Oxidation of D(−) Lactate by the Electron Transport Fraction of Azotobacter vinelandii

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D(−) Lactate oxidation in Azobacter vinelandii strain O is readily carried out by the membrane bound enzyme that concentrates in the electron transport fraction (R3). This oxidation in the R3 fraction is not dependent on externally added nicotinamide adenine dinucleotide, flavine adenine dinucleotide, or flavin mononucleotide. Phenazine methosulfate, O2, and menadione all served as good electron carriers, and the oxidation of lactate was limited to the D(−) stereoisomer. Of all the α-hydroxyacids examined, only D(−) lactate and D(−) α-hydroxybutyrate were oxidized by the R3 fraction. Paper chromatographic studies revealed that the 2,4-dinitrophenylhydrazine derivative formed from D(−) lactate oxidation was pyruvate. Pyruvate, in turn, could be further decarboxylated nonoxidatively by the R3 fraction. Spectral studies revealed that both the R3 flavoprotein and cytochrome (a2, a1, b1, c1, and c0) components were reduced by D(−) lactate. The D(−) lactic oxidase activity was sensitive to electron transport inhibitors, i.e., chlorpromazine, antimycin A, 2-n-heptyl-4-hydroxyquinoline-N-oxide, rotenone, dicumarol, and cyanide, and to a small extent to 4,4,4-trifluoro-1-(2-thienyl)-1,3-butane-dione (TFTB) and Amytal. The D(−) lactic phenazine methosulfate and menadione reductases were sensitive only to dicumarol and TFTB. Chlorpromazine was found to be a highly specific inhibitor of D(−) lactic oxidase activity, 50% inhibition occurring at 6.6 × 10−6 M.

The oxidation of lactate has long been observed in both mammalian and microbial systems. In mammalian tissue, and in some microbial systems, lactate oxidation is primarily nicotinamide adenine dinucleotide-dependent (34) and is specific for the L(+)-stereoisomer (19) although the oxidation of D(−)-α-hydroxyacids is known to occur to some extent in animal tissue (36). In addition, a flavoprotein-dependent type of lactate oxidation has been observed also which has been studied almost exclusively in microorganisms. The flavoprotein catalyzed reaction can be specific for either the L(+) or D(−) isomer of lactate. This type of flavoprotein dehydrogenase is usually associated with the intracytoplasmic membranes which also contain large concentrations of cytochromes (20, 26). Those organisms that have been reported to carry out the flavoprotein-dependent lactate oxidation are: Aerobacter aerogenes (27), Acetobacter peroxydans (5), Azotobacter vinelandii (4, 14, 20, 38), Bacillus megaterium (33), Escherichia coli (2, 13), Saccharomyces cerevisiae (1, 3, 10, 24, 30, and 31), Lactobacillus arabinosus (32), Leuconostoc mesenteroides (17), Mycobacterium phlei (6, 35), M. tuberculosis var. hominis (21), M. avium (6, 39), Propionibacterium pentosaceum (23), Pseudomonas natriegens (37), Staphylococcus aureus (22), and the ciliated protozoan, Tetrahymena pyriformis (7). A notable exception is the particulate fraction isolated from Leuconostoc mesenteroides which has been reported to carry out the oxidation of lactate in the absence of flavoprotein and pyridine nucleotide (17).

Preliminary investigations have indicated that lactate oxidation in A. vinelandii strain O is carried out by a particulate flavoprotein oxidoreductase that is associated with the electron transport fraction (4, 14, 20, 38). The Azotobacter electron transport fraction catalyzes the oxidation of reduced nicotinamide adenine dinucleotide phosphate, succinate, tetramethyl-p-phenylenediamine, and cytochrome c (4, 14, 15, 16, 20, 38) and is in many ways functionally similar to the electron transport system of mammalian mitochondria. A major difference between the two particulate electron transport systems is that in Azotobacter spp. there are several other flavoprotein-dependent oxidoreductases, which can carry out the oxidation of lactate and malate (4, 14, 20; P. Jurtshuk, C. H. Denton, and A. J. Bednarz, Bacteriol. Proc., 1967, p. 104), as well as an active hydrogenase (9). The purpose of this investigation was to examine and characterize the type of lactate oxidation that occurred in cell-free ex-
tracts of *A. vinelandii* strain O. Although it was previously shown that lactate oxidation in Azotobacter spp. was associated with the particulate fractions of cell-free extracts, the type of enzyme responsible for this oxidation has not been characterized with regard to the type of isomer oxidized, overall reaction that occurred, type of cofactors and acceptors required, and the effect of various inhibitors.

