THE SIGNIFICANCE OF CERTAIN CARBOXYLIC ACIDS AS INTERMEDIATES IN THE BIOSYNTHESIS OF CHOLESTEROL*

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INTRODUCTION

Acetic acid, acetoacetic acid, β-hydroxy-β-methylglutaric acid, β-hydroxy*iso*valeric acid, β-methylglutaconic acid, and β,β-dimethylacrylic acid have all been implicated as being precursors for the biosynthesis of cholesterol. Acetate has been shown to give rise to acetoacetate, hydroxymethylglutarate, hydroxy*iso*valerate, dimethylacrylate, β-methylglutaconate, squalene, and cholesterol[1-11]. Acetoacetate yields hydroxymethylglutarate and cholesterol[12-14]. Hydroxymethylglutarate has been shown to form acetate, acetoacetate, hydroxy*iso*valerate, dimethylacrylate, β-methylglutaconate, squalene, and cholesterol[1,4,6,12,15-17]. Hydroxy*iso*valerate can be converted to acetate, acetoacetate, hydroxymethylglutarate and cholesterol[15-18]. Dimethylacrylate forms acetate, hydroxymethylglutarate, hydroxy*iso*valerate, squalene, and cholesterol[15-18]. The conversion of β-methylglutaconic acid to cholesterol has also been demonstrated[17].

From the above it is apparent that not only can each of these acids yield cholesterol, but, also, since it is extremely probably that all of the reactions concerned are reversible, each acid can be derived from each of the others. Therefore, the nature of the early steps in the conversion of acetate to cholesterol remains in doubt. A discussion of some of the discrepancies has recently been published by BLOCH[19]. Possible pathways are as follows:

1) Acetate ⟶ Acetone ⟶ DMA ⟶ cholesterol[5,20]
2) Acetate ⟶ HMG ⟶ HIV ⟶ DMA ⟶ cholesterol[21]
3) Acetate ⟶ HMG ⟶ BMG ⟶ DMA ⟶ cholesterol
4) Acetate ⟶ HMG ⟶ BMG ⟶ cholesterol[6]

The column chromatographic procedure of ADAMSON AND GREENBERG[22], supplemented by paper chromatography, permits the isolation from an incubation mixture of each of the above acids except acetoacetate. By application of this procedure to the mixtures resulting from the incubation of ^{14}C-labeled acetate, hydroxymethylglutarate, or dimethylacrylate with various liver preparations, more complete information about the interconversions of these acids and their participation in cholesterol biosynthesis has been obtained than was hitherto known. The work reported here lends support

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to the pathway of equation 4 above, in which cholesterol synthesis is accomplished independently of the formation of β-hydroxy*iso*valerate or dimethylacrylate.

EXPERIMENTAL MATERIALS AND METHODS

The livers used were from Long-Evans male rats weighing approximately 100 g. The livers were removed as quickly as possible after decapitation of the animal, weighed, and dropped into cold buffer solution. Liver slices were prepared free hand by slicing in iced buffer. Liver homogenates and cell fractions were prepared as described by BUCHER[23,24].

The incubation medium used consisted of 0.1 *M* potassium phosphate buffer, pH 7.2, $MgCl_2$ (0.004 *M*), niacinamide (0.03 *M*), and glucose (0.02 *M*). When centrifugal cell fractionation was employed, the buffer medium contained, in addition, 0.125 *M* sucrose. 2.5 ml of the buffer medium per g wet weight of tissue were used throughout. Incubations were carried out in the Dubnoff shaking metabolic incubator for a 3 hour period in an atmosphere of air.

The radioactive substrates employed were as follows: 1-^{14}C-sodium acetate (specific activity, 24 μc/mg), 1-^{14}C-β-methylcrotonic acid (dimethylacrylic acid) (specific activity, 11.5 μc/mg) purchased from Tracerlab, and 3-^{14}C-β-hydroxy-β-methylglutaric acid, prepared by Dr. MORTON ROTHSTEIN of this Department.

