

- ⁴ Ruben, Hassid and Kamen, *Jour. Am. Chem. Soc.*, **61**, 661 (1939).
⁵ Ruben, Kamen, Hassid and DeVault, *Science*, **90**, 570 (1939).
⁶ Additional evidence to be published shortly.
⁷ McAlister, *Jour. Gen. Physiol.*, **22**, 613 (1939).
⁸ Emerson and Lewis, *Am. Jour. Botany*, **26**, 808 (1939).
⁹ Cf. van Niel, *Ann. Rev. Biochem.*, **6**, 606 (1937); Gaffron, *Ibid.*, **7**, 986 (1939).

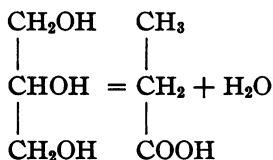
CO₂ ASSIMILATION BY PROPIONIC ACID BACTERIA STUDIED
 BY THE USE OF RADIOACTIVE CARBON

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The studies of Wood and Werkman^{1, 2, 3} as well as the work of Phelps, Johnson and Peterson⁴ have shown that propionic acid bacteria can utilize CO₂ during the fermentation of glycerol. In the absence of CO₂ this fermentation can be adequately represented by the equation:⁵



In the presence of CO₂ the formation of propionic acid is accompanied by the appearance of succinic acid in amounts closely equimolar with the quantity of absorbed CO₂.

This made it seem possible that CO₂ becomes converted into succinic acid by combination with a 3-carbon compound. The formation of succinic acid during the fermentation of pyruvate, dextrose and galactose by *Escher. coli*, particularly its dependence upon the CO₂ partial pressure,⁶ supports this view.

It is apparent that important information regarding the mechanism through which CO₂ is utilized can be obtained by the use of radioactive CO₂.⁷ We have employed this approach in a study of the fermentation of glycerol by *Propionibacterium pentosaceum*.

The bacteria were grown anaerobically in yeast extract-glycerol media in the presence of CO₂ for 3 to 6 days. For the experiments cells from 250 to 500 ml. of such cultures were centrifuged, washed and suspended in 0.5 per cent phosphate buffer at pH 7.0 with and without added substrates. The suspensions were shaken at 30°C. in the presence of N₂ and C*O₂.⁸ for

30 to 40 minutes. After centrifuging off the cells, propionic acid and succinic acid were added to the supernatant liquid to provide carriers for the small quantities of radioactive metabolic products. The volatile and non-volatile acids were separated by exhaustive steam distillation; the cells were boiled gently with acid for a few minutes and separated from the acid extract by centrifugation. Special care was taken to remove all traces of dissolved $C^{*}O_2$ before radioactivity measurements were made. The results of an experiment carried out in the presence of 2 per cent glycerol are summarized in table 1.

TABLE 1
BACTERIA + 2 PER CENT GLYCEROL + $C^{*}O_2$ FOR 40 MINUTES

	PER CENT OF TOTAL C^{*} ASSIMILATED ^a
Volatile acids	72.0
Non-volatile acids	10.0
Boiled cell extract	17.5
Cells after extraction	0.5

^a All measurements are corrected for decay and are therefore comparable.

The above table shows that 72 per cent of the $C^{*}O_2$ taken up by the cells can be recovered in the volatile acid fraction and only 10 per cent in the non-volatile acid fraction. The boiled cell extract consisted mainly of acids carried down with the cells.

The activity in the volatile fraction was found to be restricted to propionic acid by a Duclaux distillation. The C^{*} content of successive distillates is compared in table 2 with the titration values obtained with pure propionic acid distilled under the same conditions.

PER CENT VOLUME OF DISTILLATE	PER CENT RADIOACTIVITY FOUND	PER CENT ACIDITY BY TITRATION FOR PURE PROPIONIC ACID
20.0	23.0	24.5
40.0	45.0	46.7
60.0	65.0	67.0
80.0	84.0	85.1
100.0	100.0	100.0

The agreement between the radioactivity and titration measurements is remarkably good. This result excludes the presence of more than 5% of C^{*} in other fatty acids (formic, acetic, etc.).

The radioactive component of the non-volatile acid fraction was identified as succinic acid in two ways. The distribution coefficients of the non-

volatile material between water and ethyl ether by titration and radioactivity measurements are compared in table 3.

TABLE 3
DISTRIBUTION COEFFICIENTS
(10 MI. AQUEOUS PHASE + 90 MI. ETHER)

K	TITRATION	RADIOACTIVITY
	7.4	6.0

In addition, the non-volatile material was sublimed and the specific activity $\left(\frac{C^*}{\text{succinic acid}}\right)$ of the various fractions is shown in table 4.

TABLE 4
SUBLIMATION

FRACTION	SPECIFIC ACTIVITY $\left(\frac{\text{RADIOACTIVITY}}{\text{SUCCINIC ACID}}\right)$
1 (210-220°C.)	9.5
2 (220-240°C.)	9.9

No radioactive material came off below 210°C. It seems quite reasonable to conclude that the radioactivity is mainly due to succinic acid.

