Some Aspects of the Metabolism of Butyrivibrio fibrisolvens

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SUMMARY

The growth responses of an acetate-utilizing isolate of Butyrivibrio fibrisolvens to CO₂, acetate and pyruvate were determined using a chemically-defined medium. Carbon dioxide was essential for growth and both acetate and pyruvate increased growth. ¹⁴C from [1-¹⁴C]acetate was found mainly in butyrate while ¹⁴C from ¹⁴CO₂ or [1-¹⁴C]pyruvate appeared predominantly in formate and lactate. These results, together with those obtained with enzyme preparations, indicated pyruvate synthase, pyruvate-CO₂ exchange and pyruvate formate lyase to be active.

INTRODUCTION

Butyrivibrio fibrisolvens can be, numerically, an important species in the rumen population, particularly in animals fed on high-roughage diets (Bryant et al., 1958; Gilchrist & Kistner, 1962; Gouws & Kistner, 1965; Caldwell & Bryant, 1966; van Gylswyk, 1970; Latham, Sharpe & Sutton, 1971; Latham, Storry & Sharpe, 1972). This paper extends the work done on B. fibrisolvens by Joyner & Baldwin (1966) and Kistner & Kotzé (1973), who measured activities of various enzymes.

Butyrivibrio fibrisolvens is considered to be the major butyric acid-producing species in the rumen (Bryant, 1970) and many butyrivibrio isolates, of the type placed in group I by Shane, Gouws & Kistner (1969), utilize acetate in vitro. Since acetate is converted to butyrate in the rumen (Weller et al., 1967), one of the aims of the study was to establish whether a group I butyrivibrio is capable of effecting this conversion.

Bryant & Small (1956) failed to find a CO₂ requirement for a number of butyrivibrio isolates, but Gill & King (1958) found an absolute requirement for CO₂ in one group I isolate, and attributed the results of the former authors to their use of a test medium containing rumen fluid in which, despite efforts to remove it, enough bicarbonate might have remained to support growth. Van Gylswyk & Roché (1970) failed to demonstrate a requirement of group I isolates for CO₂ when using a medium containing rumen fluid. Another aim of the present work was to determine the CO₂ requirements of an acetate-utilizing isolate and, if this existed, the incorporation of CO₂ into the fermentation acids, butyrate, lactate and formate.

METHODS

Organism. The bacterium was isolate 12, characterized by van Gylswyk & Roché (1970) as belonging to group I in the classification of Shane et al. (1969).

Media. The bacterium was maintained on slopes of agar medium containing rumen fluid and cellobiose (van Gylswyk & Hoffman, 1970). The chemically-defined medium used in growth experiments was that of Roché et al. (1973) supplemented with glucose (4·5 g l⁻¹).
This medium contained NaHCO$_3$ (8 g l$^{-1}$) and the gas phase was 98% CO$_2$/2% H$_2$. For the CO$_2$-free medium, the bicarbonate and CO$_2$ were omitted and the gas phase was 98% N$_2$/2% H$_2$. Stimulatory effects of sodium acetate (4·1 g l$^{-1}$) and sodium pyruvate (5·5 g l$^{-1}$) were examined separately in media with and without CO$_2$/bicarbonate. The media were adjusted to pH 6·8. The distribution of $^{14}$C from CO$_2$, acetate and pyruvate was studied in basal medium with CO$_2$ and bicarbonate, but with glucose increased to 10 g l$^{-1}$ and cysteine-HCl. H$_2$O reduced to 250 mg l$^{-1}$.

The medium used for the production of bacteria for the enzyme tests contained (per litre): KH$_2$PO$_4$, K$_2$HPO$_4$ and NaCl, 0·9 g of each; CaCl$_2$ and MgCl$_2$.6 H$_2$O, 0·02 g of each; NaHCO$_3$, 8 g; (NH$_4$)$_2$SO$_4$, 1·5 g; glucose, 10 g; indigo carmine, 5 mg; cysteine-HCl. H$_2$O and Na$_2$S.9H$_2$O, 0·25 g of each; rumen fluid (clarified by centrifuging at 23000 g for 2 h), 400 ml. The gas phase was 98% CO$_2$/2% H$_2$.

