STUDIES ON THE METABOLISM OF *ACETOBACTER PEROXYDANS*

II. THE ENZYMIC MECHANISM OF LACTATE METABOLISM

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SUMMARY

1. Resting cells of DL-lactate-grown *Acetobacter peroxydans* oxidize D-lactate about four times as fast as the L-isomer.

2. The crude cell-free extract was separated into small particles and soluble enzymes. The particles contained all the enzymes for the oxidation of D- and L-lactate, pyruvate, ethanol and acetaldehyde to form acetate with a concomitant O_2 uptake. Cofactors were not required. The particles contained pyruvate decarboxylase, cytochromes and cytochrome oxidase. The effect of several inhibitors was studied. The particles also oxidized several primary alcohols, L-malate, fumarate and DPNH; D-lactate was oxidized about 7 times as fast as the L-isomer.

3. Soluble D-lactate and L-lactate dehydrogenases were also present. Both required artificial carriers, but the latter enzyme was very weak. There was also a soluble pyruvate decarboxylase and coenzyme-linked ethanol and acetaldehyde dehydrogenases. No evidence was found for the presence of TPN- and DPN-linked lactate dehydrogenases or lactate racemase. Acetate was not oxidized.

INTRODUCTION

Calcium lactate is one of the substrates commonly used for the differentiation of bacteria of the genus *Acetobacter*. Most of these bacteria grow very well on this substrate and their resting cells form $CaCO_3$ on calcium lactate "oxidograms"[1]. Species of the mesoxydans, oxydans and peroxydans groups of FRATEUR[1] all exhibit these properties. RAINBOW *et al.*[2,3] included acetic acid bacteria with a predominant lactate metabolism in the "*Acetobacter mobile* group", later renamed the "lactaphilics". LEIFSON[4] proposed that the present *Acetobacter* genus should be divided into two genera, *Acetobacter* and *Acetomonas*. One of the criteria in the redefinition of *Acetobacter* is the ability to oxidize lactate to CO_2 and water. There are only a few species which are unable to grow on lactate. These bacteria only oxidize it to acetate and they lack the KREBS cycle; they are members of FRATEUR's suboxydans group[1], the *Acetobacter viscosum*[2] or the glycophilic[3] group of RAINBOW *et al.*, *Acetomonas* of LEIFSON[4] or *Gluconoacetobacter* of ASAI[5]. The different names designate virtually the same group of bacteria.

Since nothing is known of the details of lactate metabolism by these bacteria

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and in view of the importance of this compound in differentiating acetic acid bacteria, a study was undertaken to obtain information of the enzymic mechanism involved. A report is presented here on the catabolism of lactate by *Acetobacter peroxydans*.

MATERIALS AND METHODS

Strain used

Acetobacter peroxydans, strain 8618 N.C.I.B. was used throughout this study. It was described previously in extenso[6].

Mass cultures

The bacteria were grown in the medium described by ATKINSON[7], with 1 % ammonium DL-lactate as the main carbon source made up as previously described[6]. A 6-l flask containing 4 l of this medium, with approx. 200 mg of Hopkins and Williams Antifoam A, was inoculated with 400 ml of a culture previously grown on a shaking machine at 30° for 2 days. Incubation was carried out at 30° for 2 days with aeration by sterile compressed air. The culture was neutralized twice a day with conc. HCl. The bacteria were centrifuged and washed twice in 0.01 *M* phosphate buffer pH 7. Yield: about 1 g/l. Four methods were used to control the purity of this crop: (1) microscopic aspect of the cells, (2) Gram negativity, (3) catalase negativity, (4) the Warburg test, in which ethanol is oxidized quickly and glucose not at all, when tested over a period of at least 2 h.

Enzyme preparations

The washed cells were disrupted in 3 ways: by grinding with alumina[8], crushing in the HUGHES block[9] or disrupting in a Raytheon Sonic Oscillator (250 W, 10 kc). No apparent difference was noted in the properties or the activity of the enzymes. The Raytheon was used currently. 4 g of cells were suspended in 60 ml 0.01 *M* phosphate buffer pH 7 and sonicated for 10–15 min. The slightly viscous suspension was centrifuged at 4° for 1 h at 10,000 rev./min in a Servall Angle Head centrifuge. The pale brown, slightly opalescent supernate will be called here the "crude extract". When centrifuged in the Spinco at 4° at 100,000 × *g* for 2 h, approx. 1 ml of particulate material sedimented. It had a gel-like appearance and a reddish violet colour. Electron microscopy showed that it consisted of small particles, with a diameter of a few hundred Å. This fraction was washed twice with 0.01 *M* phosphate buffer pH 7 in the Spinco and suspended in 6 ml of the same buffer. It will be referred to as "particles". Quite often there was a whitish layer of particulate matter, loosely sedimented on top of the "particles" after the first Spinco centrifugation. It exhibited only negligible enzyme activity and was discarded.

