JOURNAL OF BACTERIOLOGY, Jan. 1970, p. 77-83

Vol. 101, No. 1
Printed in U.S.A.

# 2,4-Diaminovaleric Acid: an Intermediate in the Anaerobic Oxidation of Ornithine by *Clostridium sticklandii*[1]

JOHN K. DYER[2] AND RALPH N. COSTILOW

*Department of Microbiology and Public Health, Michigan State University, East Lansing, Michigan 48823*

Received for publication 21 August 1969

The oxidation of ornithine in the presence of proline by crude extracts of *Clostridium sticklandii* cells was stimulated by nicotinamide adenine dinucleotide, coenzyme A, α-ketoglutarate, dimethylbenzimidazolyl cobamide (DBC) coenzyme, $MgCl_2$, and adenosine diphosphate. Deletion of various cofactors resulted in the accumulation of a new dibasic amino acid which was identified as 2,4-diaminovaleric acid. Both the oxidation of ornithine to alanine and acetate and the conversion of ornithine to 2,4-diaminovaleric acid were stimulated by addition of DBC coenzyme, and both were inhibited by intrinsic factor, an inhibitor of cobamide coenzyme-dependent reactions. This inhibition was reversed by addition of DBC coenzyme. However, the oxidation of 2,4-diaminovaleric acid was insensitive to added intrinsic factor. The data indicate that 2,4-diaminovaleric acid represents the first intermediate in the oxidation of ornithine by *C. sticklandii*.

The oxidation of L-ornithine by *Clostridium sticklandii* to ammonia, alanine, acetate, and carbon dioxide involves a primary cleavage between carbon atoms 3 and 4 (7). A high percentage of the isotope from carbon atoms 1 and 2 of uniquely labeled DL-ornithine was found in alanine, and most of that from carbon atom 5 was recovered in acetate. The carbon dioxide was derived from the carboxyl carbon and is believed to result from the oxidation of the alanine formed by the cleavage reaction. Similar cleavages were observed in lysine fermentation by *C. sticklandii* and *Clostridium* M-E (10, 11) and by *Clostridium* SB4 (6). Intact cells of *C. sticklandii* cleave lysine between carbon atoms 2 and 3 and carbon atoms 4 and 5 with equal facility (13). However, only the latter reaction was demonstrated with cell extracts (11). The products of the reactions are equimolar concentrations of acetate and butyrate and two equivalents of ammonia.

To date, the intermediates in the cleavage reactions in the lysine fermentation have been identified as 3,6-diaminohexanoate (β-lysine; 4), 3,5-diaminohexanoate (6, 15), and 3-keto-5-aminohexanoic acid (14). Another amino acid has been tentatively identified as 2,5-diaminohexanoate (12). It is thought that the latter compound may represent the primary intermediate in the cleavage between carbon atoms 4 and 5 of lysine. The conversion of β-lysine to 3,5-diaminohexanoate as well as the formation of 2,5-diaminohexanoate were shown to be cobamide coenzyme-dependent reactions (6, 15, 12).

This report presents evidence for the production of 2,4-diaminovaleric acid from ornithine by extracts of *C. sticklandii* cells. Both the overall oxidation of ornithine and the conversion of ornithine to the new basic amino acid appear to be dependent on a cobamide coenzyme. All of the evidence presented indicates that 2,4-diaminovaleric acid is the first intermediate formed in this oxidation.

## MATERIALS AND METHODS

**Culture, cultural methods, and preparation of cell extracts.** *C. sticklandii* strain HF was obtained from T. C. Stadtman. It was grown in a medium consisting of 0.6% L-arginine monohydrochloride, 0.6% L-lysine monohydrochloride, 0.6% yeast extract, 0.05% sodium thioglycollate, and 0.04 M potassium phosphate buffer (*p*H 7.5). Procedures used for the production of cells and the preparation of cell extracts were the same as described previously (7). The crude cell extracts retained most of their activities for several months at −20 C. Crude cell extracts were dialyzed for 12 hr in the cold against 0.1 M potassium phosphate buffer (*p*H 7.5) in the presence of $10^{-3}$ M dithiothreitol and 0.01 M L-ornithine.

**Reaction assays.** Reactions of 0.1 ml total volume

[1] Journal article no. 4798, Michigan Agricultural Experiment Station.

