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Coenzyme Specificity of Dehydrogenases and Fermentation of Pyruvate by Clostridia

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Summary. Four clostridial species (*C. pasteurianum*, *C. butylicum*, *C. butyricum* and *C. tetanomorphum*) grow on pyruvate. Two other species (*C. roseum* and *C. rubrum*) only ferment this compound; this is probably due to their inability to synthesize hexose phosphates from pyruvate (fructose-1,6-diphosphatase and pyruvate carboxylase are absent).

The fermentation of pyruvate by the above clostridia yields acetate, carbon dioxide, hydrogen and small amounts of compounds more reduced than acetate. Hydrogen pressure increases the amount of ethanol, butanol and butyrate formed during the fermentation of pyruvate. Since *C. roseum* and *C. rubrum* contain a ferredoxin:NADP reductase it seems likely that $NADPH_2$ is the coenzyme involved in ethanol formation. In accordance with this acetaldehyde and alcohol dehydrogenases exhibit activity with $NADPH_2$.

The glyceraldehyde-3-phosphate dehydrogenase of the clostridia under investigation is NAD specific and so is the β-hydroxy-butyryl-CoA dehydrogenase with the exception of *C. kluyveri*.

The specific activity of hydrogenase and the coenzyme specificity of NAD(P) reductase vary among the clostridial species.

When carbohydrates are fermented by clostridial species reduced pyridine nucleotides are produced in the glyceraldehyde-3-phosphate dehydrogenase reaction. The oxidized form of the pyridine nucleotides is then regenerated by converting acetyl-CoA into butyrate or other reduced compounds. In the oxidative decarboxylation of pyruvate to acetyl-CoA the electrons are transferred to ferredoxin and give rise to the evolution of hydrogen gas (Valentine, 1964). Some of the reduced ferredoxin, however, may be oxidized through the action of ferredoxin: NAD and NADP reductases and may supply additional reducing power. In order to study the participation of these reductases on the provision of reducing power for fermentative processes the fermentation of pyruvate by clostridial species was investigated.

Material and Methods

Bacteria and Cultural Conditions. a) *C. butyricum* ATCC 19398; *C. butylicum* ATCC 14823; *C. pasteurianum* ATCC 6013; *C. roseum* ATCC 17797 and *C. rubrum* ATCC 14949 were obtained from the American Type Culture Collection; b) *C. acidi-urici* from J. C. Rabinowitz, Berkeley, and c) *C. kluyveri* ATCC 8527 and *C. tetanomorphum* ATCC 15920 from H. A. Barker, Berkeley.

The microorganisms of section a) were grown in 20-l bottles containing 17 l of the following medium: 2.0% glucose; 0.6% yeast extract; 0.2% peptone; 0.2% tryptone; 0.2 mM $MgSO_4$; 0.2 mM $FeSO_4$; 0.01 mM $MnCl_2$; 0.1 mM $CaCl_2$; 0.01 mM $CoCl_2$; 0.01 mM Na_2MoO_4. The medium was autoclaved for 1 h at 120°C. After cooling it was adjusted to pH 7.6 by adding separately sterilized potassium phosphate (KPO_4) buffer, pH 7.8 to a final concentration of 50 mM. The bottles were inoculated with 5% of a well grown culture and incubated at 37°C. The cells were harvested after 16 to 18 h in a continuous centrifuge (Cepa, C. Padberg, Lahr, Baden) and stored at −20°C. *C. acidi-urici* was grown in a medium of Rabinowitz (1963), *C. kluyveri* according to Bornstein and Barker (1948) and *C. tetanomorphum* as described by Barker *et al.* (1959).

Preparation of Cell-Free Extracts. 2 g of frozen cells were suspended in 10 ml of 50 mM KPO_4 or Tris-HCl buffer, pH 7.5 containing 20 mM 2-mercaptoethanol and sonicated in a MSE ultrasonic disintegrator for 6 min in 1 min intervals at 2—4°C. Cellular debris was removed by centrifugation at 20000 rpm for 30 min. Cell protein was estimated by a modified method of La Rivière (1958); Schmidt *et al.*, (1963). Protein of cell-free extracts was estimated by the method of Lowry *et al.* (1951).

Manometry. Manometric measurements were made by conventional procedures (Umbreit *et al.*, 1964) employing double-sidearm Warburg vessels. All experiments were performed at 37°C under an atmosphere of N_2 or H_2 as indicated.

