The Lactic Acid Oxidase of the Mycobacteria

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(*Received* 28 *December* 1955)

Although most of the mycobacteria can oxidize lactate, the rate of oxidation varies considerably from one species to another (see Edson, 1951). An aerobic dehydrogenase catalysing the overall reaction (1) was originally extracted from *Mycobacterium phlei* (Edson, 1947). The enzyme was specific for the oxidation of L-lactate and did not oxidize pyruvate or acetate.

$$CH_3.CH(OH).CO_2H + O_2 = CH_3.CO_2H + CO_2 + H_2O. \quad (1)$$

Anaerobically, with methylene blue as hydrogen acceptor, the crude enzyme preparation oxidized lactate to pyruvate. Since partially purified enzyme preparations contained flavin-adenine dinucleotide (FAD), it seemed that the enzyme might be a flavoprotein (F) operating as follows:

$$CH_3.CH(OH).CO_2H + F = CH_3.CO.CO_2H + FH_2, \quad (2)$$

$$FH_2 + O_2 = F + H_2O_2, \quad (3)$$

$$CH_3.CO.CO_2H + H_2O_2 = CH_3.CO_2H + CO_2 + H_2O \quad (4)$$
(non-enzymic).

Edson (1947, 1951), however, pointed out that further purification would be needed before this hypothesis could be tested satisfactorily. A later report showed that the purified enzyme was not a typical flavoprotein (Edson & Cousins, 1953).

Since lactate-oxidizing enzymes resembling the enzyme of *Myco. phlei* have been extracted from *Mycobacterium smegmatis*, *Mycobacterium tuberculosis* var. *hominis*, *Mycobacterium tuberculosis* var. *bovis* (strain BCG) and *Mycobacterium avium* (Geronimus, Gray & Birkeland, 1949; Yamamura, Kusunose & Kusunose, 1952; Andrejew, 1954), it is likely that the enzyme is a constituent of all mycobacteria. Its presence in other organisms has not been reported, but a lactic oxidase extracted from *Lactobacillus delbruckii* is known to require FAD and free riboflavin as cofactors (Hager, Geller & Lipmann, 1954).

The purpose of the work described in this paper was to study the properties of the purified enzyme. Of the three species examined *Myco. smegmatis* proved to be the richest and most convenient source of the enzyme. After this work was completed Sutton (1954) described an extensive purification of the enzyme extracted from *Myco. phlei*.

MATERIALS AND METHODS

Organisms. Myco. smegmatis (no. 523), *Myco. phlei* (no. 525) and *Myco. stercoris* (no. 3820) were obtained from the National Collection of Type Cultures, now at the Central Public Health Laboratory, Colindale Avenue, London, N.W. 9. They were grown on beef-infusion broth containing 5% (v/v) of glycerol at 38°, harvested on the fifth day of incubation and washed with water either by centrifuging four times or by filtering on a Büchner funnel.

Cell-free extracts. Myco. phlei. A dry powder was prepared by desiccation *in vacuo* after repeated extraction with cold acetone (about 0°). Approx. 9 g. of powder was mixed into a smooth paste with 40 ml. of 0·05 M phosphate buffer, pH 6·5, and crushed for 3 hr. in the bacterial mill (Booth & Green, 1938). The mill was flushed out with the same volume of buffer and the washings were added to the extract. The gross cellular debris was removed by centrifuging in the cold room for 1 hr. at 4500 *g*, and further clarification achieved by centrifuging at 18000 *g* for 15 min. The debris could be re-extracted profitably with a further 40 ml. of buffer, which was clarified in the same way. The total yield was 90–100 ml. of a deep-yellow solution free from particulate matter but showing a pronounced Tyndall effect.

Myco. stercoris was disrupted by shaking 4 g. (wet wt.) of bacteria, 4 g. of ballotini glass beads (no. 12) and 1 ml. of 0·1 M phosphate buffer, pH 7·4, in a vibrator (H. Mickle, Hampton, Middlesex) operating for 30 min. in the cold room. The creamy mass was diluted with an equal volume of water and solid debris removed by centrifuging at 4500 *g* for 0·5 hr. A yellow opalescent solution was obtained.

Myco. smegmatis (10 g. wet wt. of bacteria suspended in sufficient 0·05 M phosphate buffer, pH 7·0, to give a total volume of 25 ml.) was disintegrated in a Raytheon 9 kcyc./sec. sonic oscillator for 30 min. The temperature of the suspension was kept between 0° and 1° by placing the apparatus in the cold room and circulating ice-water through the cup assembly at the rate of 1800 ml./min. The resulting suspension was diluted with an equal volume of water and clarified by centrifuging at 18000 *g* for 15 min. at 0°. The clear, orange-coloured supernatant showed a strong Tyndall effect.