The pale brown, sometimes greenish supernate after the first Spinco centrifugation was dialyzed overnight against 0.01 *M* phosphate buffer and will be called "soluble enzymes" or "supernate". Its activity was lost after one week at —20°.

The conventional Warburg apparatus was used. To measure oxygen uptake each vessel contained 100 μmoles of buffer, 0.2 to 1 ml of enzyme preparation, cofactors or inhibitors and 20 μmoles of substrate in the side arm. The final vol. was 3 ml. KOH was placed in the central well. The temp. was 30°. For experiments with particles no artificial carrier was necessary. With the "supernate" either 0.25 μmole of methy-

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lene blue or 2.7 μmoles of N-methyl-phenazinium methosulphate was used. For the determination of anaerobic CO_2 production, the vessels were flushed with pure N_2.

TPN- and DPN-linked dehydrogenases were measured in the Beckman spectrophotometer, model DU, at approx. 20°. Each cuvette contained 60 μmoles of pH 7.0 phosphate buffer, 0.1 to 0.5 ml of enzyme, 0.3 μmole of coenzyme added in this order followed by 10 μmoles of substrate. Where ethanol was tested as a substrate 1 mmole was employed. The final volume was 3 ml.

Cytochromes were detected with the Beck-Hartridge reversion spectroscope, equipped with a 6-V, 32-W microprojection lamp. Confirmatory cytochrome determinations were done in the Beckman spectrophotometer.

CO was prepared by dropping conc. formic acid into hot sulfuric acid and washing the gas through several KOH bottles. It was stored in a glass gasometer and mixed with pure O_2. Before flushing the manometers, the mixture was once more washed through a KOH solution to eliminate every trace of CO_2. Mixtures of O_2/N_2 were prepared in a calibrated gasometer.

Substrates

DL-lactic acid was a p.a. chemical from the Union Chimique Belge. D- and L-calcium lactates were obtained from the California Foundation for Biochemical Research. Their configuration was checked by polarimetry in 10 % ammonium molybdate. The calcium salts were converted to the sodium salts with oxalate.

RESULTS

Oxidation of lactate by resting cells

In a previous paper[6] it was mentioned that sodium DL-lactate was oxidized with the uptake of 2.7 moles of O_2/mole of substrate. Our cells oxidized D-lactate about 4 times as fast as L-Lactate. Both substrates were oxidized to the same extent, approx. 2.4 moles of O_2/mole of substrate. The oxidation of other substrates was reported previously[6].

Thick suspensions of these cells showed 3 cytochrome bands; the darkest one had its centre at 555 mμ (extending from 546 to 565 mμ). It was probably identical to that described by CHIN[10] (maximum at 554), TANENBAUM[11] (553 ± 5), and WIELAND AND PISTOR[12] (554 mμ). This band is also present in *A. pasteurianum* and *suboxydans*[13]. It appears to be a pigment differing from any other previously described cytochrome. The α-band of cytochrome a_1 has its centre at 599 mμ, extending from 587 to 613 mμ. The band at 525 mμ (from 518 to 536) was very weak. These bands disappeared readily when the suspension was oxidized by air. They were fully restored in the reduced state by the endogenous substrates after 11 min. When a few μmoles of ethanol, sodium D-lactate or pyruvate were added to an aerated suspension, the 3 cytochrome bands were reduced at once. Sodium L-lactate reduced the cytochromes only after 2–4 min.

Enzymes in the crude extract

Oxygen uptake in the Warburg apparatus: When 0.3 ml of crude enzyme (from about 15 mg of living cells) was incubated with 20 μmoles of DL-lactate in pH 7.0 phosphate buffer in the Warburg apparatus, 10 μmoles of O_2 were taken up after

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approx. 1 h. From then on the O_2 uptake continued slowly. This indicated either that D- and L-lactate were both oxidized to pyruvate followed by slow oxidation of this substrate, or that one of the isomers alone was oxidized to the acetate stage. There was always an induction period of 10–30 min which could be abolished by the addition of 0.25 to 1 μmole of methylene blue (Fig. 1). The R.Q. was 1, according to the reaction:

$$\text{lactate} + O_2 \longrightarrow \text{acetate} + CO_2$$

The pH optimum was about 6. Both D- and L-lactate were oxidized, but at different rates. D-Lactate was oxidized rapidly with the uptake of 1 O_2, whereas L-lactate gave the same end-product but about 7 times more slowly (Fig. 1).

