**An aerobic Degradation of Lysine**

**II. COFACTOR REQUIREMENTS AND PROPERTIES OF THE SOLUBLE ENZYME SYSTEM**

**Thressa C. Stadtman**

*From the Laboratory of Biochemistry, National Heart Institute, National Institutes of Health, Bethesda 14, Maryland*  

(Received for publication, March 29, 1963)

Lysine is fermented to acetate, butyrate, and ammonia (Reaction 1) by *Clostridium sticklandii* (3, 4), by the simultaneous action of two strains of *Escherichia coli* (5), and also by an as yet unidentified *Clostridium M-E* isolated from lysine-containing anaerobic enrichment cultures.  

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

From isotope studies it appears that the pathway of degradation of the amino acid in *C. sticklandii* (3), and also in *Clostridium* M-E, differs from those so far recognized in yeast, Neurospora, mammals, and certain other bacteria. Our interest in this fermentation of lysine, therefore, is centered on three main points: (a) identification of the steps that comprise the metabolic pathway, (b) the nature of the energy-yielding reactions by means of which these anaerobic microorganisms conserve the energy made available by the fermentation, and (c) elucidation of the roles of two of the required cofactors, i.e. a cobamide coenzyme (B_{12} coenzyme) and a disulfide catalyst which, in this process, might be involved in types of reactions different from those in which they are already known to function.  

Some general properties of the soluble enzyme system that carries out Reaction 1, together with balance studies and experiments demonstrating cofactor requirements, are described in the present communication.

**EXPERIMENTAL PROCEDURE**

**Materials**

Purified intrinsic factor, crystalline dimethylbenzimidazolyl-cobamide coenzyme, saccharopine, \(\alpha\-N\-acetyllysine\), \(\epsilon\-hydroxy-\alpha\-aminoacipinic acid, mesoaminopinic acid, and dithiolum acetyl phosphate were generous gifts from L. Ellenbogen, K. Folkers, H. P. Broquist, J. E. Folk, H. Vogel, E. Work, and E. R. Stadtman, respectively.  

Dilithium carbamyl phosphate was purchased from the Mann Research Laboratories; potassium pyruvate and glutaconic acid were products of the K. and K. Laboratories.

* Preliminary reports of a portion of this work have appeared elsewhere (1, 2). Paper 1 of this series is Reference 3.

1. The mud sample from which this organism was isolated was kindly collected by Drs. Betty Meggers and Clifford Evans, Smithsonian Institute, Washington, D. C., from the left bank of the Rio Daule, Ecuador.

2. V. Tarantola and T. C. Stadtman, unpublished experiments.

**Methods**

**Culture of Microorganisms and Preparation of Extracts—**

*Clostridium M-E* was cultured anaerobically in 20-liter carboys filled with a medium of the following composition: L-lysine HCl, 0.5%; Difco yeast extract, 0.5%; D-glucose, 0.2%; Na_{2}CO_{3}, 0.15%; KH_{2}PO_{4}, 0.135%; CaCl_{2}.2H_{2}O, 0.001%; MgSO_{4}.7H_{2}O, 0.02%; FeSO_{4}.7H_{2}O, 0.005%; Na_{2}S-9H_{2}O, 0.03%; and potassium ethylenediaminetetraacetate, 10^{-4} M, in distilled water. The required amounts of glucose, Na_{2}CO_{3}, and sodium sulfide were sterilized in three separate solutions which were added to the sterile, cool solution of the remaining ingredients just before inoculation. The sterile carbonate solution was neutralized with concentrated HCl just before use. The final pH of the medium is about 7. When 10% inocula are employed, the cultures attain maximal turbidity within 12 to 15 hours at 30°C. Growth is more rapid at 37°C.  

Cells were collected in a Sharples supercentrifuge, washed once in cold distilled water, and resuspended in 0.05 M Tris-HCl buffer, pH 8, containing 10^{-3} M EDTA.  

Extracts were prepared in an Amino French pressure cell and were separated from debris by centrifugation at 30,000 x g for 30 minutes. The clear brown amber solutions, containing 50 to 60 mg of protein per ml, actively catalyzed the degradation of lysine to fatty acids and ammonia. In contrast, extracts prepared in a 10 kV Raytheon magnetostriiction oscillator (5-minute exposure) from aliquots of the same batches of cells were inactive even though the amount of protein extracted was almost identical.  

*C. sticklandii* was cultured in the medium described previously (6) supplemented with 0.1% L-lysine HCl. Extracts were prepared in the French pressure cell and clarified as described above.

**Stabilization and Storage of Extracts—** After identification of some of the coenzymes required for the conversion of lysine to fatty acids (see "Results"), experiments were carried out to determine whether the enzyme system could be stabilized by the addition of any or all of these substances to the extracts before storage. Maximal stability appeared to be afforded by the addition of potassium pyruvate (5 mM) and DPN (0.5 to 1 mM). Small aliquots of the crude extracts so supplemented were stored in plastic tubes in liquid N2. Extracts of *Clostridium M E* treated in this way have been found to retain full activity for 1 year whereas those placed at -18°C lost appreciable activity within a few days. Similar cofactor supplementation and low temperature storage also improved the keeping qualities of *C. sticklandii* extracts but, in spite of these precautions, marked loss
of activity occurs during normal manipulations of enzyme preparations from this microorganism.