Assay of Fermentation Products. Alcohols and fatty acids present in deproteinized solutions of Warburg and growth experiments were determined by gas chromatography. After addition of isovaleric acid and isoamyl alcohol as internal standards the samples were saturated with $MgSO_4$ and brought to a pH of 9 to 10 with NaOH. Then they were extracted 20-times with 1 ml of ether. The combined fractions (containing the alcohols) were concentrated to a volume of 0.6 ml with a stream of nitrogen at 0°C. After removal of traces of water by solid sodium sulfate the samples were analyzed with a gas chromatograph (Perkin-Elmer, model 900) equipped with a flame ionization detector. Standard curves were obtained in the same manner. Conditions for ethanol: glas column, $^1/_4$ inch × 6 feet, Porapak QS 80—100 mesh; injector, 200°C; manifold, 230°C; oven: temperature program, 150°C—240°C with 24°C/min; initial time, 2 min. Carrier gas: nitrogen, 60 psi, flow rate, 40 ml/min; hydrogen, 20 psi; air, 30 psi. Conditions for butanol: stainless steel column, $^1/_8$ inch × 6 feet; poly-propylen-glycol, 4% on chromosorb 6/AW-DMCS, 80—100 mesh; injector, 200°C; manifold, 230°C; oven: temperature program, 55—160°C with 16°C/min. Carrier gas: nitrogen, 50 psi, flow rate, 40 ml/min; hydrogen, 20 psi; air, 30 psi.

The residues of ether extractions were brought to a pH of 2.0 by adding phosphoric acid and saturated again with $MgSO_4$. The steam-volatile acids were separated in a Markham apparatus. They were collected in 0.5 N NaOH at 0°C and were evaporated to dryness in vacuo. The remaining material was dissolved in water and brought to pH 2 with phosphoric acid (end volume 0.6 ml). The gas chromatographic analysis for acetic and butyric acid was carried out under the following conditions: glas column, $^1/_4$ inch × 6 feet; Porapak QS 80—100 mesh; injector, 230°C;

manifold, 250°C; oven temperature, 200°C isotherm. Carrier gas: nitrogen, 50 psi, flow rate, 40 ml/min; hydrogen, 20 psi; air, 30 psi. A Hitachi recorder, model 196 (chart speed, 60 or 120 mm/min) was connected with the gas chromatograph. Photostatic copies of the peaks were cut out and their weight was determined.

The determination of pyruvate was carried out with lactic dehydrogenase and $NADH_2$ according to Passoneau and Lowry (1970). Glucose was determined with glucose oxidase according to Bergmeyer and Bernt (1970).

Enzyme Assays. The assays of hydrogenase activity were performed in Warburg vessels. The evolution of hydrogen from reduced methyl viologen was measured as described by Tamiya *et al.* (1955). $Na_2S_2O_4$ was employed as reducing agent for methyl viologen. To measure the uptake of hydrogen, the dyes methyl viologen, benzyl viologen and methylene blue were used as electron acceptors. Assays were carried out according to Nicholas *et al.* (1960).

The rates of NAD- and NADP-reduction by hydrogen were measured in 1-cm Thunberg cuvettes at 366 nm in 3.00 ml of 0.1 M Tris-HCl buffer, pH 7.5 containing 2 mM NAD(P). Controls under nitrogen gas were run simultaneously. The activity of the NAD and NADP reductases was also measured manometrically under a hydrogen atmosphere. The Warburg vessels contained the components listed above and additionally in some experiments NAD and NADP regenerating systems. $NADH_2$ was oxidized with the following system: 14 mM of α-ketoglutarate; 7 mM ammonium acetate and 2.0 units of glutamate dehydrogenase; in some experiments the system of Thauer *et al.* (1971) containing 20 mM sodium pyruvate; 5 mM sodium glyoxylate and 2.0 units of lactate dehydrogenase was employed. The $NADPH_2$ consuming system consisted of 7 mM of oxidized glutathione, pH 7.0 and 1.0 unit of glutathione reductase. To reduce ferredoxin in extracts of *C. acidi-urici* a small amount of *C. pasteurianum* extract (0.02 mg/test) was added as a source for hydrogenase.

The reduction of NAD and NADP by sodium pyruvate was tested at 366 nm in a final volume of 3.00 ml according to Petitdemange *et al.* (1971): 100 mM Tris-HCl buffer, pH 7.5; 33 mM sodium pyruvate; 12 mM KH_2AsO_4 and 2 mM NAD(P). The reaction mixture was gassed with purified nitrogen for 10 min and then started by the addition of cell-free extract.