Materials. L-Lactic acid was prepared from a commercial zinc salt (Pfanstiehl Chemical Co., U.S.A.) and D-lactic acid (α_D^{20} Zn salt, +8·0°) was obtained by resolution of DL-lactic acid according to Irvine (1906). The acids were used as sodium salts, prepared by removal of the zinc with sodium carbonate. Flavin-adenine dinucleotide (FAD) was obtained from the Sigma Chemical Co., St Louis, U.S.A. The purity was assayed at 15% by the makers. Flavin mononucleotide (FMN) was obtained from L. Light and Co., England, and assayed as 87% pure by measurement of the light-absorption at 450 mμ., with $\epsilon = 12{\cdot}2 \times 10^3$ l.mole.$^{-1}$ cm.$^{-1}$ (Whitby, 1953). *iso*Riboflavin was a gift from Dr T. P. Singer.

Diphosphopyridine nucleotide (DPN) was the chromatographically purified product of the Sigma Chemical Co. Peroxidase was prepared by the method of Elliott (1932).

Analytical methods

Oxygen consumption was measured in Warburg manometers containing KOH in the inseal. Lactic acid was determined by the method of Friedemann & Graeser (1933); in the recovery of lactic acid from manometric experiments the procedure of Edson & Hunter (1947) was used. Nitrogen was determined by the titrimetric micro-Kjeldahl method of Hiller, Plazin & Van Slyke (1949). pH was measured with a glass electrode. Spectrophotometric measurements were made with a Beckman instrument, DU model.

Electrophoretic analysis of enzyme solutions was performed in a Tiselius-type apparatus (Adam Hilger, Ltd., London) equipped with a diagonal knife edge and Philpot optical system.

Enzyme activity. This was assayed by measuring O_2 uptake in the presence of excess of L-lactate (566 μmoles of DL-lactate) at pH 5·2 in a medium containing 0·04 M acetate buffer. The enzyme dilution was arranged to give an O_2 uptake of 300–700 μl. of O_2/hr. The rate of O_2 uptake was constant over the period of observation (30–60 min.).

The activity is expressed as q_{O_2} = μl. of O_2/mg. of N/hr.

RESULTS

Oxidation of lactate by extracts of mycobacteria

The experiments of Table 1 show that dialysed cell-free extracts of mycobacteria oxidize L-lactate in essentially the same way as an acetone-dried powder of *Myco. phlei* (Edson, 1947). The ratio, O_2 consumption : L-lactate disappearance, is consistent with equation (1). The reactions with extracts of *Myco. smegmatis* and *Myco. stercoris* were carried out in acetate buffer at the optimum pH (see below) and in phosphate buffer, pH 7·4, in which the reactions were slower (cf. *Myco. phlei*, Table 1) but the stoicheiometry was unaltered. On completion of manometric experiments in phosphate buffer a steam-volatile acid, identified as acetic acid by the lanthanum–iodine reaction (Edson & Hunter, 1947), was isolated from the products of each extract.

There was no significant increase of O_2 uptake in the presence of acetate, pyruvate, L-malate or α-hydroxy*iso*butyrate. It is likely that the small O_2 uptake observed in the presence of D-lactate is due to a trace impurity of the L-isomer.

Under anaerobic conditions in Thunberg tubes the extract of each organism reduced methylene blue on addition of DL-lactate, yielding pyruvate, which was detected by the nitroprusside reaction (Simon & Piaux, 1924) after deproteinization with trichloroacetic acid and removal of methylene blue with activated charcoal.

These results indicate that the same type of enzyme is present in the three species of mycobacteria.

Purification of the enzyme

Myco. smegmatis. Batches (200–300 ml., representing 100–120 g. wet wt. of bacteria) of the crude extract were dialysed to remove most of the phosphate buffer, and the pH was adjusted to 4·5 by addition of 0·33 vol. of 0·25 M acetate buffer, pH 4·5. The mixture was allowed to stand for 0·5 hr. before a heavy precipitate was removed by centrifuging. The supernatant (fraction I) was cooled to 0° and cold ethanol (−20°) added cautiously to give 40% (v/v) concentration. At the same time the temperature of the mixture was lowered to at least −7° and, after standing, the precipitate was removed by centrifuging at −7°.

Table 1. *Oxygen consumption and lactate disappearance in extracts of mycobacteria*

Warburg flasks contained 1·0 ml. of dialysed bacterial extract, 0·5 ml. of 0·2 M acetate buffer, pH 5·2 (*Myco. smegmatis* and *Myco. stercoris*) or 0·8 ml. of 0·09 M phosphate buffer, pH 7·4 (*Myco. phlei*), and 0·5 ml. of lactate solution in the side bulb. The inseal contained 0·2 ml. of 10% KOH. Gas, air. Temp., 38°. Time: 90 min. for *Myco. smegmatis* and *Myco. stercoris* and 180 min. for *Myco. phlei*.

