THE BIOSYNTHESIS OF MEVALONIC ACID

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Introduction.—Although mevalonic acid has, in recent studies, been shown to be a very efficient precursor for squalene, cholesterol, and other polyisoprene compounds, the biosynthetic origin of mevalonate has not yet been described. Previous work in this laboratory has demonstrated that $\beta$-hydroxy-$\beta$-methyl glutaryl coenzyme A (HMG CoA) can be formed in yeast and liver by the condensation of acetyl CoA and acetoacetyl CoA. In view of the structural similarities of HMG CoA and mevalonate, it could be postulated that HMG CoA might be reduced to mevalonate enzymatically via an aldehyde intermediate, as illustrated in reaction 1.

\[
\begin{align*}
\text{CH}_3 & \quad \text{CH}_2 \\
\text{H}_2\text{C} & \quad \text{CH}_2 \\
\text{O} & \quad \text{C}^{14} \\
\text{OH} & \quad \text{C}=\text{O} \\
\text{S-CoA} & \quad \text{OH}
\end{align*}
\]

3-hydroxy-3-methyl glutaryl-1-C$^{14}$CoA (HMG CoA)

\[
\begin{align*}
\text{CH}_3 & \quad \text{CH}_2 \\
\text{H}_2\text{C} & \quad \text{CH}_2 \\
\text{O} & \quad \text{C}^{14} \\
\text{OH} & \quad \text{C}=\text{O} \\
\text{H} & \quad \text{OH}
\end{align*}
\]

3-hydroxy-3-methyl-1-C$^{14}$ glutaraldehydic acid (mevaldic acid)

\[
\begin{align*}
\text{CH}_3 & \quad \text{CH}_2 \\
\text{H}_2\text{C} & \quad \text{CH}_2 \\
\text{O} & \quad \text{C}^{14} \\
\text{OH} & \quad \text{C}=\text{O} \\
\text{H} & \quad \text{OH}
\end{align*}
\]

3,5-dihydroxy-3-methyl 1-C$^{14}$-valeric acid (mevalonic acid)

Studies were undertaken to evaluate this hypothesis. Our experiments with yeast extracts have demonstrated that HMG CoA is readily reduced to mevalonate in the presence of TPNH. This finding completes a sequence of reactions for the conversion of acetate to mevalonate as follows:

acetate $\rightarrow$ acetyl CoA $\rightarrow$ acetoacetyl CoA
acetyl CoA + acetoacetyl CoA $\rightarrow$ $\beta$-hydroxy-$\beta$-methyl glutaryl CoA $\rightarrow$ mevalonic acid.

Methods.—Enzyme preparations: The incorporation of isotope from HMG-C$^{14}$-CoA into mevalonate was used to measure the synthesis of mevalonate. Fresh baker's yeast was used as a source of enzyme. Cells were ruptured by high-frequency shaking in phosphate buffer (0.1 M pH 7.6) and cell debris was removed by centrifugation at 3,200 × g. This crude enzyme solution was further treated by centrifugation for 30 min at 105,000 × g, and the resulting supernatant solution was dialyzed against phosphate buffer (0.001 M pH 7.6) for 2 hr. In some experiments extracts were used which were obtained by lysis of yeast with toluene.

Biological synthesis of labeled $\beta$-hydroxy-$\beta$-methyl glutaryl CoA: HMG-C$^{14}$-CoA was prepared by the condensation of acetyl-C$^{14}$-CoA and acetoacetyl CoA in the presence of the purified yeast condensing enzyme. When acetyl-1-C$^{14}$-CoA was used as substrate, the C$^{14}$ was located in the free carboxyl group of the resulting HMG CoA$^7$; with acetyl-2-C$^{14}$-CoA, the C$^{14}$ was in the methylene group adjacent to the free carboxyl group. Thus HMG-1- and HMG-2-C$^{14}$-CoA could conveniently be synthesized enzymatically. They were isolated by adsorption on charcoal, followed by paper chromatography. The HMG-C$^{14}$-CoA so isolated had an $R_f$ of 0.16 in ethanol:ammonium acetate, pH 4.7, and an $R_f$ of 0.41 in isobutyric acid:water:ammonia, which corresponded to the $R_f$ of synthetic HMG CoA in these solvent systems. HMG-C$^{14}$-CoA formed a hydroxamate which migrated with the same $R_f$ (0.31) as authentic HMG monohydroxamate in butanol:water. The purity of the HMG-C$^{14}$-CoA was determined in the following manner. A sample was hydrolyzed in 1.5 N KOH and the resultant free hydroxy methyl glutarate was purified by ion exchange and partition chromatography. A comparison of the C$^{14}$ content of the hydroxy methyl glutarate with that of the original HMG CoA sample afforded a measure of the purity; this varied between 70–85 per cent, i.e., 30 to 15 per cent of the C$^{14}$ present was in a form other than HMG CoA.

