* Work supported in part by a grant from the Rockefeller Foundation. The author wishes to thank Dr. David Bonner for supplying the hydroxy- and keto-acid analogues of leucine and isoleucine, and Miss Esther M. Zimmer for technical assistance in carrying out this investigation.

[1] Gray, C. H., and Tatum, E. L., these PROCEEDINGS, **30**, 404 (1944).

[2] Beadle, G. W., and Tatum, E. L., *Ibid.*, **27**, 499 (1941).

[3] Horowitz, N. H., Bonner, D., Mitchell, H. K., Tatum, E. L., and Beadle, G. W., *Amer. Nat.* (in press).

[4] Tatum, E. L., and Beadle, G. W., *Annals Mo. Botanical Garden*, **32**, 125 (1945).

[5] Roepke, R. R., Libby, R. L., and Small, M. H., *Jour. Bact.*, **48**, 401 (1944).

[6] Anderson, E. H., these PROCEEDINGS, **30**, 397 (1944).

[7] For descriptions and for further references see Robinow, C. F., *Jour. Hygiene*, **43**, 413 (1944).

[8] Binkley, F., and du Vigneaud, V., *Jour. Biol. Chem.*, **144**, 507 (1942).

[9] For a discussion of these relationships and for further references, see Shemin, D., and Rittenberg, D., *Jour. Biol. Chem.*, **158**, 71 (1945).

[10] Tatum, E. L., and Bell, T. T. (in preparation).

CARBON DIOXIDE UTILIZATION IN THE SYNTHESIS OF ACETIC ACID BY CLOSTRIDIUM THERMOACETICUM

BY H. A. BARKER AND M. D. KAMEN*

DIVISION OF PLANT NUTRITION, UNIVERSITY OF CALIFORNIA

Communicated June 20, 1945

In most carbohydrate fermentations the appearance of acetic acid or other C_2 products is accompanied by at least an equimolar quantity of carbon dioxide or other C_1 product. The carbohydrate fermentations caused by *Clostridium thermoaceticum* are a noteworthy exception to this generalization since the only product other than cell material is acetic acid.[1, 2] Specifically, glucose and xylose are acted upon by this organism to yield, respectively, about 2.65 and 2.25 moles of acetic acid per mole of carbohydrate decomposed. When pyruvate is the substrate, both acetic acid and carbon dioxide are formed but the yield of the latter is low, corresponding to about 0.5 mole per mole of pyruvate.

To account for the high yield of acetic acid and the low yield of carbon dioxide from all three substrates, it has been postulated that *Clostridium thermoaceticum*, like *Clostridium aceticum*[3, 4] and *Clostridium* acidi-urici,[5] uses carbon dioxide as an oxidizing agent in such a way that it is condensed with a second molecule and reduced to acetic acid. If this occurs in the decomposition of glucose, for example, at least two alternative mechanisms, illustrated by the following sets of equations, are possible.

I. $$C_6H_{12}O_6 + 2H_2O = 2CH_3COOH + 2CO_2 + 8H \quad (1)$$

$$8H + 2CO_2 = CH_3COOH + 2H_2O \quad (2)$$

II. $$C_6H_{12}O_6 + 6H_2O = 6CO_2 + 24H \quad (3)$$

$$6CO_2 + 24H = 3CH_3COOH \quad (4)$$

For either scheme the over-all reaction is

$$C_6H_{12}O_6 = 3CH_3COOH. \quad (5)$$

Scheme I represents the fermentation as a partial oxidation of glucose to acetic acid and carbon dioxide (equation 1) accompanied by the reduction of 2 moles of carbon dioxide to acetic acid (equation 2). The oxidation of glucose presumably follows the usual glycolytic mechanism involving pyruvic acid. Scheme II represents the fermentation as a complete oxidation of glucose (equation 3) accompanied by the reduction of 6 moles of carbon dioxide (equation 4) to acetic acid. Although Scheme II appears less likely to represent the true course of the fermentation than does Scheme I, it must nevertheless be considered as a possibility.

In this paper experiments will be described which provide direct evidence for the conversion of carbon dioxide to acetic acid and for the decomposition of glucose in accordance with Scheme I. This evidence has been obtained[6] by the use of carbon dioxide labeled with the long-lived carbon isotope, C^{14}.

Experimental Methods.—For the detection of radiation emitted by C^{14} a Geiger counter of "bell-jar" type with a thin mica window was used. The mica window had a thickness corresponding to about 3.5 mg. per cm.^{2} and an area of approximately 25 cm.^{2} It was supported by a brass grid cut to allow maximal passage of the radiation. Samples consisting of barium carbonate or barium acetate were spread thinly and evenly on duraluminum discs and dried at 100°C. These were placed about 1 mm. below the window in a standard fixed position. Variations in sensitivity of the counter were corrected for by reference to a standard sample of barium carbonate prepared in the same way. Corrections for self-absorption, necessitated by the softness of the beta rays (0.15 M. E. V.) emitted, were determined from a curve constructed from counts on standard samples of known activity and varying thickness.† The long half-life of C^{14} ($2.5 \pm 1 \times 10^4$ yrs.)[7] obviated corrections for decay. The counting circuit was of a conventional type and requires no description.