Organism	Substrate	Lactate		O_2 uptake (μmoles)	Extra O_2 uptake (μmoles)	μmoles of O_2 used/ μmole of lactate disappearing
		Added (μmoles)	Utilized (μmoles)			
Myco. smegmatis	Nil	—	—	0·0	0·0	—
	D-Lactate	64·8	1·1	0·7	0·7	—
	L-Lactate	82·8	52·7	49·0	49·0	0·93
	DL-Lactate	103·8	25·0	23·8	23·8	0·95
Myco. stercoris	Nil	—	—	0·8	—	—
	D-Lactate	64·8	—	1·6	0·8	—
	L-Lactate	82·8	58·0	54·0	53·1	0·92
	DL-Lactate	103·8	37·3	41·1	40·3	1·08
Myco. phlei	Nil	—	—	2·4	—	—
	D-Lactate	53·0	0·1	3·1	0·7	—
	L-Lactate	45·0	16·6	18·1	15·7	0·95
	DL-Lactate	102·2	17·8	20·6	18·2	1·02

The pale-yellow precipitate was extracted with 0·1 M acetate buffer, pH 5·2 (approximately equal to 0·1 vol. of the original extract) and the insoluble residue discarded. The solution (fraction II) was dialysed against water for 3–4 hr. to remove traces of ethanol and then heated at 55° for 5 min., the denatured protein being removed. The pale-yellow supernatant (fraction III) was dialysed at 2° against water. After 24 hr. most of the enzyme had precipitated, but sometimes it was necessary to leave the solution at 0° for a further 12 hr. to effect complete precipitation. The precipitate was extracted with 10–20 ml. of 0·05 M acetate buffer, pH 5·2, and any insoluble residue discarded. The supernatant is fraction IV. Repetition of dialysis and extraction of the resulting precipitate gave a pale-yellow solution (fraction V) with high lactate-oxidizing activity (q_{O_2}, approx. 200 000) which represents more than 100-fold purification of the crude extract. Preparations of this activity were used in the subsequent work on properties unless stated otherwise. The loss of enzyme during the procedure was 40–50 %.

Further purification was achieved towards the concluding stages of this investigation. The enzyme solution (fraction IV) was subjected to dialysis against 80 vol. of 0·1 M acetate buffer, pH 4·5, for a period not exceeding 24 hr. The precipitated enzyme was redissolved in 0·05 M acetate buffer, pH 5·2 (fraction VI). More prolonged dialysis caused inactivation.

The activity of the enzyme at various stages of purification is given in Table 2.

Myco. phlei. Crude extracts were purified by essentially the same method as far as fraction III. At this stage some inactive protein was removed by adsorption on alumina C_γ gel in a mixture buffered to pH 6·0 with 0·04 M phosphate buffer, and the purification was not carried further. The enzyme solution was pale yellow. *Myco. phlei* was abandoned in favour of *Myco. smegmatis* because extracts of the latter offered better yields and contained less extraneous yellow pigment.

Electrophoretic behaviour of the purified enzyme

About 50 mg. of enzyme (q_{O_2}, 200 000), corresponding to an extract of 800–900 g. of *Myco. smegmatis*, were required for a single run in the Tiselius apparatus. The ascending and descending patterns in phosphate buffer, pH 5·9, showed the presence of at least three components (Fig. 1). The small, slowly moving component (*C*) was isolated and found to be a colourless, inactive protein. The yellow pigment and the remaining protein moved with the leading peaks (*A* and *B*) whose mobilities were too similar to permit electrophoretic isolation. Component *B* contained 51 % of the total protein.

The component containing the enzyme was located by means of filter-paper electrophoresis. The enzyme solution (fraction V) was applied to strips (width 1 in.) of Whatman no. 1 paper and developed for 8 hr. with phosphate buffer, ionic strength 0·2, pH 5·9, and a current of 10 mA. Stained by the method of Grassmann & Hannig (1952), the same three protein components were sharply demarcated and well separated. A duplicate unstained strip showed that the yellow pigment was associated with component *B*. Strips (1 cm. long) were cut from the duplicate in positions corresponding to the protein bands and transferred to 0·05 M acetate buffer, pH 5·2, in Warburg manometers. In this manner it was found that the enzymic activity was contained solely in component *B*.

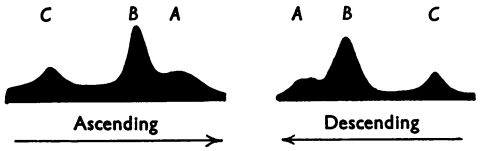

Fig. 1. Electrophoresis of a purified lactic oxidase preparation (fraction V, q_{O_2} = 200 000) from *Myco. smegmatis* in phosphate buffer, I = 0·2, pH 5·9. Protein concentration approximately 1 %. Time, 210 min.