Non-radioactive substrates and carriers included synthetic β-hydroxy-β-methylglutaric acid, β-hydroxy*iso*valeric acid, β-methylglutaconic acid, and β,β-dimethylacrylic acid, These were all prepared by Mr. DONALD MORRISON of this Department. Both the high and low melting isomers of methylglutaconic acid were prepared. It was found, however, that the low melting sample was a mixture of the two isomers and this mixture was therefore used. These non-radioactive substrates were used as diluents or trapping agents in conjunction with the radioactive substrates. They were employed in a concentration of 40 microequivalents per g of tissue, approximately 10 times the usual concentration of the radioactive substrate.

The method used for chromatography of the acidic cholesterol precursors has been described by ADAMSON AND GREENBERG[22]*, and is a modification of the method of KINNORY, TAKEDA AND GREENBERG[25]. Subsequent experience has shown that certain modifications of the published method result in more consistently satisfactory results. Substitution of NH_4OH for NaOH throughout deproteinization-desalting treatment assures complete neutralization without the accumulation of non-volatile base in the dried residues. It was found that the protein and salt can be more conveniently removed as follows: After incubation, that portion of the mixture to be used for chromatography is poured into a 12 ml graduated centrifuge tube and 5–10 microequivalents of each of the acids to be separated are added to the sample as carriers. In experiments where non-radioactive acids were added to the enzyme system before incubation, no additional carrier was added after incubation. The centrifuge tube is immersed for approximately 1 minute in boiling water to coagulate most of the protein, and this is separated by centrifugation for 5 minutes. The supernatant liquid is decanted into another centrifuge tube and the protein precipitate is washed once with 1 ml of water, again immersed in boiling water, and recentrifuged. Two volumes of acetone are then added to the combined supernatant solutions. The acetone precipitates the remainder of the protein and some of the salts. After centrifugation, the solution is poured into a 50 ml Erlenmeyer flask, made alkaline with an excess of concentrated NH_4OH, and placed in a heated vacuum desiccator for drying. To the dry residue is added one drop of 0.1 % congo red and enough 10 % HCl to give a blue reaction by the indicator (two or three drops). When the residue has dissolved, 4 ml of a mixture of acetone and ether (2:1) are added and then decanted into a centrifuge tube. The salts remaining in the flask are extracted twice more with 4 ml portions of acetone-ether and the combined extracts are centrifuged. The supernatant fluid from this centrifugation is practically salt free. NH_4OH is added in excess, and the sample is again evaporated to dryness in the vacuum desiccator. Ordinarily the dried residue from this evaporation is suitable for chromatography. Sometimes it is apparent that there is still too much salt present, in which case the final precipitation procedure is repeated. Acidification of the final sample for chromatography and its transfer to the column must be accomplished without the addition of more than 0.1 ml of water. If additional liquid is required for quantitative transfer benzene may be used.

For the work with cholesterol precursors, two solvent systems have been used as previously described[22]. In one system (designated as BE) the initial solvent is benzene and it is gradually replaced by ether as the latter is delivered into the bottom of the mixing vessel. The other solvent system (referred to as the standard system) uses benzene and, instead of ethyl ether, a mixture of

* After its submission for publication, it was found that the large titration and radioactivity peaks mistakenly identified as hydroxymethylglutarate in Fig. 2 ref. 22 actually represents β-hydroxybutyric acid. The statements therein about the high activity and recoveries of hydroxymethylglutarate are therefore erroneous.

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TABLE I

SEPARATION OF ACIDS CONCERNED IN CHOLESTEROL BIOSYNTHESIS BY TWO DIFFERENT CHROMATOGRAPHIC SYSTEMS

Eluent solvents	*Fraction number of eluted acid (100 drop fractions)*							
	DMA	*HOAC*	*BMGH*	*BMGL*	*HIV*	*SUCC*	*BHB*	*HMG*
BE system	10	58	61	67	84	130	142	178
Std. system	8	61	74	80	93	150		181

