COFACTORS OF THE PHOSPHOROCLASTIC REACTION OF CLOSTRIDIUM BUTYRICUM

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The degradation of pyruvate, in the presence of phosphate, to acetate, carbon dioxide, and hydrogen by cell-free extracts of Clostridium butylicum was described by Koepsell and Johnson (1). Later acetyl phosphate, the labile precursor of the acetate, was shown to be produced (2, 3), and phosphotransacetylase was shown to be present in the cell-free extracts (4). This reaction is, at least, superficially similar to the phosphoroclastic reaction of Escherichia (5) which produces acetyl phosphate and formate from pyruvate and phosphate. However, the Clostridium enzyme does not produce hydrogen and carbon dioxide from formate (1), nor does labeled formate exchange with pyruvate, although carbon dioxide readily does (6).

Little is known of the details of the reaction, and this study was undertaken in an attempt to relate this reaction to the other acetate-producing reactions of pyruvate. Evidence is presented that cocarboxylase (CoC), coenzyme A (CoA), and ferrous ions participate in the reaction, but that the pyruvate oxidation factor (POF) does not. A brief report of some of these results has appeared (7).

EXPERIMENTAL

Cell-Free Extract Preparation—Clostridium butyricum (an organism similar to C. butylicum), strain 6014 of the American Type Culture Collection, was grown in broth consisting of 1 per cent tryptone, 1 per cent yeast extract, 1 per cent glucose, and 0.2 per cent K₂HPO₄. 5 liter batches were inoculated with 20 ml. of an actively gassing culture and were harvested after 12 hours at 37°C. A lyophilized cell-free extract was prepared by the method of Koepsell and Johnson (1) and stored at −17°C in stoppered test-tubes.

Measurement of Reaction—Standard manometric techniques were used for the determination of carbon dioxide and hydrogen, and acetyl phos-

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phosphate was determined colorimetrically by the hydroxamic acid method of Lipmann and Tuttle (8).

The reduction of 2,2-(p-diphenylene)bis(3,5-diphenyltetrazolium chloride), neotetrazolium, by the reaction mixture was found to be a convenient method of following the reaction. The rate of formation of the soluble reduced form of the salt was followed at 540 m\(\mu\) in a Beckman DU spectrophotometer. Before addition of the enzyme solution to the reaction mixture hydrogen was bubbled from a 1 mm. capillary through the contents of each cuvette for 2 minutes. The enzyme solution was then added and mixed by bubbling hydrogen for 10 to 15 seconds. The cuvette cap was replaced as the capillary was withdrawn to prevent escape of gas, and optical density was determined at appropriate intervals. For each experiment the amount of enzyme was adjusted to give a convenient rate. The change in optical density on the reduction of 100 \(\gamma\) of neotetrazolium in a volume of 3.2 ml. by hydrosulfite was 1.06. This corresponds to 6.6 \(\mu\)l. of H\(_2\); hence the sensitivity of the method is apparent. The activity of the enzyme system varied greatly, depending on the type and length of treatments before addition to the cuvette.

**Materials**—The special chemicals used were from the following sources: CoA, 75 per cent pure, from the Pabst Laboratories, CoC from Merck and Company, diphosphopyridine nucleotide, 60 per cent pure, from the Schwarz Laboratories, triphosphopyridine nucleotide, 80 per cent pure, from the Sigma Chemical Company, neotetrazolium from the Dajac Laboratories, 2,3,5-triphenyltetrazolium chloride from the Paul-Lewis Laboratories, and riboflavin-5-phosphate from the Krischell Laboratories. We are indebted to Dr. H. G. Paul and Dr. R. Asnis for generous gifts of the nitrofurans, to Dr. I. C. Gunsalus for flavin-adenine dinucleotide, and to The Dow Chemical Company for 50 to 100 mesh Dowcx 1.