**Enzyme Assay Conditions**—The over-all lysine fermentation as catalyzed by crude freshly prepared soluble extracts is assayed in 0.5 to 0.6-ml reaction mixtures containing Tris-HCl buffer, pH 8.0, 20 μmoles; K2HPO4, 5 μmoles; potassium pyruvate, 3 to 6 μmoles; MgCl2, 3 μmoles; ADP, 3 μmoles; DPN, 0.4 μmole; and 1- or 6-C14-dl-lysine-HCl (2500 to 4000 c.p.m. per μmole), 20 μmoles. Usually 10 to 12 mg of bacterial protein must be employed to provide an adequate level of the rate-determining enzyme for this complex series of reactions. Other reactants also participating in the over-all process are indicated in pertinent sections under "Results." The samples are incubated at 30° in rubber-stoppered test tubes, 10 × 75 mm, in an atmosphere of helium for periods of 30 to 90 minutes (linearity of reaction with time is determined for each extract). About 50% inhibition of the reaction is usually observed when the gas phase of the stoppered tubes is air rather than helium. The optimal substrate concentration is in the range of 0.03 to 0.05 M. The rate of fatty acid formation falls off appreciably at higher or lower lysine concentrations. Either the D or the L isomer can be employed; presumably the extracts contain a racemase (present in excess).

In routine assays, reactions are terminated by the addition of 1 ml of 10⁻⁷ M HCl to each tube, followed at once by 0.3 g of moist Dowex 50-X3 (H⁺). Immediate mixing of the diluted reaction mixtures with the resin insures adequate acidification of the samples and precipitation of proteins. The stoppered tubes are then shaken vigorously on a shaker for 5 minutes and centrifuged, and 0.1-ml aliquots of the supernatant solutions are assayed in a scintillation spectrometer for noncational labeled products derived from labeled lysine. Under these conditions residual C¹⁴-lysine is quantitatively adsorbed on the resin. In supernatant solutions. From the specific activity of the starting substrate, the yield of labeled fatty acid products can be calculated. Results are usually expressed as micromoles of lysine decomposed.

**Other Analytical Methods**—Ammonia was recovered from reaction mixtures by diffusion in a model 120 multipurpose rotator (Scientific Industries, Inc., Springfield, Massachussetts) and estimated by reaction with Nessler's solution. Orthophosphate and 10-minute acid-labile (1 N HCl, 100°) phosphate were estimated by the Fiske-SubbaRow method (7). Phosphatase activity was determined by measuring the amounts of DPN and pyruvate were 0.6 and 3 μmoles, respectively. In addition, CoA-SH, 0.2 μmole, carbamyl-P, 7 μmoles, DMBCl*, dipyridyl and 1,10-phenanthroline, it was found that the lysine-decomposing system in fresh active extracts of _C. sticklandii_ is inhibited by the addition of these substances (Table II), suggesting that ferrous iron is a cofactor for the enzyme system from this source also. Similar inhibition of the lysine system in fresh extracts of Clostridium M-E has been observed (2). Extracts of the latter organism which require Fe⁺⁺ addition for activity are unable to decompose lysine when metal ions such as Mn⁺⁺, Co⁺⁺, or Mg⁺⁺ are substituted for Fe⁺⁺.

**Effect of Cobamide Coenzyme**—It was reported earlier that lysine fermentation to fatty acids is inhibited by purified intrinsic factor preparations and that this inhibition is overcome by the addition of cobamide coenzyme just equivalent to the cobamide-binding capacity of the intrinsic factor protein added (1, 2). Furthermore, enzyme fractions partially resolved for the coenzyme by treatment with charcoal could be reactivated with the intact coenzyme whereas light-inactivated coenzyme or free vitamin B₁₂ (cyanocobalamin) often failed to substitute. The complete reaction mixture (0.5 ml) contained the components listed under "Enzyme Assay Components" except that the amounts of DPN and pyruvate were 0.6 and 3 μmoles, respectively. In addition, CoA-SH, 0.2 μmole, carbamyl-P, 7 μmoles, DMBC* orthophosphate, 5 × 10⁴ μmole, and _C. sticklandii_ crude extract, 12.5 mg of protein, were present. Incubation time was 70 minutes. The levels of DPN and CoA-SH employed were established in preliminary experiments to be optimal for these conditions. Sufficient B₁₂ coenzyme and pyruvate (1 μmole) were supplied with the enzyme; no appreciable decrease in activity was observed when the above indicated amounts of these components were omitted.

