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Uptake and activation of acetate and butyrate in *Clostridium acetobutylicum*

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Summary. The pathway for uptake of acids during the solvent formation phase of an acetone-butanol fermentation by *Clostridium acetobutylicum* ATCC 824 was studied. ^{13}C NMR investigations on actively metabolizing cells showed that butyrate can be taken up from the medium and quantitatively converted to butanol without accumulation of intermediates. The activities of acetate phosphotransacetylase, acetate kinase and phosphate butyryltransferase rapidly decreased to very low levels when the organism began to form solvents. This indicates that the uptake of acids does not occur via a reversal of these acid forming enzymes. No short-chain acyl-CoA synthetase activity or butyryl phosphate reducing activity could be detected. Based on our results and a critical analysis of literature data on acetone-butanol fermentations, it is suggested that an acetoacetyl-CoA : acetate (butyrate) CoA-transferase is solely responsible for uptake and activation of acetate and butyrate in *C. acetobutylicum*. The transferase exhibits a broad carboxylic acid specificity. The key enzyme in the uptake is acetoacetate decarboxylase, which is induced late in the fermentation and pulls the transferase reaction towards formation of acetoacetate. The major implication is that it is not feasible to obtain a batch-wise butanol fermentation without acetone formation and retention of a good yield of butanol.

Introduction

A batch-wise acetone-butanol fermentation by *Clostridium acetobutylicum* can be divided into two distincitive stages, and acid production phase and a solvent production phase (Johnson et al. 1931). The first stage is characterized by rapid growth and formation of acetic and butyric acids that are excreted into the medium, thereby lowering its pH-value. During the second stage acetone, butanol, and ethanol are the major products formed. The production of acids ceases as well as growth. The acids formed earlier in the fermentation pass back into the cells in protonated form (Kell et al. 1981) and are reutilized for solvent production. Wood et al. (1945) have shown that butyrate added to the medium permeates the cytoplasmic membrane and is almost quantitatively converted to butanol. Due to the uptake, the pH of the medium increases slightly. The uptake has been seen as a detoxification process in response to an unfavourable environment, since the solvents are less toxic than the acids produced (Costa 1981). At the end of the fermentation the microbial activity ceases, primarily due to high levels of solvents which alter the functionality of the cell membrane (Moreira et al. 1981). It has been shown that butanol can be formed from butyryl-CoA by the action of two NADH-dependent dehydrogenases (Andersch et al. 1983). Different mechanisms have been suggested for uptake and activation of the acids produced earlier in the fermentation. Valentine and Wolfe (1960) proposed that a reversal of the two enzymatic reactions responsible for butyrate formation from butyryl-CoA, the phosphate butyryltransferase and butyrate kinase reactions, could operate during the formation of butanol from butyric acid. Doelle (1975) and Andersch et al. (1983) suggested that an acetoacetyl-CoA : acetate (butyrate) CoA-transferase plays a role in the uptake of the acids during the solvent production phase. Other possible mechanisms involve ATP-requiring acetyl-CoA and butyryl-CoA synthetases or a reversal of the acetate and butyrate kinases and a direct reduction of the acyl phosphates formed to the corresponding aldehydes. In this investigation we have studied possible mechanisms of uptake and activation of the acids produced

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during the frist stage of the acetone-butanol fermentation by *C. acetobutylicum*.

Materials and methods

Materials. Potassium salts of carboxylic acids were prepared by neutralization of the acids with potassium hydroxide. Lithium butyryl phosphate was prepared according to the method of Avison (1955). Acetoacetyl-CoA was purchased from Sigma Chemical Co. and sodium [1-^{13}C]butyrate (92 atom%) was obtained from Amersham. All other chemicals were of the best commercial quality.

Organism and growth conditions. Clostridium acetobutylicum ATCC 824 was grown at 35° C in a medium containing (g/l): $(NH_4)_2SO_4$, 2.0; K_2HPO_4, 1.0; KH_2PO_4, 0.5; $MgSO_4 \cdot 7\,H_2O$, 0.1; $FeSO_4 \cdot 7\,H_2O$, 0.015; $CaCl_2$, 0.01; $MnSO_4 \cdot H_2O$, 0.01; $CoCl_2$, 0.002; $ZnSO_4 \cdot 7\,H_2O$, 0.002; Na_2SeO_3, 0.00025; Tryptone, 2.0; Yeast extract, 1.0; Glucose, 50.

