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Crystallization and Properties of L-Lactate Oxidase from *Mycobacterium smegmatis*

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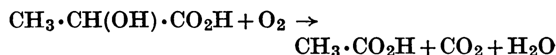
1. An original method was devised for purifying and crystallizing L-lactate oxidase from *Mycobacterium smegmatis*. 2. The crystalline enzyme exhibited a single protein component ($S^0_{20,w}$ 14·7 S) in the ultracentrifuge and also on electrophoresis on cellulose acetate strips. 3. The enzyme has a typical flavoprotein spectrum, and it was confirmed that the prosthetic group is FMN. 4. Preliminary studies indicated that the molecular weight is in the range 300000–400000. Since the minimum molecular weight was found to be 50000 ± 3000, it was concluded that L-lactate oxidase contains 6–8 moles of flavine/mole. 5. Other properties of the enzyme reported include the substrate specificity, an apparent K_m for L-lactate, the effect of several inhibitors on the enzyme activity and the pH–activity curve.

L-Lactate oxidase (decarboxylating) (EC 1.1.3.2) is a constitutive enzyme of the mycobacteria. It catalyses the oxidative decarboxylation of L-lactate to acetate (Edson, 1947; Sutton, 1954; Cousins, 1956):



$$CH_3{\cdot}CH(OH){\cdot}CO_2H + O_2 \rightarrow CH_3{\cdot}CO_2H + CO_2 + H_2O$$

Hayaishi & Sutton (1957) showed that lactate oxidase is a mono-oxygenase, the substrate serving as an internal electron donor.

Since the lactate oxidase of *Mycobacterium phlei* was insensitive to cyanide and arsenite and since FAD was detected in preparations of the enzyme, it was suggested that lactate oxidase might be an autoxidizable flavoprotein (Edson, 1947). Yamamura, Kusunose & Kusunose (1952) obtained preparations of lactate oxidase from *Mycobacterium avium* in which FAD was detected, and it was concluded that the enzyme was a flavoprotein.

Cousins (1956) purified lactate oxidase 200-fold from extracts of *Mycobacterium smegmatis*, but the nature of the prosthetic group was not resolved. The prosthetic group of lactate oxidase from *M. phlei* was eventually characterized as FMN (Sutton, 1955). Lactate oxidase was subsequently obtained in crystalline form and judged to be essentially homogeneous by ultracentrifuge and electrophoretic tests (Sutton, 1957). The molecular weight was reported as 260000, and it was estimated that lactate oxidase contained 2 moles of FMN/mole. Neither the specific activity nor the absorption spectrum of the crystalline enzyme was reported.

Since the currently accepted description of lactate oxidase is based on uncertain evidence, it was decided to improve the method for purifying lactate oxidase from *M. smegmatis* and to carry out some studies on the properties of the enzyme.

After this work was completed a new method for purifying lactate oxidase from *M. phlei* was reported together with a re-evaluation of the properties of the enzyme (Takemori, Nakazawa, Nakai, Suzuki & Katagiri, 1968). The properties of the crystalline enzyme from *M. phlei* are quite similar to the properties of the enzyme from *M. smegmatis* described in the present paper.

MATERIALS AND METHODS

Lactic acid. A.R. DL-lactic acid (British Drug Houses Ltd., Poole, Dorset) was diluted, neutralized with NaOH and heated to 80° to decompose any lactides present. The solution was adjusted to pH 5·1 and diluted to 2·0 M.

L-Lactic acid was obtained from the Sigma Chemical Co., St Louis, Mo., U.S.A. Solutions were prepared as described above, adjusted to pH 5·7 and assayed with NAD-linked L-lactate dehydrogenase (C. F. Boehringer und Soehne G.m.b.H., Mannheim, Germany) by the method of Hohorst (1963).

Pyruvic acid. Pyruvic acid, purified by vacuum distillation, was stored as a 1·0 M solution until required. Solutions of pyruvic acid were adjusted to pH 5·7 and assayed with NAD-linked L-lactate dehydrogenase by the method of Bücher, Czok, Lamprecht & Latzko (1963).

Flavines. FAD, sodium salt (B grade), was purchased from the California Corp. for Biochemical Research, Los Angeles, Calif., U.S.A. FMN, sodium salt, was a gift from

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the Sigma Chemical Co., and riboflavine was obtained from L. Light and Co., Colnbrook, Bucks.

Oxyhaemoglobin. Crystalline horse oxyhaemoglobin was prepared by the method of Heidelberger (1922) as described by Lemberg & Legge (1949).

Snake venom. A dried preparation of Russell-viper snake venom was a gift from Dr D. R. D. Shaw.

Buffers. Buffer solutions used throughout this work were prepared according to Tables compiled by Dawson & Elliott (1959). Citrate–phosphate buffers were prepared with 0·2 M-Na_2HPO_4 and 0·1 M-citrate acid.