All media were sterilized by Millipore filtration, except that used for production of bacteria for enzyme tests: in this case the basal medium was heat sterilized, but solutions of glucose and reducing agent were separately sterilized by Millipore filtration and added to the cooled medium.

**Anaerobic techniques.** Anaerobic conditions were obtained by purging media and apparatus with O$_2$-free gases containing about 2% H$_2$. The gases were passed through a 'Deoxo' catalyst cartridge (Engelhard Industries, London) to remove traces of O$_2$. Inocula were transferred with sterile syringes. Incubation temperature was 38°C.

**Effects of CO$_2$, acetate and pyruvate on growth.** Bacteria grown on three maintenance agar slopes for 22 h were collected, suspended in 8 ml CO$_2$-free medium and portions (0·2 ml) were used to inoculate each 10 ml culture. Growth was measured directly as extinction (578 nm) in culture bottles (28 ml McCartney bottles) which were previously matched. The length of the light path through the medium was about 23 mm.

**Incorporation of $^{14}$C from [1-$^{14}$C]acetate, NaH$^{14}$CO$_3$ and [1-$^{14}$C]pyruvate into the fermentation acids by growing bacteria.** Bacteria were grown in 50 or 100 ml portions of medium inoculated with 0·5 ml of a bacterial suspension (see above). The cultures were incubated for up to 5 days. Cells and slime were precipitated by adding 0·5 ml 2·5 M-H$_2$SO$_4$ and shaking, and then 1 ml 10% (w/v) Na$_2$WO$_4$.2H$_2$O solution per 10 ml culture was added. The precipitate was compacted by centrifuging at 15000 g for 20 min. Portions of the supernatant were alkalinized and evaporated. The free acids were released by adding 2·5 M-H$_2$SO$_4$ (2 ml); and acetic, butyric, lactic and formic acids in 0·5 ml of the resulting solution were separated by silicic-acid column (30 x 1 cm) chromatography using a method adapted from Ramsey (1963). Eluants (equilibrated with 0·25 M-H$_2$SO$_4$) were: benzene, 150 ml; chloroform, 100 ml; chloroform/ t-butanol (99;1, v/v), 50 ml; chloroform/ t-butanol (98;2, v/v),100 ml; chloroform/t-butanol (92;8, v/v), 150 ml. The eluates were collected in 8 ml portions, titrated to pH 9 with 0·01 M-KOH [in 90% (v/v) ethanol] while mixing the contents of the tubes with a stream of nitrogen, then pooled, evaporated to a few ml and made up to 10 ml; 1 ml of this solution was added to 15 ml scintillation fluid (Braithwaite, Glascock & Riazuddin, 1969) and $^{14}$C was estimated using a Packard Tri-Carb liquid scintillation spectrometer (model 3003). Corrections for quenching were made by the 'channels ratio' method.

**Enzyme tests.** Bacteria were grown until the pH of the medium reached 6·1, then cooled in ice water, harvested (1600 g; 25 min), resuspended in anaerobic diluent (containing minerals, reducing agent and indigo carmine, and with the gas phase as for the growth medium) and reharvested. The resulting thick paste was purged with 98% CO$_2$/2% H$_2$ and stored in solid CO$_2$.
Bacteria were treated with toluene by mixing 7 ml of the paste with 0.5 ml toluene immediately before incubation.

Cell-free extracts were prepared under 98% N2/2% He. About 7 ml of wet cells were thawed at room temperature, and then mixed with 2 ml dithiothreitol (0.05%), followed by 1 ml Tris buffer (7.27%, w/v; pH 7.6), 1 ml lysozyme (0.4%) and 1 ml EDTA solution (disodium salt; 4%, w/v; pH 7.6). The mixture was incubated at 39 °C for 30 min. Crude lysate (7 ml) was disrupted ultrasonically (Soniprobe, type 1130A; Dawe Instruments, London) for two 15 s periods separated by 1 min for cooling, and centrifuged for 20 min at 35000 g at 0 °C. The supernatant was termed cell-free extract.