Chemical synthesis of labeled $\beta$-hydroxy-$\beta$-methyl glutaryl CoA: Labeled HMG-CoA from synthetic sources was prepared in the following manner. HMG-2-C$^{14}$ was synthesized from mevalonic-2-C$^{14}$ (Isotope Specialties Co.) by oxidation with permanganate at pH 6.6. After addition of a large amount of nonisotopic mevalonate to dilute any residual mevalonate-2-C$^{14}$, HMG-2-C$^{14}$ was isolated (free of mevalonate-2-C$^{14}$) using an acid celite column. The labeled HMG was then converted to the anhydride by the method of Hilz et al., recrystallized, and allowed to react with CoASH to form racemic HMG-2-C$^{14}$-CoA. The compound was isolated by paper chromatography in isobutyric acid:water:ammonia.

Enzymatic conversion of HMG-CoA to mevalonate: The conversion of HMG-C$^{14}$-CoA to mevalonic acid was done with yeast extracts at room temperature in air. A large pool of nonisotopic mevalonate was added to trap any labeled mevalonate which was formed, and to serve as carrier for its isolation. C$^{14}$-mevalonate was isolated from reaction mixtures as the lactone by chromatography on acid celite, using acid-equilibrated chloroform for elution. This method afforded good resolution of mevalonate, HMG, and acetate. Proportionality was excellent between the lactone as determined by titration and the C$^{14}$ concentration in eluate fractions. Identity and purity of recovered mevalonate were further established by its derivatization as the dibenzylethylenediamine salt (m.p. 121–122°) and recrystalliza-
tion to constant specific activity. In some cases, the recovered mevalonate-C\textsuperscript{14} was oxidized to HMG-C\textsuperscript{14} with acid permanganate as further proof of identity. The yeast extracts contained the enzymes required for generation of TPNH from glucose-6-phosphate and TPN and DPNH from fructose-1,6-diphosphate and DPN.

**Results.**—Table 1 summarizes the results of two representative experiments in which HMG-C\textsuperscript{14}-CoA was incubated with yeast extracts. These data show a reasonably efficient conversion of HMG-C\textsuperscript{14}-CoA to mevalonate by the crude enzyme preparations. The activity of the enzyme preparation was markedly reduced by high-speed centrifugation, and further reduced by brief dialysis. The addition of a TPNH generating system restored and greatly augmented the conversion, while addition of a DPNH generating system only partially restored the activity. In experiments of this duration there was usually observed a 40–60 per cent conversion of total substrate to mevalonate when TPNH was the generated cofactor. Subsequent experiments have shown that TPNH added in substrate quantities could replace the TPNH generating system.

We have examined the possibility that HMG-C\textsuperscript{14}-CoA might first be broken down, and then be converted to mevalonic acid by an as yet unknown pathway. Two reactions have been described in which HMG CoA is enzymatically degraded. The first is by hydrolytic deacylation, as reported by Dekker et al.,\textsuperscript{15} wherein free HMG and CoASH are produced. The second is by the cleavage reaction described by Bachhawat et al.,\textsuperscript{14} in which HMG CoA is cleaved to acetyl CoA and acetoacetic acid.

Table 2 summarizes experiments designed to evaluate the possible role of HMG, acetoacetate, and acetyl CoA in the biosynthesis of mevalonate. It can be seen that when HMG-C\textsuperscript{14}-CoA was hydrolyzed under alkaline conditions to free HMG and CoASH there was no incorporation of C\textsuperscript{14} into mevalonic acid. Acetoacetic acid likewise had no effect on the conversion of HMG-C\textsuperscript{14}-CoA to mevalonate. Un-