The following assays were performed as described: alcohol dehydrogenase (Bergmeyer, 1970); NAD(P) acetaldehyde dehydrogenase (Burton and Stadtman, 1953); NAD(P) β-hydroxybutyryl-CoA dehydrogenase (Lynen and Wieland, 1955); butyryl-CoA dehydrogenase (Mahler, 1955b); glyceraldehyde phosphate dehydrogenase (Bergmeyer, 1970); fructose-1,6-diphosphatase (Racker and Schroeder, 1958); diaphorase (Mahler, 1955a).

Pyruvate carboxylase was tested by a procedure adapted from the test of Henning and Seubert (1964). 2 ml of the following components were incubated at 30°C in 2-ml Durham tubes with a plastic stopper for 10 to 30 min; 100 mM Tris-HCl buffer, pH 7.5; 5 mM $MgCl_2$; 1 mM ATP; 15 mM $KHCO_3$; 0.6 units citrate synthase; 1 mM acetyl phosphate; 5.0 units phosphotransacetylase; 0.6 mM CoASH; 10 mM sodium pyruvate. The reaction was stopped by heating for 3 min in a boiling water bath. After centrifuging, an aliquot of the clear supernatant was analyzed for citrate formed by means of the citrate lyase reaction (Dagley, 1970).

Enzymes. Phosphotransacetylase, EC 2.3.1.8; glyceraldehyde-3-phosphate dehydrogenase, EC 2.7.2.3; citrate lyase, EC 4.1.3.6; citrate synthase, EC 4.1.3.7; glucose-6-phosphate dehydrogenase, EC 1.1.1.49; glucosephosphate isomerase, EC 5.3.1.9; glutamate dehydrogenase, EC 1.4.1.3; lactate dehydrogenase, EC 1.1.1.27 and glutathione reductase, EC 1.6.4.2 were purchased from C. F. Boehringer Mannheim GmbH. (Mannheim, Germany).

Results

Growth of clostridial species on pyruvate was tested in culture media containing 0.25 to 1% sodium pyruvate. As is evident from Fig. 1 *C. pasteurianum* and *C. butyricum* utilized pyruvate for growth. *C. butylicum* and *C. tetanomorphum* behaved similarly. *C. roseum* and *C. rubrum*, however, did not grow on this substrate. The reason for this inability could be that the ferredoxin : NAD and NADP reductases were lacking in these microorganisms or that enzymes required for the synthesis of certain metabolites from pyruvate were absent. Since this would not impair a fermentative breakdown of pyruvate by these microorganisms the formation of hydrogen gas and of carbon dioxide by cell suspensions of *C. roseum* and *C. rubrum* was tested. Both species decomposed pyruvate (Fig. 2).

In the absence of the ferredoxin : NAD and NADP reductases, pyruvate should be fermented to acetate, CO_2 and H_2. The balances summarized in Table 1, however, reveal that *C. roseum* and *C. rubrum* formed also some butyrate under these conditions. The formation of compounds

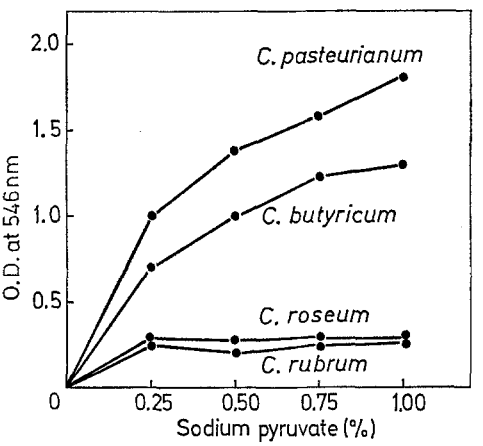

Fig. 1

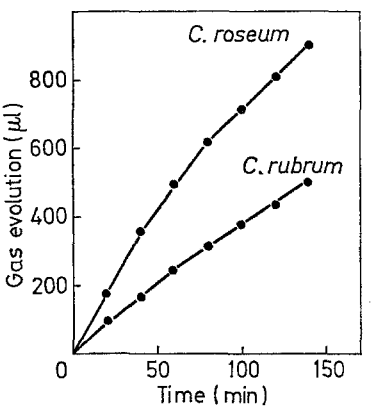

Fig. 2

Fig. 1. Growth of clostridial species on pyruvate. In addition to the components listed in Methods the growth medium contained sodium pyruvate as indicated. 10 ml of growth medium in test tubes were inoculated with 1 ml of a glucose-grown culture. After 30 h of growth at 37° C the optical density of the cultures was measured at 546 nm

