PYRUVATE OXIDATION IN DESULPHOVIBRIO DESULPHURICANS

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The metabolism of Desulphovibrio desulphuricans does not appear to have been studied extensively. Stephenson and Stickland (1931) and Sisler and ZoBell (1950) showed that the organism can use gaseous hydrogen as a reducing agent for sulfate reduction. By growing cultures for several generations in mineral salt solution containing less than one part per million of sulfate, Sisler and ZoBell (1951a) were able to show that autotrophic sulfate-reducers can utilize CO₂ as the sole hydrogen acceptor. Neither growth nor hydrogen uptake, however, was nearly as rapid in the sulfate-free media as in similar media enriched with sulfate. However, washed cells of sulfate-reducers were found to reduce fumarate considerably more rapidly than sulfate (Sisler and ZoBell, 1951a). Sisler and ZoBell (1951b) reported the fixation of nitrogen by hydrogenase producing sulfate-reducers. Postgate (1952) reported that many strains of this organism were able to grow in the absence of sulfate if pyruvate was present.

Pyruvate oxidation in bacterial extracts has been studied by several authors. In the presence of inorganic phosphate, cell-free extracts of Lactobacillus delbrueckii oxidatively decarboxylated pyruvate to acetyl phosphate and CO₂ (Lipmann, 1939), whereas an enzyme prepared from Clostridium butylicum dissimilates it to acetate, H₂, and CO₂ (Koepseil and Johnson, 1942). Cell-free extracts of Proteus vulgaris oxidize pyruvate to acetate and CO₂. By acid precipitation of such extracts, Stumpf (1945) prepared a protein fraction that required co-enzyme A and certain divalent metal ions for activity. Moyed and O’Kane (1952) separated by ammonium sulfate fractionation the pyruvicoxidase system of the cell-free extracts of P. vulgaris into two heat-labile components both of which are required for activity. No evidence for the participation of coenzyme A or acetyl co-enzyme A could be found. Korkes et al. (1951) reported a soluble enzyme, isolated from Escherichia coli, which catalyzes a diphosphothiamine dependent oxidative decarboxylation of pyruvate to acetyl coenzyme A and CO₂ in the presence of diphosphopyridine nucleotide and coenzyme A. Soluble enzyme preparations from E. coli and Streptococcus faecalis catalyze the dismutation of two molecules of pyruvate to acetyl phosphate, CO₂, and lactate when orthophosphate is present. The system is inactive in the absence of diphosphopyridine nucleotide (Korkes et al., 1950).

Recently a new coenzyme (protogen, pyruvate oxidation factor, lipoic acid, thioctic acid, or lipothiamide pyrophosphate) has been shown to be required for pyruvate oxidation by several organisms (Stokstad et al., 1949; O’Kane and Gunsalus, 1947; Gunsalus et al., 1952; Reed et al., 1951; Brockman et al., 1952; Reed and Debusk, 1952).

The present investigation deals with a cell-free, soluble enzyme preparation from D. desulphuricans which catalyzes the conversion of pyruvate to acetyl phosphate, CO₂, and ethyl alcohol and which requires inorganic phosphate for activity. The reaction can be formulated as:

\[ \text{H}_2\text{PO}_4^- + 2 \text{pyruvate} \rightarrow 1 \text{acetyl phosphate} + 2\text{CO}_2 + 1 \text{ethyl alcohol} \]

