[CONTRIBUTION FROM THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF WASHINGTON]

The Origin of Some of the Carbon Atoms of the Side Chain of C^{14}-Ergosterol[1]

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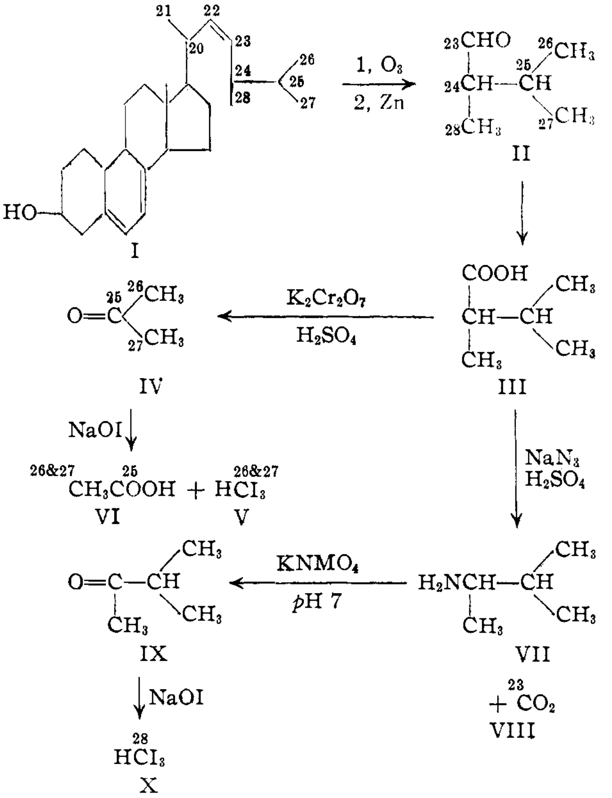
The terminal six carbon side chain unit of C^{14}-ergosterol synthesized from acetic acid-1-C^{14} by "CoA rich" yeast has been examined for its isotope distribution by a procedure not heretofore described. Carbons 23 and 25 appear to have been derived from the carboxyl group of acetic acid.

It has been shown that acetic acid can be a sole source of carbon for ergosterol formation in the *acetate-less* mutant of *Neurospora crassa*[4] and in *Saccharomyces cerevisiae*.[5] Similar observations have been made by Bloch, *et al.*,[6] on the formation of cholesterol from acetate. Inasmuch as the origin of the carbons of the isoöctyl side chain of cholesterol from the methyl or carboxyl carbon atoms of acetic acid has been established,[6c,7] data on the distribution pattern of isotopic carbon in ergosterol synthesized from acetic acid would be of considerable interest. In this way one could ascertain whether the biosynthetic pathways for ergosterol and cholesterol are similar.

Attention was directed toward the elucidation of the position of labeling in the side chain of C^{14}-ergosterol synthesized from acetic acid-1-C^{14} by "CoA rich" yeast. The first point of chemical attack was the terminal six-carbon unit of the side chain. The degradation scheme shown in the accompanying diagram was adopted. So far as is known, this represents the first stepwise degradation of this portion of the ergosterol molecule.

This procedure involved the ozonolysis of ergosterol (I), essentially by the technique described by Guiteras, *et al.*,[8] and treatment of the product with zinc dust to give isopropylmethylacetaldehyde (II), which was converted by permanganate oxidation to α,β-dimethylbutyric acid (III). Two schemes were used to determine the location of radioactivity in the various carbons of this acid. In the first procedure III was converted by dichromate oxidation to acetone (IV), which was oxidized to iodoform (V) (C_{26} and C_{27}) and acetic acid (VI) (C_{25}, C_{26} and C_{27}). In the second scheme III was converted by the Schmidt azide reaction,[9] as modified by Phares,[10] to 2-amino-3-methylbutane (VII) and CO_2, representing C_{23}. VII was oxidized to methyl isopropyl ketone (IX), which on treatment with hypoiodite gave rise to iodoform (X), representing C_{28}. Carbon 24 was determined indirectly by the difference in specific activity of the amine VII and the total specific activity of C_{25}, C_{26}, C_{27} and C_{28}.



