acetaldehyde with the liberation of carbon dioxide, thus:

SUMMARY

1. *Eremothecium ashbyii* incorporates generally-labelled L-[¹⁴C]threonine into riboflavin to the extent of 4%.

2. C-1 and C-2 of threonine are incorporated as glycine into C-4a + C-9a of riboflavin. C-3 and C-4 of threonine are not incorporated into riboflavin.

3. The enzyme threonine aldolase, splitting threonine into glycine (C-1 + C-2) and acetaldehyde (C-3 + C-4), is present in *E. ashbyii*.

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The Lactic Dehydrogenase of *Propionibacterium pentosaceum*

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The occurrence in *Propionibacterium pentosaceum* of enzymes which are able to catalyse an oxidation of lactate in the presence of fumarate has been demonstrated by the work of Barker & Lipmann (1944). The evidence presented by these authors suggested the participation in the system of a type of lactic dehydrogenase different from that occurring in animal tissues.

They worked exclusively with dried cell preparations and their attempts to extract active fractions from such cells failed. A crude preparation, which carried out a fast lactate-fumarate dismutation, was obtained by Lara (1954) by grinding frozen cells of *P. pentosaceum* with alumina, essentially as described by McIlwain (1948).

The present paper describes the methods of preparation and some properties of a fraction capable of catalysing the oxidation of lactate in the presence of several redox-dyes, and also some inhibition studies.

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MATERIALS AND METHODS

Organism and media. *P. pentosaceum* strain E. 2-1, obtained from Professor C. B. van Niel's collection, was cultured and harvested essentially as described by Barker & Lipmann (1944); tryptone, however, was omitted from the medium.

Methods. All reagent solutions were prepared with twice-distilled water, and the enzyme dilutions were made with 0.5% (w/v) bovine plasma-albumin solution (Armour). All operations with the enzyme were done in a cold room maintained at 0-4°, and the centrifugings were performed in a Servall centrifuge, model SS-1, at 25 000 g for 15 min. unless otherwise specified. Oxygen consumption was determined in a Warburg apparatus of conventional type, and the extinction determinations were made in 3 ml. cuvettes on a Beckman spectrophotometer model DU. Protein was determined by the method of Gornall, Bardawill & David (1949), with a coefficient of 0.095 (extinction at 540 mμ, 1 cm. light path, given by a solution of 1 mg. of protein/3 ml. of reaction mixture in the presence of 1.5 ml. of biuret reagent), and pyruvate was estimated by the

method of Friedemann & Haugen (1943), simplified by omitting the extraction of the phenylhydrazones.

Perchloric acid extracts were obtained from the purified enzyme preparations as follows: 60% (w/v) perchloric acid was added to the purified enzyme to obtain 0.6N final concentration. The precipitated proteins were separated by centrifuging and the supernatant was neutralized with KOH; the precipitated perchlorate was separated by filtration. One unit of perchloric acid extract is defined as the amount contained in the neutralized supernatant obtained from a solution containing initially one indophenol unit of lactic dehydrogenase.

The preparation used in the inhibition studies had a specific activity of 4.7 indophenol units/mg. of protein. The inhibitory action was estimated usually by the effect upon activity while the enzyme was being determined by the indophenol assay, in which case all the assay components plus the inhibitor were added to the cuvettes and the reaction was started by enzyme addition. Dicoumarol was dissolved in 0.01N-KOH, and in this case the results obtained were compared with blank enzyme assays in which the same amount of 0.01N-KOH was added.