MATERIALS AND METHODS

D. desulphuricans, strain “Hildenborough”, no. 8303, kindly supplied by Dr. K. R. Butlin was used in these studies. The organism was grown in 10 liter flasks in a medium containing: 0.2 per cent Na₂SO₄, 0.2 per cent MgSO₄.7H₂O, 0.35 per cent sodium lactate, 0.05 per cent K₃HPO₄, 0.4 per cent yeast extract, 0.5 per cent peptone, ferrous ammonium sulfate traces, plus distilled water to volume. The medium was adjusted to pH 7.4 to 7.6 and autoclaved for 30 minutes at 10 lb per sq in. The organism was grown initially in the medium described above but without added yeast extract and peptone. The growth thus obtained after 72 hours' incubation was used to inoculate ten volumes of
the basic medium. The flasks were filled up to the top with the medium after inoculation. The cells were harvested after 72 hours' incubation by centrifugation at 0 to 4 C in an angle head attachment of the International centrifuge at 3,500 rpm for 45 minutes. The cells were washed twice with 0.8 per cent potassium chloride solution, and the final sediment resuspended in a small quantity of 0.8 per cent potassium chloride solution. A homogeneous suspension of the cells was treated then with 8 to 10 volumes of acetone which had been cooled previously to 0 C. The precipitate was filtered quickly under suction, washed 3 to 4 times with cold acetone, and dried in a vacuum desiccator over P2O5 overnight at 0 C and then stored at -18 C.

The yield was 20 to 25 g of wet cells or 4 to 5 g of dried acetone powder per 10 liters of the medium.

Preparation of enzyme extract. The enzyme preparation was obtained by grinding 100 mg of dried acetone powder with 1 ml of 0.2 M potassium phosphate buffer, pH 6.4, for 15 to 20 minutes at 0 C. Extraction of the enzyme was carried out with 0.2 M potassium acetate buffer, pH 6.4, when phosphate-free enzyme was required. Intact cells and cellular debris were removed by centrifugation at 15,000 rpm in the high speed attachment of the International centrifuge for 30 minutes or more. The supernatant thus obtained was pale yellow in color and was used for the study of pyruvate metabolism. Usually the extract was used on the same day. The extract was dialyzed against 0.1 M potassium phosphate or acetate buffer, pH 6.4, at 0 C with stirring for 3 to 4 hours with 3 to 4 changes of the buffer solution. The acetone powder could be preserved at -18 C for a few weeks although some decrease in activity occurred during this period.

Lithium monoacetyl phosphate was prepared by reaction of isopropenyl acetate with phosphoric acid (Stadtman and Lipmann, 1950). Lithium pyruvate was prepared according to Wendel (1931). Cocarboxylase was obtained from Merck and Co., Inc. Diphosphopyridine nucleotide of 85 per cent purity was purchased from the Schwarz Laboratories, Inc.

DL-α-Lipoic acid and lipothiamide pyrophosphate were kindly supplied by Dr. L. J. Reed and thioctic acid by Dr. E. L. R. Stokstad. The coenzyme A (CoA) used in this work was a highly purified preparation (20 per cent pantothenic acid) kindly supplied by Dr. D. E. Green.

Volatile acids were collected by steam distillation after acidification to pH 2.0. Acetic acid and ethyl alcohol were estimated by the methods of Friedemann (1938) and Hutchens and Kass (1949). Lactate was determined according to Barker and Summerson (1941), pyruvate according to Straub (1936), and acetylmethylearbinol by a modification of the procedure of Barritt (1936). Inorganic phosphate was determined by the method of Fiske and Subbarow (1925) and acyl phosphate according to Lipmann and Tuttle (1945). Transacetylase activity was determined by arsenolysis in the test system described by Stadtman et al. (1951). Protein concentrations were determined by light absorption at 290 μg and 280 μg in the Beckman spectrophotometer, corrected for nucleic acid according to Warburg and Christian (1941), and by the trichloroacetic acid precipitation method of Stadtman et al. (1951).

Manometric experiments were carried out in the conventional Warburg apparatus. Glass distilled water was used in all the experiments, and the pH was determined with the glass electrode. All solutions were adjusted to pH 6.4 unless otherwise indicated. Potassium hydroxide was used for neutralization of acid solutions and potassium salts used in preference to the sodium salts since sodium salts inhibit transacetylase activity (Stadtman, 1952).

