Ferredoxin in the Phosphoroclastic Reaction of Pyruvic Acid and Its Relation to Nitrogen Fixation in Clostridium pasteurianum

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We have reported recently that nitrogen fixation in cell extracts of Clostridium pasteurianum is dependent upon transitory intermediates from pyruvate metabolism (1–3). Thus, fixation has been observed to occur only while pyruvate is being actively consumed; the mole ratio of pyruvate consumed to N₂ fixed approximated 100:1. Except for 2-ketobutyrate, no replacement for pyruvate has been found among many compounds and combinations tested. The predominant pathway of pyruvate utilization in these extracts involves oxidative decarboxylation by the so-called phosphoroclastic reaction.

$$\text{CH}_3\text{C}==\text{COOH} + \text{H}_2\text{PO}_4^- \underset{\text{enzymes}}{\rightarrow} \text{CH}_3\text{COPO}_3\text{H} + \text{CO}_2 + \text{H}_2$$

Several different enzyme systems causing the same net pyruvate reaction in various organisms have been reported (4–11). Their one common feature is the presence of pyruvic dehydrogenase activity. Although pyruvic dehydrogenase has not been identified as a single enzyme common to the several systems, it appears to be a modified carboxylase in which thiamine pyrophosphate (6–9) is closely coupled with one or more electron-transferring components. The possible presence of an electron acceptor as an integral part of the carboxylase molecule has not been identified as a single enzyme common to the several systems, it appears to be a modified carboxylase in which thiamine pyrophosphate (6–9) is closely coupled with one or more electron-transferring components. The possible presence of an electron acceptor as an integral part of the carboxylase molecule when this enzyme is constituted as pyruvic dehydrogenase has not been established, but electron transport is known to involve lipoic acid in Lactobacillus delbrueckii (9) and to involve flavin adenine dinucleotide in Escherichia coli (7, 8) and to involve flavin adenine dinucleotide in Lactobacillus delbrueckii (9). Lipoic acid is not part of the system in Clostridium butyricum (6). Terminal electron transport operates through hydrogenase, where H₂ formation occurs in various anaerobes (6, 10), whereas in other enzyme systems the electrons are absorbed in reducing various carbon compounds. Coenzyme A and phosphotransacetylase are required in E. coli (7) and various clostridia (6, 10, 11) but not in L. delbrueckii (9).

Little has been reported about the phosphoroclastic system in C. pasteurianum, and accordingly the studies reported here were undertaken particularly to explore its relationship to nitrogen fixation. The work has led to the discovery of a new electron-transferring protein, which we have named ferredoxin. It contains iron but apparently not heme or flavin groups, and it stimulates a variety of enzymic reactions in which H₂ is formed or utilized. Preliminary publications on its preparation and properties have appeared (12, 13). Confirmation and extensions have been reported by Professor D. I. Arnon (14), to whom information on ferredoxin was communicated before publication.

EXPERIMENTAL PROCEDURE

C. pasteurianum Cell Extracts— Cultures were grown and harvested by methods previously reported (2), except that the medium was modified to contain 0.01 g of MnSO₄·H₂O per liter. Cells were disrupted for preparation of extracts by the autolysis method previously described (2). Phosphate concentration was varied according to the purpose as specified in the figures and tables, but for N₂ fixation it was 0.01 to 0.02 M. Endogenous phosphate from the cells usually corresponded to an increment of 0.003 M in extracts prepared to contain 30 mg of protein per ml.

Preparation of Dialyzed Enzymes—Endogenous coenzyme A and phosphate were removed by precipitation and dialysis of the protein. A cell extract containing 20 g of protein was treated with 2.5 g of protamine sulfate and then centrifuged, and the supernatant solution was treated with solid (NH₄)₂SO₄ (70% saturation). The resulting protein precipitate thus obtained was dialyzed anaerobically at 3°C for 60 hours against 18 liters of H₂O in three changes of 6 liters each, dried by evaporation at 0.05 mm pressure in a rotating vacuum evaporator, and stored in a desiccator at –10°C for use.

Preparation of “Minus-ferredoxin” Enzymes—Ferredoxin was separated from the phosphoroclastic system by means of the DEAE-cellulose method recently described (12) or by an alternative 2-propanol method as follows. Crude cell extract corresponding to 18 g of protein in 500 ml of 0.02 M phosphate buffer, pH 6.5, at 3°C was agitated with a magnetic stirrer while 350 ml of 2-propanol precooled to –20°C were added in dropwise fashion. The protein precipitate, which represented 98% of the original quantity, was the “minus-ferredoxin” preparation. It was essentially inactive for the phosphoroclastic reaction but regained 25 to 50% of the original activity when supplied with appropriate amounts of ferredoxin or methyl viologen.

