CARBON DIOXIDE UTILIZATION BY RUMEN MICROORGANISMS

C. N. HUHTANEN, F. J. CARLETON, AND H. R. ROBERTS

National Dairy Research Laboratories, Inc., Oakdale, L. I., New York

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The requirement for carbon dioxide in the metabolism of heterotrophic microorganisms has been investigated by many workers. Early work (Valley and Rettger, 1927; Valley, 1928; Gladstone *et al.*, 1935; Wood and Werkman, 1938; Phelps *et al.*, 1939) showed the importance of CO_2 for growth of heterotrophic bacteria. Mechanisms for bacterial utilization of CO_2 were investigated with the advent of radioactive tracer techniques. Barker *et al.* (1945) showed that CO_2 was used in the formation of fatty acids by *Butyribacterium rettgeri*. More recently, Abelson *et al.* (1952*a, b*) and Bolton *et al.* (1952) demonstrated the incorporation of CO_2 into the protein and nucleic acid components of *Escherichia coli*. The investigations reported herein concern the utilization of CO_2 by some rumen microorganisms in the formation of fatty acids and proteins.

EXPERIMENTAL METHODS

The rumen bacteria used in this study were RO-L_1, described by Gall and Huhtanen (1951); RO-C1, RO-C8, RO-L_5, and RO-CR, described by Huhtanen and Gall (1952*b*). *Proteus mirabilis* and *Propionibacterium freudenreichii* were from the laboratory stock collection. All cultures were grown in a rich organic medium containing per 100 ml: peptone, 1 g; tryptone, 1 g; yeast extract, 1 g; glucose, 0.5 g; beef extract, 0.5 g; K_2HPO_4, 0.5 g; cysteine hydrochloride, 0.05 g; and $NaHCO_3$, 0.05 g. The source of the radioactive CO_2 for the experiments was $Na_2C^{14}O_3$ and was added to culture media aseptically as a Seitz filtered solution. The approximate activity was 40 μC per ml, and 6 ml were added per liter of medium. Incubation was at 37 C for *P. mirabilis*, RO-CR, RO-L_5, RO-L_1, and RO-C1. *P. freudenreichii* was incubated at 30 C. Incubation was for two days except for RO-C1 which required five days for good growth. Two per cent inocula were used and flasks closed with rubber stoppers. After growth the cells were centrifuged at 1,500 × gravity and washed three times with 1/10 growth volume of distilled water. They were then dried *in vacuo*. The dried cells were fractionated into "lipide", "nucleic acids", and "protein" according to the methods of Abelson *et al.* (1952*a*). Chromatographic procedures for the detection of amino acids were those of McFarren (1951) and McFarren and Mills (1952). Radioautograms were prepared employing Eastman Kodak "No Screen" X-ray film and an exposure time of one month.

The resting cells for the fatty acid experiments were from one liter of culture that had been centrifuged and washed once with 0.1 M pH 6.8 phosphate buffer. The cells were made up to 8 ml with the phosphate and added to the reaction vessel. An atmosphere of radioactive CO_2 was generated by the addition of concentrated sulfuric acid to 750 mg $Na_2C^{14}O_3$ in a glass enclosed system under vacuum. The substrate was introduced from a side arm. Sheffield Chemical Company sodium lactate was used at a concentration of 500 μM per vessel. The reaction was allowed to proceed 2 hours at 37 C. Flask contents were then removed and assayed for fatty acids by the method of Fairbairn and Harpur (1951).

RESULTS

The two organisms used for the determination of carbon dioxide incorporation into fatty acids were the lactate utilizers, RO-C8 and RO-C1.

In the results summarized in table 1 RO-C8 showed no CO_2 fixation while RO-C1 showed activity in all three fatty acids. This experiment with RO-C8 precludes the possibility that butyrate is formed from a C_3 and CO_2 condensation. No carbon balances were attempted.