Abbreviations used in tables and figures are: DMA, β,β-dimethylacrylic acid; HOAC, acetic acid; BMGH, high-melting isomer of β-methylglutaconic acid; BMGL, low-melting isomer of β-methylglutaconic acid; HIV, β-hydroxy*iso*valeric acid; BHB, chiefly β-hydroxybutyric acid, but succinic and lactic acids, if present, are also represented in this peak; HMG, β-hydroxy-β-methylglutaric acid.

petroleum ether and ethyl ether (1:1). The latter mixture is delivered into the mixing flask during the collection of the first 5,000 drops only. It is then replaced by ethyl ether for the remainder of the run. This replacement was found to hasten the elution of the slowest appearing acid (hydroxymethylglutaric.) The location of the acid peaks observed with the two different solvent systems is shown in Table I. It can be seen that with the BE solvent system, acetic acid and the high-melting isomer of β-methylglutaconic acid are only very slightly separated. In cases where it is not necessary to detect radioactivity in the acetate peak (*i.e.* in incubations which use acetate as the radioactive substrate) this system can be used for testing the radioactivity of the two isomers of β-methylglutaconic acid if the aqueous layers are made acidic before plating and drying. Since the acetic acid is volatile and the β-methylglutaconic acid is not, any radioactivity remaining on the planchet is due to the latter acid. The BE system was used only when it was desirable to test the radioactivity of a given acid by using two different solvent systems, or when it was desirable to separate the radioactivity of succinic acid from that of β-hydroxybutyric acid. The peak labeled BHB is a complex peak containing, with the standard system, not only β-hydroxybutyric acid and succinic acid but also lactic acid. Although the succinic-lactic peak is separable from the β-hydroxybutyric acid on a BE column, the large amounts of BHB formed by liver from the substrates employed here usually obscure the succinic acid peak. Because of the multiple nature of this peak, titration and radioactivity curves seldom coincide. In no case was any carrier hydroxybutyric lactic, or succinic acid added. All titration in this area represents endogenously formed acid.

Acetoacetate, being extremely unstable in the acid form, does not survive the deproteinization-desalting procedure. If acetoacetic acid is added directly to the column, as much as 50% of it can be recovered in a peak located in the same region as β-methylglutaconic acid. Therefore, fractions between acetic and hydroxy*iso*valeric acids were routinely acidified before plating in order that no radioactivity due to either acetate or acetoacetate would be attributed to methylglutaconic acid. It was assumed that the magnitude of the hydroxybutyric acid radioactivity peak could be taken as an approximate measure of the degree of acetoacetate formation.

*Iso*valeric acid emerged from the column very early, with the peak appearing at about 400 drops. It precedes dimethylacrylic acid and if small amounts of each acid are present, *iso*valeric acid and dimethylacrylic acids are separable.

Pyruvic acid recovery is very low, so that demonstration of its formation from acetate is difficult. On the column described here, it is not separated from hydroxy*iso*valerate, although it is eluted very slightly later. In cases where there is a possibility that ^{14}C-pyruvic acid has been formed, the radioactivity in hydroxy*iso*valerate must be confirmed by paper chromatography as described below. Fumaric acid is eluted from the column in the same fractions as the low-melting isomer of methyl-glutaconic acid. These two acids are also separable by paper chromatography. With 3 hour incubations, radioactive fumaric acid was rarely detected.

For confirmation of radioactivity of hydroxy*iso*valeric acid (in the presence of radioactive pyruvic acid) or of methylglutaconic acid (in the presence of fumaric acid), paper chromatography was employed. The solvent system used, developed by Dr. ADELBERG*, consists of a mixture of ethyl ether, benzene, formic acid, and water in the ratios of 35:15:14:10. The top layer of this mixture is used for ascending chromatography with Whatman No. 1 filter paper. The acidified sample containing less than 5 micro-equivalents of each acid, is spotted at the origin and the chromatogram is allowed to develop overnight. The paper is allowed to dry in air for one hour and is then sprayed with 0.04% bromcresol green. The spots which are thus located can then be cut from the paper, eluted, and counted.

* We are indebted to Dr. A. E. ADELBERG, Department of Bacteriology, University of California, or a description of this solvent prior to publication.