### Table 1

<table>
<thead>
<tr>
<th>Omission</th>
<th>1-C¹⁴-Lysine decomposed</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>9.0</td>
</tr>
<tr>
<td>DPN</td>
<td>0.8</td>
</tr>
<tr>
<td>CoA-SH</td>
<td>5.0</td>
</tr>
<tr>
<td>Carbamyl-P</td>
<td>3.6</td>
</tr>
<tr>
<td>ADP</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* In the tables and figures, DMBC is used as the abbreviation for dimethylbenzimidazolylcobamide.
The reaction mixtures (0.65 ml) contained the components listed under "Enzyme Assay Conditions" plus DMBE coenzyme, 5 X 10⁻⁸ μmole, French press extract, 14.2 mg of protein, chelator as indicated, and, for Experiment 1, acetyl-CoA, 0.55 μmole, or, for Experiment 2, carbamyl-P, 9 μmoles. In the absence of either acetyl-CoA or carbamyl-P only 0.5 μmole of lysine was decomposed. Incubation time was 60 minutes.

<table>
<thead>
<tr>
<th>Inhibitor added</th>
<th>1-C⁴-Lysine decomposed (μmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Experiment 1</td>
</tr>
<tr>
<td>None</td>
<td>8.1</td>
</tr>
<tr>
<td>α,α-Dipyridyl</td>
<td>2.2</td>
</tr>
<tr>
<td>1,10-o-Phenanthroline</td>
<td>2.7</td>
</tr>
</tbody>
</table>

* The final concentration of each inhibitor was 4.6 X 10⁻⁴ M.

The enzyme preparation (12.5 mg of protein, French press extract of Clostridium M-E) was incubated initially for 20 minutes in helium at 30° with the following components: Tris buffer, pH 8.1, 20 μmoles; MgCl₂, 3 μmoles; K₂HPO₄, 5 μmoles; plus potassium pyruvate, 4 μmoles; FeSO₄, 4 μmoles; and DPN, 0.4 μmole, as indicated. The samples were then chilled in ice, opened, and supplemented with the omitted cofactors plus DMBE coenzyme, 6 X 10⁻⁴μmole, and substrate. The complete reaction mixtures (0.5 ml) were incubated in helium for 20 minutes. In similar experiments with this enzyme preparation it was established that a prior incubation with buffer, Mg⁺⁺, and phosphate only was insufficient to activate the lysine system. Inclusion of DPN in the initial incubation usually proved inhibitory even if samples were further supplemented with DPN or pyruvate, or both, for the second incubation.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>1-C⁴-Lysine decomposed (μmoles)</th>
</tr>
</thead>
<tbody>
<tr>
<td>No prior incubation</td>
<td>0.7</td>
</tr>
<tr>
<td>Prior incubation with:</td>
<td></td>
</tr>
<tr>
<td>Pyruvate</td>
<td>7.8</td>
</tr>
<tr>
<td>Fe⁺⁺</td>
<td>1.5</td>
</tr>
<tr>
<td>Pyruvate + Fe⁺⁺</td>
<td>8.6</td>
</tr>
<tr>
<td>DPN</td>
<td>0.8</td>
</tr>
<tr>
<td>Pyruvate + DPN</td>
<td>0.3</td>
</tr>
<tr>
<td>Pyruvate + Fe⁺⁺ + DPN</td>
<td>0.7</td>
</tr>
</tbody>
</table>
scribed were also added, this was not true for all. In Experiment 1, Table IV, are shown the marked activating effects of either carbamyl-P or acetyl-P on the ability of the enzyme system to decompose lysine. Phosphoenolpyruvate, creatine phosphate, and phosphoramidate were inactive with this preparation. The acetyl and carbamyl phosphates apparently are not active by virtue of their ability to serve as the source of a nucleoside triphosphate because ATP, ITP, GTP, CTP, and UTP, at 0.5- to 5.0-mole levels, failed to activate the system. On the other hand, the probability that acyl phosphate is needed to generate an acyl-CoA is suggested (a) by the discovery that CoA-SH, in addition to acyl phosphate, must be added for maximal activation of some enzyme preparations of *C. sticklandii*, and (b) by the observation that acetyl-CoA (0.9 mM) replaces the requirement for both CoA-SH and acyl phosphate (Experiment 2, Table IV). Whereas 0.16 mM CoA-SH is required to saturate the enzyme system in the presence of an optimal level of carbamyl-P (Fig. 3), higher though still catalytic concentrations of acetyl-CoA (at least 0.8 mM) are necessary for maximal activation of lysine decomposition (Table V). The higher requirement for acetyl-CoA probably reflects the instability of this thioester in the relatively crude enzyme preparations used. Particularly noteworthy are the data of Table V showing that pyruvate and acetyl-CoA are both obligatory requirements of the enzymic system. With the extracts used in these experiments no prior incubation with pyruvate was necessary for maximal activity.

