β-HYDROXY-β-METHYLGLUTARYL COENZYME A REDUCTASE, CLEAVAGE AND CONDENSING ENZYMES IN RELATION TO CHOLESTEROL FORMATION IN RAT LIVER*

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SUMMARY

Investigation of the intracellular localization of the principal enzymes involved in the formation and breakdown of β-hydroxy-β-methyl-glutaryl CoA (HMG CoA) in rat liver has shown that HMG CoA condensing and cleavage enzymes are both preponderantly in the mitochondria. HMG CoA reductase, which leads to the pathway of cholesterol synthesis, is in the microsomes, and is only one twentieth as active as the cleavage enzyme which leads to acetoacetate production. In spite of this unfavorable ratio, cholesterol synthesis does occur—possibly because a small amount of condensing enzyme and most or all of the reductase are in the microsomes which are low in cleavage activity.

Stimulation of cholesterol synthesis by injection of Triton, or inhibition by fasting does not importantly alter the amount or distribution of the condensing and cleavage enzymes. However, a severe depression of reductase activity in fasting suggests that this may be a significant factor in the rate limitation of cholesterol synthesis in this condition.

INTRODUCTION

When cell-free preparations of rat liver were studied under conditions in which cholesterol formation from acetate was experimentally either stimulated or suppressed, the results pointed to the existence of a major rate-controlling step at an early stage in the biosynthetic sequence. The evidence implied that the sensitive step lay in the reaction chain somewhere between acetyl CoA and mevalonic acid, and that it was

Abbreviations used: CoA, coenzyme A; DPN and TPN, di- and triphosphopyridine nucleotides respectively; DPNH and TPNH, their reduced forms; glucose-6-P, glucose-6-phosphate; HMG CoA, β-hydroxy-β-methyl glutaryl coenzyme A; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

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mediated by an enzyme which was bound to the microsomes and dependent upon reduced pyridine nucleotide (probably TPNH)[1-4].

The pathways for the formation of cholesterol and acetoacetic acid from acetyl CoA are outlined in Fig. 1: they are identical up to the level of HMG CoA, beyond which they diverge. In the studies mentioned in the paragraph above, one method employed to suppress the conversion of acetate to cholesterol was to deprive animals of food for 24 h. Since acetoacetate formation is increased in livers of fasting animals[5,6], it can be inferred that the production of its immediate precursor, HMG CoA (see ref. 7), is not impaired in this condition. HMG CoA is also a precursor of mevalonic acid which is on the pathway leading to cholesterol. Thus the implication is that the reduction in cholesterol formation exhibited by livers of fasting animals derives not from lack of production of HMG CoA, but rather from interference with its further progress towards mevalonic acid (Fig. 1). The present investigation provides evidence in support of a lowering of HMG CoA reductase activity in such animals.

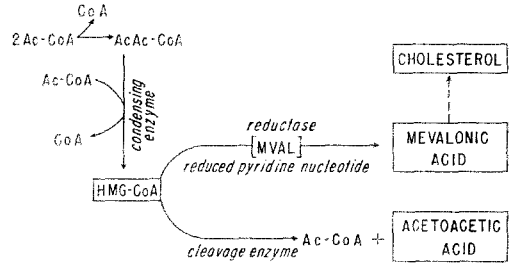

Fig. 1. Pathways for the formation of mevalonic acid and acetoacetic acid from acetyl-CoA. The following abbreviations are used: Ac-CoA, acetyl-CoA; AcAc-CoA, acetoacetyl-CoA; HMG CoA, β-hydroxy-β-methylglutaryl-CoA; MVAL, mevaldic acid.

EXPERIMENTAL

Materials and methods

Materials: We are indebted to Dr. J. KNAPPE for biosynthetic preparation of [1,3,5-^{14}C]HMG CoA as well as for mevalonic dehydrogenase and mevalonic kinase. [1-^{14}C]acetate was obtained from Kernreaktor Bau- und Betriebsgesellschaft m.B.H, Karlsruhe, and DPN, TPN, glucose-6-P and glucose-6-P dehydrogenase all from C. F. Boehringer, Söhne, Mannheim-Waldhof.