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RESULTS AND DISCUSSION

Figs. 1–4 illustrate typical chromatograms obtained by treatment of an incubation mixture as described above. The different figures are only qualitatively comparable since different amounts of tissue, substrate, and non-isotopic carriers were used in the different groups of incubations.

A summary of the information obtained from the experiments performed is given in Table II. Although only one chromatogram for each kind of experiment is represented in Table II, most of the incubations were repeated a number of times, so that each chromatogram described is representative of several trials.

TABLE II

DISTRIBUTION OF RADIOACTIVITY IN SUSPECTED INTERMEDIATE ACIDS OF CHOLESTEROL BIOSYNTHESIS

Chromatogram No.	*Rat liver preparation*	*Substrate*	*Radioactivity in recovered carrier acids*						
			HOAC[a]	*BHB*[b]	*HMG*[c]	*HIV*[d]	*BMG*[e]	*DMA*[f]	*Chol.*[g]
1[h]	+Chol. homog.[i]	Acetate-^{14}C	S[j]	+	+	+	+	+	+
2	+Chol. homog.[i]	HMG-^{14}C	+	+	S	—	+	trace	trace
3	+Chol. homog.[i]	DMA-^{14}C + cold BMG	+	+	—	+	—	S	trace
4[k]	+Chol. homog.[i]	DMA-^{14}C + cold HMG	+	+	—	+	—	S	trace
7	Slices	Acetate-^{14}C	S	+	+	+	+	+	+
8	Slices	HMG-^{14}C	+	+	S	—	+	trace	+
9[l]	Slices	HMG-^{14}C + cold DMA	+	—	S	+	+	—	+
10	Slices	HMG-^{14}C + cold HIV	+	—	S	trace	+	—	+
11	Slices	DMA-^{14}C	+	+	+	+	+	S	+
12[m]	Slices	Acetate-^{14}C —0.1 *M* phenyl butyrate	S	+	+	+	+	—	+
14	—Chol. homog.[n]	Acetate-^{14}C	S	+	+	+	—	+	—
15	—Chol. homog.[n]	DMA-^{14}C + cold HMG	+	—	—	+	—	S	—
18	Supernatant liquid	Acetate	S	+	+	+	+	+	—

a = Acetate; *b* = hydroxybutyrate: assumed to reflect radioactivity of acetoacetate; *c* = β-hydroxy-β-methylglutarate; *d* = β-hydroxy*iso*valerate; *e* = β-methylglutaconate; *f* = β,β-dimethylacrylate; *g* = cholesterol. *h* = Chromatogram of Fig. 1. *i* = Homogenate capable of synthesizing cholesterol from acetate. *j* = Substrate. *k* = Chromatogram of Fig. 2. *l* = Chromatogram of Fig. 3. *m* = Chromatogram of Fig. 4. *n* = Homogenate showing no synthesis of cholesterol from acetate.

From the figures and from Table II, certain inferences can be drawn, namely that:

(1) Neither hydroxy*iso*valerate nor dimethylacrylate is an obligatory precursor of cholesterol. It is to be observed that in some of the incubations of ^{14}C-hydroxymethylglutarate with liver slices, cholesterol synthesis occurred without appreciable formation of hydroxy*iso*valerate (chromatogram No. 8) or dimethylacrylate (chromatogram Nos. 8–10). When non-radioactive hydroxy*iso*valerate was added at the beginning of the incubation (chromatogram No. 10) the recovered acid was only very slightly radioactive. When non-radioactive dimethylacrylic acid was added as a trapping agent at the beginning of the incubation, the recovered dimethylacrylate was non-radioactive (chromatogram No. 9). The facts that these same slices were capable of synthesizing cholesterol from ^{14}C-dimethylacrylic acid (chromatogram No. 11), and that the addition of non-radioactive hydroxy*iso*valerate or dimethylacrylate depressed the specific activity of the cholesterol synthesized from ^{14}C-hydroxymethylglutarate or from ^{14}C-acetate indicate that the added non-radioactive acids were metabolically