Chemicals. Adenosine monophosphate (AMP), adenosine triphosphate (ATP), diphosphopyridine nucleotide (DPN), reduced diphosphopyridine nucleotide (DPNH), triphosphopyridine nucleotide (TPN), flavin mononucleotide (FMN), flavinadenine dinucleotide (FAD), cytochrome *c* and 2-amino-2-hydroxymethylpropane-1,3-diol (tris) buffer were obtained from the Sigma Chemical Co. (St Louis, Mo., U.S.A.). Protamine sulphate was obtained from Eli Lilly and Co., Indianapolis, Ind., U.S.A., D-(+)-lactic acid and L-(-)-lactic acid were obtained from the Delta Chemical Works, New York, U.S.A. and disodium dihydrogen ethylenediaminetetra-acetate (EDTA) was obtained from City Chemical Corp., New York, U.S.A. Phenazine methosulphate was synthesized according to Singer & Kearney (1957), 1:2-dihydroxybenzene-3:5-disulphonate was the kind gift of Dr V. Massey. Sodium pyruvate and thyroxine were obtained from F. Hoffmann-La Roche and Co., Basel, Switzerland; sodium 5-ethyl-5-isoamylbarbiturate (Amytal) was from Eli Lilly and Co. of Brazil, São Paulo, Brazil; sodium 5-methyl-5-(1-methyl-butyl)barbiturate (Nembutal) from Abbot Laboratório do Brasil Ltda., São Paulo, Brazil; pentachlorophenol from Eastmann Kodak Co., Rochester, N.Y., U.S.A.; 3-chloro-7-methoxy-9-(1-methyl-4-diethylaminobutylamino)-acridine dihydrochloride (quinacrine) and 7-chloro-4-(4-diethyl-amino-1-methylbutylamino)-quinoline diphosphate (chloro-quine) were from the Winthrop Products Inc., N.Y., U.S.A.; quinine monohydrochloride was from Pelletier Delondre et Sevaillant, Armet de Lise et Cie., Paris, France. Dicoumarol [3:3'-methylenebis-(4-hydroxycoumarin)] and *o*-iodosobenzoic acid were gifts from Dr Isaias Raw. Vitamin K₁ (aqueous emulsion) and menadione (2-methyl-1:4-naphthaquinone) diphosphate (aqueous solution) were used in the form of pharmaceutical products Kanakon and Synkavit respectively, and were provided by Products Roche Químicos e Farmacêuticos S.A., Rio de Janeiro, Brazil.

Enzyme assays

In the course of the purification and study of the properties of the enzyme several assay systems were used, involving spectrophotometric and manometric methods which are described below.

Indophenol assay. In a 3 ml. cuvette are placed 60 μ moles of potassium phosphate buffer, pH 7.3, 50 μ moles of racemic sodium lactate, 0.1 μ mole of 2:6-dichlorophenolindophenol, 5 μ moles of disodium dihydrogen EDTA and water plus enzyme to give a final volume of 3 ml. The reaction is initiated by enzyme addition and its course estimated by measurements of extinction at 600 m μ with water as a blank. Readings are taken every 30 sec. during 2 min. In the absence of lactate the extinction changes are negligible under these conditions. The amount of enzyme used is such that the range of extinction change/min. is 0.020–0.100. Under these conditions the reaction obeys zero-order kinetics until 75% of the dye is reduced, and the velocity of dye reduction is strictly proportional to the amount of enzyme added.

One indophenol unit is defined as the amount of enzyme which catalyses the reduction of 1 μ equiv. of dye/min., at room temperature (25–27°). Specific activity was measured in indophenol units/mg. of protein. The molar-extinction coefficient for 2:6-dichlorophenolindophenol used was that reported by Basford & Huennekens (1955).

Quinone assay. The conditions are the same as for the indophenol assay, 1 μ mole of 1:2-naphthaquinone-4-sulphonate being substituted for 2:6-dichlorophenolindophenol and the extinction measurements are made at 305 m μ .

One quinone unit is the amount of enzyme capable of reducing 1 μ equiv. of 1:2-naphthaquinone-4-sulphonate/min. The extinction coefficient for this substance was taken as 3.7×10^4 cm.² mole⁻¹.

Methylene blue and phenazine methosulphate assays. In the main compartment of a Warburg vessel are placed 100 μ moles of potassium phosphate buffer, pH 7.3, water and the enzyme solution. The side arm contains either 3 μ moles of methylene blue or 5.4 μ moles of phenazine methosulphate and 40 μ moles of racemic sodium lactate; total volume, 2 ml. After equilibration at 30° for 5 min. the reaction is started by tipping the contents of the side arm into the main compartment. The manometers are read 2 and 7 min. after tipping. The oxygen consumption in this 5 min. period is used in calculating enzyme activity. The amount of enzyme is adjusted so that the oxygen consumption is not higher than 8 μ l./min. Under these conditions oxygen consumption is strictly proportional to the amount of enzyme used.

One methylene blue, or phenazine, unit is defined as the amount of enzyme capable of catalysing the consumption of 1 μ l. of oxygen/min., when each dye respectively is used as hydrogen carrier.

With purified preparations (4.7 indophenol units/mg. of protein) the correspondence among the several defined units is: 1.0 indophenol unit $\equiv$ 0.7 quinone unit $\equiv$ 9.2 methylene blue units $\equiv$ 28.5 phenazine units.

