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SYNTHESIS OF ACETATE FROM CO_2 IN THE CECUM OF SOME RODENTS

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1. Introduction

When we attempted to culture methanogenic bacteria from the cecum of small rodents, such as laboratory rats and guinea-pigs, by serial dilution of cecal contents in an anaerobic rumen fluid agar medium [1], a pressure drop within the tubes was noted when the gas-phase consisted of hydrogen (80%, v/v) and carbon dioxide (20%, v/v). Yet, formation of methane was never observed. Likewise, freshly collected cecal contents did not form methane in short-term incubations, although hydrogen-gas, when added, was rapidly taken up.

To study the possible occurrence in the cecum of the fixation of H_2 and CO_2 into other microbial fermentation products than methane, experiments were carried out with cecal contents from rats, guinea-pigs and rabbits. Fixation of CO_2 in volatile fatty acids (VFA) was measured. A remarkable process, the total synthesis of acetate from CO_2 was found to occur in the cecum. This process is quantitatively important only in the absence of methanogenesis.

2. Materials and methods

Cecal contents were taken from female adult Wistar rats which had been fed a commercial pelleted ration (20% crude protein, 5% fat, 4.4% crude fiber, 53.3% residual carbohydrates, 5.5% ash and 11.8 moisture). The complete cecal contents (2.10 g and 1.77 g of wet contents, respectively) were rapidly collected and diluted in 25 ml anaerobic salt solution. The salt solution had the following percentage composition (w/v): KH_2PO_4 0.075, K_2HPO_4 0.075, NaCl 0.15, $(NH_4)_2SO_4$ 0.075, $MgSO_4 \cdot 7\ H_2O$ 0.015 and $CaCl_2$ $2\ H_2O$ 0.015. Just before use this solution had been heated to boiling and then cooled under a stream of oxygen-free carbon dioxide. Quantities of 4 ml cecal suspension were incubated for 1 h, 2 h, and 4 h under an atmosphere of hydrogen (80%, v/v) and nitrogen gas (20%, v/v) at 39°C in culture-tubes fitted with recessed butyl rubber stoppers. At the start of the incubation 150 μmol $NaH^{14}CO_3$ (14 μCi) were added by injection through the stoppers. After incubation the tubes were analyzed for VFA by gas-chromatography [2] and the presence of radioactivity in these acids was monitored with a gas proportional-counter (Packard Model 894). More accurate measurements of the radioactivity in the fermentation products formate, acetate, propionate, butyrate, lactate and succinate were obtained after separation of the acids by partition chromatography [3]. Gas-analyses were carried out on a Becker 'Permalyzer' gas-chromatograph (column Porapak Q, 80–100 μm mesh, 1000 × 4 × 6 mm) using pure N_2 as the carrier gas at a flow-rate of 30 ml/min.

Acetate was isolated from the cecal fluids by partition chromatography [3] and degraded by the Schmidt reaction as described previously [4].

For the experiment reported in table 1, cecal contents from an adult female Wistar rat were diluted in 25 ml of the anaerobic buffer described above. Portions of 4 ml, representing 0.25 g original cecal wet, were incubated for 2 h at 39°C under a 100% CO_2 atmosphere. Prior to incubation, the gas-phase in the closed tubes, to which the $NaH^{14}CO_3$ (150 μmol, 25 μCi) was added, was allowed to equilibrate with the labeled $NaHCO_3$. This was done by acidification with dilute HCl followed by neutralization with dilute NaOH. All these and further additions (cecal contents, H_2, gas or chloroform, see table 1) were introduced by injection through the stoppers. At the end of the incubation the tubes were analyzed for fermen-

TABLE 1

Distribution of radioactivity in volatile fatty acids after incubation of rat cecal contents with $NaH^{14}CO_3$

Additions	(% Label in individual VFA)				Total radioactivity in VFA (dpm × 10^{-3})
	Formate	Acetate	Propionate	Butyrate	
None (100% CO_2 gas-phase)	0.1	18.4	79.2	2.4	1068.5
517 μmol H_2	8.1	46.6	43.9	1.4	4721.1
874 μmol H_2	14.3	40.2	43.2	2.3	4457.1
517 μmol H_2 + 50 μM $CHCl_3$	26.0	18.2	54.8	1.0	4215.8

tation-gases and VFA. In parallel experiments cecal contents from three other rats were likewise diluted in anaerobic salt solution and 4 ml quantities of these suspensions were incubated for 1 h without or with chloroform ($CHCl_3$) under a 100% CO_2 atmosphere. At the end of the incubation the tubes were analyzed for fermentation-gases.