Organism. M. smegmatis (no. 523, National Collection of Type Cultures, London) was selected as a source of lactate oxidase. The culture was grown at 37° as a surface pellicle on a modified Sauton (1912) medium. The medium contained (per l.) 30g. of glycerol, 10g. of DL-lactic acid, 4g. of L-asparagine monohydrate, 2g. of citric acid monohydrate, 0·5g. of KH_2PO_4,H_2O, 0·5g. of $MgSO_4,7H_2O$, 0·05g. of ferric ammonium citrate, 0·01g. of thiamine and 1·0ml. of a solution containing 64mg. of $(NH_4)_6Mo_7O_{24},4H_2O$/l. and 2mg. of $ZnSO_4,7H_2O$/l. The medium was adjusted to pH 7·4 with NaOH.

A stock culture was maintained on 20ml. of medium in 100ml. conical flasks and subcultured after 4 days of growth.

Batches of bacteria for the enzyme purification were grown statically in 1l. Roux flasks containing 150ml. of medium. The flasks were inoculated with a loopful of a 4-day culture and incubated for 4 days. The harvest was filtered on a coarse calico cloth and packed by centrifuging. The cells were washed three times with water by thorough dispersion and centrifuging. A yield of 45–50g. wet wt. of bacterial cake/l. of medium was thus obtained.

Analytical methods

Protein. This was usually determined by the method of Lowry, Rosebrough, Farr & Randall (1951). Alternative procedures used included an ultraviolet–biuret method (Ellman, 1962) and the method of Kalckar (1947). Crystalline bovine serum albumin (Armour Pharmaceutical Co. Ltd., Eastbourne, Sussex) was used as a standard.

Fluorescence. Fluorescence of flavine solutions was measured with a Locarte fluorimeter. Filters selected were a Locarte LF/3 (primary) and a Chance OY3 (secondary). A linear relationship was established between flavine concentration and fluorescence over the range 0–0·33μg. of riboflavine/ml.

Ultracentrifugal analysis. Sedimentation velocities were determined in a Spinco model E ultracentrifuge with a standard 12·0mm. cell. Sedimentation coefficients were evaluated directly from the photographic plates by measuring the distances moved by the maximum ordinate of the gradient curve with a travelling microscope. Apparent diffusion coefficients were determined from projections obtained with a photographic enlarger. Measurements were made with respect to a reference base line, and the areas under the curves were determined with a planimeter.

Sucrose-density-gradient centrifugation. This was carried out by the method of Martin & Ames (1961). An SW 39 rotor was used with the Spinco model L ultracentrifuge.

Electrophoresis. Electrophoresis on cellulose acetate was carried out by the method of Kohn (1957).

Enzyme assay. Lactate oxidase activity was assayed manometrically or with an oxygen electrode. The standard manometric system consisted of 2·0m-moles of DL-lactate, pH 5·1, 0·5ml. of citrate–phosphate buffer, pH 5·1, and 1·0ml. of an enzyme solution sufficient to produce an oxygen uptake of 1–8μl./min. The centre cups contained 0·2ml. of 2N-NaOH. The total volume was 2·5ml. The temperature was 37°. Either DL-lactate or the enzyme was added from the side arm to start the reaction and manometer readings were taken at 5min. intervals. Studies with the purified enzyme showed that D-lactate markedly inhibited lactate oxidase (Table 6). However, oxygen uptake observed in the assay system described above was linear between 10min. and 40min. after the start of the reaction and was proportional to enzyme concentration.

A unit of enzyme activity is defined as the amount of enzyme that produces an oxygen uptake of 1μl./min. measured after the first 10min. interval of the assay.

Oxygen uptake was also followed with a Clark-type oxygen electrode fitted with an enclosed reaction chamber of capacity 2·3ml. The rate of decrease of current was proportional to enzyme concentration and consistent with the oxygen uptake observed manometrically. The oxygen electrode provided a quick method for determining relative activity.

Purification of the enzyme

Unless otherwise stated all operations were performed at 0°.

Preparation of cell-free extract. Cell-free extracts were prepared by stirring bacterial suspensions with glass beads in a Sorvall Omnimix. Each batch (up to 100g. of bacteria) contained bacteria, no. 12 Ballotini beads and water in the proportions 1:2:1 (w/w/v). The stainless-steel cup was immersed in ice-cold water and the contents were stirred for three 5min. periods (Variac setting 200) with 5min. intervals for cooling. The extract was filtered through a no. 1 sintered-glass filter with the aid of a water pump. The glass beads and debris were washed with a further 1vol. of water and the combined filtrate was centrifuged at 20000*g* for 30min. Cell-free extracts were stored at −20° until required. Yields of lactate oxidase obtained by this method were comparable with those obtained by ultrasonic oscillation.

