During experiments on the effect of cyanide on the oxidation of ethanol and butyrate by dried cell preparations of Clostridium kluyveri (3), it was observed that cyanide depressed acetyl phosphate accumulation much more than the oxygen uptake. An investigation of this phenomenon led to the discovery that cyanide at rather high levels induces a rapid enzymatic acetylation of amino acids and proteins in which acetyl phosphate serves as the acetyl donor. This paper reports the basic observations that established the nature of the cyanide-induced reaction.

EXPERIMENTAL

Influence of Cyanide on Acyl Phosphate Decomposition—When acetyl phosphate is incubated with a dried cell preparation of Clostridium kluyveri, it is decomposed slowly by both enzymatic and non-enzymatic reactions (4). Table I shows that the addition of 0.1 M cyanide to this system causes a great acceleration in the rate of acetyl phosphate disappearance. This cyanide effect can also be observed with propionyl phosphate. The enzymatic character of the reaction was established by showing that no comparable disappearance of acyl phosphates occurs when the enzyme preparation has been boiled for 3 minutes.

A possible explanation of this phenomenon is that cyanide simply catalyzes the hydrolysis of acetyl phosphate. Some support for this idea was provided by the observation that the reaction results in an increase in acidity, measured manometrically in a bicarbonate buffer system. However, this explanation was excluded by showing that the accelerated disappearance of acetyl phosphate is always accompanied by a decrease in acidity.

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1 The rate is slower at lower cyanide concentrations. The influence of cyanide concentration on the rate will be considered more fully in a subsequent paper.
the amount of volatile acid. This pointed to the conversion of acetyl phosphate to a non-volatile product.

To test this possibility, 30 μM of C\textsuperscript{14}-labeled acetyl phosphate were allowed to incubate with 50 mg of dried cells at pH 8.1 in the presence and absence of 0.1 M KCN. After 4 hours at 26°, the volatile acid was removed by steam distillation and the non-volatile residue was examined for radioactivity. In the sample with cyanide, about 25 per cent of the total C\textsuperscript{14} was found in the non-volatile fraction, whereas less than 3 per cent was found in the sample without cyanide. This established the formation of one or more non-volatile products in the reaction.

The residue from the steam distillation of the volatile acids contained a considerable amount of denatured protein. The protein was separated

\begin{table}[h]
\centering
\begin{tabular}{|l|c|c|c|}
\hline
System & Acyl phosphate & & \\
 & 0 min. & 30 min. & 130 min. \\
\hline
Acetyl phosphate & 11.7 & 10.1 & 6.7 \\
& + HCN & 11.0 & 2.7 & 0.6 \\
Propionyl phosphate & 19.4 & 18.3 & 11.8 \\
& + HCN & 17.3 & 5.0 & 0.6 \\
\hline
\end{tabular}
\caption{Influence of Cyanide on Acyl Phosphate Decomposition}
\end{table}

by centrifugation, washed twice with 0.1 M acetic acid to remove adsorbed radioacetate, and finally washed with absolute alcohol. This material contained about half of the total radioactivity of the non-volatile fraction, indicating that acetate was bound to protein as well as to non-coagulable components of the system. This conclusion was confirmed by precipitating the protein from a separate aliquot of the medium with trichloroacetic acid and washing several times with trichloroacetic acid, acetic acid, and ethanol. Again the protein derived from the cyanide-treated sample contained more than 10 times as much C\textsuperscript{14} as the control.

The fixation of C\textsuperscript{14}-labeled acetyl groups on protein suggested that the reaction might be an acetylation of free amino groups. This interpretation was strengthened by the observation that the addition of proteins and amino acids to the reaction mixture increases the rate of disappearance of acetyl phosphate and volatile acids (Table II). The greatest acceleration was observed with casein hydrolysate, which contains the
highest concentration of free amino acids. The total fixation of C\textsuperscript{14}-labeled acetyl groups was also increased by the supplements. Addition of egg albumin increased the radioactivity in the protein fraction, whereas addition of amino acids increased the radioactivity in the alcohol-soluble, non-protein fraction and significantly decreased the fixation to protein. In general the results support the view that amino acids and proteins are directly involved in the cyanide-induced disappearance of acetyl phosphate.

