Arginine and Ornithine Catabolism by *Clostridium botulinum*[1]

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ABSTRACT

Clostridium botulinum 62-A was shown to catabolize L-arginine via citrulline to ornithine, NH_3, and CO_2. The individual enzymes of the dihydrolase system were all demonstrated in extracts of cells, spores, and germinated spores. There was no liberation of urea from L-arginine, so no functional arginase enzyme is present, but there was some transamidinase activity in cell extracts. L-Ornithine was degraded at a significant rate by cells grown in an L-ornithine-supplemented medium; it was partially decarboxylated to putrescine and partially fermented to NH_3, CO_2, volatile acids, and δ-aminovaleric acid. Results from the fermentation of L-ornithine-C^{14}, -1-C^{14}, and -2-C^{14} demonstrated that essentially all of the CO_2 was derived from carbon 1, and volatile acids from carbons 2 to 5. Assays for the products of L-ornithine-C^{14} fermentation revealed that the volatile acids consisted of acetate, propionate, valerate, and butyrate (in order of decreasing concentrations), and that δ-amino-valerate was the primary reduced product. A small amount of citrulline was formed during the fermentation. The carbon and redox balances indicated that L-ornithine is fermented as a single substrate. Preliminary experiments demonstrated that the fermentation of L-ornithine is carried out by cell extracts with the production of volatile acids.

Germinating spores of *Clostridium botulinum* were shown by Costilow (3) to catabolize L-arginine with a CO_2/NH_3 value considerably greater than 0.5, which is the maximal theoretical value for the dihydrolase pathway. Thus, it was thought that this organism must contain some catabolic route(s) different from or in addition to the dihydrolase system. More recently, Perkins and Tsuji (19) reported that high levels of arginine stimulated sporulation of *C. botulinum* in a synthetic medium, and that most of the arginine was degraded by the dihydrolase system. However, analysis of their data shows that of 90 μmoles/ml of arginine disappearing from the medium in one experiment only about 70 μmoles could be accounted for in citrulline and ornithine after 7 days of incubation; and, in a second experiment, only 4 of 11 μmoles of arginine disappearing

could be accounted for in these two products. In addition, their data indicate that cell yields were increased when ornithine was substituted for arginine in a synthetic medium, and that the pH decreased considerably during growth in the medium with ornithine while it increased in the arginine-rich medium. This indicates that acid was being produced from ornithine.

The degradation of arginine by a number of bacteria via the dihydrolase system has been studied extensively (15, 22, 24), but there is little information on the fermentation of ornithine. *C. sporogenes* utilizes this amino acid as a hydrogen acceptor (30) in a Stickland reaction, converting it to δ-aminovaleric acid. Stadtman (26) found that ornithine could be substituted for arginine in a minimal medium containing formate and lysine and could support growth of an unidentified *Clostridium* (strain HF). However, no growth occurred without lysine. Analysis of a growth medium containing DL-ornithine-2-C^{14} and L-lysine demonstrated that the label appeared primarily in acetate and butyrate, and no labeled amino acids were found among the products. The degradation of ornithine was primarily oxidative, with lysine providing the neces-

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sary electron acceptor(s). There are no previous reports, to the authors' knowledge, indicating that ornithine can be fermented as a single substrate by a single organism.

The results in this report demonstrate conclusively that L-arginine is catabolized by *C. botulinum* primarily via the dihydrolase pathway, and they present considerable evidence that L-ornithine is fermented as a single substrate.

MATERIALS AND METHODS

Culture and cultural methods. C. botulinum 62-A (ATCC 7949) from the American Type Culture Collection was used for all studies. The cultural methods used were essentially the same as described previously (5). Vegetative cells were harvested from log-phase cultures (7 to 8 hr of incubation), washed three times with cold water, and suspended in 0.067 M phosphate buffer (*p*H 7.0). The 4% Trypticase-1 ppm thiamine medium was supplemented with 0.2% L-ornithine to produce cells used in studying the fermentation of this substrate. Germinated spores were produced as described by Simmons and Costilow (23).

Analytical methods. Reactions were run at 37 C in Warburg flasks which were flushed with helium. All but one component of the reaction mixtures were added to the main compartment of the vessel; the one exception was substrate, which was tipped from a side arm after thermal equilibrium. Reactions were stopped by tipping 0.2 ml of 2 M H_2SO_4 from a side arm. This released all of the CO_2, which was measured manometrically by the direct method (28). The reaction mixtures were centrifuged to remove the cells and were stored at 4 C until analyzed.

Ammonia was separated from reaction mixtures by microdiffusion in Conway plates, and determined by nesslerization by use of the procedure of Johnson (12).

Arginine, citrulline, and ornithine were determined colorimetrically by the procedures of Van Pilsum et al. (29), Spector and Jones (25), and Chinard (2), respectively, and by column chromatography using the method of Moore and Stein (17) for separation and an Auto Analyzer (Technicon Co., Chauncey, N.Y.) for analysis of the fractions by the procedure of Piez and Morris (20). The results obtained with the two methods agreed closely.

Putrescine and δ-aminovaleric acid were separated from the reaction mixture by adsorbing on a 0.6 × 8 cm column of Dowex-50 × 4, 400-mesh in the H+ form, equilibrated with water, and by eluting with 1.5 N HCl to remove δ-aminovaleric acid (7) and with 2.5 N HCl to remove putrescine (27). The fractions were analyzed as described above.

Gas chromatographic techniques were used to analyze the fermentation mixture for volatile acids. The instrument used was a model A-600 B, HY-FI gas chromatographer, with hydrogen flame ionization detector (Wilkens Instrument and Research Inc., Walnut Creek, Calif.). The columns used were charged with Carbowax (20 M), TPA on 60/80-mesh Chromosorb and HMDS (4.2 g). Steam-saturated N_2 was maintained at 12 psi, with a flow rate of 12 ml/min.