**Materials and Methods**

**Chemicals.** The following materials were obtained from the sources indicated: D(-)-lactic acid (crystalline), DL-alpha-hydroxyisobutyric acid, DL-alpha-hydroxyisovaleric acid, DL-L-alpha-hydroxyacaproic acid, Amytal, antimycin A, dicumarol, 2-n-heptyl-4-hydroxyquinoline-N-oxide (HQNO), rotenone, flavine adenine dinucleotide, flavine mononucleotide, from Sigma Chemical Co., St. Louis, Mo.; DL-L-sodium lactate, from Fisher Scientific Co., Pittsburgh, Pa.; phenazine methosulfate from Mann Research Laboratories, New York, N.Y.; 2,4-dinitrophenylhydrazine, 4,4,4-trifluoro-1-(2-thienyl)-1,3-butanedione (TFTB) from Eastman Organic Chemical Co., Rochester, N.Y.; sodium pyruvate, oxaloacetic acid, L(+)-lactic acid, DL-alpha-hydroxybutyric acid, D(+) malate, menadione (vitamin K$_3$) from Calbiochem, Los Angeles, Calif.; glycollic acid from Matheson Co., East Rutherford, N.J.; chlorpromazine hydrochloride, gift of Smith, Kline, & French, Philadelphia, Pa.

**Chemical methods.** Pyruvate was identified as the end product of D(-)-lactate oxidation by forming (29) and chromatographically characterizing the 2,4-dinitrophenylhydrazine derivative (28) in the conventional manner. Reaction-time intervals of 5 to 15 min and an enzyme concentration of approximately 0.5 mg/ml were used in studies where the keto end product of D(-)-lactate oxidation was trapped as the 2,4-dinitrophenylhydrazine derivative. Studies on reaction stoichiometry that involved the quantitative oxidation of D(-)-lactate as well as the complete decarboxylation of pyruvate required longer time intervals and greater enzyme concentrations as indicated in Table 1.

For use in manometric assays, a 1% solution (0.033 M) of phenazine methosulfate was prepared with deionized water, and a 2% solution (0.116 M) of menadione was prepared in 95% ethyl alcohol. Both solutions were stored in dark tubes and were used within 72 hr after preparation. Stock solutions of Amytal, antimycin A, HQNO, rotenone, and TFTB were prepared in 95% ethyl alcohol. A stock solution of dicumarol was prepared with 0.1 M NaOH; subsequent dilutions were carried out in deionized water. Stock solutions of chlorpromazine and potassium cyanide were made in deionized water. Protein was determined by a modification of the biuret method of Gornall, Bardawill, and David (8).

**Preparation of *A. vinelandii* O cell-free extracts.** The preparation of the Azotobacter R$_3$ fraction was described previously (15, 16). In Table 3 are summarized data showing specific activities for D(-) lactate oxidation and recovery of protein and the activity units which resulted from the fractionation procedure.

**Enzyme assays.** The D(-) lactate oxidase activity was assayed manometrically at 37 C by using air as the gas phase and conventional Warburg vessels of 15-ml capacity. The main compartment of the flask contained tris(hydroxymethyl)aminomethane (Tris)-chloride buffer (pH 8.5), 33.3 mM; the Azotobacter R$_3$ fraction, 0.33 to 0.83 mg of protein per ml; and deionized water to a total volume of 3.0 ml. The center well contained 0.2 ml of 2 M KOH solution. The side arm contained a solution of 100 μmole of D(-)-sodium lactate or 50 μmole of D(-)-sodium lactate which was added to start the reaction after the initial temperature equilibration period of 7 min.

The phenazine methosulfate reductase assay was identical to that described for the lactate oxidase assay, except that the reaction was assayed with potassium phosphate buffer (pH 7.0), 33.3 mM; and 3 mM KCN. In this assay, the phenazine methosulfate (0.2 ml of a 1% solution) was tipped from a second side arm at the same time that lactate was added to initiate the reaction.

