A Lactate Oxygenase from *Mycobacterium phlei*

IMPROVED PURIFICATION AND SOME PROPERTIES OF THE ENZYME*

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SUMMARY

A simplified method for the preparation of a large amount of crystalline lactate oxygenase from *Mycobacterium phlei* has been introduced. The crystalline enzyme exhibited one component on polyacrylamide gel electrophoresis and revealed a single homogeneous peak ($s_{20,w} = 12.5$ S) in an ultracentrifuge. The enzyme has a typical flavoprotein spectrum, and the prosthetic group was shown to be flavin mononucleotide as judged by enzymatic, paper chromatographic, and fluorometric methods. The apparent molecular weights derived from diffusion and sedimentation constants, the method of Archibald, and gel filtration were 399,000, 383,000 and 340,000, respectively. The minimum molecular weight from the analysis of flavin was 55,300 to 56,200. On the basis of these results, the conclusion is that the enzyme contains 6 to 7 moles of FMN per mole of protein. The enzyme catalyzes the oxidation of only L-lactate and L-hydroxy-n-butyrate, and is inhibited competitively by D-lactate.

The monoxygenase generally known as “lactate oxidative decarboxylase” was first crystallized from cells of *Mycobacterium phlei* by Sutton in 1957 and was shown to be a flavoprotein with FMN as the prosthetic group (1-3). Hayashi and Sutton (4) have further indicated by the use of the $^{18}$O technique that 1 atom of molecular oxygen is incorporated into the acetate formed, and that the other atom is presumed to be reduced to water, as shown in the equation below.

$$\text{CH}_3\text{CHOHCOOH} + \text{O}_2 \rightarrow \text{CH}_3\text{COOH} + \text{CO}_2 + \text{H}_2\text{O}$$

In order to provide further information about the mechanism of Reaction 1, it is essential that the enzyme be available in pure form and in large quantities. The present report describes an improved procedure for the crystallization of lactate oxygenase from *M. phlei*. It requires only a few operations and employs no chromatographic procedures. With this simplified method, relatively large amounts of the crystalline enzyme, which was apparently homogeneous in the ultracentrifuge and in gel electrophoresis, have been obtained. With the resultant crystalline enzyme, reported data (1-3) such as the value of molecular weight, the identification of flavin as FMN, the substrate specificity, as well as some kinetic properties were restudied.

EXPERIMENTAL PROCEDURE

Materials

*Growth and Preparation of Cells—Cells were cultivated in the same medium as described by Sutton (1), except that L-asparagine was satisfactorily replaced by a commercial product, L-sodium glutamate (Asahiaji, Asahi Kasei Company, Ltd., Osaka, Japan). Six 500-ml Roux bottles, each containing 120 ml of growth medium, were inoculated with a culture of *M. phlei*. After 9 days of horizontal standing at 37°, the bottles were vigorously shaken to suspend the surface growth, and the contents were then distributed to sixty 500-ml Roux bottles containing 120 ml of the same medium. The culture was further incubated for 5 days at 37°. The cells were harvested by a continuous flow centrifuge, washed twice with distilled water, and stored at -20° until used. The yield of cells was approximately 240 g (wet weight)/5 liters of culture medium.*

*Proteins Used for Molecular Weight Determinations by Gel Filtration Method—Crystalline catalase was prepared from beef liver by the method of Kitagawa and Shirakawa (5). Crystalline lactate dehydrogenase from pig heart was obtained from Boehringer und Sohne GmbH Mannheim. Apoferritin was prepared from a crystalline horse spleen ferritin* 2 according to the method described by Granick and Michaelis (6) with the following modifications. In carrying out the removal of iron, 5 ml of 1 to 2% ferritin solution was dialyzed for 16 hours at 5° against 150 ml of 1 M acetate buffer, pH 4.6, containing 2 g of sodium dithionite and 30 mg of o-phenanthroline, and then against 1 M acetate buffer, pH 4.6, until the protein solution became colorless. The

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1 The strain was kindly supplied by Dr. R. Shimizu of the Institute for Cancer Research, Kanazawa University.

2 We are most grateful to Professor Y. Yoneyama and Dr. Y. Sugita of the Kanazawa University School of Medicine, who kindly provided the preparation of a crystalline ferritin.
iron-free apoferritin thus obtained was crystallized with cadmium sulfate.