Ammonium sulphate fractionation. Undialysed cell-free extract was fractionated with $(NH_4)_2SO_4$; lactate oxidase was recovered in the fraction at 0·35–0·65 saturation. A 500ml. volume of extract was placed in a stainless-steel beaker on ice. Finely ground $(NH_4)_2SO_4$ (105g.) was added at the rate of approx. 5g./min. while the extract was stirred with a magnetic stirrer and bar. After the addition was completed stirring was continued for a further 30min. and the extract was then centrifuged at 15000*g* for 20min. Further $(NH_4)_2SO_4$ (100g.) was added to the clear brown supernatant, followed by stirring for 30min. and centrifuging at 23000*g* for 30min. The precipitate was dissolved in 0·01M-sodium acetate buffer, pH 5·4, and made up to 100ml. with buffer (fraction I).

Heat treatment. The clear brown solution obtained from the previous step (fraction I) was placed in a 250ml. conical flask and immersed in a water bath at 75–78°. The solution was swirled continuously until the temperature reached 50–52° and then the flask was transferred to a constant-temperature water bath at 54°. After 5min. at this temperature it was transferred to an ice bath. The precipitate was removed by centrifuging at 10000*g* for 10min.

Dialysis against acetate buffer. The supernatant from the previous step (fraction II) was dialysed against 80vol. (7·5–8l.) of 0·02M-sodium acetate buffer, pH4·75, for 4–4½hr. The contents of the dialysis sac were thoroughly mixed at hourly intervals. A large precipitate formed during the dialysis was recovered by centrifuging at 10000*g* for 10min. Lactate oxidase was extracted from the precipitate with 0·05M-sodium acetate buffer, pH5·4 (20ml.). To ensure complete solution of the lactate oxidase the suspended protein was homogenized with a glass rod and then left to stand for 10–15min. Most of the protein remained undissolved and was removed by centrifuging at 10000*g* for 10min., leaving a bright-yellow supernatant (fraction III) containing lactate oxidase. Fraction III could be stored frozen for many months with only slow loss of enzyme activity.

Second ammonium sulphate fractionation. Fraction III was made 0·5 saturated with $(NH_4)_2SO_4$ by the addition of an equal volume of saturated $(NH_4)_2SO_4$ soln., pH7·0. The saturated $(NH_4)_2SO_4$ soln. was added dropwise over 15min. and stirring was continued for a further 15min. A small amount of inactive protein was removed by centrifuging at 15000*g* for 15min. Lactate oxidase was precipitated from the supernatant by lowering the pH to 5·1. After a further 15min. of stirring the precipitate was recovered by centrifuging at 23000*g* for 30min., and this was dissolved as described below.

Crystallization. Lactate oxidase was crystallized from 1·0M-sodium acetate buffer, pH5·4. The bright-yellow precipitate from the previous step was dissolved in a minimum volume of buffer. Solution was facilitated by warming the suspension to 30° and carefully adding buffer until a clear solution was obtained. When this solution (fraction IV) was left to cool in the refrigerator for 1–2hr. a dense precipitate of crystalline lactate oxidase with a marked sheen formed.

Recrystallization. The crystalline enzyme was recovered by centrifuging at 10000*g* for 10min. and the precipitate was dissolved as described above. Lactate oxidase was recrystallized from this solution (fraction V) and the precipitate was stored as a frozen suspension at −20° in 1·0M-sodium acetate buffer, pH5·4. The suspension of recrystallized enzyme is fraction VI.

The results of a typical purification of lactate oxidase are summarized in Table 1. The specific activity of preparations of the recrystallized enzyme varied from 650 to 720.

Lactate oxidase crystallized from 1·0M-sodium acetate, pH5·4, as fine needles, which are best observed by phase-contrast microscopy. Larger crystalline needles were obtained when the enzyme was recrystallized on a microscope slide (Fig. 1). Sutton (1957) and Takemori *et al.* (1968) obtained square crystalline plates of lactate oxidase from *M. phlei* by using different conditions for crystallization from those employed in this work.

RESULTS

Ultracentrifugal analysis. Preparations of recrystallized enzyme exhibited a single symmetrical moving boundary on sedimentation in the ultracentrifuge (Fig. 2). Sedimentation coefficients were calculated by a method described by Schachman (1957). Studies at several protein concentrations showed that, though the rate of

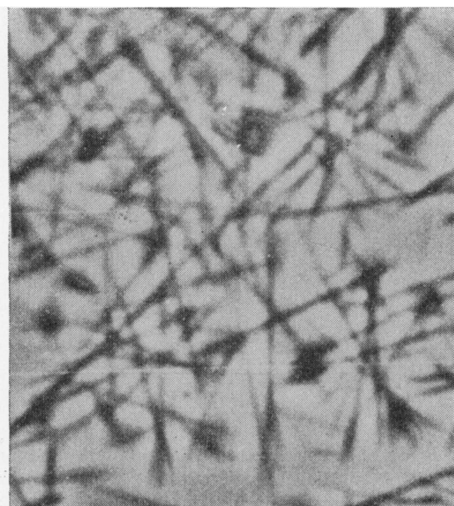

Fig. 1. Crystals of L-lactate oxidase prepared on a microscope slide (phase-contrast micrograph; × 900).