Preparation of crude extracts. The cells were harvested by centrifugation and supended in 50 mM MOPS-KOH, pH 7.0 [2-(N-morpholino)propane-sulfonic acid] containing 1 mM dithiothreitol. The cells were disintegrated by sonication and after centrifugation the crude extracts were desalted using prepacked Pharmacia PD-10 columns. Where indicated, this procedure was carried out under anaerobic conditions in an anaerobic glove box (Forma Scientific, USA).

Analytical methods. Protein concentrations were estimated by the method of Bradford (1976) using gamma globulin as the reference protein.

NMR studies. Cultures of 1l each were grown for the ^{13}C NMR experiments. Cells were harvested by centrifugation in the solvent production phase. The pellets were washed once and resuspended in an equal volume of a medium containing (g/l): $K_2HPO/_4$, 1.0; KH_2PO_4, 1.0; $(NH_4)_2SO_4$, 2.0; Glucose, 80. The pH of this medium was adjusted to 5.0 using phosphoric acid. Finally 50 mM sodium [1-^{13}C]butyrate and 10% v/v D_2O were added to the cell suspension which was pipetted into 10 mm NMR tubes. The NMR spectra were measured on a Bruker WP-200 nuclear magnetic resonance spectrometer operating at 50.3 MHz for ^{13}C. The spectra are Fourier transforms of the accumulated free induction decays obtained using radiofrequency pulses with a flip angle of 45° and a repetition time of 2.5 s. In order to make the signal intensities comparable, the broadband decoupling was turned on only during the aquisition of the free induction decays (a few hundred milliseconds). Thereby also an excessive heating of the sample was avoided. In these heterogeneous samples it was not possible to obtain line widths of less than 25 Hz. However, the internal deuterium field lock was used during the experiments to avoid systematic field drift. The peaks in the spectra were identified by comparison with the natural abundance ^{13}C resonances of the compounds.

Enzyme assays. Phosphate acetyltransferase (EC 2.3.1.8) and phosphate butyryltransferase (EC 2.3.1.19) activities were determined in the acyl-CoA forming direction using the method of Bergmeyer et al. (1963).

Acetate kinase (EC 2.7.2.1) and butyrate kinase (EC 2.7.2.7) were assayed in the acyl phosphate forming direction as described by Rose (1955).

For assay of CoA-dependent acetyl-CoA synthetase (EC 6.2.1.1) and butyryl-CoA synthetase (EC 6.2.1.2) activities, the method for assay of kinases described by Rose (1955) was used under anaerobic conditions. The acetate and butyrate kinase activities, respectively, were used as blanks. To measure acyl-CoA synthetase activity 2 mM CoA was added to the reaction mixture.

Acetoacetyl-CoA : acetate (butyrate) CoA-transferase (EC 2.8.3.-) activity was determined by following the disappearance of the enolate form of acetoacetyl-CoA as described by Sramek and Frerman (1975). The assay mixture (1.0 ml) contained 175 mM Tris-HCl, pH 7.5; 20 mM $MgCl_2$; 2 mM dithiothreitol; 0.1 mM acetoacetyl-CoA and desalted crude extract. The reaction was initiated by the addition of 100 mM of the potassium salt of the carboxylic acid substrate. The molar extinction coefficient in this assay was 8,000 $M^{-1} \cdot cm^{-1}$ (Stern 1956).

The reduction of butyryl phosphate by various reductants was assayed under anaerobic conditions by determination of remaining butyryl phosphate by the hydroxamate method of Rose (1955). The assay mixture (1.0 ml) contained 100 mM potassium phosphate pH 6.0 or pH 7.0; 5 mM dithiothreitol; 5 mM butyryl phosphate; desalted crude extract and reductant. The different reductants used were NADH, 1 mM; NADPH, 1 mM; and hydrogen. The cofactors were incubated both in the presence and in the absence of hydrogen. When hydrogen was used, the test tubes were flushed with hydrogen gas for 5 min. All samples were incubated for 20 min at 30° C.