Preparation of Apolactate Oxygenase—Crystalline lactate oxygenase was dissolved in 0.1 M Tris-HCl buffer, pH 8.0, at the concentration of 4 mg of protein per ml, and saturated ammonium sulfate solution was added to 0.6 saturation. The pH of the solution, kept at 0° in an ice bath, was adjusted to 2.5 with 1 N HCl and immediately centrifuged at 11,000 × g for 10 min. The protein precipitate was dissolved in 0.1 M Tris-HCl buffer, pH 8.0, and allowed to stand in an ice bath for 30 min. The acid-ammonium sulfate treatment and the centrifugation were repeated two additional times, and the final protein precipitate was dissolved in the same buffer.

Other Materials—Salicylate hydroxylase was prepared according to the method described by Katagiri et al. (7), and the flavin-free apoenzyme was obtained by a method similar to the one described by Warburg and Christian (8). FMN and riboflavin were purchased from Wako Pure Chemical Company, Osaka, Japan. FAD was kindly supplied by Dr. E. Ohmura, Takeda Research Laboratories, Osaka. Each flavin was purified when necessary by chromatography on DEAE-cellulose columns, as described by Rao et al. (9). D- and L-Lithium lactates were purchased from Miles Laboratories, Inc., Elkhart, Indiana. dl-α-Hydroxy-n-butyric acid, dl-β-hydroxy-n-butyric acid, α-hydroxy-isobutyric acid, and glycicolic acid were procured from Tokyo Kasei, Ltd. Tokyo, Japan. Sephadex G-200 and DEAE-Sephadex A-50, were obtained from Pharmacia. Phosphate buffer was prepared from K₂HPO₄ and KH₂PO₄. Glass-distilled water was used throughout except for culture medium which was prepared with tap water.

Methods

Assay of Enzymatic Activities The activity of the enzyme was assayed at 30° by measuring the rate of oxygen uptake with the use of the conventional Warburg manometric technique. The cuvette contained 40 μmoles of potassium phosphate buffer, pH 6.0, 150 μmoles of L-lactate, as well as an appropriate amount of the enzyme in a total volume of 2.0 ml; and 0.1 ml of 20% KOH was placed in the center well. The reaction was started by the addition of lactate from the side arm. Under the standard conditions, the reaction was linear with time although a lag appeared during the initial 5-min period, and the rate was strictly proportional to the amount of crystalline enzyme in concentrations up to 12 μg without any correction as reported by Sutton (1) (Fig. 1). In all enzyme assays, 1 unit was defined as the amount of enzyme which consumed 1 μ mole of molecular oxygen per min under the conditions of the assay. The specific activity was expressed in units per mg of protein.

Sedimentation and Diffusion—Sedimentation studies were performed with a Hitachi model UCA-1 type ultracentrifuge. The sedimentation constant, calculated from a plot of the logarithm of the boundary distances from the rotation axis versus time, was reduced to the value at the standard condition of 20° in water. In the analysis of molecular weight by the method of Archibald (10), the most satisfactory rotor speed was 57,000 rpm. Diffusion studies were made by the schlieren cylindrical lens method with a Neurath type cell (11). Diffusion was followed over a period of 6 hours. The diffusion constant was calculated and corrected to standard conditions by the method summarized by Watanabe, Kawade, and Isono (12).

Absorption and Fluorescence Measurement—Measurements of absorption spectrum were performed in a Hitachi model EPH-3 spectrophotometer or with a Hitachi Perkin-Elmer model 130 spectrophotometer, with the use of a cuvette with a 1-cm light path. Fluorescence was measured with a Farrand recording spectrofluorometer equipped with the 150-watt direct current xenon arc lamp as the exciting source.

Protein Determinations—Protein was determined in the crude preparation by the procedure of Lowry et al. (13) with crystalline bovine serum albumin (Sigma) as a standard. In the crystalline enzyme, the protein concentration was estimated spectrophotometrically from absorbances at 280 μm or 454 μm in 0.1 M phosphate buffer, pH 7.0. The absorbance indexes (A(280 μm protein/ml)-1) at 280 and 454 μm were 2.04 and 0.226, respectively.

Gel Electrophoresis—Polyacrylamide gel electrophoresis was carried out according to the method of Davis (14), and the gel was stained for protein with 1% Amido black in 7% acetic acid. Destaining was accomplished in 7% acetic acid.

Paper Chromatography—The descending technique was employed with the use of Whatman No. 1 paper with l-butiloacetic acid-H₂O (4:1:5), pyridine-H₂O (2:1), and 5% Na₂HPO₄.