### Table 1

**The Incorporation of β-Hydroxy-β-Methyl-Glutaryl-1-C\textsuperscript{14} Coenzyme A into Mevalonic Acid**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Enzyme Preparation</th>
<th>Pyridine Nucleotide Generating System</th>
<th>Total C\textsuperscript{14} in Mevalonic Acid, cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Crude</td>
<td>None</td>
<td>7,320</td>
</tr>
<tr>
<td></td>
<td>105,000 × g supernatant</td>
<td></td>
<td>1,200</td>
</tr>
<tr>
<td></td>
<td>Same, dialyzed</td>
<td>None</td>
<td>885</td>
</tr>
<tr>
<td></td>
<td>Same, dialyzed</td>
<td>DPNH + TPNH</td>
<td>25,400</td>
</tr>
<tr>
<td>2</td>
<td>Dialyzed 105,000 × g supernatant</td>
<td></td>
<td>483</td>
</tr>
<tr>
<td></td>
<td>Same</td>
<td>DPNH + TPNH</td>
<td>19,700</td>
</tr>
<tr>
<td></td>
<td>Same</td>
<td>DPNH</td>
<td>1,690</td>
</tr>
<tr>
<td></td>
<td>Same</td>
<td>TPNH</td>
<td>22,200</td>
</tr>
</tbody>
</table>

A separate enzyme preparation was used in each experiment. The reaction mixture contained 1.3 millimoles of phosphate buffer, pH 7.6, HMG-1-C\textsuperscript{14}-CoA (50,000 cpm, approximately 0.05 \textmu{}mole\textsuperscript{14}), 50 \textmu{}mole Mg\textsuperscript{2+}, 300 \textmu{}mole nicotinamide, 40–50 mg yeast protein, 100 \textmu{}mole unlabeled mevalonate, and where indicated 9 \textmu{}mole of DPN and 100 \textmu{}mole of fructose-1,6-diphosphate as a DPNH generating system and/or 9 \textmu{}mole TPN and 100 \textmu{}mole of glucose-6-phosphate as a TPNH generating system. Incubation time 2 hr, final volume was 13.7 ml. Mevalonate-C\textsuperscript{14} counted as the recrystallized dibenzylethylene diamine salt.

* Specific radioactivity is estimated from the specific activity of the acetic-1-C\textsuperscript{14}-anhydride used in making acetyl-1-C\textsuperscript{14}-CoA, the source of the HMG-1-C\textsuperscript{14}-CoA. The incorporation of 25,000 counts into mevalonate represents a yield of mevalonate in the range of 0.025 \textmu{}mole.
labeled acetyl CoA markedly depressed the level of radioactivity in mevalonate. The addition of acetyl-1-C\textsuperscript{14}-CoA however produced a similar depression suggesting an inhibition of the overall reaction rather than dilution of the isotope. Furthermore, substitution of equivalent acetyl-1-C\textsuperscript{14}-CoA for HMG CoA as substrate resulted in no detectable incorporation of C\textsuperscript{14} into mevalonate. In Experiment 3, Table 2, it was found that the addition of CoASH to a system which converts HMG-2-C\textsuperscript{14}-CoA to mevalonate resulted in a depression of the conversion.

### TABLE 2

**The Effect of Metabolites of \( \beta \)-Hydroxy-\( \beta \)-Methyl Glutaral Coenzyme A on the Conversion of \( \beta \)-Hydroxy-\( \beta \)-Methyl Glutaral Coenzyme A to Mevalonic Acid**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Substrate</th>
<th>Total C\textsuperscript{14} in Mevalonate, cpm</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>HMG-2-C\textsuperscript{14}-CoA (0.05 ( \mu )mole)</td>
<td>3,680</td>
</tr>
<tr>
<td></td>
<td>HMG-2-C\textsuperscript{14}-CoA (0.05 ( \mu )mole) + acetoacetate (24 ( \mu )moles)</td>
<td>3,680</td>
</tr>
<tr>
<td></td>
<td>HMG-2-C\textsuperscript{14}-CoA (0.05 ( \mu )mole) + acetoacetate (96 ( \mu )moles)</td>
<td>3,670</td>
</tr>
<tr>
<td></td>
<td>HMG-2-C\textsuperscript{14}-CoA (0.05 ( \mu )mole) hydrolyzed in alkali</td>
<td>0</td>
</tr>
<tr>
<td>2</td>
<td>HMG-1-C\textsuperscript{14}-CoA</td>
<td>12,000</td>
</tr>
<tr>
<td></td>
<td>HMG-1-C\textsuperscript{14}-CoA + acetyl CoA (5 ( \mu )moles)</td>
<td>2,000</td>
</tr>
<tr>
<td></td>
<td>HMG-1-C\textsuperscript{14}-CoA + acetyl-1-C\textsuperscript{14}-CoA (0.3 ( \mu )mole)</td>
<td>&lt;200</td>
</tr>
<tr>
<td></td>
<td>Acetyl-1-C\textsuperscript{14}-CoA (0.05 ( \mu )mole, 50,000 cpm)</td>
<td>9,100</td>
</tr>
<tr>
<td>3</td>
<td>HMG-2-C\textsuperscript{14}-CoA</td>
<td>4,040</td>
</tr>
<tr>
<td></td>
<td>HMG-2-C\textsuperscript{14}-CoA + CoASH (5 ( \mu )moles)</td>
<td>306</td>
</tr>
</tbody>
</table>