Purification procedure

Crude extract. The cells, harvested from the medium by centrifuging and washed with water, were frozen, ground with twice their weight of alumina-301 (Aluminium Co. of America) and the paste was extracted with a portion of 0.1M-potassium phosphate buffer, pH 7.0, equal to three times the volume of the cells. The alumina and cell debris were then separated by centrifuging at 10 000 g for 15 min., and the supernatant was collected.

First ammonium sulphate fractionation. To 100 ml. of the crude cell extract obtained as described above, 17.6 g. of

solid $(\text{NH}_4)_2\text{SO}_4$ is added with stirring, and in small portions. Stirring is continued for 15 min. and the precipitated proteins are separated by centrifuging and discarded. A further 8.6 g. of $(\text{NH}_4)_2\text{SO}_4$ is added to the supernatant in the same manner. The precipitate formed is collected by centrifuging and dissolved in 0.1M-sodium citrate buffer, pH 5.3, to give a solution containing about 25 indophenol units/ml.

Protamine treatment. To the solution obtained in the previous step is added an equal volume of 0.1M-tris buffer, pH 8.8, and then 0.7 vol. of 0.5% (w/v) protamine sulphate solution. After stirring for 10 min. the precipitate is separated by centrifuging and discarded. The supernatant is brought to pH 6.0 by the addition of 0.2M-sodium acetate buffer, pH 4.0

Second ammonium sulphate fractionation. The solution obtained in the previous step is again fractionated with solid $(\text{NH}_4)_2\text{SO}_4$, the fraction separating between 25 and 30% of saturation being collected by centrifuging and dissolved in 0.1M-sodium citrate, pH 5.3, to give approx. 15 indophenol units/ml.

Isoelectric precipitation and ethanol fractionation. To the solution obtained in the previous step 0.2 vol. of twice-distilled water, and then 0.8 vol. of 0.2M-sodium acetate buffer, pH 4.0, are added (final pH is now between 4.5 and 4.7). The inert material is separated by centrifuging, the solution is placed in the cold room at -10° and its temperature is lowered until the first ice crystals begin to appear. Then 98% (w/v) ethanol, cooled to -20° , is slowly added through a capillary pipette with stirring until a 10% solution is obtained. At the end of the addition the temperature of the solution is about -15° and the precipitate formed is separated by centrifuging at -15° ; the precipitate is dissolved in 0.1M-sodium citrate, pH 5.3, and dialysed against the same buffer (containing 0.01M-EDTA) for 1 hr.

RESULTS

Purification and properties of the enzyme

Purification procedure. Table 1 shows the purification achieved by the procedure described above.

Absence of pyridine nucleotide moiety. The following evidence indicates that the lactic dehydrogenase from *P. pentosaceum* is independent of pyridine nucleotide coenzymes.

1. When purified preparations of specific activity 4.7 were dialysed against 0.025M-sodium citrate, pH 5.3, for 7 hr. a 33% decrease in activity occurred. The addition of DPN to the indophenol system in concentrations of up to 0.34 mM in the

assay of these fractions did not restore the initial activity.

2. Relatively large amounts of lactic dehydrogenase (0.3 indophenol unit) failed to reduce either DPN or TPN in the presence of lactate. Similarly, DPNH was not oxidized by the addition of sodium pyruvate.

3. Malic dehydrogenase plus malate failed to bring about an increase in extinction at 340 m μ when added to either the purified *P. pentosaceum* lactic dehydrogenase preparations or to boiled juices (Kochsaft) obtained from these.

4. The dehydrogenase activity is not affected by 20 min. pre-incubation in 0.25M-hydroxylamine or by the addition of this substance to the indophenol assay system in concentrations of 50 mM.

5. In the crude *P. pentosaceum* extracts DPNH disappeared rapidly, as measured by decrease in extinction at 340 m μ , and this decrease was identical both aerobically and anaerobically as well as in the presence or absence of pyruvate.

Specificity. Among several hydroxy acids tested, lactate was the only effective hydrogen donor observed: α -hydroxyisobutyric acid, β -hydroxybutyric acid and malic acid gave completely negative results. DPNH does not serve as hydrogen donor in the assay systems used.

A stereospecificity was observed and the relative velocities for the different lactates at a concentration of 1.67 mM in the assay system were as follows: racemic lactate, arbitrarily taken as 10; D-(–)-lactate, 9.3; L-(+)-lactate 3. The cause of the residual activity in the presence of L-(+)-lactate has not been determined.