Ferredoxin—Preparation and 60-fold purification of ferredoxin were performed by the methods recently described (12, 13). Assays were based on its ability to catalyze formation of acetyl phosphate in pyruvate oxidation in the phosphoroclastic preparations (12), also the formation of H₂ from Na₂S₂O₄ in hydrogenase preparations (12, 15).
Assay of Acetyl Phosphate or Acetate Formation from Pyruvate

Assays for phosphoroclastic or pyruvic dehydrogenase activity were conducted in test tubes without excluding air. A typical reaction mixture consisted of 10 mg of protein as minus-ferredoxin enzyme preparation, 110 μmoles of sodium pyruvate, 0.13 μmole of coenzyme A, 50 μmoles of potassium phosphate buffer, pH 6.5, up to 0.2 mg of ferredoxin, depending upon its activity (or 200 μg of methyl viologen), and H₂O to 1 ml. The mixture was incubated for 15 minutes at 30°; the reaction was terminated by addition of 1 ml of 2 M neutral NH₄OH. Acetyl phosphate was measured as acetylamidic acid by the method of Lipmann and Tuttle (16). Acetate was determined by the acetate kinase method (17). The amount of ferredoxin fraction used was chosen to give an optical density of 0.1 to 0.3 at 530 μg in a Lumetron colorimeter in this procedure. Ferredoxin assays were made with the same batch of minus-ferredoxin enzymes, which was dried and stored at -10° to preserve it as a standard for comparisons.

Assay of H₂ from Pyruvate and O₂ Uptake—Standard manometric techniques were used (18). The standard reaction mixture, in the main compartment of a Warburg flask, contained 200 μmoles of sodium pyruvate, 100 μmoles of potassium phosphate, pH 6.5, 60 units of CoA, and up to 0.5 mg of ferredoxin or 200 μg of methyl viologen. The side arm contained 5 to 10 mg of protein as a minus-ferredoxin enzyme preparation. Total volume was 3 ml. Incubation was at 30°. The center well contained 0.2 ml of 20% KOH. The gas phase was argon or N₂ for H₂ production experiments, and an atmosphere of air was used to measure O₂ uptake. When CO₂ formation was measured, alkali was omitted from the center well, and the atmosphere was CO to inhibit hydrogenase and block H₂ formation.

Other Assays—Protein was determined by the biuret method (19) or the Lowry modification of the Folin procedure (20). N₂ fixation was assayed by a modified micro-Conway NH₃ titration (21). Pyruvate was determined by the 2,4-dinitrophenylhydrazone method of Friedemann and Haugen (22).

Materials—Coenzyme A (300 units per mg), glutathione, and 2-mercaptoethanol were purchased from California Corporation for Biochemical Research. Methyl viologen and benzyl viologen were purchased from British Drug Houses, Ltd.

RESULTS

Phosphoroclastic Cleavage of Pyruvate—Experiments on the mechanism of pyruvate oxidation by C. pasteurianum showed that coenzyme A served as the initial acceptor for the acetyl group generated from pyruvate. Other thiol were found to serve as acetyl acceptors (Table I), but only in the presence of catalytic amounts of coenzyme A.

Experiments freed from coenzyme A by dialysis (for 60 hours at 3° under N₂) did not catalyze the reduction of electron-accepting dyes such as methylene blue or benzyl viologen unless coenzyme A was added; these results suggest that a suitable acetyl acceptor is required for oxidation to occur. Our data on the phosphoroclastic reaction of C. pasteurianum indicated that it is closely similar to that of the other butyric clostridia on which reports have been published (6, 10, 23-25).

Role of Ferredoxin in Electron Transport—Preliminary reports have been published on the preparation and properties of ferredoxin, a new electron-transferring protein that stimulates the phosphoroclastic reaction as well as various other biological oxidation-reductions requiring electrons at voltages near that of the H₂ electrode (12, 13). It was obtained for the first time in the course of fractionation experiments on cell extracts of C. pasteurianum during the present studies.

