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# LACTIC DEHYDROGENASES OF *PSEUDOMONAS NATRIEGENS*

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## ABSTRACT

WALKER, HAZEL (University of Georgia, Athens), AND R. G. EAGON. Lactic dehydrogenases of *Pseudomonas natriegens*. J. Bacteriol. **88**:25-30. 1964.—Lactic dehydrogenases specific for D- and L-lactate were demonstrated in *Pseudomonas natriegens*. The L-lactic dehydrogenase showed considerable heat stability, and 40% of the activity remained in extracts after heating at 60 C for 10 min. An essential thiol group for enzyme activity was noted. The results of these experiments were consistent with the view that lactate was dehydrogenated initially by a flavin cofactor and that electrons were transported through a complete terminal oxidase system to oxygen. The intracellular site of these lactic dehydrogenases was shown to be the cell membrane. It was suggested that the main physiological role of these lactic dehydrogenases is that of lactate utilization.

The marine bacterium, *Pseudomonas natriegens*, was reported to produce acidic products when cultivated aerobically in media containing glucose. These acidic products consist mainly of acetic, lactic, and pyruvic acids (Payne, Eagon, and Williams, 1961). Although this is reminiscent of "coliform-type" of heterolactic fermentation, there is the major difference that *P. natriegens* forms these products aerobically. Similarly, *P. natriegens* was demonstrated to utilize predominantly an aerobic Embden-Meyerhof pathway, even though enzymes of the hexose monophosphate pathway were also detected in extracts (Eagon and Wang, 1962). Reduced nicotinamide adenine dinucleotide (NADH) formed during the operation of the Embden-Meyerhof pathway was presumably oxidized through participation of the cytochrome system with oxygen as the terminal electron acceptor. At the same time, evidence was obtained for the operation of the Krebs tricarboxylic acid cycle.

With the foregoing in mind, the question of how lactic acid is metabolized by *P. natriegens* appeared to be pertinent. Although lactic dehydrogenases derived from animal sources are considered to be nicotinamide adenine dinucleotide (NAD)-linked (Singer and Kearney, 1954), lactic dehydrogenases from many microorganisms are not NAD-linked. Some examples are those of *Acetobacter peroxydans* (De Ley and Schel, 1959), *Escherichia coli* (Haugaard, 1959), *Tetrahymena pyriformis* (Eichel and Rem, 1962), and *Saccharomyces cerevisiae* (Nygaard, 1961). Similarly, preliminary evidence indicated that lactic dehydrogenase of *P. natriegens* also was not NAD-linked (Lobley and Eagon, 1961). Thus, the experiments described in this communication were undertaken to characterize the lactic dehydrogenase of *P. natriegens*.

## MATERIALS AND METHODS

*Cultivation of organism and preparation of cell-free extracts. P. natriegens* was cultivated at 30 C on a rotary shaker in a basal salts-sea salt medium containing dehydrated nutrient broth and glucose as previously described (Eagon and Wang, 1962), modified by the addition of 3 g of dehydrated yeast extract per liter.

Cell-free extracts were prepared by sonic oscillation and were dialyzed as previously described (Eagon and Wang, 1962). Protein determinations of the extracts were made with the Folin reagent described by Lowry et al. (1951).

*Preparation of cell membranes.* Cells of *P. natriegens* were suspended in 0.03 M tris(hydroxymethyl)aminomethane (tris) buffer (pH 8.0) containing lactose to 0.2 M and 200 $\mu$g/ml of ethylenediaminetetraacetic acid (EDTA). This suspension was adjusted to an optical density of 0.5 to 0.6 at 420 m$\mu$. Lysozyme was added to a concentration of 30 $\mu$g/ml and the mixture was incubated at 30 C for 20 min. The resulting spheroplasts were then osmotically ruptured by the sudden addition of 4 volumes of cold, distilled water, and this reaction mixture was centrifuged at 34,000 $\times$ *g* for 15 min. The supernatant fluid was decanted and discarded. The loosely packed top layer of the pellet, which was composed of

cell membranes, was carefully separated from the densely packed lower layer of cellular debris. The membranes were then washed three times by resuspending in 0.052 M $MgCl_2$ and collected by centrifugation. After the final wash, the membranes were resuspended in 0.1 M tris buffer (pH 7.5). Electron microscopy revealed both intact and fragmented membranes.

