dinitrophenylhydrazones of the above ketones have been made by direct methods in this Laboratory. Previous negative results may be accounted for by the tendency of the derivatives to supersaturate in solvents until seed crystals were available, by relatively unfavorable equilibria, and perhaps by difficulty in distinguishing readily between derivative and reagent. A modified qualitative test, to be published elsewhere, apparently helps to surmount these difficulties. In the qualitative test, a more highly concentrated solution of the reagent is used, and the derivative is washed thoroughly with hot, 10% hydrochloric acid.

The formation of 2,4-dinitrophenylhydrazones of some hindered ketones suggests the possibility of the formation and isolation of other derivatives such as the oximes. Report on this work will be made later.

Experimental[2]

2,4,6-Trimethylacetophenone-2,4-DNH.—The derivative was made by refluxing a mixture of 1 g. each of ketone and 2,4-dinitrophenylhydrazine, 5 ml. of concentrated hydrochloric acid, 5 ml. of water and 40 ml. of ethanol. As the solution cooled, seeds from a previous smaller run of a qualitative nature were added. The crystals obtained were washed thoroughly with hot, 10% aqueous hydrochloric acid and then with water. The yield was 1.2 g., 57%, of amber-colored, transparent prisms of m.p. 148–149° (previous softening) after two recrystallizations from a mixture of 25 ml. of methanol and 4 ml. of ethyl acetate.

Anal. Calcd. for $C_{17}H_{18}N_4O_4$: N, 16.37. Found: N, 16.21.

4-*t*-Butyl-2,6-dimethylacetophenone-2,4-DNH.—This compound, made in a similar manner, was recrystallized from methylcyclohexane as small, yellow-orange needles, m.p. 164.5–166° (previous softening); nearly quantitative crude yield.

Anal. Calcd. for $C_{20}H_{24}N_4O_4$: N, 14.57. Found: N, 14.46.

Recrystallization from methanol gave yellow needles of wide melting range (150–162°). After being evacuated at 2 mm. and 78°, the yellow needles melted at 163.5–164.5 (previous softening).

2,6-Dimethylacetophenone-2,4-DNH.—The derivative was made in 28% yield from 0.75 g. of 2,6-dimethylacetophenone.[3] From the filtrate, 0.5 g., 66% of ketone, was recovered by extraction with petroleum ether (b.p. 30–60°). The derivative was recrystallized twice from methylcyclohexane as yellow needles, m.p. 158.5–159.5°.

Anal. Calcd. for $C_{16}H_{16}N_4O_4$: N, 17.07. Found: N, 16.90.

Benzoylmesitylene[4] failed to give a 2,4-DNH, or a readily isolable one, under the above conditions and under the forcing conditions (concentrated sulfuric acid and dioxane) of Josten.[5]

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DEPARTMENT OF CHEMISTRY
VANDERBILT UNIVERSITY
NASHVILLE 5, TENN.

(2) All melting points are corrected. Analyses were by Clark Microanalytical Laboratory, Urbana, Ill.

(3) This ketone was made in two steps from 2,6-dimethylaniline (Distillation Products, Inc.). The yield of 2,6-dimethylbenzonitrile, m.p. 88.5–89.5°, was 16%. We are indebted to Mr. James R. Cox, Jr., and Mr. C. G. Carlile for this synthesis. The addition of the nitrile to methylmagnesium iodide in anisole gave a 34% yield of ketone, b.p. 69–70° at 2 mm., $n^{25}D$ 1.5132.

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The Biosynthesis of Radioactive β-Hydroxyisovaleric Acid in Rat Liver[1–3]

BY JOSEPH L. RABINOWITZ

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Previous reports by this and other laboratories on the biosynthesis of cholesterol have established the formation of β-hydroxy-β-methylglutaric acid,[4,5] β-methylglutaconic acid,[6] and senecioic acid[7,8] from labeled acetate in cell-free homogenates[9,10] and particle-free extracts of rat liver.[11] Further investigation has revealed the presence of another radioactive acid which has been found to be β-hydroxyisovaleric acid. It is probable that this acid was derived from HMG by an enzymatically catalyzed decarboxylation. β-Hydroxyisovaleric acid was isolated from cell-free homogenates and particle-free extracts of rat liver,[4,11,12] after incubation with 2-C^{14}-NaOAc or with 3′-C^{14}-HMG by means of carrier technique. Results are shown in Table I and the details are described in the experimental