Electron transport in the phosphoroclastic reaction of C. pasteurianum was investigated with the aid of procedures permitting separation of the cell extracts into two fractions, one containing ferredoxin and the other containing the balance of the phosphoroclastic system in reconstitutables condition. Procedures for preparing the phosphoroclastic enzymes free of ferredoxin are described in “Experimental Procedure” and in previous publications (12, 13). When these preparations were made with sufficient speed and protection from air, as much as 90% of their original phosphoroclastic activity could be restored by adding either ferredoxin or methyl viologen in appropriate amounts. However, preparations obtained under more convenient working conditions usually regained no more than 50% of their original activity when supplied with these substances. Typical results are presented in Figs. 1 to 3. Failure to regain 100% of the original activity was attributed to sensitivity to

**Table I**

<table>
<thead>
<tr>
<th>Thiol</th>
<th>Thiolacete ester formed*</th>
</tr>
</thead>
<tbody>
<tr>
<td>2-Mercaptoethanol</td>
<td>5.6</td>
</tr>
<tr>
<td>2-Mercaptoacetic acid</td>
<td>2.9</td>
</tr>
<tr>
<td>Glutathione</td>
<td>0.7</td>
</tr>
<tr>
<td>2-Mercaptoethylamine</td>
<td>0.0</td>
</tr>
</tbody>
</table>

* Expressed as micromoles formed per mg of protein per hour.
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FIG. 2. Catalysis of phosphoroclastic reaction by ferredoxin (Fd). A, The mixture contained 4.12 mg of protein as minus-ferredoxin enzymes preparation obtained by the DEAE-cellulose method (12), 60 units of CoA, 200 μmoles of sodium pyruvate, 100 μmoles of potassium phosphate buffer, ferredoxin as indicated, and H₂O to 1 ml, at pH 6.5. Incubation was at 30° for 15 minutes in air. B, The mixture contained 10.2 mg of protein as the minus-ferredoxin enzyme preparation obtained by the 2-propanol method, 200 μmoles of sodium pyruvate, 200 μmoles of potassium phosphate, 60 units of CoA, 0.41 mg of ferredoxin, and H₂O to 3 ml, at pH 6.5. The center well contained 0.2 ml of 20% KOH. Incubation was at 30° under argon.

FIG. 3. Catalysis of phosphoroclastic reaction by ferredoxin (Fd). Mixtures contained the indicated weight of protein as minus-ferredoxin enzyme preparation by the DEAE-cellulose method (12), 60 units of CoA, 200 μmoles of sodium pyruvate, 50 μmoles of potassium phosphate buffer, ferredoxin as indicated, and H₂O to 1 ml, at pH 6.5. Incubation was at 30° for 15 minutes in air.

Air, which was particularly noticeable in hydrogenase and in pyruvic dehydrogenase. Exposure to air for 30 minutes at 30° resulted in complete and irreversible loss of pyruvate-oxidizing activity. The optimal pH for the phosphoroclastic reaction in these preparations was approximately pH 6.1 with ferredoxin and about pH 6.7 with methyl viologen.

Since methyl viologen, an oxidation-reduction dye, was able to restore phosphoroclastic activity in these preparations, their deficiency was seen to be in electron transport. Because ferredoxin likewise restored phosphoroclastic activity, the function of ferredoxin was seen to be in electron transport of the phosphoroclastic reaction.

Reduction of ferredoxin is accompanied by a decrease in spectral absorption in the vicinity of 400 μM. This property has been employed to investigate details of its operation in electron transport. Ferredoxin appeared to be 50 to 60% in the reduced form under steady state conditions during the phosphoroclastic reaction according to the observed decrease in optical density at 420 μM (Fig. 4). The maximal decrease in optical density obtainable (0.16 optical density unit per 150 units of ferredoxin per 3 ml in Fig. 4) was estimated by admitting CO, which imposes a reversible inhibition on hydrogenase (3). CO does not inhibit the activity of ferredoxin or pyruvic dehydrogenase, as indicated in Fig. 4, where reduction of ferredoxin and methylene blue by pyruvic acid occurred equally rapidly under either CO or argon. The results of the foregoing experiments indicate that ferredoxin functions between pyruvic dehydrogenase and hydrogenase rather than between hydrogenase and H₂; thus

Pyruvic dehydrogenase → ferredoxin → hydrogenase → H₂

Whether other electron carriers participate in this sequence cannot be answered from present information.