Fig. 2. Formation of hydrogen plus carbon dioxide from pyruvate by cell suspensions of *C. roseum* and *C. rubrum*. Warburg vessels contained washed cells (4 to 6 mg of protein) in 3 ml of the buffer described in Table 1. After attaining anaerobic conditions by passing a stream of nitrogen through the vessels the reactions were started by tipping in 0.2 ml of 0.5 M Na-pyruvate from the side bulbs. Controls with KOH in the center well were run simultaneously and showed that 50% of the gas evolved consisted of carbon dioxide

more reduced than acetate was pronounced in experiments under hydrogen pressure. Then, *C. roseum* produced only minor amounts of acetate and converted pyruvate predominantly into ethanol, butyrate and butanol (Table 2). Similar results were obtained with *C. pasteurianum*. Suspensions of *C. formicoaceticum* on the other hand fermented pyruvate rapidly

Table 1. Dissimilation of pyruvate by cell suspension of Clostridia

Product	Products formed in moles/100 moles of pyruvate			
	C. pasteurianum	*C. butyricum*	*C. roseum*	*C. rubrum*
Acetate	92.7	66.3	81.0	84.9
Butyrate	3.6	5.9	16.2	12.9
Butanol	—	—	2.1	—
Carbon dioxide	101.8	96.9	108.3	77.7
Hydrogen	98.9	68.5	76.7	74.4

The fermentations were carried out in Warburg vessels in a total volume of 3.0 ml at 37°C.

The vessels contained in the main compartment; 100 mM sodium citrate buffer, pH 6.5; 2 mM $MgSO_4$; 0.2 mM $FeSO_4$; 0.01 mM $MnCl_2$; 0.1 mM $CaCl_2$; 0.01 mM $CoCl_2$; 0.01 mM Na_2MoO_4; 4 mM KH_2PO_4; 20 mM 2-mercaptoethanol; washed cell suspensions, 2—6 mg protein/vessel. The side bulb contained 100 μmoles of sodium pyruvate. The center cup contained 0.2 ml of 20% KOH and a strip of folded filter paper for CO_2 absorption or 0.2 ml of water for measurements of $CO_2 + H_2$ evolution. The vessels were gassed with nitrogen for 10 min and, after attainment of equilibrium, the content of the side bulb was mixed with that of the main compartment. After 2 to 3 h the fermentations were stopped by added phosphoric acid to give a final pH of 2. During this time 40—60 μmoles of pyruvate were fermented. The concentrations of substrate and products were determined as described in Methods.

Table 2. Effect of hydrogen pressure on the fermentation of pyruvate by *C. roseum*

Product	Products formed in moles/100 moles of pyruvate	
	4 atm H_2	32 atm H_2
Acetate	9.9	7.7
Butyrate	26.0	20.4
Butanol	9.0	15.7
Ethanol	13.5	30.1

The fermentations were carried out in 100-ml stainless steel vessels which contained 10 ml of the basal medium described in Table 1 and 400 μmoles of sodium pyruvate in a glass beaker. The autoclaves were flushed with purified hydrogen for 10 min. 1 ml of cell suspension was added and the hydrogen pressure desired was established. During incubation at 37°C the fermentation solutions were stirred by means of a magnetic stirrer. After 5 h of incubation the autoclaves were depressurized and the fermentations were stopped by adding phosphoric acid to give a final pH of 2. The concentrations of substrate and products were determined as described in Methods.

but did not yield increased amounts of reduced compounds under hydrogen pressure. Since this microorganism is devoid of hydrogenase this excluded a non-enzymatic action of hydrogen in the experiments with *C. roseum* and *C. pasteurianum*.

Since under hydrogen pressure, the production of ethanol by *C. roseum* and *C. pasteurianum* was pronounced the coenzyme specificity of related hydrogenation and dehydrogenation reactions was investigated. These experiments were extended to other clostridial species.