The discovery (14) that acetate kinase catalyzes the synthesis of acetyl-P from carbamyl-P and acetate explains how carbamyl-P can replace acetyl-P for generation of the acetyl-CoA required for lysine degradation. Both lysine-fermenting organisms were activated by a 28-minute prior incubation with pyruvate as described in the legend of Fig. 2. DMBC coenzyme, $5 \times 10^{-6}$ μmole, was also included. In the second incubation with substrate the reaction mixtures (0.55 ml) contained, in addition, DPN, 0.4 μmole, FeSO₄, 3 μmole, and dithiolum carbamyl-P or dithiolum acetyl-P as indicated. For Experiment 2 a *C. sticklandii* extract (13.4 mg of protein) was employed; there was no prior incubation with pyruvate. The complete reaction mixtures (0.61 ml) contained the same components as Experiment 1 except that iron was omitted; further additions were made as indicated. Incubation times for both experiments were 60 minutes.

![Figure 2. Increase in activity of lysine-decomposing system as a function of time of prior incubation with pyruvate.](image)

**Fig. 2.** Increase in activity of lysine-decomposing system as a function of time of prior incubation with pyruvate. During the prior incubation in helium at 30° the reaction mixtures contained Tris buffer, pH 7.5; 20 μmole; MgCl₂, 3 μmole; K₂HPO₄, 5 μmole; potassium pyruvate, 4 μmole; ADP, 3 μmole; and crude Clostridium M-E enzyme, 0.2 ml, 10.8 mg of protein. Duration of prior incubation was as indicated. Then DPN, 0.4 μmole, 1-Cl⁵-lysine, 20 μmole, and FeSO₄, 3 μmole, were added. The second incubation period was 70 minutes. Parallel samples supplemented with ADP during the second incubation rather than the first or incubated initially in open tubes rather than in helium exhibited identical activities.

**Table IV**

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Addition to complete system</th>
<th>1-C⁵-Lysine decomposed, μmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>None</td>
<td>1.2</td>
</tr>
<tr>
<td></td>
<td>Carbamyl-P, 7 μmoles</td>
<td>12.5</td>
</tr>
<tr>
<td></td>
<td>Acetyl-P, 7 μmoles</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>Carbamyl-P, 3.5 μmoles, + acetyl-P, 3.5 μmoles</td>
<td>10.9</td>
</tr>
<tr>
<td></td>
<td>Hydrolyzed* carbamyl-P, 7 μmoles</td>
<td>1.9</td>
</tr>
<tr>
<td>2</td>
<td>None</td>
<td>0.6</td>
</tr>
<tr>
<td></td>
<td>Acetyl-P, 3 μmoles; 6 μmoles</td>
<td>1.6; 2.9</td>
</tr>
<tr>
<td></td>
<td>Carbamyl-P, 3 μmoles; 6 μmoles</td>
<td>1.3; 2.7</td>
</tr>
<tr>
<td></td>
<td>CoA-SH, 0.1 μmole</td>
<td>1.8</td>
</tr>
<tr>
<td></td>
<td>CoA-SH, 0.1 μmole, + acetyl-P, 3 μmoles; 6 μmoles</td>
<td>6.8; 8.1</td>
</tr>
<tr>
<td></td>
<td>CoA-SH, 0.1 μmole, + carbamyl-P, 3 μmoles; 6 μmoles</td>
<td>5.5; 10.5</td>
</tr>
<tr>
<td></td>
<td>CoA-SH, 0.1 μmole, + acetyl-P, 3 μmoles, + carbamyl-P, 3 μmoles</td>
<td>8.9</td>
</tr>
<tr>
<td></td>
<td>Acetyl-CoA, 0.5 μmole</td>
<td>8.8</td>
</tr>
</tbody>
</table>

* Heated for 5 minutes at 100°, pH 7.

![Figure 3. Dependence of enzymic activity on CoA-SH concentration.](image)

**Fig. 3.** Dependence of enzymic activity on CoA-SH concentration. The experimental conditions and enzyme were the same as those given for Experiment 2 of Table IV. Carbamyl-P, 6 μmoles, was present in each sample.
isms are rich in acetate kinase; as shown in Table VI, either the unfractionated lysine system or a purified acetate kinase preparation from C. sticklandii was found to catalyze the conversion of carbamyl-P and acetate to acetyl-P. The latter was estimated and identified chromatographically (10) as its hydroxamate. It is to be noted that ADP (2 mm) and MgCl₂ (6 to 10 mm), which are normal cofactors for acetate kinase, were also required for acetyl-P formation from carbamyl-P and acetate.

The acetyl-P, either added directly or produced enzymically from added carbamyl-P and acetate (always present in crude bacterial extracts, 1 to 5 mm), is finally used for the synthesis of acetyl-CoA by the action of phosphate acetyltransferase, which is present in extracts of both lysine-decomposing organisms.

In the crude enzyme systems studied, carbamyl-P was a somewhat more efficient activator of lysine decomposition than acetyl-P. Maximal activation with either phosphate ester was observed at about 10 mM final concentrations. Mixtures of the two esters gave intermediate values, suggesting that the higher concentrations of acyl phosphate were inhibitory.