Table 2. *Activities of fractions obtained in the purification of lactic oxidase* (Myco. smegmatis)

Warburg flasks contained 1·5 ml. of a suitable dilution of enzyme preparation, 0·5 ml. of 0·2 M acetate buffer, pH 5·2, and 0·5 ml. of DL-lactate solution (556 μmoles) in the side bulb. The inseal contained 0·2 ml. of 10 % KOH. Gas, O_2. Temp., 38°. Time, 1 hr. q_{O_2} = μl. of O_2/mg. of N/hr.

Preparation	O_2 uptake (μl.)	Nitrogen (μg./ml.)	Nitrogen (μg./flask)	Activity (q_{O_2})
Unfractionated dialysed extracts	274–446	1050–1200	210–240	1300–1900
Fraction II	677	360	36	18 800
Fraction III	704	560	22	32 000
Fraction IV	400	84	3·3	121 000
Fraction V	308	79	1·6	192 000
Fraction VI	331	56	1·1	301 000

* In the absence of lactate the O_2 uptake of the purified preparations was negligible.

The most active preparation of the lactic acid oxidase made from *Myco. phlei* showed three similar peaks and the same distribution of pigment when subjected to electrophoresis in the Tiselius apparatus.

Absorption spectrum of the enzyme

The original suggestion that the lactic oxidase of the mycobacteria might be an autoxidizable flavoprotein containing FAD was not substantiated when the first purified preparations were made from *Myco. phlei* and *Myco. smegmatis* (Cousins, 1951; Edson & Cousins, 1953). The absorption spectra were different from those of typical flavoproteins.

During the course of purification of the enzyme from *Myco. smegmatis* it was noticed that absorption at 403 mμ. increased relatively to absorption at 276 mμ. Fig. 2 shows the absorption spectrum of fraction V. The band with the maximum at 403 mμ. has a small inflexion at 375–380 mμ., and $E_{403\ m\mu.}/E_{276\ m\mu.}$ is 0·14–0·17. An intensification of colour, which occurs with increased pH, is accompanied by increased absorption at 403 mμ. and a general widening of the band.

The increase in absorption at 403 mμ. during purification suggested that a pigment with an absorption band in this region might be connected with lactic oxidase activity. This hypothesis was discounted when fraction VI (q_{O_2}, 300 000) became available. Fraction VI, dissolved in phosphate buffer, pH 7·1, gave a faintly yellow solution with the absorption spectrum shown in Fig. 3. The absorption at 403 mμ. has decreased relatively to that at 276 mμ. in spite of considerable increase in enzymic activity. There was no significant absorption at wavelengths higher than 490 mμ. The soluble chromoprotein remaining in solution after dialysis at pH 4·5 was faintly yellow and had negligible enzymic activity, although the quotient $E_{403\ m\mu.}/E_{276\ m\mu.}$ had increased to 0·33.

After removal of the inactive chromoprotein from fraction V, absorption at 380 and 450 mμ. is more apparent (Fig. 3). The quotients $E_{275\ m\mu.}/E_{450\ m\mu.}$ and $E_{375\ m\mu.}/E_{450\ m\mu.}$ are 15·6 and 1·7 respectively (fraction VI). These values are much higher than the corresponding quotients for purified flavoproteins, e.g. 10·5 and 1·0 for L-amino acid oxidase (Singer & Kearney, 1950*a*, *b*).

The FAD content of enzyme solutions was assayed manometrically at several stages of purification (Table 3) by the D-amino acid oxidase test of Warburg & Christian (1938), with commercial FAD as a standard. Significant amounts of combined FAD are present in the crude extract and the concentration increases in fraction II; but the apparent absence of FAD from fraction VI cannot be regarded as conclusive evidence of its total absence, since the amount present after denaturation of the protein by brief heating at 100° and pH 5·2 may have been below the limit of sensitivity of the assay.

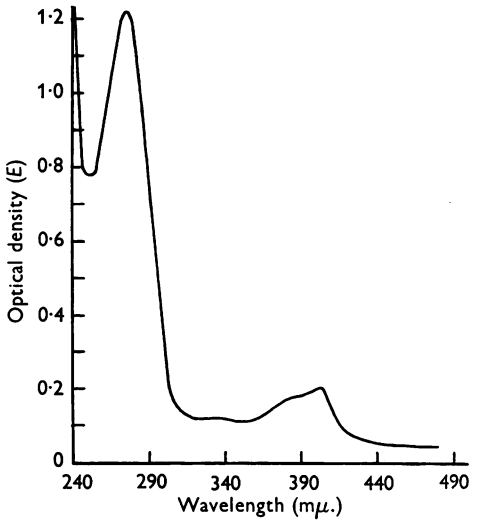

Fig. 2. Absorption spectrum of a partially purified preparation (fraction V, $q_{O_2}=200\,000$) of the lactic oxidase (*Myco. smegmatis*) in 0·05 M acetate buffer, pH 5·2.