Animals: Young adult male Sprague-Dawley rats were allowed stock diet and water *ad lib.* Animals treated with Triton WR1339 received 1 ml of 10 % aqueous solution per 100 g of body weight intravenously 24 h before removal of the liver. Fasting animals comprised those deprived of food for 24 h.

Preparation of liver: Rats were stunned by a blow on the head and decapitated. Livers were excised, chilled thoroughly in crushed ice, rinsed in medium and put through an ice-cold tissue press. The liver pulp was homogenized as previously described[8]. For determination of HMG CoA condensing and cleavage enzymes, the medium consisted of 0.25 *M* sucrose. For other determinations it was composed of 0.1 *M* potassium phosphate buffer pH 7.4, 0.004 *M* magnesium chloride, and 0.03 *M* nicotinamide.

The supernatant fluid obtained from centrifugation of whole homogenates for 10 min at 5,000 × *g* was employed for routine incubations. It consisted principally of microsomes and soluble cell components, and will be designated S-5000. In more detailed cell fractionation studies a crude "nuclear" fraction (containing in addition to nuclei, red blood cells, unbroken liver cells and cell debris) was sedimented by centrifugation for 10 min at 600–700 × *g* and a mitochondrial fraction at 9000 × *g*. These procedures were carried out in a cold room at 4° in a Servall centrifuge, type SS1. A microsomal fraction was obtained after 1 h at 105,000 × *g* in a Spinco preparative ultracentrifuge. Each fraction was washed once by resuspension and recentrifugation. The supernatant fluid from the final centrifugation was considered to comprise the soluble cell components.

Cholesterol biosynthesis: S-5000 preparations were incubated for 2 h aerobically with [1-^{14}C]acetate, DPN, TPN and glucose-6-P. After alkaline hydrolysis cholesterol was extracted with petroleum ether, precipitated with digitonin, and its radioactivity determined in an end window counter with an efficiency of approx. 8 % (see ref. 2).

Acetoacetate formation: S-5000 preparations were incubated with labeled acetate as above. After addition of acetoacetate as carrier they were deproteinized with trichloroacetic acid and strongly acidified with HCl. The [^{14}C]acetone was collected by steam distillation in a solution of 2,4-dinitrophenylhydrazine, and the resulting hydrazone extracted with carbon tetrachloride[9]. This extract was washed thoroughly with solutions of sodium acetate and sodium bicarbonate and water, and concentrated by evaporation *in vacuo*. An aliqout was plated for determination of its radioactivity. Enzyme-free control preparations, serving as blanks, showed that no [1-^{14}C]acetate contaminated the final product.

HMG CoA Condensing and cleavage enzymes: Livers were homogenized and fractioned in 0.25 *M* sucrose. The fractions were then rehomogenized for 1 min in 0.05 *M* potassium phosphate buffer at pH 7.5 in a high speed (approx. 35,000 rev./min) blendor-type homogenizer (Edmund Bühler, Co., Tübingen), while cooled in a saline bath at —6 to —10°. In the case of the microsomes the potassium salt of EDTA was added in a final concentration of 10^{-3} *M* to promote further lysis of the particles. These procedures were found necessary for activation of particle-bound portions of the enzymes (see below). The assays were carried out by the methods previously described[7]. Preparations were incubated with Tris buffer, sodium sulfide, coenzyme A, reduced glutathione, lithium acetyl phosphate and *Cl. kluyveri* extract, the latter to provide for generation of acetyl CoA and acetoacetyl CoA. For assay of condensing enzyme an excess of cleavage enzyme from beef liver was added, and for assay of cleavage enzyme an excess of condensing enzyme from baker's yeast. The beef liver extract was free from condensing enzyme activity and the yeast extract was free from cleavage enzyme activity. At the end of a 30-min incubation the amount of acetoacetate formed was determined colorimetrically[10]. Each liver fraction was run at a minimum of two concentrations; results were considered acceptable if they fell within ± 10 % of the mean. (At extremely low levels of enzyme activity the error was somewhat larger.) A unit of enzyme activity was considered to be the amount yielding 1 μmole of acetoacetate under the conditions of this assay. Specific activity was defined as units/mg of protein.

