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CARBON DIOXIDE UTILIZATION IN THE SYNTHESIS OF ACETIC AND BUTYRIC ACIDS BY BUTYRIBACTERIUM RETTGERI

BY H. A. BARKER, M. D. KAMEN* AND VICTORIA HAAS

DIVISION OF PLANT NUTRITION, UNIVERSITY OF CALIFORNIA, BERKELEY

Communicated October 9, 1945

Butyribacterium rettgeri causes a modified butyric acid type fermentation of lactate, the main products being carbon dioxide and acetic and butyric acids.[1] This fermentation is remarkable because of the low yield of carbon dioxide and the high yield of fatty acids. In the usual type of butyric acid fermentation, such as is carried out by *Clostridium saccharobutyricum*,[2] for example, not less than one mole of carbon dioxide and not more than one mole of C_2 derivatives (acetic acid, butyric acid $\times$ 2, etc.) are formed per mole of triose. *B. rettgeri*, however, produces only 0.4 mole of carbon dioxide and up to 1.2 moles of C_2 derivatives per mole of lactate. These unusual yields suggest that this organism utilizes part of the carbon dioxide produced from lactate for the synthesis of fatty acids. This possibility has been investigated using the long-lived radioactive carbon isotope, C^{14}, as a tracer to follow the transformations of carbon dioxide, with the results reported below.

Experimental.—To investigate the possible conversion of carbon dioxide into fatty acids, *B. rettgeri* was allowed to grow in a medium containing lactate and C^*O_2. (C^* indicates carbon labeled with C^{14}.) The substrate used and the products formed were estimated quantitatively and the C^{14} content of each compound was determined.

A basal medium of the following composition in g. per 100 ml. was used: sodium lactate 0.5–2.0, Difco yeast extract 0.3, yeast autolysate 0.3,

$(NH_4)_2SO_4$ 0.05, K_2HPO_4 0.1, KH_2PO_4 0.4, $MgSO_4.7H_2O$ 0.02, cysteine hydrochloride 0.05, $FeSO_4.7H_2O$ 0.0005, pH 6.2. A sterile solution of labeled sodium carbonate was added to the basal medium after autoclaving to give a final pH of 7.0–7.6. After inoculating with *B. rettgeri*, strain 32, samples were removed to determine the initial lactate and carbon dioxide. An Oxsorbent seal was then applied to remove oxygen. Cultures were incubated at 30–35°C. until growth ceased and the products of the fermentation were estimated quantitatively, separated and their C^{14} contents determined. The separation of the fatty acids was accomplished by the distillation procedure of Schicktanz, *et al.*[3] The method of determining C^{14} activity has already been described.[4]

TABLE 1

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

(Experiment 1)

COMPOUND	MM/10 ML.	CTS./MIN./MM	TOTAL CTS./MIN.
Lactic acid, decomposed	0.463	...	...
Carbon dioxide, initial	0.115	33,800	3,890
Carbon dioxide, final	0,298	4,800	1,430
Acetic acid	0,252	2,470	620
Butyric acid	0.177	6,350	1,120

The results of a typical experiment are given in table 1. It can be seen that approximately 63% of the C^{14} was lost from the carbon dioxide during the fermentation of 0.46 mM of lactate per 10 ml. Of the total C^{14} lost, about 25% and 46% was recovered as acetic and butyric acids, respectively. In another experiment the corresponding values were 26% and 53%, while, in addition, 1% of the C^{14} was recovered as caproic acid, 7% as trichloracetic acid insoluble cell material and 3% as unidentified nonvolatile compounds. The total recovery of transformed C^{14} varied from 80 to 90%.

The distribution of C^{14} in the acetic acid was determined by the decarboxylation of the barium salt as previously described[4] after the purity of the product was established by a Duclaux distillation. The data given in table 2 show that both methyl and carboxyl groups contain the isotope,

TABLE 2

DISTRIBUTION OF C^{14} IN ACETIC ACID

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

CARBON ATOM	EXPT. 2	EXPT. 3
Carboxyl	56.5 ± 2	57 ± 1
Methyl	43.5 ± 2	43 ± 1

though the amount is not the same in the two positions. The carboxyl group contains significantly more C^{14} than the methyl group.

