a 50% increase in incorporation into HMG, but higher concentrations (10^{-2} M) were markedly inhibitory. As in the previous system, addition of Mg^{2+} (Table I) or omission of EDTA (Table II) resulted in decreased activity. Addition of a TPNH generating system lead to decreased incorporation of ^{14}C into HMG. Incorporation of [^{14}C]Ac–CoA into HMG was linear with time for 1 hr with a further increase, but at a slower rate, occurring during the second hour. The rate of incorporation was also proportional to microsomal protein content.

Optimal conditions were thus as follows: microsomal fraction from 800 mg of tissue was incubated for 1 hr with 1 μmole of [^{14}C]Ac–CoA, 10 μmoles of EDTA, 7 μmoles of dithiothreitol, and 300 μmoles of potassium phosphate buffer, pH 7.0, in a volume of 7.0 ml. Under these conditions, inclusion of AcAc–CoA, also a substrate for HMG–CoA condensing enzyme, resulted in a marked depression of [^{14}C]Ac–CoA incorporation into HMG. This effect of AcAc–CoA has been noted previously (Rudney, 1957). Consequently, AcAc–CoA was not added to these preparations and the incorporation of [^{14}C]Ac–CoA into HMG measured the sum of AcAc–CoA thiolase and HMG–CoA condensing enzyme activities.

C. [^{14}C]ACETYL–CoA → HMG (SOLUBLE SYSTEM). Incorporation of [^{14}C]Ac–CoA into HMG occurred not only with whole microsomes, but also with a soluble (105,000*g* supernatant) fraction. In this system, dithiothreitol (10^{-3} M) inhibited incorporation into HMG. As with the previous two systems, addition of Mg^{2+} (Table I) or omission of EDTA (Table II) resulted in decreased activity. EDTA (3×10^{-4} M) was optimal. In contrast to the microsomal system (above), addition of a TPNH generating system had no effect on incorporation into HMG. Although rates of incorporation of [^{14}C]Ac–CoA into HMG were proportional to protein content, amounts derived from more than 30 mg of tissue were inhibitory. Incorporation was also linear for 2 hr. As with the microsomal system, addition of 1 μmole of AcAc–CoA depressed incorporation into HMG by 95%.

Optimal conditions, then, were as follows: soluble fraction derived from 12 mg of tissue was incubated for 1 hr with 1 μmole of [^{14}C]Ac–CoA, 2 μmoles of EDTA, and 30 μmoles of potassium phosphate buffer, pH 7.0, in a volume of 7.0 ml.

HMG–CoA Reductase. Three systems were developed for assay of HMG–CoA reductase.

A. [1-^{14}C]ACETATE → HMG + MEVALONIC ACID. Incorporation of [^{14}C]Ac into mevalonic acid requires the presence of both soluble fraction and microsomes; when microsomes were omitted, incorporation into HMG was unimpaired, but virtually no incorporation of ^{14}C into mevalonic acid was obtained. Neither an EDTA extract of microsomes nor the microsomal membranes remaining after EDTA extraction could substitute for this requirement for whole microsomes.

TABLE III: Nucleotide Requirements for Incorporation into HMG and Mevalonic Acid.[a]

	Substrate	Soluble Fraction	Microsomes	TPNH Generating System	Incorporation of Substrate (mμmoles) HMG	Mevalonic Acid
A	[1-^{14}C]Ac	+		None	58.5	
		+		TPN^+ + G6P[b] + G6PD[b]	61.5	
B	[1-^{14}C]Ac	+	+	None	41.0	
		+	+	TPN^+ + G6P + G6PD	25.3	
C	[1-^{14}C]Ac	+	+	None	58.5	30.0
		+	+	G6P	57.0	26.0
		+	+	G6P + G6PD	57.0	30.5
		+	+	TPN^+	46.8	62.0
		+	+	TPN^+ + G6PD	47.2	88.0
		+	+	TPN^+ + G6P + G6PD	57.5	104.5
D	[^{14}C]Ac–CoA		+	G6P + G6PD + TPN^+, 2 μmoles	72.0	13.9
			+	G6P + G6PD + TPN^+, 10 μmoles	54.5	26.9
E	[^{14}C]HMG–CoA		+	None		0.1
			+	G6P + G6PD + TPN^+, 0.6 μmole		5.3
			+	G6P + G6PD + TPN^+, 2 μmoles		11.8
			+	TPNH, 10 μmoles		12.0
			+	DPN, 0.6 μmole + ethanol, 10 μmoles		0.1
			+	DPNH, 10 μmoles		0.4
F	[^{14}C]HMG–CoA		+	G6P + G6PD + TPN^+, 0.6 μmole		9.5
			+	G6P + G6PD + TPN^+, 2 μmoles		13.4
			+	G6P + G6PD + TPN^+, 10 μmoles		17.8
			+	G6P + G6PD + TPN^+, 50 μmoles		12.4

[a] Conditions as described in text. [b] Abbreviations: G6P for glucose 6-phosphate and G6PD for glucose 6-phosphate dehydrogenase.