Similarly, for the menadione reductase assay, the main compartment contained Tris-chloride buffer (pH 8.0), 33.3 mM; KCN, 3 mM; and the final concentration of the Azotobacter R$_3$ fraction ranged from 0.67 to 1.0 mg of protein per ml. Menadione (0.03 ml of a 2% solution in 95% ethyl alcohol) was added initially to the main compartment. All other features of the assay were identical to that of the oxidase assay.

Specific activities were expressed routinely in micro-liters of O$_2$ uptake per hour per milligram of protein at 37 C.

**Spectral studies.** For difference spectra studies (reduced minus oxidized), the Azotobacter R$_3$ fraction was suspended in 0.02 M phosphate buffer, pH 7.5, at a concentration of 10 mg of protein per ml. Steady-state reduction was achieved by adding D(-)-sodium lactate at a final concentration of 0.33 M. Chemical reduction was achieved by adding a few crystals of dithionite.

**Results**

**Stoichiometry of reaction.** The oxidation of lactate is carried out by the Azotobacter R$_3$ electron transport fraction according to the following overall reaction:

\[
\begin{align*}
\text{COOH} & \rightarrow \text{COOH} \\
2 \text{HC}==\text{OH} + \text{O}_2 & \rightarrow \text{C}=\text{O} + 2 \text{H}_2\text{O} \\
\text{CH}_3 & \text{CH}_3
\end{align*}
\]

Paper chromatography studies on the 2,4-dinitrophenylhydrazones formed from the end product verified that pyruvate was a major end product of the reaction. The 2,4-dinitrophenylhydrazine derivative of the unknown end product, as well as that of pyruvate, a-ketoglutarate, and oxaloacetate, were resolved and identified by paper chromatography by using three solvent systems previously described (28).

Assuming the complete enzymatic oxidation of...
that trapped uptake, pyruvate, indicating nonoxidatively, in turn, Pyruvate, was oxidized completely, by lactate to pyruvate, 2 μmoles of lactate are oxidized by 1 μmole of oxygen to 2 μmoles each of pyruvate and water. This reaction stoichiometry was readily confirmed (Table 1). For these studies, an excess of the Azotobacter R3 fraction was added to the reaction used to insure complete and rapid oxidation of 10 μmoles of D(−) lactate. Further studies on the reaction stoichiometry revealed that lactate can also be decarboxylated by the particulate Azotobacter R3 fraction (Table 1). When 10 μmoles of lactate was oxidized completely, 9.8 μmoles of CO2 was formed. In a similar study, when 10 μmoles of pyruvate was used as substrate, there was no oxygen uptake, although 9.1 μmoles of carbon dioxide was liberated after acidification.

This series of studies strongly suggested that the electron transport system of A. vinelandii readily oxidizes lactate to pyruvate which can be trapped as 2,4-dinitrophenylhydrazine derivative Pyruvate, in turn, can be further decarboxylated nonoxidatively, indicating the presence of a pyruvic decarboxylase in the Azotobacter cell-free extract. Preliminary observations indicated that CO2, acetaldehyde, and acetoin are the probable end products of this nonoxidative decarboxylation of pyruvate.

**Oxidation of D(−) lactic acid and other α-hydroxyacids.** The lactic oxidase in the Azotobacter R3 fraction also was examined for its ability to oxidize D(−) lactate as well as other α-hydroxy-acid substrates (Table 2). D(−) Lactate was oxidized readily at a rate of 244 μliters of O2 consumed per hr per mg of protein, whereas the L(+) isomer was inactive. The racemic mixture of D,L-lactate was oxidized at a rate of 249 μliters of O2 uptake per hr per mg of protein, a specific activity equivalent to that observed for the D(−) stereoisomer. Of the series of other α-hydroxyacid substrates tested, only D,L-α-hydroxybutyrate was oxidized. The activity noted for this substrate represented 81% of the activity obtained for D(−) lactate. No other α-hydroxyacid tested was oxidized by the Azotobacter R3 fraction. Glycollate (or α-hydroxyacetate) was not oxidized, which indicated that the D(−) lactic oxidase of the R3 fraction required at least three carbons for activity. D(+)-Malate, the unnatural isomer of the acid, also was not oxidized, indicating that an addition of a carboxyl group to the third carbon atom of D(−) α-hydroxyacid renders this substrate inactive. The L(−) malate can be oxidized at appreciable rates by the Azotobacter R3 electron transport (4, 14, 20; J.urtshuk, C. H. Denton, and A. J. Bednarz, Bacteriol. Proc., p. 104, 1967).