The approximate distribution of C^{14} in butyric acid was determined by a combination of two methods: (1) the oxidation of the ammonium salt with hydrogen peroxide as described by Wood, *et al.*,[5] and (2) the decarboxylation of barium butyrate.

Wood, *et al.*, have shown that the acetone formed in the H_2O_2 oxidation of butyric acid is derived from the alpha, beta and gamma carbon atoms. By further oxidizing the acetone with alkaline iodine solution, iodoform and acetic acid are formed. The iodoform originates from the alpha and gamma positions in butyric acid and may be used to determine the average activity of these two carbon atoms. The acetic acid originates from the alpha or gamma and beta positions. When the molar activities of the iodoform and acetic acid are known, the activity of the carboxyl carbon in acetic acid, which is equivalent to the beta position in butyric acid, can be calculated by difference.

Decarboxylation of barium butyrate was used to determine the activity of the carboxyl carbon. The reaction was carried out at about 400°C. Control experiments with carboxyl-labeled butyric acid, prepared by Grignard synthesis,[6] showed that approximately half (actually 52%) of the carboxyl activity appeared in the barium carbonate formed by decarboxylation. The reaction is therefore reliable for quantitatively determining the amount of C^{14} in the carboxyl group of the fermentation butyric acid.

TABLE 3

DISTRIBUTION OF C^{14} IN BUTYRIC ACID

(Experiment 3)

CARBON ATOM	PERCENTAGE OF TOTAL C^{14}
Carboxyl	39.3
Alpha and gamma (average)	30.8
Beta	29.9

The results presented in table 3 demonstrate that all four positions in butyric acid contain carbon derived from carbon dioxide. As in acetic acid, the distribution of C^{14} in butyric acid is not entirely uniform. The carboxyl carbon atom contains significantly more C^{14} than the atoms in other positions. The appearance of more C^{14} in carboxyl carbon relative to alpha and gamma carbon was to be expected, since butyric acid is probably formed (see below) by a condensation of two molecules of acetic acid or derivatives thereof,[5, 7] and the methyl group of the acetic acid from which the alpha and gamma carbon atoms would be derived, contains less C^{14} than the carboxyl group. On the same basis, the beta carbon atom of butyric acid should have the same C^{14} content as the carboxyl group, which is not in accordance with the observed result. A possible explanation for this apparent discrepancy is that the reported C^{14} content of the beta carbon atom may be in error. Due to the fact that this value is calcu-

lated by difference from the data for acetic acid and iodoform derived from the oxidation of acetone, it is probably less reliable than the values for the other carbon atoms.

Having demonstrated the conversion of carbon dioxide to fatty acids and cell material, it seemed desirable to determine the total quantity of carbon dioxide formed per mole of lactate decomposed. It is obvious that the total carbon dioxide production ($CO_{2\,total}$) must exceed the observed or net carbon dioxide production ($CO_{2\,obs.}$) by the amount used for synthetic reactions ($CO_{2\,used}$), i.e.,

$$CO_{2\,total} = CO_{2\,obs.} + CO_{2\,used.} \qquad (1)$$

The carbon dioxide used and therefore the total carbon dioxide produced can be calculated from data, such as is presented in table 1, showing the decrease in C^{14} content of the carbon dioxide during the fermentation of a known amount of lactate, if it is assumed that the formation of carbon dioxide from lactate is the only process causing a dilution of the C^*O_2. More specifically, it must be assumed that no exchange of C^{14} occurs between carbon dioxide and any of its reduction products such as acetic and butyric acids.

The absence of such exchange was verified by an experiment in which lactate was fermented in the presence of acetic acid labeled in both the methyl and carboxyl groups; the carbon dioxide was unlabeled. After the fermentation, no C^{14} (2 ± 3 cts./min.) could be detected in carbon dioxide, while about 57% of the C^{14} was present in butyric acid (445 ± 10 cts./min.). Incidentally, this result also proves that butyric acid is formed from acetic acid.

Another assumption involved in the calculation is that the carbon dioxide inside and outside the cells is in isotopic equilibrium at all times. No direct test of the validity of this assumption has been attempted. If isotopic equilibrium does not exist, the C^{14} content of carbon dioxide inside the actively metabolizing cells will be lower than that outside. Such a condition would result in a lowering of the calculated total carbon dioxide production since the utilization of C^*O_2 and hence its dilution would be less than anticipated.