Reduction of ferredoxin by pyruvate in the C. pasteurianum system depends on coenzyme A but not on phosphate (Fig. 5). In preparations from which coenzyme A had been removed, no detectable reduction of ferredoxin by pyruvate occurred until coenzyme A was added, whereupon reduction occurred almost immediately. Results were the same with or without phosphate present. Coenzyme A did not itself reduce ferredoxin either alone or in the presence of the enzymes (minus pyruvate). These results are in accord with the observation already mentioned that coenzyme A is required for both the formation of acetyl phosphate and the reduction of dyes by pyruvate. Coenzyme A and ferredoxin appear to be functioning together in enabling pyruvic dehydrogenase to act, but whether they function simultaneously or one before the other cannot be decided from present results.

Oxygen as Electron Acceptor in Pyruvate Oxidation—The coenzymes or other functional units in pyruvic dehydrogenase from C. pasteurianum have not been identified. However, on the basis of information on the corresponding enzyme from other organisms (6-9), it presumably contains thiamine pyro-
phosphate for carboxylase activity and a coupled electron acceptor. Also, on the basis of such analogy, the decarboxylated substrate-enzyme complex presumably would be represented by hydroxylsulfhydrylamine pyrophosphate (26-32) or the equivalent acetyl derivative with electrons transferred to a closely coupled electron acceptor, possibly an integral part of the enzyme. Ferredoxin apparently is not essential for pyruvic dehydrogenase to be active in pyruvate oxidation. Pyruvic dehydrogenase preparations stripped of ferredoxin proved capable of reducing various dyes and \( \text{O}_2 \) when supplied with pyruvate and suitable acetyl acceptors.

Experiments on pyruvate oxidation with \( \text{O}_2 \) as terminal electron acceptor have shed light on the mode of action of ferredoxin. In a typical experiment, 36 \( \mu \)moles of acetyl phosphate were formed in 1 hour when a mixture of 12 mg of protein as \( C. \text{pasteurianum} \) minus-ferredoxin enzyme preparation, 100 \( \mu \)moles of sodium pyruvate, and 0.2 mg of ferredoxin was shaken in air. An atmosphere of 80% CO did not inhibit acetyl phosphate formation in this reaction. A control run without ferredoxin showed a slow \( \text{O}_2 \) uptake, but no acetyl phosphate was formed; in place of acetyl phosphate, the product was 5 \( \mu \)moles of acetate, which indicates that oxidation of pyruvate in the absence of ferredoxin apparently resulted in the intermediate formation of an unstable acetyl derivative that was hydrolyzed to acetate. Pyruvate conversions were regularly lower in mixtures not containing ferredoxin than in those to which ferredoxin had been added, because pyruvate oxidation activity declined rapidly and disappeared when exposed to air unless ferredoxin was present. In mixtures containing ferredoxin, an initial burst of \( \text{H}_2 \) production occurred. Cessation of \( \text{H}_2 \) formation was due to loss of hydrogenase activity, no doubt by action of \( \text{O}_2 \), because \( \text{H}_2 \) production could be recommenced by fresh additions of hydrogenase.

These observations indicate that pyruvic dehydrogenase is capable of donating electrons from pyruvate to any of a wide variety of acceptors; however, ferredoxin is the preferred acceptor over \( \text{O}_2 \) or reducible dyes such as methylene blue, and ferredoxin also transfers electrons preferentially to hydrogenase rather than to \( \text{O}_2 \). Ferredoxin stabilizes pyruvic dehydrogenase, but not hydrogenase, against inactivation by air.

**Relation of Phosphoroclastic Reaction to Nitrogen Fixation**

The rate of \( N_2 \) fixation in the cell extracts proved to be governed in part by the kind and concentration of acetyl acceptor employed for pyruvate oxidation. Maximal rates of fixation as measured by \( \text{NH}_3 \) titration (21) were obtained in preparations containing phosphate at 0.01 to 0.02 \( \text{M} \) concentrations. Fixation rates declined at both higher and lower concentrations, but the rate of pyruvate metabolism increased with increasing phosphate ions up to at least 0.075 \( \text{M} \) (Table II and Figs. 6 and 7). Superoptimal phosphate concentrations also had the effect of imposing an induction period on nitrogen fixation, during which time no detectable \( \text{NH}_3 \) was synthesized in spite of the vigorous rate of pyruvate metabolism (Fig. 7). The same conditions also favored higher steady state concentrations of acetyl phosphate, previously found to be a fixation inhibitor (2).