This same pattern of substrate specificity for α-hydroxyacids is reminiscent of that reported for the D(−) lactic cytochrome c reductase from aerobically grown S. cerevisiae (12). In contrast, it has been reported (10, 30) that a large number of D(−) α-hydroxyacids were capable of being oxidized by the soluble D(−)-α-hydroxyacid dehydrogenase isolated from anaerobically grown S. cerevisiae.

**Effects of flavine adenine dinucleotide and flavine mononucleotide on D(−) lactate oxidation.** Since other known D- and L-lactic acid oxidoreductases are flavoprotein enzymes, they possess either flavine adenine dinucleotide or flavine mononu-

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**Table 1. Reaction stoichiometry for the oxidation of D(−) lactate and decarboxylation of pyruvate by the Azotobacter R3 fraction**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Conc (μmoles/vessel)</th>
<th>O2 consumedab (μmoles)</th>
<th>Theory (%)</th>
<th>CO2 evolvedb (μmoles)</th>
<th>Theory (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Actual</td>
<td>Theory</td>
<td>Actual</td>
<td>Theory</td>
</tr>
<tr>
<td>D(−) Lactate</td>
<td>10.0</td>
<td>4.7</td>
<td>5.0</td>
<td>95</td>
<td>9.8</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>10.0</td>
<td>0.0</td>
<td>0.0</td>
<td>0</td>
<td>9.1</td>
</tr>
</tbody>
</table>

* Protein concentration of the R3 fraction used was 7.3 mg/ml; reaction time was 3 hr.
* Protein concentration of the R3 fraction used was 6.8 mg/ml; reaction time was 30 min.

**Table 2. Comparative study on the ability of Azotobacter R3 fraction to oxidize D(−) lactate and other related α-hydroxyacid substrates**

<table>
<thead>
<tr>
<th>Substratea</th>
<th>Conc (μm)</th>
<th>Oxidase activity Specific activity</th>
<th>Activity</th>
</tr>
</thead>
<tbody>
<tr>
<td>D(−) Lactate</td>
<td>16.7</td>
<td>244</td>
<td>100</td>
</tr>
<tr>
<td>D,L-Lactate</td>
<td>33.3</td>
<td>249</td>
<td>102</td>
</tr>
<tr>
<td>D,L-α-Hydroxybutyrate</td>
<td>33.3</td>
<td>198</td>
<td>81</td>
</tr>
</tbody>
</table>

* The following substrates were not oxidized: L(+)- lactate (16.7 mM), D,L-α-hydroxyisobutyrate (33.3 mM), D,L-α-hydroxyisovalerate (33.3 mM), D,L-α-hydroxycaproate (33.3 mM), glycollate (33.3 mM), and D(+)- malate (33.3 mM).
*Expressed in microliters of O2 uptake per hour per milligram of protein (37 C).
cleotide as prosthetic groups (3, 11, 25). A study was undertaken to determine the effect of these two flavine cofactors on D(-) lactate oxidation by the Azotobacter R3 fraction. Neither flavine adenine dinucleotide nor flavine mononucleotide stimulated this oxidation, regardless of the terminal acceptor used (phenazine methosulfate, menadione, or molecular O2). No attempt was made to identify the types of flavine components present in the Azotobacter R3 fraction, since the spectral characteristics of this fraction reveal the presence of a large concentration of flavoprotein (16).