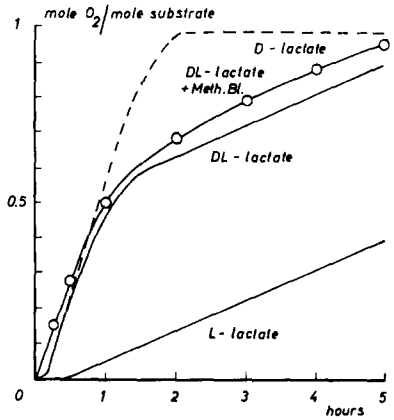

Fig. 1. Oxidation of lactates by crude cell-free extracts of *A. peroxydans*. Content of the Warburg vessels, see text.

20 μmoles of sodium pyruvate were quickly decarboxylated, probably to acetaldehyde, with the formation of 18 μmoles of CO_2 followed by a slow O_2 uptake corresponding to the conversion of acetaldehyde to acetate.

20 μmoles of ethanol were oxidized with the uptake of 20 O_2 and the medium was turned distinctly acid at the end of the expt.

Sodium acetate was not oxidized under these conditions. 1.5 μmoles of TPN and DPN, added separately or together, did not affect any of the oxidation rates or the overall O_2 uptake.

TPN- and DPN-linked dehydrogenases: D-Lactate was oxidized very slowly by TPN in glycine–NaOH buffer pH 9 or 10 after an induction phase of several minutes. Later the explanation was (see below) found to be that the substrate was first oxidized by the particles to acetaldehyde, which is the real substrate for the oxidation. There was no reaction with DPN and L-lactate was not oxidized. The reverse reaction, in phosphate buffer pH 7.0 with 10 μmoles of pyruvate and 0.3 of μmole of either DPNH or TPNH, showed that no reduction of pyruvate occurred.

Pyruvate in phosphate buffer pH 7.0 was quickly oxidized by TPN. The TPNH formation was preceded by an induction period of about 1 min, suggesting the formation of acetaldehyde. There was a negligible increase in O.D. with DPN. 1 μmole of ethanol was oxidized by either TPN or DPN. It was occasionally observed that the DPNH formation did not begin immediately after addition of the ethanol, but

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that there was a distinct lag of 2–3 min. The explanation is not known. We cannot agree with TANENBAUM's[11] statement that ethanol dehydrogenase in this species is TPN-specific. On the other hand, ATKINSON[14] reported that the ethanol dehydrogenase reacts with DPN. Acetaldehyde was oxidized by a TPN-specific dehydrogenase, explaining the induction phase in the D-lactate and pyruvate oxidation.

Absence of lactate racemase: 0.5 ml of the crude enzyme in $M/30$ phosphate buffer pH 6 was placed in the upper bulb of a Thunberg tube containing 50 μmoles of sodium D-lactate in 0.5 ml. In other tubes 0.15 μmole of TPN and DPN were placed. In the control tubes either D-lactate or the enzyme was omitted. The tubes were evacuated (to prevent oxidation of lactate), and the contents were mixed and incubated overnight at 30°. At the end of the incubation time an equal vol. of 3.5 % perchloric acid was added, the proteins were removed by centrifugation and L-lactate was determined in an aliquot in the spectrophotometer with crystalline L-lactic dehydrogenase (Boehringer). No trace of L-lactic acid was found. There was thus no racemase for the reactions:

$$\text{D-lactate} \rightleftharpoons \text{L-lactate}$$

$$\text{or D-lactate} \overset{\text{DPN}}{\underset{\text{TPN}}{\rightleftharpoons}} \text{pyruvate} \overset{\text{DPN}}{\underset{\text{TPN}}{\rightleftharpoons}} \text{L-lactate}$$

The crude extract was separated by ultracentrifugation into a particulate fraction and soluble enzymes and each fraction was investigated separately in a further attempt to localize and to study the above enzymes.