Test system. Enzyme activity was determined manometrically by measuring the evolution of CO2 with a test system containing, except when otherwise indicated, 0.1 M potassium phosphate, pH 6.4, 40 units of coenzyme A, 15 μmoles of cysteine, 100 μg of cocarboxylase, 50 μg of MgCl2, 20 μmoles of lithium pyruvate, enzyme solution 6 to 7 mg protein, and water to 2 ml. The reaction was started by tipping in pyruvate from the side arm, and readings were taken every 5 minutes (or 10 minutes). The rate of CO2 evolution was calculated from the second 10 minute reading and expressed as microliters of gas evolved per hour. In all cases when total CO2 was measured, the results were corrected for bound CO2 by tipping in sulfuric acid from the side arm except when acetyl phosphate was determined in the test solution. The rate remains essentially linear till all the pyruvate is consumed. Endogenous gas evolution in control flasks in which the
pyruvate solution was replaced by water was negligible. No acyl phosphate formation could be detected when the enzyme was incubated in the absence of pyruvate.

**Experimental Results**

The enzymes responsible for pyruvate oxidation were extracted by 0.2 M potassium phosphate buffer, pH 6.4, and do not appear to be associated with macro particles which readily settle down on centrifugation. A clear cell-free supernatant was obtained after centrifugation for 10 minutes at 15,000 rpm in the high speed attachment of the International centrifuge. The supernatant retained 80 to 85 per cent of activity even after 60 minutes’ centrifugation at 15,000 rpm.

**Balance study.** Preliminary experiments with the dialyzed bacterial extract indicated that there was rapid anaerobic evolution of gas from pyruvate only when inorganic phosphate was also added to the test system. When the reaction was carried out in the presence of phosphate, the solution gave a positive test by the ferric hydroxyam acid method of Lipmann and Tuttle (1945), indicating the formation of an acyl phosphate. Lactate and acetyl methyl carbinol were not formed, and there was no gas evolution when the center well of the Warburg cup contained potassium hydroxide, showing that no H₂ was formed and that the gas evolved was CO₂.

These experiments showed that the reaction did not involve “phosphoroclastic” cleavage of pyruvate to acetyl phosphate, CO₂, and H₂ or dismutation to lactate, acetyl phosphate, and CO₂. Moreover stoichiometric experiments showed the formation of one mole of CO₂ from one mole of pyruvate. In addition one mole of acyl phosphate was formed also from two moles of pyruvate as shown in table 1.

These results could best be explained on the assumption that the enzyme extract catalyzed the formation of one mole each of ethyl alcohol and acetyl phosphate and two moles of CO₂ from two moles of pyruvate. This was confirmed by the following large scale experiment.

The reaction was carried out with dialyzed bacterial extract in Warburg flasks under the experimental conditions described in table 2. As a control a duplicate flask was used with an identical test solution except that it contained no pyruvate. After CO₂ evolution had ceased, the reaction mixture was acidified by tipping in dilute sulfuric acid to release bound CO₂ and to hydrolyze acyl phosphate. An aliquot of the test solution was then steam distilled and acetic acid estimated in the distillate both by titration according to Friedemann (1938) and by the lanthanum nitrate method of Hutchens and Kass (1949) which is specific for acetate and propionate.

A second aliquot of the test solution was neutralized to about pH 8.0 and the volatile neutral products removed by distillation. The distillate gave a positive iodoform test. Ethyl alcohol was estimated in the distillate by oxidation to acetate with acid dichromate followed by

### Table 1

<table>
<thead>
<tr>
<th>Reaction Mixture</th>
<th>CO₂ Evolved</th>
<th>Acetyl Phosphate Formed</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>µmoles</td>
<td>µmoles</td>
</tr>
<tr>
<td>Complete system</td>
<td>11.8</td>
<td>5.44</td>
</tr>
<tr>
<td>- Cysteine</td>
<td>11.6</td>
<td>4.10</td>
</tr>
<tr>
<td>- Coenzyme A</td>
<td>9.1</td>
<td>3.55</td>
</tr>
<tr>
<td>- Cysteine, - Coenzyme A</td>
<td>8.8</td>
<td>2.78</td>
</tr>
</tbody>
</table>

The complete system contained 6 mg protein of dialyzed enzyme extract in 0.2 M potassium phosphate buffer, pH 6.4; lithium pyruvate, 20 µmoles; cocarboxylase, 100 µg; MgCl₂, 50 µg; coenzyme A, 40 units; cysteine, 15 µmoles; water to 2 ml. Gas phase, nitrogen; temperature, 34 C; time, 60 minutes.