Dithiothreitol (10^{-3} M) had no consistent effect on incorporation. Mg^{2+} was required (Table I), but concentrations higher than 10^{-3} M inhibited HMG–CoA reductase. The Mg^{2+} requirement was not present if EDTA was omitted from the system, suggesting that endogenous Mg^{2+} is present in adequate concentration unless removed by chelation. Omission of EDTA decreased incorporation into both HMG and mevalonic acid (Table II). For mevalonic acid, a partial requirement for a TPNH generating system, consisting of TPN^+, glucose 6-phosphate, and glucose 6-phosphate dehydrogenase, was shown (Table III); TPN^+ appeared to be the major component required. There was no significant increase in HMG in the absence of TPNH.

In contrast to increased incorporation with time into HMG in the case of soluble fraction alone, when microsomes were also present HMG incorporation reached a peak at 30 min followed by a plateau (Figure 1). Incorporation into mevalonic acid was linear for 2 hr, however, suggesting that the rate of HMG formation equaled that of conversion of HMG–CoA into mevalonic acid.

A mevalonic acid pool was necessary in order to trap [^{14}C]-mevalonic acid formed from [^{14}C]Ac. In preliminary experiments, it was shown that (1) incorporation of Ac into cholesterol was completely prevented by dilution when 25 μmoles of mevalonic acid was added and (2) maximum disappearance of mevalonic acid during incubation was 5 μmoles. Since a pool size of 50 μmoles was routinely used, it may be assumed that all synthesized mevalonic acid was trapped, and that mevalonic acid lost as a result of further reactions during incubation would not greatly affect the final value for total incorporation.

Optimal conditions for this assay were as follows: soluble fraction from 160 mg of tissue and microsomal fraction from 800 mg of tissue were incubated for 1 hr with 5 μmoles of mevalonic acid (potassium salt), 20 μmoles of glucose 6-phosphate (dipotassium salt), 10 μmoles of TPN^+, 0.5 unit of glucose 6-phosphate dehydrogenase, and 250 μmoles of potassium phosphate buffer, pH 7.0, in a volume of 7 ml. When the specific activity of AcAc was determined AcAc formation was optimized by incubating the soluble fraction from 320 mg of tissue and microsomes from 800 mg of tissue with 10 μmoles of [2-^{14}C]Ac, 0.2 μmole of CoA, 10 μmoles of ATP, 50 μmoles of $MgCl_2$, 50 μmoles of mevalonic acid, 20 μmoles of glucose 6-phosphate, 10 μmoles of TPN^+, 0.5 unit of glucose 6-phosphate dehydrogenase, and 250 μmoles of potassium phosphate buffer, pH 7.0, in a volume of 7.0 ml.

B. [^{14}C]ACETYL-CoA → HMG + MEVALONIC ACID. As in the previous system, incorporation into mevalonic acid required the presence of microsomes; substitution by soluble fraction or a microsomal EDTA extract produced incorporation into HMG only. ATP was not required. Addition of 10^{-3} M dithiothreitol resulted in fourfold increased incorporation; 10^{-2} M was inhibitory. In the absence of TPN$^+$, very little incorporation was observed; optimal incorporation was at 10^{-3} M TPN$^+$ (Table III). Linear increases in incorporation into mevalonic acid proportional to microsomal content or to duration of incubation indicated that enzyme content, rather than substrate or cofactors, was rate limiting.

Presence or absence of a TPNH generating system had no effect on incorporation into HMG in systems containing soluble fraction only (Table IIIA). However, if microsomes were present so that reduction of HMG-CoA to mevalonic acid occurred (Table IIIB, C, D) addition of a TPNH generating system led to inconsistent changes in HMG and increased incorporation into mevalonic acid. In Table IIIC there was no change, while in B and D, addition of a TPNH generating system led to decreased incorporation of substrate into HMG.

Optimal conditions for this assay were as follows: microsomal fraction from 800 mg of tissue was incubated for 1 hr with 1 μmole of [^{14}C]Ac-CoA, 7 μmoles of dithiothreitol, 10 μmoles of EDTA, 50 μmoles of mevalonic acid, 20 μmoles of glucose 6-phosphate, 10 μmoles of TPN$^+$, 0.5 unit of glucose 6-phosphate dehydrogenase, and 300 μmoles of potassium phosphate buffer, pH 7.0, in a volume of 7 ml.

C. [^{14}C]HMG-CoA → MEVALONIC ACID. As with the previous systems used to assay HMG-CoA reductase, the microsomal fraction was necessary. Dithiothreitol was required in high concentration for protection of reductase activity, and was optimal at 10^{-2} M. ATP was not required. Addition of Mg^{2+} or omission of EDTA led to decreased incorporation. Microsomes could be stored at −70° for 48 hr with no loss of activity.

The reaction was dependent on TPNH or a TPNH generating system (Table III). DPNH would not substitute for this requirement. Unlike the partial requirement demonstrated with [^{14}C]Ac as substrate, probably related to the presence of cofactors in the soluble fraction, no reduction of HMG-CoA occurred without addition of TPNH to the microsomal system. A linear increase in incorporation of HMG-CoA into mevalonic acid was demonstrated with increased incubation time or microsome content.