Sugar Analysis—The flavin-free apoenzyme was used as the starting material for sugar analysis. The crystalline enzyme was heated at 80° for 20 min to release flavin from the enzyme protein. The mixture was rapidly cooled and centrifuged at 13,000 × g for 10 min. The precipitate was washed once with distilled water and dried in a drying oven at 60°. The dried precipitate was analyzed for hexose by the method described by Johansen et al. (16) with a mannose standard in which the reaction mixture was incubated for 45 min at 80°. For the determination of hexosamine, 10 mg of the dried material were hydrolyzed for 8 hours with 0.5 ml of 6 N HCl at 100° in a sealed tube, and the hexosamine content was determined by the Elson-Morgan method modified by Belcher, Nutten, and Sambrook (17) with a glucosamine standard.

Metal Analysis—The crystalline enzyme was dialyzed for 8 hours against 0.05 M phosphate buffer, pH 7.0, containing 10⁻⁴ M

3 The procedure used for sugar analysis was checked with egg albumin as a standard. This protein was found to contain 2.2% hexose and 1.4% hexosamine in agreement with the values cited by François, Marshall, and Neuberger (16) and Johansen, Marshall, and Neuberger (16).

4 We wish to thank Dr. K. Terada and Mr. M. Akamatsu of the Department of Chemistry, Kanazawa University, for their kind collaboration in performing metal analysis.
EDTA with three changes of the outer solution, and then for 24 hours against the deionized distilled water. No inactivation of the enzyme was observed during the dialysis. The dialyze was lyophilized, and the dried material was analyzed for metals by a Shimazu model QL 170 quartz spectrophotograph equipped with a Shimazu universal current source.

RESULTS AND DISCUSSION

Purification of Lactate Oxgenase

All purification steps were carried out at 0–5°C unless otherwise specified. A typical protocol is presented in Table I.

Extraction—The cells (200 g, wet weight) were ground mechanically in a mortar with twice their weight of aluminum oxide (Wako W. 800) for 30 min and mixed with 3 volumes of distilled water. The resultant slurry was centrifuged at 13,000 × g for 30 min, and the supernatant fraction was saved. The sediment was reground for 10 min, mixed with 2 volumes of distilled water, and again centrifuged for 30 min at 13,000 × g. The sediment, consisting of cellular debris and alumina, was discarded. The supernatant fractions from the two centrifugation steps were combined (total volume of 930 ml).

First Ammonium Sulfate Fractionation—To the crude extract obtained in the previous step, 24.3 g of solid ammonium sulfate per 100 ml (0.40 saturation) were added slowly and with constant stirring. After 30 min of additional stirring, the precipitate was separated by centrifugation (13,000 × g for 30 min) and discarded. Then 16.5 g of ammonium sulfate per 100 ml (0.65 saturation) were added to the supernatant solution, and the mixture was centrifuged at 77,500 × g for 30 min. The precipitate was dissolved in a small volume of 0.1 M phosphate buffer, pH 7.0. The turbid solution (135 ml) was then dialyzed overnight at 5°C against 8 liters of 0.1 M phosphate buffer, pH 7.0.

DEAE Sephadex Treatment—DEAE Sephadex A-50 (150 g, wet weight) which had been buffered with 0.1 M phosphate buffer, pH 7.0, and collected by filtration through sintered glass, was added to the enzyme solution obtained in the previous step. The mixture was stirred mechanically for 1 hour. To the mixture, 100 ml of 0.1 M phosphate buffer, pH 7.0, containing 1 m NaCl were added for each 100 ml of the dialyzed solution, and the stirring was continued for 1 hour. The enzyme solution was separated by filtration through sintered glass. The gel was washed three times with a small volume of 0.1 M phosphate buffer, pH 7.0, containing 0.5 m NaCl, and the washings were added to the original filtrate.

Second Ammonium Sulfate Fractionation. To the combined enzyme solution (385 ml), 43.0 g of ammonium sulfate per 100 ml were added with constant stirring. After standing for 30 min, the mixture was centrifuged at 13,000 × g for 30 min. The precipitate was dissolved in 50 ml of 0.1 M phosphate buffer, pH 6.0. In this step the enzyme solution was viscous and turbid.

Third Ammonium Sulfate Fractionation. To the enzyme solution, 5.5 g of ammonium sulfate per 100 ml were slowly added with stirring. The precipitate, which was allowed to accumulate overnight, was collected and dissolved in a small volume of 0.1 M phosphate buffer, pH 6.0, to give 64 ml of a turbid solution.