Enzymes in the particulate fraction

Lactate oxidation: The particles oxidized DL-lactate to the same extent as the crude enzyme. Methylene blue did not stimulate and even inhibited the reaction by 10–20 %. The stimulation of the crude extract by methylene blue was thus due to the effect on the soluble enzyme. This fraction oxidized D-lactate readily and L-lactate five to seven times more slowly (Fig. 2). Both substrates were oxidized with the uptake of 1 O_2/mole of substrate. The oxidations were neither enhanced by TPN or DPN nor inhibited by $3.3 \cdot 10^{-4}$ M atebrine. Since methylene blue had also no stimulating

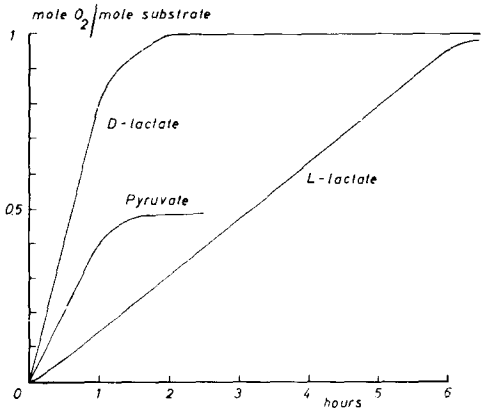

Fig. 2. The oxidation of some substrates by particles. Content of the Warburg vessels: 20 μmoles substrate, $M/30$ phosphate buffer pH 6.0, 0.3 ml particles, Temp. 30°.

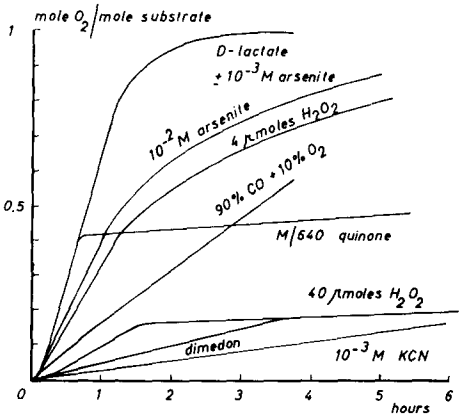

Fig. 3. The effect of some inhibitors on the oxidation of D-lactate with particles. Content of the Warburg vessels as in Fig. 2.

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effect, it was concluded that no dissociable flavin coenzyme was involved in the enzyme chain. Analysis of the particles, solubilized with desoxycholate, showed that they contained tightly bound flavins which either did not participate in the electron transfer, or could not be reached by atebrine. The oxidation of both lactate isomers was inhibited by CO. Mixtures of 90 % CO + 10 % O_2 or 95 % CO + 5 % O_2 were used; in the control expts. the CO was replaced by N_2. The oxidation by 95 %. These results showed that both lactates were oxidized ultimately by way of cytochromes and cytochrome oxidase, probably a_2, according to CHIN[10]. The oxidation of lactates was not inhibited by $5 \cdot 10^{-3}$ *M* urethane but was inhibited by approx. 20 % by $5 \cdot 10^{-3}$ *M* B.A.L. A SLATER factor was thus not involved. Neither $5 \cdot 10^{-3}$ *M* amytal, which is a specific reagent for the presence of pyridine nucleotides in the respiratory chain, nor 10^{-2} *M* *o*-phenanthroline had any effect. The effects of arsenite, H_2O_2, *p*-benzoquinone and dimedon are represented in Fig. 3. *M*/640 quinone stopped the reaction after 0.5 O_2 had been consumed. Analysis of the content of the Warburg vessels by the method of FRIEDEMANN AND HAUGEN[15] showed that both lactates had been quantitatively converted into pyruvate. H_2O_2 was not detectable as an end-product. 40 μmoles of H_2O_2 inhibited the reaction and also the D-lactate oxidase, possibly owing to the presence of a peroxidase. 10^{-2} *M* Arsenite inhibited only the last phase of the oxidation, presumably through the inhibition of the acetaldehyde oxidation. All these inhibitors behaved in the same way with D- and L-lactates.

Pyruvate metabolism: These particles contained a carboxylase. Anaerobically, 18 μmoles of CO_2 were formed from 20 μmoles of pyruvate; the reaction proceeded to completion in the presence of 25 mg of dimedon. The anaerobic CO_2 production was completely blocked by *M*/640 *p*-benzoquinone but 10^{-2} *M* arsenite had no effect.

Aerobically, pyruvate was oxidized with the uptake of 0.5 O_2 according to the equation:

$$\text{pyruvate} + 0.5\ O_2 \longrightarrow \text{acetate} + CO_2$$

Dimedon, *p*-benzoquinone and 40 μmoles of H_2O_2 inhibited O_2 uptake almost completely but 10^{-3} *M* arsenite had no effect, although 10^{-2} *M* arsenite inhibited the

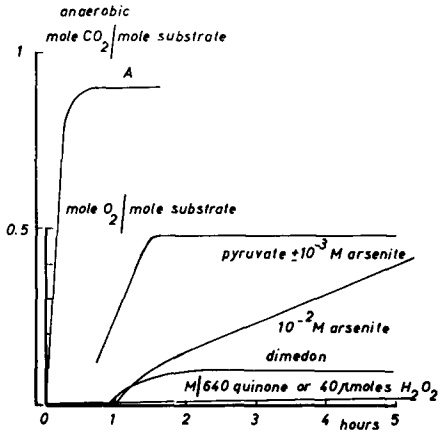

Fig. 4. The effect of some inhibitors on the oxidation of pyruvate with particles. Content of the Warburg vessels as in Fig. 2. The initial O_2 uptake could not be measured because of the strong CO_2 formation. The curve A represents the anaerobic CO_2 formation.