[2] Present address: College of Dentistry, The University of Nebraska, Lincoln, Neb. 68503.

were conducted as described by Costilow and Laycock (3). Ornithine and the unknown basic amino acid produced from it were separated from the neutral amino acids and anions by adsorption on small Dowex 50W-X4, 200- to 400-mesh, $Na^+$-form columns at *p*H 7.0. They were then eluted with 1 M $NH_4OH$, dried under vacuum, and resolved by ascending chromatography on Whatman 3MM paper with *n*-propanol-pyridine-water (1:1:1) solvent.

**Isolation of $^{14}$C-labeled unknown.** A reaction mixture of 5 ml containing 25 mM tris(hydroxymethyl)aminomethane (Tris)-chloride (*p*H 7.5), 3 mM potassium phosphate buffer (*p*H 7.5), 20 mM $^{14}$C-L-ornithine (specific activity, 13,100 counts/min per μmole), 2 μM dimethylbenzimidazolylcobamide (DBC) coenzyme, 2 mM adenosine diphosphate (ADP), and crude cell extract (65 mg of protein) was incubated under helium in the dark at 37 C for 2 hr. The reaction was stopped by the addition of an equal amount of 10% trichloroacetic acid. The protein was removed by centrifugation, and the trichloroacetic acid in the supernatant fluid was extracted with ether (3 ×). The basic amino acids were separated from the neutral amino acids, as described above. The solution containing the basic amino acids was desalted by use of a Dowex 50W-X4, 200- to 400-mesh, $H^+$-form column. After washing, the basic amino acids adsorbed to the column were eluted with 1 M $NH_4OH$. The eluate was evaporated to dryness at reduced pressure to remove excess ammonia and reconstituted to a 1-ml volume with a chloroform-methanol-15% ammonium hydroxide (40:40:10) mixture. The amino acids were then separated by column chromatography by using silicic acid (SilicAR CC-4, 100 to 200 mesh; Mallinckrodt Chemical Works, St. Louis, Mo.), developed with a chloroform-methanol-15% ammonium hydroxide mixture as described in the legend to Fig. 2. Fractions were monitored for their $^{14}$C content and the identity of each established by chromatography on Whatman 3MM paper with *n*-propanol-pyridine-water (1:1:1) solvent. The fractions containing the new basic amino acid were pooled and evaporated under reduced pressure. A second lot of this unknown was produced and purified in the same manner using $^{14}$C-ornithine with a specific activity of 7,370 counts/min per μmole.

**Analytical methods.** Protein was determined by the method of Lowry et al. (9) by using crystalline bovine serum albumin as a standard. The scintillation fluid described by Bray (1) was used for all aqueous samples, and a toluene-based fluid (3) was used for paper strips and for $^{14}CO_2$ collected in hydroxide of hyamine. A Tri-Carb liquid scintillation spectrometer (model 314; Packard Instrument Co., Inc., Downers Grove, Ill.) was used for measuring radioactivity.

**Chemicals.** Uniformly labeled $^{14}$C-L-ornithine was obtained from New England Nuclear Corp., Boston, Mass. Intrinsic factor (highly concentrated, 10 × average, without $B_{12}$) was purchased from Nutritional Biochemicals Corp., Cleveland. The DBC coenzyme was generously supplied by H. A. Barker, Dept. of Biochemistry, University of California, Berkeley.

## RESULTS

**Cofactors required for the oxidation of ornithine.** The effects of various cofactors on the activity of a dialyzed cell extract were assessed by their deletion individually and by groups. The deletion of all cofactors resulted in essentially no activity, and the deletion of nicotinamide adenine dinucleotide (NAD) alone reduced the total utilization of ornithine by about 85% (Table 1). Significant effects on the utilization of ornithine and on the accumulation of oxidized products were also observed on deletion of coenzyme A (CoA), α-ketoglutarate, DBC coenzyme, $MgCl_2$, and adenosine diphosphate, in order of their effect. The deletion of either pyruvate or adenosine triphosphate (ATP) had no effect. The addition of $FeSO_4$ (5 mM) to a reaction mixture containing all of the other cofactors failed to enhance the utilization of ornithine.