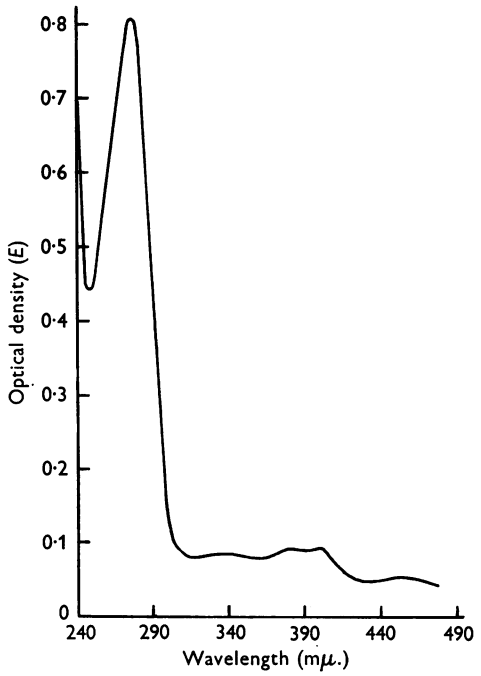

Fig. 3. Absorption spectrum of a purified preparation (fraction VI, $q_{O_2}=301\,000$) of the lactic oxidase (*Myco. smegmatis*) in phosphate buffer, pH 7·1.

Some properties of the enzyme

The enzyme solution can be stored in the frozen state for 3 months without loss of activity. The enzyme is relatively stable to heating for 5 min. at 50°, but above 65° activity is destroyed rapidly.

Optimum pH. Fig. 4 shows the variation of activity over the pH range 3·6–8·0. The enzyme is stable and highly active over a wide pH range but shows a peak of activity at pH 5·2–5·3. It may be significant that electrophoretic observations indicated that the isoelectric point of the enzyme is near pH 5. At pH 2·8 and 10·1 (glycine buffers) the enzyme is quite inactive.

The pH–activity curves of the purified enzyme from *Myco. phlei* and of the extracts of *Myco. stercoris* were almost identical in shape when determined under the same conditions.

Effects of inhibitors. The rate of oxidation of lactate in acetate buffer at pH 5·2 was not affected by 0·01 M arsenite, fluoride, hydroxylamine, urethane, azide or *iso*nicotinic acid hydrazide; 0·01 M iodoacetate, 0·001 M-HCN and octyl alcohol (saturated) produced inhibitions of 9, 5 and 6 %, respectively.

An unexpected inhibition was brought about by the addition of flavins. Table 4 shows that FMN reduces O_2 uptake by 24 % and FAD by 18 % in the course of 1 hr. The inhibition by FAD is progressive, the rate of O_2 uptake at the end of 1 hr. being less than 50 % of the original rate. Although the significance of inhibition by an impure preparation of FAD is doubtful, a similar inhibition was noticed with other preparations known to contain FAD (heat-inactivated bacterial extract or a neutralized HCl-extract made from acetone-dried *Myco. smegmatis*). The addition of 50 μg. of riboflavin or of *iso*riboflavin did not affect O_2 consumption.

Anaerobic oxidation of lactate. Cell-free extracts and acetone-dried powders made from *Myco. phlei* reduce methylene blue anaerobically in the presence of lactate, forming pyruvate (Edson, 1947). It was found that crude extracts of *Myco. smegmatis* also give pyruvate under these conditions, but purified preparations of the lactic acid oxidase do not reduce methylene blue, or do so relatively slowly at high enzyme concentration.

The ability of the enzyme preparations to reduce methylene blue in presence of lactate is inversely related to the degree of purification, suggesting progressive removal of some non-diffusible hydrogen carrier. Since this carrier can be partially replaced by free flavins, it may be a flavoprotein (Table 5). Flavoprotein is removed during purification, especially in the precipitate at pH 4·5.

There is no change in the colour or absorption spectrum of the purified enzyme when it is incubated anaerobically with 550 μmoles of DL-lactate

Table 3. *FAD content of enzyme preparations of* Myco. smegmatis

FAD was measured by the manometric assay of Warburg & Christian (1938) with commercial FAD as a standard.