HMG CoA Reductase: Measurement of HMG CoA reductase was carried out only on washed microsomes (see below). These were incubated for 1 h with K_2HPO_4, re-

duced glutathione, potassium salt of EDTA, glucose-6-P, glucose-6-P dehydrogenase, TPN, DPN, serum albumin, mevalonic dehydrogenase, and [1,3,5-^{14}C]HMG CoA. The pH was adjusted to approx. 7 with 2 *N* KOH. After incubation the reaction was stopped by heating for 2.5 min in a boiling water bath. Carrier mevalonic acid was added, and an excess of Dowex 50 (H form), and the mixture was then extracted overnight in a continuous extractor with peroxide-free ether. After addition of an excess of KOH solution the ether was distilled off *in vacuo*. The residue, containing any [^{14}C]mevalonic acid that had formed, was neutralized and reincubated with magnesium salt of EDTA, potassium bicarbonate, potassium fluoride, serum albumin, ATP and mevalonic kinase. After 2 h incubation, Dowex 50 (H form) was added, and the mixture again extracted continuously with ether overnight. The phosphomevalonic acid formed from [^{14}C]mevalonic acid remained in the aqueous phase, while residual [^{14}C]HMG was removed by the ether. Determination of the radioactivity in the aqueous phase therefore gave a measure of the amount of HMG CoA that was reduced[11]. Microsome-free blanks were run with each experiment.

Mevaldic acid disappearance: Mevaldic acid disappearance was measured as an index of mevalonic acid dehydrogenase activity. S-5000 preparations were incubated for 30 min with potassium phosphate buffer at pH 7.0, potassium fluoride, glucose-6-P, glucose-6-P dehydrogenase, TPN and mevaldic acid. The mixture was deproteinized with trichloroacetic acid, and the residual mevaldic acid determined colorimetrically by reaction with 2,4-dinitrophenylhydrazine[12].

Protein: Protein was determined by the biuret reaction[13].

RESULTS

HMG CoA condensing and cleavage enzymes: Initial experiments were carried out to determine the intracellular distribution of HMG CoA condensing and cleavage enzymes in normal liver. At first the liver fractions in 0.25 *M* sucrose were tested directly, but the resulting values were too low, since an approximately 10-fold increase in activity of both enzymes occurred in particulate prepatations that were stored at —15° (Table IA). However, the activity of particle-free supernatant fluid obtained from centrifugation at 105,000 × *g* did not increase during storage under similar conditions.

In order to activate the bound enzymes, various methods were investigated for breaking up the particles. Preparation of an acetone powder followed by extraction with phosphate buffer yielded lower values than homogenization alone. Repeated freezing and thawing resulted only in doubling the activity of condensing enzyme, compared to the 10-fold increase obtained in the same preparation on storage at —15°. High frequency oscillation with ballotini produced only two-thirds as much activation as the final method. Addition of surface-active agents was also unsuccessful: Triton WR-1339 in a final concentration of 1 % had no effect, and cholate at 0.1–1 % final concentration was strongly inhibitory. The most convenient and reproducible technique, and the one ultimately adopted for the standard assay procedure, was homogenization for 1 min in the Bühler apparatus, as described above. Although not ideal, the results indicated that a high degree of activation was obtainable under these conditions without unduly rapid destruction of the rather unstable condensing enzyme (Table IB). Confidence in this technique is engendered by the data in Tables II and III,

which show that the sum of the activities of the various cellular fractions, in units/g of liver, were in reasonable accord with the level in the unfractionated homogenate.

TABLE I

ACTIVATION OF HMG CoA CONDENSING AND CLEAVAGE ENZYMES

Data are expressed as enzyme specific activities. For determination of condensing enzyme 20 μmoles of lithium acetyl phosphate, 70 μmoles of Tris buffer pH 7.9, 7 μmoles of Na_2S, 0.3 μmole of CoASH, 3 μmoles of reduced glutathione, 4 μmoles of $MgCl_2$, 7 μmoles of dipotassium-magnesium salt of EDTA, 0.01 ml of *Cl. kluyveri* extract, plus approximately 1 unit of purified HMG CoA cleavage enzyme from beef liver (specific activity 12 units/mg protein) were incubated for 30 min at 37° with aliquots of liver preparations in a final volume of 0.7 ml. For cleavage enzyme the conditions were the same, except that approximately 1 unit of condensing enzyme from a toluene autolysate of baker's yeast (specific activity 0.6 units/mg), or enriched preparations thereoff, was substituted for the beef liver preparation.