Attempts were made to partially purify the enzymes involved by ammonium sulfate precipita-

TABLE 1. *Effect of various cofactors on the oxidation of ornithine by a dialyzed extract*[a]

| Component omitted | Ornithine utilized | Oxidized products | |
|---|---|---|---|
| | | Alanine | Anions[b] |
| | mM | mM | mM |
| None | 13.8 | 8.2 | 13.0 |
| ADP | 10.2 | 6.4 | 9.6 |
| ATP | 13.8 | 8.0 | 12.7 |
| ADP, ATP | 9.4 | 5.8 | 8.3 |
| NAD | 2.1 | 0.7 | 3.0 |
| CoA | 5.2 | 2.6 | 4.1 |
| DBC coenzyme | 8.5 | 5.4 | 10.9 |
| Pyruvate | 14.1 | 8.7 | 12.4 |
| α-Ketoglutarate (α KG) | 6.5 | 3.7 | 6.2 |
| Pyruvate, α KG | 7.6 | 5.5 | 6.3 |
| $MgCl_2$ | 9.2 | 6.1 | 8.6 |
| All of the above | 0.9 | 0.4 | 0.2 |

[a] The complete reaction mixture contained 25 mM Tris-chloride (*p*H 7.5), 3 mM potassium phosphate (7.5), 20 mM $^{14}$C-L-ornithine (specific activity, 11,600 counts/min per μmole), 2.5 mM dithiothreitol, 2.5 mM nicotinamide adenine dinucleotide (NAD), 2 mM adenosine diphosphate (ADP), 2 mM adenosine triphosphate (ATP), 1 mM coenzyme A (CoA), 1 μM dimethylbenzimidazolylcobamide (DBC) coenzyme, 10 mM sodium α-ketoglutarate, 10 mM potassium pyruvate, and fresh dialyzed cell extract (0.6 mg of protein) in a total volume of 0.1 ml. Omissions from individual reactions were as indicated. Incubation was for 1 hr under argon in the dark at 37 C.

[b] Values calculated on the basis of acetate.

tion to assess more rigorously the effects of various cofactors, but this treatment led to inactivation. Also, attempts to inactivate any bound cobamide coenzyme by exposure of an extract to a tungsten lamp for periods up to 5 hr were not successful. However, ornithine utilization was dramatically inhibited by addition of relatively high levels of intrinsic factor (Table 2). The inhibition observed with 600 μg of intrinsic factor was completely reversed by addition of 6 μg of DBC coenzyme. This amount of coenzyme partially reversed the inhibition noted with 800 μg of intrinsic factor, but had no effect when 1 mg of the inhibitor was added.

**Accumulation of an unknown amino acid.** A radioactive ninhydrin-positive compound with an $R_F$ slightly higher than ornithine on chromatograms developed with butyl alcohol-acetic acid-water (60:15:25) was noted in some reaction mixtures from which cofactors were omitted. Electrophoresis of these mixtures at a number of *p*H values indicated that the compound was a basic amino acid similar to ornithine. Since it was likely that this compound was an intermediate in the oxidation, we investigated the effect of various cofactors on its accumulation. Of those tested, only DBC coenzyme and ADP increased the amount of the unknown observed (Table 3). The effects of these two cofactors appeared to be additive with both crude and dialyzed cell extracts. None of the unknown compound was found when the reaction mixture was supplemented with ADP, NAD, and α-ketoglutarate. However, when pyruvate was substituted for α-ketoglutarate in such a reaction mixture, there was no observable effect on the amount of unknown accumulated.

The involvement of a $B_{12}$-coenzyme in the reaction(s) converting ornithine to the unknown compound was further indicated on addition of intrinsic factor to reaction mixtures. Addition of 1,000 μg of this preparation inhibited the accumulation of the unknown by about 80% (Table 4). Addition of DBC-coenzyme (2 μM) reversed the inhibition very significantly.

With crude cell extracts, the accumulation of the unknown amino acid was nearly maximum after 10 to 20 min of incubation, although there was some increase in the amount present through

TABLE 2. *Effect of intrinsic factor and dimethylbenzimidazolylcobamide (DBC) coenzyme on ornithine utilization.*[a]

| Intrinsic factor added | DBC coenzyme added | Ornithine utilized |
|---|---|---|
| μg | μg | mM |
| 0 | 0 | 15.7 |
| 300 | 0 | 15.5 |
| 500 | 0 | 16.1 |
| 600 | 0 | 0.9 |
| 800 | 0 | 0.8 |
| 1,000 | 0 | 1.1 |
| 600 | 6 | 16.7 |
| 800 | 6 | 11.4 |
| 1,000 | 6 | 0.9 |