The data obtained on the specific activity of the various carbons of the six-carbon unit of the side chain of C^{14}-ergosterol are given in Table I. A comparison of the specific activity of the ergosterol, 2555 c./min./mmole C, and the α,β-dimethylbutyric acid, 1490 c./min./mmole C, indicates that there is most probably a higher concentration of isotope in the nucleus than in the side chain. As is evident from the data in Table I the radioactivity in the terminal six-carbon unit was located almost exclusively in carbons 23 and 25. The calculated specific activity values in Table I were based on the assumption that only two of the carbon atoms of sodium α,β-dimethylbutyrate were radioactive. If this assumption was correct, then each of the two radioactive carbon atoms should have a specific activity close to the predicted value. A comparison of the theoretical value of 4,470 c./min./mmole C with that of 4,200 c./min./mmole C for C_{23} and 4,080 c./min./mmole C for C_{28} showed this prediction to be true. Apparently both of these carbons are derived solely from the carboxyl group of acetic acid. It would appear that the close

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[2] Part of the thesis submitted by Salih J. Wakil to the Graduate School, University of Washington, September, 1952, in partial fulfillment of the requirements for the degree of Doctor of Philosophy in Biochemistry.

[3] Bursary Student of Ministry of Education of Iraq.

[4] R. C. Ottke, E. L. Tatum, I. Zabin and K. Bloch, *J. Biol. Chem.*, **189**, 429 (1951).

[5] D. J. Hanahan and S. J. al-Wakil, *Arch. Biochem. Biophys.*, **37**, 167 (1952).

[6] (a) K. Bloch and D. Rittenberg, *J. Biol. Chem.*, **145**, 625 (1942); (b) K. Bloch and D. Rittenberg, *ibid.*, **159**, 45 (1945); (c) H. Little and K. Bloch, *ibid.*, **183**, 33 (1950).

[7] J. Wüersch, R. L. Huang and K. Bloch, *ibid.*, **195**, 439 (1952).

[8] A. Guiteras, Z. Nakamiya and H. H. Inhoffen, *Ann.*, **494**, 116 (1932).

[9] K. F. Schmidt, *Z. angew. chem.*, **36**, 511 (1923); *Ber.*, **57**, 704 (1924).

[10] E. F. Phares, *Arch. Biochem. Biophys.*, **33**, 173 (1951).

agreement of the specific activities of C_{23} and C_{25}, *i.e.*, 4,200 and 4,080 c./min./mmole C, supports the assumption that a given carbon atom of a sterol is derived from either a carboxyl or methyl carbon atom of acetic acid but not from both. These results are in essential agreement with the observations of Wüersch, *et al.*,[7] who found that carbons 20, 23 and 25 of the isoöctyl group of cholesterol were derived from the carboxyl group of acetic acid. Although carbon 28 contained a detectable s.a. 150 c./min./mmole C, it is believed at present that little significance can be attached to this observation. Comparison of this value with the data obtained on carbons 23 and 25 support this conclusion.

TABLE I

DISTRIBUTION OF THE CARBOXYL GROUP OF $CH_3C^{14}OOH$ IN THE SIDE CHAIN OF C^{14}-ERGOSTEROL

	Found c./min./ mmole C	Calculated,[a] c./min./mmole active C
Ergosterol	2555	
Na α,β-dimethylbutyrate	1490	4470[b]
2-Amino-3-methylbutane	850	4250[c]
C_{23}[d]	4200	4200
C_{24}[e]	0	0
C_{25}[f]	4080	4080
C_{25}, C_{26}, C_{27}[g]	1317	3950[g]
C_{26}, C_{27}	0	0
C_{28}	150	0

[a] This value is based on the assumption that only two carbon atoms of Na α,β-dimethylbutyrate are active. Each of the calculated values is based on the same assumption. [b] Calculated on basis that two carbon atoms are active (dilution factor is 3). [c] Calculated on basis that one carbon atom is active (dilution factor is 5). [d] Obtained as CO_2. [e] Calculated by difference from the specific activity of the amine VII and the sum of C_{25}, C_{26}, C_{27} and C_{28}. [f] Analyzed as acetic acid. [g] Obtained as acetone and specific activity calculated on basis that one carbon atom is active (dilution factor is 3).