Glutathione could be used as a replacement for phosphate in the medium without inhibiting \( N_2 \) fixation appreciably (Table III). However, 2-mercaptoethanol, which appeared to be 8 times as effective as glutathione for pyruvate oxidation, was

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**Fig. 5.** Coenzyme A requirement in reduction of ferredoxin by pyruvate. Reductions were performed at 30° in 1-cm silica cuvettes (3 ml) with caps designed to hold 0.3 ml. The cuvette contained 150 \( \mu \)moles of ferredoxin, 0.5 mg of protein as minus-ferredoxin enzyme preparation obtained by the DEAE-cellulose method (32), 200 \( \mu \)moles of sodium pyruvate, either 140 \( \mu \)moles of potassium phosphate or, in the phosphate-free experiment, 140 \( \mu \)moles of sodium cacodylate, and CoA as indicated in a total volume of 3 ml, at pH 6.5. The blank contained all ingredients except CoA. The gas phase was CO to prevent reoxidation of ferredoxin by hydrogenase. Optical density was measured in a Lumetron colorimeter.

**Fig. 6.** Time course of \( N_2 \) fixation and pyruvate oxidation in 0.012 \( \text{M} \) phosphate medium.

**TABLE II**

Relative responses of pyruvate oxidation and \( N_2 \) fixation to phosphate concentration

<table>
<thead>
<tr>
<th>Phosphate concentration</th>
<th>Specific activity*</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Pyruvate oxidation</strong></td>
<td><strong>NH₃ synthesis</strong></td>
</tr>
<tr>
<td>0.012</td>
<td>7.71</td>
</tr>
<tr>
<td>0.075</td>
<td>10.8</td>
</tr>
</tbody>
</table>

*Expressed as micromoles of pyruvate consumed or \( \text{NH}_3 \) formed per mg of protein per hour.
**Figure 7.** Time course of N₂ fixation and pyruvate oxidation in 0.075 M phosphate medium.

### Table III

**Effects of various acetyl acceptors on N₂ fixation**

The standard system, in a 25-ml flask under N₂ at 1 atmosphere, contained 35 mg of protein as a dialyzed cell extract, 100 μmoles of the acetyl acceptor indicated, 420 μmoles of sodium pyruvate, 8 μmoles of CoA, and H₂O in a total volume of 4 ml at pH 6.5. The reaction was initiated by tipping in pyruvate, and the mixture was incubated for 2 hours at 30°C with shaking.

<table>
<thead>
<tr>
<th>Acetyl acceptor</th>
<th>Changes</th>
<th>N₂ fixation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>H₃PO₄</td>
<td>-</td>
<td>18.4</td>
</tr>
<tr>
<td>Glutathione</td>
<td>CoA</td>
<td>10.8</td>
</tr>
<tr>
<td>Glutathione</td>
<td>+ 2 μmoles of H₂AsO₄⁻</td>
<td>0.8</td>
</tr>
<tr>
<td>Glutathione</td>
<td>Pyruvate</td>
<td>3.8</td>
</tr>
<tr>
<td>Glutathione</td>
<td>-</td>
<td>0.0</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>- CoA</td>
<td>1.2</td>
</tr>
<tr>
<td>2-Mercaptoethanol</td>
<td>- CoA</td>
<td>0.0</td>
</tr>
<tr>
<td>None</td>
<td></td>
<td>0.4</td>
</tr>
</tbody>
</table>

*Expressed as micromoles of NH₃ formed.

### Table IV

**Effect of arsenate on N₂ fixation**

The complete system was as in Table I, but without the thiol.

<table>
<thead>
<tr>
<th>Additions</th>
<th>N₂ fixation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 μmoles</td>
<td></td>
</tr>
<tr>
<td>H₃PO₄</td>
<td>8.8</td>
</tr>
<tr>
<td>H₂PO₄⁻</td>
<td>5.6</td>
</tr>
<tr>
<td>H₂AsO₄⁻</td>
<td>0.0</td>
</tr>
<tr>
<td>H₃PO₄ + 20 H₂AsO₄⁻</td>
<td>0.6</td>
</tr>
<tr>
<td>H₃PO₄ + 2 H₂AsO₄⁻</td>
<td>3.4</td>
</tr>
<tr>
<td>H₂PO₄⁻ + 10 ATP</td>
<td>1.7</td>
</tr>
<tr>
<td>H₂PO₄⁻ + 10 ATP + 2 H₂AsO₄⁻</td>
<td>3.5</td>
</tr>
</tbody>
</table>

*Expressed as micromoles of NH₃ formed.