The K_m for HMG-CoA was 1.5×10^{-5} M, and substrate was routinely added at a concentration fourfold higher than K_m in order to approach V_{max} and ensure steady-state conditions with saturating concentrations of HMG-CoA. Assurance that substrate will not be rate limiting is important in order to determine maximum rates at which available pathways can be traversed under the experimental conditions with a continuous supply of substrate (Landau *et al.*, 1963).

Optimal conditions for this assay were as follows: microsomal fraction from 800 mg of tissue was incubated for 1 hr with 400 mμmoles of [^{14}C]HMG-CoA, 70 μmoles of dithiothreitol, 10 μmoles of EDTA, 50 μmoles of mevalonic acid, 20 μmoles of glucose 6-phosphate, 10 μmoles of TPN$^+$, 0.5 unit of glucose 6-phosphate dehydrogenase, and 300 μmoles of potassium phosphate buffer, pH 7.0, in a volume of 7.0 ml.

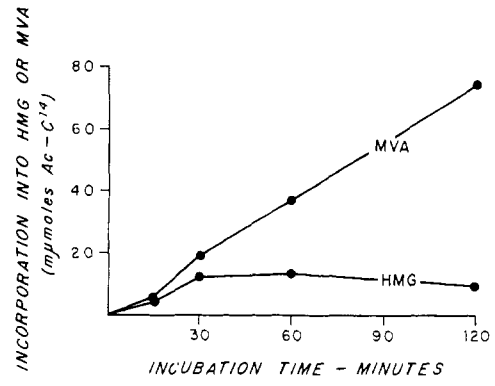

FIGURE 1: Time course of incorporation of [^{14}C]acetate into HMG and mevalonic acid. Incubations were performed for periods of 15, 30, 60, and 120 min. Contents of incubation flasks are outlined in text. Each point represents the mμmoles of [^{14}C]Ac incorporated into HMG or mevalonic acid.

Determination of HMG and Mevalonic Acid Synthesis. In all of the methods utilized here, both incorporation and synthesis must be considered. Incorporation of ^{14}C-labeled substrate into HMG or mevalonic acid may not reflect the rate of synthesis if unlabeled endogenous substrate dilutes the radioactivity of labeled substrate or intermediate pools. This is true in these relatively crude systems because of the possibility of Ac-CoA formation from oxidation of fatty acids. Thus, decreased incorporation of [^{14}C]Ac into HMG may result from a decreased rate of synthesis, or from increased dilution of ^{14}C of [^{14}C]Ac-CoA as a result of unlabeled endogenous substrates entering the Ac-CoA pool. It is conceivable that relatively large pools of Ac-CoA, AcAc-CoA, and HMG-CoA may be formed from both labeled [^{14}C]Ac and unlabeled sources; consequently, rates of synthesis cannot be determined in the absence of knowledge of the specific activity of these intermediate pools.

We have used the specific activity of the intermediate AcAc as an indicator, since its specific activity will be determined by that of Ac-CoA and AcAc-CoA, and therefore will bear a known relationship to the specific activity of the products HMG and mevalonic acid. The formazan derivative of [^{14}C]-AcAc can also be isolated and counted, permitting specific activity determination. We found that three recrystallizations were essential to obtain constant specific activity.

The model used is depicted in Figure 2. It is assumed that the only sources of AcAc formation are deacylation of AcAc-CoA or cleavage of HMG-CoA to form Ac-CoA and AcAc (HMG-CoA-AcAc lyase, E.C. 4.1.3.4.), and that nonlabeled substrates enter intermediate pools prior to HMG-CoA formation. There is a possibility that leucine may contribute directly to the HMG-CoA pool in this system. Since studies of other workers indicate that leucine is almost completely converted into ketone bodies, the specific activity of AcAc will also reflect the contribution of leucine to the HMG-CoA pool. It is also assumed that the endogenous AcAc-CoA and HMG-CoA pools at the beginning of the incubation are relative to the amount formed during the incubation; this is indicated by the fact that AcAc specific activity during incubation with [^{14}C]Ac reached a plateau within 30 min and then remained constant.

Under these conditions, specific activity of AcAc will equal

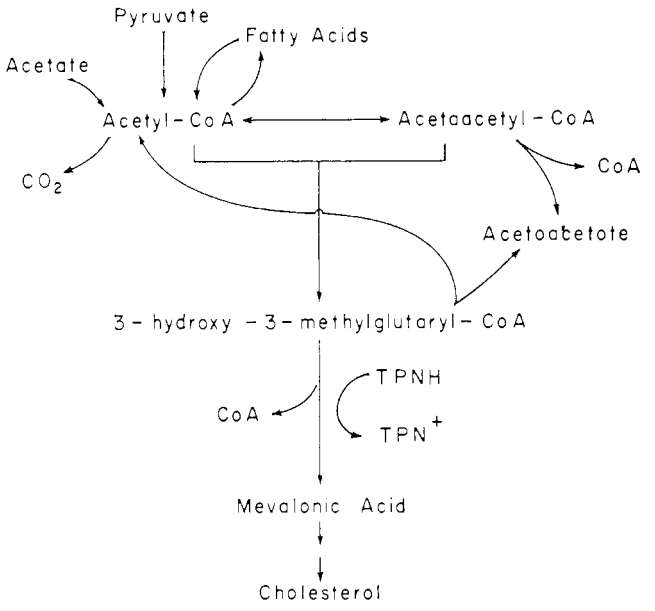

FIGURE 2: Abbreviated scheme of reactions involved in synthesis of 3-hydroxy-3-methylglutaryl-CoA and mevalonic acid.