Results and discussion

^{13}C NMR studies

^{13}C NMR is a noninvasive technique for investigations of metabolism and changes in concentrations of metabolites in living cells. Anaerobic organisms are especially suitable for NMR investigations, since it is easy to maintain anaerobic conditions throughout the experiments.

In an attempt to detect possible intermediates in the metabolic pathway from butyrate to butanol during uptake of butyrate in *C. acetobutylicum*, ^{13}C NMR spectra were recorded of cells harvested in the solvent formation phase of the fermentation. Anaerobicity was maintained during the whole experiments, where enriched [1-^{13}C] butyrate was added to the actively metabolizing cell suspensions. Figure 1 shows a 12.5-kHz spectrum recorded 2 h after the

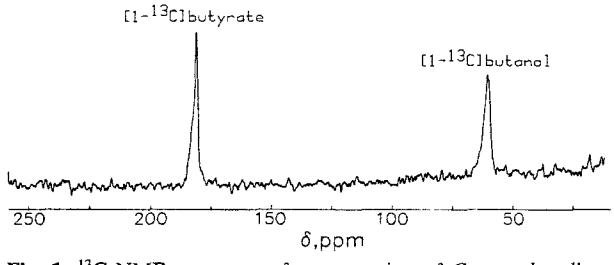

Fig. 1. ^{13}C NMR spectrum of a suspension of *C. acetobutylicum* cells at 5° C, recorded 2 h after the addition of 50 mM [1-^{13}C]butyrate. The spectrum represents an accumulation of 700 free induction decays. The shifts are given relative to tetramethylsilane

addition of ^{13}C-butyrate to the system. It was not possible to detect any other peaks than those of [1-^{13}C]butyrate and [1-^{13}C]butanol. No traces of intermediates could be found when all free induction decays obtained during the complete reaction were added to decrease the detection limit of ^{13}C species present in a steady state. The detection limit of ^{13}C-metabolites in these experiments was estimated to be approximately 1 mM. An especially close examination was performed in the spectral regions where resonances from [1-^{13}C]butyraldehyde and [1-^{13}C]butyryl phosphate could be expected, but no additional peaks could be found.

^{13}C NMR spectra of the labeled compounds in suspensions of *C. acetobutylicum* as a function of time are plotted in Fig. 2. It shows the changes in concentrations of the [1-^{13}C]butyrate added and the [1-^{13}C]butanol formed during a 90-min experiment. Butyrate was quantitatively converted to butanol by the cells which were in the solvent formation phase. Wood et al. (1945) showed, by analyzing the end products of an acetone-butanol fermentation, that 84% of the ^{13}C-butyrate added to the medium of metabolizing *C. acetobutylicum* cells was converted to butanol.

The results presented here show a quantitative conversion of the added butyrate to butanol during the solvent formation phase, without accumulation of intermediates in the pathway.