[a] Reaction mixtures contained 50 mM Tris-chloride (*p*H 7.5), 3 mM potassium phosphate (*p*H 7.5), 20 mM $^{14}$C-L-ornithine (specific activity 11,600 counts/min per μmole), 2.5 mM dithiothreitol, 2 mM $MgCl_2$, 2.5 mM adenosine diphosphate, 1 mM coenzyme A, 2.5 mM nicotinamide adenine dinucleotide, 10 mM sodium α-ketoglutarate, dialyzed cell extract (0.7 mg of protein), and, where indicated, intrinsic factor (Nutritional Biochemicals Co., 10 ×, without $B_{12}$) and DBC coenzyme. Reactions were incubated 2 hr in the dark under argon at 37 C.

TABLE 3. *Effect of cofactors on accumulation of unknown $^{14}$C-amino compound produced from $^{14}$C-ornithine*[a]

| Addition | $^{14}$C-unknown formed[b] | |
|---|---|---|
| | Crude extract | Dialyzed extract |
| None | 2.3 | 1.0 |
| NAD | 2.0 | 0.9 |
| ADP | 3.0 | 1.3 |
| ATP | | 0.9 |
| CoA | | 1.0 |
| DBC coenzyme | 3.5 | 1.8 |
| Pyridoxal phosphate | 2.2 | 1.0 |
| Pyruvate | | 1.0 |
| α-Ketoglutarate | | 0.8 |
| ADP, DBC coenzyme | 4.1 | 2.4 |
| ADP, NAD, pyruvate | 2.4 | |
| ADP, NAD, α-ketoglutarate | 0 | |
| $MgCl_2$ | | 1.0 |

[a] All reaction mixtures of 0.1 ml total volume contained 25 mM Tris-chloride (*p*H 7.5), 3 mM potassium phosphate (*p*H 7.5), 20 mM $^{14}$C-L-ornithine (specific activity, 11,600 counts/min per μmole), and either crude extract (1.8 mg of protein) or dialyzed extract (0.6 mg of protein). The following were added where indicated: 2.5 mM nicotinamide adenine dinucleotide (NAD), 2.5 mM adenosine diphosphate (ADP), 2.5 mM adenosine triphosphate (ATP), 1 mM coenzyme A (CoA), 2 μM dimethylbenzimidazolyl cobamide (DBC) coenzyme, 10 μg pyridoxal phosphate, 10 mM potassium pyruvate, 10 mM sodium α-ketoglutarate, and 2 mM $MgCl_2$. Incubation was for 1 hr under argon in the dark at 37 C.

[b] Calculated as equivalent to specific activity of ornithine.

1 hr of incubation (Fig. 1). Between 20 to 30% of the radioactivity in the $^{14}$C-ornithine added was detected in the unknown compound after 1 hr of incubation with either substrate concentration. This may have resulted from the attainment of the reaction equilibrium or from utilization of the unknown in subsequent reactions.

TABLE 4. *Effect of intrinsic factor and dimethylbenzimidazolylcobamide (DBC) coenzyme on the accumulation of the unknown compound*[a]

| Intrinsic factor | DBC coenzyme | Unknown[b] |
|---|---|---|
| μg | μM | mM |
| | | 2.7 |
| | 2.0 | 3.2 |
| 600 | | 2.1 |
| 1,000 | | 0.8 |
| 600 | 2.0 | 2.6 |
| 1,000 | 2.0 | 2.1 |

[a] Reaction mixtures contained 25 mM Tris-chloride (*p*H 7.5), 3 mM potassium phosphate (*p*H 7.5), 20 mM $^{14}$C-L-ornithine (specific activity, 9,700 counts/min per μmole), 2.5 mM adenosine diphosphate, crude cell extract (1.7 mg of protein), and 2 μM DBC coenzyme and intrinsic factor where indicated in a total volume of 0.1 ml. They were incubated in the dark at 37 C under argon for 1 hr.

[b] Calculated as equivalent to specific activity of ornithine.

**Separation and identification of the unknown amino acid.** Two lots of the $^{14}$C-unknown were produced from $^{14}$C-ornithine as described in Materials and Methods. The $^{14}$C-unknown was separated along with ornithine from the neutral and anionic compounds by use of ion-exchange resins and was then separated from residual ornithine on a silicic acid column. An example is shown in Fig. 2. Peak I contained the $^{14}$C-unknown and peak II contained the residual $^{14}$C-ornithine. The fractions from peak I were pooled and evaporated to dryness under reduced pressure. A single ninhydrin-positive spot was obtained on paper chromatography and paper ionophoresis of this material. These solutions were used for characterization.