### Table II

**Influence of Nitrogenous Compounds on Acetyl Group Fixation**

The reaction mixture contained 100 μM of lithium acetyl phosphate, 400 μM of potassium cyanide, 400 μM of sodium bicarbonate, 25 μM of acetate-1-C\textsuperscript{14} (75,000 c.p.m.), 50 mg. of dried cells (Lot I), and 100 mg. of the indicated supplements in a total volume of 3.5 ml. The cyanide was added 15 minutes after the other components to permit equilibration of labeled acetate with acetyl phosphate in the absence of cyanide. The mixture was then incubated for 2 hours at 26° \textit{in vacuo}. After removing samples for acetyl phosphate estimation, the protein was precipitated with 25 volumes of 95 per cent ethanol, washed with alcohol containing dilute acetic acid, and finally dissolved in aqueous alkali for C\textsuperscript{14} estimations. The alcohol-soluble, non-protein fraction was acidified and steam-distilled repeatedly with additions of unlabeled acetate to remove labeled acetate completely. C\textsuperscript{14} estimations were carried out on the neutralized residue.

<table>
<thead>
<tr>
<th>Supplement</th>
<th>Final acetyl phosphate</th>
<th>Final volatile acid</th>
<th>14C in Protein ppt.</th>
<th>14C in Non-protein fraction</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>33.6 μM</td>
<td>128 μM</td>
<td>7,500 c.p.m.</td>
<td>2,900 c.p.m.</td>
</tr>
<tr>
<td>Casein hydrolysate</td>
<td>0.0 μM</td>
<td>79 μM</td>
<td>3,100 c.p.m.</td>
<td>42,500 c.p.m.</td>
</tr>
<tr>
<td>Peptone</td>
<td>2.4 μM</td>
<td>93 μM</td>
<td>6,700 c.p.m.</td>
<td>31,800 c.p.m.</td>
</tr>
<tr>
<td>Egg albumin</td>
<td>20.0 μM</td>
<td>123 μM</td>
<td>10,600 c.p.m.</td>
<td>2,900 c.p.m.</td>
</tr>
</tbody>
</table>

An acetylation of amino groups by acetyl phosphate, according to Reaction 1, should liberate 1 mole of acid per mole of the acetyl derivative

\[
\text{Acetyl phosphate} + \text{NH}_4^+ - R - \text{COO}^- \rightarrow \text{acetyl-NH} - R - \text{COO}^- + \text{H}^+ + \text{phosphate}
\]

formed. The formation of acid was demonstrated manometrically by measuring carbon dioxide formation in a bicarbonate buffer system. With acetyl phosphate, enzyme, and casein hydrolysate, 5.6 μM of acid were formed during 45 minutes in the absence of cyanide, whereas 30.6 μM were formed in the presence of 0.15 M cyanide. With cyanide and without casein hydrolysate, 21.7 μM of acid appeared. It is evident that the addition of cyanide to the bacterial preparation and acetyl phosphate results
in a great increase in acid production. The further addition of casein hydrolysate causes a smaller but still significant effect. Acid production therefore can be used as an indication of the reactivity of amino compounds.

To find out whether the reactivity of casein hydrolysate is due to all amino acids or to specific components of the preparation, twenty-one amino acids were tested in groups for ability to accelerate the disappearance of acetyl phosphate in the presence of cyanide. Group A contained glycine, DL-alanine, DL-valine, L-leucine, DL-norleucine, and DL-isoleucine; Group B contained DL-serine, DL-threonine, DL-methionine, L-cysteine, and L-cystine; Group C contained L-aspartic acid and L-glutamic acid; Group D contained DL-phenylalanine, L-tyrosine, DL-tryptophan, L-histidine, L-proline, and L-hydroxyproline; and Group E contained L-arginine and DL-lysine. With the exception of Group C, all groups showed a reactivity equal to or greater than that of casein hydrolysate. With Group C, containing aspartic and glutamic acids, the disappearance of acetyl phosphate was less than with casein hydrolysate, but was significantly greater than the control without any amino acid supplement. Evidently the cyanide-induced reaction is relatively non-specific.

