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

PETER JURTSHUK AND LAHOMA HARPER

Department of Microbiology, The University of Texas, Austin, Texas 78712

D(−) Lactate oxidation in *Azobacter vinelandii* strain O is readily carried out by the membrane bound enzyme that concentrates in the electron transport fraction (R_3). This oxidation in the R_3 fraction is not dependent on externally added nicotinamide adenine dinucleotide, flavine adenine dinucleotide, or flavine mononucleotide. Phenazine methosulfate, O_2, 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 R_3 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 R_3 fraction. Spectral studies revealed that both the R_3 flavoprotein and cytochrome (a_2, a_1, b_1, c_4, and c_5) 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 mitochrondria. 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), D,L-α-hydroxyisobutyric acid, D,L-α-hydroxyisovaleric acid, D,L-α-hydroxycaproic 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.; D,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, D,L-α-hydroxybutyric acid, D(+) malate, menadione (vitamin K₃) 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-dinitrophenylhydrazone 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 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* R₃ 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(−) lactic 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 (*p*H 8.5), 33.3 mM; the *Azotobacter* R₃ 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 μmoles of DL-sodium lactate or 50 μmoles 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 lactic oxidase assay, except that the reaction was assayed with potassium phosphate buffer (*p*H 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.
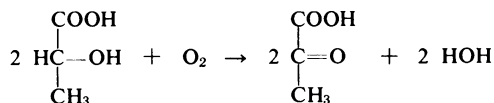
Similarly, for the menadione reductase assay, the main compartment contained Tris-chloride buffer (*p*H 8.0), 33.3 mM; KCN, 3 mM; and the final concentration of the *Azotobacter* R₃ fraction ranged from 0.67 to 1.0 mg of protein per ml. Menadione (0.03 ml of a 2% solution in 95% ethyl alcohol) was added 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 microliters of O₂ uptake per hour per milligram of protein at 37.C.

Spectral studies. For difference spectra studies (reduced minus oxidized), the *Azotobacter* R₃ fraction was suspended in 0.02 M phosphate buffer, *p*H 7.5, at a concentration of 10 mg of protein per ml. Steady-state reduction was achieved by adding DL-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* R₃ electron transport fraction according to the following overall reaction:

$$2 \; \underset{\underset{\text{CH}_3}{|}}{\overset{\overset{\text{COOH}}{|}}{\text{HC}}}{-}\text{OH} \;+\; \text{O}_2 \;\rightarrow\; 2 \; \underset{\underset{\text{CH}_3}{|}}{\overset{\overset{\text{COOH}}{|}}{\text{C}}}{=}\text{O} \;+\; 2 \; \text{HOH}$$

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, α-ketoglutarate, and oxaloacetate, were resolved and identified by paper chromatography by using three solvent systems previously described (28).

Assuming the complete enzymatic oxidation of

TABLE 1. *Reaction stoichiometry for the oxidation of* D(−) *lactate and decarboxylation of pyruvate by the Azotobacter* R_3 *fraction*

Substrate	Concn (μmoles/vessel)	O_2 consumed[a] (μmoles)		Theory (%)	CO_2 evolved[b] (μmoles)		Theory (%)
		Actual	Theory		Actual	Theory	
D(−) Lactate	10.0	4.7	5.0	95	9.8	10.0	98
Pyruvate	10.0	0.0	0.0	0	9.1	10.0	91

[a] Protein concentration of the R_3 fraction used was 7.3 mg/ml; reaction time was 3 hr.

[b] Protein concentration of the R_3 fraction used was 6.8 mg/ml; reaction time was 30 min.

TABLE 2. *Comparative study on the ability of Azotobacter R_3 fraction to oxidize* D(−) *lactate and other related α-hydroxyacid substrates*

Substrate[a]	Concn	Oxidase activity	
		Specific[b] activity	Activity
	mM		%
D(−) Lactate	16.7	244	100
D,L-Lactate	33.3	249	102
D,L-α-Hydroxy-butyrate	33.3	198	81

[a] 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).