that of AcAc–CoA; the specific activity of HMG–CoA or other derivatives of HMG, and of mevalonic acid, will be 1.5 that of AcAc–CoA. According to the model, altered incorporation of substrate into HMG or mevalonic acid in the absence of a change in AcAc specific activity indicates an altered rate of HMG or mevalonic acid formation. Knowing the specific activity of acetoacetate and the total incorporation of [^{14}C]Ac into HGM or mevalonic acid it is possible to calculate the amounts of HMG and mevalonic acid synthesized. Thus, the rate of HMG synthesis may be calculated and compared with the rate of [^{14}C]Ac incorporation into HMG using four experimentally determined values: (1) total incorporation of ^{14}C into HMG (cpm); (2) total content of AcAc formed (mμmoles); (3) total incorporation of ^{14}C into AcAc (cpm); (4) specific activity of initial [^{14}C]Ac (cpm/mμmole).[3]

AcAc specific activity remained constant over a wide range of values for AcAc formation (Table IV) suggesting that the specific activity of this substance did reflect that of intermediate precursors. In the experiment of Table IV, AcAc specific activity was 16% of the theoretical maximum, *i.e.*, of the value expected if [^{14}C]Ac were the only precursor of AcAc. This indicates considerable formation from unlabeled sources

[3] (A) [^{14}C]Ac incorporated into HMG (mμmoles)

$$= \frac{\text{total incorporation of } ^{14}\text{C into HMG (cpm)}}{\text{sp act. of added [}^{14}\text{C]Ac (cpm/m}\mu\text{mole)}}$$

(B) Specific activity of [^{14}C]AcAc (cpm/mμmole)

$$= \frac{\text{total incorporation into AcAc (cpm)}}{\text{total content of AcAc (m}\mu\text{moles)}}$$

(C) Specific activity of HMG = 1.5 specific activity of AcAc
(D) HMG synthesis (mμmoles)

$$= \frac{\text{total incorporation of } ^{14}\text{C into HMG (cpm)}}{\text{specific activity of HMG (cpm/m}\mu\text{mole)}}$$

TABLE IV: Acetoacetate Formation and Incorporation from [2-^{14}C]Acetate.[a]

EDTA 10 μmoles	$MgCl_2$ 30 μmoles	mμmoles of Acetoacetate Formed	Specific Activity (cpm/mμmole) of Acetoacetate
+	+	318	262
0	0	634	230
0	+	891	245

[a] The soluble fraction was used. Initial [2-^{14}C]Ac specific activity was 760 cpm/mμmole. Conditions as described in text.

and points to the importance of considering the dilution factor in any determination of synthesis.

Protein Binding of HMG. The possibility that HMG, when synthesized, is protein bound by analogy to intermediates of fatty acid synthesis (Majerus *et al.*, 1964) was considered.

A. Following a 2-hr incubation with [1-^{14}C]Ac in a system competent to form HMG, two aliquots of medium were removed. One aliquot was centrifuged at 150,000*g* for 3.5 hr at 3°, and the supernatant was decanted. Unlabeled HMG was added as carrier to the supernatant as well as to the other aliquot that had not been centrifuged. There was no difference in ^{14}C incorporation into [^{14}C]HMG in the two aliquots, indicating that no [^{14}C]HMG was bound to the protein that had been centrifuged.

B. Following a 2-hr incubation with a similar system, the protein was precipitated with HCl (final concentration 1 N HCl) and separated from supernatant by centrifugation. The precipitate was exhaustively washed with acetone–ether–1 N HCl (20:5:1) until the wash contained less than 20 cpm. Unlabeled HMG was added to the remaining precipitate, to the original supernatant, and to the final wash, and HMG purification was performed. No ^{14}C was incorporated into HMG in the final wash, indicating that the washed protein precipitate was not contaminated by [^{14}C]HMG in adherent supernatant. Incorporation into HGM of protein precipitate (*i.e.*, protein-bound HMG) was 4.4% and 5.0% of incorporation into soluble HMG in two experiments.

C. Incubation of a similar system as in A was performed with tissue inside a dialysis sac immersed in buffer. Aliquots of medium outside the sac were taken at intervals, and [^{14}C]-HGM was found to be released in a linear relationship with time for 2 hr. The material inside the sac was then exhaustively dialyzed for 24 hr at 3°. Considerable ^{14}C remained bound to protein (*i.e.*, ^{14}C concentration inside the sac greatly exceeded the concentration in outer medium). When unlabeled HMG was added to the sac contents, and HMG purified, no ^{14}C was incorporated into HMG. This did not rule out the possibility that HMG may have been gradually released during the prolonged dialysis.