The bacteria were grown in a medium of the following composition in g. per 100 ml.: glucose 0.15–0.7, tryptone 0.5, yeast extract 0.5, pH 6.6, phosphate buffer 0.9, $(NH_4)_2SO_4$ 0.05, $MgSO_4 \cdot 7H_2O$ 0.01, sodium thioglycollate 0.05, $Na_2C^*O_3$ about 0.2 (* indicates carbon labeled with C^{14}). The phosphate buffer and labeled sodium carbonate were added after autoclaving. In a typical experiment the total volume of medium was about 12 ml. Oxygen was removed by means of an Oxsorbent seal and

the culture tube was closed with a ground-glass stopper to prevent loss of carbon dioxide. After incubating for 3 to 6 days at 55°C. cultures were analyzed and the C^{14} content of the carbon dioxide and the fermentation products was determined.

Results.—In table 1 are presented data from an experiment such as that described above. The glucose concentration was 0.7 g. per 100 ml.

TABLE 1

THE FERMENTATION OF GLUCOSE IN THE PRESENCE OF C^*O_2
Experiment 1

SUBSTANCE	MG./10 ML.	CTS./MIN./MG.	TOTAL CTS./MIN.
Glucose fermented	54.0	...	.....
Initial carbon dioxide as $BaCO_3$	(22.3)	117	2610 ± 50
Final carbon dioxide as $BaCO_3$	22.3	5.7	128 ± 20
Acetic acid formed as $BaAc_2$	101.6	19.9	2020 ± 40
Cell material (residue from T. C. A. extraction)	2.5	12.8	32 ± 4
Trichloracetic acid extract of cells	4.5	1.5	7 ± 4
Non-volatile cell-free material	...	...	96 ± 30
Total C^{14} activity in products			2155
C^{14} recovered in per cent			2155/2482 × 100 = 87

It can be seen that most (94%) of the C^{14} disappeared from the added carbon dioxide during the fermentation and a large part of it (81%) was recovered in acetic acid. Adequate evidence that the C^{14} was actually present in acetic acid rather than some associated compound was provided by distilling the volatile acid, using a modified Duclaux method, and determining the specific activity of the barium acetate derived from successive distillation fractions. The specific activity was found to be the same in all fractions within the limits of experimental accuracy (table 2).

TABLE 2

SPECIFIC ACTIVITIES OF DUCLAUX FRACTIONS OF ACETIC ACID
Experiment 1 (total volume 110 ml.)

DUCLAUX FRACTION	CTS./MIN./MG. $BaAc_2$
0– 40 ml.	17.9
40– 80 ml.	18.9
80–110 ml.	18.7

A small part of the C^{14} was also present in the cells after removal of acetic acid and carbon dioxide, and in the non-volatile fraction of the medium after removal of the cells. It is noteworthy that the quantity of C^{14} per mg. of dry cells was of the same order of magnitude as in the barium acetate. It seems reasonable to conclude that a considerable part of the cell material was synthesized from carbon dioxide.

Only about one-fifth of the C^{14} in the cells could be extracted with 4% trichloracetic acid; the remainder must have been present in proteins,

lipoids and other acid-insoluble forms. The small yield of acid-extractable C^{14} was due possibly to the relatively long incubation which was continued for several days after growth ceased. The bacterial cells may have autolyzed, releasing soluble constituents. This could account for the considerable amount of non-volatile C^{14} present in the medium after removal of the bacteria. The actual weight of this fraction could not be estimated because of the large amount of inorganic salt present; for the same reason the C^{14} content of this fraction could be only roughly determined.

To determine the distribution of C^{14} in the acetic acid, its barium salt was first decarboxylated according to the equation

$$(CH_3COO)_2Ba = CH_3COCH_3 + BaCO_3.$$

In control experiments with synthetic CH_3C^*OOH, the barium carbonate was shown to originate from the carboxyl carbon of acetic acid (table 3, column 2). Hence, this method was used to estimate the C^{14} content of the carboxyl carbon. To determine the C^{14} content of the methyl group, the acetone derived from the decarboxylation was oxidized with alkaline iodine to iodoform and acetic acid. The iodoform, which was shown to originate from the methyl group of acetic acid (table 3, column 2), was oxidized to carbon dioxide and converted to barium carbonate for counting C^{14}.