Table 3 summarizes the hydrogenase activity of eight clostridial species as measured by hydrogen uptake with three acceptors and by hydrogen evolution from reduced methyl viologen. *C. pasteurianum* was exceptional in that hydrogen evolution proceeded with ten times the activity of the other species. Furthermore, hydrogen uptake, especially with methylene blue as acceptor was extraordinary fast (Fig. 3). Most species exhibited little activity in hydrogen uptake. The reaction with methylene blue was inhibited and stopped after a 5 min as shown in Fig. 4 for extracts of *C. rubrum*.

The specific activities of the ferredoxin : NAD and NADP reductases in clostridial species are summarized in Tab. 4. The activities were determined with hydrogen or pyruvate as electron donors for ferredoxin. Petitdemange *et al.* (1971) observed that the reductases were found in higher activity when pyruvate was employed as reducing agent. It can be seen that all species except *C. tetanomorphum* contained a ferredoxin : NADP reductase. This enzyme was not inhibited by $NADPH_2$ as indicated by the finding that a NADP regenerating system (glutathione reductase) did not enhance the rates observed. The NAD specific reductase was present in *C. pasteurianum*, *C. tetanomorphum* and *C. acidi-*

Table 3. Specific activity of hydrogenase in clostridial species

Microorganism	H_2 evolution	H_2 uptake		
		Methyl viologen	Benzyl viologen	Methylene blue
	(μmoles/min · g protein)	(μmoles/min · g protein)		
C. pasteurianum	2462	1488	2817	19122
C. tetanomorphum	60	37	50	+[a]
C. butylicum	325	72	73	+
C. butyricum	237	79	64	+
C. roseum	205	48	18	+
C. rubrum	98	19	18	+
C. acidi-urici	0	0	0	0
C. kluyveri	222	211	833	1733

[a] Hydrogen uptake stopped after a few minutes.

urici in high activity. This enzyme could not be demonstrated in *C. roseum* and *C. rubrum*. In order to assay this enzyme in extracts of *C. pasteurianum* and of *C. butyricum* the addition of a $NADH_2$ consuming system was necessary. This was consistent with findings of Jungermann *et al.* (1971). It is evident from Table 5 that the reduction of NADP by extracts of *C. roseum* as measured with pyruvate as electron donor was dependent on the presence of ferredoxin and CoA.

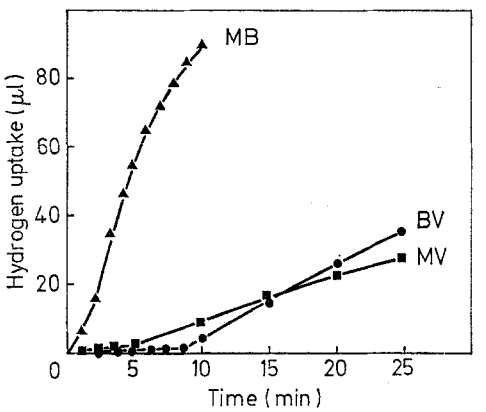

Fig. 3. Uptake of hydrogen by extracts of *C. pasteurianum* with methyl viologen (*MV*), benzyl viologen (*BV*) and methylene blue (*MB*) as electron acceptors. The assay procedure is described in Methods. Cell-free extract was added as follows: 0.016 mg of protein (methylene blue); 0.028 mg of protein (benzyl viologen); 0.030 mg of protein (methyl viologen)

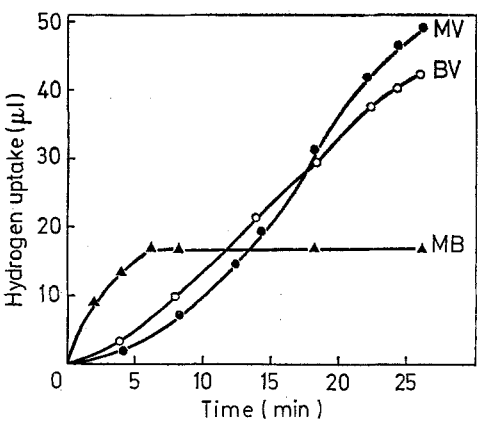

Fig. 4. Uptake of hydrogen by extracts of *C. rubrum* with methyl viologen, benzyl viologen and methylene blue as electron acceptors. Warburg vessels contained 0.2 ml of cell-free extract of *C. rubrum* (20 mg of protein/ml)

Table 4
Specific activity of ferredoxin: NAD and NADP reductases in clostridial species

Microorganism	Pyruvate as electron donor		Hydrogen as electron donor	
	NAD	NADP	NAD	NADP
	(μmoles/min · g protein)		(μmoles/min · g protein)	
C. pasteurianum	*n*	50.0	16.4	20.9
C. tetanomorphum	26.7	*n*	21.7	*n*
C. butylicum	6.9	4.2	4.8	3.8
C. butyricum	*n*	5.2	2.0	4.7
C. roseum	*n*	4.4	*n*	*n*
C. rubrum	*n*	5.4	*n*	*n*
C. acidi-urici	—	—	24.7	16.4
C. kluyveri	2.5	17.7	4.0	39.0

n = not detectable; for assay conditions see Methods.

