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RADIOACTIVE CARBON AS AN INDICATOR OF CARBON DIOXIDE UTILIZATION. IX. THE ASSIMILATION OF CARBON DIOXIDE BY PROTOZOA

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Previous publications from these laboratories have demonstrated the widespread occurrence of carbon dioxide utilization by living cells.[1–7] A survey of the known cases[8] has made it extremely likely that in all cases the absorption leads primarily to the formation of a carboxylic acid in which the carbon dioxide molecule becomes incorporated as a carboxyl group.

So far, the class of protozoa had not yet been investigated in this respect. It is, however, noteworthy that Rahn[9] has recently published evidence for the absolute necessity of CO_2 in the development of these organisms, a fact which is entirely in line with the general conclusions regarding the rôle of this compound in metabolism, expressed in the previous paper of this series.[8]

Experiments by one of us (J. O. T.) with pure cultures of the holotrichous ciliate, *Tetrahymena geleii*, have shown that under anaerobic conditions a sugar fermentation occurs in which the main metabolic products are lactic, acetic and succinic acids, accompanied by carbon dioxide. Furthermore, it was ascertained that suspensions of the protozoan in a phosphate-bicarbonate buffer can take up measurable amounts of carbon dioxide. In view of the work of Wood and Werkman,[10–13] Carson and Ruben[2, 3] and others, it seemed probable that this assimilation would be responsible for the production of succinic acid. Since it is difficult, if not impossible, due to the net evolution of CO_2 to demonstrate conclusively the participation of carbon dioxide in the succinic acid formation we have studied the anaerobic metabolism of *Tetrahymena geleii* in the presence of radioactive $C^{11}O_2$.*

The ciliate was grown in yeast extract media with 2% glucose under conditions of adequate aeration. The cells were centrifuged for use in the experiments, washed and resuspended in phosphate buffer (pH 7.5) with 1%

glucose. The suspension was then shaken in the presence of $C^{11}O_2$ in an oxygen-free atmosphere for 30 minutes at 30°C. Following the addition of small amounts of lactic, acetic, pyruvic, succinic and fumaric acids as carriers the suspension was rapidly boiled in order to stop further metabolism, and to drive off dissolved CO_2. Complete elimination of residual $C^{11}O_2$ was, as usual, accomplished by addition of $NaHCO_3$, acidification and further boiling. Hereafter the suspensions were centrifuged, the cells resuspended in H_2O and again thrown down. Measurements of the insoluble cellular material at this time showed it to be practically without radioactivity.

Pyruvic and fumaric acids were precipitated from separate aliquots of the original supernatant solution with 2,4-dinitrophenylhydrazine and mercurous nitrate, respectively. These precipitates, also, showed but little activity.

From another aliquot of the original supernatant the volatile acids were removed by a vacuum distillation, and the activities of the distillate and the residue measured separately. The latter fraction contains the known products, succinic and fumaric acids.

The relative activities of the various fractions are assembled in table 1.

TABLE 1

RELATIVE ACTIVITIES OF THE FRACTIONS OBTAINED FROM A SUSPENSION OF *Tetrahymena geleii*, INCUBATED FOR 30 MINUTES IN THE PRESENCE OF $C^{11}O_2$

FRACTION	ACTIVITY IN % OF TOTAL C^*O_2 REDUCED[a]
Fumaric acid	< 4
Pyruvic acid	< 0.2
Volatile acid	< 0.1
Non-volatile residue	98
Cell material	2

[a] All values corrected for decay, hence strictly comparable.

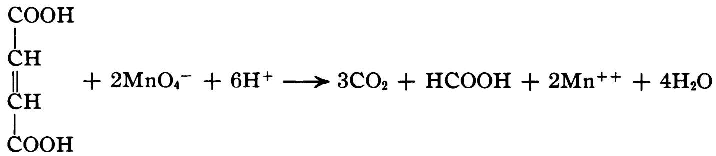
The uptake of $C^{11}O_2$ was surprisingly large; during the incubation period about 35% of the available supply had been reduced by the protozoa. It even reached 50% in a subsequent experiment. It is important to note that the two principal products of the fermentation, lactic and acetic acid, were completely inactive, and only a small fraction of the activity was contained in the cell material after one washing with H_2O. The non-volatile residue, containing the succinic and fumaric acids, accounted for practically all the activity. Boiling with acid permanganate reduced this activity only slightly. It may be pointed out that the activity of the fumaric acid fraction may have been caused partly by co-precipitated mercurous succinate.