In another experiment the ability of individual amino acids to react in the acetyl phosphate-cyanide system was tested. The data of Table III show that all five of the amino acids tested, namely L-leucine, DL-lysine, DL-serine, L-proline, and glycine, are active. Lysine appears to react more rapidly than the neutral amino acids.

Isolation of Acetylglycine—The nature of the reaction between acetyl phosphate and glycine was established by isolating and characterizing the reaction product. 6 mM of lithium acetyl phosphate, 6 mM of glycine, 1.3 mg. of acetate-1-C\textsuperscript{14} containing about 6 \( \mu \)c. of C\textsuperscript{14}, 12 mM of sodium cyanide adjusted to pH 8 with sulfuric acid, and 3 gm. of dried cells of Lot R were present in 120 ml. of 0.025 M potassium phosphate buffer, pH 8.0. The radioactive acetate was added to serve as a label for the reaction product. The digest was incubated in vacuo for 15 hours at 26°. Approximately 1.75 m.eq. of volatile acid disappeared during the incubation.

6 volumes of ethanol were added to the reaction mixture to remove protein, and the supernatant was concentrated to a small volume, acidified with sulfuric acid to pH 2, and evaporated to dryness in vacuo to remove radioactive acetic acid. A second evaporation after adding 2 ml. of 0.1 N acetic acid removed the last traces of labeled volatile acid. The residue was dissolved in water, neutralized to pH 8 with barium hydroxide, and centrifuged. The supernatant and washings were evaporated to dryness and extracted six times with 10 ml. of 95 per cent ethanol. The extract,
containing 89 per cent of the non-volatile radioactive products, was evaporated to remove alcohol and dissolved in water, and the barium salts were converted to the free acids by addition of an equivalent amount of sulfuric acid. After removal of the barium sulfate, the aqueous solution was exhaustively extracted with butanol. The extract was freed of butanol, dissolved in water, and, after treatment with Norit, was filtered and evaporated to dryness, yielding 294 mg. of a crystalline product. This material was twice sublimed at a pressure of about 100 μ and a temperature of 160–200°. The crystalline sublimate was shown to be acetylglucine. The melting point and mixed melting point with synthetic acetylglucine were 206.5–207°; acid equivalent weight 118.3 (theory,

TABLE III
Reactivity of Some Amino Acids

The reaction mixture contained 400 μM of potassium cyanide, 400 μM of sodium bicarbonate, 100 μM of lithium acetyl phosphate, 50 mg. of dried cells (Lot I), and either 50 μM of the L isomer or 100 μM of the DL isomer of the indicated amino acid in 3.5 ml. of water. Volatile acid determinations were made after incubating 2 hours at 26°.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Final volatile acid</th>
<th>Δ volatile acid</th>
</tr>
</thead>
<tbody>
<tr>
<td>None added</td>
<td>104</td>
<td>-22</td>
</tr>
<tr>
<td>L-Leucine</td>
<td>82</td>
<td>-48</td>
</tr>
<tr>
<td>DL-Lysine</td>
<td>77</td>
<td>-27</td>
</tr>
<tr>
<td>DL-Serine</td>
<td>77</td>
<td>-23</td>
</tr>
<tr>
<td>L-Proline</td>
<td>81</td>
<td>-27</td>
</tr>
<tr>
<td>Glycine</td>
<td>77</td>
<td></td>
</tr>
</tbody>
</table>

117.1); nitrogen content 11.92 per cent (theory, 11.95 per cent). A sample of the product was hydrolyzed by heating in a sealed tube with 2 N NaOH for 4 hours at 95°. The ratio of amino nitrogen (2) to glycine (1) to acetic acid in the hydrolysate was found to be 1:0.99:0.88.

The yield of pure acetylglucine in the above isolation was about 60 per cent of that to be expected on the basis of the amount of volatile acid that disappeared during the reaction. This proves that acetylglucine is a major product of the reaction between acetyl phosphate and glycine in the presence of cyanide, but does not exclude the formation of other products in substantial amounts.