### Table 2

<table>
<thead>
<tr>
<th>Pyruvate</th>
<th>Acetate Formed</th>
<th>Ethyl Alcohol Formed</th>
<th>CO₂ Evolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>Initial</td>
<td>µmoles</td>
<td>µmoles</td>
<td>µmoles</td>
</tr>
<tr>
<td>Final</td>
<td>0</td>
<td>280</td>
<td>257</td>
</tr>
<tr>
<td>Theory</td>
<td>0</td>
<td>280</td>
<td>257</td>
</tr>
</tbody>
</table>

The reaction mixture was carried out in six Warburg flasks each containing 10 mg protein of dialyzed enzyme extract in 0.2 M potassium phosphate buffer, pH 6.4; lithium pyruvate, 100 µmoles; MgCl₂, 50 µg; cocarboxylase, 100 µg; diphosphopyridine nucleotide (DPN), 100 µg; water to 3 ml. Gas phase, nitrogen; temperature, 34 C.
TABLE 3
Transacetylase activity of Desulphovibrio desulphuricans enzyme extract

<table>
<thead>
<tr>
<th>REACTION MIXTURE</th>
<th>ACETYL PHOSPHATE DECOMPOSED IN 10 MINUTES</th>
<th>µmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>- Enzyme</td>
<td></td>
<td>0.00</td>
</tr>
<tr>
<td>- Arsenate</td>
<td></td>
<td>0.25</td>
</tr>
<tr>
<td>- Cysteine</td>
<td></td>
<td>1.10</td>
</tr>
<tr>
<td>- Coenzyme A</td>
<td></td>
<td>0.65</td>
</tr>
<tr>
<td>- Cysteine, - Coenzyme A</td>
<td></td>
<td>0.58</td>
</tr>
<tr>
<td>Complete system</td>
<td></td>
<td>2.20</td>
</tr>
</tbody>
</table>

The complete system contained 1 mg protein of dialyzed enzyme extract in 0.2 M potassium phosphate buffer, pH 6.4; lithium monoacetyl phosphate, 10 µmoles; MgCl₂, 5 µmoles; cysteine, 15 µmoles; potassium arsenate, 50 µmoles; coenzyme A, 40 units. Water to 1 ml.

TABLE 4
Effect of pH on the dissimilation of pyruvate by enzyme preparation of Desulphovibrio desulphuricans

<table>
<thead>
<tr>
<th>pH</th>
<th>µL CO₂ evolved.</th>
</tr>
</thead>
<tbody>
<tr>
<td>5.3</td>
<td>8</td>
</tr>
<tr>
<td>5.8</td>
<td>20</td>
</tr>
<tr>
<td>6.4</td>
<td>122</td>
</tr>
<tr>
<td>7.0</td>
<td>101</td>
</tr>
<tr>
<td>7.7</td>
<td>62</td>
</tr>
<tr>
<td>8.0</td>
<td>17</td>
</tr>
<tr>
<td>9.0</td>
<td>6</td>
</tr>
</tbody>
</table>

Each Warburg flask contained 6 mg protein of enzyme extract in 0.2 M potassium phosphate of indicated pH; lithium pyruvate, 20 µmoles; MgCl₂, 50 µg; cocarboxylase, 100 µg; coenzyme A, 40 units; cysteine, 15 µmoles. Total volume, 2 ml. Gas phase, nitrogen; temperature, 34 C; time, 30 minutes.

determination of acetate according to the two methods described above. No lactate and acetyl-methylcarbinol could be detected.

The results given in table 2 show that stoichiometric amounts of acetate, alcohol, and CO₂ were formed from pyruvate as expected from equation (1).

