The role of glutamine in the 1-carbon transfer reaction is yet to be elucidated.

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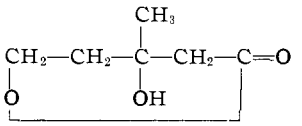
THE UTILIZATION OF β-HYDROXY-β-METHYL-δ-VALEROLACTONE IN CHOLESTEROL BIOSYNTHESIS

Sir:

Wolf, *et al.*,[1] have shown recently that the structure of the growth-promoting factor for *Lactobacillus acidophilus* (ATCC 4963) discovered by Skeggs, *et al.*,[2] and obtained by Wright, *et al.*,[3] is β-hydroxy-β-methyl-δ-valerolactone



The structural similarity of this lactone to β-hydroxy-β-methylglutaric acid prompted us to investigate its possible role in cholesterol metabolism. To this end we have studied cholesterol synthesis in cell-free rat liver homogenates using the technique described by Bucher[4] and modified by Rabinowitz and Gurin.[5]

Material prepared from distillers' solubles, as well as synthetic DL samples, suppress the incorporation of 1-C^{14}-acetate into cholesterol (expts. 1, 2, Table I). Since such an effect is open to several interpretations, β-hydroxy-β-methyl-δ-valerolactone (DVA) labeled in the 2-position with C^{14} was synthesized[6] and used in a second experiment. The lactone-C^{14} was incorporated into cholesterol to such a degree as to indicate preferential utilization of the 2-carbon of the lactone over the 1-carbon of acetate for sterol synthesis (expt. 2, Table I).

In the light of these observations, and the experiments of Rabinowitz and Gurin,[5] of Bloch, *et al.*,[7] and of Rudney,[8,9] indicating that β-hydroxy-β-methylglutaric acid (HMG) and β,β-dimethylacrylic acid (DMA) may be incorporated into cholesterol, experiments were conducted to determine the relative efficiency of HMG, DMA and DVA in contributing isotope for cholesterol biosynthesis.[10] The results of one of these experiments are shown in Table II.

(1) D. E. Wolf, C. H. Hoffman, P. E. Aldrich, H. R. Skeggs, L. D. Wright and K. Folkers, THIS JOURNAL, **78**, 4499 (1956).

(2) H. R. Skeggs, L. D. Wright, E. L. Cresson, G. D. E. MacRae, C. H. Hoffman, D. E. Wolf and K. Folkers, *J. Bact.*, in press.

(3) L. D. Wright, E. L. Cresson, H. R. Skeggs, G. D. E. MacRae, C. H. Hoffman, D. E. Wolf and K. Folkers, THIS JOURNAL, in press.

(4) N. L. R. Bucher, THIS JOURNAL, **75**, 498 (1953).

(5) J. L. Rabinowitz and S. Gurin, *J. Biol. Chem.*, **208**, 307 (1954).

(6) Prepared for us by Dr. C. S. Miller of this laboratory.

(7) K. Bloch, L. C. Clark and I. Harary, *J. Biol. Chem.*, **211**, 687 (1954).

(8) H. Rudney, THIS JOURNAL, **76**, 2595 (1954).

(9) H. Rudney, *ibid.*, **77**, 1698 (1955).

(10) We wish to acknowledge our indebtedness to Dr. S. Gurin, Department of Physiological Chemistry, School of Medicine, University of Pennsylvania, Philadelphia, and to Dr. J. L. Rabinowitz, Radioisotope Unit, Veterans Administration Hospital, Philadelphia, for generous gifts of both natural and radioactive HMG and DMA.

TABLE I

INCORPORATION OF ACETATE AND OF β-HYDROXY-β-METHYL-δ-VALEROLACTONE (DVA) INTO CHOLESTEROL IN RAT LIVER HOMOGENATES

Each flask contained 5 ml. of liver homogenate, and 1 mg. each of ATP and DPN. Labeled or non-labeled substrates were added as indicated. Final volume was 9.5 ml. Gas phase was 95% O_2–5% CO_2. In a given experiment all flasks contained aliquots of the same liver preparation. Incubation was carried out at 37° for 4.5 hours. Cholesterol was isolated and counted as the digitonide.

Expt.	Substrates NaOAc	Substrates DVA	Activity added total c.p.m. × 10^5	Recovered cholesterol c.p.m./mg. C
1	0.16 mg., 1-C^{14}	....	8.7	1,280
	0.16 mg., 1-C^{14}	80 units[a] concentrate	8.7	570
2	0.10 mg., 1-C^{14}	....	1.15	4,800
	0.10 mg., 1-C^{14}	0.92 mg., synthetic, non-labeled	1.15	2,180
	0.10 mg. non-labeled	0.92 mg., 2-C^{14}	1.15	28,400

[a] One unit of activity has since been shown to be equivalent to 0.010 mg. of DL synthetic material.

TABLE II

ACTIVITY OF HMG, DMA, AND β-HYDROXY-β-METHYL-δ-VALEROLACTONE (DVA) IN CHOLESTEROL BIOSYNTHESIS BY RAT LIVER HOMOGENATES

Protocol as in Table I. All flasks contained aliquots from one pool of liver homogenate. "Corrected" values are calculated to DVA as standard. Each compound was added at the level of 6 μM. per flask. Total cholesterol estimated by a modification of the method of Abell, *et al.*[11]

Substrates	Activity added total c.p.m. × 10^4	C^{14} found c.p.m./mg. C.	Cholesterol recovered C^{14} corrected c.p.m./mg. C	Total mg./flask
3′-C^{14}-HMG	33.0	400	139	1.61
4-C^{14}-DMA	4.9	1,350	3,170	1.64
2-C^{14}-DVA	11.5	33,700	33,700	1.77