TABLE I

INCORPORATION OF 2-C^{14}-NaOAc INTO CHOLESTEROL AND β-HYDROXYISOVALERIC ACID BY RAT LIVER HOMOGENATES

Each flask contained 5 ml. of cell-free homogenate; the additions were 1 mg. each of AMP, DPN and 2-C^{14}-NaOAc (1.5×10^5 c.p.m./mg. C). Following incubation for 3 hours at 37°, 0.5 mg. of carrier cholesterol and 30 mg. of carrier HIV was added to each flask. Gas phase was O_2.

	Radioactivity recovered as $BaCO_3$, c.p.m./mg. C				
Experiment	HIV	Cholesterol	Cu salt of HIV	Ag salt of HIV	Paper chromatographed HIV
1	1590	220	1410	1540	1430
2	1970	375	..	..	..

part. In Table II the incorporation of different substrates into HIV is shown for both cell-free homogenate and aqueous extracts of rat liver. As expected, HMG proved to be a much better precursor of HIV than acetate. The distribution of isotope in HIV (derived from 2-C^{14}-HOAc) paralleled the observations of other workers in the field.[5,8] The results of the degradation of HIV obtained from 3′-C^{14}-HMG pointed to the utilization of some intact HMG; but also to a great deal of breakdown and equilibration of HMG with HOAc and probably AcAcOH prior to utilization. Table III shows the results obtained. Although a relatively small amount of C^{14} is found in carbons 1 and 3 in

(1) Supported by a grant from the American Heart Association.

(2) The radioactive materials were obtained on allocation from the United States Atomic Energy Commission.

(3) The following abbreviations are used: AMP = adenosine-5′-monophosphate; ATP = adenosinetriphosphate; DPN = diphosphopyridine nucleotide; HMG = β-hydroxy β-methylglutaric acid; MG = β-methylglutaconic acid; SA = senecioic acid; HIV = β-hydroxyisovaleric acid; NaOAc = sodium acetate; AcAcOH = acetoacetic acid.

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TABLE II

BIOSYNTHESIS OF β-HYDROXYISOVALERIC ACID FROM 2-C^{14}-NaOAc AND 3′-C^{14}-HMG BY RAT LIVER

Each flask contained 5 ml. of enzyme. The additions were 1 mg. each of AMP, DPN and substrates. Following incubation for 3 hours at 37°, 30 mg. of HIV carrier was added to each flask. Gas phase was O_2. In each system aliquots of the same enzyme system were used.

System	Experiment	Radioactivity recovered, c.p.m./mg. C in HIV Precursors: 2-C^{14}-NaOAc (1.5×10^5 c.p.m./MgC)	3′-C^{14}-HMG (1.0×10^4 c.p.m./MgC)
Cell-free homogenate	1	1110	1830
	2	770	710
Particle-free extract	3	640	710
	4	890	700

HIV obtained biosynthetically from HMG this can be accounted for by the formation of some radioactive CO_2. The carboxylation reaction[12] would thus account for the possible formation of carboxyl labeled AcAcOH.

TABLE III

DISTRIBUTION OF C^{14} IN BIOSYNTHETIC HIV[a] OBTAINED FROM 2-C^{14}-NaOAc AND 3′-C^{14}-HMG

β-Hydroxyisovaleric acid	Radioactivity recovered as $BaCO_3$, c.p.m./mg. C: HIV from 3′-C^{14}-HMG precursor	HIV from 2-C^{14}-HOAc precursor
COOH	50– 60 (3%)	110–130 (5%)
CH_2	190–230 (14%)	690–760 (30%)
HCOH	100–120 (7%)	80–100 (4%)
CH_3 CH_3	560–600 (75%)	780–840 (61%)
Total oxidation found	300–320	510–530
Total oxidation calcd.	240	590

[a] Pooled materials obtained from cell-free homogenates and particle-free systems.

Experimental

β-Hydroxyisovaleric acid[13–15] was prepared by a modification of Saizew's[13] method; dimethylallylcarbinol was ozonized in ethyl acetate. The index of refraction of HIV obtained by each synthetic method as well as the biosynthetic HIV was identical, $n^{20}D$ 1.435.