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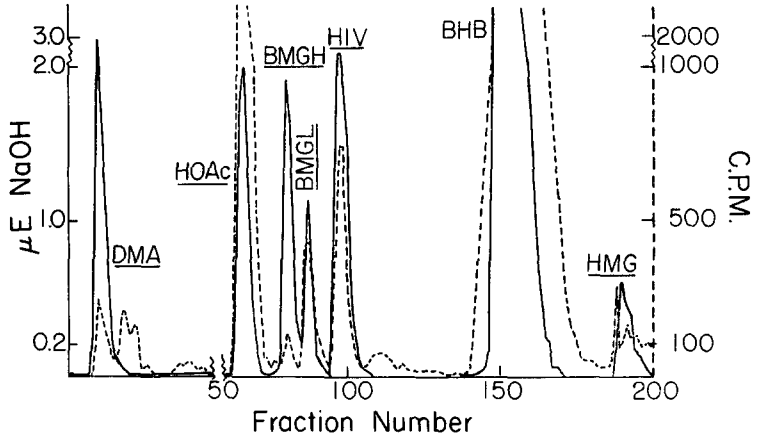

Fig. 1. Chromatogram of carboxylic acids isolated from incubation of liver homogenate with sodium acetate-1-^{14}C. Abbreviations for acids given in footnote to Table I. Solid lines represent titrations, broken lines radioactivity, in all figures. Standard system eluent employed in chromatography of experiments represented in the figures.

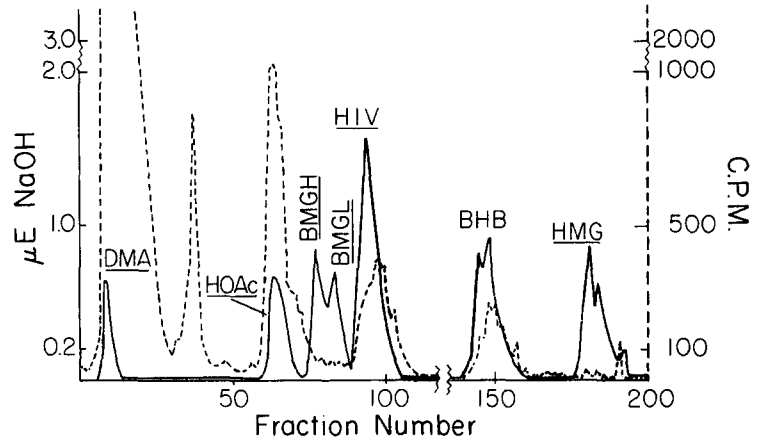

Fig. 2. Chromatography of carboxylic acids isolated from incubation of liver homogenate with β,β-dimethylacrylic acid-1-^{14}C and unlabeled β-hydroxy-β-methylglutaric acid.

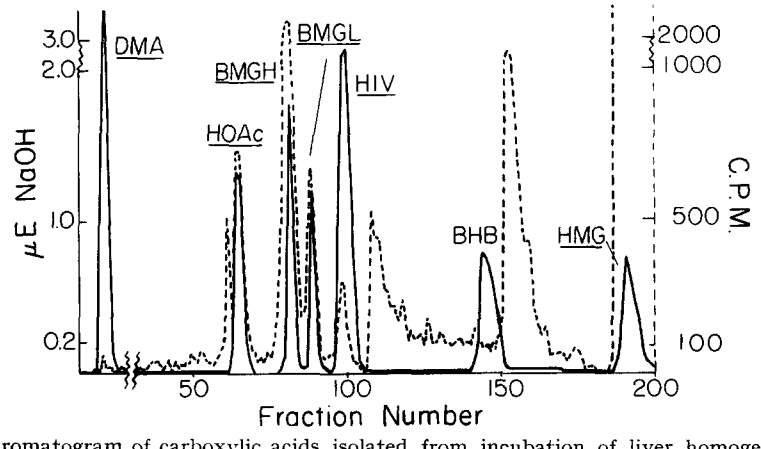

Fig. 3. Chromatogram of carboxylic acids isolated from incubation of liver homogenate with β-hydroxy-β-methylglutaric acid-3-^{14}C and unlabeled β,β-dimethylacrylic acid.