A number of studies were carried out with cecal contents from guinea-pigs and rabbits using the incubation procedure described above. Estimates of the rate of acetate synthesis from CO_2 were made by measuring the rate of incorporation of $NaH^{14}CO_3$ and by comparing the rates of acetate synthesis in the absence and in the presence of the inhibitor $CHCl_3$.

Rumen fluid was obtained from a fistulated lactating dairy-cow few a diet of grass, hay and concentrates. The incubation procedure with $NaH^{14}CO_3$ was as described above for the experiment reported in table 1, except that quantities of undiluted rumen fluid were used at 4 ml/tube.

3. Results and discussion

It was found that radioactivity was incorporated into acetate, propionate and butyrate when cecal contents of 2 rats were incubated with $NaH^{14}CO_3$ under an atmosphere of hydrogen (80%, v/v) and nitrogen (20%, v/v). Of the total radioactivity incorporated in these three acids after 2 h incubation, acetate contained 69% and 64%, propionate 23% and 20% and butyrate 8% and 16% for the two rats, respectively. Acetate was found to be uniformly labeled (50.1% label in methyl group). Based on the rate of uptake of H_2, as measured in one of these incubations (35 μmol H_2/g wet cecal contents/h), it was calculated that maximally 8.75 μmol acetate were synthesized from H_2 and CO_2/g wet cecal contents/h, when it it assumed that all of the H_2 taken up was used for acetate synthesis. It is likely, however, that some H_2 was consumed in other reactions as well, e.g., in the formation of butyrate from acetate. Based on the rate of incorporation of labeled bicarbonate into acetate, the rate of acetate synthesis from CO_2 was calculated to be 7.68 μmol acetate/g wet cecal contents/h. When the label in butyrate (probably formed from labeled acetyl groups) is also considered, 8.53 μmol acetate were formed/g wet cecal contents/h. The rate of incorporation of label from bicarbonate into acetate plus butyrate was perfectly linear up to at least 4 h of incubation.

Formation of acetate from H_2 and CO_2 in enrichment cultures inoculated with mud has been observed many years ago [5]. The microorganism responsible for this conversion, according to the reaction:

$$4\ H_2 + 2\ CO_2 \rightarrow CH_3COOH + 2\ H_2O \qquad [6]$$

was isolated and named *Clostridium aceticum* [7]. The organism was lost but a related bacterium was later isolated by El Ghazzawi [8] and named *Cl. formico-aceticum* [9]. This organism differed from *Cl. aceticum* by its inability to grow on H_2 plus CO_2 and also by the fact that H_2 did not stimulate the production of acetate in the presence of organic-energy sources. However, another Gram-positive anaerobe which oxidizes hydrogen and reduces carbon dioxide to acetate has recently been isolated from fermenting sea-weed [10]. This organism more closely resembles *Cl. aceticum*. The organisms that are responsible for the synthesis of acetate from CO_2 in the rat cecum may be related to these isolates but their presence in the intestines of animals has as yet not been described.

In the synthesis of acetate from CO_2 by *Cl. formicoaceticum* and the metabolically very similar *Cl. thermoaceticum* CO_2 is first reduced to formate by formate dehydrogenase and this formate carbon is to become the methyl group of acetate [11,12]. The conversion of formate into acetate is carried out be converting the formate to 10-formyltetrahydrofolate which is subsequently reduced to 5-methyltetrahydrofolate and thereafter the methyl group is transferred to an enzyme-bound corrinoid, upon which finally carboxylation of the methyl groups occurs to form acetate. This latter process as well as the transmethylation from methyltetrahydrofolate to the corrinoid enzyme can be inhibited by alkyl-halides [13]. Since formate is an intermediate in the total synthesis of acetate from CO_2 by the organisms mentioned, it was decided to analyze for this acid too in further experiments as well as for a few other acidic fermentation end-products (lactate, succinate).