TABLE 3

DISTRIBUTION OF C^{14} IN SYNTHETIC AND FERMENTATION ACETIC ACID

(The figures give the percentage of the total C^{14} in each atom)

CARBON ATOM	SYNTHETIC CH_3C^*OOH	FERMENTATION ACETIC ACID EXPT. 1	EXPT. 3
Methyl	0 ± 3	49 ± 2	40 ± 2
Carboxyl	100 ± 2	51 ± 2	60 ± 2

Data from two experiments are given in table 3, columns 3 and 4. It can be seen that in experiment 1 the C^{14} in the acetic acid produced by fermentation was almost equally distributed between the methyl and carboxyl groups as would be expected if a total synthesis of acetic acid from carbon dioxide had occurred. In experiment 3, a different result was obtained; about 50% more C^{14} was present in the carboxyl than in the methyl group. The difference in C^{14} distribution between the two experiments appears to be significant but we have no explanation for it. Further work will be necessary to elucidate the relation between the experimental conditions and the isotope distribution in acetic acid. There is, however, a possible explanation for the fact that in experiment 3 the carboxyl group had a higher C^{14} content than the methyl group. The synthesis of acetic acid from carbon dioxide by *Cl. thermoaceticum* may occur in two ways, one involving the fixation of carbon dioxide in both the methyl and carboxyl groups, the other involving only fixation in the carboxyl group.

The relative rates of the two processes would then determine the distribution of the isotope. A fixation of carbon dioxide exclusively in the carboxyl group of acetic acid has been observed by Slade, *et al.*,[8] using *Aerobacter indologenes* and *Clostridium welchii*.

Having established the conversion of carbon dioxide to acetic acid, it seemed desirable to find out how much carbon dioxide was produced as an intermediate in the glucose fermentation. This can be calculated from a knowledge of the C^{14} contents of the initial and final carbon dioxide if it is assumed that the formation of carbon dioxide from sugar is the only process which causes dilution of the labeled carbon dioxide. One specific reaction which must not occur is an exchange of C^{14} between carbon dioxide and preformed acetic acid.

The evidence against a reversible exchange of C^{14} between carbon dioxide and acetic acid is of two types. First, a wide variation in the molar ratio of the C^{14} contents of acetic acid and carbon dioxide was observed in different experiments. The range was from 0.95 (experiment 2, table 4) to 2.26 (experiment 1, table 1). A rapid exchange of C^{14} between the two compounds should result in a constant ratio. Secondly, an experiment was performed in which glucose was fermented in the presence of acetate, labeled in both the methyl and carboxyl positions, and ordinary carbon dioxide. At the beginning of the experiment 0.215 mM of acetic acid giving 726 ± 15 cts./min. was present per 10 ml. of medium. After the fermentation 0.385 mM of acetic acid giving 706 ± 15 cts./min. was recovered. The final carbon dioxide (0.049 mM) gave only 9.8 ± 1 cts./min., or about 1% of the C^{14} initially added as acetate. From this result it must be concluded that whereas a conversion of acetic acid carbon to carbon dioxide does occur the rate of the reaction is so slow as to be negligible under the conditions of these experiments.

To calculate the intermediate carbon dioxide production we shall assume then that the only pertinent reactions are the following:

$$(a) \quad \text{glucose } (C^{12}) \rightarrow CO_2\ (C^{12})$$

$$(b) \quad CO_2\ (C^{12} + C^{14}) \rightarrow \text{acetic acid } (C^{12} + C^{14}).$$

The carbon dioxide will always consist of a mixture of $C^{14}O_2$ and $C^{12}O_2$. Let x be the quantity of $C^{14}O_2$ per unit volume at any time during the fermentation and let x_0 be the initial, and x_f the final $C^{14}O_2$. Further, let V represent the amount of $C^{12}O_2$ plus $C^{14}O_2$ converted to acetic acid at any time; V also equals the amount of $C^{12}O_2$ formed from glucose since there is no net change in carbon dioxide. Va is the constant amount of carbon dioxide present throughout the fermentation and V_f is the total carbon dioxide formed or utilized during the experiment.