Table 5. The dependence of NADP reduction of *C. roseum* on the presence of ferredoxin and coenzyme A

Conditions	NADP reduction (μmoles/min · g protein)
Crude extract	4.4
DEAE-cellulose treated extract + 0.05 mM CoA	0.5
DEAE-cellulose treated extract + 0.5 mg ferredoxin	0
DEAE-cellulose treated extract + 0.05 mM CoA and 0.5 mg ferredoxin	7.3

In order to remove ferredoxin a cell-free extract of *C. roseum* was passed through a DEAE-cellulose column (1 cm in diameter × 3 cm) equilibrated against 50 mM Tris-HCl buffer, pH 7.5. The ferredoxin employed was prepared from *C. acidi-urici* following the procedure of Mortenson (1964).

Table 6. Coenzyme specificity of dehydrogenases

Microorganism	Enzyme activities in μmoles/min · mg of protein			
	GAP-DH[a]		AcH-DH	
	NAD	NADP	NAD	NADP
C. pasteurianum	5.3	*n*	*n*	0.004
C. tetanomorphum	0.5	*n*	0.004	0.004
C. butylicum	3.0	*n*	0.003	0.012
C. butyricum	0.7	*n*	*n*	0.009
C. roseum	4.0	*n*	0.002	0.013
C. rubrum	5.6	*n*	0.005	0.017
C. kluyveri	—	—	0.175	0.110

[a] GAP-DH = glyceraldehyde-3-phosphate dehydrogenase; AcH-DH = acetal-hydroxybutyryl-CoA dehydrogenase;

From the experiments described so far it can be assumed that reduced ferredoxin which is formed during pyruvate fermentation by *C. roseum* and *C. rubrum* is used to evolve hydrogen and to reduce NADP. Since ethanol was preferentially formed under hydrogen pressure the coenzyme specificity of the dehydrogenases involved in the fermentative metabolism was determined. The results of Table 6 reveal that during glucose fermentation NAD was reduced in the glyceraldehyde-3-phosphate dehydrogenase reaction. All clostridial species except *C. kluyveri* contained an active NAD specific β-hydroxybutyryl-CoA dehydrogenase. The diaphorase of all species exhibited activity with both coenzymes. Therefore, $NADH_2$ seemed to be the preferred hydrogen donor for butyrate synthesis from glucose. Acetaldehyde dehydrogenase and alcohol dehydrogenase were present in low activity. As already shown by Burton and Stadtman (1953) *C. kluyveri* contained a NAD specific alcohol dehydrogenase, and the aldehyde dehydrogenase reacted with both coenzymes. In most other species, especially in *C. roseum* and *C. rubrum* more activity was found with NADP as coenzyme. These results were in agreement with the finding that in these species the formation of ethanol from pyruvate was favoured by hydrogen pressure.

Although the experiments in cell-free systems explained the results obtained with respect to the fermentative breakdown of pyruvate by *C. roseum* and *C. rubrum* the exact reason why these species do not grow on pyruvate had yet to be found. Fig. 5 illustrates that cultures of *C. roseum* in a medium containing glucose and pyruvate decomposed both compounds readily but stopped growing after exhaustion of glucose. *C. pasteurianum* could grow on pyruvate also in the absence of glucose (Fig. 6). Therefore, it seemed likely that *C. roseum* was not able to syn-

involved in clostridial fermentations

A-DH		BOH-CoA-DH		B-CoA-DH	Diaphorase	
NAD	NADP	NAD	NADP	DCPIP	NAD	NADP
0.003	0.002	10.1	0.06	10.4	716.4	145.7
n	0.003	23.6	0.13	2.8	718.3	306.5
0.005	0.020	37.0	0.16	6.3	641.0	126.3
n	0.054	30.6	0.25	5.4	189.8	19.1
0.022	0.450	35.8	0.12	5.8	531.3	106.3
0.008	0.023	41.6	0.15	6.9	175.9	82.1
0.050	*n*	1.7	24.7	75.5	$14 \cdot 10^3$	$17 \cdot 10^3$

dehyde dehydrogenase; A-DH = alcohol dehydrogenase; BOH-CoA-DH = β-B-CoA-DH = butyryl-CoA dehydrogenase.