Several dyes act as hydrogen acceptors, as indicated in the description of the assay methods. The relative efficiency of these dyes, throughout the course of purification, is nearly constant, as shown by the data of Table 2. Besides these, indigo tetrasulphonate (1.33 mM), toluidine blue (0.5 mg./ml.) and azure I (0.5 mg./ml.) were also effective, but the rates of consumption were 7, 8 and 17% respectively of that obtained with methylene blue. Indigo disulphonate (1.33 mM), indigo trisulphonate (1.33 mM), Nile blue (0.5 mg./ml.) and cresyl violet (0.5 mg./ml.) were completely ineffective.

Table 1. *Purification of lactic dehydrogenase from Propionibacterium pentosaceum*

The activities were measured by the indophenol assay.

Fraction	Total indophenol units	Specific activity (units/mg. of protein)	Total protein (mg.)
Crude extract	1880	0.5	3980
First $(\text{NH}_4)_2\text{SO}_4$ fractionation	900	1.2	770
Protamine supernatant	642	1.0	650
Second $(\text{NH}_4)_2\text{SO}_4$ fractionation: 25–30% saturation	216	2.8	78
Ethanol fractionation: 0–10% ethanol	166	5.7	29

Table 2. Comparison of activities observed with the various assay systems, shown with the several fractions obtained in the course of purification of *Propionibacterium pentosaceum* lactic dehydrogenase

Specific activity is expressed in indophenol units/mg. of protein. The activities were assayed by the different methods as described in the text.

Fraction	(1) Specific activity	(2) Indophenol units/ml.	(3) Quinone units/ml.	(4) Phenazine units/ml.	(5) Methylene blue units/ml.	(3) (2)	(4) (2)	(5) (2)
Crude extract	0.5	5.3	3.5	162	72	0.66	30.5	13.6
After first $(\text{NH}_4)_2\text{SO}_4$ fractionation	1.0	21.1	13.3	655	200	0.62	31.0	9.4
After protamine treatment and second $(\text{NH}_4)_2\text{SO}_4$ fractionation	2.6	12.7	9.0	380	116	0.70	30.0	9.2
After ethanol fractionation	4.1	12.3	8.7	374	112	0.70	30.4	9.1

Table 3. Relation between the amount of dye reduced and pyruvate formed during lactate oxidation by *Propionibacterium pentosaceum* lactic dehydrogenase

Variable amounts of 2:6-dichlorophenolindophenol (as indicated) were completely reduced by excess of lactate (20 μ moles) in the presence of 0.64 indophenol unit of lactic dehydrogenase (specific activity, 2.6), in a system containing 100 μ moles of potassium phosphate buffer, pH 7.3, in a total volume of 2 ml. The pyruvate in a portion of the medium was assayed directly and the results were corrected for blanks: (a) without dye; (b) without enzyme.

Expt. no.	Indophenol reduced (μ mole)	Pyruvate formed (μ mole)	Pyruvate Dye
1	0.53	0.54	1.02
2	1.06	1.15	1.08
3	1.58	1.70	1.08

Fig. 1. Determination of the Michaelis constant of lactic dehydrogenase. The activity was measured by the indophenol assay with and without addition of ammonium sulphate, the concentration of D-(-)-lactate being varied as indicated. Enzyme of specific activity 4.7 was used. O, Absence of ammonium salts; ●, presence of 10 mM-ammonium sulphate. pH 7.3 and temp. 26°.

Fig. 2. Effect of varying phenazine methosulphate concentration on lactic dehydrogenase activity. Activity was measured by the phenazine methosulphate assay with solutions of varying concentrations of phenazine: 0.13 indophenol unit of purified enzyme of specific activity 4.7 was used per Warburg vessel. Temp. 30°.

Reaction products. The stoichiometry of the reaction studied is the following:

It was verified by paper chromatography (Cavallini, Frontali & Toschi, 1949) that pyruvic acid is the only keto acid formed under the assay conditions. The number of moles of pyruvate formed and the number of moles of reduced dye produced are nearly equal (Table 3).

Michaelis constants. The dependence of the rate of reduction of 2:6-dichlorophenolindophenol on the concentration of D-(-)-lactate is shown by the data presented in Fig. 1, from which it can be calculated that K_m for lactate in the absence of $(\text{NH}_4)_2\text{SO}_4$ is 51.6 μM at pH 7.3 and at 26°.