The preparation of tissue slices, cell-free homogenates and aqueous extracts of rat liver has been described earlier.[9–11] The only modification involved the preparation of the aqueous extract of the mitochondrial fraction. This fraction was treated with 3 volumes of H_2O and rehomogenized for 30 seconds instead of the previous method which involved intermittent shaking for 45 minutes. The buffers utilized, co-factors and methods of separation have all been described.[4–8] The purity of the isolated HIV was demonstrated by the constant activity of the original preparation as compared to the activity shown by the Ag and Cu salts[13] as well as the eluate obtained after paper chromatography.[12] (None of these preparations absorbed bromine; therefore, contamination of HIV with SA was considered minimal.) Incubation of inactive enzyme preparations with C^{14}-labeled substrates yielded no radioactive HIV.

The degradation of HIV was accomplished by the utilization of several standard methods. The Schmidt reaction yielded the carboxyl carbon as CO_2. Dehydration of HIV to SA was accomplished with H_2SO_4[15]; the product was then sublimed and subsequently ozonized in ethyl acetate, yielding acetone and oxalic acid. The acetone fragments obtained from this ozonization was isolated with Denige's reagent. The resulting Hg–acetone complex was dissolved in acid and the acetone distilled; an aliquot of the distillate was treated with NaOI; the resulting iodoform was isolated, recrystallized and oxidized to CO_2. The remaining NaOAc fragment (derived from the acetone) was isolated and an aliquot of this material was oxidized to CO_2; another aliquot of this acetate was converted into the Ba salt and pyrolyzed to yield the carboxyl carbon of acetate as $BaCO_3$. This carbon was derived from the carbonyl group of the acetone. The oxalic acid was converted to its Ag salt and directly oxidized to CO_2. (The only carbon not directly isolated was carbon 2. This value was obtained from the difference in specific activities between the carboxyl carbon of HIV and the oxalic acid carbons.)

Cholesterol was isolated as the digitonide and plated as such. Most of the other materials were oxidized to CO_2 and radioassayed as $BaCO_3$ on a flow counter and corrected to infinite thinness. Variations in the activity of different derivatives of the same sample were within statistical counting error.

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RADIOISOTOPE UNIT, VETERANS ADMINISTRATION HOSPITAL
PHILADELPHIA, PENNSYLVANIA, AND
DEPARTMENT OF PHYSIOLOGICAL CHEMISTRY
SCHOOL OF MEDICINE
UNIVERSITY OF PENNSYLVANIA

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Crystallized N,N′-Diacetylchitobiose[1]

BY F. ZILLIKEN, G. A. BRAUN, C. S. ROSE AND P. GYÖRGY

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Among the partial breakdown products of chitin, N,N′-diacetylchitobiose was described for the first time in 1931.[2] It was obtained as an amorphous product having a melting point above 185° from decalcified chitin by acetolysis[2] followed by saponification of the crystalline hexa-*O*-acetyl-di-N-acetylchitobiose with KOH. The known structure of chitin[3,4] leads one to expect an N,N′-diacetylchitobiose to be a 2-acetamido-2-deoxy-4-(2-acetamido-2-deoxy-β-D-glucopyranosyl)-D-glucose.

Upon saponification of hexa-*O*-acetyl-di-N-acetylchitobiose, m.p. 290–291° and $[\alpha]^{26}D$ 55.3°, with dry methanolic ammonia, an amorphous product was obtained. It was demonstrated by means of paper chromatography to consist of five different components.

After chlorination of the paper, followed by spraying with an alcoholic solution of benzidine dihydrochloride[5] there were in addition to N,N′-diacetylchitobiose (IV, R_{lac} = 1.49) one faster (V, R_{lac} = 2.38) and 3 slower moving spots (I, R_{lac} = 0.51; II, R_{lac} = 0.66; III, R_{lac} = 0.96) visible, indicating that all five components possess the –NH–CO– group. The fastest moving spot V was identified as acetamide. Upon spraying with aniline oxalate I, II, III and IV gave a positive test, all four spots were negative to ninhydrin.

Chromatography of this amorphous mixture on a charcoal/Celite column[6] using aqueous ethanol of

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