Improved synthesis of 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA)

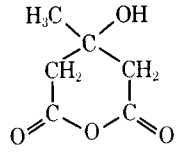
A. I. LOUW*, I. BEKERSKY, and E. H. MOSBACH

Department of Laboratory Diagnosis, Public Health Research Institute of the City of New York, Inc., and the Bureau of Laboratories, New York City Department of Health, New York 10016

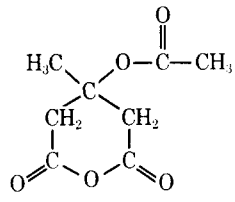
ABSTRACT An improved method for the chemical synthesis of HMG-CoA is described. The procedure is designed to prevent the formation of 3-acetoxy-HMG-CoA.

SUPPLEMENTARY KEY WORDS 3-acetoxy-3-methylglutaric anhydride · 3-hydroxy-3-methylglutaric anhydride · 3-acetoxy-3-methylglutaryl CoA

THE REDUCTION of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) to mevalonate is catalyzed by the enzyme HMG-CoA reductase (EC 1.1.1.34). This reaction is believed to be an important rate-determining step in the biosynthesis of cholesterol in the liver (1–4) as well as in the intestinal mucosa (5). The chemical synthesis of HMG-CoA, the substrate of HMG-CoA reductase, from HMG was first described by Hilz, Knappe, Ringelmann and Lynen (6) in 1958 and has since been employed by many investigators (e.g. 7, 8). It consists of converting HMG to 3-hydroxy-3-methylglutaric anhydride (I) by treatment of a benzene solution of HMG with excess acetic anhydride under reflux for 1 hr. The crystalline anhydride (I) (needles, mp 102–103°C) is then allowed to react with reduced coenzyme A in aqueous solution under mild conditions (in the cold, pH 7.5) to form the thioester (6). The solution of HMG-CoA so produced can be used directly (7, 8) or lyophilized, or a purer product can be obtained by paper chromatography ([7], solvent system not reported).



I



II

When searching the literature we noted that in 1953 Adams and Van Duuren (9) refluxed HMG ("dicrotalic acid") with acetic anhydride in benzene for 2 hr and obtained 3-acetoxy-3-methylglutaric anhydride (II) (prisms, mp 85°C). We therefore reinvestigated the synthesis of HMG-CoA and particularly of the intermediate anhydride (I). We have found that the reaction of HMG with acetic anhydride under the conditions specified by Hilz et al. (6) does not lead to the formation of I but results in the acetylated product II. By restudying the synthesis of I and II under various conditions we arrived at the conclusion that the results of Hilz et al. (6) and of Adams and Van Duuren (9) differ because the former probably employed acetic anhydride of high acetic acid content. On the basis of these experiments we have worked out an improved synthesis of I (and of HMG-CoA) by careful control of temperature and acetic anhydride concentration.

MATERIALS

3-Hydroxy-3-methylglutaric acid (mp 108°C) was purchased from Mann Research Laboratories, Inc., New York, and was dried before use in synthetic procedures for 24 hr over $CaSO_4$ at 0.01 mm Hg. 3-Hydroxy-3-methylglutaric-3-^{14}C acid and acetic-^{3}H anhydride were purchased from New England Nuclear Corp., Boston, Mass. Benzene (Merck and Co., Inc., Rahway,

Abbreviations: HMG, 3-hydroxy-3-methylglutaric acid.

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Please address requests for reprints to Dr. E. H. Mosbach, Public Health Research Institute, 455 First Avenue, New York, New York 10016.

N. J.) was dried over sodium. Acetic anhydride (Merck) was assayed for acetic acid by extracting it with ice-cold distilled water and titrating the acetic acid with N KOH and phenolphthalein as indicator (10). Acetic acid (Merck) and coenzyme A (free acid, chromatopure, P-L Biochemicals, Inc., Milwaukee, Wisc.) were used without further purification. The melting points reported herein are uncorrected.

METHODS

Paper Chromatography

HMG-CoA and 3-acetoxy-HMG-CoA were separated by ascending paper chromatography on Whatman filter paper No. 3 MM (in the direction of the grain of the paper) with the solvent system *n*-butanol–acetic acid–water 5:2:3, for 4 hr at 24° ± 1°C. Coenzyme A, HMG, and 3-acetoxy-HMG served as reference compounds. The latter was obtained by hydrolysis of 3-acetoxy-3-methylglutaric anhydride (II) in water under the conditions used in the preparation of 3-acetoxy-HMG-CoA, except that coenzyme A was omitted from the reaction mixture. This solution was used as a source of 3-acetoxy-HMG without further purification. The free acids were detected by spraying the air-dried chromatogram with *p*-dimethylaminobenzaldehyde in acetic anhydride (11). HMG appeared as a light brown spot, and the 3-acetoxy-HMG appeared as a more intense strawberry-colored spot.