The affinity of the enzyme for the several hydrogen acceptors was also determined. In the manometric-assay conditions, the influence of phenazine

methosulphate on reaction rate is shown by the data of Fig. 2, and K_m for phenazine methosulphate is $640\text{ }\mu\text{M}$. Under the same conditions, if methylene blue is used as acceptor, hardly any variation is observed in the velocity of lactate oxidation, when the dye concentration varies from 0.5 mM to 3.33 mM . Independence of reaction velocity on dye concentration was also observed with 2:6-dichlorophenolindophenol ($14\text{ }\mu\text{M}$ – $50\text{ }\mu\text{M}$) and 1:2-naphthoquinone-4-sulphonate (0.5 mM – 1.0 mM). Thus in the last three cases mentioned K_m is equal to or nearly equal to zero.

pH dependence. The effect of pH on reaction velocity was studied manometrically with methylene blue as the hydrogen acceptor. Fig. 3 shows that the optimum pH is 7.7.

Stability. The enzyme seemed to be fairly stable when kept at $0\text{--}4^\circ$ in solutions of pH 5.3–5.8. The drop in activity was only 2% when solutions (5–10 indophenol units/ml.) in 0.1 M-sodium citrate, pH 5.3, containing 0.01 M -EDTA, were kept frozen for 1 month at -25° . Dilute enzyme (0.4 indophenol unit/ml.) in this same citrate buffer containing EDTA can be kept at 0° for 10 min. without any loss in activity. If, however, EDTA is not added an 8% drop in activity is observed.

The enzyme is thermolabile, activity being completely destroyed by boiling for 3 min., and only 15% of the activity remains after heating to 47° for 3 min., as measured by the indophenol assay.

Prosthetic group. The spectrum of the best preparation obtained is shown in Fig. 4. It shows

general absorption in the visible range, and the shoulder at $420\text{ m}\mu$ is due to contamination with cytochrome *b*, which is abundantly present in the crude preparations (Lara, 1959).

Many attempts to split the enzyme by the procedures of Horecker (1950) and of Theorell & Maelhy (1950) failed. The addition of 6% (w/v) perchloric acid to solutions of the enzyme in 20% (w/v) $(\text{NH}_4)_2\text{SO}_4$, to produce final concentrations of 6 mN – 0.1 N , led to white precipitates which, when dissolved in 0.1 M -sodium citrate buffer, pH 5.3, showed from 0 to 60% of the original activity. Restoration of the activity was not obtained by addition of FMN, FAD or boiled juices obtained from purified enzyme.

Activators. Several ions activate the enzyme, as measured by the indophenol assay. The effect of the NH_4^+ ion is particularly striking (Table 4). This

Fig. 3. Effect of pH on lactic dehydrogenase activity. Activities were measured by the methylene blue assay with 0.25 indophenol unit of purified enzyme of specific activity 4.1 . Each Warburg vessel contained $100\text{ }\mu\text{moles}$ of buffers of varying pH in place of the one regularly used (citrate, pH 5.3–6.0; phosphate, pH 6.7–7.7; tris-HCl, pH 7.7–8.5; carbonate, pH 8.85 and 8.3). Temp. 30° .

Fig. 4. Absorption spectra of purified lactic dehydrogenase. $\bigcirc$, Enzyme in equilibrium with air; $\times$, enzyme in the presence of sodium lactate. Main figure: solution of purified enzyme of specific activity 4.7 , 1.6 indophenol units/ml.; insert: solution of purified enzyme of specific activity 5.5 , concentration 16.4 indophenol units/ml.

Table 4. Activation of *Propionibacterium pentosaceum* lactic dehydrogenase by ammonium salts

The activities were measured by the indophenol assay, with enzyme of specific activity 3.1 . The salts were included in the assay system in the concentration indicated. The activity in the absence of the salts was taken arbitrarily as 100.

Concn. (M)	Relative activity			
	$(\text{NH}_4)_2\text{SO}_4$	Na_2SO_4	NH_4Cl	NaCl
—	100	100	100	100
0.01	122	103	120	104
0.05	160	117	146	105
0.10	202	111	194	111
0.20	228	126	257	106
0.50	266	123	348	100

is not merely due to an increase in the ionic strength, since the activation caused by Na_2SO_4 or NaCl is either very small or non-existent as compared with the corresponding ammonium salts.

Table 5. *Activation of Propionibacterium pentosaceum lactic dehydrogenase by several substances*

The activities were measured as described in Table 4.

Activator	Concn. (mM)	Relative activity
None	—	100
Magnesium sulphate	5.0	126
Magnesium sulphate	10.0	129
Potassium cyanide	1.0	109
Potassium cyanide	3.3	125
Sodium fluoride	50.0	126
isoNicotinic acid hydrazide	10.0	118
Potassium malonate	10.0	151

Table 6. *Action of some inhibitors on the dehydrogenation of lactate catalysed by purified preparations from Propionibacterium pentosaceum*

All experiments were conducted under the conditions described for the indophenol assay, the inhibitory substances being incorporated in the assay solution in the concentrations indicated. The observed result is therefore due to the action of the inhibitor during the 2 min. required to conduct the assay.