Experimental

I. **Preparation of Acetic Acid-1-C^{14}.**—Carboxyl labeled acetic acid was synthesized by the Grignard technique as described by Lemmon.[11]

II. **Preparation of C^{14}-Ergosterol.**—Labeled ergosterol was prepared by incubation of "CoA rich" yeast with acetic acid-1-C^{14}. For use in the experiments described below, 12.0 mg. of ergosterol, s.a. 150,000 c./min./mg. was dissolved in 100 ml. of warm 3:1 ethyl alcohol–benzene mixture and approximately 10 g. of non-radioactive ergosterol was added. The mixture was allowed to cool and the crystals were collected on a filter. They were recrystallized once more from the same solvent system; m.p. 163–164°; specific activity, 187 c./min./mg. or 2,555 c./min./mmole C.

III. **Degradation of Ergosterol to Isopropylmethylacetaldehyde.**—Eight grams of ergosterol, s.a. 2,555 c./min./mmole C, was suspended in 80 ml. of aldehyde-free acetic acid.[12] Five to six per cent. ozone was passed through the mixture at room temperature until the ergosterol went into solution and the ozone was present in excess, as indicated by starch–iodide paper reaction.[8] During the reaction period of 1.5 hours, the temperature of the mixture increased to 35–40°. At the end of the ozonization, the solution was cooled to 10° and 10 g. of zinc dust added, with constant stirring, over a period of one hour. The mixture was stirred for an additional hour, then filtered by suction and the residue washed successively three times with 10 ml. of glacial acetic acid. The filtrate was transferred to a 1-liter round-bottom flask, 400 ml. of distilled water added and the mixture distilled slowly until no more oil drops came over (*ca.* 100 ml.). The distillate was neutralized with sodium hydroxide to *p*H 7.0 and redistilled. The distillate (about 30 ml.) contained the aldehyde; yield 1.2 g. (determined by conversion to sodium α,β-dimethylbutyrate). The aldehyde was identified through the following derivatives.

1. **Semicarbazone.**—This derivative was prepared by the addition of a semicarbazide–hydrochloric acid solution (neutralized with sodium bicarbonate) to a sample of the aldehyde. Immediately after vigorous shaking, white, needle-shaped crystals appeared, which were removed by vacuum filtration. The compound was recrystallized three times from water and twice from 1:1 benzene–petroleum ether. The crystals were dried at room temperature *in vacuo* for 24 hours over P_2O_5; m.p. 109–110°.[13] The melting point did not change upon sublimation at 0.001 to 0.005 mm. pressure and at 80°. *Anal.*[14] Calcd. for $C_7H_{15}ON_3$ (157): C, 53.50; H, 9.62; N, 26.70. Found: C, 53.58; H, 9.58; N, 26.66.[13]

2. **2,4-Dinitrophenylhydrazone.**—This compound was prepared in the usual manner.[15] Needle-shaped crystals were obtained and were recrystallized from 1:1 petroleum ether–benzene; m.p. 124–125°, reported value 124–125°.[13]

3. **α,β-Dimethylbutyramide.**—The aldehyde was oxidized with potassium permanganate to the acid.[15] The acid was extracted with diethyl ether, dried over anhydrous sodium sulfate and the ether evaporated under a fine stream of air. A colorless liquid was obtained. Calcd. for α,β-dimethylbutyric acid: neut. equiv., 116.0; found, neut. equiv., 114.0. The acid was neutralized with sodium hydroxide and the sodium salt obtained as crystals by concentration of this solution. The compound was recrystallized once from water; specific activity, 65.0 c./min./mg. or 1,490 c./min./mmole C.

Thirty milligrams of the above acid was refluxed with 0.5 ml. of thionyl chloride for 20 minutes. The reaction mixture was poured into 0.5 ml. of ice-cold concentrated ammonia solution. The precipitated amide was collected by vacuum filtration and purified by repeated crystallizations from water and from 1:1 petroleum ether–benzene; m.p. 129–130°, reported value 129–130°[13]; specific activity, 79.0 c./min./mg. or 1512 c./min./mmole C.

IV. **Degradation of Sodium α,β-Dimethylbutyrate.** 1. **Carbons 23 and 28.**—One-half millimole (69 mg.) of sodium α,β-dimethylbutyrate was subjected to the Schmidt sodium azide reaction,[9,16] as modified by Phares.[10] Carbon 23, liberated in a 95% yield as CO_2, was trapped in sodium hydroxide and precipitated as barium carbonate. The C^{14} of the barium carbonate was analyzed in the usual manner; specific activity, 4,200 c./min./mmole C.