HMG–CoA Formation. To determine if [^{14}C]HMG in the incubation medium was formed as free HMG or as the CoA ester, advantage was taken of the fact that HMG–CoA is adsorbed by charcoal or Celite. A series of experiments were performed.

A. Following a 2-hr incubation with [1-^{14}C]Ac in a system competent to form both HMG and mevalonic acid, the protein was precipitated with trichloracetic acid and removed by centrifugation, and the supernatant was added to acid-treated charcoal (pH 4–5) kept in ice. This was thoroughly mixed for 15 min, the charcoal was centrifuged, and the supernatant removed. The charcoal precipitate was washed twice with 5 ml of 0.02 N HCl, the washings were combined with the original supernatant, and unlabeled HMG as carrier was added. Two additional washings of the charcoal precipitate with water were performed, and unlabeled HMG was added to the precipitate. HMG–CoA was eluted from the charcoal and hydrolyzed by KOH addition. In two experiments, 10% and 7.5% of the total [^{14}C]HMG was found in the fraction that had adhered to charcoal, with the rest in the supernatant and washes. These are minimum values for HMG–CoA, since some deacylation may occur during mixing and washing.

A similar experiment was performed in which free HMG and HMG–CoA were separated by Celite (as described below). Free HMG had 91% of the ^{14}C of the total HMG, indicating that 9% of the HMG was present as the CoA ester. Again, this must be considered a minimum value since some HMG–CoA may undergo spontaneous deacylation on Celite.

B. Following a 30-min incubation using soluble 105,000 *g* supernatant fraction only, the incubation medium was divided into two aliquots. One aliquot was acidified and carrier was added followed by Celite chromatography. Free HMG was eluted with ether. The other aliquot was hydrolyzed by KOH for 1 hr, followed by identical treatment with Celite, and represented total HMG. Under these conditions, in which reduction to mevalonic acid was not possible, Celite retained 89% of the [^{14}C]HMG indicating that a minimum of 89% of the HMG was present as the CoA ester. With a shorter duration of incubation, less HMG–CoA undergoes deacylation; with omission of microsomes, the pathway involving reduction to mevalonic acid is not available.

Discussion

Numerous studies have suggested that regulation of cholesterol synthesis occurs at a site on the biosynthetic pathway prior to mevalonic acid formation. The step involving TPNH-dependent reduction of HMG–CoA to form mevalonic acid has been generally accepted as the probable regulatory site, and it has been suggested that a variety of diverse stimuli that influence cholesterol synthesis may all act at this single point.

While localization at the HMG–CoA reductase step has been based on several experimental findings, all the methods used in previous studies have been dependent on changes in the rate of incorporation of labeled substrate into products. While this has been referred to as synthesis, in fact it is only a measure of incorporation. This use of incorporation studies for comparison of synthetic rates is invalid, since altered pool sizes with dilution of labeled intermediates by unlabeled endogenous precursors can influence incorporation independent of any changes in synthesis. Changes in incorporation into products will reflect altered synthesis only if specific activity of precursors remains unchanged.

This problem is exemplified by the demonstration that certain factors influence incorporation of [^{14}C]Ac into cholesterol, but have no effect on incorporation of [^{14}C]mevalonic acid into cholesterol (Bucher *et al.*, 1959; Gould and Popjak, 1957). This has been interpreted as indicating a site of action between Ac and mevalonic acid. The further finding that incorporation into fatty acids is not inhibited by cholesterol feeding (Tomkins *et al.*, 1953) places the control step beyond Ac–CoA, a common precursor for cholesterol and fatty acids. These studies give only a general localization of the site of action. In addition, they are complicated by the fact that an alternative explanation for the data exists, *i.e.*, increased entry of unlabeled substrate at a pre-mevalonic acid site. An altered rate of fatty acid oxidation or glycolysis would lead to changes in pool size with a corresponding change in specific activity of Ac–CoA. On the other hand with exogenous [^{14}C]mevalonic acid as the radioactive precursor and a relatively small proportion of Ac–CoA entering the mevalonic acid pool, changes in Ac–CoA pool size leading to corresponding changes in mevalonic acid formation would not result in large changes in specific activity of [^{14}C]mevalonic acid. Clearly, methods for measuring HMG and mevalonic acid synthesis are required.

Sufficiently sensitive methods are presently unavailable for chemical quantification of HMG and mevalonic acid content in tissues; furthermore, the necessity of adding a mevalonic acid pool to stop the reaction at this stage mitigates against quantitative analysis of endogenous mevalonic acid. Spectrophotometric methods are available for assaying yeast HMG–CoA condensing enzyme (Stewart and Rudney, 1966) and HMG–CoA reductase (Durr and Rudney, 1960); however, these methods are not sufficiently sensitive for mammalian systems. Furthermore, they require a high degree of purification for removal of interfering components. This would remove cofactors and inhibitors that may be important in control; relatively crude preparations are more relevant for physiological or pathological studies.

The problem of pool dilution could be overcome by removing endogenous substrates and intermediate pools from the incubation mixture. Again, this would require a high degree of purification and, in addition, it would not be possible to be certain that all potential substrates are removed.