Table 1. *Purification of L-lactate oxidase*

	Fraction	Volume (ml.)	Total enzyme (units)	Protein (mg./ml.)	Sp. activity (units/mg. of protein)
	Crude extract	500	95500	9·6	19·8
I	Solution of fraction precipitated by $(NH_4)_2SO_4$ (0·35–0·65 satn.)	100	68800	23·2	29·7
II	Supernatant after heat treatment	94	60900	17·4	37·2
III	Solution of extract after dialysis against acetate buffer, pH4·75	20	19200	7·5	128
IV	Solution of fraction precipitated by $(NH_4)_2SO_4$ (0·5 satn.), pH5·1	3·3	19000	10·6	542
V	Crystalline L-lactate oxidase	4·8	18700	6·25	624
VI	Recrystallized enzyme	5·8	18000	4·5	690

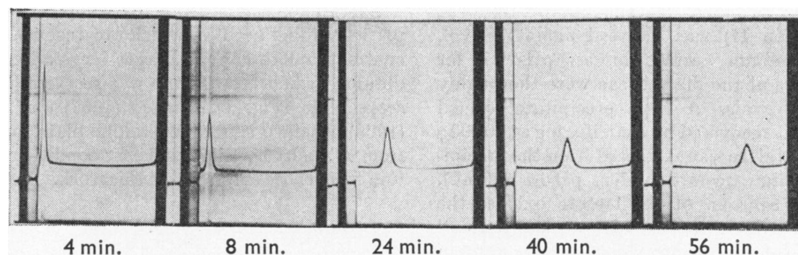

Fig. 2. Sedimentation analysis of recrystallized L-lactate oxidase. Recrystallized L-lactate oxidase (specific activity 720) was dialysed against a solution of 0·2M-NaCl–0·01M-sodium phosphate buffer, pH 7·0, for 24hr., and the dialysed enzyme was diluted to a protein concentration of 4·0mg./ml. with the same buffered saline. The temperature was 20°. The rotor speed was 50740rev./min. Time was measured from the instant when the accelerating rotor was at 34400rev./min.

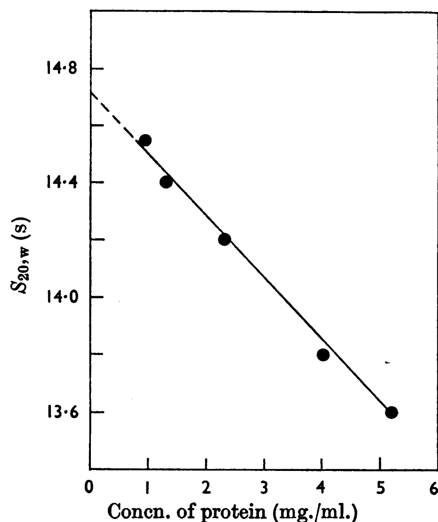

Fig. 3. Effect of protein concentration on the sedimentation coefficient. A solution of recrystallized L-lactate oxidase was prepared as described in Fig. 2. Dilutions were prepared in the buffered NaCl soln. (Fig. 2) and examined in the ultracentrifuge (at 20° and at 50740rev./min.). Apparent sedimentation coefficients were corrected for the viscosity of the solvent.

sedimentation was constant throughout each analysis, the apparent sedimentation coefficient was dependent on protein concentration (Fig. 3). The sedimentation coefficient, $S^0_{20,w}$, was found to be 14·7s.

An apparent diffusion coefficient was also evaluated from the sedimentation-velocity data by using an equation given by Schachman (1957). Although a reference base line was used the analysis was still subject to considerable error. The apparent diffusion coefficient, $D^0_{20,w}$ (app.), obtained was $3{\cdot}95 \times 10^{-7}$cm.^{2}sec.$^{-1}$.

Electrophoresis. Recrystallized lactate oxidase was analysed electrophoretically at pH 5·9, 7·6 and 8·1 in 0·05M-sodium phosphate buffer on cellulose acetate strips. A sample (25–30μg.) of protein was applied to each strip (3μl./strip) as a streak, and a constant current of 1½mA/strip was applied for 1hr. Protein was located by staining with 0·2% Ponceau S prepared in 3% (w/v) trichloroacetic acid. Lactate oxidase migrated as a single band at the three pH values tested.

