Intermediary Metabolism in *Clostridium acetobutylicum*: Levels of Enzymes Involved in the Formation of Acetate and Butyrate

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The levels of seven intermediary enzymes involved in acetate and butyrate formation from acetyl coenzyme A in the saccharolytic anaerobe *Clostridium acetobutylicum* were investigated as a function of time in solvent-producing batch fermentations. Phosphate acetyltransferase and acetyl kinase, which are known to form acetate from acetyl coenzyme A, both showed a decrease in specific activity when the organism reached the solvent formation stage. The three consecutive enzymes thiolase, β-hydroxybutyryl-coenzyme A dehydrogenase, and crotonase exhibited a coordinate expression and a maximal activity after growth had ceased. Only low levels of butyryl coenzyme A dehydrogenase activity were found. Phosphate butyryltransferase activity rapidly decreased after 20 h from 5 to 11 U/mg of protein to below the detection limit (1 mU/mg). Butyrate no longer can be formed, and the metabolic flux may be diverted to butanol. Butyrate kinase showed a 2.5- to 10-fold increase in specific activity after phosphate butyryltransferase activity no longer could be detected. These results suggest that the uptake of acetate and butyrate during solvent formation can not proceed via a complete reversal of the phosphate transferase and kinase reactions. The activities of all enzymes investigated as a function of time in vitro are much higher than the metabolic fluxes through them in vivo. This indicates that none of the maximal activities of the enzymes assayed is rate limiting in *C. acetobutylicum*.

The saccharolytic anaerobe *Clostridium acetobutylicum* is known to utilize several different carbohydrates as the sole source of carbon and energy (22, 44). The main end products of the fermentation are acetate, butyrate, acetone, butanol, ethanol, carbon dioxide, and hydrogen (15). The typical acetone-butanol fermentation can be divided into two distinctive phases. In the acidogenic phase acetate and butyrate are formed together with carbon dioxide and hydrogen. During this stage the organism grows rapidly and the pH drops to 4 to 4.5 due to the accumulation of acids in the medium (15). The pH then remains at a fairly constant level throughout the remainder of the fermentation. In the second phase the growth slows down, acids are taken up from the medium and metabolized, and acetone, butanol, ethanol, carbon dioxide, and hydrogen are formed (3, 8, 29). Although many investigators (7, 11, 23, 25, 28, 43, 45, 46) since Speakman (32) have studied the biochemistry of the acetone-butanol fermentation, little is known about the intermediary metabolism and its regulation and the factors responsible for the shift from acid production to acid metabolism and neutral solvent formation. In this investigation we report on the metabolic pathways of acetate and butyrate formation from acetyl coenzyme A (acetyl-CoA) in *C. acetobutylicum* grown on glucose. The variations in specific activities of the enzymes responsible for the production of these acids from acetyl-CoA are presented as a function of fermentation time. The studies were carried out on typical solvent-producing, butylic, batch fermentations.

MATERIALS AND METHODS

**Materials.** The lithium salt of butyryl phosphate was prepared by the method of Avison (1). Potassium butyrate was prepared by neutralization of butyric acid with potassium hydroxide. *Clostridium kluyveri* cells were a kind gift from T. C. Stadtman, National Institutes of Health. All co-factors, CoA thioesters, and electron transport dyes were purchased from Sigma Chemical Co. All other materials were obtained from commercial sources and were of the highest available purity.

**Bacterial strain and growth conditions.** *C. acetobutylicum* ATCC 824 was obtained from the American Type Culture Collection. The culture was maintained as spores on sterile soil. The cells were grown in a medium containing the following (grams per liter): (NH₄)₂SO₄, 2.0; K₂HPO₄, 1.0; KH₂PO₄, 0.5; MgSO₄·7H₂O, 0.1; FeSO₄·7H₂O, 0.015; CaCl₂, 0.01; MnSO₄·H₂O, 0.01; CoCl₂·6H₂O, 0.002; ZnSO₄·7H₂O, 0.002; Na₂SeO₃, 0.00025; tryptone, 2.0; yeast extract, 1.0; glucose, 50. The stock culture was heat shocked at 90°C for 5 min in 200 ml of growth medium and incubated in an anaerobic box (Forma Scientific; model 1024) at 37°C for 24 h. The volume of the culture was scaled up to 20 liters in two successive transfers.