The enzyme reaction mixtures (Table 2) were incubated at 39 °C (30 min) and the reactions were stopped by immersion in boiling water (10 min). Unlabelled acetate (150 μmol), butyrate (300 μmol), formate (25 μmol) and lactate (100 μmol), as carriers, were added before the alkaline mixtures were evaporated to dryness. The acids were separated on a silicic-acid column (75 x 1 cm) using the following sequence of solvents: benzene, 50 ml; chloroform, 100 ml; chloroform/t-butanol (99:1, v/v), 150 ml; chloroform/t-butanol (98:2, v/v), 100 ml; chloroform/t-butanol (95:5, v/v), 100 ml; chloroform/t-butanol (92:8, v/v), 250 ml. The extent of incorporation of label into the different acids was estimated as described above.

Sources of biochemicals and labelled compounds. Biochemicals were from Boehringer, and the 14C-labelled compounds from The Radiochemical Centre, Amersham.

RESULTS AND DISCUSSION

Growth was rapidly initiated in media containing NaHCO3 plus CO2 (Fig. 1). Both acetate and pyruvate stimulated growth, though pyruvate had a greater effect than acetate. In media free of NaHCO3 and CO2, there was an extended lag: acetate stimulated growth more than pyruvate but almost no growth occurred without acetate or pyruvate. The low rate of growth in CO2-free media may be due to a slow endogenous production of CO2 by the inoculum.

When incorporation of 14C from media containing [1-14C]acetate, NaH14CO3 or [1-14C]pyruvate into acetate, butyrate, formate and lactate by growing cells was followed (Table 1), butyrate was found to be extensively labelled from acetate, showing that extracellular acetate is a precursor of butyrate. The recovery of label in the fermentation acids was 87% while 6% was retained on the silicic acid column. The remainder was probably incorporated into cells but was not measured. Butyrate was formed from both acetate supplied and acetate produced from glucose, because the molar specific activity of butyrate was less than twice that of the acetate supplied. The labelling of lactate from acetate suggests the existence of a pyruvate synthase system (Bachofen, Buchanan & Arnon, 1964). Label in formate cannot arise from the hydrolytic splitting of [2-14C]pyruvate produced from [1-14C]acetate by pyruvate synthase, since formate would come from C-1 of pyruvate. Hence formate must be formed from acetate by other means. When the specific activities of acetate and lactate are compared it appears that about half the lactate was due to pyruvate synthesis. An amount of CO2 equivalent to that of acetate must have been used to produce pyruvate. Thus the extent of CO2 fixation is appreciable.

Label from NaH14CO3 appeared mostly in formate and lactate thus providing further evidence for activity of pyruvate synthase. Specific activities of formate and lactate were similar and so these acids could have been derived from the same pyruvate pool. A small amount of label appeared in acetate and butyrate. Preliminary work had shown that a small
Fig. 1. Growth of *Butyrivibrio fibrisolvens* (isolate 12) in defined medium containing glucose (△), glucose and acetate (○), or glucose and pyruvate (●), each in the presence or absence of CO₂. Acetate and pyruvate were equimolar.

Table 1. Recovery of acids and incorporation of ¹⁴C from [¹-¹⁴C]acetate, NaH¹⁴CO₃ or [¹-¹⁴C]pyruvate into these acids by *B. fibrisolvens* (isolate 12) grown in chemically-defined medium containing 0·8% NaHCO₃ and 1% glucose with a gas phase of 98% CO₂/2% H₂