These results make it probable, therefore, that the $C^{11}O_2$ had actually been used in the production of succinic acid. For a convincing demonstration a characterization of the nature of the radioactive substance was carried out in an additional experiment in the following manner.

The protozoa suspension, prepared in the same manner as before, and after having been shaken in the presence of $C^{11}O_2$ for 30 minutes at 30°C., was again acidified, boiled and centrifuged. The supernatant, upon the addition of about 20 mg. carrier succinic acid, was neutralized (final pH 7.17), and subjected to the action of a succino-dehydrogenase preparation from beef heart in the presence of air.† The dehydrogenation of the succinic acid was followed manometrically, and was complete in 30 minutes at 37°C. The enzyme preparation was then precipitated by the addition of trichloroacetic acid, and the precipitate removed by centrifugation. The supernatant, which now contained the original succinic acid in the form of fumaric acid, was divided into three aliquots which were used for the following tests:

1. The total radioactivity was measured in one aliquot directly. It showed that 50% of the available $C^{11}O_2$ had been assimilated.
2. In the second aliquot the fumaric acid was precipitated with mercurous nitrate, the precipitate filtered, washed, dried and its activity determined.
3. The third fraction was treated with $KMnO_4$ in 1.5 N H_2SO_4 at 40°C. in a stream of nitrogen. The gas evolved was passed through $Ba(OH)_2$ solution, in which a copious precipitate of $BaCO_3$ occurred. This also was filtered, washed, dried and weighed, and its activity was measured on weighed portions. With the solution remaining after the permanganate treatment a steam distillation was performed, and the distillate, after having been made alkaline, measured in the Geiger counter.

The oxidation of fumaric acid under the conditions of the test proceeds in accord with the equation



$$\begin{array}{l}\mathrm{COOH}\\ |\\ \mathrm{CH}\\ \|\\ \mathrm{CH}\\ |\\ \mathrm{COOH}\end{array} + 2\mathrm{MnO_4^-} + 6\mathrm{H^+} \longrightarrow 3\mathrm{CO_2} + \mathrm{HCOOH} + 2\mathrm{Mn^{++}} + 4\mathrm{H_2O}$$

It has been demonstrated (Allen and Ruben[15]) that the formic acid arises exclusively from the methine carbons of the fumaric acid. Thus, a comparison of the activities of the oxidation products, CO_2 and formic acid, permits of an unambiguous localization of the C^{11} atoms in the fumaric, and, therefore, in the original succinic acid. Table 2 presents the results.

From the first experiment (table 1) it has emerged that the amount of radioactive fumaric acid in the fermentation solution is certainly not above 4%. The very large quantity of this substance in a solution subjected to the highly specific action of succino-dehydrogenase thus shows conclusively that at least 83% of the $C^{11}O_2$ reduced had been converted by *Tetrahymena geleii* into succinic acid. This value must be considered as a lower limit because unfortunately the quantity of mercurous fumarate was large, so that considerable self-absorption of the C^{11} positrons seriously interfered with an accurate radioactivity measurement. This is borne out by the fact that the determination of C^{11} in the $BaCO_3$, carried out with a smaller amount (thinner sample), yielded a higher relative activity, undoubtedly the result of a decreased self-absorption.

A comparison of the values for the carbonate and formic acid fractions clearly proves that the labeled carbon was present in the fumaric acid only in the carboxyl groups. Together with the evidence given above, this implies that the carbon dioxide assimilated during the anaerobic sugar metabolism of *Tetrahymena geleii* is converted mainly into succinic acid, and is present exclusively in the carboxyl groups of this substance.