Crystallization. To the enzyme solution (approximately 79 mg of protein per ml), solid ammonium sulfate was added slowly until a turbid solution became viscous. The solution was then stored at 5°C. The enzyme could be crystallized from such a viscous solution without difficulty. By means of a microscope, the crystals could be easily observed as bright yellow transparent plates. Crystallization began after several hours and continued for 1 to 2 days. If the mixture was stored at 5°C for several days, the yellow crystals settled, leaving a turbid solution. The crystals were collected in a centrifuge (2800 × g for 10 min), and the viscous turbid supernatant was discarded. The crystals were dissolved in a minimum volume of 0.1 M phosphate buffer, pH 6.0, by allowing the solution to come to room temperature for about 30 min. The insoluble materials were removed by centrifugation and discarded. To the supernatant solution (30 ml), solid ammonium sulfate was added until permanent turbidity was observed. The enzyme started to crystallize almost immediately and the solution had a silky sheen upon swirling. After standing overnight, the crystals were collected, dissolved in 0.1 M phosphate buffer, pH 6.0, and recrystallized by the addition of ammonium sulfate. The above procedure was repeated two additional times to remove any residual impurity. After the fourth recrystallization, the specific activity became 50.3 and remained unchanged upon further recrystallization. The yield from 200 g of cells was about 150 mg of enzyme crystallized four times. The crystals are shown in Fig. 2. The final suspension of crystals was stored at 5°C. Samples were centrifuged and dissolved as needed. When stored as a suspension, the crystalline enzyme was quite stable for several months.

Evidences for Homogeneity of Enzyme

Homogeneity of the crystalline enzyme was judged by several criteria. Polyacrylamide gel electrophoresis resulted in the appearance of a single zone of protein (Fig. 3). Upon high speed sedimentation of the enzyme in the analytical ultracentrifuge, a single component appeared which spread little during sedimentation (Fig. 4). Fig. 5 shows the elution pattern of the enzyme upon gel filtration on Sephadex G-200. A single protein peak was observed which was capable of being superimposed on the peaks with respect to enzyme activity and absorptions at 454 and 280 mg, indicating homogeneity by this criterion.

Identification of Prosthetic Group as FMN

The early finding of Sutton (2, 3) that the prosthetic group of lactate oxidase appears to be flavin mononucleotide was confirmed from three different lines of evidence. (a) Paper chromatography of the enzyme flavin with three different solvent

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein</th>
<th>Activity</th>
<th>Specific Activity</th>
<th>Yield</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude extract</td>
<td>930</td>
<td>8,690</td>
<td>15,400</td>
<td>1.76</td>
<td>100</td>
</tr>
<tr>
<td>First ammonium sulfate</td>
<td>135</td>
<td>6,380</td>
<td>13,500</td>
<td>2.11</td>
<td>88</td>
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<tr>
<td>Second ammonium sulfate</td>
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<td>6,010</td>
<td>12,500</td>
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<tr>
<td>Third ammonium sulfate</td>
<td>64</td>
<td>5,090</td>
<td>10,600</td>
<td>2.08</td>
<td>89</td>
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<tr>
<td>First crystallization</td>
<td>30</td>
<td>2,660</td>
<td>10,400</td>
<td>3.94</td>
<td>68</td>
</tr>
<tr>
<td>Second crystallization</td>
<td>20</td>
<td>695</td>
<td>10,400</td>
<td>15.3</td>
<td>68</td>
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<td>7.0</td>
<td>210</td>
<td>10,200</td>
<td>48.5</td>
<td>80</td>
</tr>
<tr>
<td>Fourth crystallization</td>
<td>5.4</td>
<td>154</td>
<td>7,780</td>
<td>30.3</td>
<td>51</td>
</tr>
</tbody>
</table>

5 A part of the enzyme activity remained in the supernatant if the mixture was centrifuged at a low speed.
that seen with FMN. However, FAD was completely ineffective. This was in contrast to the report of Sutton (2), who stated that FAD was as effective as FMN and riboflavin.

**Metal and Sugar Analyses of Crystalline Enzyme**

The analysis of metals by an emission spectrograph showed that both iron and copper were not present in the enzyme preparation. This result is consistent with the recent observation from

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6 We are indebted to Dr. Y. Kishida of the Department of Biology, Kanazawa University, for the photomicrograph.

7 The flavin analyses were carried out with the supernatant obtained after the apoprotein had been precipitated either by heating the enzyme in the presence of 0.5% ammonium sulfate at 80° for 15 min or by adding trichloroacetic acid to a concentration of 10%.