Absorption spectrum. Lactate oxidase had a typical flavoprotein absorption spectrum with maxima at 276, 375 and 452mμ (Fig. 4). The spectrum also included a pronounced shoulder at about 480mμ and a less pronounced inflexion at about 420mμ.

Pure preparations of lactate oxidase had an absorption minimum at 400mμ. This was of particular interest because it indicated that the chromoprotein present in Cousins's (1956) preparations was absent from crystalline preparations.

Addition of excess of DL-lactate to lactate oxidase bleached the enzyme (Fig. 5). There were no changes in the spectrum that would suggest the formation of a long-wavelength intermediate as has been observed with such flavoproteins as D-amino acid oxidase and lipoyl dehydrogenase (Massey & Gibson, 1964).

Identification of the prosthetic group. Solutions of lactate oxidase did not exhibit flavine fluorescence, and the flavine was tightly bound to the protein. The prosthetic group was released by heating a neutral solution of the enzyme for 3min. in a boiling-water bath. A sample of the heat-liberated prosthetic group was analysed spectrophotometrically. The absorption maxima had shifted from 452 to 448mμ and from 276 to 265mμ, but there was no detectable change in extinction at 450mμ. In Table 2 the extinction ratios of the prosthetic group are compared with those of standard values for FAD and FMN. The free flavine was also analysed by descending paper chromatography. The results are summarized in Table 3. Samples of FAD, FMN and the prosthetic group were treated

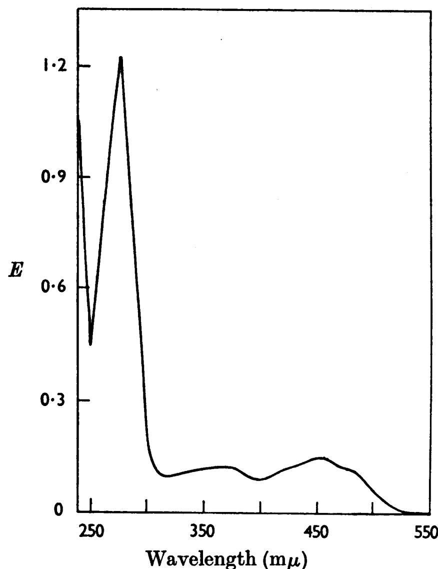

Fig. 4. Absorption spectrum of lactate oxidase. The cuvette contained 0·855mg. of recrystallized enzyme (specific activity 720) and 60μmoles of sodium phosphate buffer, pH7·0. The total volume was 1·5ml.

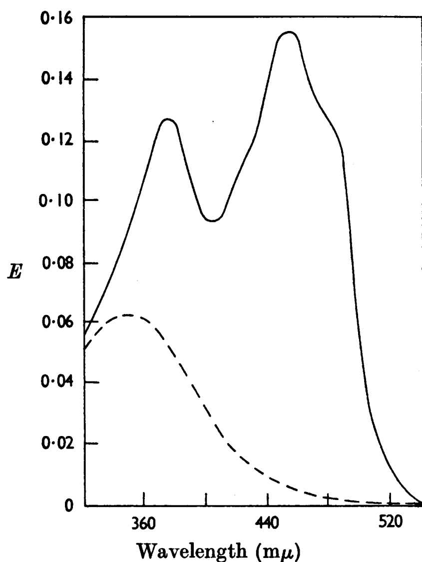

Fig. 5. Absorption spectrum of lactate oxidase: oxidized and reduced forms. Crystalline lactate oxidase (1·05mg.; specific activity 590) and sodium phosphate buffer, pH7·0 (60μmoles), were added to a stoppered cuvette. The total volume was 1·5ml. The cuvette was evacuated and then flushed with nitrogen, and this cycle was repeated twice. ——, Original spectrum; ----, spectrum (corrected for dilution) after the addition of 200μmoles of DL-lactate from the hollow stopper.

Table 2. *Extinction ratios of the heat-liberated prosthetic group*

The extinction ratios of the supernatant containing the flavine liberated by heat treatment as described in the text were measured. The original solution of lactate oxidase contained 0·9mg. of recrystallized enzyme (sp. activity 690) and 100μmoles of sodium phosphate buffer, pH7·0, in a final volume of 1·5ml. The standard extinction ratios of FAD and FMN are quoted from Whitby (1953).

Flavine	E_{375}/E_{450}	E_{260}/E_{450}
Prosthetic group	0·87	2·53
FMN	0·85	2·22
FAD	0·82	3·27

Table 3. *Paper chromatography of the heat-liberated prosthetic group*

The heat-liberated prosthetic group was analysed by descending chromatography on Whatman no. 1 paper with two solvent systems (Huennekens & Felton, 1957): (1) 5% (w/v) Na_2HPO_4; (2) butan-1-ol–acetic acid–water (4:1:5, by vol.; lower phase). The chromatograms were developed in darkness, and the flavines were located by their fluorescence under u.v. light. Traces of decomposition products were detected from all the flavines, but no FAD was detected in the prosthetic-group preparation. Previously reported values are given in parentheses.