TABLE V

Dependence of C. sticklandii lysine system on both acetyl-CoA and pyruvate

The reaction mixtures (0.03 ml) contained the components listed under "Enzyme Assay Conditions" except that pyruvate (4 μmoles) was added only as indicated. Each sample also contained DMBC coenzyme, 5 × 10⁻⁴ μmole; in the absence of this added factor only 4 μmoles of lysine were decomposed rather than 7.5 μmoles (last line of table). The enzyme (14.2 mg of protein) was a crude French press extract of C. sticklandii. Incubation time was 60 minutes.

<table>
<thead>
<tr>
<th>Additions</th>
<th>1-C¹⁴-Lysine decomposed μmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td>0.4</td>
</tr>
<tr>
<td>Pyruvate</td>
<td>0.6</td>
</tr>
<tr>
<td>Acetyl-CoA, 0.82 mm</td>
<td>0.8</td>
</tr>
<tr>
<td>Pyruvate + acetyl-CoA, 0.41 mm</td>
<td>3.1</td>
</tr>
<tr>
<td>Pyruvate + acetyl-CoA, 0.82 mm</td>
<td>7.5</td>
</tr>
</tbody>
</table>

TABLE VI

Requirements for acetyl-P formation from carbamyl-P and acetate

The reaction mixtures, in 0.5-ml volumes, contained Tris buffer, pH 8.0, 20 μmoles; dilithium carbamyl-P, 10 μmoles; potassium acetate, 100 μmoles; 2-mercaptoethanol, 3 μmoles; MgCl₂, 3 μmoles except where indicated otherwise; ADP, as indicated; and crude extract or purified acetate kinase as shown. Incubation time was 20 minutes at 30°.

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Acetyl phosphate formed* by Crude C. sticklandii extract, 0.005 ml</th>
<th>Purified acetate kinase, 2 units</th>
</tr>
</thead>
<tbody>
<tr>
<td>No ADP</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>ADP, 0.5 μmole</td>
<td>2.7</td>
<td>4.8</td>
</tr>
<tr>
<td>ADP, 1.0 μmole</td>
<td>6.05</td>
<td>1.0</td>
</tr>
<tr>
<td>ADP, 3.0 μmole</td>
<td>6.05</td>
<td>1.0</td>
</tr>
<tr>
<td>ADP, 3 μmole; no Mg²⁺</td>
<td>0.23</td>
<td>0.2</td>
</tr>
</tbody>
</table>

* Estimated spectrophotometrically as the hydroxamate (10) after the addition of 720 μmoles of neutralized hydroxylamine at the end of the incubation.

Product Stoichiometry—The relative yields of ammonia and labeled fatty acid products from lysine-C¹⁴ have been determined in dialyzed extracts of Clostridium M-E. In Table VII it can be seen that approximately 2 equivalents of ammonia appear for each micromole of C¹⁴-acetate formed from 1-C¹⁴-lysine or of

<table>
<thead>
<tr>
<th>Experiment</th>
<th>Substrate</th>
<th>Cofactor omitted</th>
<th>Products formed*</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td>NH₃</td>
</tr>
<tr>
<td>1</td>
<td>1-C¹⁴-Lysine</td>
<td>None</td>
<td>20.4</td>
</tr>
<tr>
<td>2</td>
<td>1-C¹⁴-Lysine</td>
<td>None</td>
<td>20.2</td>
</tr>
<tr>
<td>3</td>
<td>1-C¹⁴-Lysine</td>
<td>None</td>
<td>12.4</td>
</tr>
<tr>
<td>4</td>
<td>1-C¹⁴-Lysine</td>
<td>None</td>
<td>20.2</td>
</tr>
</tbody>
</table>

* Dashes indicate that analyses were not performed. Where values are given for both fatty acids in the same sample, the hydroxamates were prepared and examined for C¹⁴ after separation by paper chromatography (see "Methods").
C⁴ butyrate formed from 6 C⁴ lysine. Furthermore, when the two different radioactive substrates are decomposed in parallel reaction mixtures, the yield of labeled acetate from the carboxyl end of the molecule is the same as the yield of labeled butyrate from the e carbon end of the molecule. In Experiments 1 and 3 of Table VII the complementary fatty acid formed in each reaction mixture, isolated as its hydroxamate (10), was found to be nonradioactive. In fact, none of the soluble enzyme preparations, from either of the two Clostridia employed, have been found to catalyze any other type of cleavage of the lysine carbon skeleton. This is in contrast to results obtained with fresh cell suspensions and many lots of dried cells (2, 3) which catalyze, in addition, the reciprocal type of cleavage. The data of Experiment 2 of Table VII show that omission of Blz coenzyme from the reaction mixture results in no selective inhibition of product formation. In other experiments that will not be described in detail, similar results are obtained when a dye such as methyl viologen is employed as alternate electron acceptor or when Amytal and arsenite are employed as inhibitors; parallel inhibition of all three products is observed and no evidence of a partial inhibition was obtained. In addition, the reciprocal type of cleavage. The data of Experiment 1, Table VIII, except that ADP, 1 µmole, orthophosphate, 11 µmoles, 2-deoxy-d-glucose, 25 µmoles, and Sigma hexokinase, 0.5 mg, were also present in each sample.