The remarkable result that so much of the radioactive carbon is found in propionic acid might be explained by assuming a reversible reaction $\text{CH}_3\text{CH}_2\text{COOH} + \text{CO}_2 \rightleftharpoons \text{COOHCH}_2\text{CH}_2\text{COOH}$. Experiments were performed to test this hypothesis by the addition of propionic acid and succinic acids to cell suspensions in the presence of C^*O_2 . In order to reduce the amount of glycerol and reserve products in the cell material, the bacteria were centrifuged from the medium in which they were grown, and suspended in phosphate buffer for 3 hours; this procedure was then repeated, and immediately before exposure to C^*O_2 they were again washed. The results of this experiment are shown in table 5.

TABLE 5
CELL SUSPENSION IN PHOSPHATE BUFFER EXPOSED TO C^*O_2 FOR 30 MINUTES

SUBSTRATE	TOTAL C^* ASSIMILATED (ARBITRARY UNITS)	RADIOACTIVITY OF VOLATILE ACID NON-VOL. ACID
Nothing	5.3	0.7
0.4 per cent Na propionate	4.8	0.78
0.2 per cent Na succinate	4.5	0.2
2.0 per cent glycerol	100.0	3.1

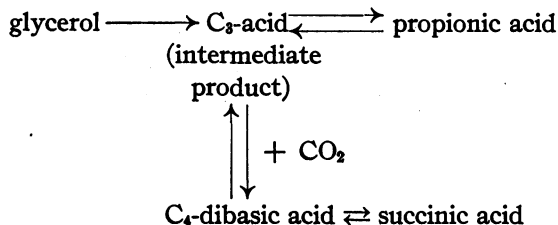
In a further experiment the cells were suspended in phosphate buffer for 7 hours and then centrifuged and washed as above. The results are summarized in table 6.

TABLE 6
CELL SUSPENSION IN PHOSPHATE BUFFER EXPOSED TO C*O₂ FOR 30 MINUTES

SUBSTRATE	TOTAL C* ASSIMILATED (ARBITRARY UNITS)	RADIOACTIVITY OF VOLATILE ACID NON-VOL. ACID
Nothing	2.3	0.41
2 per cent glycerol	100.0	3.0, 3.8
2 per cent glycerol + 0.4 per cent Na propionate	85.0	2.4

The addition of propionic acid in the absence of glycerol has little if any effect on the distribution of the C* between the propionic and succinic acids or on the total C* taken up. This is evidence against the formation of succinic acid from CO₂ and propionic acid. The presence of glycerol not only has an enormous effect on the C*O₂ uptake, but also increases the ratio of radioactive carbon in propionic acid and succinic acid. It seems quite likely in view of these results that propionic and succinic acids are formed via reactions between CO₂ and glycerol or intermediate products arising during its fermentation. Pyruvic acid, which could be a possible intermediate, was found to be inactive (<0.5%).

The data so far presented could find an explanation on the basis of a mechanism such as:



In this case one would expect the radioactive carbon atom to be located in the carboxyl groups. Further experiments are in progress to test this hypothesis.

The results of the above experiments are of general interest also in connection with a major problem encountered in tracer experiments with labeled carbon, namely, the synthesis of radioactive molecules starting with C*O₂. In many cases synthetic organic methods result in poor yields despite time-consuming and painstaking effort. In such instances the use of appropriate microorganisms to perform the desired synthesis may be highly desirable. We may cite the above experiments as an example. In 30 minutes the bacteria converted over 80 per cent of the assimilated C*O₂ into propionic and succinic acids, which are thus made readily available for tracer experiments.

It is a pleasure to thank Dr. M. D. Kamen and Professor E. O. Lawrence

of the Radiation Laboratory for their interest, and for making the radioactive carbon available. We are indebted to Professor C. B. van Niel and Professor H. A. Barker for much advice and assistance.

¹ *Biochem. Jour.*, **30**, 48 (1936).

² *Ibid.*, **32**, 1262 (1938).

³ *Ibid.*, **34**, 7 (1940).

⁴ *Biochem. J.*, **33**, 726 (1939).

⁵ C. B. van Niel, *The Propionic Acid Bacteria*, Haarlem (1928).

⁶ Elsdon, *Biochem. Jour.*, **32**, 187 (1938).

⁷ Ruben, Hassid and Kamen, *Jour. Am. Chem. Soc.*, **61**, 661 (1939).

⁸ The symbol C* will be used to represent carbon labeled by the radioactivity of some of its atoms. The isotope is C¹¹ and was produced in the Berkeley cyclotron; it has a half-life of 21 minutes.

THE REDUCTION OF RADIOACTIVE CARBON DIOXIDE BY METHANE-PRODUCING BACTERIA

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Several years ago¹ it was shown that the formation of methane in the fermentation of ethyl and butyl alcohols and of butyric acid by impure cultures of methane-producing bacteria is accompanied by the disappearance of an equi-molecular quantity of carbon dioxide. In the fermentation of ethyl alcohol, for example, the reaction can be rather closely described by the equation:



The same reaction has recently been shown² to be carried out by pure cultures of *Methanobacterium Omelianskii*.

As to the mechanism of the above reaction, it seemed quite likely that the acetic acid arises by an oxidation of ethyl alcohol, carbon dioxide being simultaneously reduced to methane. This would be in accordance with the view originally advanced by Professor C. B. van Niel that the methane fermentation of organic as well as of inorganic compounds is a process of oxidation by means of carbon dioxide which can be represented by the general equation:



where H₂A is an oxidizable molecule and A is its oxidation product. Conclusive proof in favor of this mechanism, however, was lacking. Furthermore, no direct experimental evidence could be obtained in support of the