Development of assay and enzyme kinetics. The effects of various concentrations of D(-) lactate on the activity of the Azotobacter R3 fraction by using the three different electron acceptors are shown in Fig. 1. This composite figure shows the comparative activities obtained when the three electron acceptors are used as oxidants. Specific activity is plotted as a function of D(-) lactate concentration. The highest specific activities and Vmax value were obtained when phenazine methosulfate was used as electron acceptor. The natural acceptor, molecular oxygen, also gave a relatively high Vmax value for lactate oxidation, whereas menadione gave the lowest value. All acceptors appeared to be saturated at approximately 1.5 mM of D(-) lactate. First-order kinetics were displayed by the D(-) lactic oxidase system at concentration levels approaching and slightly more than the 1.5 mM level of D(-) lactate, whereas the assay with phenazine methosulfate and menadione exhibited first-order kinetics at a much lower substrate concentration level, approximately 0.75 mM. At higher D(-) lactate concentrations, the oxidation rate increased only gradually with increasing substrate concentration and only for the oxidase and menadione reductase assays. Analyses by the double-reciprocal plot (insert, Fig. 1) for the oxidase assay gave a Km value for D(-) lactate of 0.003 M and a Vmax value of 714 μliters of O2 uptake per hr per mg of protein at 37 C. The phenazine methosulfate reductase had a Km value for D(-) lactate of 0.002 M and a Vmax value of 909. The menadione reductase exhibited the lowest Km value for D(-) lactate, or 0.0008 M and a Vmax value of 333. The

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**Fig. 1.** Effect of D(-) lactate concentration on the lactic oxidase, phenazine methosulfate reductase, and menadione reductase in the Azotobacter R3 fraction.
final D(-) lactate concentration selected for use in all three assay systems was 0.0167 M. This concentration was approximately 6, 8, and 20 times the \( K_m \) value for the lactic oxidase, phenazine methosulfate reductase, and menadione reductase, respectively.

**Distribution of enzymatic activities for \( \mu \)(-) lactate oxidation in cell-free extracts.** A representative fractionation scheme showing the distribution of activity units for \( \mu \)(-) lactate oxidation in cell-free extracts is shown in Table 3. These fractions were obtained by differential centrifugation from sonically disrupted resting cells of *A. vinelandii*. The residue fractions are designated "R" and the supernatant fractions are designated "S." The assays developed for the three electron acceptors (molecular \( O_2 \), phenazine, methosulfate, and menadione) were performed on each of the fractions. The \( S_1 \) fraction or the first supernatant fraction was collected after centrifugation of the sonic homogenate at 4,300 \( \times g \) for 10 min. This removed all unbroken whole cells, as well as the large cellular debris matter. Centrifugation of the \( S_2 \) fraction at 37,000 \( \times g \) for 20 min gave two fractions, the \( S_3 \) and \( R_3 \), which contained 80\% and 13\% protein, respectively. Further centrifugation of the \( S_3 \) fraction at 144,000 \( \times g \) for 2 hr separated the \( S_3 \) from the \( R_3 \) fraction. The former contained 50\% of the remaining protein, and the latter contained 38\%. The highest activity for lactate oxidation was found in the \( R_3 \) particulate fraction, which exhibited approximately a two- to threefold purification when compared to the original \( S_1 \) extract. The \( S_2 \) fraction exhibited little or no activity for \( \mu \)(-) lactate oxidation, regardless of the electron acceptor used. All subsequent analyses revealed that the greatest number of activity units were recovered in the particulate \( R_3 \) fraction. The percentage of recovery of enzyme units in the \( R_3 \) fraction (from the \( S_1 \) fraction) was approximately 64\% for the \( \mu \)(-) lactic oxidase, 91\% for the phenazine methosulfate reductase assay, and 49\% for the menadione reductase assay.

**Difference spectrum of the Azotobacter \( R_3 \) fraction.** Studies on the steady-state reduction that directly affects the oxidation-reduction components of the Azotobacter \( R_3 \) (Fig. 2) verified that a substantial proportion of the electron transport

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**Table 3. Distribution of activity units for \( \mu \)(-) lactate oxidation in fractions obtained by differential centrifugation from sonically disrupted resting cells of Azotobacter vinelandii**

<table>
<thead>
<tr>
<th>Azotobacter fractions</th>
<th>Protein recovery (%)</th>
<th>Specific activity* and recovery of activity units</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>( O_2 )</td>
</tr>
<tr>
<td>( S_1 )</td>
<td>100</td>
<td>91</td>
</tr>
<tr>
<td>( S_2 )</td>
<td>80</td>
<td>80</td>
</tr>
<tr>
<td>( R_2 )</td>
<td>13</td>
<td>102</td>
</tr>
<tr>
<td>( S_3 )</td>
<td>100</td>
<td>80</td>
</tr>
<tr>
<td>( R_3 )</td>
<td>50</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>80</td>
<td>193</td>
</tr>
</tbody>
</table>

*Expressed in microliters of \( O_2 \) uptake per hour per milligram of protein (37 C).