Expt. 1—Each flask contained 60 mg of yeast protein (dialyzed crude toluene lyate), 200 \( \mu \)moles of glucose-6-phosphate, 9 \( \mu \)moles of TPN, 150 \( \mu \)moles of Mg\textsuperscript{++}, 300 \( \mu \)moles of nicotinamide, 600 \( \mu \)moles of potassium phosphate buffer pH 7.6, 100 \( \mu \)moles of MVA was present as a trapping pool. The HMG CoA was prepared synthetically, and the specific activity was 480,000 cpm/\( \mu \)mole. The hydrolyzed HMG CoA was prepared by incubating 0.05 ml (0.05 \( \mu \)mole) HMG CoA with 0.1 ml of 3.5 N KOH for 1 hr at 30\( ^\circ \). It was then neutralized with 3.5 N H\textsubscript{2}SO\textsubscript{4}. The same amounts of 3.5 N KOH and H\textsubscript{2}SO\textsubscript{4} were added to the other flasks. Incubation time was 1 hr at 25\( ^\circ \), final volume in each flask 12 ml.

Expt. 2—Where indicated, 0.05 \( \mu \)mole of biosynthetic HMG CoA was present (50,000 cpm). All flasks contained TPN and glucose-6-phosphate. Incubation, supplemental additions, and isolation were as indicated in Table 1.

Expt. 3—Each flask contained 50 \( \mu \)moles glucose-6-phosphate, 4.5 \( \mu \)mole TPN, 75 \( \mu \)moles Mg\textsuperscript{++}, 300 \( \mu \)moles of nicotinamide, 300 \( \mu \)moles potassium phosphate buffer pH 7.6, 120 mg of yeast protein (crude toluene lyate). 0.12 \( \mu \)mole HMG-2-C\textsuperscript{14}-CoA (biosynthetic, 134,000 c.p.m./\( \mu \)mole), and 100 \( \mu \)moles of mevalonic acid. Final volume 6.0 ml. Incubation time: 30 min at 20\( ^\circ \).

From these results it would appear that HMG, acetoacetate, and acetyl CoA are not direct precursors of mevalonate but that HMG must be activated to HMG CoA before it can be converted to mevalonate. Furthermore, there is an apparent inhibition of the conversion of HMG CoA to mevalonate by free CoASH and some thiol esters of CoASH.

In order to establish whether the free carboxyl group or the thiol-esterified carboxyl group of HMG CoA was reduced in the conversion to mevalonate, biosynthetic labeled HMG CoA was incubated with the yeast enzyme and the TPNH generating system as outlined in Table 1. The mevalonate was converted to the barium salt and subjected to pyrolysis under vacuum at 500\( ^\circ \). CO\textsubscript{2} obtained from the residual BaCO\textsubscript{3} provided a sample of the carboxyl group of mevalonate. It was found that mevalonate obtained from HMG-1-C\textsuperscript{14}-CoA yielded C\textsuperscript{14}O\textsubscript{2}, while the CO\textsubscript{2} from HMG-2-C\textsuperscript{14}-CoA was unlabeled. These results show that the free carboxyl group of HMG CoA remains intact during the reduction, and consequently that the reduction occurs of the thiol-esterified group of HMG CoA, as indicated in Reaction 1.