Table IX

Lysine-dependent esterification of orthophosphate

The enzyme (7.9 mg of protein) was a supernatant solution prepared as described in Table VIII. The reactants and conditions were those described for Experiment 1, Table VIII, except that ADP, 1 µmole, orthophosphate, 11 µmoles, 2-deoxy-d-glucose, 25 µmoles, and Sigma hexokinase, 0.5 mg, were also present in each sample.

<table>
<thead>
<tr>
<th>Incubation time</th>
<th>µmoles</th>
</tr>
</thead>
<tbody>
<tr>
<td>30 min</td>
<td>-4.0</td>
</tr>
<tr>
<td>60 min</td>
<td>-6.5</td>
</tr>
</tbody>
</table>

AMP, 5 µmoles, +
ADP, 0.5 µmole

Orthophosphate

-4.1 -6.4

1-C⁴-Acetate

+4.1 +6.4

ATP Synthesis—The data of phosphate balance studies (Tables VIII and IX) show that when ADP and orthophosphate are present lysine decomposition occurs with a concomitant synthesis of ATP. Whereas crude French pressure cell extracts are equally active with either ADP or AMP plus a low level of ADP (Experiment 2, Table VIII) and even exhibit fair activity in the absence of added adenylates, the soluble supernatant fraction obtained by centrifugation at 104,000 X g for 4 hours, requires addition of substrate levels of ADP for activity (Experiment 1, Table VIII). The inability of AMP and a catalytic level of ADP to activate the supernatant fraction suggests that adenylyl kinase was sedimented with the particulate fraction. The close correspondence between lysine decomposed and phosphate esterified by the supernatant enzyme suggests that phosphatases were also sedimented. The stoichiometry of the reaction with respect to 1-C⁴-acetate formation is shown in Table IX. For this experiment, hexokinase and 2-deoxy-d-glucose, in the presence of a catalytic level of ADP, were employed to trap the ATP generated. In parallel samples containing P³₂-labeled orthophosphate, equivalent amounts of P³₂-labeled organic phosphate accumulated. The phosphorylated product was identified chromatographically as 2-deoxyglucose 6-phosphate. In reaction mixtures lacking a trapping system and containing P³₂-labeled orthophosphate, the substrate-dependent synthesis of P³₂-labeled ATP was demonstrated. From these data it appears that 1 equivalent of orthophosphate is esterified for each mole of lysine fermented to fatty acids and ammonia, and hence the equation of the over-all fermentation can be amended as shown in Reaction 2.

Lysine + 2 H₂O + HPO₄⁻ + ADP

→ 2 NH₄⁺ + acetate + butyrate + ATP (2)

Substrate Specificity—Aside from the d and the L isomers of lysine which are fermented at equal rates by the unfractonated enzyme preparations, the only other compound which has been found to be actively decomposed to fatty acids and ammonia in the complete reaction mixture is α-N-acetyl-L-lysine. Although the latter derivative gives rise to the expected products from the lysine moiety plus 1 extra equivalent of acetate from the N-acetyl group, it does not appear to be an intermediate. Thus, in an experiment wherein 1-C⁴-lysine of high specific activity was fermented in the presence of unlabeled α-N-acetylysinine under conditions such that both compounds were partially decomposed, the residual α-N-acetylysinine isolated from the reaction mixture by paper chromatography was found to contain no C⁴. This would suggest that the acetylated lysine is first attacked by a hydrolase which converts it to free lysine and that the latter then is fermented in the normal fashion. A preliminary survey of the substrate specificity of the hydrolase attacking α-N-acetyl-L-lysine indicates that the following are
not decomposed: e-N-acetyl-dl-lysine, N-acetylglutamic acid, N-acetyl-
\( \alpha \)-aminocaproic acid, N-acetyl-\( \alpha \)-aminobutyric acid, N-acetyl-
\( \gamma \)-aminobutyric acid, N-acetyltryptophan, and N-acetylvaline.

The failure to observe either acetate formation or liberation of
ammonia (Reaction 2) is a balanced oxidation-reduction reaction
which results in the net yield to the organism of 1 equivalent of
high energy ester phosphate per mole of amino acid decomposed.
It is possible, therefore, that it may have a
role in the over-all reaction and normally decays some derivative
of lysine which has undergone modification at the \( \epsilon \) carbon
end of the molecule.

The following other mono- and dibasic amino acids and derivat-
ives of lysine have been tested in the soluble lysine-decomposing
enzyme system and found to be inert: dl-ornithine, 2,4-diamino-
butyrate, mesodiaminopimelic acid, dl 5-hydroxylysine, dl-
phenylacetic acid, dl-\( \epsilon \)-hydroxy-\( \alpha \)-aminocaproic acid, dl-\( \alpha \)-aminoadipic acid, dl-\( \alpha \)-aminobutyric acid, e-aminocaproic acid,
dl-isoleucine, l-leucine, \( \gamma \)-aminobutyric acid, glycine, dl-
threonine, dl-\( \alpha \)-alanine, \( \delta \)-aminovulnic acid, and sorbic acid
(hexadienoic acid).