**Fermentations.** The butylic fermentations were carried out at 37°C in a jacketed 100-liter stainless steel substrate tank (AB Taby Rostfria, Taby, Sweden) with a 90-liter working volume with the medium described above. Glucose was autoclaved separately from other components of the medium. Before inoculation anaerobic conditions were maintained by sparging the medium with nitrogen. The initial pH before inoculation was 6.5. When temperature equilibrium had been reached the medium was inoculated with a 10% (vol/vol) inoculum from the 20-liter culture. The pH was not controlled during the fermentation. Growth was followed by measuring the optical density at 540 nm. One optical density unit at 540 nm corresponded to 0.14 g of cells (dry weight) per liter.

**Samples.** Samples (1 liter) were withdrawn at the times indicated in Fig. 2 and centrifuged at 15,000 × g for 20 min. Samples of the supernatants were filtered and stored at −20°C for gas-liquid chromatographic analyses. The har-
vested cells were immediately frozen and stored in liquid nitrogen until they were used for the preparation of cell-free extracts.

**Preparation of cell-free extracts.** The cells harvested from 1-liter samples were thawed in 4 ml of 50 mM 2-(N-morpholine) propanesulfonic acid–KOH (pH 7.0) containing 1 mM 1,4-dithiothreitol. The cell suspensions were sonicated four times for 30 s at 45% of maximum power output at 0°C with a Branson B-12 sonifier. Cellular debris was removed by centrifugation at 40,000 × g for 30 min at 4°C. All superna- 
tants were desalted by gel permeation chromatography on Phenyl-PD-10 columns equilibrated with 50 mM 2-(N
dithiothreitol)propanesulfonic acid–KOH (pH 7.0) containing 1 mM 1,4-dithiothreitol. The desalted extracts were immediately frozen and stored at −75°C until used for enzyme assays and protein determinations.

**Protein determination.** Protein concentrations in crude extracts were estimated by the dye-binding method of Bradford (6). Determinations of the protein content of whole cells were made by the biuret method as described by Herbert et al. (14). In both methods gamma globulin was used as the reference protein. For the calculations of the fluxes of metabolites in the cells a value of 0.7 mg of protein per mg of cell dry weight was used.

**Analysis of fermentation products.** The concentrations of acetone, butan-2anol, ethanol, acetic, and butyric acids were determined by gas-liquid chromatography with a Perkin-Elmer 3920B gas chromatograph equipped with a flame ionization detector. The instrument was fitted with a Teflon column (6 ft [ca. 1.8 m] by 0.085 in. [ca. 0.22 cm], inner diameter) packed with Chromosorb 101 (80-100 mesh; E. Merck, A. G. Darmstadt, West Germany). The 0.5-μl samples, acidified with metaphosphoric acid to ensure protonation of the acids, were injected at an injector temperature of 180°C. The analyses were run isothermally at a column temperature of 180°C and a detector temperature of 215°C. The nitrogen carrier gas flow rate was 30 ml/min. External standards containing all five fermentation products to be separated were used.

**Electrophoretic analysis.** Disc gel electrophoresis of crude extracts from different stages of the fermentations was performed with 7.5% polyacrylamide gels under non-denaturing conditions by a modification of the method of Laemmli (17).

Enzyme activity staining of butyrate kinase after electrophoresis of crude extracts was carried out by the hydroxamate method of Rose (27) with the assay mixture described below for the enzyme assays. The gel was incubated in 60 ml of reaction mixture for 20 min at 25°C. Then the reaction mixture was discarded, and the gel was immersed in 100 ml of FeCl₃ reagent until a brown color developed.

