11979

Position of the Carbon Dioxide-Carbon in Propionic Acid Synthesized by *Propionibacterium.**

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The assimilation of carbon dioxide by heterotrophic bacteria was first established by Wood and Werkman[1] with the propionic acid bacteria. Recently the stable isotope of carbon (C^{13})[2] and radioactive carbon (C^{11})[3] have been used as tracers of the carbon dioxide fixed in the fermentation of glycerol. The fixed carbon has been shown to be predominantly in the products propionic and succinic acids. Wood, *et al.,*[2] demonstrated, in addition, that the fixed carbon in succinic acid is exclusively in the carboxyl group. These authors suggested that all the carbon dioxide is fixed by the following reaction

$$CO_2 + HCH_2 \cdot CO \cdot COOH \longrightarrow COOH \cdot CH_2 \cdot CO \cdot COOH$$

and that propionic acid containing fixed carbon is derived from the 4-carbon compounds. Accordingly, the fixed carbon of propionic acid would be in the carboxyl group. Therefore, the report by Carson, *et al.,*[4] of fixed carbon occurring in the α- and β-carbons as well as in the carboxyl carbon of propionic acid is of particular interest. These results imply that, in addition to the mechanism shown by Wood, *et al.,*[2] the propionic acid bacteria have another method of fixing carbon dioxide. The latter method might involve the synthesis of the entire chain from 1-carbon compounds, since the fixed carbon is suggested by Carson, *et al.,*[4] to be distributed among the 3 carbons of propionic acid. This observation places in question the present schemes of fermentation, for the implication is that the substrate

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[1] Wood, H. G., and Werkman, C. H., *J. Bact.*, 1935, **30**, 332; *Biochem. J.*, 1936, **30**, 618.

[2] Wood, H. G., Werkman, C. H., Hemingway, Allan, and Nier, A. O., *J. Biol. Chem.*, 1940, **135**, 789; 1941 submitted.

[3] Carson, S. F., and Ruben, S., *Proc. Nat. Acad. Sci.*, 1940, **26**, 422.

[4] Carson, S. F., Foster, J. W., Ruben, S., and Kamen, M. D., *Science*, 1940, **92**, 433.

may first be broken down to 1-carbon compounds and then synthesized to the final products of fermentation. While it is clear that autotrophic forms of life must perform such a synthesis, it does not seem necessary for heterotrophic forms of life to break down the substrate to a 1-carbon compound and then resynthesize it to final products. We have, therefore, reinvestigated the problem. Contrary to the report of Carson, *et al.*,[4] we find the fixed carbon is exclusively in the carboxyl group of propionic acid.

The experimental procedure was as follows: Propionic acid containing fixed carbon enriched with C^{13} was obtained from the fermentation of glycerol as previously described.[2] The volatile acids in the steam distillate contained approximately 3.0 g of propionic acid. The acids were neutralized, evaporated to 5 ml, acidified with 10 N sulphuric acid and the mixture was extracted three times with 5 ml portions of ether. The ether solution was dehydrated with calcium chloride and then distilled in a small fractionating column. The fraction containing propionic acid (136-141°C, mostly at 140°C) weighed 2.1 g and was degraded by the following reactions:

$$CH_3 \cdot CH_2 \cdot COOH \xrightarrow{\text{P and Br, then } H_2O} CH_3 \cdot CHBr \cdot COOH$$

$$CH_3 \cdot CHBr \cdot COOH \xrightarrow{AgOH} CH_3 \cdot CHOH \cdot COOH$$

$$CH_3 \cdot CHOH \cdot COOH \xrightarrow{KMnO_4} CH_3CHO + CO_2$$

$$CH_3CHO \xrightarrow{I_2 \text{ (alkali)}} CHI_3 + HCOONa$$

The above conversion was carried out as follows:

The reaction mixture after α-bromination[5] was made alkaline to litmus with silver oxide and then acid with hydrochloric acid to precipitate the silver and give free lactic acid. The filtrate solution was oxidized with potassium permanganate[2] and the resulting aldehyde and carbon dioxide were collected in bisulfite and alkali respectively. The recovery was about 40% (11.2 mM of acetaldehyde determined by iodine-titration of bound sulfite and 12.0 mM of carbon dioxide). Part of the aldehyde solution was oxidized[6] to carbon dioxide for mass spectrographic determination[7] of its C^{13} content. The remainder was converted into iodoform and formic acid.[8] The excess iodine in the iodoform reaction was titrated with thiosulfate and the mixture was then filtered. The iodoform was

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[5] Cohen, J. B., *Practical Organic Chemistry*, 1924, p. 110, Macmillan, London.