A preliminary experiment to determine CO_2 incorporation into bacterial protoplasm is shown in table 2. Surprisingly, the lactic acid producing bacteria (RO-L_5, RO-CR and RO-L_1) showed a great tendency to assimilate CO_2 while RO-C1, an organism closely related to the propionic acid bacteria, showed only a slight degree of CO_2 incorporation into the cells. *P. freudenreichii* showed only a little greater activity than RO-

TABLE 1

CO_2 incorporation into fatty acids by rumen bacteria

Radioisotope Assay	RO-C1	RO-C8
	ct/min	*ct/min*
Carbon dioxide (start)	3,085,130	2,328,400
Carbon dioxide (recovered	3,035,273	2,258,626
Carbon dioxide (used)	49,857	69,774
Acetic acid	7,000	none
Propionic acid	8,300	none
Butyric acid	none	none
$>C_4$ acids	31,300	none
Fatty Acid Assay		
	μM	*μM*
Acetic acid	18.85	none
Propionic acid	53.12	0.48
Butyric acid	none	41.04
$>C_4$ acids	23.00	21.60

Approximately 90 mg cells in 8 ml 0.1 M pH 6.8 phosphate. Substrate 500 μM lactate. Incubation 3 hours at 37 C in atmosphere of $C^{14}O_2$.

TABLE 2

CO_2 incorporation into bacterial protoplasm by four rumen bacteria and two other types

Culture	Wt mg	ct/min/mg
Propionibacterium freudenreichii	243	4,625
Proteus mirabilis	43	1,701
RO-L_5	281	138,000
RO-CR	142	125,000
RO-C1	430	1,520
RO-L_1	110	25,858

Cultures grown in rubber stoppered flasks containing Seitz filtered $Na_2C^{14}O_3$ solution added aseptically after autoclaving.

C1. *P. mirabilis*, an example of a facultative organism, showed only a small degree of CO_2 fixed into the cellular material.

A second experiment was then carried out using only culture RO-L_5. After centrifuging and washing the cells were lyophilized. Recovery was 308 mg. The specific activity of the cells was 81,411 counts/minute/mg. The "nucleic acid" extract, based on original cell weight, showed a specific activity of 29,683 counts/minute/mg cells. No attempt was made to identify the radioactive components of this extract. "Lipide" extraction revealed negligible activity in that portion.

The extracted cells were then hydrolyzed and the amino acids determined by paper chromatography. The following amino acids were identified: aspartic acid, glutamic acid, serine, glycine, threonine, alanine, phenylalanine, proline, lysine, arginine, valine, isoleucine, hydroxyproline, tyrosine, methionine, cystine, and tryptophan. Radioautograms revealed five active amino acids: aspartic acid, glutamic acid, threonine, phenylalanine and arginine. The major part of the activity was in aspartic acid.

DISCUSSION

The importance of a soluble source of CO_2 in culture media for rumen bacteria was shown by Huhtanen and Gall (1952*a*). The experiments reported here show that CO_2 may be used in the formation of the fatty acids in the rumen and for bacterial protoplasm. A soluble source of CO_2 appears to be important for *in vitro* cellulose digestion (unpublished data). The saliva secreted by the animal into the rumen may therefore play a vital metabolic role, in addition to acting as a buffer.

The experiments of Abelson *et al.* (1952*a*) were made using *Escherichia coli* cells in the logarithmic phase of growth. They reported that six amino acids became significantly radioactive: aspartic acid, glutamic acid, threonine, arginine, lysine, and proline. Our investigations revealed RO-L_5 to use CO_2 in synthesizing the first four of these and, in addition, phenylalanine. It is interesting that RO-L_5 should use CO_2 at all since it was grown from a relatively small inoculum and in a medium containing adequate amounts of organic nutrients. RO-L_5 is a homofermentative lactic acid producing bacterium and has not been shown to require CO_2 in its culture medium. These data do not prove that this organism has an absolute requirement for CO_2 although CO_2 is metabolized when present in the culture medium.

SUMMARY

The incorporation of CO_2 into fatty acids by a rumen bacterium was shown.

The incorporation of CO_2 into bacterial protoplasm was investigated. About 35–40 per cent of the activity in the cells of RO-L_5 could be

accounted for in the "nucleic acid" extract; the remainder was in the "protein" fraction.

Most of the activity in the "protein" fraction was found in five amino acids: aspartic acid, glutamic acid, threonine, arginine, and phenylalanine.

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