Acetyl phosphate was not identified directly, but the experiment described above on the estimation and identification of acetate as one of the products of the reaction shows that acetyl phosphate was formed during the reaction.

Acetaldehyde. The dismutation of acetaldehyde by the bacterial extract was studied in bicarbonate-CO₂ buffer with or without inorganic phosphate. If the dismutation occurs according to equation (2), one mole of CO₂ should be evolved for every mole of acetate owing to the formation of an acidic group. If the reaction involves phosphorolysis according to equation (3), one mole of CO₂ should be absorbed for every mole of acetyl phosphate formed owing to the loss of one acid equivalent.

(2) \[ 2\text{CH}_3\text{CHO} + \text{H}_2\text{O} \rightarrow \text{CH}_3\text{CH}_2\text{OH} + \text{CH}_3\text{COOH} \]

(3) \[ 2\text{CH}_3\text{CHO} + \text{H}_2\text{PO}_4^- \rightarrow \text{CH}_3\text{CH}_2\text{OH} + \text{CH}_3\text{CO}^{-}\cdot\text{O}^{-}\cdot\text{PO}_4^-\]

But there was no evolution or absorption of CO₂; acetyl phosphate also was not formed. Negative results were obtained with acetaldehyde concentrations varying from 0.002 to 0.01 M. These results suggest that free acetaldehyde is not utilized by the enzyme or that it is inhibitory in the concentrations used.

**Acetyl phosphate formation.** Cysteine and coenzyme A had an activating effect on acetyl phosphate formation, especially when aged extracts were used (table 1). In some experiments acetyl phosphate formation could not be detected, possibly due to hydrolysis.

Transacetylase, which catalyzes the formation of acetyl phosphate from acetyl coenzyme A and inorganic phosphate, also was found to be present in the bacterial extracts as determined by the arsenolysis test of Stadtman et al. (1951). Added acetyl phosphate did not disappear in significant amounts in the absence of arsenate, showing the absence of acetyl phosphatase.

Transacetylase activity was markedly stimulated both by cysteine and coenzyme A (table 3). **Effect of pH on enzyme activity.** The relationship between reaction velocity and pH is shown in table 4. In phosphate buffer systems, the rate shows a marked change with pH between 5.8 to 7.7 with the optimum at 6.4. Beyond these limits the rate of CO₂ evolution falls off rapidly with practically no activity at pH 5.3 and 9.0.

**Effect of substrate concentration.** There was no change in the initial velocities when the pyruvate concentration was varied from 0.001 M to 0.0125 M indicating that the enzyme system is easily saturated by the substrate.

**Effect of phosphate concentration.** The effect of the concentration of added inorganic phosphate on the rate of CO₂ evolution is shown in table 5. Only a slight effect was observed when the phos-
Effect of phosphate concentration on pyruvate dissimilation by dialyzed Desulphovibrio desulphuricans enzyme preparation

<table>
<thead>
<tr>
<th>PHOSPHATE CONCENTRATION</th>
<th>μL CO₂ evolved</th>
<th>mg CO₂ evolved</th>
</tr>
</thead>
<tbody>
<tr>
<td>M/3</td>
<td>97.6</td>
<td>264.7</td>
</tr>
<tr>
<td>M/6</td>
<td>275.8</td>
<td>209.3</td>
</tr>
<tr>
<td>M/12</td>
<td>395.3</td>
<td>350.3</td>
</tr>
<tr>
<td>M/48</td>
<td>70.5</td>
<td>55</td>
</tr>
<tr>
<td>M/192</td>
<td>192</td>
<td>0</td>
</tr>
<tr>
<td>M/960</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

Each cup contained 6 mg protein of dialyzed enzyme in 0.1 M potassium acetate buffer, pH 6.4; lithium pyruvate, 25 μmoles; MgCl₂, 50 μg; co-carboxylase, 100 μg; DPN, 100 μg; plus inorganic phosphate in indicated concentrations. Total volume, 3 ml. Gas phase, nitrogen; temperature, 34 C; time, 60 minutes.