[6] Osburn, O. L., and Werkman, C. H., *Ind. Eng. Chem.*, Anal. Ed., 1932, **4**, 421.

[7] Nier, A. O., *Rev. Sci. Instruments*, 1940, **2**, 212.

[8] Goodwin, L. F., *J. Am. Chem. Soc.*, 1920, **42**, 39.

TABLE I.

Fraction of propionic acid	% C^{13}
Entire molecule	1.35
α and β-carbon (CH_3CHO)	1.07
α-carbon ($HCOONa$)	1.08
β-carbon (CHI_3)	1.08
COOH-carbon (CO_2)	1.77
COOH-carbon (calculated value)*	1.87

*$2 \times 1.09 + X = 3 \times 1.43$. $X = \%$ C^{13} in COOH-carbon.

dried for 24 hours at room temperature over calcium chloride and then oxidized to carbon dioxide by dry combustion. Formic acid in the filtrate was removed by steam distillation and oxidized to carbon dioxide with mercuric oxide.[9] The formic acid and iodoform were formed in equimolar quantities but the yield was only 50% on the basis of the acetaldehyde. From 4.0 mM of acetaldehyde 2.0 mM of iodoform and 2.1 mM of carbon dioxide were obtained. The iodoform reaction with acetaldehyde is not quantitative.[10] However, since the iodoform and formic acid were formed in equimolar amounts, these compounds undoubtedly arise from the β and α carbons respectively of the acetaldehyde. The data from the above procedure are given in Table I.

It is to be remembered in considering the data in Table I that all natural carbon contains approximately 1.09% C^{13}. The value 1.35 for propionic acid indicates there is carbon present in the molecule which was derived from fixed-carbon dioxide in which the C^{13} content had been artificially increased to serve as a tracer. The experimental variation in the mass spectrographic analysis is about ±0.02. Therefore the value 1.35 is clearly indicative of fixed carbon dioxide. The α and β carbons of the propionic acid contained the normal complement of C^{13}, evidently there was no fixed carbon in these positions. The carboxyl carbon, on the other hand, contained a concentration of C^{13} above that of normal carbon. The fixed carbon from $C^{13}O_2$ was obviously present in this position. The agreement between the experimental and calculated values, on the basis that all the fixed carbon is contained in the carboxyl group substantiates this conclusion. This fact is in agreement with the authors' concept of the mechanism of carbon dioxide-fixation through synthesis with 3-carbon and 1-carbon compounds. The discrepancy between our results and those of Carson, *et al.*,[4] calls for further investigation.

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[9] Osburn, O. L., Wood, H. G., and Werkman, C. H., *Ind. Eng. Chem.*, Anal. Ed., 1933, **5**, 247.

[10] Hatcher, W. H., and Mueller, W. H., *Trans. Roy. Soc. Canada*, 1929, Ser. 3, **23**, Sect. 3, 35.

There are two possibilities, either the chemical reactions used are unreliable or the synthetic reactions of the bacteria differed under the respective experimental conditions. That the stepwise degradation used in our investigation was reliable is shown by the quantitative recovery of the C^{13} in the carboxyl group. We are at present endeavoring to determine the reliability of the chemical reactions used by Carson, *et al.*,[4] by degrading chemically synthesized propionic acid containing C^{13} in the carboxyl group.

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11980

Repeated Attacks of Influenza.

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Recent reports[1, 2, 3] have established the principle that illnesses which on the basis of their similar clinical symptoms would be classified together as influenza can be caused by antigenically different viruses. The same principle must be taken into account when repeated attacks of influenza occur in an individual; that is, in a person who previously has had influenza a subsequent attack may be due to infection with a virus that is antigenically different from the one responsible for the earlier illness. That examples of that sort do occur will be shown by the results of the following experiments in which samples of serum obtained over a period of 4 to 5 years from persons who had had more than one attack of influenza were tested against two antigenically different viruses, the *TM* virus[1, 3] and the *PR8* strain of the virus of epidemic influenza.[4]

Patient A had 2, and Patient B had 3 attacks of influenza during the period of observation. The serums, which were 14 from each patient, included samples obtained at the time of onset of the symp-

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[1] Magill, T. P., PROC. SOC. EXP. BIOL. AND MED., 1940, **45**, 162.

[2] Francis, T., Jr., *Science*, 1940, **92**, 405.

[3] Magill, T. P., and Tyndall, M., in press.

[4] Francis, T., Jr., *Science*, 1934, **80**, 457.