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FIG. 2. A phase contrast photomicrograph of crystalline lactate oxidase. 6 X 2300

systems revealed a yellow, fluorescent spot with the same mobility as authentic FMN. (b) The fluorescence of the enzyme flavin at 530 mp was fairly identical with that of authentic FMN when activated by light having wave lengths of 450, 375, and 260 mp. (c) The enzyme flavin was inactive with the aposalicylate hydroxylase from a pseudomonad which is specifically reactivated by FAD (18).

As a significant reactivation of the apoenzyme could not be demonstrated by Sutton (2), attempts were made to reactivate the apoenzyme with various kinds of flavin. The addition of FMN or flavin isolated from the enzyme* restored the specific activity to the extent of 60 to 80% of the original level, depending upon the experimental conditions; however, the initial activity was not recovered to completion. This might be due to the denaturation of part of the apoenzyme. In fact, the apoenzyme was unstable at neutral pH, and some precipitate appeared after several hours. As shown in Fig. 6, the apoenzyme was also reactivated by riboflavin, but the activity was much lower than

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Fig. 3. Polyacrylamide gel electrophoresis pattern of lactate oxidase. A sample of 7.5 µg dissolved in 0.1 M phosphate buffer, pH 7.0, was applied to a column (0.7 X 4.8 cm) and the run was made at 1.6 ma per tube for 60 min.

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Fig. 4. Ultracentrifugal patterns of lactate oxidase in 0.1 M phosphate buffer, pH 7.0. Sedimentation is from left to right. The numbers indicate the time in minutes after the rotor attained full speed (46,000 rpm). The average rotor temperature was 24.8°, and the protein concentration, 1.2%.
A molecular weight of 399,000 was calculated from the sedimentation pattern of the enzyme.

It is not clear whether the trace sugars are genuine components of the enzyme. However, it is not known whether the changes in absorbance were due to the presence of flavins. Aliquots were taken for measurement of activity.

The reactivation of the apoenzyme was performed by incubation with flavins over a period of 30 min at 0°C. The incubation mixture (0.7 ml) contained 10 μmoles of Tris-HCl buffer, pH 8.0; 10 μmoles of L-cysteine; 226 μg of apoenzyme; and indinonate (a thiol-reducing agent) was effective in the reactivation of the apoenzyme. The addition of glutathione or cysteine increased the degree of reactivation by about 20 to 30%.

**Molecular Weight**

From the sedimentation pattern, it was calculated that $s_{20,w}$ was $12.5 \times 10^{-13} \text{ cm}^2 \text{ g}^{-1} \text{ sec}^{-1}$ and that $D_{20,w}$ was $3.01 \times 10^{-12} \text{ cm}^2 \text{ sec}^{-1}$. A molecular weight of 399,000 was calculated from the sedimentation and diffusion data based on the assumption of a partial specific volume of 0.75. As the sedimentation constant was in agreement with Sutton's value, the main source of error in Sutton's lower value of molecular weight (260,000) was a high diffusion constant, which might be due to a slight inhomogeneity of the preparation. The molecular weight of the enzyme, further determined by the method of Archibald, was found to be 383,000. An estimate of the molecular weight was also made by the method of gel filtration as described by Andrews (22). As shown in Fig. 7, the elution peak of the enzyme was situated between catalase and apoferritin, and the elution volume was 143 ml. From the position of the elution volume of the enzyme on the standard curve, the molecular weight was estimated to be 340,000. In order to check the possibility that some kind of polymerization might take place during the crystallization, the crude enzyme preparation was applied to gel filtration under the same conditions that were used with the crystalline enzyme, except that the elution volume was determined by measurement of the enzyme activity. Only one single peak with the elution volume of 143 ml was observed, indicating that no conformational changes occurred during the purification process.

To determine a minimum molecular weight, the FMN content was calculated from the molecular absorption coefficient at 454 nm, and the protein content was estimated either by the biuret method, by Kjeldahl nitrogen (assumed to be 16% of the protein), or by dry weight. As shown in Table II, the weight of the protein per mole of FMN was 55,000 to 56,000. On the basis of these results, the conclusion is that the enzyme contains 6 to 7 moles of FMN per mole of protein. The minimum molecular weight had previously been reported by Sutton (3) to be 125,700. Sutton's value is much higher than that obtained either by the biuret method, by nitrogen content, or by dry weight. The main source of error in Sutton's higher value might be due to a faulty spectrophotometric estimation of the protein content as shown in Table II.