\(^b\) Calculated by multiplying total protein concentration by specific activity.
components, viz., the cytochromes and flavoprotein, were reduced by both D(-) lactate and dithionite. Reduction of cytochromes $a_1 + a_2$ (590 and 629 $\mu m$, respectively), cytochrome $b_1$ (561 and 531 $\mu m$), and cytochromes $c_1 + c_2$ (553 and 523 $\mu m$) was readily accomplished by the addition of lactate. However, a much smaller percentage of total flavoprotein appeared to be reduced by lactate. The trough at 457 $\mu m$ was characteristic of bleaching obtained for flavoprotein and possibly nonheme iron reduction. Complete reduction of all the oxidation-reduction components was obtained by the addition of a few crystals of sodium dithionite.

Effects of inhibitors. The effects of various electron transport inhibitors on the D(-) lactic oxidase activity in the $R_3$ fraction are shown in Fig. 3. Chlorpromazine proved to be the most potent inhibitor, 50% inhibition occurring at a concentration of $6.6 \times 10^{-4}$ M. Complete inhibition with chlorpromazine was obtained at a concentration of $1.0 \times 10^{-4}$ M. Neither the L-malic oxidase nor the succinoxidase were so sensitive to chlorpromazine. Similar inhibition patterns for the D(-) lactic oxidase were observed with antimycin A, HQNO, and cyanide. With antimycin A, 50% inhibition occurred at approximately $5 \times 10^{-4}$ M; with HQNO and cyanide, 50% inhibition occurred at a level of approximately $10^{-4}$ M. Comparative studies were made on the D(-) lactic oxidase and succinoxidase in the same Azotobacter $R_3$ preparations, and it was observed that the latter was consistently 10 times more sensitive to HQNO and cyanide. This suggests that there might be two different pathways with electron transport chains, one predominantly for D(-) lactate oxidation and the other for succinate oxidation.

Rotenone showed a peculiar inhibition pattern which was nonlinear in comparison to the other inhibitors tested. Extrapolation of the rotenone curve shows a theoretical 50% inhibition at $2.5 \times 10^{-4}$ M. This unusual type of inhibitor response by rotenone can perhaps be best explained by its insolubility in aqueous systems at high concentrations.

Dicumarol and TFTB appeared to be the poorest inhibitors of D(-) lactic oxidase. These inhibitors displayed 50% inhibitions at $4.5 \times 10^{-4}$ M and $6.0 \times 10^{-4}$ M, respectively. One interesting aspect of this study was that TFTB, a specific inhibitor of succinate oxidation, inhibited the D(-) lactic oxidase in the Azotobacter $R_3$ fraction at the same concentration at which it inhibited succinoxidase.

The most effective inhibitors of the D(-) lactic oxidase activity were chlorpromazine, antimycin A, HQNO, and cyanide.

The same electron transport inhibitors (Fig. 3) were tested for their ability to inhibit the D(-) lactic phenazine methosulfate and menadione reductase activities. The concentrations of inhibitors used for this study were those which inhibited the oxidase by 50%. Only dicumarol and TFTB inhibited the D(-) lactic phenazine methosulfate and menadione reductase; the inhibition observed was approximately equal to that noted for the oxidase assay. This suggests that both dicumarol

![Fig. 3. Patterns of inhibition observed with various electron transport inhibitors on the D(-) lactic oxidase of the Azotobacter $R_3$ fraction.](http://jb.asm.org/Downloaded from http://jb.asm.org/on October 20, 2017 by guest)
and TFTB inhibit the D(−) lactate oxidation at or close to the flavoprotein site, whereas all other inhibitors function at a site beyond the flavoprotein region.