Compounds more oxidized than lysine were tested in reaction mixtures supplemented with liver alcohol dehydrogenase and isopropyl alcohol; for those more reduced, liver alcohol dehydrogenase and acetone were added. In most
instances ammonia liberation was employed as assay, but addi-
tionally the reaction mixtures containing the 6 carbon compounds
related to lysine, \( \gamma \)-aminobutyrate, and glycine were examined
for fatty acid formation by reaction with the fatty acid kinase of

C. sticklandii. e-Aminocaproate, \( \alpha \)-aminoadipate, \( \epsilon \)-hydroxy-\( \alpha \)-
aminocaproate, and glyoxylate were tested in pool-type experiments
wherein they were incubated in complete reaction mixtures con-
taining radioactive lysine and subsequently were isolated and
examined for radioactivity. In no instance did any of these
compounds become radioactive under conditions where active
lysine decomposition to fatty acids was observed. By these
criteria all of the compounds listed above were inert in the en-
zyme system when lysine decomposition was occurring, and
hence none appear to be free intermediates.

**Activation by Protein Fraction Obtained by Gel Filtration with
Sephadex G-100**—During the course of the studies on lysine
fermentation by Clostridium M-E, some extracts were prepared
that exhibited very low specific activities in spite of supplementation
with all of the above mentioned cofactors. Only upon the
addition of 2 to 3 mg of protein of another very active Clostridium
M-E crude extract (a level, by itself, insufficient to catalyze any
detectable over-all reaction) was good activity on lysine ob-
served. If the small amount of the active extract was heated at
100° for 5 minutes before its addition, its ability to enhance the
activity of the poor extract was destroyed. Crude extracts of
C. sticklandii proved to be even better sources of the heat-labile
material required by the deficient Clostridium M-E extracts, and
at little as 0.005 to 0.01 ml of the crude extract (0.2 to 0.4 mg of
protein) served to restore activity to the normal level. This
activation routinely represented a 5- to 10-fold increase in specific
activity of the over-all enzyme system. Determination of some
of the general properties of the heat-labile activating substance
revealed it to be nondialyzable and precipitable with ammonium
sulfate.

When subjected to gel filtration on Sephadex columns, the
active component was found to be excluded from the grades of
Sephadex designed to retain only low molecular weight solutes
(G-25 and G-50) and retained on Sephadex G-100 and G-200.

The pattern of elution from a Sephadex G-100 column (Fig. 4) of
the active material present in a 0.4 to 0.75 saturated ammonium
sulfate fraction shows that the active fraction came off as a
symmetrical peak after about 60% of the total protein already
had emerged. The fraction active in the lysine system also
separated from an iron reductase (15); the latter was displaced
from the Sephadex G-100 column immediately preceding the
salt front. The active material is also distinguishable from
ferredoxin as judged by the fact that a highly purified preparation
of this iron-containing protein failed to stimulate lysine decom-
position by deficient Clostridium M-E extracts when tested at
levels which were not inhibitory to the properly supplemented
system. Further purification and characterization of the active
component from C. sticklandii are currently in progress.

**Discussion**

The anaerobic degradation of lysine to fatty acids and am-
monia (Reaction 2) is a balanced oxidation-reduction reaction
which results in the net yield to the organism of 1 equivalent of
high energy ester phosphate per mole of amino acid decomposed.
In view of the demonstrated requirement of the system for
acetyl-CoA, it is possible that two energy-rich esters are actually

\[ \text{C. sticklandii. e-Aminocaproate, } \alpha \text{-aminoadipate, } \epsilon \text{-hydroxy-} \alpha \text{-aminocaproate, and glyoxylate were tested in pool-type experiments wherein they were incubated in complete reaction mixtures containing radioactive lysine and subsequently were isolated and examined for radioactivity. In no instance did any of these compounds become radioactive under conditions where active lysine decomposition to fatty acids was observed. By these criteria all of the compounds listed above were inert in the enzyme system when lysine decomposition was occurring, and hence none appear to be free intermediates.}