Another approach is the addition to the system of a sufficient excess of labeled substrate, such that endogenous dilution is relatively insignificant. This is only of value in a single step reaction where no intermediates can be diluted, and this approach has been used in some of the systems described. However, an unknown amount of dilution may still occur, and addition of high concentrations of CoA esters may lead to inhibition of the reactions as demonstrated for yeast systems (Stewart and Rudney, 1966).

The indirect approach described here in which an intermediate, AcAc, that can be measured both chemically and isotopically bears some known relationship to the products, HMG and mevalonic acid, permits calculation of specific activity and therefore synthesis of the products.

Changes in AcAc formation have also been offered as evidence that the regulatory site in cholesterol synthesis is the step catalyzed by HMG–CoA reductase. Many workers have noted that under conditions of altered cholesterol or mevalonic acid synthesis, AcAc formation was either unaltered (Siperstein and Guest, 1960) or changed in the opposite direction (Bucher *et al.*, 1960; Siperstein and Fagan, 1966). These workers assumed that HMG–CoA was a precursor of AcAc and β-hydroxybutyrate *via* HMG–CoA cleavage. They concluded that HMG–CoA production was not influenced and

therefore the control point must be beyond HMG–CoA (Bucher *et al.*, 1960). However, it is clear that there are at least two enzymatic mechanisms concerned with the production of AcAc from AcAc–CoA. The above assumption is valid only if the two-stage sequence known as the "HMG shunt" (Lynen, 1958) is the sole pathway for AcAc generation under physiological conditions. Evidence has been accumulating that direct deacylation of AcAc–CoA is also a significant mechanism for AcAc generation (Burch and Triantafillou, 1968; Segal and Menon, 1961). Another consideration is that studies of AcAc formation have been performed with mitochondrial preparations that may bear little relationship to the situation in the intact cell where a variety of changes in other cell fractions may influence AcAc generation. Nonetheless, if deacylation of AcAc–CoA were the major (or only) pathway in the intact cell for AcAc generation, then HMG–CoA condensing enzyme could represent a control site for cholesterol synthesis. This reaction is irreversible and is uniquely situated, influencing the concentration of Ac–CoA available for oxidation or fatty acid synthesis, of AcAc–CoA for ketone synthesis, and of HMG–CoA for sterol synthesis. Consequently, on a theoretical basis, one could make a case for control at this point.

Demonstration of altered incorporation of [^{14}C]Ac into mevalonic acid has added further evidence for a premevalonate control point (Siperstein and Fagan, 1964, 1966) although this gives only a general localization. The additional finding that this is unaccompanied by similar changes in incorporation of [^{14}C]Ac into HMG (Siperstein and Fagan, 1966) has led to the conclusion that the regulatory site lies between HMG–CoA and mevalonic acid. This may be an invalid conclusion for two reasons. First, free HMG is probably not an intermediate in mevalonic acid synthesis, but a product of HMG–CoA deacylation. If deacylation of a relatively small fraction of biosynthesized HMG–CoA occurred, free HMG might not be proportional to the amount of HMG–CoA formed. Secondly, if the rate-limiting and control step were in fact HMG–CoA condensing enzyme, with reductase present in excess, changes in the rate of HMG–CoA generation might only be reflected in altered quantities of mevalonic acid. The time studies (Figure 1) showing that HMG does not accumulate beyond 30 min while mevalonic acid continues to be formed suggests that HMG–CoA formation limits the amount of mevalonic acid that can be formed under these particular conditions. Therefore, simply measuring incorporation of substrate into HMG when HMG and HMG–CoA are not end products does not adequately determine the rate of HMG–CoA generation. To determine if rate control is exerted prior to formation of a product, the reaction must be restricted to formation of that product; this necessitates prevention of further reactions of HMG–CoA and free HMG if one is to rule out an effect prior to HMG–CoA formation. Use of an HMG trapping pool, similar to that used for stopping the reaction at the mevalonic acid stage, is clearly inadequate, since free HMG is not an intermediate. The reaction can be stopped, however, at the HMG–CoA stage by removing microsomes, thereby removing the enzyme catalyzing the reduction of HMG–CoA, or by omitting TPNH. AcAc formation can be minimized by the addition of EDTA. Under these conditions, HMG–CoA will accumulate and saponification will yield free HMG derived from HMG, HMG–CoA, and possibly other ester forms.

By omitting HMG–CoA reductase and its required coenzyme, three systems were developed for selective study of HMG–CoA condensing enzyme, differing in the initial substrate or the tissue fraction used. Either [^{14}C]Ac or [^{14}C]Ac–CoA was used as substrate; the latter measures HMG–CoA condensing enzyme and β-ketothiolase. With [^{14}C]Ac as substrate, however, the sum of acetate activation, β-ketothiolase, and HMG–CoA condensing enzyme activity is measured. An absolute requirement for the soluble fraction, related to the need for acetate activating enzyme, is present; this is not needed with [^{14}C]Ac–CoA as substrate. To avoid pool dilution, AcAc–CoA was not used in the systems described here. Consequently, β-ketothiolase activity is also required for generating AcAc–CoA. Stewart and Rudney (1966) have shown that HMG–CoA condensing enzyme and β-ketothiolase in yeast appear to be closely associated. These workers were unable to physically separate the two enzyme activities, but could show differential inhibition by proteolytic enzymes, iodoacetamide, or heat.