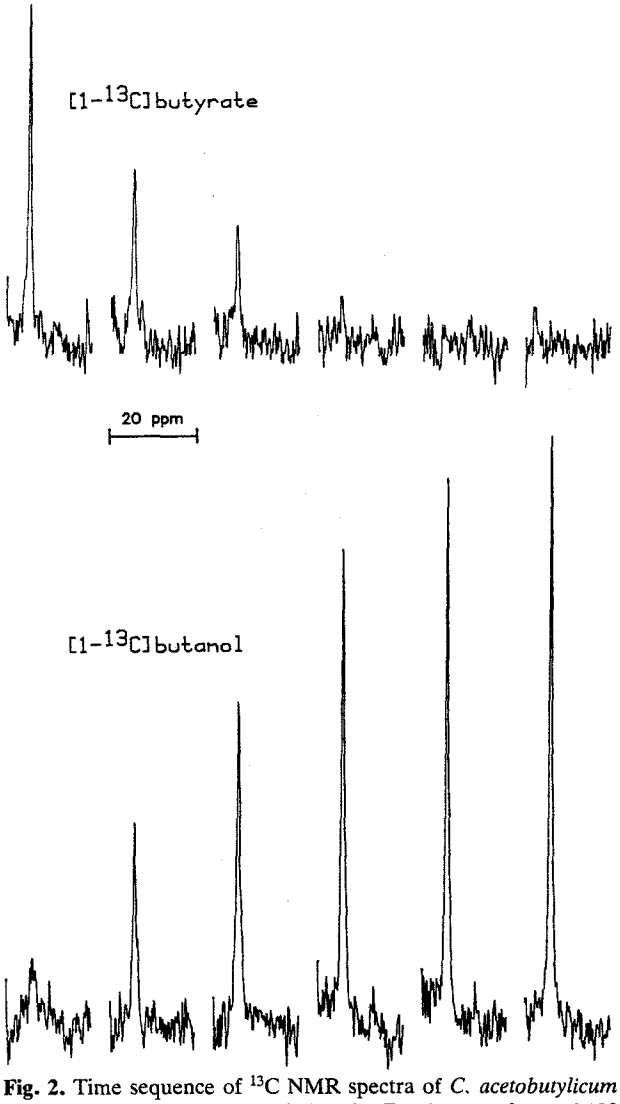

Fig. 2. Time sequence of ^{13}C NMR spectra of *C. acetobutylicum* cells at 25° C, each spectrum being the Fourier transform of 120 free induction decays. The corresponding spectra were recorded at 15-min intervals, beginning immediately after the addition of 50 mM [1-^{13}C]butyrate

Enzyme levels

The levels of phosphate acetyltransferase, acetate kinase, phosphate butyryltransferase, and butyrate kinase were determined in batch fermentations in order to investigate the possibility of uptake of acids by a reversal of the enzymes above as suggested by Valentine and Wolfe (1960). The results are shown in Fig. 3. The phosphate acyltransferases were assayed in the acyl-CoA forming direction and the kinases were determined in the acyl phosphate forming direction. It can be seen that all activities except that of butyrate kinase decrease rapidly as the fermentation reaches the solvent production phase after 15–17 h. This pattern of variation in enzyme activities was obtained in all three fermentations investigated. Similar results were published recently (An-

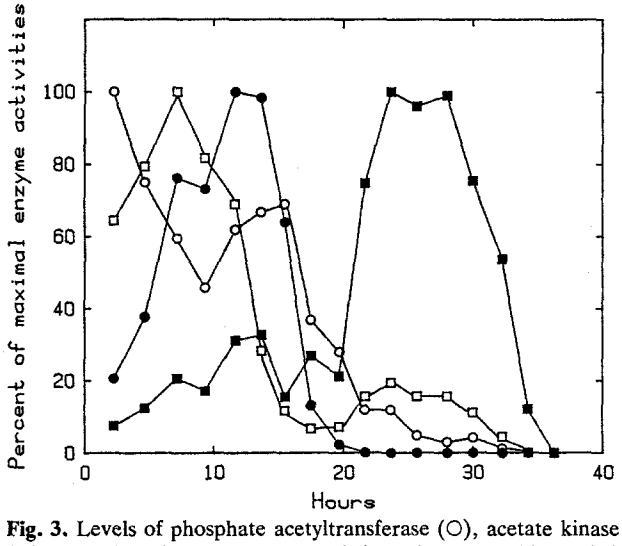

Fig. 3. Levels of phosphate acetyltransferase (○), acetate kinase (□), phosphate butyryltransferase (●), and butyrate kinase (■) during a batch fermentation of *C. acetobutylicum*. Enzyme activities were measured as described in Materials and methods. The enzyme levels are expressed as percent of the maximal activities found for each enzyme

dersch et al. 1983) although the changes in activities were less pronounced and no increase in butyrate kinase activity was reported. The authors used a different growth medium and a different strain of *C. acetobutylicum*, DSM 1732, compared to that used in our studies, the type strain ATCC 824 (DSM 792), which could explain the discrepancies found. The large decreases found in phosphate acetyltransferase, acetate kinase, and phosphate butyryltransferase activities suggest that the uptake does not occur by a complete reversal of the acid forming pathways.