The $R_F$ values of the unknown were consistently higher than those for ornithine in the five solvent systems tested (Table 5). However, the best separation from ornithine was obtained in the two solvents containing pyridine. Ionophoretically, the unknown compound was virtually identical to ornithine over a *p*H range from 2.0 to 7.5 (Table 5). The unknown did migrate slightly ahead of ornithine at *p*H 2.0, but good separation was not achieved.

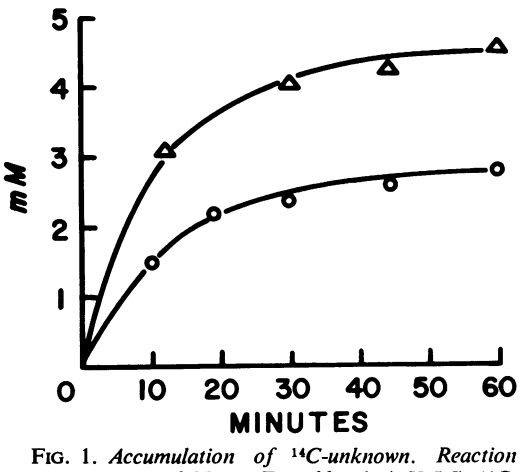


FIG. 1. *Accumulation of $^{14}$C-unknown. Reaction mixtures contained 25 mM Tris-chloride (pH 7.5), $^{14}$C-L-ornithine ($10^5$ counts/min per μmole), 2.5 mM ADP, 2 μM DBC coenzyme, and fresh crude cell extract in a total volume of 1 ml. One reaction mixture (○) contained 10 mM $^{14}$C-L-ornithine and 4 mg of extract protein, and the other mixture (△) contained 20 mM substrate and 40 mg of extract protein. They were incubated in the dark under argon at 37 C, and 50 μliter-samples were removed at the intervals indicated. These samples were mixed with 50 μliters of 0.2 M formic acid.*

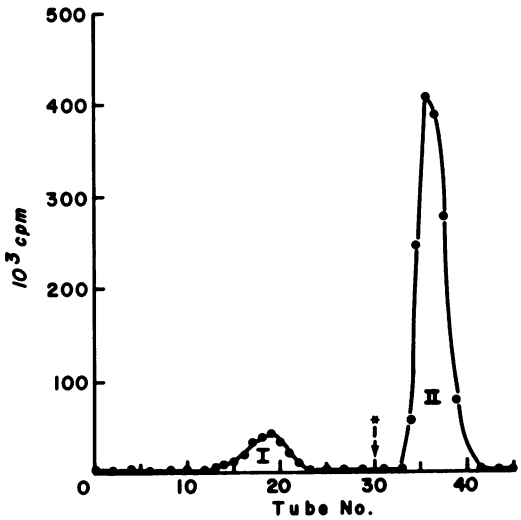


FIG. 2. *Separation of $^{14}$C-unknown from $^{14}$C-ornithine on a silicic acid column. The bed volume of the column was 1.4 × 36 cm. The silicic acid was equilibrated with $CHCl_3$-$CH_3OH$-15% $NH_4OH$ (40:40:10), and this solvent was used for elution of the unknown. Fractions (3 ml) were collected to the point marked by the asterisk and arrow. The proportions of solvent components were changed at that point to 36:41:20, and the volume of fractions was increased to 10 ml.*

TABLE 5. *Comparison of the chromatographic and electrophoretic properties of the unknown compound and ornithine*

| Solvent | Unknown | L-Ornithine |
|---|---|---|
| Chromatography[a] (*RF*) | | |
| Butyl alcohol-acetic acid-water (60:15:25) | 0.18 | 0.12 |
| Propanol-pyridine-water (1:1:1) | 0.42 | 0.22 |
| Phenol-water-ammonia (100:-25:1) | 0.84 | 0.78 |
| Methanol-water-pyridine (20:5:1) | 0.46 | 0.16 |
| Chloroform-methanol-15% $NH_4OH$ (40:40:10) | 0.14 | 0.03 |
| Electrophoresis (cm from origin[b]) | | |
| 0.2 M Formic acid, pH ~ 2.0, 42.5 volts/cm, 45 min | 23.4 | 21.9 |
| 0.1 M Sodium formate, *p*H 3.0, 42.5 volts/cm, 45 min | 17.8 | 17.8 |
| 0.12 M Sodium acetate, *p*H 4.2, 42.5 volts/cm, 30 min | 11.4 | 11.4 |
| 0.05 M Potassium phthalate, *p*H 5.7, 32.0 volts/cm, 1.5 hr | 17.0 | 17.0 |
| 0.05 M Potassium phosphate, *p*H 7.5, 21.0 volts/cm, 45 min | 8.3 | 8.3 |