	R_F	
Flavine	Solvent 1	Solvent 2
Riboflavine	0·29 (0·30)	0·39 (0·30)
FMN	0·48 (0·48)	0·13 (0·13)
FAD	0·35 (0·35)	0·07 (0·05)
Prosthetic group	0·48	0·12

with a solution of snake-venom extract that contains a phosphodiesterase; only the FAD control showed a net increase in fluorescence intensity. Thus analysis of the heat-liberated flavine confirmed that the prosthetic group of the mycobacterial lactate oxidase is FMN (Sutton, 1955; Takemori *et al.* 1968).

Minimum molecular weight. Since the extinction at 450mμ was not altered when FMN was released from the native enzyme, it was assumed that the molar extinction coefficient of lactate oxidase at 450mμ was the same as that of free FMN (ϵ_{450} 12200; Whitby, 1953). Measurements based on several preparations indicated that there was 1 mole of FMN/49500 ± 1500g. of enzyme protein. The specific extinction coefficients, $E_{1\,cm.}^{1\%}$, of recrystallized lactate oxidase at 280 and 452mμ were 21·9 and 2·62 respectively. The protein concentration for these measurements was determined by the method of Lowry *et al.* (1951).

Sutton (1957) reported that the minimum molecular weight of lactate oxidase was 125700;

protein was determined by the ultraviolet-absorption method of Kalckar (1947) in this work. To solve this discrepancy over the minimum molecular weight, protein was determined on a dry-weight basis, by a modified biuret method (Ellman, 1962) and by the ultraviolet-absorption method (Kalckar, 1947). The results, summarized in Table 4, confirmed the validity of protein determinations obtained by the Lowry method, and showed that measurements based on the ultraviolet absorption are twice the value expected. Takemori *et al.* (1968) measured the protein content of crystalline lactate oxidase from *M. phlei* by several methods; they found that the ultraviolet-absorption method gave erroneous results. The minimum molecular weight was found to be in the range 55300–56200.

Molecular weight. The molecular weight of lactate oxidase in solution was evaluated from a sucrose-density-gradient analysis and from the sedimentation-velocity ultracentrifugal data.

Crystalline horse oxyhaemoglobin (sedimentation coefficient 4·4s; Svedberg & Peterson, 1940) was used as a standard in the sucrose-gradient studies. The sedimentation coefficient of crystalline lactate oxidase was found by this method to be 12·0s. An approximate molecular weight was obtained by applying the equation $S_1/S_2=(M_1/M_2)^{\frac{2}{3}}$, where S_1 and S_2 are sedimentation coefficients and M_1 and M_2 are the respective molecular weights; the value obtained was 300000.

The molecular weight determined from the ultracentrifuge data was 341000, assuming that the partial specific volume was 0·73ml./g. This value quoted should only be considered as a tentative value. However, if the molecular weight of lactate oxidase is within the range 300000–400000, each mole of enzyme contains 6–8 moles of FMN.

Stoicheiometric reduction of the enzyme with L-*lactate.* Lactate oxidase (1·2mg. of recrystallized enzyme, specific activity 630) was incubated anaerobically at pH 7·0 with 24mμmoles of L-lactate in a sealed cuvette. The changes in the absorption spectrum were consistent with conversion of the flavine into the fully reduced form. After 1hr. of incubation the extinction at 450mμ had decreased by 85% of the original reading and no further changes were observed. It was estimated that 20mμmoles of flavine were reduced during the incubation.

Substrate specificity. Of the eight compounds tested only DL-α-hydroxybutyrate and DL-3-phenyllactate were oxidized slowly. No activity was observed towards glycollate, tartronate, L-malate, DL-glycerate or D-lactate.

Effect of pH *on the rate of lactate oxidation.* The effect of pH on the rate of L-lactate oxidation is shown in Fig. 6. The pH–activity curve was similar to that reported by Cousins (1956), but the pH for optimum activity, pH 5·7–5·8, was 0·5pH unit higher than reported by Cousins (1956). Sutton (1954) found that the pH for optimum activity was between pH 5·6 and 6·0. Takemori *et al.* (1968) reported a pH optimum of 6·0.

Effect of L-*lactate concentration on the activity of lactate oxidase.* An apparent K_m for L-lactate at 37° with air-saturated solutions was determined from a Lineweaver–Burk plot; the value obtained was 25mM. It was also confirmed that pyruvate competitively inhibits the oxidation of lactate. The apparent K_i for pyruvate was found to be

Table 4. *Protein determination of lactate oxidase*

Protein determinations were made on a preparation of recrystallized lactate oxidase (specific activity 720) by several methods as described in the text. Crystalline bovine serum albumin was used as a standard in estimations by the Lowry method and by the ultraviolet–biuret method. Flavine concentration was estimated spectrophotometrically, assuming that the molar extinction of lactate oxidase at 452mμ was identical with the molar extinction of FMN at 450mμ. ϵ_{450} for FMN, 12200 (Whitby, 1953).