The calculation of $CO_{2\,used}$ and $CO_{2\,total}$ follows the method developed for the acetic acid fermentation caused by *Clostridium thermoaceticum*[4] with the exception that the quantity V representing the carbon dioxide concentration throughout the fermentation is variable instead of constant. At any instant, more carbon dioxide is being produced from the decomposition of lactate than is being used in synthesis; consequently, a net increase in carbon dioxide concentration is observed. Let x be the quantity of C^*O_2 per unit volume at any time during the fermentation, x_i and x_f denoting the initial and final C^*O_2 concentrations. V will now represent the

total concentration of carbon dioxide ($C^*O_2 + CO_2$) present at any time. For every mole of carbon dioxide observed to be produced ($CO_{2\ obs.}$) as a result of the breakdown of lactate, A moles of $CO_2 + C^*O_2$ are converted to acetic acid. The decrease in C^*O_2 concentration ($-\Delta x$) is given by the expression

$$-\Delta x = (A\,\Delta V/V + \Delta V)\cdot x. \qquad (2)$$

The differential form of this relation

$$-dx/x = A\,dV/V \qquad (2a)$$

is integrated between the limits x_i and x_f for x, and V_i and V_f for V, where V_i and V_f denote the initial and final observed concentrations of carbon dioxide.

Thus

$$-\int_{x_i}^{x_f} dx/x = \ln x_i/x_i = A\int_{V_i}^{V_f} dV/V = A \ln V_f/V_i \qquad (3)$$

or

$$A = \log x_i/x_f/\log V_f/V_i. \qquad (3a)$$

Using the data of table 1 where $V_i = 0.115$ mM, $V_f = 0.298$ mM, $x_i =$ 3,890 cts./min., $x_f =$ 1,430 cts./min., and the lactate decomposed is 0.463 mM,

$$A = \log 2.72/\log 2.59 = 0.435/0.413 = 1.05$$

and $CO_{2\ total} = 0.395$ mM $+ 0.395\cdot 1.05$ mM $= 0.81$ mM/mM lactate. In three other experiments the values of A ranged from 0.91 to 1.02 and the values of $CO_{2\ total}$ from 0.62 to 0.81. The lower values were obtained from experiments in which higher concentrations of lactate were fermented (1.0–1.3 mM/10 ml.). However, the data are too few to permit generalization concerning the relation between the quantity of lactate fermented and the functions in question.

Although the above results demonstrate that much more carbon dioxide is produced than accumulates, nevertheless the calculated total carbon dioxide production appears to be significantly lower than one mole per mole of lactate that would be expected on theoretical grounds. This result may well be due to the fact that the carbon dioxide inside the fermenting cells is not in isotopic equilibrium with the carbon dioxide in the outside medium. It has already been noted that lack of isotopic equilibrium would lower the calculated carbon dioxide utilization and total production below the true values.

From the data obtained in these experiments, the fermentation of lactate by *B. rettgeri* can be fitted into the following simplified reaction scheme if we disregard the minor quantitative discrepancies noted above.

$$CH_3CHOHCOOH + H_2O = CH_3COOH + CO_2 + 4H \quad (4)$$

$$2CO_2 + 8H = CH_3COOH + 2H_2O \quad (5)$$

$$2CH_3COOH + 4H = CH_3CH_2CH_2COOH + 2H_2O \quad (6)$$

Reaction (4) represents the oxidation of lactic acid to approximately one mole each of carbon dioxide and acetic acid; the primary product may well be acetyl phosphate or some other C_2 compound convertible into acetic acid rather than the acid itself. Reaction (5) represents the condensation and reduction of two moles of carbon dioxide to acetic acid; this reaction occurs in such a way that both the methyl and carboxyl groups are derived from carbon dioxide. Reaction (6) represents the condensation and reduction of two moles of acetic acid or some related compound to butyric acid. Neither reaction (5) nor (6) goes to completion since they compete for the available hydrogen from reaction (4). The caproic acid which is produced in small yield may be formed by a further condensation of the same type as reaction (6), possibly involving butyric acid and a C_2 compound.[7]

In conclusion it may be pointed out that *B. rettgeri* is the first non-sporulating bacterium and the fourth anaerobe[4, 8, 9] that has been shown to cause a total synthesis of acetic acid from carbon dioxide.

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

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

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