[a] All chromatograms were on Whatman 3MM paper except for the one with chloroform-methanol-15% $NH_4OH$ solvent which was a thin-layer chromatogram on Silica Gel G.

[b] Both compounds migrated as cations.

TABLE 6. *Chloramine-T oxidation and C-terminal methyl determinations of basic intermediate and ornithine*

| $^{14}$C-labeled compound | Added | $^{14}$C in product | |
|---|---|---|---|
| | | Theoretical | Observed |
| | *μmoles* | *counts/min* | *counts/min* |
| Chloramine-T oxidation[a] | | | |
| $^{14}$C-unknown | 1.0 | 1,474 | 1,386 |
| $^{14}$C-L-ornithine | 1.0 | 2,580 | 2,800 |
| Terminal methyl determination[b] | | | |
| $^{14}$C-unknown | 1.0 | 2,948 | 2,835 |
| $^{14}$C-L-ornithine | 1.0 | 0 | 0 |

[a] Reaction mixtures for the chloramine-T oxidation in Warburg vessels contained 0.8 ml of 0.2 M sodium acetate buffer (*p*H 4.2) and 1.5 ml of 10% (w/v) chloramine-T solution. Either $^{14}$C-L-ornithine (12,800 counts/min per μmole) or the $^{14}$C-labeled unknown (7,370 counts/min per μmole) was placed in the side arm. The center well contained 0.4 ml of 1 M hydroxide of hyamine in methanol. After temperature equilibration, oxidation was allowed to proceed for 1 hr at 30 C.

[b] Acid chromate oxidation for C-terminal methyl group determination was effected in sealed ampoules containing 2 ml of chromic acid-$H_2SO_4$ reagent, 25 μliters of 2 N acetic acid, and either $^{14}$C-L-ornithine (12,800 counts/min per μmole) or $^{14}$C-labeled unknown (7,370 counts/min per μmole). The ampoules were treated and measured quantitatively for radioactivity as described by Dekker and Barker (6).

The above data demonstrated that the unknown compound was a dibasic amino acid very similar to ornithine. Since it is known that the early reactions in lysine fermentations involve the migration of the amino groups to form 3,6-diaminohexanoate, 3,5-diaminohexanoate, and possibly 2,5-diaminohexanoate, a similar reaction was most suspect in the formation of this compound. Thus, experiments were conducted to determine the position of the amino groups on the unknown. Oxidation with chloramine-T demonstrated that the unknown was an α-amino acid (Table 6). Essentially stoichiometric amounts of radioactivity were released by the oxidation. Oxidation with acid chromate demonstrated the presence of a C-terminal methyl group on the unknown compound, indicating that the migration involved the δ-amino group. These data indicated that the compound must be either 2,3- or 2,4-diaminovalerate. The possibility of the amino groups being present on adjacent carbons was eliminated by exposing the $^{14}$C-unknown to periodate oxidation (8). No degradation of the unknown occurred under these conditions. Thus, it is most probable that the unknown compound was 2,4-diaminovaleric acid.

**Oxidation of 2,4-diaminovaleric acid by cell extracts.** The extent of oxidation of 2,4-diaminovaleric acid was about the same as that observed with ornithine (Table 7). Similar amounts of alanine and of anions were formed from the two substrates after 1 and 2 hr of incubation in reaction mixtures with crude cell extracts. The reaction from ornithine to 2,4-diaminovaleric acid is obviously reversible since significant amounts of ornithine were formed in reaction mixtures in which an excess of the latter compound was used as substrate. Addition of intrinsic factor to reaction mixtures inhibited the oxidation of ornithine greatly, but had no significant effect on the oxidation of 2,4-diaminovalerate. Thus, the conversion of ornithine to 2,4-diaminovaleric acid appears to be the only cobamide coenzyme-dependent reaction in the oxidation of ornithine.