**Discussion**

There are at least four known classes of flavoprotein-dependent lactic oxidoreductases that have been studied in microbial systems. One type, which catalyzes the enzymatic oxidation of the L(+) isomer of lactate, has been characterized in aerobically grown yeast (1) and in the *Myco-

*bacterium* sp. (6, 35). In yeast, this enzyme initially concentrates in the particulate fraction and is referred to as L(+) lactic dehydrogenase (EC 1.1.2.3) or the “cytochrome b₅₅₃” type preparation. Appleby and Morton (1) were able to crystallize this “flavohemoprotein” by use of butanol and acetone fractionation procedures. One of the prosthetic groups was identified as protoheme and flavine mononucleotide. Much less, however, is known about the soluble flavoprotein L(−)-lactic oxidative decarboxylase (EC 1.1.3.2) of *M. phlei* (35). This enzyme carries out the direct oxidation of L-lactate (by molecular oxygen), forming acetate and CO₂. Studies on the crystalline enzyme have revealed that pyruvate competitively inhibits lactate oxidation and that flavine mononucleotide is the other of the prosthetic groups present on the enzyme.

The oxidation of the D(−) stereoisomer of lactate can occur also via the flavoprotein-type oxidoreductases. In addition to *Azotobacter* spp. (4, 14, 20, 38), oxidation specific for the D(−) lactate isomer has been reported in *L. mesen-

*teroides* (17), *P. pentosaceum* (23), and *S. cerevisiae* (10, 24, 31). Two enzymes of this class have been examined in microorganisms, i.e., the D(−) lactic cytochrome c reductase (EC 1.1.2.4) and the D(−) α-hydroxyacid dehydrogenase (EC 1.1.99.6). In cell-free homogenates, the former is found in the particulate fraction of aerobically grown *S. cerevisiae*, namely, that having electron transport function. The latter, or the D(−) α-hydroxyacid dehydrogenase, has been found in the soluble fraction of anaerobically grown yeast (3, 30) as well as in animal tissues (36). Extensive studies on the D(−) lactate cyto-

*ochrome c reductase revealed that it oxidizes solely D(−) lactate and D(−) α-hydroxybutyrate. The prosthetic group for D(−) lactic cytochrome c reductase is flavine adenine dinucleotide, and it also contains bound Zn²⁺, whose concentration is stoichiometric with the flavine (25, 30). It has been referred to as the “metalloflavoprotein” type enzyme. In contrast, the D(−) α-hydroxyacid dehydrogenase is capable of oxidizing most other D(−) α-hydroxyacids, in addition to D(−) lac-

tate and D(−) α-hydroxybutyrate. The prosthetic group for the D(−) α-hydroxyacid dehydrogenase is also flavine adenine dinucleotide, and the purified enzyme is believed to contain divalent metal ions (3, 30).

The oxidation of D(−) lactate by the *Azotobacter* Rs fraction exhibits certain similarities and differences when compared to the two microbial D(−) lactic oxidoreductases described. The D(−) lactic cytochrome c reductase isolated specifically from aerobically grown *S. cerevisiae* most closely resembles the flavoprotein dehydrogenase of *A. vinelandii*. In both microorganisms, enzymatic activity is located in the respiratory particles capable of carrying out electron transport function. Further similarity is noted in the substrate specificity pattern. Of all the α-hydroxyacids tested, both the *Azotobacter* and *Saccharomyces* particulate enzymes were capable of oxidizing only D(−) lactate and D(−)-α-hydroxybutyrate (Table 2). This specificity suggests that the D(−) lactic oxidase of the *Azotobacter* Rs fraction is not the “general” type of D(−)-α-hydroxyacid dehydro-

genase that is found in the soluble fraction in *S. cerevisiae* grown under anaerobic conditions (3).

Stimulation of D(−) lactate oxidation by either flavine adenine dinucleotide or flavine mononucleotide could not be demonstrated at the level of purification achieved for the *Azotobacter* Rs fraction. However, studies on the highly purified yeast D(−)-lactic cytochrome c reductase of aerobic yeast indicated that the flavine adenine dinucleotide is the functional flavoprotein moiety which was reversibly dissociated from the apo-

enzyme (12).