These data indicate that only 0.16% of the isotope of 3′-C^{14}-HMG and 3.8% of the radioactivity of 4-C^{14}-DMA are incorporated into cholesterol. In contrast, 43.4% of the isotope of 2-C^{14}-DVA appears in the sterol. If the utilization by liver tissue is restricted to only one of the optical isomers of synthetic factor (as is the case with *Lactobacillus acidophilus*), then it appears that virtually all of the biologically available DVA-C^{14} must have been transferred to sterol. It has been reported that while some HMG can be utilized *in toto* for the synthesis of β-hydroxyisovaleric acid (and presumably cholesterol), a considerable amount of HMG undergoes cleavage to acetate and acetoacetate.[12,13] On the other hand, the great extent of conversion of the C^{14} carbon of DVA into cholesterol suggests that the major pathway of cholesterol biosynthesis from this compound is direct, as opposed to cleavage to smaller molecules.

It thus appears that β-hydroxy-β-methyl-δ-va-

(11) L. L. Abell, B. B. Levy, B. B. Brodie and F. E. Kendall, *J. Biol. Chem.*, **195**, 357 (1952).

(12) B. K. Bachhawat, W. G. Robinson and M. J. Coon, THIS JOURNAL, **76**, 3098 (1954).

(13) J. L. Rabinowitz, *ibid.*, **77**, 1295 (1955).

lerolactone is either the important biological precursor of isoprene units, or is converted by some relatively minor biochemical transformation to an "active isoprene" unit. In either case, this compound offers promise in the study not only of steroid biogenesis but also of other natural substances that arise completely or in part by the condensation of isoprenoid units, *e.g.*, the carotenes, the tocopherols and rubber.

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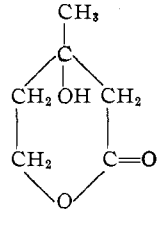
β-HYDROXY-β-METHYL-δ-VALEROLACTONE (DIVALONIC ACID), A NEW BIOLOGICAL FACTOR

Sir:

A new acetate-replacing factor for lactobacilli has been identified by structural degradation and synthesis as β-hydroxy-β-methyl-δ-valerolactone (I). The discovery of this substance and its preparation in highly purified form has been described.[1,2] In aqueous solution, the substance gave an acidic reaction. The potentiometric titration curve rose sharply on addition of alkali, and then drifted in the manner characteristic of lactones. A back-titration of the alkaline solution gave a typical neutralization curve with an equivalent weight of 128 and a *p*H $^1/_2$ value of 4.3 which indicates an acid strength intermediate between that of an unsubstituted carboxylic acid such as acetic acid and a stronger α-hydroxy acid such as lactic acid.

The infrared spectrum of the substance in chloroform gave clear evidence for the presence of hydroxyl-function (2.90–2.95 μ) and δ-lactone function (5.78 μ), but no indication of a carboxyl group. When the substance was dissolved in morpholine and its infrared spectrum recorded at intervals over a period of forty-eight hours, the band ascribed to the δ-lactone function (5.78 μ) slowly decreased in intensity while a band due to carboxyl-function (6.1 μ) appeared and then increased.

Reactions were carried out on the factor with acetic anhydride and benzoyl chloride but non-crystalline products were obtained; reaction with *p*-nitrobenzoyl chloride and pyridine gave decomposition.



I

Amides were formed with ammonia and benzylamine, but they were not obtained crystalline. However, a crystalline amide was obtained with benzhydrylamine which could be used for final critical purification and for analysis; m.p. 92–93°, $[\alpha]^{20}D$ −2.0° (*c* = 20 mg./ml. in ethanol). (Calcd. for $C_{19}H_{23}NO_3$: C, 72.82; H, 7.40; N, 4.47. Found: C, 72.70, 72.60; H, 7.17, 7.07; N, 4.74.) Analysis for the presence of a C-methyl group in the benzhydrylamide gave results suggestive of one terminal methyl group (calcd. for 1 C-methyl, 4.80. Found: C-methyl, 5.9).

Both the lactone and its benzhydrylamide were examined for the presence of adjacent hydroxyl groups by reaction with alkaline periodate. No periodate was consumed, which indicated that a glycol structure was not present. With alkaline iodine no iodoform was produced from either the lactone or its benzhydrylamide which indicated the absence of the grouping: CH_3CHOH.

Acetylation of the benzhydrylamide with acetic anhydride in pyridine gave a monoacetate; m.p. 104–105°, $[\alpha]^{20}D$ +1.6° (*c* = 45 mg./ml. in ethanol) (calcd. for $C_{21}H_{25}NO_4$: C, 70.97; H, 7.09; acetyl, 12.1. Found: C, 70.70, 70.80; H, 7.09, 6.87; acetyl, 11.4).

The structure β-hydroxy-β-methyl-δ-valerolactone (I) is compatible with these known facts. This structure was confirmed by synthesis. Partial reduction of β-hydroxy-β-methylglutaric acid, a compound previously known as a possible precursor of sterols,[3,4] gave DL-β-hydroxy-β-methyl-δ-valerolactone. The benzhydrylamides of the synthetic DL-lactone and the factor prepared from distillers solubles had identical infrared spectra. Hydrolysis of the synthetic benzhydrylamide gave the DL-lactone which was one-half as active microbiologically as the factor prepared from distillers solubles.

The generic name divalonic acid is being used to designate the corresponding β,δ-dihydroxy-β-methylvaleric acid. The function of divalonic acid in steroid synthesis is presented in the accompanying paper.[5]

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(5) P. A. Tavormina, M. H. Gibbs and J. W. Huff, THIS JOURNAL, **78**, 4498 (1956).