**Enzyme assays.** Unless otherwise indicated, all enzymes were assayed in air at 30°C in masked 1-ml cuvettes in a Gif- 
fract 2600 or a Shimadzu UV-120-02 spectrophotometer. At the dilutions of crude extracts used in the assays, NADH oxidase activity was negligible. One unit of enzyme activity is defined as the amount of enzyme catalyzing the conversion of 1 μmol of reactant to products per min. All of the enzymes assayed as a function of time in crude extracts were stable to repeated freezing and thawing and storage at −75°C during the course of this investigation.

Phosphate acetyltransferase (acetyl-CoA:orthophosphate acetyltransferase, EC 2.3.1.8) and phosphate butyryltransferase (butyryl-CoA:orthophosphate butyryltransferase, EC 2.3.1.19) were assayed by measuring the formation of acetyl-CoA from acyl phosphate. The extinction coefficient used was 4,440 M⁻¹ cm⁻¹ at 233 nm. The 1.0-ml reaction mixture was a modification of that described by Bergmeyer et al. (4) and contained 100 mM Tris-hydrochloride (pH 8.0), 100 mM KCl, 0.6 mM CoA, and 10 mM lithium acyl phosphate. The reaction was initiated by the addition of extract. Phosphate butyryltransferase was also assayed in the butyryl phosphate-forming direction. The disappearance of the thioester bond was measured spectrophotometrically at 233 nm. The 1.0 ml reaction mixture contained 100 mM potassium phosphate (pH 7.0) and extract. The addition of 0.1 mM butyryl-CoA initiated the reaction. The assay was linear with activities up to 0.05 absorbancy units per min. To determine possible thioesterase activity, controls containing 50 mM 2-(N-morpholine)propanesulfonic acid–KOH (pH 7.0), 0.1 mM butyryl-CoA, and extract were run.

Acetate kinase (ATP:acetate phosphotransferase, EC 2.7.2.1) and butyrate kinase (ATP:butyrate phosphotransferase, EC 2.7.2.7) were assayed by the hydroxamate method of Rose (27). The absorbances were measured at 540 nm by using blanks where ATP had been omitted. Acetyl hydroxamate was used for preparation of all standard curves as the extinction coefficients of acetyl hydroxamate and butyryl hydroxamate are essentially identical (19). To investigate the reversibility of butyrate kinase the enzyme was also assayed in the butyrate-forming direction in a coupled assay with hexokinase and glucose 6-phosphate dehydrogenase (18). The 1.0-ml reaction mixture contained 75 mM Tris-hydrochloride (pH 8.0), 6 mM ADP, 10 mM MgCl₂, 2 mM glucose, 1 U of hexokinase, 0.2 mM NADP⁺, 1 U of glucose 6-phosphate dehydrogenase, and rate-limiting amounts of enzyme. The reaction was initiated by the addition of 7 mM butyryl phosphate. The controls contained assay mixture where ADP had been omitted.

Determination of thiolase (acetyl-CoA:acetyl-CoA C-acetyltransferase, EC 2.3.1.9) activity were made in the direction of CoA-dependent acetocetyl-CoA cleavage. The decrease in absorbance at 303 nm was measured spectrophotometrically (36). The 1.0-ml reaction mixture contained 100 mM Tris-hydrochloride (pH 8.0), 1 mM 1,4-dithiothreitol, 10 mM MgCl₂, 50 μM acetoacetyl-CoA, and extract. The addition of 2.0 mM coenzyme A initiated the reaction. The molar extinction coefficient used was 14,000 M⁻¹ cm⁻¹ (37).

β-Hydroxybutyryl-CoA dehydrogenase (1,3-hydroxyacyl-CoA:NAD⁺ oxidoreductase, EC 1.1.1.35) activity was assayed by a modification of the method of Madan et al. (21). The 1.0-ml assay mixture contained 50 mM 2-(N-morpholine)propanesulfonic acid–KOH, pH 7.0, 1 mM dithiothreitol, 0.2 mM NADH, and extract. The addition of 75 μM acetoacetyl-CoA initiated the reaction.