It was assumed that the butyrate kinase could participate in the ATP-requiring activation of butyrate to butyryl phosphate due to the increase in specific activity. Crude desalted extracts from different stages of the acetone-butanol fermentation, prepared under anaerobic conditions, were assayed in an anaerobic glove box for a disappearance of butyryl phosphate. NADH, NADPH and hydrogen were tried as reductants as described in Materials and methods. In no case a disappearance of butyryl phosphate could be detected. The physiological function of the increase in butyrate kinase activity remains unknown.

Other known enzymes for activation of acetate and butyrate are the ATP and CoA requiring acyl-CoA synthetases (Mahler et al. 1953). Crude, desalted extracts of solvent producing cells were assayed under anaerobic conditions for acetyl-CoA and butyryl-CoA synthetase activities as described in Materials and methods. No ATP-dependent acyl-CoA synthetase activities could be found in these assays.

Acetoacetyl-CoA : acetate (butyrate) CoA-transferase

The existence of an acetoacetyl-CoA : acetate CoA transferase was first postulated by Doelle (1975) and an acetoacetyl-CoA : acetate (butyrate) CoA-transferase was recently shown to be present in *C. acetobutylicum* (Andersch et al. 1983). These authors suggest that the enzyme plays a partial role in the activation of short-chain fatty acids at the expense of acetoacetyl-CoA during the solvent formation phase of a fermentation. This transferase is present during the whole fermentation and its specific activity increases during solvent production.

Beside the normal uptake of acetate and butyrate during a batch-wise fermentation, it has been reported that propionate, when added to the culture medium of actively metabolizing cells, can be converted to propanol (Blanchard and MacDonald 1935). This prompted us to investigate the carboxylic acid specificity of the transferase from *C. acetobutylicum*. The results are summarized in Table 1. These experiments were carried out using desalted crude extracts. All carboxylic acids were added as their potassium salts. The rates are expressed relative to acetate. Sodium ions were found to inhibit the transferase and it exhibited no activity with the corresponding sodium salts. The inhibition of an acetoacetyl-CoA : acetate (butyrate) CoA-transferase by sodium ions has not been reported previously. This transferase, like other acetoacetyl-CoA : acetate (butyrate) CoA-transferases previously described (Sramek and Frerman 1975; Barker et al. 1978) exhibits a rather broad substrate specificity. It can utilize straight chain C_1-C_7 carboxylic acids and also branched chain carboxylic acids. It showed no activity with benzoate and the dicarboxylic acids tested. It is interesting to note the high relative rate exhibited with formate as substrate.

Table 1. Substrate specificity of the CoA-transferase in crude extracts

Carboxylic acid[a] (100 mM)	Relative rate
Formate	87
Acetate	100
Propionate	89
Butyrate	85
Valerate	73
Hexanoate	56
Heptanoate	5
Octanoate	< 0.1
Nonanoate	< 0.1
Isobutyrate	77
DL-2-methylbutyrate	64
Isovalerate	57
Isohexanoate	48
Vinylacetate	61
Crotonate	61
Benzoate	< 0.1
Fumarate[b]	< 0.1
Maleate	< 0.1
Malonate	< 0.1
Succinate	< 0.1

Assay methods are described in Materials and methods
[a] All carboxylic acids were added as their potassium salts
[b] The concentration of potassium fumarate was 20 mM

A mechanism that involves a CoA-transferase for uptake of acetate and butyrate is energetically more attractive than a reaction scheme comprising ATP-consuming reactions catalyzed by butyrate and acetate kinases or acyl-CoA synthetases. In a CoA-transferase reaction the energy of the thioester bond is conserved and transferred to the acids without the expense of ATP. The reaction catalyzed by the transferase in *C. acetobutylicum*, acetoacetyl-CoA + acetate (butyrate) $\rightleftharpoons$ acetoacetate + acetyl-CoA (butyryl-CoA), has an equilibrium con-

Table 2. Analysis of literature data on acetone-butanol fermentations by *C. acetobutylicum*