*Starch-gel electrophoresis.* Zone electrophoresis of cell-free extracts in starch gel to demonstrate lactic dehydrogenase patterns was carried out according to the technique of Lindsay (1963), modified by the use of 0.03 M borate buffer (pH 8.6). The reaction mixture for the visualization of lactic dehydrogenase was modified by the omission of NAD and by the use of tris buffer (pH 8.3).

*Lactic dehydrogenase assay.* Lactic dehydrogenase activity of the extracts was determined either by measuring the rate of pyruvate formation from lactate or by measuring the rate of reduction of dyes by the Thunberg technique.

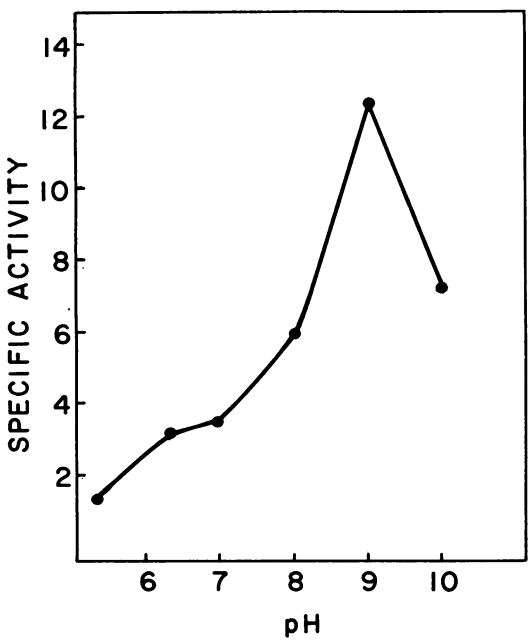


FIG. 1. *Optimal pH of lactic dehydrogenase in extracts of Pseudomonas natriegens determined by the Thunberg technique. The buffer systems employed were as follows: acetate buffer (pH 5.4); phosphate buffer (pH 6.4 and 7.0); tris buffer (pH 8.0 and 9.0); glycine buffer (pH 10.0). Specific activity: per cent reduction per min per mg of protein (corrected for endogenous enzyme activity).*

For the former method, the following reaction mixture was employed: 100 μmoles of tris buffer (pH 9.0); 10 μmoles of D- or L-lactate; 0.1 ml of diluted extract containing 0.2 to 0.3 mg of protein; total volume, 1.0 ml. The reaction was carried out at 37 C. Pyruvate was assayed according to the method of Friedemann and Haugen (1943), and the resulting "chromogen" was measured with a Klett-Summerson colorimeter with filter no. 42. Under these experimental conditions, pyruvate was found not to be further metabolized by extracts. Thus, its accumulation with respect to time was a valid measure of lactic dehydrogenase activity.

The Thunberg technique was carried out according to the method suggested by Burris (1951).

## RESULTS

*Optimal pH.* The Thunberg technique, which uses methylene blue as the hydrogen acceptor, was employed to determine the optimal pH of lactic dehydrogenase. Optimal activity occurred at pH 9.0 (Fig. 1). Reagent grade DL-lactate (J. T. Baker Chemical Co., North Phillipsburg, N.J.) was used for these experiments.

*Time-rate experiments.* Formation of pyruvate is linear with respect to time (Fig. 2). Activity was noted for both D- and L-lactate (Mann Research Laboratory, New York, N.Y.). The rate of reaction was not altered by the addition of NAD, nicotinamide adenine dinucleotide phosphate (NADP), flavin adenine dinucleotide (FAD), or flavin mononucleotide (FMN) to the reaction mixture. Furthermore, passage of extract through a column of acid-washed Norit A did not alter activity.

*Evidence for two enzymes.* The difference in activities for D- and L-lactate suggested the presence of more than one lactic dehydrogenase, of a lactate racemase, or of a nonspecific lactic dehydrogenase. The results of thermal inactivation experiments (Fig. 3) indicated that activity for D-lactate is rapidly lost when the extract is heated at 60 C. Heating for 6 min at 60 C destroyed 99% of the activity for D-lactate, whereas only 20% of the activity for L-lactate was lost. L-Lactate dehydrogenase, therefore, showed considerable heat stability, and 40% of the activity remained after heating at 60 C for 10 min. These results, therefore, confirmed the presence of two enzyme systems.