**Stability**—The lyophilized cell-free extract, although stable in powder form under a vacuum or refrigeration, was found to be extremely labile in solution. When solutions of the preparation (10 mg. per ml. in 0.033 M phosphate, pH 6.5) were allowed to stand exposed to air in an ice bath, only 5 to 25 per cent of the original activity remained after 3 hours. If the air was replaced with an atmosphere of hydrogen or nitrogen, approximately 60 per cent of the activity remained. This significant loss of activity in the cold and in the absence of oxygen was not prevented by addition of either cysteine, thioglycolate, or sulfide, but up to 80 per cent of the activity could be preserved for 3 hours by potassium ascorbate or ferrous sulfate in a concentration of 1 mg. per ml. The addition of potassium ascorbate during the preparation of cell-free extracts yielded extracts more active \((Q_{H_2} 70 to 80)\) than those prepared without potassium ascorbate \((Q_{H_2} 40 to 50)\). The addition of ferrous sulfate to extracts prepared
without reducing agents doubled the reaction rate, whereas extracts prepared with ascorbate did not exhibit this stimulation.

Hydrogen Acceptors—In order to simplify the reaction a search was made for hydrogen acceptors which would by-pass the mechanism of molecular hydrogen formation and yet allow production of carbon dioxide and acetyl phosphate. The compounds were tested by observing whether they were reduced by the reaction mixture. The substances which were tested are listed in Table I. The active compounds prevented the evolution of molecular hydrogen as long as the oxidized form of the compound was present. This relation for 5-nitro-2-furaldehyde semicarbazone (Furacin) is illustrated in Fig. 1 and indicates that when the oxidized form has been reduced molecular hydrogen production proceeds at a rate similar to that in the control flask. Therefore, the reduced compound has no inhibitory effect on the rate of the reaction.

The products of the reaction in the presence of Furacin or tetrazolium salts were determined, and the data are presented in Table II. Although no manometer change occurs when hydrogen is diverted to the acceptor and CO₂ is absorbed by KOH, the expected amount of acetyl phosphate is formed. Furacin was found to be ideal for use in such measurements. The low solubility of the oxidized form allowed an excess of the solid to be added to the flasks, and neither the oxidized nor reduced form interfered with determinations of acetyl phosphate. To measure the formation of acetyl phosphate in the presence of tetrazolium salts, 2,3,5-triphenyltetrazolium chloride was chosen because the reduced form, which would interfere at 540 mp, was removed with the protein precipitate in the acetyl phosphate determination.

The addition of hydrogen acceptors sometimes stimulated the rate of

<table>
<thead>
<tr>
<th>Compounds Tested As Hydrogen Acceptors in Phosphoroclastic Reaction</th>
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<tbody>
<tr>
<td><strong>Inactive</strong></td>
</tr>
<tr>
<td>Fumarate</td>
</tr>
<tr>
<td>Nitrate</td>
</tr>
<tr>
<td>Sulfite</td>
</tr>
<tr>
<td>Thiosulfate</td>
</tr>
<tr>
<td>Formaldehyde</td>
</tr>
<tr>
<td>Hydroxylamine</td>
</tr>
<tr>
<td>Diposphopyridine nucleotide</td>
</tr>
<tr>
<td>Triphosphopyridine</td>
</tr>
</tbody>
</table>
the reaction. In the experiment in Table II the addition of Furacin produced almost a 3-fold increase in acetyl phosphate and carbon dioxide.

![Diagram showing the effect of varying amounts of Furacin on hydrogen production. Each cup contained 0.033 M phosphate buffer, pH 6.5, 10 mg. of enzyme preparation No. 9, and 100 μM of pyruvate; final volume 3.0 ml. and KOH in center well. Incubated at 37° in a nitrogen atmosphere. Furacin was added in solution and evaporated to dryness at 60° prior to other additions.]

**Figure 1** Effect of varying amounts of Furacin on the reaction as measured by hydrogen production. Each cup contained 0.033 M phosphate buffer, pH 6.5, 10 mg. of enzyme preparation No. 9, and 100 μM of pyruvate; final volume 3.0 ml. and KOH in center well. Incubated at 37° in a nitrogen atmosphere. Furacin was added in solution and evaporated to dryness at 60° prior to other additions.