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uptake considerably (Fig. 4). Quinone inhibited aerobic CO_2 production completely. The other inhibitors had no influence on this reaction.

pH optimum for the oxidation of several substrates: The optimum pH was about 6 for most substrates, and about 5 for ethanol (Table I). The explanation for this

TABLE I

Each Warburg vessel contained $M/30$ buffer, 0.2 ml particles, 20 or 30 μmoles substrate, KOH in the central well, temp. 30°. The results are expressed as μmoles O_2 taken up after 60 min. The reaction rates with the different substrates are not comparable since the table is a compilation from several experiments. For CH_3CHO oxidation, 0.1 ml particles was used and the temp. was 24°.

pH	*4*	*5*	*6*	*7*	*8*	*9*	*10*	*11*	*12*
Na-D-lactate	2.0	15.3	18.6	15.0	3.8	0.8	0		
	1.4	14	16.7	9.0	3.5	1.2	0	0	0
Na-L-lactate	1.2	4.9	10.2	5.9	3.0	0.7	0	0	0
Na-pyruvate:									
O_2	1.1	6.0	6.6	3.3	0.8				
CO_2	1.9	12.1	13.6	7.4					
Acetaldehyde	2.5	5.5	6.9	5.9	3.3	1.6		0	
Ethanol	2.5	7.9	7.2	0.2	0				
	4.0	14.2	8.7	1.5	0	0	0		

behaviour could be readily observed; at pH 4 the particles precipitated and coagulated and partial precipitation also occurred at pH 5. Above pH 7 the particles started to disintegrate, the suspension becoming clear, and at pH 9 they were completely dissolved. Thus the pH optimum of the entire particle was measured instead of that of the individual enzyme systems.

Ferricyanide as acceptor (Fig. 5): This substance was a good acceptor. Both D- and L-lactate gave 5.1–5.2 CO_2, ethanol 3.9 CO_2, and pyruvate 3.0 CO_2. The end-product in each case was thus again acetate.

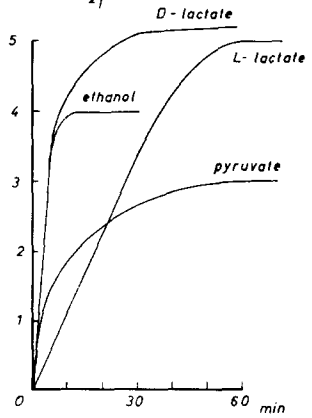

Fig. 5. Ferricyanide as an acceptor. Content of the Warburg vessels (30°): 9.5 mg $NaHCO_3$, 5 μmoles substrate, 0.2 ml ferricyanide solution (5 ml 10% $K_3Fe(CN)_6$ and 1 ml 0.16 M $NaHCO_3$), 0.4 ml particles in the side-arm. Final vol. 3 ml. Atmosphere: pure CO_2.

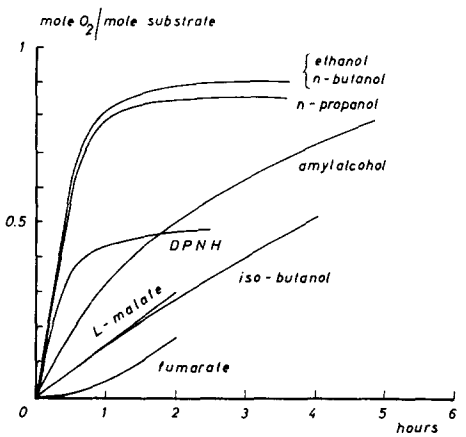

Fig. 6. The oxidation of several substrates by particles. Content of the Warburg vessels as in Fig. 2.

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Oxidation of other substrates (Fig. 6): These particles oxidized the primary alcohols, ethanol, *n*-propanol, *n*-butanol, iso-butanol and amyl alcohol, to the corresponding acids. *Sec.*-butanol, iso-propanol and methanol were not oxidized, although the secondary alcohols were oxidized by the intact cells, probably with the coenzyme-linked dehydrogenase. L-Malate and fumarate were slowly oxidized but citrate, formate, acetate and glucose were not. DPNH was oxidized according to:

$$DPNH + H^+ + 0.5\ O_2 \rightarrow DPN^+ + H_2O$$

H_2O_2 was not detectable with titanium sulfate.