\[ \text{Activation by Protein Fraction Obtained by Gel Filtration with Sephadex G-100—During the course of the studies on lysine fermentation by Clostridium M-E, some extracts were prepared that exhibited very low specific activities in spite of supplementation with all of the above mentioned cofactors. Only upon the addition of 2 to 3 mg of protein of another very active Clostridium M-E crude extract (a level, by itself, insufficient to catalyze any detectable over-all reaction) was good activity on lysine observed. If the small amount of the active extract was heated at 100° for 5 minutes before its addition, its ability to enhance the activity of the poor extract was destroyed. Crude extracts of C. sticklandii proved to be even better sources of the heat-labile material required by the deficient Clostridium M-E extracts, and at little as 0.005 to 0.01 ml of the crude extract (0.2 to 0.4 mg of protein) served to restore activity to the normal level. This activation routinely represented a 5- to 10-fold increase in specific activity of the over-all enzyme system. Determination of some of the general properties of the heat-labile activating substance revealed it to be nondialyzable and precipitable with ammonium sulfate. When subjected to gel filtration on Sephadex columns, the active component was found to be excluded from the grades of Sephadex designed to retain only low molecular weight solutes (G-25 and G-50) and retained on Sephadex G-100 and G-200. The pattern of elution from a Sephadex G-100 column (Fig. 4) of the active material present in a 0.4 to 0.75 saturated ammonium sulfate fraction shows that the active fraction came off as a symmetrical peak after about 60% of the total protein already had emerged. The fraction active in the lysine system also separated from an iron reductase (15); the latter was displaced from the Sephadex G-100 column immediately preceding the salt front. The active material is also distinguishable from ferredoxin as judged by the fact that a highly purified preparation of this iron-containing protein failed to stimulate lysine decomposition by deficient Clostridium M-E extracts when tested at levels which were not inhibitory to the properly supplemented system. Further purification and characterization of the active component from C. sticklandii are currently in progress.}

\[ \text{Discussion—The anaerobic degradation of lysine to fatty acids and ammonia (Reaction 2) is a balanced oxidation-reduction reaction which results in the net yield to the organism of 1 equivalent of high energy ester phosphate per mole of amino acid decomposed. In view of the demonstrated requirement of the system for acetyl-CoA, it is possible that two energy-rich esters are actually}

\[ \text{These initial experiments were carried out in collaboration with Dr. John K. Hardman, whose able assistance is gratefully acknowledged.}

\[ \text{A generous gift from J. E. Carnahan, DuPont Research Institute, Wilmington, prepared from Clostridium pasteurianum (16)}

\[ \text{L. Blankenship and T. C. Stadtman, unpublished experiments.} \]
formed in the reaction but that one is consumed in an activation step at each turn of the cycle. In view of the sensitivity of the enzyme system to Amytal and arsenite (2) and the necessity for DPN addition, it seems likely that the electron transfer process is mediated by flavin, disulfide, and DPN-linked catalysts. The cobamide coenzyme requirement for lysine decomposition may, conceivably, be diagnostic of an isomerization such as occurs in the fermentation of glutamate by Clostridium tetanomorphum (17), and this possibility cannot be excluded until the exact fate of carbon atoms 3, 4, and 5 of lysine are known; i.e., do these become carbon atoms 1, 2, and 3, respectively, of butyrate? Alternatively, the cobamide may also serve as electron carrier in the over-all oxidation-reduction reaction. The identity of the natural cobamide in Clostridium M-E has not been established but C. sticklandii, which is particularly rich in cobamide coenzyme (18), seems to synthesize almost exclusively the adenine cobamide derivative. The C. sticklandii protein which is retained by Sephadex G-100 and activates deficient Clostridium obtains to suggest that this iron protein has any role in the ferredoxin, but, so far no experimental evidence has been obtained for that one is consumed in the activation step or destroyed by arsenolysis.

It is of some general interest, in view of the apparent role of the over-all metabolic pathway employed. Numerous balance experiments performed in the absence of one or more required cofactors or in the presence of effective metabolic inhibitors have shown, in every case, lowered yields of ammonia, acetate, and butyrate but maintenance of normal stoichiometry. The addition of DPN-linked electron donor or electron acceptor systems or oxidized or reduced dyes has similarly failed to upset the oxidation-reduction balance, thereby leading to an accumulation of intermediates. Hydroxylamine cannot be employed as a trapping agent for acyl intermediates because, at levels sufficient to be effective, it completely prevents the decomposition of lysine.

**SUMMARY**

Lysine is cleaved under anaerobic conditions by soluble extracts of Clostridium sticklandii and Clostridium M-E to 1 mole each of butyrate and acetate and 2 moles of ammonia. Linked to this balanced oxidation-reduction process is the esterification of 1 mole of orthophosphate per mole of amino acid fermented; the acceptor is adenosine diphosphate, and adenosine triphosphate is synthesized.

Cofactor additions required for activity of most unfraccionated enzyme preparations include a B⁺ coenzyme such as dimethylbenzimidazolecobamide coenzyme, diphosphopyridine nucleotide, ferrous iron, pyruvate, and acetyl coenzyme A (or coenzyme A—SE plus acetyl or carboxyl phosphate).

From inhibitor studies a flavin (Amytal sensitivity) and a disulfide catalyst (inhibition by 5 × 10⁻⁴ m arsenite) also are implicated in the over-all reaction mechanism.

**REFERENCES**

Anaerobic Degradation of Lysine: II. COFACTOR REQUIREMENTS AND PROPERTIES OF THE SOLUBLE ENZYME SYSTEM
Thressa C. Stadtman


Access the most updated version of this article at http://www.jbc.org/content/238/8/2766.citation

Alerts:
• When this article is cited
• When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts

This article cites 0 references, 0 of which can be accessed free at http://www.jbc.org/content/238/8/2766.citation.full.html#ref-list-1