It has previously been shown that incorporation of substrate into HMG is greater with EDTA microsomal extracts than with whole microsomes (Rudney, 1957). We have shown that incorporation of Ac or Ac–CoA into HMG by the soluble fraction and incorporation of HMG–CoA into mevalonic acid by microsomes were enhanced by addition of EDTA. By contrast, Mg^{2+} addition inhibited these reactions. It has been shown that 5×10^{-3} M Mg^{2+} is required for incorporation of Ac into nonsaponifiable compounds (Knauss *et al.*, 1959). Since these high concentrations inhibit the systems described here, they are probably required for reactions beyond mevalonic acid. One possible mechanism of action of Mg^{2+} and EDTA might be an effect on pool dilution; if EDTA prevented endogenous precursors from entering the Ac–CoA pool, less AcAc precursor would be available but specific activity of Ac–CoA would increase resulting in increased incorporation into HMG and mevalonic acid. However, EDTA or Mg^{2+} had no influence on AcAc specific activity (Table IV), indicating that altered incorporation reflected altered synthesis.

Other workers have shown that Mg^{2+} is required for, and EDTA inhibits, HMG–CoA cleavage enzyme activity (Bachhawat *et al.*, 1955). Under some circumstances, Mg^{2+} stimulates and EDTA inhibits AcAc–CoA deacylation (Drummond and Stern, 1960). We have found that Mg^{2+} enhances and EDTA inhibits AcAc formation from Ac or Ac–CoA. However, formation of AcAc when AcAc–CoA or HMG–CoA were added to a soluble system was not affected by Mg^{2+} or EDTA. This apparently contradictory result may be a reflection of the fact that purified cleavage enzyme from beef liver shows complex interrelationships with Mg^{2+}, EDTA, and thiols (M. J. Kornblatt and H. Rudney, unpublished studies). The effect of EDTA might also be related to nonenzymatic lipid peroxidation, known to occur rapidly with tissue homogenates and particularly with isolated microsomes (Lynn, 1965). Peroxidation occurs in the presence of Fe^{2+} in concentrations of 10^{-5} M or higher; these are present in reagents used in the present work. EDTA, a strong chelator of Fe^{2+}, will prevent peroxidation. Thus it would appear that the effect of Mg^{2+} and EDTA is complex and further work is required to sort out the various factors that could be involved.

Porter (1961) has previously demonstrated conversion of Ac to HMG–CoA in soluble pigeon liver systems. Our studies show that the conditions of incubation, including the presence

or absence of microsomal HMG-CoA reductase, determine whether free HMG or HMG-CoA is formed by HMG-CoA condensing enzyme. First, time studies show continuous formation of HMG when microsomal reductase is absent; with addition of microsomes and a TPNH generating system no further net HMG formation occurs after 30 min while mevalonic acid formation continues to increase with time (Figure 1). Secondly, studies using charcoal and Celite to adsorb HMG-CoA reveal that virtually all HMG was in the free form when microsomal reductase was present. In the absence of microsomes, however, when mevalonic acid could not be formed, most HMG accumulated as the CoA ester.

HMG-CoA can follow several pathways (Rudney, 1963), and it would appear that a relatively small HMG-CoA pool exists if other pathways are available. Deacylation gives rise to free HMG which does not undergo reduction to mevalonic acid and whose only known fate is reactivation to HMG-CoA (Burch *et al.*, 1964). With microsomal reductase present, HMG-CoA is removed by reduction to mevalonic acid. Cleavage of HMG-CoA generates Ac-CoA and AcAc. However, with a sufficiently short incubation period, and in the absence of microsomes, a pool of HMG-CoA accumulates.

The important reaction with respect to cholesterol synthesis is reduction to mevalonic acid, catalyzed by HMG-CoA reductase. By measuring the products HMG, AcAc, and mevalonic acid, some indication of the relative importance of the various pathways can be obtained. To clearly show that HMG-CoA reductase is the key regulatory step as claimed by other workers, it is necessary to directly demonstrate formation of mevalonic acid from HMG-CoA. This has been shown in yeast extracts (Durr and Rudney, 1960), but in mammalian systems most studies have been indirect, as outlined earlier, and it has been suggested that HMG-CoA may not be an intermediate. Brodie *et al.* (1963) have demonstrated an alternate pathway for mevalonic acid formation in pigeon liver involving malonyl-CoA as substrate and related to the pathway for fatty acid synthesis. In their scheme, HMG-CoA is not an intermediate, but intermediates including AcAc and HMG are enzyme bound. Siperstein and Fagan (1966) suggested that a similar pathway exists in rat liver based on a failure to obtain incorporation of [^{14}C]HMG or [^{14}C]HMG-CoA into mevalonic acid. By contrast, our studies support the role of HMG-CoA as an intermediate. As outlined above, incorporation of [^{14}C]Ac into HMG-CoA in the absence of reductase, and the failure of HMG-CoA to accumulate in the presence of reductase, suggest that HMG-CoA is the substrate for mevalonic acid formation. Unlike Siperstein and Fagan (1966), we have also obtained direct incorporation of [^{14}C]HMG-CoA into mevalonic acid. Recently, Linn (1967b) has also demonstrated the presence of HMG-CoA reductase in rat liver microsomes and has shown that free [^{14}C]HMG will not serve as substrate.