Inhibitors	Concn. (mM)	Inhibition (%)
Sodium pyruvate	0.3	21
	1.7	47
	8.3	74
Sodium oxalate	0.1	24
	0.2	43
	1.0	74
<i>p</i> -Chloromercuribenzoate	1.0	19
	2.0	64
	3.0	98
Monoiodoacetate	6.0	26
	8.2	44
Urethane	10.0	26
Amytal	10.0	44
Nembutal	10.0	9
	50.0	73
Quinacrine	0.1	30
	1.0	83
Chloroquine	1.0	20
	0.4	50
Quinine	1.0	72
	0.02	16
Dicoumarol	0.1	49
	1.0	96
Vitamin K ₁	0.2	18
	5.0	59
Pentachlorophenol	0.1	19
	1.0	65
Thyroxine	0.2	19
Hydrazine	5.0	30
	20.0	64

The results shown in Fig. 1 indicate that the NH_4^+ ion alters the affinity of the enzyme for lactate.

Results obtained which indicate activation by other substances are presented in Table 5.

Metal components. The purified enzyme seems to be independent of metals for its action. Cyanide and fluoride activate (Table 5) whereas EDTA (0.1 M), 8-hydroxyquinoline (1 mM), sodium azide (1 mM) and 1:2-dihydroxybenzene-3:5-disulphonate (5 mM) do not decrease the activity when included in the indophenol assay system.

Action of inhibitors

Effect of pyruvate and oxalate. Table 6 shows the effect of pyruvate and oxalate on the lactic dehydrogenase activity. Kinetic studies of these inhibitions (Fig. 5) indicate that pyruvate inhibits non-competitively (Lineweaver & Burk, 1934), whereas oxalate acts uncompetitively (Burk, 1944).

Effect of sulphhydryl reagents. Monoiodoacetate, *p*-chloromercuribenzoate and *o*-iodosobenzoate have strong inhibitory actions on the enzyme (Table 6). It was also found that the inhibition produced by both *p*-chloromercuribenzoate and *o*-iodosobenzoate is potentiated when they are pre-incubated with the enzyme in the presence of lactate (Table 7).

Heavy-metal ions (Hg^{2+} , Ag^+ and Cu^{2+} at 0.1 mM concentration) led to total inactivation of the enzyme.

Effect of narcotics. Urethane, Amytal and Nembutal are able to inhibit the dehydrogenase, as shown in Table 6.

Effect of flavin antagonists. Quinacrine (Atabrin), chloroquine and quinine have been described as

Fig. 5. Effect of sodium pyruvate and sodium oxalate on lactic dehydrogenase. Activity was measured at 26° by the indophenol assay with enzyme of specific activity 4.7 in the absence (○) and in the presence of 0.333 mM-sodium pyruvate (Δ) or 0.5 mM-sodium oxalate (●).

flavoprotein inhibitors (Haas, 1944; Wright & Sabine, 1944; Hellerman, Lindsay & Bovarnick, 1946). Data presented in Table 6 show that the preparations under study are sensitive to relatively low concentrations of these antimalarial drugs. These inhibitions could not be reversed by the addition of boiled juices prepared from the enzyme. Also FMN (0.1–1.0 μ M) and FAD (0.1–1.0 μ M) were ineffective. A reversion was obtained, however, on the addition of perchloric acid extracts, which were prepared as described in the Materials and Methods section. The results obtained are shown in Table 8. If the extracts were irradiated for 30 min. with ultraviolet light, their capacity for reversing the inhibition was completely lost (Table 8).

The inhibition by quinine is uncompetitive (Burk, 1944) (Fig. 6).

Table 7. Inhibition of lactic dehydrogenase by *p*-chloromercuribenzoate and *o*-iodosobenzoate, in the presence and absence of lactate

To 0.6 unit of lactic dehydrogenase (specific activity 3.5) in 0.1 ml. were added 0.8 ml. of 0.5% bovine plasma-albumin solution, the inhibitors and lactate to obtain the concentrations indicated. The final volume was made to 1 ml. in all cases by addition of water. Incubation time: 5 min.; temperature: 0°. The activity remaining at the end of incubation was determined by the indophenol assay.