Uptake		Formation		Formation of acetone	Reference
Acetate (mM)	Butyrate (mM)	Acetone (mM)	Butanol (mM)	Uptake of acids (mole %)	
20.0	15.0	35.0	52.0	100	Johnson et al. (1931)
0	13.1	12.0	39.2	92	Davies and Stephenson (1941)
2.5	7.4	10.3	94.5	104	Maddox (1980)
41.8	30.8	60.2	98.6	83	Costa (1981)
18.0	50.0	75.0	150.0	110	Bahl et al. (1982)
11.7	18.2	34.4	40.5	115	Doerner et al. (1982)
18.4	19.4	37.8	62.1	100	Martin et al. (1982)
12.5	30.5	41.2	91.0	96	Andersch et al. (1983)
40.1	28.5	60.2	121.5	88	Costa and Moreira (1983)
8.4	43.3	56.8	135.0	110	Martin et al. (1983)
6.0	23.0	30.0	50.0	103	Nishio et al. (1983)
23.4	26.2	39.6	94.5	80	Ounine et al. (1983)
5.1	16.5	18.2	58.3	85	Hartmanis (unpubl. results)
				$\bar{x} = 97 \pm 11$	

stant of between 18 and 46, corresponding to a $\Delta G^{o\prime}$ of −7.1 to −9.6 kJ/mol. The data were calculated from Thauer et al. (1977) and Jencks (1973). Due to the rather unfavourable thermodynamics of this reaction a subsequent reaction is needed which is able to pull the reaction towards the formation of acetoacetate. In *C. acetobutylicum* the pulling mechanism is suggested to be the decarboxylation of acetoacetate to acetone by acetoacetate decarboxylase. The calculated $\Delta G^{o\prime}$ for this reaction is −26.2 kJ/mol, corresponding to an equilibrium constant of $4 \cdot 10^4$ at 25° C. The data were calculated from Thauer et al. (1977) and Burton and Krebs (1953). Although the K_m for acetoacetate is high, 8 mM (Davies 1943), this is sufficient for an effective pulling of the transferase reaction in the direction of acetoacetate formation. Acetoacetate decarboxylase can therefore be considered the key enzyme in the pathway of acetone formation from acetoacetyl-CoA. The decarboxylase is induced rather late in the fermentation when growth ceases (Autor 1970; Andersch et al. 1983). Further no uptake of acetate and butyrate from the medium occurs until the decarboxylase is induced, although the CoA-transferase is present earlier in the fermentation (Andersch et al. 1983). These facts indicate that the transferase-decarboxylase pathway is solely responsible for the uptake of acetate and butyrate during solvent formation. In order to further verify this, a survey of the literature for batch-wise fermentations of *C. acetobutylicum* was made. The results are summarized in Table 2. The data for uptake of acids and formation of solvents were calculated from figures in papers published since 1931, showing the time course of acetone-butanol fermentations. The millimolar formation of acetone and butanol was calculated for the same time interval of each fermentation as the uptake of acetate and butyrate. If the transferase reaction is the major uptake and activation mechanism, then the molar ratio of formation of acetone to uptake of acids, expressed as percent, should equal 100. The literature data in Table 2 show a mean value of 97 with a standard deviation of 11. Those values that exceed 100 can be explained by the fact that there may still be some formation of acids during the beginning of the solvent production phase. The lowest value found was 80. This is probably due to an underestimation of the concentration of acetone in the medium. At 30° C the vapour pressure of acetone is much higher than the vapour pressures of acetic acid, butyric acid, and butanol (Doerner et al. 1982). If the samples are not kept cold and analyzed immediately after sampling, considerable errors can occur in the determination of acetone.

The results obtained in this investigation show that the acetic acid and butyric acid formed during the first stage of the acetone-butanol fermentation, are taken up and activated to their respective CoA thiolesters via an acetoacetyl-CoA : acetate (butyrate) CoA-transferase. This mechanism for the activation of the acids causes an equimolar formation of acetoacetate. The acetoacetate formed is then decarboxylated by acetoacetate decarboxylase to yield acetone. The decarboxylation provides the pulling mechanism for the uptake of acids from the medium.

The implication of these results is that there can not be any uptake of acetate and butyrate from the medium without a concomitant formation of acetone.

This means that the formation of acetone and the formation of butanol are metabolically coupled in such a way that it is impossible to obtain a batch-wise butanol fermentation without formation of acetone and retention of a good yield of butanol.

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