TABLE 2

RELATIVE ACTIVITIES OF THE VARIOUS FRACTIONS AND ALIQUOTS

FRACTION	ACTIVITY IN % OF TOTAL C^*O_2 REDUCED
Fumaric acid as mercurous fumarate	83
CO^2 produced during $KMnO^4$ oxidation of second aliquot	89
Formic acid fraction of fumarate oxidation	1

Since the mechanism for the formation of succinic acid with the above properties has been adequately dealt with elsewhere[8, 12, 13] it need not be further discussed at this place.

We are indebted to Professor E. O. Lawrence and members of the Radiation Laboratory for making these experiments possible.

Summary.—Experiments with radioactive carbon dioxide have shown that the formation of succinic acid during the anaerobic sugar metabolism of the holotrichous ciliate, *Tetrahymena geleii*, involves the assimilation of carbon dioxide. Since the radioactivity of the succinic acid so produced is strictly limited to the carboxyl groups, the results support the current concepts of the mechanism of succinic acid formation.

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[*] The half-life of C^{11} is 20.5 minutes; the production and quantitative determination of C^{11} have been described elsewhere.[14]

[†] We are indebted to Mr. Bartley Carden for the enzyme preparation and for directions concerning its use.

[1] Ruben, S., and Kamen, M. D., *Proc. Nat. Acad. Sci.*, **26**, 418 (1940).

[2] Carson, S. F., Ruben, S., Kamen, M. D., and Foster, J. W., *Ibid.*, **27**, 475 (1941).

[3] Carson, S. F., and Ruben, S., *Ibid.*, **26**, 422 (1940).

[4] Barker, H. A., Ruben, S., and Kamen, M. D., *Ibid.*, **26**, 426 (1940).

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

[6] Carson, S. F., Foster, J. W., Ruben, S., and Barker, H. A., *Ibid.*, **27**, 229 (1941).

[7] Foster, J. W., Carson, S. F., Ruben, S., and Kamen, M. D., *Proc. Nat. Acad. Sci.*, **27,** 590 (1941).

[8] van Niel, C. B., Ruben, S., Carson, S. F., Kamen, M. D., and Foster, J. W., *Ibid.*, **28,** 8 (1942).

[9] Rahn, O., *Growth*, **5,** 197 (1941).

[10] Wood, H. G., and Werkman, C. H., *Biochem. Jour.*, **30,** 48 (1936).

[11] Wood, H. G., and Werkman, C. H., *Ibid.*, **32,** 1262 (1938).

[12] Wood, H. G., and Werkman, C. H., *Ibid.*, **34,** 7, 129 (1940).

[13] Wood, H. G., and Werkman, C. H., Hemingway, A., and Nier, A. O., *Jour. Biol. Chem.*, **139,** 483 (1941).

[14] Ruben, S., Kamen, M. D., and Hassid, W. Z., *Jour. Am. Chem. Soc.*, **62,** 3443 (1940).

[15] Allen, M. B., and Ruben, S., *Ibid.* (in press).

THE ENERGY EQUATION FOR A VISCOUS COMPRESSIBLE FLUID

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One of the more important problems discussed in any text book on Dynamic Meteorology is the calculation of the energy of atmospheric motions, and the conversion of potential and thermal energy to the kinetic energy of the motion of the winds. This problem was first discussed by Margules[1] and has since then been discussed by Bjerknes,[2] Brunt[3] and Haurwitz.[4] Rossby has also discussed this work in his lectures on Dynamic Meteorology. It is therefore of considerable interest to note that the development of the energy equation for a viscous compressible fluid by all these latter writers is in error through the omission of terms involving the viscous stresses. On the other hand, this matter is correctly handled in Margules' original paper.

The energy equation for this case can be obtained easily from the dynamical equations of motion for a viscous fluid. This is, using the Cartesian tensor notation,

$$\frac{Du_i}{Dt} = -\frac{1}{\rho}\frac{\partial p}{\partial x_i} - \frac{\partial V}{\partial x_i} + \frac{1}{\rho}\frac{\partial}{\partial x_j}(\tau_{ij}) \qquad (1)$$

where x_i are rectangular coördinates, u_i is the fluid velocity vector, ρ is the fluid density, V is the gravitational potential and τ_{ij} is the viscous stress tensor. Upon taking the scalar product of the velocity vector with this, one obtains, since $\frac{\partial V}{\partial t} = 0$,