Results of an experiment with rat cecal contents, presented in table 1, show (a) that the formation of acetate from CO_2 was greatly stimulated by the addition of H_2, (b) that labeled formate was produced from labeled CO_2 and (c) that inhibition of acetate synthesis from CO_2 could be obtained with a low concentration of the simple alkyl-halide chloroform ($CHCl_3$), while in the presence of this inhibitor a further accumulation of formate occurred. Methane was not produced. Incorporation of label was not seen when the cecal contents were heated for 10 min at 70°C prior to incubation. No label was incorporated into succinate or lactate. The rate of acetate synthesis from CO_2 in the presence of added H_2 was calculated to be 6 μmol acetate/g wet cecal contents/h. The stimulation of acetate synthesis by added H_2 suggests that the actual in vivo rates of H_2 supply may be rate-limiting and, therefore, actual rates of acetate synthesis in the cecum probably are much lower than in H_2-supplemented incubations. As can be calculated from the data given in the first 2 lines of table 1, acetate synthesis from CO_2 in the absence of added H_2 was about 11 times lower than in the first H_2-supplemented incubation.

Inhibition of acetate synthesis from CO_2 by alkyl halides such as $CHCl_3$ resulted in an increased net-evoluation of gaseous H_2. It was thought that a rapid estimate of the actual rate of acetate synthesis from CO_2 could easily be obtained by comparing the rates of H_2 evoluation of freshly samples cecal contents in the absence and presence of a low concentration of $CHCl_3$. Rates of H_2-evolution by rat cecal contents from three different animals were 550 and 2387, 2160 and 3616, 660 and 2540 nmol H_2-gas/g wet cecal contents/h as measured after 1 h of incubation without and with 50 μM $CHCl_3$, respectively. The differences in net H_2 production rates can be related to actual rates of acetate synthesis of 0.46, 0.36 and 0.47 μmol acetate/g wet cecal contents/h, according to stoichiometry of the reaction shown above. These rates were slightly lower than the rates measured from incorporation of $^{14}CO_2$ in acetate in the cecal samples in parallel incubations, being 0.54, 0.43 and 0.58 μmol acetate/g wet cecal contents/h, respectively.

The following hypotheses may explain the lower rates of acetate synthesis observed in the inhibitor experiments: (a) formate accumulated in the presence of $CHCl_3$ (see table 1), (b) synthesis of acetate from CO_2 might not be completely blocked by 50 μM

TABLE 2

Distribution of radioactivity in acidic fermentation products after incubation of bovine rumen fluid with $NaH^{14}CO_3$

Additions	(% Label in individual acids)					Total radioactivity in acids (dpm × 10^{-3})
	Formate	Acetate	Propionate	Butyrate	Lactate	
None (100% CO_2 gas-phase)	0.1	5.0	91.3	3.6	0	302.3
517 μmol H_2	76.8	4.0	15.5	2.0	1.7	3117.2
874 μmol H_2	75.4	3.7	13.4	6.7	1.8	3103.1
517 μmol H_2 + 50 μM $CHCl_3$	80.0	0.9	15.0	4.1	0	3428.8

$CHCl_3$ (see table 1), (c) the incubation time was too long or (d) the stoichiometry of the H_2-production differed from the equation shown above. This latter phenomenon has also been observed in the case of inhibition of rumen-methanogenesis by methane analogues [14]. This may be caused by direct inhibition of H_2-production by $CHCl_3$ or by the fact that other H_2-consuming reactions become more important when the H_2-concentration is raised.

Further studies showed that acetate synthesis from CO_2 occurred in a similar way in the cecum of guinea-pigs and rabbits. Rates of acetate synthesis as measured by incorporation of $NaH^{14}CO_3$ were found to range 0.3–0.8 μmol acetate/g wet cecal contents/h or 1.5–5.1 μmol/g dry cecal material/h. Measurements based on inhibitor experiments (50 μM $CHCl_3$) again gave 20–30% lower estimates. In some rabits a slight formation of methane was observed and in these animals the rates of incorporation of CO_2 into acetate in the cecal contents were much deduced. Similarly, methanogenic rumen contents obtained from fistulated cattle incorporated labeled CO_2 rapidly into formate but acetate synthesis from CO_2 was a minor process (table 2). It is suggested that the methanogenic bacteria are capable of competing very succesfully for molecular hydrogen with the as yet unknown organisms that synthesize acetate from CO_2.

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