*Inhibitor studies.* The results in Table 1 indicate the effect of various inhibitors on lactic dehydrogenase enzymes in extracts of *P. natriegens*. With the exception of *p*-chloromercuribenzoic acid (*p*CMB), these inhibitors are known to inhibit at specific points in the terminal oxidase system. Inhibition by antimycin A could be reversed with phenazine methosulfate (PMS). The results are consistent, therefore, with the view that lactate is dehydrogenated initially by a flavin cofactor, and that electrons are passed through a complete terminal oxidase system to oxygen. Dehydrogenation of D- and L-lactate was inhibited to similar extents.

Inhibition by *p*CMB pointed to the existence of an essential thiol group for enzyme activity. This inhibition was fully reversed by the reducing agents, glutathione or cysteine.

*Natural and artificial electron and hydrogen acceptors.* The results in Table 2 indicate the hydrogen or electron acceptors capable of functioning in the lactate dehydrogenase reactions. These experiments were carried out with the Thunberg technique. They revealed that dyes with positive potentials served readily as acceptors. On the other hand, doubtful reduction of $K_3Fe(CN)_6$ was observed. Whether this was due to its high potential or to other factors could not be discerned from these experiments.

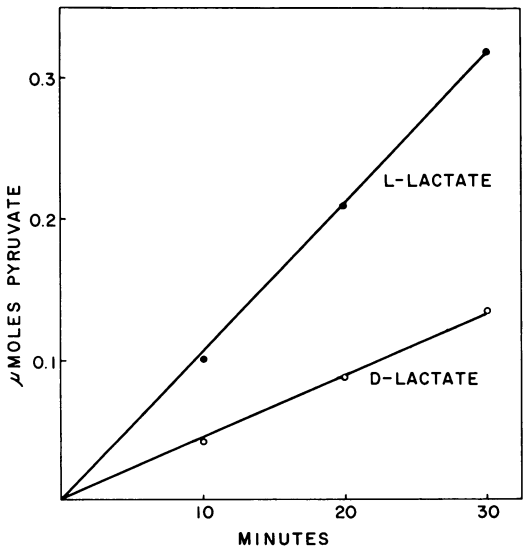


FIG. 2. *Time-rate of lactic dehydrogenase enzyme systems in extracts of Pseudomonas natriegens. Protocol is given in text.*

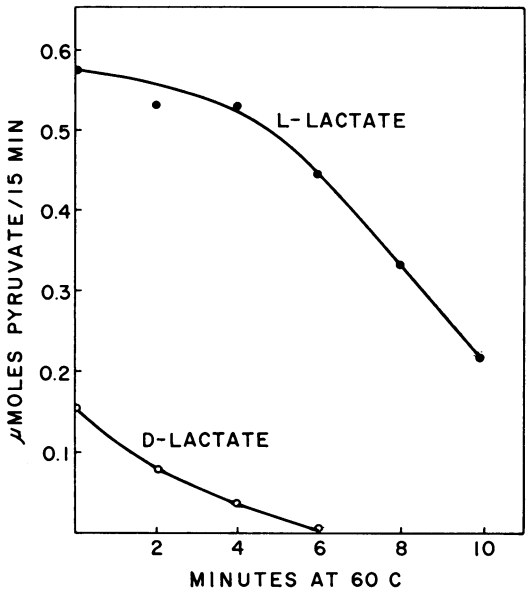


FIG. 3. *Thermal inactivation experiments. Extracts of Pseudomonas natriegens were heated at 60 C for the time intervals indicated. Lactic dehydrogenase activity was measured as μmoles of pyruvate produced in 15 min according to the assay procedure given in the text.*