Method	Protein (mg./ml.)	Minimum mol.wt. (μg. of protein/ μmole of flavine)
Lowry method	2·8	47000
Dry wt.	3·2	53000
Ultraviolet–biuret method	3·1	51000
Kalckar method	6·6	109000

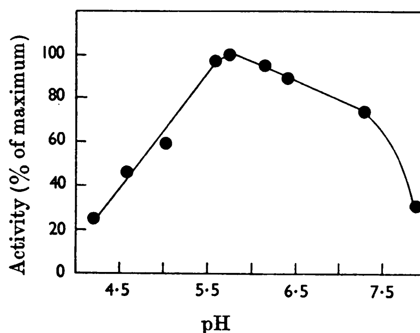

Fig. 6. Effect of pH on the activity of lactate oxidase. Each Warburg flask contained 1·7ml. of sodium citrate–phosphate buffer of the pH indicated, 0·3ml. of 2·0M-L-lactate and 0·1ml. of a solution containing 5·6μg. of recrystallized enzyme (specific activity 560) in the side arm. The total volume was 2·3ml. The temperature was 37°. The pH of the solution in the main compartment was checked before and after each experiment. Results are expressed as a percentage of the activity observed at pH 5·75.

Table 5. *Effect of metal chelators, inhibitors and flavines on lactate oxidase*

Warburg flasks contained 0·5ml. of citrate–phosphate buffer, pH 5·1, 11 μg. of recrystallized enzyme (specific activity 450–500) and 1·0ml. of 2·0M-DL-lactate, pH 5·1, in the side arm. The total volume was 2·3ml. The temperature was 37°. The compounds tested were added to the main compartment. The flasks were equilibrated at 37° for 10min. before the reaction was initiated. The results are expressed as percentage inhibition.

	Concn. (mM)	Inhibition (%)
EDTA	0·8	0
Phenanthroline	0·8	5·7
8-Hydroxyquinoline	0·8	11·8
Arsenite	0·8	0
Glutathione	0·8	4·8
Cysteine	0·8	0
p-Chloromercuribenzoate	0·04	51
p-Chloromercuribenzoate	0·02	28
Quinacrine	0·4	−14
Sodium Amytal	0·8	6
Sodium phenylbarbital	0·8	3
FAD	0·04	−1·7
FMN	0·084	49
FMN	0·042	22
Riboflavine	0·04	8

Table 6. *Inhibition of lactate oxidase by some α-hydroxy acids*

Warburg flasks contained 1·5ml. of citrate–phosphate buffer, pH 5·7, and 0·1ml. of enzyme solution (specific activity 600) containing 5·9 μg. of recrystallized enzyme in the side arm. The total volume was 3·2ml. The temperature was 37°. The α-hydroxy acids tested were included with L-lactate (156mM) in the main compartment. Solutions of the α-hydroxy acids, including L-lactate, were adjusted to pH 5·7. The results are expressed as percentage inhibition.

	Concn. (mM)	Inhibition (%)
D-Lactate	52	43·5
	5·2	14
DL-Glycerate	156	35
	15·6	5·8
Tartronate	31·3	92·6
	6·3	51·6
L-Malate	78	0
DL-Alanine	62·5	0

14·5mM. These values were obtained from manometric experiments at pH 5·7 with the recrystallized enzyme. Sutton (1957) reported an apparent K_m for L-lactate of 17mM and an apparent K_i for pyruvate of 1·1mM. The apparent K_m for L-lactate given by Takemori *et al.* (1968) was 25mM.

Maximum specific activity and catalytic-centre activity. The maximum specific activity of lactate oxidase was calculated from manometric experiments carried out at pH 5·7 in the presence of excess of L-lactate. Preparations of recrystallized enzyme with a standard specific activity of 700 had a maximum specific activity of 74–76 μmoles of oxygen/min./mg. of protein at 37°. If the enzyme contains 1 μmole of FMN/50000 μg. of protein and there is one flavine group in each catalytic centre, then the corresponding catalytic-centre activity at 37° was 3700–3800 μmoles/min./μmole of flavine; the catalytic-centre activity at 22° was 1600.

Inhibitors. L-Lactate oxidase was unaffected by most of the reagents tested (Table 5). *p*-Chloromercuribenzoate at relatively high concentrations inhibited the enzyme. Cousins (1956) reported that free FAD and FMN inhibited the enzyme. It was confirmed that FMN caused a marked inhibition. FAD did not inhibit significantly (Table 5). It is possible that FMN was a major contaminant in the preparations of FAD (15% pure) used by Cousins (1956), and this would account for the inhibition.