TABLE 7. *Oxidation of 2,4-diaminovalerate and ornithine by cell extracts*[a]

| Substrate | Incubation time | Substrate utilized | Products formed | | | |
|---|---|---|---|---|---|---|
| | | | Ornithine | 2,4-Diamino-valerate | Alanine | Anions[b] |
| | hr | mM | mM | mM | mM | mM |
| Expt 1 | | | | | | |
| $^{14}$C-2,4-diaminovalerate | 1 | 7.9 | 2.1 | | 3.2 | 5.2 |
| | 2 | 9.1 | 1.7 | | 4.2 | 6.7 |
| $^{14}$C-L-ornithine | 1 | 6.5 | | 0.4 | 2.7 | 5.8 |
| | 2 | 8.3 | | 0.2 | 3.2 | 8.1 |
| Expt 2 | | | | | | |
| $^{14}$C-2,4-diaminovalerate | | | | | | |
| Control | 1 | 4.5 | 0.1 | | 4.0 | 5.1 |
| Plus IF | 1 | 4.5 | 0.1 | | 3.7 | 4.9 |
| $^{14}$C-L-Ornithine | | | | | | |
| Control | 1 | 4.6 | | | 3.7 | 5.6 |
| Plus IF | 2 | 0.9 | | | 0.1 | 0.9 |

[a] Reaction mixtures contained 25 mM Tris-chloride (*p*H 7.5), 3 mM potassium phosphate (*p*H 7.5), either $^{14}$C-labeled 2,4-diaminovalerate (specific activity, 7,370 counts/min per μmole) or $^{14}$C-L-ornithine (specific activity, 9,700 counts/min per μmole) as indicated (10 mM in experiment 1 and 5 mM in experment 2), 2.5 mM nicotinamide adenine dinucleotide, 20 mM sodium α-ketoglutarate, 20 mM L-proline, and crude cell extract (1.8 mg of protein) in a total volume of 0.1 ml. Intrinsic factor (IF), 1 mg, was added where indicated. The reaction mixtures were incubated in the dark at 37 C under argon.

[b] Values calculated on the basis of acetate.

## DISCUSSION

The data presented in this paper indicate that the initial reaction involved in the oxidation of ornithine by *C. sticklandii* is mediated by a mutase enzyme which is cobamide coenzyme-dependent. The reaction results in the migration of the terminal amino group to the adjacent carbon atom forming 2,4-diaminovalerate, analogous to the initial reactions involved in the degradation of lysine by *C. sticklandii* and *Clostridium* M-E (15) and by *Clostridium* SB-4 (6). Cell extracts of these clostridia cleave lysine between carbon atoms 2 and 3, although studies with intact cells indicate that lysine may be degraded between carbons 2 and 3 and carbons 4 and 5 with equal facility (10). Ornithine degradation by *C. sticklandii* has been shown to result in alanine containing carbons 1 and 2, and in acetate containing carbon 5, indicating that the primary cleavage of ornithine occurs between carbons 3 and 4 (7). The first intermediate in lysine fermentation by cell extracts is formed by a migration of the α-amino group to carbon atom 2 forming L-3,6-diaminohexanoic acid (L-β-lysine) (3). The reaction appears to be mediated by a pyridoxal phosphate-dependent mutase. L-β-Lysine is then converted to 3,5-diaminohexanoic acid (6, 15). The migration of the ε-amino group is dependent on the presence of a cobamide coenzyme. Stadtman and Tsai (12) have tentatively identified another possible intermediate in the lysine fermentation as 2,5-diaminohexanoate which was produced from D-α-lysine by a cobamide coenzyme-dependent reaction system. They proposed that this reaction may represent the first step leading to the cleavage of lysine between carbon atoms 4 and 5. On the basis of the evidence presented herein, we propose a similar reaction as the first step in the reaction system leading to the cleavage of ornithine between carbon atoms 3 and 4. The cell extracts used in this study contained a very active ornithine racemase, so we have no information as to whether the substrate is D- or L-α-ornithine.