Inhibitor	Concn. (mM)	Inhibition (%)	
		With 50 mM-lactate	Without lactate
<i>p</i> -Chloromercuribenzoate	0.2	33	16
<i>o</i> -Iodosobenzoate	1.0	30	5
	2.0	36	2
	5.0	54	36

Table 8. Inhibition of lactic dehydrogenase by quinacrine and quinine, and reversal by perchloric acid extracts

The activity determinations were made by the indophenol assay, the inhibitors and perchloric acid extract (see Materials and Methods section) being added to the assay reagents before enzyme addition.

Inhibitor	Concn. (mM)	Perchloric acid extract units	Inhibition (%)
Quinacrine	0.1	—	38
	0.1	0.3	23
	0.1	0.6	13
	0.1	0.3*	38
Quinine	0.2	—	36
	0.2	1.3	12
	0.4	—	41
	0.4	1.3	16

* This extract was irradiated with ultraviolet light for 30 min. (Mineralight SL-2537, Ultraviolet Products Inc., South Pasadena, Calif., U.S.A.).

Fig. 6. Effect of dicoumarol and quinine on lactic dehydrogenase. Activity was measured at 26° by indophenol assay with enzyme of specific activity 4.7 in the absence (○) and presence of 40 μ M dicoumarol (●) or 100 μ M-quinine (×).

Fig. 7. Effect of pH on the inhibition of lactic dehydrogenase by quinacrine and dicoumarol. All experiments were made with enzyme of specific activity 4.7. The data for pH 7.3 were obtained by the regular indophenol assay. For other pH values, phosphate, pH 5.9 and 6.6, and tris buffer, pH 8.0 and 8.8, were substituted for the phosphate solution employed normally in the assay, the buffer concentration being always the same. For either inhibitor the concentration was 100 μ M and percentage inhibition at a given pH was expressed by taking arbitrarily the activity in the absence of inhibitor as 100. The actual extinction changes per minute in the absence of inhibitor were: pH 5.9, 0.031; pH 6.6, 0.050; pH 7.3, 0.052; pH 8.0, 0.044; pH 8.8, 0.026. ○, Quinacrine; ●, dicoumarol.

Effect of dicoumarol and vitamin K₁. The sensitivity of the dehydrogenase to dicoumarol and vitamin K₁ is shown in Table 6. A kinetic study of the dicoumarol inhibition was carried out (Fig. 6) and the results obtained indicate that dicoumarol acts uncompetitively (Burk, 1944).

Menadione in concentrations up to 0.5 mM did not show any effect on enzyme activity, either in the presence or absence of dicoumarol. In contrast, vitamin K₁ and dicoumarol seem to interact in some manner, for it was observed that the combined inhibition produced by vitamin K₁ (0.44 mM) and dicoumarol (0.1 mM) was only 41%, whereas either of these substances alone would inhibit 31% and 48% respectively.

Effect of other substances. Data in Table 6 show that the preparations are inhibited by pentachlorophenol, thyroxine and hydrazine. Several other substances were also found to be without effect on the enzyme activity; among these are 2:6-dichlorophenolindophenol (when the concentration in the assay system was not greater than 0.1 mM), 2:4-dinitrophenol, adenine, FAD, FMN, ADP, ATP, hydroxylamine and the chelating agents mentioned under metal components above.

Effect of pH on quinacrine and dicoumarol inhibitions. Fig. 7 shows the pH dependency of the inhibition produced by quinacrine and dicoumarol.

DISCUSSION

Although much effort was spent in searching for purification methods, the best method obtained resulted in only a 15-fold enrichment in activity. The relation of the activities observed with the various electron acceptors is nearly constant throughout the purification. This might indicate that only one enzyme is necessary for the reduction of all dyes. It might also be possible that something like the 'electron-transferring flavoprotein' of Crane & Beinert (1956) is operative in *P. pentosaceum*. This hypothesis is attractive because it could explain the difficulty experienced in purifying the enzyme. No evidence for this has been obtained, however.

The enzyme is very likely independent of freely dissociable coenzymes. The evidence presented by Barker & Lipmann (1944), based on thermodynamic considerations, had already led to this conclusion, which is supported by the results presented in this paper. The enzyme is specific for D-(-)-lactate and in this respect resembles that found in *Escherichia coli* (Szulmajster, Grunberg-Manago & Delavier-Klutcho, 1953) and differs from that found in muscle (Kubowitz & Ott, 1943) and yeast (Boeri, Cutolo, Luzzati & Tosi, 1955).