### Table V

**Effects of reducible dyes on N₂ fixation**

The reaction mixture, in a 25-ml flask under N₂ at 1 atmosphere, contained 35 mg of protein as cell extract, 30 μmoles of potassium phosphate, and 420 μmoles of sodium pyruvate in H₂O to a total volume of 4 ml at pH 6.5. The reaction was initiated by tipping in pyruvate, and the mixture was incubated at 30°C for 1 hour with shaking.

<table>
<thead>
<tr>
<th>Additions</th>
<th>N₂ fixation*</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>8.8</td>
</tr>
<tr>
<td>Methylene blue, 200 μg</td>
<td>7.0</td>
</tr>
<tr>
<td>Benzyl viologen, 200 μg</td>
<td>0.0</td>
</tr>
<tr>
<td>Methyl viologen, 100 μg</td>
<td>0.0</td>
</tr>
<tr>
<td>Methyl viologen, 50 μg</td>
<td>3.2</td>
</tr>
<tr>
<td>Methyl viologen, 20 μg</td>
<td>4.2</td>
</tr>
</tbody>
</table>

*Expressed as micromoles of NH₃ formed.

The reaction mixture, in a 25-ml flask under N₂ at 1 atmosphere, contained 35 mg of protein as cell extract, 30 μmoles of potassium phosphate, and 420 μmoles of sodium pyruvate in H₂O to a total volume of 4 ml at pH 6.5. The reaction was initiated by tipping in pyruvate, and the mixture was incubated at 30°C for 1 hour with shaking.

**Discussion**

The enzyme system for the phosphoroclastic reaction of pyruvic acid in C. pasteurianum appears to be composed of pyruvic dehydrogenase, hydrogenase, and phosphotransacetylase, together with coenzyme A and the electron-transferring agent ferredoxin (12, 13). The three enzymes were found to be present in sufficient concentrations to account for the observed rate of pyruvate oxidation. The mechanism of reaction as visualized from present findings is outlined in Fig. 8.

Pyruvic dehydrogenase appeared to have a specific requirement for coenzyme A; however, other components of the system could be replaced with artificial substitutes. Thus, pyruvate oxidation was obtained when phosphate was replaced by various other acetyl acceptors, including arsenate, 2-mercaptoethylamine, 2-mercaptoacetate, and glutathione. The requirement for coenzyme A was not relieved by employing these reagents. Ferredoxin only 10% as effective as glutathione for fixation (Tables I and III). Coenzyme A was required for nitrogen fixation just as it was for the phosphoroclastic reaction when these thiols were employed in place of phosphate as acetyl acceptors.

Arsenate, which promotes the phosphoroclastic rate but not acetyl phosphate formation, has been reported to inhibit N₂ fixation (33). At low levels, the arsenate effect appeared to be partially reversed by phosphate; also, the inhibitory effects of ATP (2) were less marked with arsenate present than with phosphate (Table IV).

Methyl viologen (E° = -440 mV), which can replace ferredoxin as catalyst for the phosphoroclastic reaction, is a potent inhibitor of N₂ fixation in C. pasteurianum cell extracts (Table V). By contrast, methylene blue (E° = 11 mV) is neither a fixation inhibitor (Table V) nor a phosphoroclastic catalyst, although in substrate amounts it does support pyruvate oxidation by serving as electron acceptor (Fig. 4). Benzyl viologen (E° = -350 mV) inhibited N₂ fixation (Table V) and was a weak catalyst for the phosphoroclastic reaction, substituting for ferredoxin.

The significance of these observations for the mode of coupling between pyruvate metabolism and N₂ fixation remains to be understood. Clearly, the states of both the acetyl-accepting and electron-accepting branches of the phosphoroclastic mechanism have influence over the rate of N₂ fixation.
doxin, which serves with hydrogenase for \( \text{H}_2 \) formation, could be replaced by catalytic amounts of methyl viologen without altering the normal reaction pattern. Furthermore, ferredoxin and hydrogenase could be jointly replaced in their role of terminal electron transport by substrate amounts of benzyl viologen, methylene blue, neotetrazolium chloride, or \( \text{O}_2 \); under these conditions, pyruvate oxidation was not accompanied by \( \text{H}_2 \) formation. Coenzyme A stimulated pyruvate oxidation in all instances. In fact, if pyruvate and pyruvic dehydrogenase can reduce ferredoxin without aid of coenzyme A, the amount of ferredoxin reduced was not detectable spectrophotometrically under conditions that gave readily observable reduction with coenzyme A added. Accordingly, coenzyme A appeared to be a requisite for both acetyl transfer and electron transfer, which suggests that the two could be concerted processes.