<table>
<thead>
<tr>
<th>Additions per 100 ml medium</th>
<th>10 mmol Na [¹-¹⁴C]acetate*</th>
<th>NaH¹⁴CO₃† + 5 mmol Na acetate</th>
<th>5 mmol Na [¹-¹⁴C]pyruvate‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acids recovered</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Acetate (mmol/100 ml culture)</td>
<td>7·36 (2·64 utilized)</td>
<td>3·29 (1·71 utilized)</td>
<td>2·92</td>
</tr>
<tr>
<td>Butyrate (mmol/100 ml culture)</td>
<td>6788</td>
<td>182</td>
<td>248</td>
</tr>
<tr>
<td>Lactate (mmol/100 ml culture)</td>
<td>13551</td>
<td>96</td>
<td>147</td>
</tr>
<tr>
<td>Formate (mmol/100 ml culture)</td>
<td>1·18</td>
<td>1·43</td>
<td>0·66</td>
</tr>
<tr>
<td>Lactate (mmol/100 ml culture)</td>
<td>847</td>
<td>1·197</td>
<td>1·046</td>
</tr>
<tr>
<td>Lactate (mmol/100 ml culture)</td>
<td>1·46</td>
<td>2·28</td>
<td>6·89</td>
</tr>
<tr>
<td>Lactate (mmol/100 ml culture)</td>
<td>3236</td>
<td>1·071</td>
<td>811</td>
</tr>
</tbody>
</table>

Final specific activities: * 10·712 x 10² d.p.m./mmol; † not determined (6 μCi/100 ml); ‡ 8·632 x 10² d.p.m./mmol.

Amount of CO₂ could be included in the acetate peak despite acidification with strong acid, but label in the butyrate fraction shows that some CO₂ must have been incorporated into acetate. This result, together with the finding that label from [¹-¹⁴C]acetate enters formate, suggests that CO₂ exchanges with the carboxyl group of acetate (Ljungdahl & Wood, 1969). ¹⁴CO₂ so derived from [¹-¹⁴C]acetate could enter pyruvate and thence formate.

When pyruvate replaced acetate in the medium the proportions of the fermentation acids were changed. Although pyruvate appeared to be converted mainly to lactate, the specific activity of lactate was only about 10% of that of the added pyruvate. This could have been due to dilution of exogenous with endogenously produced pyruvate, but the possible production of pyruvate from the available glucose was calculated to be too low.
Table 2. Percentage recovery of $^{14}$C in acetate, butyrate, formate and lactate when cell-free extract of B. fibrisolvens (isolate 12) was incubated with $^{14}$C-labelled pyruvate, bicarbonate or acetyl-CoA

The reaction mixtures contained: Cell-free extract pretreated with lysozyme and EDTA, 3 ml; dithiothreitol, 10 mg; deionized water, to 5.5 ml; plus either (i) Na [1-14C]pyruvate, 4 μmol (0.2 pCi); CoA, 1 μmol; phosphotransacetylase, 2 u.; Na glyoxylate, H$_2$O, 4 μmol; Na arsenate, 10 μmol; or (ii) NaH$^{14}$CO$_3$, 2 μmol (1 μCi); acetyl-CoA, 2 μmol; or (iii) [1-14C]acetyl-CoA, 2 μmol (1 μCi); NaHCO$_3$, 2 μmol; NADH, 2 μmol. The gas phase was H$_2$.

<table>
<thead>
<tr>
<th></th>
<th>Acetate</th>
<th>Butyrate</th>
<th>Formate</th>
<th>Lactate</th>
</tr>
</thead>
<tbody>
<tr>
<td>(i) Na [1-14C]pyruvate</td>
<td>0</td>
<td>0</td>
<td>8.7</td>
<td>12.2</td>
</tr>
<tr>
<td>(ii) NaH$^{14}$CO$_3$</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>(iii) [1-14C]acetyl-CoA</td>
<td>91.6</td>
<td>5.6</td>
<td>91.6</td>
<td>5.6</td>
</tr>
</tbody>
</table>

Table 3. Recovery of $^{14}$C in the acids produced by B. fibrisolvens (isolate 12) treated with toluene and incubated with NaH$^{14}$CO$_3$ and an acetyl-CoA regenerating system or NaH$^{14}$CO$_3$ and pyruvate