Several α-hydroxy acids, including D-lactate, DL-glycerate and tartronate (α-hydroxymalonate), inhibited lactate oxidase (Table 6).

Activity of lactate oxidase with redox dyes. Cousins (1956) and Sutton (1954) showed that purified lactate oxidase did not utilize methylene blue or tetrazolium as electron acceptor. However, Sutton (1955) reported that 2,6-dichlorophenol-indophenol served as an electron acceptor.

Several redox dyes were tested with lactate oxidase under anaerobic conditions. Although more than five times the quantity of enzyme required for a standard manometric assay was used, the rate of dye reduction was slow (Table 7). Phenol blue and 2,6-dichlorophenol-indophenol were the most efficient acceptors. Ferricyanide was not reduced. Other dyes tested were reduced slowly over the course of many hours.

Enzyme stability. Suspensions of crystalline enzyme, pH 5·4, were stable for at least 1 week at 0°. A dilute solution of recrystallized lactate oxidase (0·11mg./ml.) at pH 7·0 had lost 30% of its activity after 3 weeks at 0°. Recrystallized lactate oxidase stored as a frozen suspension at −20° had lost 30% of its activity after 6 months.

Alteration in enzyme activity on storage. As described above, a slow loss of enzyme activity (total units) occurred when recrystallized preparations were stored at −20°. During this storage the enzyme also underwent a reversible alteration in its catalytic activity.

Oxygen uptake catalysed by fresh crystalline preparations in the first 10min. interval of the standard manometric assay at 37° was somewhat

Table 7. *Activity of lactate oxidase with redox dyes*

Anaerobic cuvettes contained 1·5 ml. of 0·2 M-sodium phosphate buffer, pH 7·0, 0·5 ml. of 2 M-DL-lactate, pH 7·0, and dye solution sufficient to give an initial extinction value within the range 0·7–0·95. Enzyme (0·1 ml. containing 28 μg. of protein; specific activity 720) was placed in the hollow stopper. The final volume was 3·0 ml. The temperature was 20°. The cuvettes were flushed with N_2 and evacuated. After the initial readings were taken, the cuvettes were tipped and the rate of reduction was followed for 1 hr.

Dye	Wavelength (mμ)	E_0' at pH 7·0 (v)	Rate of reduction (E unit/hr.)
2,6-Dichlorophenol-indophenol	600	0·217	0·320
Phenol blue	655	0·216	0·370
Cresol-2,6-dichlorophenol-indophenol	585	0·181	0·085
Toluylene blue	655	0·125	<0·01
1-Naphthol-2,6-dibromoindophenol	615	0·119	<0·01

variable, but it approached the linear rate subsequently observed. However, oxygen uptake catalysed by the stored preparations in the first 10 min. interval of assays at 37° was only half that observed in the following 10 min. intervals. When the stored enzyme was assayed at 22° the maximum rate of oxygen uptake was not attained until 25–30 min. after the start of the reaction. This lag suggests that the stored enzyme undergoes a reversible inactivation. This phenomenon is not due to repeated freezing and thawing. Preincubation of the altered enzyme in the absence of substrate at temperatures from 20° to 40° did not effect a reactivation.

DISCUSSION

This work confirms an earlier report by Sutton (1954) that the mycobacterial lactate oxidase is a flavoprotein and that the prosthetic group is FMN. Further, the physical and enzymic properties of lactate oxidase from *M. smegmatis* are similar to, indeed almost identical with, the properties of the enzyme purified from *M. phlei* by Takemori *et al.* (1968).

Precise characterization of the molecular weight of lactate oxidase requires further studies, but the results obtained from ultracentrifugal analysis and the sucrose-gradient analysis place the molecular weight in the range 300000–400000. This suggests that lactate oxidase contains 6–8 moles of flavine/mole. Takemori *et al.* (1968) found that the molecular weight was in the range 340000–399000 and they concluded that the enzyme contains 6–7 moles of flavine/mole.

The change in enzymic activity that lactate oxidase undergoes on storage at −20° is an unusual phenomenon. It is possible that this type of alteration is due to some reversible change in protein conformation.

Unlike the lactate oxidase from *Diplococcus pneumoniae* (Udaka, Koukol & Vennesland, 1959), the α-hydroxy acid oxidase from *Tetrahymena pyriformis* (Eichel, 1966) and other NAD-independent lactate dehydrogenases, the mycobacterial lactate oxidase exhibits a marked specificity for both substrate and electron acceptors. Further, if pyruvate is formed during the catalysis it is present only as an enzyme-bound intermediate (Cousins, 1956; Sutton, 1957).

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