The role of ferredoxin in electron transport was indicated by its equivalency with the oxidation-reduction dye, methyl viologen, for reconstituting phosphoroclastic activity in extracts from which ferredoxin had been removed. The site of ferredoxin in the electron transport sequence was located between pyruvic dehydrogenase and hydrogenase on the basis of effects obtained with \( \text{CO} \) and \( \text{O}_2 \). Thus, \( \text{CO} \) inhibited the formation of \( \text{H}_2 \) and the oxidation of reduced ferredoxin by hydrogenase; \( \text{CO} \) did not inhibit the reduction of ferredoxin or of dyes by pyruvate, and it did not inhibit oxidation of reduced ferredoxin by \( \text{O}_2 \). The preferred pathway for electrons from pyruvic dehydrogenase was through ferredoxin even when \( \text{O}_2 \) was the terminal electron acceptor. Under aerobic conditions, which permitted a choice of terminal electron acceptors, ferredoxin passed electrons to hydrogenase in preference to \( \text{O}_2 \) until hydrogenase was no longer active. Both pyruvic dehydrogenase and hydrogenase were sensitive to \( \text{O}_2 \) and quickly lost activity on exposure to air unless ferredoxin was present. Ferredoxin stabilized pyruvic dehydrogenase, but not hydrogenase, against the destructive effects of \( \text{O}_2 \). These observations were taken as evidence that (a) ferredoxin is intermediate to pyruvic dehydrogenase and hydrogenase in the electron transport sequence, (b) neither ferredoxin nor pyruvic dehydrogenase is appreciably sensitive to \( \text{CO} \), and (c) ferredoxin may combine with pyruvic dehydrogenase to form a complex in which an otherwise sensitive site on the enzyme is protected from inactivation by \( \text{O}_2 \). Other reports on the properties and functions of ferredoxin are forthcoming.

Nitrogen fixation activity proved to be sensitive to some but not all reagents that modified the course or rate of the phosphoroclastic reaction. Fixation was highly sensitive to phosphate ion concentration, with the optimum at 0.01 to 0.02 m, but phosphate could be replaced by glutathione without adverse effect on fixation. In a similar experiment with 2-mercaptoethanol, however, which gave an 8-fold higher phosphoroclastic rate, the fixation rate was only 10% of that obtained with glutathione. Also, arsenate, which stimulated the rate of pyruvate oxidation but altered the qualitative course of reaction to form acetate in place of acetyl phosphate, was a potent inhibitor for fixation (33). Dyes with strongly negative oxidation-reduction potentials, especially methyl viologen and benzyl viologen, inhibited nitrogen fixation whereas others, such as methylene blue, did not. The mechanism of coupling between pyruvic acid metabolism and nitrogen fixation in these preparations clearly is sensitive to the state of the phosphoroclastic system with respect to both its electron-accepting and acetyl-accepting segments. These relationships are the subject of continuing investigations.

**SUMMARY**

Nitrogen fixation is linked with pyruvic acid metabolism in cell extracts of *Clostridium pasteurianum*, where pyruvate utilization occurs primarily by the phosphoroclastic reaction. Studies undertaken to clarify the dependence of nitrogen fixation on pyruvate metabolism have established that the phosphoroclastic reaction, which for this organism has not been previously resolved, is dependent upon the recently discovered electron-transferring agent, ferredoxin, together with pyruvic dehydrogenase, hydrogenase, phosphotransacetylation, and coenzyme A. Ferredoxin serves in the transport of electrons from pyruvic dehydrogenase to hydrogenase for \( \text{H}_2 \) formation. Pyruvic dehydrogenase exhibited a specific requirement for coenzyme A, but substitutions could be made for other components of the system without deleterious effects on pyruvate oxidation. \( \text{N}_2 \) fixation was highly sensitive to most such changes, however; the responses were such as to suggest that the pyruvate-oxidizing system is able either to provide or to deny requirements of the \( \text{N}_2 \)-fixing system.

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