Soluble enzymes

Lactate metabolism: DL-Lactate was not oxidized in the absence of methylene blue; in its presence 20 μmoles of substrate consumed 10 μmoles of O_2. D-Lactate was slowly oxidized in the presence of methylene blue with the uptake of 1 mole of O_2 per mole of substrate. TPN and DPN were again without effect. N-methyl-phenazinium methosulfate was a better carrier than methylene blue (Fig. 7), the oxidation proceeding at least three times faster. 150 μg of riboflavine alone or in the presence of methylene blue had no effect, whereas $3.3 \cdot 10^{-4}$ *M* atebrine inhibited the oxidation markedly, although not completely. From the initial induction period of the oxygen uptake curve, atebrine appeared to eliminate part of the methylene blue by association and thus the inhibition could not be interpreted as an intervention by a flavin. Indeed, when phenazine was used as the carrier, atebrine had no effect, even up to concns. of 10^{-2} *M*. The oxidation of D-lactate was not inhibited by 10^{-2}

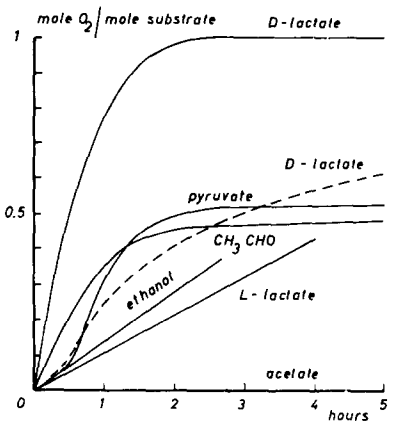

Fig. 7. The oxidation of some substrates by soluble enzymes of *A. peroxydans* in the presence of phenazine (full lines) or methylene blue (broken line). Content of the Warburg vessels, see text. There was no O_2 uptake in the absence of carriers.

or 10^{-3} *M* arsenite or by 4 μmoles of H_2O_2 but 40 μmoles of H_2O_2 inhibited the reaction almost completely. *M*/640 Freshly sublimed quinone stopped the reaction after 0.5 O_2 had been taken up. The FRIEDEMANN AND HAUGEN[15] reaction showed the accumulation of the theoretical amount of pyruvate. 25 mg of dimedon/Warburg vessel inhibited the reaction by 60 % or more. The picture was thus essentially the same as for the particles, except that here 10^{-2} *M* arsenite and 4 μmoles H_2O_2 did not inhibit. The oxidation of D-lactate was not inhibited by 10^{-2} *M* EDTA, and hardly at all by 10^{-3} *M* KCN or 10^{-3} *M* *p*-chloromercuribenzoate. Metal ions, heavy metal enzymes or –SH enzymes thus appear not to be involved.

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TABLE II

pH OPTIMUM FOR THE OXIDATION OF D-LACTATE BY O_2

The Warburg vessels contained in 3 ml $M/30$ buffer: 0.5 ml soluble enzyme, 0.25 μmole methylene blue or 2.7 μmoles phenazine, 30 μmoles Na-D-lactate in the side arm and KOH in the central well. Temp. 30°. The results are expressed as μmoles O_2 uptake after 1 h.

pH	*4*	*5*	*5.5*	*6*	*6.5*	*7*	*8*	*9*	*10*
Buffer	*Acetic acid/acetate*			*Phosphate*				*NH_4Cl/NH_4OH*	
				Methylene blue					
	0	4.8	—	5.3	—	3.9	1.8	0.3	0
				Phenazine					
	1.2	5.1	6.1	5.6	5.0	4.0	1.3	0.2	0.1

TABLE III

pH OPTIMUM OF THE SOLUBLE D-LACTATE DEHYDROGENASE

Different buffers 0.02 M final, as given below, were used. Each Thunberg tube contained 20 μmoles Na-D-lactate, 0.15 μmole methylene blue; 0.1 ml enzyme in the upper bulb. Final vol. 3 ml. The tubes were entirely immersed in a water bath at 30° and equilibrated for 10 min. The results are expressed as decoloration time in minutes.

pH	*4*	*4.5*	*5*	*5.5*	*6*	*6.5*	*7*	*7.5*	*8*	*9*	*10*	*11*
Buffer	*Acetic acid/acetate*				*Phosphate*				*Tris-HCl*	*Glycine/NaOH*		
	> 90		30		12		9		11	33	> 90	> 90
	> 90		58		24		21		23	58	> 90	> 90
		> 120	56	23	24	20	23	26.5	37	150	> 150	

TABLE IV

USE OF SEVERAL ACCEPTORS FOR THE OXIDATION OF D-LACTATE IN THE THUNBERG TUBE

Conditions as before; pH 6; 0.15 μmole of the acceptor; 30°.