TABLE 1. *Effect of inhibitors on lactic dehydrogenase activity in extracts of Pseudomonas natriegens with D- and L-lactate as substrates*

| Inhibitor | Final concn | Per cent inhibition | |
|---|---|---|---|
| | | L-Lactate | D-Lactate |
| | *M* | | |
| Amytal | $5 \times 10^{-3}$ | 36 | 44 |
| Atabrine | $10^{-3}$ | 25 | — |
| HOQNO* | $5 \times 10^{-4}$ | 73 | 60 |
| Antimycin A | $10^{-4}$ | 65 | 76 |
| KCN | $10^{-3}$ | 88 | 79 |
| $NaN_3$ | $10^{-2}$ | 55 | 62 |
| *p*CMB† | $10^{-3}$ | 64 | — |

* HOQNO: 2-*n*-heptyl-4-hydroxyquinolin-N-oxide.

† *p*CMB: *p*-chloromercuribenzoic acid.

When NADH was substituted for lactate as substrate, FAD was readily reduced, indicating that the experimental procedure employed was satisfactory. An important observation was the greater affinity shown for PMS by the D-lactic dehydrogenase system, indicating a major difference between D- and L-lactic dehydrogenase

enzyme systems. Other acceptors served equally well for both systems.

*Starch-gel electrophoresis.* Results of zone electrophoresis of extracts in starch gel for a period of 5.5 hr are shown in Fig. 4. Visualization of the lactic dehydrogenase systems revealed only a single band which had migrated approximately 1 cm from the electrophoretic origin toward the cathode. Both D- and L-lactic dehydrogenase systems were present in this single band. This band, furthermore, was composed of many dark, purple particles giving a stippled appearance rather than an evenly, homogeneously stained area. This suggested that the lactic dehydrogenase activity was located in a subcellular particulate fraction in the extract rather than as a "soluble" enzyme system.

TABLE 2. *Natural and artificial electron acceptors for lactic dehydrogenase enzyme systems in extracts of Pseudomonas natriegens with* D- *and* L-*lactate as substrates*

| Electron acceptor | $E_0'$ (at pH 7) | Specific activity* | |
|---|---|---|---|
| | | L-Lactate | D-Lactate |
| | *v* | | |
| Neutral red | −0.325 | 0 | 0 |
| Janus green | −0.225 | 0 | 0 |
| Flavin adenine dinucleotide | −0.226 | 0 | 0 |
| Flavin mononucleotide | −0.185 | 0 | 0 |
| 2,3,5-Triphenyltetrazolium | −0.080 | <1† | <1† |
| Nitro blue tetrazolium | −0.050 | 1 | 1 |
| Methyl viologen | −0.050 | <1 | — |
| Methylene blue | +0.011 | 13 | 12 |
| Phenazine methosulfate | +0.080 | 16 | 49 |
| 2,6-Dichlorophenol indophenol | +0.218 | 12 | 11 |
| $K_3Fe(CN)_6$ | +0.360 | <1† | <1† |

* Specific activity: per cent reduction per min per mg of protein (corrected for endogenous enzyme activity).

† Reduction of these substances occurred slowly, requiring several hours.

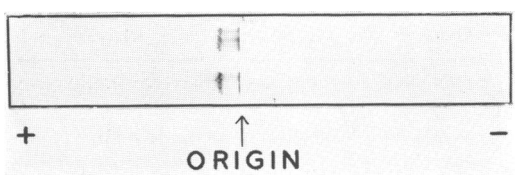


FIG. 4. *Zone electrophoresis of extracts of Pseudomonas natriegens in starch gel. The lactic dehydrogenase systems are represented by the stippled dark bands near the electrophoretic origin. Two transverse slits were cut in the gel, and filter paper strips saturated with extract were inserted. See text for protocol.*

TABLE 3. *Evidence that lactic dehydrogenase systems are located in cell membranes of Pseudomonas natriegens with* D- *and* L-*lactate as substrates*

| Enzyme fraction | Specific activity* | |
|---|---|---|
| | L-Lactate | D-Lactate |
| Crude sonic extract | 65† | 27† |
| Lysozyme supernatant | 0 | 0 |
| | 35 | 4 |
| | 36 | — |
| Cell membranes | 173 | 125 |
| | 506 | 225 |
| | 696 | — |

* Specific activity: μmoles of pyruvate per min per mg of protein × 1,000.