### Table II

<table>
<thead>
<tr>
<th>Products</th>
<th>H₂</th>
<th>CO₂</th>
<th>Acetyl PO₄</th>
</tr>
</thead>
<tbody>
<tr>
<td>Preparation No. 10........................</td>
<td>4.0</td>
<td>4.1</td>
<td>4.9</td>
</tr>
<tr>
<td>&quot; 10 + excess Furacin</td>
<td>0.0</td>
<td>12.8</td>
<td>13.0</td>
</tr>
<tr>
<td>&quot; 10 + &quot; KOH in center well</td>
<td>0.0</td>
<td>0.0</td>
<td>12.7</td>
</tr>
<tr>
<td>Preparation No. 16, KOH in center well</td>
<td>10.3</td>
<td>0.0</td>
<td>10.8</td>
</tr>
<tr>
<td>&quot; 16 + 10 μM 2,3,5-triphenyltetrazolium chloride, KOH in center well</td>
<td>1.0</td>
<td>0.0</td>
<td>11.4</td>
</tr>
</tbody>
</table>

Each flask contained 10 mg. of enzyme preparation, 0.033 M PO₄, pH 6.5, 100 μM of pyruvate, and hydrogen acceptor when added; total volume per flask 3.2 ml. Furacin was added as the solid to appropriate flasks.

The magnitude of the stimulation depends on the method of preparation of the enzyme solution. Solutions prepared with ascorbate and protected from oxidation show little or no stimulation, as in the tetrazolium experiment in Table II. However, solutions which have been exposed to air in the absence of reducing agents may show as great as a 10-fold stimulation. This suggests that the mechanism which produces molecular hy-
drogen is the most labile to oxidation, since its replacement restores activity to partially inactivated preparations.

The reduced forms of the flavins, riboflavin, riboflavin-5-phosphate (FMN), and flavin-adenine dinucleotide (FAD), are readily oxidized by air. This made possible the use of oxygen as the ultimate hydrogen acceptor. The reaction rate in the presence of 300 μ of FMN was rapid (75 ml. of O₂ uptake per 10 mg. of enzyme per 10 minutes) but fell off completely after 10 minutes. The addition of 3 mg. of potassium ascorbate and 1 mg. of ferrous sulfate to the flask permitted oxygen uptake at a constant rate for at least 30 minutes. The P:O ratio ((moles of acetyl phosphate produced)/(atoms of oxygen used)) in a typical experiment was 0.95, which indicates that the oxidation of FMN produces water not hydrogen peroxide. In support of this conclusion, no peroxide could be detected by the sensitive test of Main and Shinn (9), nor was the oxygen uptake diminished by the addition of catalase. Anaerobically the enzyme will oxidize reduced FMN, but no hydrogen is produced. This seems to be caused by hydrogen acceptors present in the enzyme solution.

No change in optical density at 340 μm was observed during the course of the reaction whether in the presence or in the absence of diphosphopyridine or triphosphopyridine nucleotide. The reduced nucleotides were oxidized by the enzyme preparation, but no hydrogen was evolved. The failure of the enzyme system to produce lactate from pyruvate in the presence of diphosphopyridine nucleotide and lactic dehydrogenase provides further evidence that diphosphopyridine nucleotide is not reduced.

Hydrogen uptake could not be demonstrated when the hydrogen acceptors were incubated with enzyme and buffer in an atmosphere of hydrogen. This confirms and extends the observation of Koepsell and Johnson (1), who observed no hydrogen uptake in the presence of methylene blue, a relatively poor hydrogen acceptor in the forward reaction. Nevertheless, as first observed by Lipmann and Tuttle (3), a hydrogen atmosphere inhibits the forward reaction about 40 per cent.