The liberated amine, 2-amino-3-methylbutane, was distilled from a bath of temperature of 105–115°, into 0.2 *N* sulfuric acid solution. The mixture was concentrated to 3–5 ml. and an aliquot removed for C^{14} analysis; specific activity, 850 c./min./mmole C. The remaining amine sulfate solution was adjusted to *p*H 7.0, and 5 ml. of 5% potassium permanganate solution was added. The mixture was stirred at room temperature for 30 minutes and then made acidic with sulfuric acid and steam distilled. The distillate was trapped in 5 ml. of 1 *N* sodium hydroxide and excess iodine solution added. The precipitated iodoform was centrifuged, washed several times with distilled water and dried. This iodoform, derived from carbon 28, was analyzed for its C^{14} content; specific activity, 150 c./min./mmole C.

2. **Carbons 25, 26 and 27.**—One mmole (138 mg.) of sodium α,β-dimethylbutyrate was placed in a 30-ml., two-

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(11) R. M. Lemmon, in Calvin, *et al.*, "Isotopic Carbon," John Wiley and Sons, Inc., New York, N. Y., 1949, p. 272.

(12) Aldehyde-free acetic acid was prepared by distilling acetic acid three times over chromic acid.

(13) The reported melting point of this compound in the literature is 129–130° (F. Reindel and Kipplan, *Ann.*, **493**, 181 (1932)); see also reference (8), Guiteras, *et al.*: the aldehyde in this case also had been prepared by a similar procedure from ergosterol. On the basis of the other derivatives coinciding with the literature values, it is believed that the compound on hand was isopropylmethylacetaldehyde and that an error had occurred in the original reporting of the melting point value.

(14) Microanalyses were performed by Elek Micro Analytical Laboratories, 4763 West Adams Boulevard, Los Angeles, California.

(15) R. L. Shriner and R. C. Fuson, "The Systematic Identification of Organic Compounds," 3rd edition, John Wiley and Sons, Inc., New York, N. Y., 1948, p. 171.

(16) (a) R. Adams, "Organic Reactions," Vol. III, Chap. 8, John Wiley and Sons, Inc., New York, N. Y., 1946; (b) C. Schuerch and E. H. Huntress, THIS JOURNAL, **71**, 2233 (1949).

necked, pear-shaped distilling flask (A), which was fitted with a 30-ml. dropping funnel containing 20 ml. of 0.11 *M* potassium dichromate in 18 *N* sulfuric acid, and a water cooled condenser. Four hundred milligrams of 2,4-dinitrophenylhydrazine in 15 ml. of 4 *N* sulfuric acid was placed in a 60-ml. pear-shaped receiving flask (B), which was connected to a carbon dioxide trap. A slow stream of nitrogen (2 to 3 bubbles per second) was passed through the apparatus and 10 ml. of the dichromate solution was added. The reaction mixture was maintained at room temperature for 10 to 15 minutes. Flask A was then heated at 70° by means of a water-bath. Within 5 to 10 minutes crystals of acetone 2,4-dinitrophenylhydrazone appeared in the receiving flask (B). To ensure complete oxidation an additional 10 ml. of dichromate solution was added to the reaction mixture and the reaction continued for an additional 15 minutes.

The hydrazone was collected on a filter paper and crystallized from 95% alcohol. The crystals were dissolved in petroleum ether and any insoluble residue removed by filtration. The petroleum ether solution was concentrated to dryness *in vacuo* and the hydrazone crystallized twice from 1:1 petroleum ether–diethyl ether mixture, yielding 20 mg. of needle-shaped crystals, m.p. 125–126°, no depression on admixture with an authentic sample of acetone 2,4-dinitrophenylhydrazone. The yield from sodium α,β-dimethylbutyrate was 8.5%. *Anal.* Calcd. for $C_9H_{10}O_4N_4$ (238): C, 45.42; H, 4.20; N, 23.50. Found: C, 45.47; H, 4.27; N, 23.47.

Twenty milligrams of the radioactive acetone 2,4-dinitrophenylhydrazone was diluted with an equal amount of nonradioactive hydrazone and dissolved in 2 *N* sulfuric acid solution. The hydrazone solution was slowly distilled and the distillate was trapped in 7.5 ml. 1 *N* sodium hydroxide solution. After about 15 ml. of distillate had been collected, the trap was detached and excess iodine solution added to the distillate.[15] The iodoform was collected by centrifugation, washed with distilled water and analyzed for its C^{14} content; specific activity, 0.00 c./min./mmole C. This iodoform was derived from both C_{26} and C_{27}.