The possible formation of other compounds was investigated thoroughly by the application of methods of paper chromatography and radioautography to enzymatic products obtained by the use of radioactive acetyl phosphate and radioactive glycine, respectively. In an experiment with
labeled acetate, the reaction mixture contained 50 μM of acetyl phosphate, 50 μM of glycine, 20 μM of C14-labeled sodium acetate, 20 mg. of dried cells of Lot R, and 100 μM of sodium cyanide-sulfuric acid per ml. of 0.02 M phosphate buffer, pH 8. The total volume was 0.3 ml. and the total radioactivity was 2 μc. After 6 hours incubation, a 20 μl. aliquot of the deproteinized and desalted mixture containing about 0.14 μc. of C14 was used for a two-dimensional paper chromatogram with phenol-water and butanol-propionic acid-water as developing solvents. A radioautograph was made from the paper chromatogram to locate labeled compounds. Only a single spot was found in the radioautograph and its position was identical with that of synthetic radioactive acetylglycine. Radioactive acetate does not give a spot on such chromatograms because it volatilizes from the paper during development. Other amino acids were present in the enzyme preparation and presumably were acetylated to some extent, but the amount of each acetyl derivative must have been too small, of the order of 0.5 per cent of the acetylglycine assuming random acetylation, to cause detectable blackening of the film. It may be concluded that acetylglycine is the main stable, non-volatile acetate derivative formed in the reaction mixture. Other acetate derivatives, if formed, must be present in amounts less than 1 per cent of the acetylglycine.

The experiment with labeled glycine was carried out in the same manner, with about the same quantity of C14. The radioautographs showed two large spots corresponding to glycine and acetylglycine and several much smaller spots. Most of the latter were shown to be due to impurities in the synthetic radioglycine used in the experiment. However, one of the minor spots seems to be due to a product of the enzymatic reaction. The compound has not been identified, but, since it gives a purple color with ninhydrin, it is probably a glycine derivative containing a free amino group.

**Isolation of Acetylleucine**—A crystalline product of the reaction of L-leucine and acetyl phosphate was isolated from a reaction mixture similar to that used for the preparation of acetylglycine. The compound, obtained in 20 per cent yield based on the amount of acetyl phosphate decomposed, was identified as acetylleucine by the following properties: m.p. 185–186° (Synge (5) reported 186–188° for acetyl-D-leucine); acid equivalent weight 175; theory 173; total nitrogen 8.2 per cent; theory 8.1 per cent. The possible formation of other products was not investigated.

**DISCUSSION**

The evidence presented in this paper establishes the fact that dried cell preparations of *Clostridium kluyveri* catalyze an acetylation of amino acids in the presence of a high concentration of cyanide. The enzymatic
character of the reaction is indicated by heat inactivation experiments. The reaction is relatively non-specific, since it occurs with many amino acids and with proteins such as egg albumin.

The physiological significance of the reaction is not clear. Since in enzyme preparations the reaction is dependent upon the presence of cyanide, it certainly cannot occur in the living cell unless a normal cell component is able to substitute for cyanide. Actually the available data from balance experiments on growing cultures make it unlikely that acetylated amino acids are formed or, at least, accumulate in vivo in amounts comparable to those observed in enzyme preparations supplied with cyanide. An alternative interpretation is that the enzyme system responsible for amino acid acetylation in the presence of cyanide normally catalyzes some other acetyl transfer reaction, such as the synthesis of a C₄ precursor of butyric acid. Cyanide may act upon a product or an intermediate of such a reaction so as to induce an unphysiological acetylation of amino acids. The final step in the reaction series may be enzymatic or non-enzymatic.

SUMMARY

The addition of cyanide at rather high concentrations greatly accelerates the decomposition of acetyl phosphate in the presence of dried cell preparations of Clostridium kluyveri. The increased rate of decomposition is due to a relatively non-specific cyanide-induced enzymatic acetylation of amino acids and proteins. Acetylglycine and acetylleucine have been isolated and identified as products of the reaction with glycine and leucine, respectively.

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CYANIDE-INDUCED ACETYLATION OF AMINO ACIDS BY ENZYMES OF CLOSTRIDIUM KLUYVERI
E. R. Stadtman, J. Katz and H. A. Barker


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