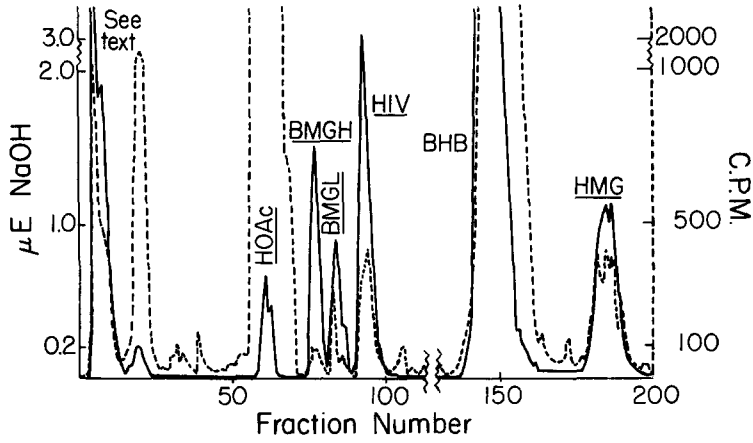

Fig. 4. Chromatogram of carboxylic acids isolated from incubation of liver slices with sodium acetate-1-^{14}C and unlabeled 0.01 *M* α-phenylbutyrate.

active. This being the case, it seems impossible that in these incubations radioactive dimethylacrylic or hydroxy*iso*valeric acid could have been formed during the course of cholesterol synthesis without contaminating the non-radioactive pool of acid already present.

(2) β-Methylglutaconic acid may be an obligatory cholesterol precursor. While direct proof for such a statement is difficult to obtain, the data of Table II provide indirect support for it. In every case where cholesterol synthesis was observed, radioactive β-methylglutaconic acid was detected (chromatogram Nos. 7–11). In other cases, where cholesterol was not synthesized, no radioactive methylglutaconic acid was formed (chromatogram Nos. 3, 4, 14, 15). RABINOWITZ AND GURIN[6] similarly observed that no radioactive methylglutaconic acid could be recovered from a particle-free extract after an incubation, with ^{14}C-acetate, during which no ^{14}C-cholesterol was formed; radioactive β-methylglutaconic acid was obtained from similar extracts which did form cholesterol from acetate.

From the chromatographic data, no conclusion can be reached as to which isomer of β-methylglutaconic acid is a cholesterol precursor, since radioactivity was not consistently observed in one isomer that was unaccompanied by radioactivity in the other.

(3) β-Hydroxy*iso*valerate or dimethylacrylate apparently can yield acetate without the intermediary formation of hydroxymethylglutarate. Reactions involving the formation of dimethylacrylic acid or hydroxy*iso*valeric acid by the condensation of acetic acid with some three carbon unit such as acetone have been suggested previously[5,20,21] and their occurrence has not been disproved. If ^{14}C-acetate is used as the substrate, the pattern of labeling in the derived five carbon acid will be the same whether it is formed via acetone or hydroxymethylglutarate. Use of labeled acetone does not furnish definitive proof as to whether the suggested reaction occurs, because acetone apparently forms acetate with great facility[26]. RABINOWITZ[4] found that hydroxymethylglutarate was a more efficient precursor of hydroxy*iso*valerate than was acetate. However, from the degradation of the hydroxy*iso*valerate derived from hydroxymethylglutarate, he concluded that although some of the hydromethyl-

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glutarate was used as an intact unit, there was a great deal of breakdown and equilibration with acetate. Perhaps the strongest argument against formation of a five carbon acid by condensation of three and two carbon units is the fact that the demonstrated reversible formation of hydroxy*iso*valerate from acetate via hydroxymethylglutarate[4,15,16] appears to accomplish the same result, and the proposed reaction would therefore be redundant. However, the work reported here provides support for some formation of acetate from dimethylacrylate independently of hydroxymethylglutarate synthesis. The most obvious mechanism for this formation would be a reversal of the proposed 2-carbon plus 3-carbon condensation.