The enzyme preparation and other reagents used in the concentrations indicated contained 6 μmoles of inorganic phosphate as determined by Fiske and Subbarow method (1925).

Phosphate concentration was M/192, and a decided effect at M/48. The optimum phosphate concentration was found to be M/12. Above M/6 phosphate concentration, the rate of pyruvate breakdown was depressed.

Arsenate and pyrophosphate were found to replace inorganic phosphate in facilitating the dissimilation of pyruvate by the dialyzed enzyme (table 6). The bacterial extract does not contain pyrophosphatase as an inorganic phosphate was formed after incubation of the extract with pyrophosphate.

Cofactors. Attempts to prepare the pyruvic dissimilating enzyme in a form which was inactive without added Mg⁺⁺, co-carboxylase, and diphosphopyridine nucleotide were unsuccessful although some of the enzyme activity was lost during these procedures. These studies include prolonged dialysis against buffers varying in pH from 4.5 to 8.0 and repeated precipitation of the enzyme at pH 4.5. The possibility was open, however, that the coenzymes were present in a firmly bound form. Marked stimulation of activity by coenzyme A and cysteine was obtained with dialyzed enzyme. But this stimulation of activity was observed only occasionally, probably due to the presence of hydrogen sulfide in the preparation. However, preparations of the enzyme show a pronounced dilution effect, i.e., doubling the enzyme concentration more than doubles the reaction rate. Co-carboxylase, magnesium, diphosphopyridine nucleotide, thiocar or lipoic acid, lipothiamide pyrophosphate, or combinations of these had no effect on the reaction rate (table 7).

DISCUSSION

The conversion of pyruvate to alcohol, acetyl phosphate, and CO₂ by cell-free extracts of D. desulphuricans does not appear to involve the formation of free acetaldehyde as an intermediate followed by its dismutation to alcohol and ace-
tate or acetyl phosphate. The absence of carboxylase activity similar to that of yeast is shown by the fact that pyruvate is not decarboxylated in the absence of inorganic phosphate. Acetaldehyde also is not utilized as a substrate by the extract either in the presence or absence of inorganic phosphate. The primary product is possibly an enzyme-acetaldehyde or coenzyme-acetaldehyde complex which is oxidized further to the acetate level without appreciable dissociation to form free acetaldehyde.

The requirement of phosphate for the metabolism of pyruvate by the bacterial extract suggests the formation of acetyl coenzyme A as an intermediate which is converted to acetyl phosphate and coenzyme A in the presence of inorganic phosphate by transacetylase (Korkes et al., 1951). This is confirmed by the ability of arsenate to replace phosphate. But the effect of pyrophosphate in replacing phosphate has not hitherto been reported. The effect is not due to hydrolysis of pyrophosphate to inorganic phosphate since the extracts show no appreciable pyrophosphatase activity. A compound which gives a positive reaction in the Lipmann’s ferric-hydroxamic acid test accumulates during pyruvate conversion in the presence of pyrophosphate which is being studied further.

ACKNOWLEDGMENTS

The author is indebted to Dr. M. Damodaran and Dr. V. Jagannathan for their advice and many helpful suggestions.

SUMMARY

A soluble enzyme system from Desulphovibrio desulphuricans which catalyzes the conversion of two moles of pyruvate to one mole of acetyl phosphate, one mole of ethyl alcohol, and two moles of CO₂ has been described. The system required inorganic phosphate for pyruvate dissimilation. Pyrophosphate and arsenate could replace inorganic phosphate.

The reaction was most rapid at pH 6.4. The optimum phosphate concentration was M/12. Acetaldehyde was not utilized as a substrate.

ADDENDUM

During the preparation of this manuscript, a report by Dr. K. R. Butlin (1953) and his colleagues appeared in which they have described the dissimilation of pyruvate to alcohol, acetate, and CO₂ by nonproliferating live cells of a strain of D. desulphuricans.

REFERENCES


O’Kane, D. J., and Gunsalus, I. C. 1947 Ac-