It is of interest that if allowance is made for the fact that synthetic HMG CoA consists of at least 50 per cent of the unnatural optical isomers of HMG CoA the conversion to mevalonate (Experiment 1, Table 2) was almost as good as with
enzymatically generated HMG CoA. Dituri et al.\textsuperscript{16} reported that labeled HMG CoA made by the procedure of Bachhawat et al.\textsuperscript{14} was not incorporated by liver preparations into squalene. In view of the fact that mevalonate can be readily converted to squalene we are unable to explain the failure to obtain incorporation. There is a possibility that the liver preparations may contain the HMG CoA cleavage\textsuperscript{14} and deacylating\textsuperscript{15} enzymes which would tend rapidly to destroy HMG CoA. These enzymes do not appear to be present to any great extent in our yeast preparations.

In order to establish whether the reduction of HMG CoA to mevalonic acid could be reversed, mevalonate-2-C\textsuperscript{14} (1 \(\mu\)mole, 500,000 cpm) was incubated with TPN, CoASH, oxidized glutathione, and yeast extract. Since there is a large amount of glutathione reductase in this enzyme preparation, it was expected that glutathione would favor HMG CoA formation through reoxidation of the TPNH formed in the oxidation of the mevalonate. However, no isotope could be detected in carrier HMG CoA, indicating that the reaction is not readily reversible. These results might explain the observation of Popjak\textsuperscript{17} that labeled mevalonic acid did not appear to give rise to labeled acetyl units when incubated with liver homogenate preparations.

We have not been able to establish whether the aldehydic acid, mevaldic acid, is intermediate between HMG CoA and mevalonic acid, although Wright et al.\textsuperscript{18} and Lynen\textsuperscript{19} have described the conversion of mevaldic acid to mevalonate, and added mevaldate has been reported to depress incorporation of isotope from acetyl-C\textsuperscript{14} into cholesterol.\textsuperscript{20} The addition of unlabeled mevaldate or of semicarbazide to our system did not significantly depress incorporation of isotope from HMG-C\textsuperscript{14}-CoA into mevalonate. In addition, no isotope could be detected in the re-isolated aldehyde. This raises the possibility that the aldehyde intermediate may exist as a thio-hemiacetal of CoASH, or be bound to enzyme.

The evidence presented indicates that mevalonic acid is formed by the reduction of HMG CoA. The role of HMG CoA as a key intermediate in the biosynthesis of terpenes and sterols from acetate now becomes apparent, since it represents the first point of synthesis of the branched chain structure which is subsequently reduced to mevalonate. The fact that the reduction appears to be relatively irreversible suggests that once the 2-carbon units have condensed to form HMG CoA and are reduced to mevalonate they may not re-enter the 2-carbon pool, but rather they must proceed toward polyisoprene biosynthesis. Similar findings on the reduction of HMG CoA to mevalonate have been cited briefly by Lynen et al.\textsuperscript{21}

Summary.—Evidence is cited for the existence in yeast of a pathway for the enzymatic synthesis of mevalonic acid from \(\beta\)-hydroxy-\(\beta\)-methyl glutaryl coenzyme A. This conversion would seem to be direct, and not involve any of the known degradation products of \(\beta\)-hydroxy-\(\beta\)-methyl glutaryl coenzyme A. The reduction is maximal in the presence of reduced triphosphopyridine nucleotide, and is not readily reversible; the reduction occurs on the thiol-esterified carboxyl group of \(\beta\)-hydroxy-\(\beta\)-methyl glutaryl coenzyme A. Addition of free mevaldic acid does not decrease the incorporation of isotope from \(\beta\)-hydroxy-\(\beta\)-methyl glutaryl coenzyme A into mevalonic acid. It therefore seems probable that the free aldehyde is not an intermediate of the reaction. Demonstration of this conversion provides a complete pathway for the synthesis of mevalonic acid from acetate in yeast.
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† Work completed during tenure of a Cleveland Area Heart Society Research Fellowship. Present address: Department of Medicine and Biochemistry, University of Pennsylvania School of Medicine, Philadelphia 4, Pennsylvania.

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♀ Senior Research Fellow, S.F. 199, Public Health Service.

|| The following abbreviations are used: HMG CoA, 3-hydroxy-3-methyl glutaryl coenzyme A; HMG, 3-hydroxy-3-methyl glutaric acid; CoASH, reduced coenzyme A; DPN and TPN, di- and triphosphopyridine nucleotide; DPNH and TPNH, reduced di- and triphosphopyridine nucleotide.