Acceptor	*E_0'*	*Decoloration time*
Thionine	+0.062	4 min
Cresyl blue	+0.031	6
Methylene blue	+0.011	15.5
Nile blue	−0.116	partially
Gallophenine	−0.142	partially
Cresyl violet	−0.167	∞
Neutral blue	−0.192	∞

L-Lactate was not oxidized in the presence or absence of methylene blue. With phenazine a slow oxidation occurred with the uptake of 1 O_2 after 5 h. 10^{-3} M Arsenite had no effect while 10^{-2} M arsenite inhibited only slightly. Dimedon and 40 μmoles of H_2O_2 blocked the reaction almost completely. Quinone stopped the reaction when 0.6 O_2 were taken up and the FRIEDEMAN AND HAUGEN[15] test showed that L-lactate had been quantitatively converted into pyruvate.

The pH optimum with both carriers was 5.5–6 (Table II). Methylene blue was a good acceptor in anaerobic conditions. The reaction had a broad pH optimum from 5.5–7.5 (Table III). Other acceptors could also be used (Table IV).

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Pyruvate metabolism: A carboxylase was present, for in a N_2 atmosphere 17 μmoles of CO_2 were evolved from 20 μmoles of pyruvate. The reaction did not go to completion because acetaldehyde is a well-known inhibitor of carboxylase. In the presence of 25 mg of dimedon or of 40 μmoles of H_2O_2 the reaction went to completion. This was another strong indication that a soluble peroxidase was present. Quinone inhibited the anaerobic CO_2 production by at least 80 % but 10^{-2} or 10^{-3} *M* arsenite had no effect (Fig. 8).

Aerobically, in the absence of an artificial carrier, pyruvate also gave CO_2. In the presence of methylene blue about 0.83 mole of CO_2 was formed, and 0.3 of mole O_2/mole of substrate taken up. The decomposition did not go to completion because

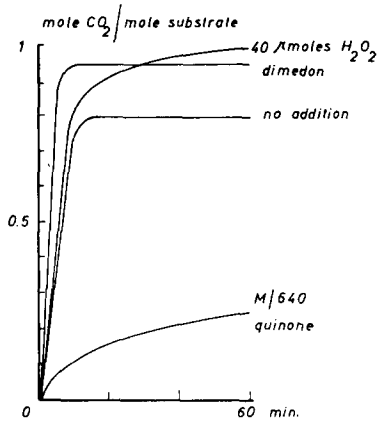

Fig. 8. Anaerobic CO_2 production from pyruvate in the presence of soluble enzymes and influence of some inhibitors. Content of the Warburg vessels as before. N_2 atmosphere.

the enzymes were slowly inactivated. The oxidation was preceded by the formation of practically all of the CO_2 during the first 10-min period. In the presence of phenazine, the oxidation proceeded faster and the theoretical amount of 0.5 O_2 was taken up; all of the CO_2 was again evolved in the initial period. 10^{-2} and 10^{-3} *M* arsenite did not inhibit oxidation of pyruvate whereas quinone and 40 μmoles of H_2O_2 blocked it completely. Dimedon stopped the reaction when about 40 % of the theoretical amount of O_2 was taken up. None of the inhibitors except quinone prevented the aerobic CO_2 formation.

Metabolism of other substrates: Acetaldehyde was oxidized in the presence of phenazine with the uptake of the theoretical amount of 0.5 O_2. This oxidation was not affected by quinone, 4 μmoles of H_2O_2 or 10^{-3} *M* arsenite, but was inhibited by about 10–20 % by 10^{-2} *M* arsenite. In contrast to the opinion sometimes expressed in the literature, dimedon is not a good trapping agent for acetaldehyde. It inhibited the reaction by about 50 % and stopped it when about 70 % of the acetaldehyde was oxidized. Ethanol was oxidized in the presence of phenazine with the theoretical uptake of 1 O_2. Acetate oxidation did not occur and was not stimulated by any of the carriers or cofactors mentioned in this paper.

Ferricyanide as an acceptor: D-Lactate gave 5.2 CO_2, ethanol, 4 and pyruvate, 3.0 CO_2. L-Lactate was oxidized much more slowly than in the case of the particles. These results agreed closely with the theoretical values to be expected for the conversion of these substances into acetate.