Coenzyme A and its esters, HMG-CoA and 3-acetoxy-HMG-CoA, were detected under UV radiation. For radioactivity measurements the pertinent areas of the chromatograms were cut out and eluted with distilled water according to the procedure of Edstrom (12); aliquots of the eluate were counted in a Beckman LS 200 B liquid scintillation counter using 0.5% 2,5-diphenyloxazole and 10% naphthalene in dioxane as scintillation fluid. Suitable corrections were made for background and quenching. Doubly-labeled compounds were counted by internal standardization (13).

Spectrophotometry

IR absorption measurements of HMG, 3-hydroxy-3-methylglutaric anhydride (I), and 3-acetoxy-3-methylglutaric anhydride (II) were made in a Perkin–Elmer model 421 grating spectrophotometer. UV absorption measurements at 412 nm were done with a Beckman DU-2 spectrophotometer.

Chemical Analysis

Reduced coenzyme A was determined before and after hydrolysis (0.1 N KOH for 1 hr at 25°C) of HMG-CoA and 3-acetoxy-HMG-CoA by the method of Ellman (14) as modified by Penefsky and Warner (15) but without addition of ATP.

EXPERIMENTAL PROCEDURE AND RESULTS

3-Hydroxy-3-methylglutaric-3-^{14}C Anhydride (I)

3-Hydroxy-3-methylglutaric-3-^{14}C acid (125 mg, 0.77 mmole, 60.85 μc/mmole) was dissolved in 1.0 ml of acetic acid, and anhydrous benzene (2.8 ml) and acetic anhydride (0.4 ml, 3.85 mmoles) were added in this order. The mixture was stirred at 37°C (in an incubator) for 16 hr. The solvents and reagent were removed by vacuum distillation (24°C, 10 mm Hg), whereupon crystallization occurred in the distilling flask. The crude crystalline material was crystallized twice from anhydrous benzene to yield fine needles; 91.0 mg (83% of theoretical), mp 101–102°C (lit. 102–103°C [5]), 60.85 μc/mmole.

Analysis: $C_6H_8O_4$ (144.12);
calculated: C, 50.00; H, 5.60
found: C, 50.10; H, 5.61

The IR spectrum of the above (0.5 mg/200 mg KBr) showed a hydroxyl band at 3500 cm^{-1} ascribable to weak hydrogen bonding resulting from single-bridge dimers (16). There were two anhydride bands, one at 1805 cm^{-1} and a second at 1750 cm^{-1}. In HMG the absorption due to the hydroxyl function extends over a broad spectrum with an alcoholic hydroxyl band at 3215 cm^{-1} and a bonded hydroxyl band at 2660 cm^{-1}. There was a carbonyl band at 1710 cm^{-1} (9).

3-Acetoxy-^3H-3-methylglutaric-3-^{14}C Anhydride (II)

A mixture of 3-hydroxy-3-methylglutaric-3-^{14}C acid (125 mg, 0.77 mmole, 60.85 μc/mmole), anhydrous benzene (2.8 ml), and acetic-^3H anhydride (1.4 ml, 15 mmoles, 401.4 μc/mmole) were boiled under reflux for 1 hr. The solvent and reagent were removed by vacuum distillation (22°C, 10 mm Hg). Crystallization of the resulting syrup from anhydrous ether resulted in prisms; 98 mg (68% of theoretical), mp 85°C (lit. 85°C [10]), ^{14}C:60.8 μc/mmole; ^3H: 200.7 μc/mmole.

Analysis: $C_8H_{10}O_5$ (186.2);
calculated: C, 51.61; H, 5.41
found: C, 51.82; H, 5.46

The above compound (1.0 mg/150 mg KBr) showed no IR absorption band for the hydroxyl function at 3500 cm^{-1}. It exhibited the characteristic cyclic anhydride absorption at 1800 cm^{-1} and 1750 cm^{-1} and an ester carbonyl band at 1720 cm^{-1}. These values were in accordance with those reported by Adams and Van Duuren (9).

