give pregnan-3α,17α-diol-11,20-dione 3-acetate, m.p. 195–198°, [α]D +50.3° (chloroform), and by hydrolysis, pregnan-3α,17α-diol-11,20-dione, m.p. 199.5–200.5°, [α]D + 33.9° (chloroform).

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The Biosynthesis of Radioactive Cholesterol, β-Methylglutaconic Acid and β-Methylcrotonic Acid by Aqueous Extracts of Liver[1]

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It has been previously reported that aqueous particle-free extracts of rat liver which possess the ability to incorporate C^{14}-labeled acetate or pyruvate into cholesterol[2] can also synthesize β-hydroxy-β-methylglutaric[3] and β-methylcrotonic acids.[4] Further investigation revealed the presence of another radioactive acid which has now been found to be *trans*-β-methylglutaconic acid (Table I). It is probable that this acid is derived from β-hydroxy-β-methylglutaric acid (HMG) by an enzymatically catalyzed dehydration. The formation of these two acids is consistent with the metabolic pathway

$$CH_3COCH_2COOH + CH_3COOH \longrightarrow CH_3C(OH)(CH_2COOH)_2 \longrightarrow CH_3C(CH_2COOH)=CHCOOH$$

Evidence for the mechanism of formation of the Coenzyme A derivative of HMG has recently been obtained by Robinson, Bachhawat and Coon.[5] The above postulated pathway undoubtedly also involves the participation of Coenzyme A.

C^{14}-Methyl-labeled HMG is incorporated into cholesterol by surviving liver slices, homogenates, or particle-free extracts of rat liver (Table II). When compared with 2-C^{14}-acetate it was found to be equally well incorporated. Similarly, the incorporation of HMG into β-methylglutaconic and β-methylcrotonic acids was equal to that observed with 2-C^{14}-acetate (Table III).

The role of these substances as precursors of cholesterol is under further investigation.

Experimental

Particle-free extracts (7 ml.) of rat liver,[3] homogenates (5 ml.), or tissue slices (1 g.) were incubated with 1 mg. each of adenosinetriphosphate, diphosphopyridine nucleotide and with 2-C^{14}-potassium acetate (1 mc./mmole) or C^{14}-methyl-HMG (0.08 mc./mmole). The labeled HMG was prepared from 2-C^{14}-acetate by the method of Klosterman.[6] The gas phase was 95% O_2–5% CO_2, and the time of incubation was 3 hours at 37°. The conditions used were those found to be optimum for cholesterol biosynthesis.[3] Following the incubation period, 34 mg. of carrier methylglutaconic acid, 20 mg. of β-methylcrotonic acid or 1 mg. of carrier cholesterol were added to separate incubation flasks. Each specimen was saturated with KCl, acidified to *p*H 2 with metaphosphoric acid, and extracted continuously with ether for 24–48 hours. The ether solution was evaporated to dryness and kept in a desiccator over KOH for 24 hours. The residue was extracted with 10% acetic acid and again extracted continuously with ether. The ether extract was afterward evaporated to dryness. For the separation of either β-methylcrotonic acid or β-methylglutaconic acid, the oily residue was transferred to a vacuum micro sublimation apparatus and heated with a small micro burner at 1 millimeter of mercury until sublimation ceased. A pale yellow crystalline sublimate was obtained. This material was dissolved in a few milliliters of absolute ether, decolorized by warming with charcoal and petroleum ether added to the filtrate to incipient turbidity. When β-methylglutaconic acid was isolated, the material obtained (4–5 mg.) melted at 114–115°. A mixed melting point with an authentic sample[7] showed no depression. The sublimed acid was plated and assayed for radioactivity; the counts were corrected to infinite thinness. The acid was diluted with carrier and subsequently converted to α,β-dibromo-β-methylglutaric acid[8] which was recrystallized (m.p. 145°). A mixed melting point with authentic dibromo-β-methylglutaric acid showed no change. The β-bromo-β-methylglutaric derivative[9] was also prepared (m.p. 129°); a mixed melting point with an authentic sample was unchanged. Subsequent radio-assay yielded the expected activity. Cholesterol was isolated as the digitonide and plated.[3] Incubations with inactive preparations of particle-free extracts of rat liver yielded no radioactive β-methylglutaconic acid. Variations in the activity of different derivatives of the same sample were within counting errors.

TABLE I

INCORPORATION OF 2-C^{14}-ACETATE INTO β-METHYLGLUTACONIC ACID BY AQUEOUS EXTRACTS OF RAT LIVER

Experiment	Radioactivity recovered in β-Methylglutaconic acid, c.p.m./mg. C	β-Bromo-β-methylglutaric acid, c.p.m./mg. C	α,β-Dibromo-β-methylglutaric acid, c.p.m./mg. C
1	354	302	..
2	791	..	..
3	269	..	234

TABLE II

INCORPORATION OF 2-C^{14}-ACETATE AND METHYL-C^{14}-HMG INTO CHOLESTEROL

Precursor	Radioactivity recovered[a] Aqueous extract	Homogenate	Slices
2-C^{14}-Acetate	0.0073	0.0081	0.0095
3′-C^{14}-β-Hydroxy-β-methylglutaric acid	.0086	.0093	.0071

[a] Recovered radioactivity expressed as μmoles of precursor incorporated = total recovered counts/counts per μmole of precursor. Aliquots of same extract, homogenate and slices obtained from same liver were employed.

TABLE III

INCORPORATION OF C^{14}-LABELED HMG AND 2-C^{14}-ACETATE INTO CHOLESTEROL, β-METHYLGLUTACONIC AND β-METHYLCROTONIC ACIDS BY PARTICLE-FREE EXTRACTS OF RAT LIVER

Precursor	Radioactivity recovered[a] Cholesterol	β-Methylglutaconic acid	β-Methylcrotonic acid
2-C^{14}-Acetate	0.003	0.183	0.095
3′-C^{14}-β-Hydroxy-β-methylglutaric acid	.005	.134	.094

[a] Radioactivity expressed as μmoles of precursor incorporated in recovered product (see Table II).

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