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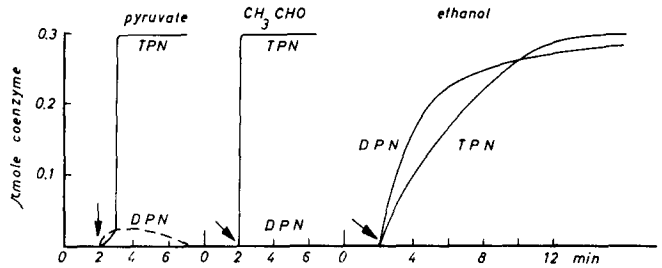

Fig. 9. Oxidation of several substrates by TPN or DPN in the presence of soluble enzymes. Content of the Beckman cuvettes, see text.

TPN- and DPN-linked dehydrogenases: The dehydrogenases, which were present in the crude extract, were all found in the soluble fraction. The absence of TPN- or DPN-linked D- or L-lactate dehydrogenases was confirmed.

Cytochromes: Cytochromes were present in the supernate. The 3 bands could easily be seen in the Beck-Hartridge reversion spectroscope. The maximum of the Soret peak was at 418 mμ, as measured in the spectrophotometer. The amount of cytochromes in the particulate fraction was however much greater.

DISCUSSION

The particles from *A. peroxydans* contain a complete set of enzymes for the oxidation of D- and L-lactate, pyruvate, ethanol and acetaldehyde to acetate. By the use of appropriate inhibitors it can be shown that both lactates are oxidized by way of pyruvate and acetaldehyde; ethanol is oxidized by way of acetaldehyde. The particles thus excrete acetate and CO_2. They also contain enzymes for the transfer of electrons from the lactate, ethanol and acetaldehyde dehydrogenases to oxygen by way of cytochromes and cytochrome oxidase. They thus constitute a complete functional unit for the preparation of acetate from the C_3 and C_2 compounds. No evidence was found for a pyruvate oxidase. Flavins may also be present in the electron transfer chain and this point is now under investigation. There was no evidence for the intervention of TPN, DPN or a SLATER factor in the oxidation chain. The particles also oxidize *n*-propanol, *n*-butanol, isobutanol, amyl-alcohol, L-malate, fumarate and DPNH.

An unusual aspect is the presence of the D-lactate oxidase; for the enzymes involved in the production and decomposition of lactate are usually specific for the L-configuration. They are either soluble and linked to DPN (muscle) or particulate and linked to flavins and cytochromes (yeast). D-Lactic oxidizing enzymes are unusual, although their existence might be expected in lactic acid bacteria, because many of these micro-organisms make D-lactic acid. It is remarkable that the particulate D-lactate oxidase from *A. peroxydans* bears much resemblance to the simular enzyme from rat-liver and kidney mitochondria[16], *e.g.* it also does not require added cofactors and the endproduct is pyruvate. The pH optimum is slightly different; MAHLER's enzyme functions best at pH 6.8 to 7.8, and ours at pH 6.

The soluble enzymes effect the same overall series of reactions. An artificial carrier is required. Both lactates are again decomposed by way of pyruvate and acetaldehyde and ethanol is oxidized by way of acetaldehyde. It is possible that the

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active soluble D-lactate dehydrogenase and the weak L-lactate dehydrogenase, as well as the soluble pyruvate decarboxylase, are different from the particulate enzymes. However, they may possibly be derived from broken particles caused by sonication. The latter view is strengthened by the similar effect of most of the inhibitors on both the particulate and the soluble enzymes. $M/640$ Quinone is an excellent inhibitor for pyruvate decarboxylase and dimedon in excess was found to trap acetaldehyde only incompletely, while 40 μmoles of H_2O_2 inhibited the oxygen uptake most probably by the presence of a peroxidase. 10^{-2} M Arsenite had hardly any effect on the soluble enzymes, but partially inhibited the particulate enzymes, presumably through the acetaldehyde oxidase. No evidence was found for a lipoic acid-dependent pyruvate oxidase. Acetone preparations of *Escherichia coli*[17,18] contain a D-lactate dehydrogenase, which is comparable to our soluble enzyme, because it requires methylene blue as acceptor. For ethanol oxidation there is a dual pathway: a particulate ethanol and acetaldehyde oxidase, and a soluble system consisting of a TPN- and DPN-linked ethanol dehydrogenase and a TPN-linked acetaldehyde dehydrogenase. These findings confirm the results of PRIEUR[19].

The results with the crude extracts may be regarded as the joint actions of the particles and the supernate. The oxidation of substrates by the crude extract in the absence of an artificial carrier is obviously caused by the particles. When methylene blue stimulates the oxidation of some substrate, it corresponds to the activity of the soluble enzyme.

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