3-Hydroxy-3-methylglutaryl-3-^{14}C Coenzyme A

Coenzyme A (31 mg, 33 μmoles) was dissolved in 2.0 ml of ice-cold water while nitrogen was slowly bubbled through the solution, which was stirred with a magnetic stirrer. The pH was adjusted to approximately 7.5 with 1 N KOH. Saturated $KHCO_3$ solution (0.2 ml) was added as a buffer. 3-Hydroxy-3-methylglutaric-3-^{14}C anhydride (5.0 mg, 35 μmoles, 60.85×10^{-3} μc/μmole) was added slowly with stirring until the nitroprusside test (17) for free sulfhydryl groups was negative. The pH of the solution was adjusted to 5.5 (pH meter) with 2 N HCl. The final volume was 3.0 ml. The solution was stored at −20°C.

Paper Chromatography. The R_f values observed were: principal spot, 0.35 (HMG-CoA); traces, 0.17 (coenzyme A) and 0.76 (3-hydroxy-3-methylglutaric-3-^{14}C acid). The spot at 0.35 showed a positive nitroprusside test only after treatment of the chromatogram with methanolic NaOH (18).

Chemical Analysis. Color could be produced with Ellman's reagent (14) only after hydrolysis of an aliquot of the solution with KOH. The chromatographic and analytical data were considered evidence for the presence of the thioester, HMG-CoA.

Yield. Radioactivity assay of spots from the paper chromatogram indicated a yield of 75% and chemical determination a yield of 74% based on reduced coenzyme A.

3-Acetoxy-^3H-3-methylglutaryl-3-^{14}C Coenzyme A

The synthesis was performed as above except that 3-acetoxy-^3H-3-methylglutaric-3-^{14}C anhydride (5.0 mg, 27 μmoles, ^{14}C: 60.85×10^{-3} μc/μmole; ^3H: 200.7×10^{-3} μc/μmole; [$^3H/^{14}C$ = 3.3]) and coenzyme A (24.1 mg, 25.5 μmoles) were employed. The final volume was 3.0 ml, and the solution was stored at −20°C.

Paper Chromatography. The observed R_f values were: principal spot, 0.51 (3-acetoxy-HMG-CoA); traces, 0.17 (coenzyme A), 0.76 (3-hydroxy-3-methylglutaric-3-^{14}C acid), and 0.87 (3-acetoxy-^3H-3-methylglutaric-3-^{14}C acid). The spot at 0.51 gave a positive nitroprusside test after it was sprayed with methanolic NaOH (18).

Radioactivity Measurements. The $^3H/^{14}C$ ratio of 3-acetoxy-^3H-3-methylglutaric-3-^{14}C anhydride was 3.19. This ratio remained unchanged (3.14) in 3-acetoxy-^3H-3-methylglutaryl-3-^{14}C coenzyme A, which indicated that within the experimental error there was no appreciable hydrolysis of the acetyl group during the formation of 3-acetoxy-HMG-CoA.

Yield. By radioactivity assay of spots from the paper chromatogram, the yield of 3-acetoxy-HMG-CoA was 79%, calculated on the basis of reduced coenzyme A used. This value decreased to 49% after storage of the reaction mixture at −20°C for 10 days and occasional thawing and refreezing.

TABLE 1 EFFECT OF ACETIC ACID CONTENT OF REACTION MIXTURE ON THE FORMATION OF 3-ACETOXY-1-^{14}C-3-METHYLGLUTARIC ANHYDRIDE (II)*

Expt.	Acetic-1-^{14}C Anhydride	Acetic Acid	Amount of II Formed
	mmoles	*mmoles*	%
1	14.9	—	68†
2	1.7‡	—	37.8§
3	1.55‡	21.9	7.7§
4	2.3	20.6	21§

* 3-Hydroxy-3-methylglutaric acid (125 mg, 0.77 mmole), acetic-1-^{14}C anhydride (0.27 μc/mmole), and anhydrous benzene (2.8 ml) were boiled under reflux for 1 hr. The amounts of acetic-1-^{14}C anhydride and acetic acid added to the reaction mixture were varied as indicated. The reagent and solvent were removed by vacuum distillation. The residual oil was taken up in a small amount of anhydrous ethyl ether, and anhydrous hexane was added until no further precipitation occurred. This precipitate (estimated to contain 84–100% of the reaction products) represents a mixture of I and II. Attempts to obtain pure I and II from these mixtures by crystallization resulted in low yields of I.