[b] Expressed in microliters of O_2 uptake per hour per milligram of protein (37 C).

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* R_3 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* R_3 fraction (Table 1). When 10 μmoles of lactate was allowed to be oxidized completely, 9.8 μmoles of CO_2 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 CO_2, acetaldehyde, and acetoin are the prob-

able end products of this nonoxidative decarboxylation of pyruvate.

Oxidation of D(−) *lactic acid and other α-hydroxyacids.* The lactic oxidase in the *Azotobacter* R_3 fraction also was examined for its ability to oxidize D(−) lactate as well as other α-hydroxyacid substrates (Table 2). D(−) Lactate was oxidized readily at a rate of 244 μliters of O_2 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 O_2 uptake per hr per mg of protein, a specific activity equivalent to that observed for the D(−) stereoisomer. Of the series of other α-hydroxy-acid 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* R_3 fraction. Glycollate (or α-hydroxyacetate) was not oxidized, which indicated that the D(−) lactic oxidase of the R_2 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* R_3 electron transport (4, 14, 20; P. Jurtshuk, 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-

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 V_{max} value were obtained when phenazine methosulfate was used as electron acceptor. The natural

acceptor, molecular oxygen, also gave a relatively high V_{max} 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 K_m value for D(−) lactate of 0.003 M and a V_{max} value of 714 μliters of O₂ uptake per hr per mg of protein at 37 C. The phenazine methosulfate reductase had a K_m value for D(−) lactate of 0.002 M and a V_{max} value of 909. The menadione reductase exhibited the lowest K_m value for D(−) lactate, or 0.0008 M and a V_{max} value of 333. The
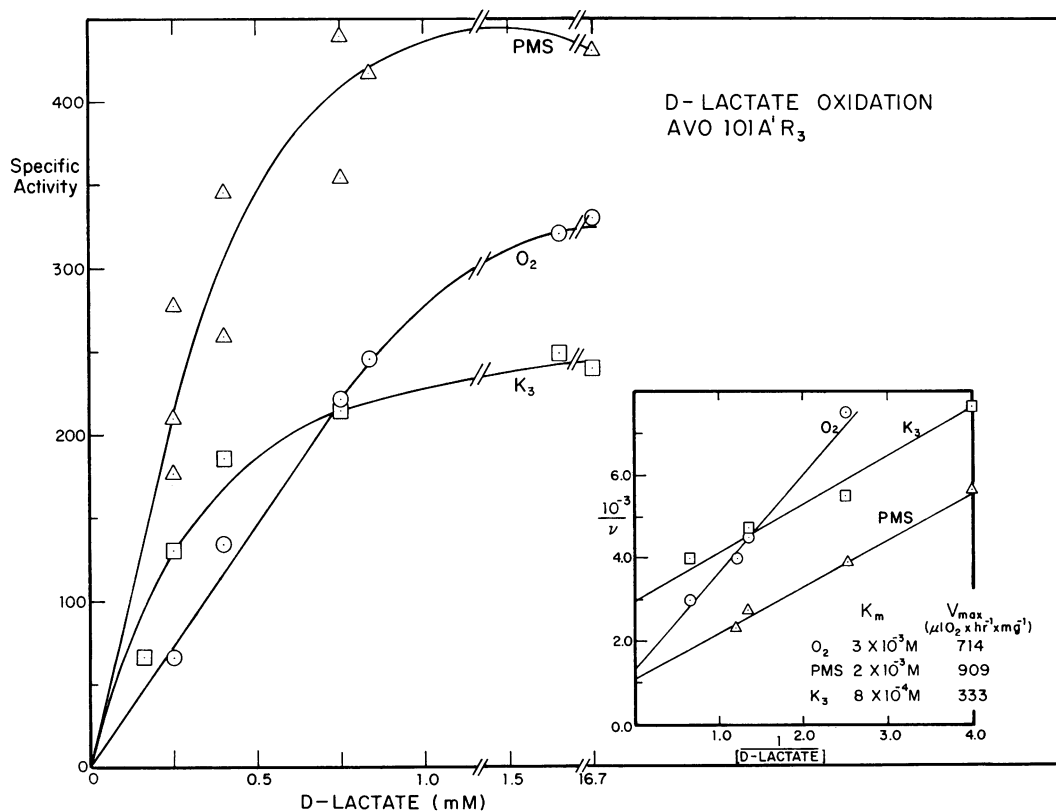