The following evidence all supports the conclusion that 2,4-diaminovalerate is truly an intermediate in the reactions leading to the cleavage of ornithine. The compound was accumulated in reaction mixtures only on omission of one or more cofactors essential for the maximal formation of alanine and acetate. Both the degradation of ornithine to alanine and acetate and the formation of 2,4-diaminovalerate were stimulated by DBC coenzyme, and were inhibited by intrinsic factor. In both instances, the inhibition was partially reversed by addition of DBC coenzyme. In contrast, intrinsic factor had no significant effect on the oxidation of 2,4-diaminovalerate. Finally, 2,4-diaminovalerate was degraded by cell extracts to alanine and an anion(s) (presumably acetate) to about the same extent as ornithine. The reversibility of the reaction from

ornithine to 2,4-diaminovalerate was indicated in the latter experiment. However, no significant amount of the latter compound was accumulated in complete reaction mixtures when excess ornithine was added as substrate. Thus, there is only a remote possibility that the formation of this compound represents a side reaction in the system.

Rimerman and Barker (14) demonstrated that the oxidative deamination of 3,5-diaminohexanoate by extracts of *Clostridium* SB-4 cells was dependent on NAD and an $\alpha$-keto acid. This reaction resulted in the formation of 3-keto-5-aminohexanoate. It was accumulated when CoA was deleted from otherwise complete reaction mixtures, and it may be the intermediate involved in a thiolytic cleavage of lysine. If true, one might suspect a similar intermediate to be involved in the oxidative cleavage of 2,4-diaminovaleric acid. Since this occurs between carbon atoms 3 and 4, the most likely compound would be 2-amino-4-ketovalerate. Attempts are being made to trap such a compound.

ACKNOWLEDGMENT

This investigation was supported by Public Health Service grant 5-ROI-AM10791-03 from the National Institute of Arthritis and Metabolic Diseases.

LITERATURE CITED

1. Bray, G. A. 1960. A simple efficient liquid scintillator for counting aqueous solutions in a liquid scintillation counter. Anal. Biochem. 1:279–285.
2. Bray, R. C., and T. C. Stadtman. 1968. Anaerobic degradation of lysine. III. $^{15}N$ studies on the conversion of lysine to 3,5-diaminohexanoate. J. Biol. Chem. 243:381–385.
3. Costilow, R. N., and L. Laycock. 1968. Proline as an intermediate in the reductive deamination of ornithine to $\delta$-aminovaleric acid. J. Bacteriol. 96:1011–1020.
4. Costilow, R. N., O. M. Rochovansky, and H. A. Barker. 1966. Isolation and identification of $\beta$-lysine as an intermediate in lysine fermentation. J. Biol. Chem. 241:1573–1580.
5. Crestfield, A. M., and F. W. Allen. 1955. Improved apparatus for zone electrophoresis. Anal. Chem. 27:422–423.
6. Dekker, E. E., and H. A. Barker. 1968. Identification and cobamide coenzyme-dependent formation of 3,5-diaminohexanoic acid, an intermediate in lysine fermentation. J. Biol. Chem. 243:3232–3237.
7. Dyer, J. K., and R. N. Costilow. 1968. Fermentation of ornithine by *Clostridium sticklandii*. J. Bacteriol. 96:1617–1622.
8. Jackson, E. L. 1944. Periodic acid oxidation. Organic Reactions 2:341–375.
9. Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265–275.
10. Stadtman, T. C. 1955. On the anaerobic degradation of lysine, p. 493–495. *In* W. D. McElroy and B. Glass (ed.), Symposium on amino acid metabolism. The Johns Hopkins Press, Baltimore.
11. Stadtman, T. C. 1963. Anaerobic degradation of lysine. II. Cofactor requirements and properties of the soluble enzyme system. J. Biol. Chem. 238:2766–2773.
12. Stadtman, T. C., and L. Tsai. 1967. A cobamide coenzyme dependent migration of the $\epsilon$-amino group of D-lysine. Biochem. Biophys. Res. Commun. 28:920–926.
13. Stadtman, T. C., and F. H. White, Jr. 1954. Tracer studies on ornithine, lysine, and formate metabolism in an amino acid fermenting *Clostridium*. J. Bacteriol. 67:651–657.
14. Rimerman, E. A., and H. A. Barker. 1968. Formation and identification of 3-keto-5-aminohexanoic acid, a probable intermediate in lysine fermentation. J. Biol. Chem. 243:6151–6160.
15. Tsai, L., and T. C. Stadtman. 1968. Anaerobic degradation of lysine. IV. Cobamide coenzyme dependent migration of an amino group from carbon 6 of $\beta$-lysine (3,6-diaminohexanoate) to carbon 5 forming a new naturally occurring amino acid, 3,5-diaminohexanoate. Arch. Biochem. Biophys. 125:210–225.