Crotonase (1-3-hydroxyacyl-CoA hydrolyase, EC 4.2.1.17) was assayed by determining the decrease in absorbance at 263 nm due to hydration of the conjugated double bond of crotonoyl-CoA as described by Stern (35). The extinction coefficient of crotonoyl-CoA was 6,700 M⁻¹ cm⁻¹ (20). The 1.0-ml reaction mixture contained 100 mM Tris-(hydrochloride (pH 7.6) and extract. The addition of 0.15 mM crotonoyl-CoA initiated the reaction.

For the assay of butyryl-CoA dehydrogenase (butyryl-CoA:NAD⁺ oxidoreductase, EC 1.3.99.2) three different methods were tried. In the first method, where butyryl-CoA is oxidized to crotonoyl-CoA, the reduction of iodonitrotetrazolium [2-(4-iodophenyl)-3-(4-nitrophenyl)-5-phenyldiazotetrazolium] and following spectrophotometrically at 492 nm (9). The 1.0 ml reaction mixture contained 100 mM potassium phosphate (pH 7.0), 0.15% Triton X-100, 0.2 mM Meldolablau (zinc chloride salt of 8-dimethylmino-2,3-ben-
zophenoxazine), 0.3 mM iodonitrotetrazolium, and the crude extract. The stoppered cuvettes were flushed with hydrogen gas for 2 min, and the reaction was initiated by the addition of 0.1 mM butyryl-CoA. The extinction coefficient was 19,400 M⁻¹ cm⁻¹ (9). The necessary controls without butyryl-CoA and without enzyme were performed. In the second method the oxidation of prerreduced Safranin O was followed spectrophotometrically at 520 nm (30). The assays were carried out in an anaerobic box in an atmosphere consisting of 85% N₂, 10% H₂, and 5% CO₂. In the third method the reduction of 2,6-dichlorophenolindophenol was followed (10).

**RESULTS AND DISCUSSION**

In the acetone-butanol fermentation of glucose by various saccharolytic clostridia the final fatty acid and neutral solvent products are derived from acetyl-CoA by the series of reactions outlined in Fig. 1. Although there is considerable information about the relative amounts of products formed and pH changes during the course of the fermentations, the factors that control the shift from acid production to neutral solvent production are not well understood. In the present study the levels of enzymes that catalyze a number of the individual steps of the overall process (Fig. 1) were measured as a function of time in C. acetobutylicum cells from a butylic fermentation without pH control (Fig. 2). Crude extracts prepared from cells harvested at the times indicated in Fig. 2 were desalted and assayed for individual enzyme activities as described above.

A more butyric type of fermentation, where the pH was maintained at 6 during the first 10 h by the addition of potassium hydroxide, was also investigated. The end products of this fermentation were predominantly butyric and acetic acids. No major differences in the levels of the investigated enzymes were found between the butyric and the butylic fermentations.

**Formation of acetate from acetyl-CoA.** Two enzymes, phosphate acetyltransferase and acetate kinase, which catalyze reactions 1 and 2, respectively, of Fig. 1, are involved in acetate formation from acetyl-CoA. These enzymes have been suggested to exist in all anaerobic bacteria that utilize acetyl-CoA to synthesize ATP via substrate level phosphorylation (38).

**Phosphate acetyltransferase.** The variations in levels of phosphate acetyltransferase during the course of the butylic fermentation are shown in Fig. 3. The enzyme was measured in the direction of acetyl-CoA formation, which is the reverse of the in vivo reaction that leads eventually to acetate formation. The activity showed a marked decrease after 13 h, when the acetate formation rate slowed down and growth ceased. Phosphate acetyltransferase from C. acetobutylicum shows similarities with the enzyme from C. kluyveri with respect to the influence of monovalent cations on activity (33). Our investigations showed that the C. acetobutylicum enzyme was activated by K⁺ and NH₄⁺ and was inhibited by Na⁺. Half-maximal activation was obtained by 60 mM K⁺ in the form of KCl.