	1000 × g supernatant from homogenate in 0.25 M sucrose	
	Fresh	*5 days at —15°*
Condensing enzyme	0.006	0.078
Cleavage enzyme	0.063	0.53

	Bühler homogenate (uncentrifuged) in 0.05 M phosphate buffer			
	1 min	*2 min*	*3 min*	*4 min*
Condensing enzyme	0.067	0.061	0.051	0.055
Cleavage enzyme	0.94	0.97	0.89	0.95

It is evident from the values obtained in whole homogenates of normal livers that there was considerable variation among animals with respect to both cleavage and condensing enzymes (Tables II and III). However, the cleavage enzyme was of the order of 10 times more active than the condensing enzyme.

The intracellular distribution of the two enzymes was very similar: nearly one-third of the activity appeared in the crude "nuclear" fraction, two-thirds in the mitochondrial and a small amount in the soluble fraction. The levels in the microsomes, especially in the case of the more labile condensing enzyme, were too low to be determined reliably by this method of assay.

When animals were subjected to fasting, which strongly suppresses cholesterol formation, or were injected with Triton WR-1339, which greatly enhances it, the levels of condensing and cleavage enzymes in the small series of animals studied did not appear to rise importantly above the normal range. In any case it is clear that the slight changes that did occur, whether real or attributable to statistical variation, were in no way parallel to, or commensurate with, the enormous differences in conversion of acetate to cholesterol exhibited by S-5000 preparations from similar animals (Table IV).

The distribution of condensing and cleavage enzymes among the cellular fractions in the treated animals also remained closely similar to the normal pattern (Tables II and III).

HMG CoA reductase: Relative to cleavage enzyme, the activity of cholesterol-forming enzymes was very low. For example, when S-5000 from normal liver was incubated with [1-^{14}C]acetate under conditions favorable for cholesterol synthesis,

TABLE II

DISTRIBUTION OF HMG CoA CONDENSING ENZYME IN CELLULAR FRACTIONS

Enzyme activities are expressed as units/g of fresh liver. Specific activities are expressed as units/mg of protein. Values in parentheses are percentages of activity attributable to each fraction if the sum of the activities is taken as 100%. Procedures as in Table I.

	Homogenate	*Crude "nuclei"*	*Mitochondria*	*Microsomes*	*Supernatant*
		Normal			
	9.4				
	11.0				
	26.7				
	24.2				
		4.6	9.6		
		5.7	13.1		
		3.6	10.4		
	9.7	4.4	8.6	—	0.4
	12.3	3.6	7.9	—	0.4
	11.7	2.8	8.4	—	0.4
Average units/g	15.0	4.1 (29%)	9.7 (68%)	—	0.4 (3%)
Average specific activity	0.073	0.068	0.182	< 0.003	0.007
		Triton-treated			
	21.8				
		7.3	16.1		
	37.1	7.4	23.4	0.2	5.8
Average units/g	29.5	7.4 (22%)	19.8 (60%)	0.1 (0.3%)	5.8 (18%)
Average specific activity	0.138	0.125	0.325	0.006	0.08
		Fasting			
	18.9				
		6.9	14.5		
		9.1	23.5		
	26.3	11.9	29.1	0.08	4.5
Average units/g	22.6	9.3 (26%)	22.4 (62%)	0.08 (0.2%)	4.5 (12%)
Average specific activity	0.086	0.149	0.382	0.005	0.059

1.80 μmoles of acetate were converted to acetoacetate while only 0.086 μmoles were converted to cholesterol, a ratio favoring the cleavage pathway of 20 to 1. Because of this overwhelming tendency towards cleavage, it was not feasible to measure the HMG CoA reductase activity in S-5000 preparations, with the limited supply of labeled HMG CoA available. However, it had already been shown that the rate-controlling step in cholesterol formation is dependent upon the microsomes[2] which, as indicated above, contain very little cleavage enzyme. Accordingly, determinations of HMG CoA reductase were carried out only on washed microsomes. In an attempt to amplify the resulting differences, liver microsomes from animals stimulated by injection of Triton were compared with those from animals depressed by fasting.