Preparation	Nitrogen (mg./ml.)	FAD (μg./ml.)	μg. of FAD/mg. of N
Crude extract	1·58	4·5	2·8
Fraction II	0·13	1·6	12·3
Fraction IV	0·56	3·7	6·6
Fraction VI	0·09	0·0	—

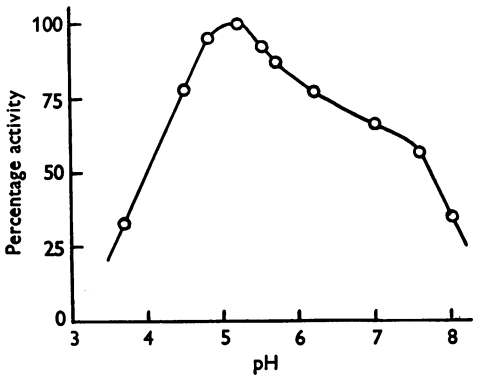

Fig. 4. Optimum pH of the purified lactic oxidase (fraction V) from *Myco. smegmatis*. The activities were determined by measurement of O_2 uptake and expressed as a percentage of the activity at pH 5·2. Warburg flasks contained 2 ml. of buffered enzyme solution and 0·5 ml. of DL-lactate solution (125 μmoles). The inseal contained 0·2 ml. of 10 % KOH. Gas, air. Temp. 38°. The following buffers (final concn. 0·05 M) were used: glycine–HCl, pH 3·6; acetate, pH 4·3–5·5; phosphate (Sörensen), pH 5·7–8·0.

Table 4. *Effect of flavins on the lactic oxidase of* Myco. smegmatis

Warburg flasks contained 1·0 ml. of enzyme solution (fraction V) buffered with 0·1 M acetate buffer, pH 5·1, 0·5 ml. of DL-lactate solution (556 μmoles) in the side bulb and 0·5 ml. of water (or flavin solution). The inseal contained 0·2 ml. of 10 % KOH. Gas, air. Temp. 38°.

Time after mixing (min.)	O_2 uptake (μl.)		
	Flavin absent	FMN*	FAD†
10	65	55	70
20	134	108	137
30	200	161	195
40	265	209	244
50	327	255	286
60	390	297	318

* Amounts of 50 μg. per flask of flavin mononucleotide (FMN) added.

† Amounts of 30 μg. per flask of flavin-adenine dinucleotide (FAD).

Table 5. *Anaerobic reduction of methylene blue by bacterial extracts and enzyme fractions* (Myco. smegmatis) *in the presence of lactate*

Evacuated Thunberg tubes contained enzyme solution (sufficient to give a manometric O_2 uptake of 3800 μl./hr.), 0·5 ml. of 0·2M acetate buffer, pH 5·2, 0·5 ml. of 1:5000 methylene blue solution and other additions with water to make 2·5 ml. Temp., 38°. DL-Lactate added, 556 μmoles

Preparation	Additions	Reduction time (min.)
Bacterial extract (dialysed)	Nil	>420
	Lactate	6
Enzyme fraction II	Nil	>420
	Lactate	210
Enzyme fraction V	Nil	>420
	FMN (25 μg.)	>420
	FAD (25 μg.)	>420
	Lactate	230
	Lactate + FMN (25 μg.)	85
	Lactate + FAD (25 μg.)	91

in 0·04M acetate-buffered medium at pH 5·2. In this test the cuvette containing the solution, and a control in which the lactate solution was replaced by an equal volume of water, were gassed with N_2 and the lids sealed before incubation for 30 min. at 38°.

Inactivation of the enzyme and attempted reactivation. Prolonged dialysis (3 days at 2°) against frequent changes of water produced no change in colour and little loss of activity, and electrodialysis against 0·05M acetate buffer, pH 4·5, or 0·05M phosphate buffer, pH 7·5, had no effect; but dialysis against 0·02N-HCl or 0·1M acetate buffer, pH 3·5, or against alkaline buffers (0·05M secondary phosphate or 0·1M glycine buffer, pH 8·9) resulted in complete or partial inactivation depending on the time of dialysis. When the enzyme was treated with 0·1N-HCl at 0° the protein was precipitated and yellow pigment released. Treatment with $(NH_4)_2SO_4$ between pH 1·5 and 3·0 had a similar effect. It appeared that any treatment which caused separation of the pigment resulted in loss of activity.

Attempts to restore activity to the decolorized protein by addition of glutathione, methylene blue, DPN, FAD, FMN and the yellow pigment obtainable from enzyme preparations were unsuccessful. Bacterial extract or purified enzyme inactivated by heat (100° for 5 min.) had no effect. The addition of FAD (25 μg./ml.) in combination with the following ions also gave negative results: Fe^{3+}, Mg^{2+}, Mn^{2+}, Zn^{2+}, Cu^{2+}, Co^{2+}, MoO_4^{2-}.

Mechanism of reaction

The mechanism originally proposed for the oxidation of lactate and illustrated by equations (2)–(4) involves the formation of H_2O_2. Yamamura *et al.* (1952), who studied the lactic oxidase of *Myco. avium*, considered that H_2O_2 could not be involved because the enzyme was not inhibited by catalase and did not promote the coupled oxidation of ethanol in the presence of catalase. Sutton (1954), working with a purified lactic oxidase from *Myco. phlei*, came to the same conclusion.