† Average values from several experiments.

*Location of lactic dehydrogenase activity in cell membranes.* Attempts to purify lactic dehydrogenases by ammonium sulfate precipitation were not successful, in that activity was found in all fractions with little purification. The observation was also made that a considerable portion of the activity was located in particles sedimented by high-speed centrifugation of extracts. This evidence, plus evidence from electrophoretic experiments, suggested that the lactic dehydrogenase systems may be located in the cell membrane. Results in Table 3 confirm that these systems are located in the cell membrane. Little activity was observed in the supernatant fraction (i.e., lysozyme supernatant fluid) after removal of cell membranes from the reaction mixture containing osmotically ruptured spheroplasts. Results from three experiments with L-lactate and two experiments with D-lactate as substrates are shown.

*Anaerobic experiments.* Lactic dehydrogenase systems in extracts prepared from *P. natriegens* cultured anaerobically had the same characteristics as did those from aerobically cultured

microorganisms. No additional cofactor requirements were noted, and the enzyme systems appeared to have unaltered activities.

## DISCUSSION

The results of these studies point to the existence of two lactic dehydrogenases in *P. natriegens* specific for D- and L-lactate. Although these two enzyme systems were shown to have similar characteristics, differences were noted in their degree of heat lability and in their affinity for PMS as an electron acceptor.

No evidence was found for the participation of NAD as cofactors. Neither dialysis of extracts nor the addition of NAD to the dialyzed extracts altered the rate of reaction. Similarly, neither reduction of FAD nor of FMN was observed when lactate was used as substrate. Reduction of these cofactors would have been highly probable if NAD were involved. On the other hand, this evidence in no way detracts from the supposition that lactate is dehydrogenated initially by enzyme-bound FAD or FMN, because transhydrogenation between enzyme-bound and added FAD or FMN would be unlikely.

Results from experiments with specific respiratory inhibitors indicated that a complete respiratory chain was involved. Inhibition was noted with Atabrine and Amytal. The former is considered to be a specific inhibitor for flavoprotein (Hellerman, Lindsay, and Bovarnick, 1946), and the latter was reported to inhibit near the lowest member of the respiratory chain (Chance, 1957), either between substrate and flavoprotein (Packer, 1958) or between flavoprotein and coenzyme Q (Hatefi et al., 1962). Participation of the cytochrome cofactors was indicated by inhibition with antimycin A and 2-*n*-heptyl-4-hydroxyquinolin-N-oxide (HOQNO), which inhibit between cytochromes *b* and *c*, and by inhibition with KCN and $NaN_3$, which are inhibitors of cytochrome oxidase (Chance, 1957; Hatefi et al., 1962; Packer, 1958). Thus, the lactic dehydrogenases of *P. natriegens* appear to be hemeflavoproteins similar to those of certain other microorganisms. These lactic dehydrogenases, exemplified by the yeast enzyme, were shown to contain flavoprotein and cytochrome $b_2$ and, correspondingly, to reduce cytochrome *c* (Morton, 1961).

Participation of the terminal oxidase system suggested that enzyme systems of the cell membrane were involved. Similarly, experiments with zone electrophoresis of extracts in starch gel suggested that the lactic dehydrogenases were located in a subcellular "particulate" fraction rather than occurring as "soluble" enzymes. These observations were confirmed when 95 to 100% of the lactic dehydrogenase activity was shown to be present in isolated cell membranes. Similarly, "aerobic" lactic dehydrogenases from other bacteria were reported to be located in the cell membrane (Smith, 1961).

These results give little indication of the physiological role played by the lactic dehydrogenases of *P. natriegens*. At the same time, the mechanism of lactic acid synthesis remains obscure. Although *P. natriegens* was reported to produce lactic acid, recent experiments indicate that, under fully aerobic conditions, less than 1% of the glucose actually catabolized is converted to lactic acid (*unpublished data*). Thus, lactic acid would appear not to be an important or essential product of *P. natriegens* metabolism. Furthermore, other experiments showed that resting cells can utilize lactate without a period of inducement. When all the data are considered, the argument can be advanced that the main physiological role of the lactic dehydrogenases of *P. natriegens* is that of lactate utilization.

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