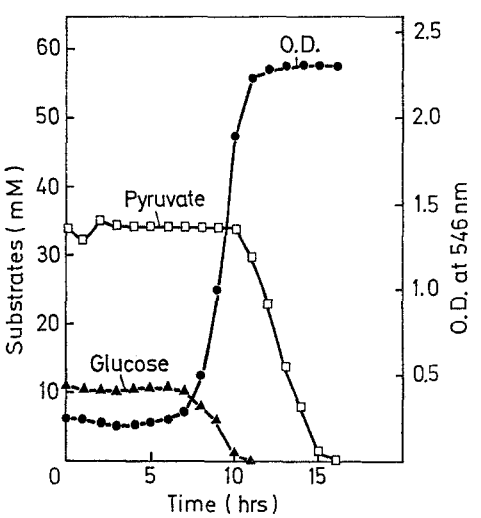

Fig. 5

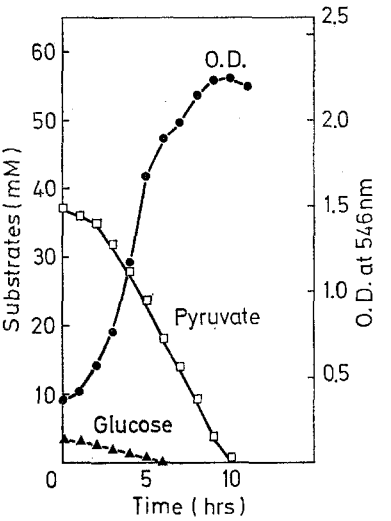

Fig. 6

Fig. 5. Growth and consumption of glucose and pyruvate by *C. roseum*. The experiment was carried out in 1-l flasks under a nitrogen atmosphere at 37° C. Samples were removed at the times indicated and analyzed for substrate concentrations and optical density

Fig. 6. Growth and consumption of glucose and pyruvate by *C. pasteurianum*. Conditions as in Fig. 5

Table 7. Specific activity of fructose-1,6-diphosphatase and pyruvate carboxylase in cell-free extracts of *C. pasteurianum* and *C. roseum*

Enzyme	Specific activity (μmoles/min · g protein)		
	C. pasteurianum		*C. roseum* Glucose + Pyruvate
	Glucose	Pyruvate	
Fructose-1,6-diphosphatase	27.8	14.7	< 1
Pyruvate carboxylase	2.5	5.6	< 0.5

Fructose-1,6-diphosphatase: assay conditions as in Methods; controls were run without substrate.

Pyruvate carboxylase: controls were run with the omission of pyruvate or citrate synthase or acetyl phosphate, CoA and phosphotransacetylase. Without pyruvate or citrate synthase, citrate formation was not detectable. The omission of the acetyl-CoA generating system resulted in a 50% inhibition of the reaction. In the presence of avidin (50 μg per assay) a 40% inhibition of the reaction was observed.

thesize all the enzymes required for growth on pyruvate. Two enzymes—fructose-1,6-diphosphatase and pyruvate carboxylase—were tested in cells of *C. roseum* grown on glucose and pyruvate and in cells of *C. pasteurianum*. Both enzymes were present in the latter microorganism but not in *C. roseum* (Table 7). It, therefore, is apparent that this bacterium is not able to carry out gluconeogenesis. Similar or related reasons may also be responsible for the incapability of *C. rubrum* to utilize pyruvate for growth.