In the present work with a purified lactic oxidase (q_{O_2} = 200 000) from *Myco. smegmatis*, it was also found that catalase was not inhibitory and that coupled oxidation of ethanol or *p*-phenylenediamine did not occur in the presence of catalase. Although this may be strong evidence against the participation of H_2O_2 in the reaction, it is not conclusive in the absence of positive evidence for a different mechanism. With horse-radish peroxidase and *p*-aminobenzoic acid as an indicator system, Edson & Cousins (1953) detected traces of H_2O_2 during the oxidation of lactate by the purified enzyme.

A similar experiment based on a method of Kenten & Mann (1952) gave a measurement of the H_2O_2 reacting with peroxidase. The experiment and its controls are shown in Table 6. On completion of the manometric measurement of O_2 uptake the Warburg flasks were cooled in ice. Saturated hydrazine sulphate solution (0·2 ml.) was added to the side bulb, the manometers were reassembled and gassed with N_2, and the hydrazine was added after equilibration. The evolution of N_2 was complete in 10 min. and amounted to 27 μl. (equivalent to 1·2 μmoles of H_2O_2) in the flask containing the complete system (no. 4). There was no evolution of N_2 in the other flasks.

Pyruvate was also determined. The contents and washings from a duplicate set of flasks were deproteinized with 6 ml. of 10 % (w/v) trichloroacetic acid, bringing the total volume from each flask to 11 ml. After filtration, 3 ml. portions were used for pyruvate estimation by the method of Elgart & Nelson (1941). The results (Table 6) show that small amounts of pyruvate were present in all the flasks in which oxidation of lactate occurred but the

Table 6. *Measurement of hydrogen peroxide during lactate oxidation by the lactic oxidase of* Myco. smegmatis

Warburg flasks contained 0·4 ml. of enzyme solution (fraction V), 0·8 ml. of 0·2M pyrophosphate buffer, pH 7·1, various additions and water to give 2·8 ml. in each. The inseal contained 0·2 ml. of 10% KOH. Gas, air. Temp., 38°.

Additions	Flask no. 1	2	3	4	5	6	7*	8
DL-Lactate, 0·2M (ml.)	0·4	0·4	0·4	0·4	0·4	—	0·4	0·4
Peroxidase solution (3 mg. of protein/ml.) (ml.)	—	0·5	—	0·5	—	0·5	0·5	—
p-Cresol, 0·001M (ml.)	—	—	0·3	0·3	—	0·3	0·3	0·3
$MnSO_4$, 0·1M (ml.)	—	—	—	0·2	0·2	0·2	0·2	0·2
Pyruvate, 0·2M (ml.)	—	—	—	—	—	0·4	—	—
O_2 uptake (μl./220 min.)	564	552	560	670	661	7	5	628
Pyruvate formed (μmoles)	0·3	0·7	—	0·9	0·4	—	0·0	—

* In flask no. 7 the enzyme solution had been previously inactivated by heating.

amount was greatest in the complete system. Traces of pyruvate can usually also be detected by the nitroprusside test after the oxidation of large quantities of lactate without any further additions to the enzyme preparation.

The complete experiment shows that 1·2 μmoles of H_2O_2 and 0·9 μmole of pyruvate were detected during the oxidation of about 30 μmoles of L-lactate.

DISCUSSION

It has been shown that extracts made from three species of mycobacteria contain an enzyme which catalyses the aerobic oxidation of L-lactate according to equation (1). The best preparation of this lactic oxidase, made from *Myco. smegmatis*, probably represents a purification of about 150-fold. The assay, based on the nitrogen content of dialysed and precipitated materials, assumes that the non-protein nitrogen is small in proportion to the total nitrogen and that the reaction velocity bears a linear relationship to enzyme concentration.

Prosthetic group. The original work on the enzyme of *Myco. phlei* indicated that FAD might be the prosthetic group. Since the reaction is an oxidative decarboxylation, Preisler & Hunter (1949) suggested that the lactic oxidase might be analogous to the 'malic enzyme' of Ochoa, Mehler & Kornberg (1948). Further investigation threw doubts on the flavin nature of the lactic oxidase but indicated that it did not need pyridine nucleotides or diphosphothiamine (Cousins, 1951). On the other hand, Yamamura *et al.* (1952), who extracted a similar enzyme, 'lactic oxidase I', from *Myco. avium*, considered that FAD was the prosthetic group. In a study of the lactic oxidase of *Myco. phlei*, Sutton (1954) found that the specific activity ratios for O_2 consumption and CO_2 formation are the same throughout a procedure which achieves at least 30-fold purification. He concluded that oxidation and decarboxylation of lactate are associated with a single protein unit. Sutton (1954) found no spectroscopic evidence for the presence of FAD in the purified enzyme which was not inhibited by quinacrine or chloroquine.