† Recrystallized product, representing minimum yield.

‡ Further reduction of acetic-1-^{14}C anhydride concentration produced mixtures of starting material (HMG), I, and II.

§ Yield based on radioactivity data.

Several attempts to synthesize 3-hydroxy-3-methylglutaric anhydride (I) according to the method of Hilz et al. (6) proved unsuccessful. The procedure consistently yielded 3-acetoxy-3-methylglutaric anhydride (II) as indicated by IR measurements, melting point, elemental analysis, and radioactivity data (incorporation of acetic-1-^{14}C anhydride of known specific activity into the final product). This result might have been predicted since the reaction conditions reported by Hilz et al. (6) were essentially the same as those of Adams and Van Duuren (9) for the synthesis of 3-acetoxy-3-methylglutaric anhydride (II). It occurred to us that Hilz et al. (6) might have employed acetic anhydride containing a relatively high proportion of acetic acid. In order to investigate this possibility, we repeated the synthetic procedure described by Hilz et al. (6) with acetic acid added to the reaction mixture, as indicated in Table 1.

When the synthesis of the anhydride is carried out near the boiling point of benzene, it is necessary to reduce the concentration of acetic anhydride and to increase the concentration of acetic acid in order to prevent the formation of the acetylated anhydride (II). The presence of this anhydride, even in relatively low proportions, in the reaction mixture interferes with the crystallization of the desired nonacetylated product (HMG-anhydride, and greatly reduces its yield (Table 1).

DISCUSSION

These experiments suggest the possibility that when HMG-CoA is prepared chemically via the cyclic anhy-

dride, the acetylated derivative (3-acetoxy-HMG-CoA) may be formed if the relative amounts of acetic anhydride and acetic acid are unfavorable. That some workers previously may have synthesized 3-acetoxy-HMG-CoA rather than HMG-CoA seems likely in view of our observation that the 3-acetoxy group is not lost during formation of the thioester bond between 3-acetoxy-3-methylglutaric anhydride (II) and coenzyme A. The improved method of synthesis of 3-hydroxy-3-methylglutaric anhydride (I) described in the present paper overcomes this difficulty.

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References

1. Gould, R. G., and E. A. Swyryd. 1966. *J. Lipid Res.* **7:** 698.
2. Siperstein, M. D., and M. J. Guest. 1960. *J. Clin. Invest.* **39:** 642.
3. Bucher, N. L. R., P. Overath, and F. Lynen. 1960. *Biochim. Biophys. Acta.* **40:** 491.
4. Siperstein, M. D., and V. M. Fagan. 1966. *J. Biol. Chem.* **241:** 602.
5. Dietschy, J. M. 1968. *J. Clin. Invest.* **47:** 286.
6. Hilz, H., J. Knappe, E. Ringelmann, and F. Lynen. 1958. *Biochem. Z.* **329:** 476.
7. Linn, T. C. 1967. *J. Biol. Chem.* **242:** 984.
8. Linn, T. C. 1967. *J. Biol. Chem.* **242:** 990.
9. Adams, R., and B. L. Van Duuren. 1953. *J. Amer. Chem. Soc.* **75:** 2377.
10. Fieser, L. F., and M. Fieser. 1967. Reagents for Organic Synthesis. John Wiley & Sons, Inc., New York. 3.
11. Nordmann, J., and R. Nordmann. 1969. *In* Chromatographic and Electrophoretic Techniques. I. Smith, editor. William Heinemann Medical Books, Ltd., London. **1:** 371.
12. Edstrom, R. D. 1968. *Anal. Biochem.* **26:** 204.
13. Okita, G. T., J. J. Kabara, F. Richardson, and G. V. LeRoy. 1957. *Nucleonics.* **15:** 111.
14. Ellman, G. L. 1959. *Arch. Biochem. Biophys.* **82:** 70.
15. Penefsky, H. S., and R. C. Warner. 1965. *J. Biol. Chem.* **240:** 4694.
16. Bellamy, L. J. 1958. The Infrared Spectra of Complex Molecules. John Wiley & Sons, Inc., New York. 99–100.
17. Grunert, R. R., and D. H. Phillips. 1951. *Arch. Biochem. Biophys.* **30:** 217.
18. Stadtman, E. R. 1957. *Methods Enzymol.* **3:** 938.