Phenazine methosulfate was found to be a good electron acceptor for the D(−) lactate oxidation in the *Azotobacter* Rs fraction; this acceptor also serves as a good electron carrier for the purified yeast D(−) lactic cytochrome c reductase. The *Kₘ* and *Vₒₘₖₖ* values for phenazine methosulfate reduction with the *Azotobacter* Rs fraction were 0.00015 M and 385 μl/h per mg of protein, respectively. The latter value corresponds to 0.578 μmoles of D(−) lactate oxidized per min per mg of protein. The corresponding values for phenazine methosulfate reduction with the highly purified yeast D(−) lactic cytochrome c reductase were 0.00545 M and 1,670 μmoles of D(−) lactate oxidized per min per mg of protein. The comparable yeast particle (from which the enzyme is isolated), or the equivalent of the *Azotobacter* Rs electron transport fraction, exhibited a specific activity of 0.4 μmoles of D(−) lactate oxidized per min per mg of protein (12).

In comparing the capability of the *Azotobacter* Rs fraction to use various electron acceptors for
D(−) lactate oxidation, it was noted that most of the electron carriers tested served as acceptors. Menadione, particularly, was found to be an effective electron acceptor, giving a \( V_{\text{max}} \) value of 1.5 μmoles of D(−) lactate oxidized per min per mg of protein at infinite menadione concentration. In addition to phenazine methosulfate and menadione, 2,6-dichlorophenolindophenol, ferricyanide, and methylene blue all served as good electron acceptors, whereas nitrotetrazolium blue and cytochrome c functioned poorly as acceptors. It is probable that this response pattern to various electron acceptors will change when the D(−) lactic oxidoreductase of \( A. \ vinelandii \) is purified to the extent achieved for \( S. \ cerevisiae \). The sites at which some of these acceptors react may be lost or modified on further purification.

The specificity for electron acceptors observed for the Azotobacter \( R_3 \) electron transport system is different from that reported for the yeast D(−) lactic cytochrome c reductase (30). Menadione, ferricyanide, 2,6-dichlorophenolindophenol were all inactive and only phenazine methosulfate and cytochrome c served as electron acceptors for the purified yeast enzyme. In this respect, the Azotobacter \( R_3 \) and yeast enzyme systems differed markedly. It was observed that, with the exception of molecular O\(_2\) and phenazine methosulfate, the specificity for electron acceptors by the Azotobacter \( R_3 \) fraction more closely resembled that reported for the D(−) a-hydroxyacid dehydrogenase found in anaerobically grown \( S. \ cerevisiae \) (30); whereas, with regard to substrate specificity, the Azotobacter \( R_3 \) system more closely resembled the D(−) lactic cytochrome c reductase of aerobically grown yeast.

D(−) Lactate oxidation in the Azotobacter \( R_3 \) fraction appears to be carried out by the electron transport system. This fact is borne out by the effect of the various electron transport inhibitors on the D(−) lactic oxidase activity (Fig. 3). Among the most effective inhibitors were ampicillin A, HQNO (18), and cyanide, all known inhibitors of electron transport. The involvement of the electron transport system is further substantiated by the data presented in Fig. 2, where steady-state reduction studies show that almost all of the oxidation-reduction components of the \( A. \ vinelandii \) electron transport chain are reduced by D(−) lactate. This is particularly true of the cytochrome components \((a_3, a_1, b_1, c_1, \) and \( c_0 \)) which are substantially reduced by lactate, when compared to the extent of reduction achieved by dithionite. In contrast, only a small percentage of the flavoprotein appears to be reduced by the addition of lactate, suggesting that the lactic flavoprotein oxidoreductase is probably present as a minor component in the \( A. \ vinelandii \) \( R_3 \) electron transport system.

One unexpected finding was the sensitivity of the D(−) lactate oxidation to chlorpromazine. The oxidase activity was inhibited 50% at a \( 6.6 \times 10^{-4} \) M concentration of chlorpromazine. This concentration level had no effect on the D(−) lactic phenazine methosulfate or menadione reductase activities, thus indicating that it acts somewhere after the flavoprotein site.

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**LITERATURE CITED**