Cocarboxylase Requirement—The dependence of this phosphoroclastic reaction on CoC was revealed by alkaline ammonium sulfate precipitation at 4°. The solution to be precipitated contained 10 mg. of powdered extract, 1 mg. of ferrous sulfate, 1 mg. of potassium ascorbate, and 0.5 ml. of 0.2 mM K₂HPO₄ per ml. To this solution in an ice bath was added saturated ammonium sulfate at pH 8.0 to 0.8 saturation. The precipitate was removed by centrifugation and dissolved in water to which had been added ascorbate and ferrous iron in the above concentration. The resulting enzyme solution was used directly without dialysis and was found to reduce tetrazolium upon the addition of CoC. These data are shown in Fig. 2 and indicate the low levels of CoC which are necessary for maximal
rate. Even in the presence of the added reducing agents it has not been possible to fractionate this enzyme system beyond a few crude precipitations.

Coenzyme A Requirement—Koepsell and Johnson (1) noted a stimulation of the reaction by fresh liver extract. In our hands this stimulation is reproduced by the addition of CoA. In order to demonstrate this requirement more specifically, the enzyme system was separated into two parts by acid precipitation in the cold. To a solution containing 10 mg. of powdered extract, 1 mg. of potassium ascorbate, and 1 mg. of ferrous sulfate per ml., 0.1 N acetate buffer at pH 4.0 was added dropwise until no further precipitation occurred. The precipitate was suspended in water to the original volume. Both precipitate and supernatant liquid were required in order to reduce tetrazolium rapidly. The precipitate contained most of the phosphotransacetylase as tested by the arselenalysis method of Stadtman and Barker (10). The slow rate of reduction caused by the supernatant liquid in tris(hydroxymethyl)aminomethane buffer (Tris) is shown in Fig. 3. The addition of CoA in fairly large amounts permits a
more rapid reaction, but a very rapid reaction occurs when arsenate and smaller amounts of CoA are added. These data suggest that acetyl CoA is formed and accumulates in the absence of phosphate, or is converted to CoA and acetate by the action of arsenate and the small amount of phosphotransacetylase present in the supernatant solution.

Iron Requirement—Since Dowex treatment of the enzyme preparation by the method of Stadtman et al. (4) resulted in loss of activity which could not be restored by addition of CoA, a search was made for other co-factors which might be removed from the extract by this treatment. The powdered extract dissolved in water (10 mg. per ml.) gave a definite test for iron with 2,2'-bipyridine. After an 8 to 15 minute treatment of the enzyme solution with  \( \frac{3}{4} \) volume of Dowex in an ice bath, no color with bipyridyl was apparent. The addition of both CoA and ferrous ions to the Dowex-treated extract restored activity. Data from a typical experiment (Fig. 4) show that the short Dowex treatment removes iron more efficiently than CoA. Of the other divalent metals tested only cobaltous ions were found to have any stimulatory action when added to the Dowex-treated extract. These data are presented in Fig. 5 and indicate that manganous, nickelous, zinc, and magnesium ions do not stimulate the reaction. Ferric ions similarly had no stimulatory effect on the treated extract. This metallic ion requirement is the same as that reported for the aldolase of Clostridium perfringens (11). The relation of the con-

![Figure 4](image-url)
centration of ferrous ions to the reaction rate is illustrated in Fig. 6. Rate studies with concentrations of ferrous sulfate beyond 0.17 μM per ml. of system were not performed because of the heavy precipitate formed with phosphate in the reaction mixture.