The evidence bearing on this point is seen in chromatogram Nos. 3, 4 and 15. In these instances, 1-^{14}C-dimethylacrylic acid was incubated with liver homogenates. In the latter two cases, the incubation mixture also contained non-radioactive hydroxymethylglutarate. Re-isolation of the hydroxymethylglutarate after the incubation period of three hours revealed no radioactivity in this acid. In all three cases, however, the acetic acid was definitely labeled. If this acetate was formed by way of hydroxymethylglutarate, the newly formed hydroxymethylglutarate must have remained completely isolated from the non-radioactive acid pool, both during the incubation and during the subsequent treatment of the sample. Such an occurrence seems unlikely, but not impossible. BACHHAWAT, ROBINSON AND COON[15] could not demonstrate the formation of hydroxymethylglutarate from hydroxy*iso*valerate without the use of malate for inhibition of the cleavage enzyme which forms acetoacetate and acetate from hydroxymethylglutarate. Since hydroxymethylglutarate, when newly formed, is probably in the form of a coenzyme A derivative, and may also be chemically distinct from the free acid because of combination with enzyme, it could conceivably split to acetoacetate and acetate without any formation of free hydroxymethylglutarate.

It should be pointed out that the activity of the acetate in these cases is much less than in comparable incubations with liver slices which were capable of forming both hydroxymethylglutarate and cholesterol from labeled dimethylacrylate (chromatogram No. 11). This decreased conversion of dimethylacrylate to acetate in the case of homogenates is also reflected by a greatly decreased radioactivity in the β-hydroxybutyrate. That the amount of acetate formed is relatively insignificant is further demonstrated by the virtual (but not complete) failure, with dimethylacrylate as substrate, of cholesterol synthesis by homogenates which are capable of forming cholesterol from acetate. These facts, taken together, may be an indication that normally the formation of acetate from dimethylacrylate independently of hydroxymethylglutarate synthesis, if it occurs, represents a pathway of minor significance.

(4) α-Phenylbutyric acid, while inhibiting cholesterol synthesis, does not inhibit the formation by liver slices of any of the acids observed here (chromatogram No. 12). Indeed the radioactivity of β-hydroxybutyric acid and of hydroxymethylglutarate were unusually high, suggesting inhibition of some step subsequent to the formation of these acids. In addition, a radioactivity peak corresponding to the usual position of crotonic acid was found to be of unprecedented magnitude. Since crotonic acid is formed from β-hydroxybutyrate and the latter was very radioactive in this case, it is assumed that the peak does represent crotonic acid although it was not further identified. The radioactivity of the recovered dimethylacrylate is uncertain because of the large titration peak for the α-phenylbutyric acid which obscures completely the titra-

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tion for dimethylacrylate. There is a peak of radioactivity in this area which corresponds more nearly to the usual position of *iso*valeric acid than that of dimethylacrylate. It is reasonable to expect that both of these acids would be labeled in this instance.

STEINBERG AND FREDRICKSON[27] concluded that the effect of α-phenylbutyrate was to inhibit the formation of acetyl-coenzyme A or else its condensation to form acetoacetyl-coenzyme A. Such a conclusion is not compatible with the result reported here.

SUMMARY

Results obtained by the application of a chromatographic method for the isolation, from a single incubation mixture, of various acidic cholesterol precursors are presented. Evidence is given which indicates that:

1. Neither β.β-dimethylacrylic acid nor β-hydroxy*iso*valeric acid is an obligatory percursor of cholesterol.

2. β-Methylglutaconic acid may be such a precursor.

3. β.β-Dimethylacrylic acid and/or β-hydroxy-*iso*valeric acid can, to a limited extent, yield acetate in liver homogenates, without the intermediate formation of β-hydroxy-β-methylglutaric acid.

4. α-Phenylbutyric acid probably inhibits cholesterol synthesis by influencing some step beyond the steps leading to the formation of the acids studied here.

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