TABLE III

DISTRIBUTION OF HMG CoA CLEAVAGE ENZYME IN CELLULAR FRACTIONS

See above Table II.

	Homogenate	*Crude "nuclei"*	*Mitochondria*	*Microsomes*	*Supernatant*
		Normal			
	123				
	185				
	175				
	105				
		33	100		
		30	85		
		26	83		
	107	41	52	0.25	2.5
	102	36	52	0.80	4.0
	139	33	102	0.70	2.3
Average units/g	134	33 (28 %)	79 (68 %)	0.58 (0.5 %)	2.9 (3 %)
Average specific activity	0.66	0.54	1.48	0.04	0.05
		Triton-treated			
	110				
		56	110		
	195	48	117	1.2	8.0
Average units/g	153	52 (30 %)	114 (65 %)	1.2 (0.7 %)	8.0 (5 %)
Average specific activity	0.71	0.89	1.87	0.06	0.11
		Fasting			
	147				
		37	64		
		56	133		
	300	64	175	2.0	15.0
Average units/g	224	52 (27 %)	124 (64 %)	2.0 (1 %)	15.0 (8 %)
Average specific activity	0.88	0.84	2.06	0.13	0.19

TABLE IV

CONVERSION OF ACETATE TO CHOLESTEROL BY LIVER PREPARATIONS

2.0 ml of S-5000 preparations were incubated for 2 h at 37° with 20 μmoles of glucose-6-P, 2 μmoles each of TPN, and DPN and 5 μmoles of [1-^{14}C]acetate (2.5 μC) in a final volume of 2.45 ml.

Normal		*Fasting*		*Triton treated*	
Cholesterol counts/min	*(ac → ch) µmoles*	*Cholesterol counts/min*	*(ac → ch) µmoles*	*Cholesterol counts/min*	*(ac → ch) µmoles*
5,390	0.075	0*	—	18,700	0.260
6,400	0.089	5	—	19,250*	0.268
6,200	0.086	16**	—	19,600**	0.272
8,640	0.119				

* S-5000 prepared from same livers as microsomes in Expt. No. 1, Table V.
** S-5000 prepared from same liver as microsomes in Expt. No. 2, Table V.

As shown in Table V, microsomes from the Triton-treated animals were highly active in reducing HMG CoA to mevalonic acid; S-5000 preparations from the same livers were similarly active in the total synthesis of cholesterol from acetate (Table IV, footnote). In the first experiment in Table V doubling the concentration of microsomes did not increase the total conversion of HMG CoA to mevalonate. Hence even at the lower microsomal concentration the amount of HMG CoA added may not have been quite sufficient to demonstrate maximal activity. In the second experiment, however, the HMG CoA could not have been limiting since doubling its concentration did not increase mevalonate formation. The capacity of microsomes to reduce HMG CoA thus appeared to be of the order of $3-4 \cdot 10^{-3}$ μmoles/mg of microsomal protein/h in livers from Triton-treated animals.

TABLE V

REDUCTION OF HMG CoA BY MICROSOMES FROM LIVERS OF FASTING AND TRITON-TREATED RATS

For determination of HMG CoA reductase activity, washed microsomes were incubated for 1 h at 37° with 20 μmoles of K_2HPO_4, 5 μmoles each of reduced glutathione and potassium salt of EDTA, 0.3 μmole each of DPN and TPN, 8 μmoles of glucose-6-P, 0.3 mg of serum albumin, glucose-6-P dehydrogenase, mevalonic acid dehydrogenase, and [^{14}C]HMG CoA (0.01 μmole containing approximately 6000 counts/min). After adjustment of the pH to 7.0–7.2, with 2 *N* KOH the final volume was approx. 0.7 ml.