Purification of lactic oxidase of *Myco. smegmatis* as far as fraction V (Table 2) gives a pale-yellow solution, the absorption spectrum of which is not that of a typical flavoprotein (Fig. 2). A modification of the procedure led to the separation of two pale-yellow proteins with different absorption spectra. One had a distinct maximum at 403 mμ. and was almost devoid of lactate oxidizing ability; the other (fraction VI), showing the spectrum of Fig. 3, was the most active preparation obtained. The spectrum of fraction V is clearly due to the presence of these two proteins; and the low maximum at 403 mμ. in fraction VI indicates that the preparation is not entirely free from the inactive chromoprotein.

Although the removal of the inactive protein made some absorption at 380 and 450 mμ. more apparent (Fig. 3), the quotients $E_{275\ m\mu.}/E_{450\ m\mu.}$ and $E_{375\ m\mu.}/E_{450\ m\mu.}$ are not those that would be expected in a purified flavoprotein. The ratios are of the same order as those obtained by Sutton (1954) for the purified enzyme of *Myco. phlei*. Moreover, FAD could not be detected in fraction VI (Table 3) and the enzyme was partially inhibited by addition of FMN or FAD (Table 4). From the high activity of fraction VI it is concluded that FAD is not the prosthetic group although the possibility of some other flavin being present is not excluded. The possible role of a metallo-flavin prosthetic group has not been explored.

Mechanism of reaction. Catalase failed to inhibit the oxidation of lactate and to promote coupled oxidation of ethanol in the presence of the purified enzyme. This is presumptive evidence against the participation of hydrogen peroxide according to equations (3) and (4), in spite of the formation of pyruvate anaerobically. Yamamura *et al.* (1952), who made similar observations with preparations

from *Myco. avium*, attributed the formation of pyruvate under anaerobic conditions to a DPN-linked enzyme, 'lactic oxidase II', and considered that hydrogen peroxide and pyruvate were not intermediates in the oxidation catalysed by 'lactic oxidase I'. Other observations opposed to the mechanism of equations (3) and (4) are reported in this paper and by Sutton (1954). Hydroxylamine and other keto acid fixatives and pyruvate do not inhibit the enzyme.

It has been shown, however, that small quantities of hydrogen peroxide and pyruvate are detectable during the course of lactate oxidation (Table 6). This might be explained if hydrogen peroxide formed in the main reaction interacts preferentially with pyruvate even when catalase or peroxidase is added. This could occur on the enzyme surface where close spatial relationships might permit the postulated intermediates to react rapidly in the direction of acetate formation. In this event the intermediates would not be freely diffused in solution. If the area of enzymic activity were relatively inaccessible to ox-liver catalase or horse-radish peroxidase (especially to the former, which is the larger molecule), the formation of hydrogen peroxide and pyruvate would not be easily detected. On the other hand, it is possible that the oxidative decarboxylation is a reaction of unknown type and that the hydrogen peroxide and pyruvate detected are formed in a reaction of secondary importance.

Dialysed extracts of *Myco. smegmatis* reduce methylene blue readily in the presence of lactate, although the purified enzyme does not. Yamamura *et al.* (1952) attribute these observations to the presence of 'lactic oxidase II' in unfractionated extracts. Since the oxygen consumption of unfractionated dialysed preparations is not increased by addition of DPN and methylene blue (the test applied by Yamamura *et al.*), the 'lactic oxidase II' does not seem to be present in *Myco. smegmatis*. Moreover, flavoprotein is removed during purification, and the ability to reduce methylene blue is partly restored by addition of free flavins (Table 4). These facts suggest that loss of ability to reduce methylene blue is due to removal of a flavoprotein mediating hydrogen and electron transfer to the dye.

The related problems of the prosthetic group and the mechanism of reaction are unlikely to be solved without purer enzyme preparations. The first obstacle is the difficulty of cultivating a sufficient quantity of bacteria under ordinary laboratory conditions.

SUMMARY

1. Extracts of *Mycobacterium smegmatis*, *Myco. stercoris* and *Myco. phlei* contain similar lactic oxidases which catalyse the oxidation of L-lactate to acetate, carbon dioxide and water in the presence of molecular oxygen.

2. The enzyme of *Myco. smegmatis* has been purified to a considerable degree. A purified fraction (q_{O_2}, 200 000) has been resolved into two yellow proteins, one without activity towards lactate and the other with enhanced activity (q_{O_2}, 300 000).

3. The properties of the purified enzyme have been studied, and the nature of the prosthetic group and the reaction mechanism are discussed.

The author wishes to express his indebtedness to Professor N. L. Edson for his interest and helpful advice throughout this work, and to the W. H. Travis Trustees for financial assistance.

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