**Acetate kinase.** The physiological role of acetate kinase during the first phase of the acetone-butanol fermentation is to catalyze the phosphorylation of ADP and the synthesis of acetate from acetyl phosphate. In this study acetate kinase was assayed in the direction of acetate phosphate formation. The specific activities of the enzyme as a function of time are shown in Fig. 3. The specific activity exhibited a maximum after only 4 h and dropped by 90% before growth ceased and
hydroxystearyl-CoA dehydrogenase, and crotonase. They all showed maximum activity after 17 h of fermentation. The reason for the high specific activities toward the end may be a reflection of the change in pH during the course of the fermentation. Berndt and Schlegel (5) have determined the pH optimum for thiolase from Clostridium pasteurianum to be 8.1 in the condensation direction. To our knowledge no pH optima for clostridial NADH-dependent β-hydroxybutyryl-CoA dehydrogenase and crotonase have been published. The pH of the interior of the cell follows the external pH to a certain extent throughout the fermentation (26). This creates an unfavorable milieu for thiolase and possibly also for the two other enzymes. Thereby more enzyme may be needed to compensate for the decrease in enzyme activities due to a low pH value. A drop in pH may be an important factor for the regulation of these three consecutive enzymes.

**Butyryl-CoA dehydrogenase.** Butyryl-CoA dehydrogenase has been shown to be present in several clostridia, such as Clostridium butylicum and C. kluyveri (2). In desalted crude extracts of C. acetobutylicum, however, we found only extremely low activities. Butyryl-CoA dehydrogenase could not be assayed by the 2,6-dichlorophenolindophenol (10) or the Safranin O assays (30). No activity was exhibited with the physiological electron donors NADH and NADPH. Activity could only be demonstrated with the Meldolablau assay (9) at pH 7.0 (0.1 M potassium phosphate). This assay worked only after the reaction mixture had been flushed with hydrogen gas, and then the enzyme showed a sluggish and nonlinear activity. In 0.1 M potassium phosphate (pH 6.2), only traces of activity could be found, and in 0.1 M Tris-hydrochloride (pH 8.0), no activity was detected. To test the assay procedures, extracts from C. kluyveri were prepared as described above, and the specific activity of butyryl-CoA dehydrogenase was determined by the Meldolablau and Safranin O assays. The activities were 0.8 and 0.7 U/mg, respectively. This was more than 100-fold higher than the activities found in C. acetobutylicum extracts. Due to poor reproducibility, no determinations of the time dependence of butyryl-CoA dehydrogenase in C. acetobutylicum were made. The reason for the low activity is not known. One possibility may be that the enzyme is oxygen sensitive and is inactivated during sonication and preparation of the desalted crude extracts.

**Butyrate formation from butyryl-CoA.** Saccharolytic clostridia form butyryl phosphate from butyryl-CoA via phosphate butyryltransferase (41). Butyrate is formed from butyryl phosphate with concomitant phosphorylation of ADP. The enzyme catalyzing this reaction is butyrate kinase (39, 40).