Expt. No.	*Rats*	*Microsome protein present (mg)*	*Labelled HMG CoA added (μmoles)*	*(HMG CoA → MVA) by microsomes*	
				Total activity (counts/min)	*μmoles/mg/protein/h (μmoles × 10⁻³)*
1	Triton	1.5	0.01	3480	3.9
		3.0	0.01	3430	1.9
	Fasting	1.8	0.01	0	0
		3.6	0.01	0	0
2	Triton	1.5	0.01	2850	3.2
		1.5	0.02	2860	3.2
	Fasting	1.1	0.01	40	0.06
		1.1	0.02	27	0.04

The preparations from fasting animals exhibited grossly impaired capacities to synthesize cholesterol from acetate and to reduce HMG CoA (Tables IV and V). The failure to demonstrate reductase activity could indicate either actual inhibition or loss of the enzyme itself, or an increase in HMG CoA cleavage enzyme sufficient to mask the reductase by elimination of its substrate. However, we have already shown that the activity and distribution of cleavage enzyme in terms of total capacity (*i.e.* after disruption of the particles in the Bühler homogenizer) do not differ significantly in fasting, normal and Triton-treated preparations. Further, we examined undisrupted mitochondria from similar animals to find whether fasting *per se* resulted in activation of bound cleavage enzyme. The results (Table VI) were somewhat equivocal in that the lowest as well as the highest values obtained in these intact particles were in the fasting series, but 3 out of 4 fell within the normal range. Although it cannot be entirely ruled out, the data lend little support to the idea that the cleavage enzyme in fasting animals is in a particularly active state. These findings therefore suggest that there is an actual inhibition or loss of HMG CoA reductase in the fasting animal.

The reduction of HMG CoA to mevalonic acid has been found to be mediated by

TABLE VI

CONDENSING AND CLEAVAGE ENZYME ACTIVITIES IN INTACT AND DISRUPTED MITOCHONDRIA FROM LIVERS OF NORMAL, FASTING AND TRITON-TREATED RATS

Procedures as in Table I.

Expt. No.	Normal		Triton		Fasting	
	Intact specific activity	Disrupted specific activity	Intact specific activity	Disrupted specific activity	Intact specific activity	Disrupted specific activity
			Condensing enzyme			
1	0.002	0.093	0.002	0.065	0.002	0.085
2	0.001	0.034	0.001	0.045	0.002	0.049
3	0.001		0.002		0.001	
4	0.001		0.002		0.005	
			Cleavage enzyme			
1	0.007	0.82	0.004	1.02	0.005	0.96
2	0.006	0.97	0.005	1.12	0.004	1.43
3	0.049		0.029		0.041	
4	0.049		0.029		0.077	

a single enzyme in yeast, and mevaldic acid is not an intermediate in this reaction[11,14]. Although the same condition may obtain in mammalian tissues, the possibility of a two-step reaction involving mevaldic acid has not yet been ruled out (Fig. 1). Accordingly we measured the disappearance of mevaldic acid as an index of mevalonic dehydrogenase activity. Comparison of normal, Triton-treated and fasting animals with respect to conversion of acetate to cholesterol by S-5000 preparations yielded 8,640 counts/min in the normal, 18,700 counts/min in the Triton-treated, and 5 counts/min in the fasting respectively. Incubation of 0.5-ml aliquots of these same preparations for 30 min with mevaldic acid resulted in relatively insignificant differences: 0.256 μmoles disappeared from the normal, 0.282 from the Triton-treated and 0.266 from the fasting.

DISCUSSION

Although there are other pathways for its formation[15–17], it is probable that a major portion of the hepatic HMG CoA is produced from acetyl CoA and acetoacetyl CoA by the action of the condensing enzyme. This enzyme appears to be preponderantly associated with the mitochondria; approximately two-thirds of the condensing activity appeared in this fraction and nearly an additional third occurred in the crude "nuclear" fraction which contained enough contaminating mitochondria to account for most if not all of the activity there. The small portion of condensing enzyme found in the soluble fraction was probably derived largely from particles disrupted during homogenization and fractionation. Negligible activity was demonstrated in microsomes. However, RUDNEY, using isotopic techniques which are far more sensitive than the biochemical methods that we employed, was able to detect this enzyme and isolate it from washed microsomes, showing that at least a small fraction of it is bound to these particles. It is important to note that these sensitive isotopic tests did not demonstrate the overwhelmingly high concentration of condensing enzyme present in the mitochondrial fraction, because the high activity of cleavage enzyme prevented