**Fig. 5.** Gel filtration of lactate oxygenase from Sephadex G-200. A sample of 23 mg was applied to a Sephadex G-200 column (2.6 × 50 cm) equilibrated with 0.05 M phosphate buffer, pH 7.0, containing 0.1 M KCl. Elution was carried out with the same buffer, and 3-ml fractions were collected at a flow rate of about 16 ml per hour. $O$, $A_{454}$; $\Delta$, $A_{280}$; $\square$, enzyme in units per ml.

**Fig. 6.** Effect of flavins on the reactivation of lactate oxygenase apoenzyme. The reactivation of the apoenzyme was performed by incubation with flavins over a period of 30 min at 0°C. The incubation mixture (0.7 ml) contained 10 μmoles of Tris-HCl buffer, pH 8.0; 10 μmoles of L-cysteine; 226 μg of apoenzyme; and indicated amounts of flavins. Aliquots were taken for measurement of activity. $O$, FMN; $\bullet$, flavin from the enzyme; $\bigcirc$, riboflavin; $\blacktriangle$, FAD.

Hayashi's laboratory which showed that metals are absent in the following monooxygenases which contain a flavin prosthetic group: salicylate hydroxylase (19), lysine oxygenase (19, 20), and imidazolacetate monooxygenase (19, 21).

A very small amount of sugar was found in the enzyme preparation. Hexose and hexosamine were detected in the amounts of 0.158 ± 0.004 and 0.120 ± 0.005%, respectively. These values correspond to 0.50 ± 0.02 mole of hexose and 0.38 ± 0.02 mole of hexosamine per 56,000 g of protein. However, it is not yet present clear whether the trace sugars are genuine components of the enzyme.
Mineral molecular weight of lactate oxygenase

The FMN content was calculated from the absorbance at 454 μm with the molecular extinction coefficient, 12.5 mm⁻¹ cm⁻¹.

<table>
<thead>
<tr>
<th>Method</th>
<th>Weight of protein per mole of FMN</th>
</tr>
</thead>
<tbody>
<tr>
<td>Dry weight</td>
<td>55,300</td>
</tr>
<tr>
<td>Nitrogen</td>
<td>55,800</td>
</tr>
<tr>
<td>Biuret</td>
<td>50,200</td>
</tr>
<tr>
<td>Spectrophotometry</td>
<td>129,500</td>
</tr>
</tbody>
</table>

* The enzyme solution, dialyzed for 48 hours against distilled water with an occasional change of the outer solution, was then dried to a constant weight in a drying oven at 60°.

** Protein nitrogen was determined by the Kjeldahl method.

The protein concentration was calculated from the absorbances at 280 and 260 μm with Kalckar’s equation (27).

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**Absorption Spectrum**

The absorption spectrum of lactate oxygenase is presented in Fig. 8. The peaks are situated at 375 and 454 μm, and shoulders occur at 480 and 430 μm. The addition of lactate to the enzyme under anaerobic conditions produced a bleaching of the visible color. The molar extinction coefficient at various peak wave lengths was calculated on the basis of direct analysis6 of FMN released from the protein moiety. The flavin of lactate oxygenase was quantitatively liberated by heating the enzyme in the presence of 0.5% ammonium sulfate at 80° for 15 min, followed by cooling to room temperature or acidification with trichloroacetic acid to a final concentration of 10%. The absorbance indexes (ε₄₈₅(FMN)⁻¹) at 280, 375, and 454 μm were 113.0, 7.06, and 12.5, respectively.

**Substrate Specificity and Kinetic Properties**

Of the various hydroxy acids tested, only L-lactate and α-hydroxy-n-butyrate served as substrates for lactate oxygenase, whereas the compounds such as α-hydroxy-isobutyrate, DL-β-hydroxy-n-butyrate, and glycolate were inactive. The apparent Kᵢ value for L-lactate was 2.5 × 10⁻³ M at pH 6.0. D-Lactate was almost inactive as a substrate for the enzyme. When L-lactate was used as a substrate, the activity was competitively inhibited by n-lactate (Fig. 9), so that DL-lactate was not suitable as a substrate for enzymatic studies. An apparent Kᵢ of 5.2 × 10⁻³ M for d-lactate was obtained. The pH optimum for the reaction with L-lactate was 6.0 in 0.1 M phosphate buffer.

**Acknowledgments**—We wish to thank Professor T. Isemura and Dr. K. Kakiuchi of the Institute for Protein Research, Osaka University, for the physicochemical measurements of molecular weight.

**REFERENCES**