At least three other lines of evidence indicate that the pathway utilizing malonyl-CoA suggested by Brodie does not play a significant role in mammalian systems. First, Fimognari (1964) has shown that Ac-CoA is a more efficient precursor of mevalonic acid than malonyl-CoA. Furthermore, she has shown that a biotin-dependent reaction is not required for mevalonic acid synthesis. Neither avidin nor biotin influences incorporation of Ac into mevalonic acid, and the reaction does not depend on the presence of CO_2. This is in agreement with previous studies by Fletcher and Myant (1960) and Bloomfield and Bloch (1960).

Secondly, fat feeding blocks the synthesis of fatty acids at a site prior to malonyl CoA formation (Bortz *et al.*, 1963) but leads to increased cholesterol synthesis (Bortz, 1967). Finally, our studies show that very little of the HMG formed is bound to protein. However, a small pool of HMG-enzyme may exist, with a relatively rapid turnover *via* transacylation to HMG-CoA or conversion into free HMG. Similarly, with respect to the reductase, we have not ruled out the possibility that HMG-CoA may be converted into HMG-protein or other intermediate which in turn serves as the actual precursor of mevalonic acid.

Involvement of acyl carier protein in this pathway has also been suggested (Majerus *et al.*, 1964). Its role has been demonstrated in mammalian fatty acid synthesis by Vagelos *et al.* (1965), and Rudney *et al.* (1966) have shown that AcAc-ACP can act as a substrate for yeast HMG-CoA condensing enzyme. However, its significance in mammalian sterol synthesis has not yet been determined. The relatively small amount of HMG that is protein bound in our studies indicates that relatively little could be bound to ACP. In a single experiment, using conditions optimal for reduction of HMG-CoA to mevalonic acid, no reduction of HMG-ACP occurred. At the completion of incubation, all ^{14}C remained as HMG or its esters; 68% precipitated with trichloroacetic acid, while the rest was free HMG. While this fails to support a role of ACP in mammalian sterol synthesis, the ACP used was isolated from *Escherichia coli*, and species differences may account for this failure.

We have defined the requirements and developed a direct and quantitative assay for HMG-CoA reductase. Other direct estimates of HMG-CoA reductase include those of Bucher *et al.* (1960) and Regen *et al.* (1966), showing that factors influencing incorportaion of Ac into cholesterol also change HMG-CoA reductase activity in the same direction. In Bucher's studies, a purified preparation of "mevalonic dehydrogenase" was added, presumably from yeast (Bucher *et al.*, 1960). In the studies of Regen *et al.* (1966), 25% of ^{14}C substrate was in material other than HMG-CoA. Furthermore, substrate was added at K_m concentration; considering competing reactions, including the considerable activity of cleavage enzyme in these preparations, it is possible that all substrate may have been utilized during the 1-hr period of incubation, making it impossible to compare rates of reduction.

Two other systems were developed for indirect assay of HMG-CoA reductase, differing in the initial substrate used. With Ac or Ac-CoA as substrate, other enzymatic steps in addition to HMG-CoA reductase were included, permitting comparison of the single reductase step with the entire segment. Requirements for mevalonic acid formation included the presence of a TPNH generating system and of microsomes. In contrast to the work of Siperstein and Fagan (1964), virtually no mevalonic acid was synthesized by the soluble fraction. Addition of dithiothreitol increased reductase activity, but inhibited HMG-CoA condensing enzyme. This latter effect may relate to the thiol requirement for HMG-CoA cleavage, whereby HMG-CoA will be preferentially split to Ac-CoA and AcAc rather than deacylated (Bachhawat *et al.*, 1955).

In the systems described, conditions have been optimized

so that all components but enzyme protein are present in excess. Using these approaches, rates of conversion of Ac into both HMG and mevalonic acid can be measured and the relative amounts formed under various conditions determined, while complementary systems can be used to study each step individually. In this manner, one can study regulation of early steps in cholesterol synthesis and determine the relative importance of steps catalyzed by HMG-CoA condensing enzyme or HMG-CoA reductase, either of which might represent an appropriate regulatory site on theoretical grounds.

It should be emphasized that enzymatic activity as measured here represents the maximum capacity of the tissue to utilize the substrates under the conditions given. However, enzymatic activity or content is only part of a more general picture in control of cholesterol synthesis. In the intact cell, the concentrations of substrate and/or cofactors, rather than enzyme activity, may influence the rate of these reactions. Therefore, physiological effects may be the consequence of altered concentrations of Ac-CoA secondary to altered rates of glycolysis, free fatty acid availability, or oxidation; of altered rates of TPNH generation or ATP hydrolysis; or of competitive changes in utilization of Ac-CoA for other pathways.

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