Discussion

The utilization of pyruvate by clostridial species has been described by several authors. Langlykke *et al.* (1937) showed that pyruvate is fermented by *C. butylicum* and Cohen and Cohen-Bazire (1948) reported that acetate and butyrate are the main products of pyruvate fermentation by *C. saccharobutyricum*. Recently, Lee and Ordal (1967) studied the growth of *C. thermosaccharolyticum*. This bacterium is not able to grow on pyruvate alone but utilizes this compound in the presence of glucose. It, therefore, resembles *C. rubrum* and *C. roseum* in this respect. Since pyruvate alone is fermented without growth it is indicated that *C. rubrum* and *C. roseum* lack enzymes necessary for the synthesis of certain metabolites from pyruvate. In accordance with this a fructose-1,6-diphosphatase and a pyruvate carboxylase could not be detected in cell-free extracts of these microorganisms. These enzymes, however, are present in extracts of *C. pasteurianum* which belongs to the group of pyruvate-utilizing clostridia. The fermentation of pyruvate by *C. butyricum* yields acetate, butyrate, hydrogen and carbon dioxide (Cohen and Cohen-Bazire, 1948). The same is shown for *C. roseum* and *C. rubrum*. Under hydrogen pressure the fermentation is shifted to more reduced compounds, such as ethanol and butanol. A similar effect of hydrogen pressure on the fermentation of glucose by *C. pasteurianum* was already reported by Eliasberg in 1930. It is conceivable that hydrogen pressure increases the portion of reduced ferredoxin which is oxidized by transfering electrons to pyridine nucleotides. A corresponding NADP specific reductase could be demonstrated in *C. roseum* and *C. rubrum*. The coenzyme specificity of this enzyme and of the enzymes involved in the conversion of acetyl-CoA into ethanol is in agreement with our finding that hydrogen pressure favours the formation of ethanol. NAD reductase or transhydrogenase activity could not be found in these bacteria. Therefore, it seems likely that the low activity of β-hydroxybutyryl-CoA dehydrogenase with $NADPH_2$ is involved in butyrate synthesis. It is also possible that the NAD reductase of these microorganisms is extremely unstable and escapes detection in cell-free extracts.

The comparative study of the coenzyme specificity of the dehydrogenases of several clostridia reveals some interesting points. When growing

on carbohydrates clostridia produce $NADH_2$ in the glyceraldehyde-3-phosphate dehydrogenase reaction. $NADH_2$ is then consumed by the reduction of acetoacetyl-CoA to β-hydroxybutyryl-CoA and by butyryl-CoA formation from crotonyl-CoA (probably with the participation of flavoproteins). *C. kluyveri* is exceptional since it contains a NADP specific β-hydroxybutyryl-CoA dehydrogenase as reported recently (Hillmer and Gottschalk, 1972). It also contains an unusually high diaphorase activity which is consistent with the high flavin content of *C. kluyveri* reported by Peel (1958). The diaphorase has been purified to homogeneity by Kaplan *et al.* (1969); it reacts with either $NADH_2$ or $NADPH_2$.

Peck and Gest (1957) found that the hydrogenase of *C. butylicum* shows relatively poor ability to activate molecular hydrogen and to catalyze the reduction of dyes. This is true for most clostridial species. *C. pasteurianum* and *C. kluyveri* hydrogenases only exhibit higher activity in benzyl viologen and methylene blue reduction than in H_2 evolution. For the enzyme from *C. pasteurianum* which has been purified to homogeneity recently (Nakos and Mortenson, 1971) this confirms data of Peck and Gest (1956). It is surprising that the *C. kluyveri* enzyme exhibits considerable ability to activate molecular hydrogen. This is, however, in agreement with previous work on the enzyme in which hydrogen uptake and reduction of pyruvate has been used as an assay procedure for this enzyme (Fredricks and Stadtman, 1965).

That higher reductase activities can be measured with pyruvate as reducing agent than with molecular hydrogen has been reported by Petitdemange *et al.* (1971). *C. tetanomorphum* and *C. acidi-urici* contain very active NAD reductases. These enzymes seem to supply $NADH_2$ for reductive processes. Barker (1937) found that *C. tetanomorphum* forms small and variable amounts of hydrogen during glutamate fermentation. This means that a considerable portion of reduced ferredoxin from the degradation of pyruvate is oxidized by butyrate formation. *C. acidi-urici* is devoid of hydrogenase and has to regenerate the oxidized form of ferredoxin for further breakdown of pyruvate by other reactions than the evolution of hydrogen. The NAD reductase very likely participates in this process. The corresponding enzyme of other clostridia may function as $NADH_2$-ferredoxin reductase. The ferredoxin dependent reductive carboxylation of acetyl-CoA with $NADH_2$ as electron donor has been shown in cell-free extracts of *C. kluyveri* (Gottschalk and Chowdhury, 1969). The evolution of hydrogen from $NADH_2$ by cell-free extracts of *C. kluyveri*, *C. pasteurianum* and *C. butylicum* could be demonstrated by Decker and his collaborators (Thauer *et al.*, 1969; Jungermann *et al.*, 1971).

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