The highest reaction velocity is observed with phenazine methosulphate as the acceptor, and the

following relationship was found relative to 1:2-naphthaquinone sulphonate (1.0): phenazine methosulphate, 3.6; 2:6-dichlorophenolindophenol, 1.4; methylene blue, 1.2.

The activating action of NH₄⁺ ion seems to alter the affinity of the enzyme towards lactate. Similar phenomena have been described in the literature for other enzymes (Mahler, Raw, Molinari & Amaral, 1958; Huennekens, Basford & Gabrio, 1955; Carpenter, 1951). The effect observed with the other activators might be similar to that of ethylenediaminetetra-acetic acid, i.e. they might act by withdrawing heavy metals which are deleterious to the enzyme.

All attempts to obtain evidence for the existence of flavins as prosthetic groups in this enzyme failed. The presence of flavins is by no means excluded, and the absence of a typical flavin spectrum might easily be due to interference by the presence of the haemoprotein, as shown with other enzymes (Horecker, 1950; Mahler, Sarkar, Vernon & Alberty, 1952).

The data presented indicate that the lactate dehydrogenation catalysed by the preparations studied depends on SH groups. The fact that the enzyme incubated with lactate is more sensitive to *p*-chloromercuribenzoate than when incubated with pyruvate might mean that more free SH groups are produced in the first case, and suggests that the SH groups might be involved in electron transport in this case.

The reversal of the inhibition effected by quinine and quinacrine by addition of perchloric acid extracts obtained from the purified enzyme indicates the necessity of a cofactor. The nature of the substance involved is unknown, but its character is unlikely to be inorganic since ultraviolet irradiation and boiling seem to destroy it. These results are also important because they exclude the possibility that the inhibition observed might be due to a chelating effect of either quinine or quinacrine. The possibility that the extracts do contain a flavin of constitution different from FAD or FMN is by no means eliminated.

Few soluble enzymes are known to be inhibited by dicoumarol (Wosilait & Nason, 1954; Martius, 1955; Martius & Marki, 1957; Mahler *et al.* 1958) and the preparations here studied constitute an addition to this group. Through the work performed by Martius & Nitz-Litzow (1953, 1954*a, b*) and by Wosilait & Nason (1954) it became evident that dicoumarol acts by virtue of its quinone structure. These observations, and the ones reported in this paper in connexion with the interaction between dicoumarol and vitamin K₁, make it probable that the enzymes of *P. pentosaceum* catalysing the reduction of 2:6-dichlorophenolindophenol by lactate may require a cofactor of a quinone nature.

The results obtained in the study of the pH effect on dicoumarol and quinacrine inhibition permit the formulation of a hypothesis about the binding of these substances on the enzyme surface. Thus according to the theory of Dixon (1953), and since dicoumarol is an acid, the negative slope observed in curve 1 of Fig. 7 might be interpreted as indicating a union of dicoumarol with some positive group in the enzyme. Similarly, quinacrine might be bound to a negative group.

The preparations studied resemble other known lactic dehydrogenases (Quastel & Wooldridge 1928; Etling, 1953) in being inhibited by oxalate and by narcotics; hydrazine inhibits, but isonicotinic acid hydrazide and hydroxylamine are without action.

SUMMARY

1. Preparations capable of oxidizing lactate to pyruvate in the presence of suitable hydrogen acceptors were obtained from *Propionibacterium pentosaceum*, by grinding frozen cells with alumina, followed by extraction with dilute phosphate buffer.

2. Several assay methods are described and a purification procedure is presented whereby a 15-fold concentration of the activity is obtained.

3. The requirements for half-saturation of the enzyme were found to be: D-(-)-lactate ($51.6 \mu\text{M}$), phenazine methosulphate (0.64 mM). The enzyme had infinite affinity for methylene blue, 2,6-dichlorophenolindophenol and 1:2-naphthaquinone-4-sulphonate.

4. The optimum pH for lactate oxidation was found to be 7.7 at 30° .

5. The preparations are activated by NH_4^+ , CN^- , F^- , Mg^{2+} ions and malonate and are thermostable.

6. The preparations are inhibited by pyruvate, oxalate, thiol reagents, narcotics, quinacrine, chloroquine, quinine, dicoumarol, vitamin K_1 , pentachlorophenol, thyroxine and hydrazine.

7. The inhibition of quinacrine is reversed by perchloric acid extracts obtained from the purified enzyme, but not by boiled juices prepared from it or by flavinadenine dinucleotide or flavin mononucleotide.

8. The effect of pH on the inhibition by quinacrine and dicoumarol was studied.

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