FIG. 1. *Effect of D(−) lactate concentration on the lactic oxidase, phenazine methosulfate reductase, and menadione reductase in the Azotobacter R₃ fraction.*

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 D(−) lactate oxidation in cell-free extracts. A representative fractionation scheme showing the distribution of activity units for D(−) 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 × g for 10 min. This removed all unbroken whole cells, as well as the large cellular debris matter. Centrifugation of the S_1 fraction at 37, 000 × g for 20 min gave two fractions, the S_2 and R_2, which contained 80% and 13% protein, respectively. Further centrifugation of the S_2 fraction at 144,000 × 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_3 fraction exhibited little or no activity for D(−) 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 D(−) 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
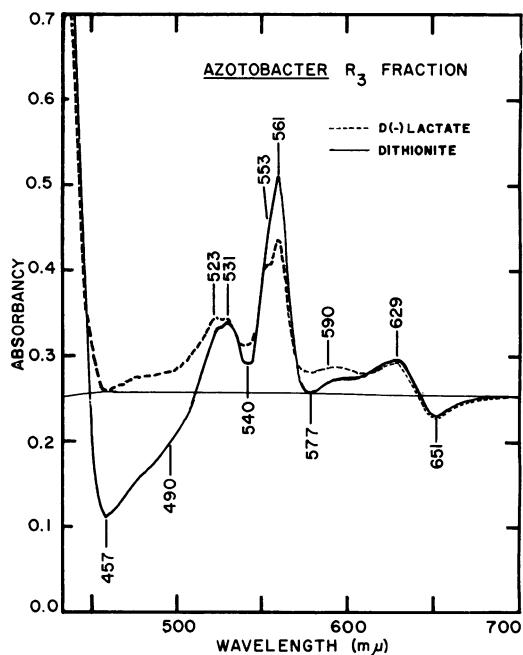

FIG. 2. *Difference spectrum (reduced minus oxidized) exhibiting the steady-state reduction of the redox components in the Azotobacter R_3 electron transport fraction. Dashed line shows the reduction achieved by the addition of D(−) lactate; solid line represents the reduction by dithionite. Concentration of the R_3 fraction was 10 mg of protein in 1.0 ml of 0.02 M phosphate buffer, pH 7.5.*

TABLE 3. *Distribution of activity units for D(−) lactate oxidation in fractions obtained by differential centrifugation from sonically disrupted resting cells of Azotobacter vinelandii*

A zotobacter fractions	Protein recovery (%)	Specific activity[a] and recovery of activity units					
		O_2	Total units[b] (%)	PMS	Total units[b] (%)	K_3	Total units[b] (%)
S_1	100	91	100	114	100	94	100
S_2	80	80	70	103	72	95	81
R_2	13	102	14	191	22	107	15
S_2	100	80	100	103	100	95	100
S_3	50	0	0	39	19	0	0
R_3	38	193	91	344	126	152	60

[a] 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 flavo-
protein, were reduced by both D(−) lactate and
dithionite. Reduction of cytochromes $a_1 + a_2$
(590 and 629 mμ, respectively), cytochrome
b_1 (561 and 531 mμ), and cytochromes $c_4 + c_5$ (553
and 523 mμ) 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 mμ was
characteristic of bleaching obtained for flavo-
protein 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 elec-
tron 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 × 10⁻⁶ M. Complete inhibi-
tion with chlorpromazine was obtained at a con-
centration of 1.0 × 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 approxi-
mately 5 × 10⁻⁵ M; with HQNO and cyanide,
50% inhibition occurred at a level of approxi-
mately 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 sug-

gests that there might be two different pathways
with electron transport chains, one predominantly
for D(−) lactate oxidation and the other for suc-
cinate oxidation.