accumulation of HMG CoA upon which these tests depend. In the method that we employed, interference by cleavage enzyme was not a factor. It can be calculated from RUDNEY'S data[18] that in his system 0.003 μmoles of HMG were formed from acetate/mg of microsome protein/30 min. This level is at the borderline of detectability by our method of measuring condensing enzyme. We found the mitochondria to have a specific activity of 0.182 μmoles/mg, or 60 times the activity determined by RUDNEY in the microsomes.

The pattern of distribution of cleavage enzyme in the cellular fractions was closely similar to that of the condensing enzyme, indicating that it too functions mainly in the mitochondria. Possibly acetoacetate production is primarily a mitochondrial function, carried out by the condensing and cleavage enzymes that are abundant in these particles, whereas the small fraction of the condensing enzyme that is in the microsomes produces the HMG CoA which is converted to mevalonic acid by the microsomal reductase. This latter HMG CoA would be more readily available to the reductase because of the low level of cleavage enzyme in the microsomes. Such compartmentation could explain how cholesterol synthesis from acetate can occur in a tissue with a many times greater potential capacity to split HMG CoA than to synthesize it or reduce it to mevalonic acid.

The high level of HMG CoA reductase in microsomal preparations from Triton-treated and the low level from fasting rat livers is in accord with the incorporation of acetate into cholesterol in such preparations. For the Triton-treated animals we found that 0.003–0.004 μmole of HMG CoA were reduced/mg of microsomal protein/h when measured directly. For comparison, S-5000 preparations containing approx. 80 mg/of protein, approx. 30 % of which was microsomal, converted 0.26 μmole of acetate to cholesterol, which is equivalent to approx. 0.002 μmole of HMG CoA reduced/mg of microsomal protein/h. Since these two sets of values for reduction of HMG CoA are of the same order of magnitude, the assumption appears valid that most if not all of the reductase is bound to the microsomes.

It has been shown that mevaldic acid can be converted to mevalonic acid by rat liver *in vitro*[19, 20], and also that addition of unlabeled mevaldic acid to a homogenate suppresses the conversion of [^{14}C]acetate to cholesterol as effectively as unlabeled mevalonic acid[21]. Further, [^{14}C]mevaldic acid is converted to steroid by rat liver homogenates much more efficiently than acetate*. In a few experiments we measured the disappearance of mevaldic acid when incubated with S-5000 preparations and found insignificant differences between normal, fasting and Triton-treated animals. This finding suggests that if in liver, unlike yeast, the reduction of HMG CoA occurs in two stages with mevaldic acid as an intermediate, the first rather than the second reductive step is the critical one.

From the evidence presented above, the diminution in cholesterol formation from acetate that is found in livers of fasting rats appears to be associated with a decrease in HMG CoA reductase acitvity. This finding is no doubt relevant to the operation of the physiological control of hepatic cholesterol synthesis, but it should be emphasized that comparison of isolated systems with those operating in the intact animal involves the risk of minimizing variables that are readily eliminated *in vitro* and yet may be paramount *in vivo*. The partially inactive state of the condensing and cleavage enzymes in intact particles is a case in point. Its physiological significance cannot be prop-

* We are indebted to Dr. J. GRANT for this unpublished information.

erly evaluated on the basis of the maximum capacities which are measured after the particles have been disrupted. Alterations in fragility of particles in altered physiological states may interfere with accurate determinations in non-disrupted particles. This may partially explain the variability in our own experiments, as well as the findings of MIGICOVSKY that mitochondria from fasting animals contain an inhibitor of cholesterol synthesis[22]. Thus the final limitation in the whole animal may be effectively brought about either by alteration of a critical step on the direct pathway, or of some ancillary step operating to alter the availability of essential substrates or cofactors. Whether the lowering of reductase activity in fasting is coincident with or consequent to alterations in these ancillary steps requires further study.

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