The reaction mixtures contained: NaH$^{14}$CO$_3$, 100 μmol (1 μCi); phosphotransacetylase, 50 u.; lysozyme, 10 mg; toluene-treated bacteria, 7.5 ml; plus either (i) acetyl phosphate, 250 μmol; CoA, 2.5 μmol; reduced glutathione, 100 μmol; deionized water, 1.5 ml; or (ii) Na pyruvate, 250 μmol; acetyl phosphate, 125 μmol; CoA, 1.25 μmol; ATP, 40 μmol; NADH, 10 μmol; MnCl$_2$.4H$_2$O, 20 μmol; MgCl$_2$.6H$_2$O, 50 μmol; KCl, 400 μmol; deionized water, 2.6 ml. The gas phase was H$_2$.

Results are expressed as d.p.m. per 2 ml supernatant of reaction mixtures.

<table>
<thead>
<tr>
<th></th>
<th>(i) NaH$^{14}$CO$_3$ plus acetyl-CoA regenerating system</th>
<th>(ii) NaH$^{14}$CO$_3$ plus pyruvate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Acetate</td>
<td>810</td>
<td>949</td>
</tr>
<tr>
<td>Butyrate</td>
<td>318</td>
<td>592</td>
</tr>
<tr>
<td>Formate</td>
<td>498</td>
<td>1813</td>
</tr>
<tr>
<td>Lactate</td>
<td>320</td>
<td>7703</td>
</tr>
<tr>
<td>Recovery (%)</td>
<td>1.3</td>
<td>7.3</td>
</tr>
</tbody>
</table>

Extraction of $^{14}$C in carboxylate, formate and lactate when cell-free extract of B. fibrisolvens (isolate 12) was incubated with $^{14}$C-labelled pyruvate, bicarbonate or acetyl-CoA could explain the discrepancy. The exchange of CO$_2$ with the carboxyl group of pyruvate seemed more likely and is characteristic of systems in which pyruvate synthesis occurs (Buchanan & Arnon, 1969). The labelled CO$_2$ released could have exchanged with the carboxyl group of acetate giving the small amount of label which was again found in acetate and butyrate.

Extracts catalysed the reduction of pyruvate to lactate (Table 2) in the absence of added NADH or NADPH. Joyner & Baldwin (1966) and Kistner & Kotzé (1973) found very low activities for lactic dehydrogenase in extracts of B. fibrisolvens despite the fact that this species produces lactate. This could mean that pyruvate reduction depends on an electron carrier other than NADH or NADPH.

Extracts contained the enzymes necessary for the synthesis of butyrate from acetate (Table 2). However, no label from NaH$^{14}$CO$_3$ was found in any of the fermentation acids, showing the absence of pyruvate synthase. The mechanisms responsible for inclusion of $^{14}$C from $^{14}$CO$_2$ into acetate and from [1-14C]acetate into formate were also absent. No incorporation of $^{14}$CO$_2$ into formate would indicate the absence of formate dehydrogenase (Thauer, Rupprecht & Jungermann, 1970). The formation of H$^{14}$COOH from [1-14C]pyruvate must thus have been due to pyruvate formate lyase activity. This is contrary to the conclusions of Miller (1975, and personal communication) who failed to detect incorpora-
tional evidence that it is formed via pyruvate formate lyase and not formate dehydrogenase. Formate is produced by direct reduction of CO₂. Increased incorporation of pyruvate synthesis is catalysed by an insoluble fraction. In this, toluene-treated bacteria also incorporated 14C from 14CO₂ into acetate and butyrate. This led to extensive labelling of lactate which again pointed to exchange of CO₂ with the carboxyl group of pyruvate. The increased incorporation of CO₂ into HCOOH in the presence of pyruvate provides additional evidence that it is formed via pyruvate formate lyase and not formate dehydrogenase. The test was repeated with avidin added to the reaction mixture so that biotin, a cofactor of pyruvate carboxylase, would be inactivated. This did not reduce the extent of labelling of acetate and butyrate. Hence it seems that label from 14CO₂ does not enter acetate by way of the reductive carboxylic acid cycle.

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REFERENCES


Metabolism of Butyrivibrio fibrisolvens