The supernatant remaining after separation of the iodoform was made acidic with dilute sulfuric acid and the excess iodine was titrated with sodium thiosulfate. The mixture was steam distilled and the distillate was neutralized with 0.1 *N* sodium hydroxide. A salt of an acid was obtained. The free acid was identified as acetic acid by its Duclaux distillation constant; specific activity of sodium salt, 49.8 c./min./mg. or 2,040 c./min./mmole C.

Radioactivity Measurements.—The counting equipment consisted of a Tracerlab Autoscaler and Flow Gas Counter. The carbon 14 content of a sample was determined by counting either a direct mount of the material on copper or aluminum disks, or by combustion of the sample to CO_2 and conversion to barium carbonate, which was then counted in the usual manner. All samples were counted a sufficient length of time to ensure less than a 4% counting error.

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Reaction Rates by Distillation. IV. The Effect of Changes in Structure on the Rate of the Baeyer Condensation

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It has been found that the Baeyer condensation proceeds smoothly in 80 to 90% yield when a benzene solution of an activated aromatic compound such as dimethylaniline or anisole, an aromatic aldehyde and *p*-toluenesulfonic acid is refluxed in an apparatus equipped with a Dean–Stark water trap. The reaction has been followed by observing the rate at which the by-product water collects and the rates of condensation of the *p*-$(CH_3)_2N$, CH_3O, CH_3, H, Cl and NO_2 benzaldehydes with dimethylaniline have been correlated quantitatively *via* Hammett's "ρ–σ" treatment. It is shown that the first of the two consecutive reactions involved in the Baeyer condensation is the rate-controlling one.

In previous papers of this series, it has been shown that etherifications, alkylations and Knoevenagel condensations may be carried out to advantage by refluxing a benzene solution of the reactants and catalyst in an apparatus equipped with a Dean–Stark trap to remove the by-product water as it forms.[2] These reactions may, furthermore, be followed quantitatively by observing the rate at which the water collects. The results of the extension of the method to the Baeyer condensation using *p*-toluenesulfonic acid as the catalyst are reported here. It is commonly considered that this condensation proceeds *via* the consecutive reactions[3,4]

$$p\text{-}RC_6H_4CHO + C_6H_5R' \longrightarrow p\text{-}RC_6H_4CHOHC_6H_4R'\text{-}p \quad (1)$$

$$p\text{-}RC_6H_4CHOHC_6H_4R'\text{-}p + C_6H_5R' \longrightarrow p\text{-}RC_6H_4CH(C_6H_4R'\text{-}p)_2 + H_2O \quad (2)$$

Dimethylaniline ($R' = N(CH_3)_2$) was found to react with various *p*-substituted benzaldehydes to give the expected products in high yield. The results of relative rate studies in which the *p*-substituent, R, was varied are shown in Table I.

TABLE I

THE CONDENSATION OF *p*-RC_6H_4CHO WITH $C_6H_5N(CH_3)_2$

R	$t_{0.5}$, min.	Yield,[a] %	$k \times 10^5$, l./mole/min.
NO_2	170	89	195 ± 4.0
Cl	475	83	69.8 ± 0.7
H	600	85	54.5 ± .8
CH_3	860	80	38.1 ± .8
CH_3O	1500	85	22.4 ± .5
$(CH_3)_2N$	5420	77	6.25 ± .1

[a] These are the yields of pure recrystallized product.

The tabulated rate constants were calculated on the assumption that the reactions are second order, *i.e.*, first order with respect to both the benzaldehyde and the dimethylaniline (see Experimental for details). In Fig. 1 it is shown that the increase in reaction rate with decrease in the ability of R

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(1) From the Ph.D. thesis of L. Q. Green, September, 1948.

(2) For the preceding article in this series see E. F. Pratt and E. Werble, THIS JOURNAL, **72**, 4638 (1950). It is evident that the distillation method should be applicable to the quantitative determination of the wide variety of functional groups which have been shown to undergo a reaction to give the theoretical yield of water.

(3) L. P. Hammett, "Physical Organic Chemistry," McGraw-Hill Book Co., Inc., New York, N. Y., 1940, p. 348.

(4) G. E. K. Branch and M. Calvin, "The Theory of Organic Chemistry," Prentice-Hall, Inc., New York, N. Y., 1944, pp. 482-483.