Phosphate butyryltransferase. Phosphate butyryltransferase was partially purified from C. acetobutylicum by Gavard et al. (11). They demonstrated that two different enzymes are responsible for the formation of butyryl phosphate and acetyl phosphate from butyryl-CoA and acetyl-CoA, respectively. They reported a phosphate butyryltransferase activity of 0.11 U/mg of protein in crude extracts. The presence of this enzyme in C. acetobutylicum was also shown by Valentine and Wolfe (41). In our investigation phosphate butyryltransferase activity was assayed in both directions with identical activity profiles as a result. Specific activities of 11 U/mg were measured in the butyryl-CoA-forming direction (Fig. 5). The activity was not growth related and reached its maximum after only 5 h. It then decreased rapidly to a value below the detection limit, 1 mU/mg, while the cells still were actively growing. From the data in Fig. 5 it becomes evident that no more butyrate can be formed from butyryl-CoA via
actively metabolizing culture of C. acetobutylicum, 84% of the label was found in butanol (45). The mechanism of butyric acid uptake and conversion to butanol is, however, still unknown. It is assumed that the transport of acetic and butyric acids across the cytoplasmic membranes of clostridia is effected by passive diffusion of the uncharged forms of these acids (38). Acetic acid has been shown to cross the membrane of C. pasteurianum in the protonated form (16). During the solvent formation phase the activity of butyrate kinase was still high, but the phosphate butyryltransferase activity was very low or not even detectable (Fig. 5). Therefore no more butyryl-CoA could be formed from butyryl phosphate. Instead a direct reduction of butyryl phosphate to butyraldehyde and further to butanol may be possible. Stadtman and Barker (34) demonstrated an enzymatic reduction of butyryl phosphate to butanol by using dried cells of C. kluyveri and hydrogen gas as the reductant. Although the direct reduction of butyryl phosphate to butanol was not demonstrated, this potential mechanism for the reutilization of butyric acid is metabolically more attractive than a complete reversal of both the butyrate kinase and phosphate butyryltransferase steps. We are presently investigating possible mechanisms for the uptake and activation of acetic acid and butyric acid in C. acetobutylicum.

**Metabolic fluxes.** In an attempt to determine any possible enzyme limitations for the metabolic fluxes through the seven enzymes investigated, the fluxes in vivo were compared with the enzyme capacities measured in vitro. Despite the ambiguities of such a comparison when the in vivo activities and regulating mechanisms are not known, it may indicate possible rate-limiting steps in the metabolic pathways leading to acetate and butyrate formation. The specific metabolic fluxes that are brought about in vivo through the action of the investigated enzymes were estimated from the product formation rates and protein content of the cells. As an approximation, the fluxes through phosphate acetyltransferase and acetate kinase reactions were estimated from acetate formation rates. Butyrate, butanol, and acetone formation rates were used for calculation of the fluxes through the thiolase reaction. The fluxes through the β-hydroxybutyryl-CoA dehydrogenase and crotonase reactions were determined from the formation rates of butyrate and butanol. Butyrate formation rates were used for calculation of the fluxes through the phosphate butyryltransferase and butyrate kinase-catalyzed steps. It should be noted that all enzyme activities were not measured under optimal conditions that gave maximal activities. Therefore the degree of utilization of the enzymes in vivo is, in most cases, even lower than is shown in Table 1. The activities of the enzymes in vitro are much higher than the fluxes, attributable to them in vivo, which means that in this fermentation

### TABLE 1. Specific metabolic fluxes in C. acetobutylicum in vivo

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>% Utilization</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phosphate acetyltransferase</td>
<td>0.3–10</td>
</tr>
<tr>
<td>Acetate kinase</td>
<td>1.2–11.5</td>
</tr>
<tr>
<td>Thiolase</td>
<td>0.3–2.8</td>
</tr>
<tr>
<td>β-Hydroxybutyryl-CoA dehydrogenase</td>
<td>0.3–4.3</td>
</tr>
<tr>
<td>Crotonase</td>
<td>0.01–1.1</td>
</tr>
<tr>
<td>Phosphate butyryltransferase</td>
<td>0.08–1.3</td>
</tr>
<tr>
<td>Butyrate kinase</td>
<td>0.002–0.7</td>
</tr>
</tbody>
</table>

* Percentage of measured specific enzyme activities in vitro during the butylic batch fermentation shown in Fig. 2.
the enzymes are used to a low extent. Despite the approximations made, the data in Table 1 indicate that there is no restriction of the fluxes due to enzyme shortages. This implies that none of the maximal enzyme activities measured seems to be rate limiting in the cells during an acetone-butanol fermentation.

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