Rotenone showed a peculiar inhibition pattern
which was nonlinear in comparison to the other
inhibititors tested. Extrapolation of the rotenone
curve shows a theoretical 50% inhibition at 2.5
× 10⁻⁴ M. This unusual type of inhibitor response
by rotenone can perhaps be best explained by its
insolubility in aqueous systems at high concen-
trations.

Dicumarol and TFTB appeared to be the poor-
est inhibitors of D(−) lactic oxidase. These inhibi-
tors displayed 50% inhibitions at 4.5 × 10⁻⁴ M
and 6.0 × 10⁻⁴ M, respectively. One interesting
aspect of this study was that TFTB, a specific in-
hibitor of succinate oxidation, inhibited the D(−)
lactic oxidase in the *Azotobacter* R_3 fraction at
the same concentration at which it inhibited suc-
cinoxidase.

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 menadine re-
ductase activities. The concentrations of inhibi-
tors 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
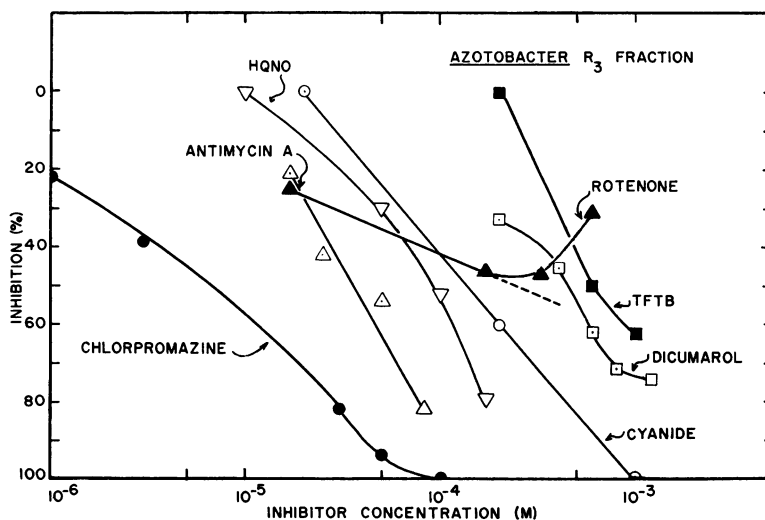oxidase assay. This suggests that both dicumarol

FIG. 3. *Patterns of inhibition observed with various electron transport inhibitors on the* D(−) *lactic oxidase of
the Azotobacter* R_3 *fraction.*

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 *Mycobacterium* 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 b_2" type preparation. Appleby and Morton (1) were able to crystallize this "flavohemoprotein" by use of butanol and acetone fractionation procedures. Its prosthetic groups were 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 CO_2. Studies on the crystalline enzyme have revealed that pyruvate competitively inhibits lactate oxidation and that flavine mononucleotide is one 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. mesenteroides* (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 cytochrome 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 Zn^{++}, whose concentration is stoichiometric with the flavine (25, 30). It has been referred to as a "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* R_3 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* R_3 fraction is not the "general" type of $D(-)$-α-hydroxyacid dehydrogenase 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* R_3 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 apoenzyme (12).

Phenazine methosulfate was found to be a good electron acceptor for the $D(-)$ lactate oxidation in the *Azotobacter* R_3 fraction; this acceptor also serves as a good electron carrier for the purified yeast $D(-)$ lactic cytochrome c reductase. The K_m and V_{max} values for phenazine methosulfate reduction with the *Azotobacter* R_3 fraction were 0.00015 M and 385 μliters of O_2 uptake per hr 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 0.00445 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* R_3 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* R_3 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_{max} value of 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 exeption 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(−) α-hydroxyacid dehydrogenase found in anaerobically grown *S. cervisiae* (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 antimycin 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, whereby 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_2, a_1, b_1, c_4, and c_5) 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 6.6×10^{-6} 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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