It is well known that saccharolytic members of the genus Clostridium produce chiefly lactic acid in an iron-deficient medium, instead of the usual mixture of hydrogen, CO₂, solvents, and acetic, lactic, and butyric acids (12, 13). Apparently, in iron deficiency, the pyruvate produced by glu-

\[ \text{Pyruvate Oxidation Factor} - \text{Cells grown in a medium deficient in POF similar to that of O'Kane and Gunsalus (14) carry out the phosphoroclastic reaction at a rate comparable to that exhibited by cells grown in natural media. Assays for the POF by the growth method of Lytle and O'Kane (15) and by the manometric assay of Gunsalus et al. (16) on hot water extracts and acid hydrolysates of active enzyme preparations prepared from cells grown in deficient media yield uniformly low results. For example, a preparation with a } Q_{H₃} \text{ of 89 was prepared for assay by heating at 120° for 1 hour in 2 N H₂SO₄. A manometric assay of the hydrolysate, with } dl-\alpha\text{-lipoic acid as a standard (1 unit equals approximately 4 mµgm.} \]
of dl-α-lipoic acid), showed the presence of less than 0.05 unit of POF per mg. of enzyme. Assays have been done on several different preparations and have been confirmed by analysis in another laboratory. This low content of pyruvate oxidation factor activity contrasts to the high level found in animal pyruvic oxidase (212 to 1180 protogen units per mg. of protein) (17) and α-ketoglutarate oxidase (170 units per mg. of protein) (18), and to the high amount required by Streptococcus faecalis for normal pyruvate oxidation (about 1 unit per mg. of dried cells) (16).

![Graph](image)

**FIG. 6.** Relation of the concentration of ferrous ions to the rate of the reaction. Each cuvette contained, in a total volume of 3.2 ml., 0.033 M phosphate buffer, 100 μM of pyruvate, 300 μM of potassium ascorbate, 10 units of CoA, 20 μM of cocarboxylase, Dowex-treated enzyme preparation, and ferrous ions as ferrous sulfate, and was incubated at 25°C.

Inhibitors—Arsenite, a potent inhibitor of the Streptococcus pyruvate oxidase (19), inhibits the phosphoroclastic reaction only 6 per cent at 0.01 M. In contrast to the phosphoroclastic reaction of Escherichia (20), 200 μM per ml. of streptomycin sulfate do not inhibit the Clostridium phosphoroclastic reaction.

**DISCUSSION**

The requirement for cocarboxylase, coenzyme A, and divalent metal suggests that the phosphoroclastic reaction is similar to the oxidative de-

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1 We are indebted to V. L. Lytle and H. D. Barner of this laboratory for many of the assays and to W. Razzell and Dr. I. C. Gunsalus of the University of Illinois for the assay cited.
carboxylation of pyruvate, as found in *S. faecalis* and *E. coli* (21), since these reactions, in addition to having acetyl phosphate and carbon dioxide as end-products, also require the same cofactors. On the basis of these data, it is easy to visualize the phosphoroclastic reaction as an oxidative decarboxylation which uses a mechanism that produces molecular hydrogen rather than one that reduces oxygen as a means of disposing of electrons. The failure to demonstrate a pyridine nucleotide requirement, characteristic of the oxidative decarboxylations, is readily explained by assuming an unknown different mechanism of electron transport.

However, the failure to find levels of the pyruvate oxidation factor comparable to those found in the oxidative decarboxylation systems seriously weakens the analogy. The *Clostridium* phosphoroclastic reaction differs from that of *Escherichia* in being insensitive to streptomycin. Thus the relationship of this phosphoroclastic reaction to the other reactions of pyruvate must await further research.

Perhaps the most puzzling aspect of this reaction is the failure to produce hydrogen from reduced hydrogen acceptors which are oxidized by the reaction mixture, and the failure to utilize hydrogen in the presence of acceptors which can be reduced by the forward reaction. Further study of the hydrogen-producing system is needed, but attempts at purification have so far been frustrated by its lability.

**SUMMARY**

The phosphoroclastic reaction of *Clostridium butyricum* requires co carboxylase, coenzyme A, and ferrous ions as cofactors. The pyruvate oxidation factor is present in small amounts only and appears not to be involved in the reaction. Various hydrogen acceptors may conveniently be used to simplify the reaction, but no hydrogen uptake can be demonstrated with these compounds.

**BIBLIOGRAPHY**