When a small quantity (ΔV) of $C^{14}O_2$ + $C^{12}O_2$ is converted to acetic

acid and an equal amount of $C^{12}O_2$ is formed from glucose, the decrease in $C^{14}O_2$ ($-\Delta x$) is given by the expression

$$-\Delta x = \frac{\Delta V}{Va + \Delta V} \cdot x$$

which means that the $C^{14}O_2$ (x) is decreased by a fraction equal to the carbon dioxide used (ΔV) divided by the total carbon dioxide present ($Va + \Delta V$). Dividing by x we get

$$\frac{-\Delta x}{x} = \frac{\Delta V}{Va + \Delta V}.$$

In the limit as ΔV is decreased

$$\frac{-dx}{x} = \frac{dV}{Va + dV} = \frac{dV}{Va}.$$

Integrating this expression between the limits x_0 and x_f for x, and 0 and V_f for V, we find

$$-\int_{x_0}^{x_f} \frac{dx}{x_1} = \ln \frac{x_0}{x_f} = 1/Va \int_0^{V_f} dV = \frac{V_f}{Va}$$

or

$$V_f = 2.3 \cdot Va \log x_0/x_f.$$

V_f has the same units as Va. V_f must be divided by the quantity of glucose fermented (= moles of acetic acid formed divided by 2.65) to give carbon dioxide production per unit of glucose.

When this method of calculation is applied to the data of experiment 1 (table 1) where $x_0 = 117$, $x_f = 5.7$, $Va = 0.113$ mM and the glucose decomposed is 0.3 mM, a result of 1.14 moles of carbon dioxide per mole of glucose fermented is obtained. This figure almost certainly errs on the low side because of the likelihood that x_f was raised by contamination with C^*O_2 from the gas phase of the culture vessel. Such contamination might easily have been important in this experiment due to the very low activity of the final carbon dioxide and the high activity of the initial carbon dioxide, some of which may have remained in the gas phase or in the Ox-sorbent seal during the fermentation. When x_f is very small in relation to x_0, a relatively slight increase in x_f will cause a disproportionately large decrease in the factor $\log x_0/x_f$ and therefore in V_f.

To obtain a more reliable value for carbon dioxide production two additional experiments were performed in which the ratio x_0/x_f was kept small by limiting the amount of glucose fermented. In this way errors due to C^{14} contamination were made insignificant. From the data of these experi-

ments (table 4) the intermediate carbon dioxide production was calculated to be 2.32 (experiment 2) and 2.19 (experiment 3) moles per mole of glucose fermented.

TABLE 4

THE FERMENTATION OF GLUCOSE IN THE PRESENCE OF C^*O_2

Experiments 2 and 3

	EXPT. 2		EXPT. 3	
COMPOUND	mM/10 ml.	cts./min./mM $\times 10^{-3}$	mM/10 ml.	cts./min./mM $\times 10^{-3}$
Carbon dioxide, initial	(0.191)	28.5 ± 0.5	(0.136)	25.5 ± 0.4
Carbon dioxide, final	0.191	10.0 ± 0.3	0.136	6.9 ± 0.3
Acetic acid, final	0.258	9.5 ± 0.2	0.245	8.6 ± 0.2
Glucose fermented	0.086	.....	0.081	.....

The above results definitely exclude a mechanism such as that implied in reactions (3) and (4). The agreement with the value of 2 to be expected on the basis of reactions (1) and (2) is very satisfactory in the light of the possibility that reactions other than those postulated may be contributing to the dilution of the C^*O_2. It should be noted in this connection that only about 85% of the glucose is accounted for as acetic acid; the remainder goes into cell material and unidentified non-volatile compounds.

It may be concluded that the "acetic acid fermentation" of glucose by *Cl. thermoaceticum* involves a partial oxidation of the substrate to two moles each of acetic acid and carbon dioxide followed by a reduction and condensation of the carbon dioxide to a third mole of acetic acid. *Cl. thermoaceticum* is the third species of Clostridium that has been shown to use carbon dioxide in this way.

The authors are greatly indebted to Prof. E. O. Lawrence and members of the staff of the Radiation Laboratory and to Dr. T. H. Norris of the Department of Chemistry for facilities used in the preparation of C^{14}.

* Present address: Mallinckrodt Institute of Radiology, Washington University, St. Louis, Mo.

† The authors are indebted to Dr. T. H. Norris of the Dept. of Chemistry of the University of California for the data on self-absorption.

[1] Fontaine, F. E., Peterson, W. H., McCoy, E., Johnson, M. J., and Ritter, G. J., *Jour. Bact.*, **43**, 701–715 (1942).

[2] Barker, H. A., these PROCEEDINGS, **30**, 88–90 (1944).

[3] Wieringa, K. T., *Leeuwenhoek*, **3**, 1–11 (1936).

[4] Wieringa, K. T., *Ibid.*, **6**, 251–262 (1939–1940).

[5] Barker, H. A., Ruben, S., and Beck, J. V., these PROCEEDINGS, **26**, 477–482 (1940).

[6] Ruben, S., and Kamen, M. D., *Phys. Rev.*, **57**, 549 (1940).

[7] Kamen, M. D., unpublished data.

[8] Slade, H. D., Wood, H. G., Nier, A. O., Hemingway, A., and Werkman, C. H., *Jour. Biol. Chem.*, **143**, 133–145 (1942).