We injected 5-μliter samples onto the column with a micropipette. Standard graphs were prepared by injecting known amounts of volatile acids onto the columns prior to the test runs.

Radioisotope studies. The C^{14}-labeled substrates were obtained from New England Nuclear Corp., Boston, Mass. They were fermented in Warburg vessels as described above, except that 0.2 ml of 10% KOH was added in the center well to absorb the CO_2. After stopping the reaction with H_2SO_4, the incubation was continued until the manometer readings indicated that all of the CO_2 was trapped. The KOH was quantitatively transferred to a vial with washings and made to a volume of 2 ml. Samples were then counted for radioactivity.

The reaction mixture was transferred to a centrifuge tube with washing and made to a total volume of 4 ml, and the cells were centrifuged out. Anions and cations were separated by passing duplicate 1-ml samples through columns (0.5 × 6 cm) of Dowex-50W × 4, 200- to 400-mesh, in the H+ form, equilibrated with water. The anions were washed through with 6 ml of water, and the cations were eluted with 1 M NH_4OH.

The volatile acids were steam-distilled from the total anions, neutralized with NH_4OH, and evaporated to dryness under vacuum. They were dissolved in 5 ml of water, placed in a conical 10-ml centrifuge tube, and evaporated to dryness again by use of a rotary Evapo-mix (Buchler Instruments Inc., Fort Lee, N.J.). These were dissolved in 0.1 ml of water, and the individual volatile acids in 20-μliter samples were separated from each other by paper chromatography (14). These were located on the paper with standards, cut out, and eluted with water, and the radioactivity in each was determined.

The alkaline solution of cations was evaporated to dryness to remove the NH_3, was dissolved in 0.5 ml of water, and was separated from 10-μliter samples by paper electrophoresis using the procedure of Crestfield and Allen (4). Electrophoresis at 65 v/cm for 20 min in 0.125 M sodium acetate buffer (*p*H 4.3) was used to separate ornithine, putrescine, δ-aminovaleric acid, and citrulline. Arginine and ornithine are found in a single spot. Samples were run in duplicate along with the appropriate standards and located by spraying duplicate paper strips with ninhydrin. The individual spots were cut out and eluted with water, and subsamples were counted. Ornithine was separated from arginine by elution of the appropriate area after electrophoresis of 40 μliters of reaction mixture as described above, evaporated to dryness, dissolved in 0.1 ml water, and 20-μliter samples were run on descending paper chromatograms using butanolacetic acid-water (4:1:5) solvent. Ninhydrin was used to develop the papers, and the amino acids were identified by comparison with standards. No arginine was found in the reactions in which radioactive L-ornithine was used as substrate.

In experiments with cell extracts, counts were made of the concentrates of cations and volatile acids without further separation. However, to determine the residual ornithine level for correction, we submitted the samples of the cations to electrophoresis.

Radioactivity was measured with a Packard Tri-

carb liquid scintillation spectrometer, model 317, by use of the scintillation solution of Bray (1) and was corrected for background. Use of internal standards demonstrated that there was no significant quenching in the samples counted.

Enzyme assays. Extracts from cells and spores were prepared as described by Simmons and Costilow (23). Protein was measured by the method of Lowry et al. (16) with crystalline bovine albumin as standard. Arginase was assayed according to the method described by Greenberg (6); arginine deiminase and citrullinase, by the methods of Oginsky (18); ornithine transcarbamylase and carbamylphosphokinase, according to the procedures of Jones (13); and transamidinase, by the method described by Ratner (21).

RESULTS

L-Arginine catabolism. Preliminary experiments demonstrated that resting cells of *C. botulinum* produced CO_2 rapidly from both arginine and citrulline, and at a slow but significant rate from ornithine (Fig. 1). Thus, it appeared that the primary reaction sequence with arginine as sub-
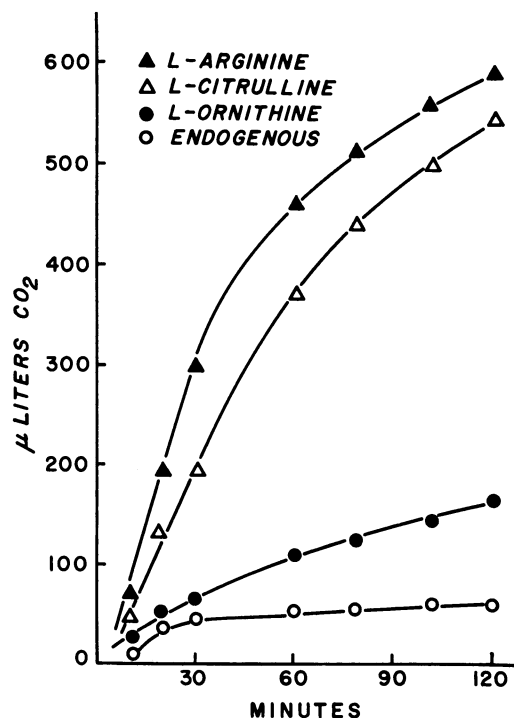
strate was the dihydrolase system. Further evidence of this is presented in Fig. 2. In the presence of NaF, citrulline and NH_3 accumulated in equimolar levels with arginine as substrate; with citrulline as substrate, NH_3 and CO_2 were produced at nearly equimolar levels.

The enzymes of the arginine dihydrolase system, arginine deiminase and citrullinase (ornithine transcarbamylase plus carbamylphosphokinase), were all demonstrated in extracts of cells, endospores, and germinated endospores (Table 1). It is of interest that the carbamylphospho-
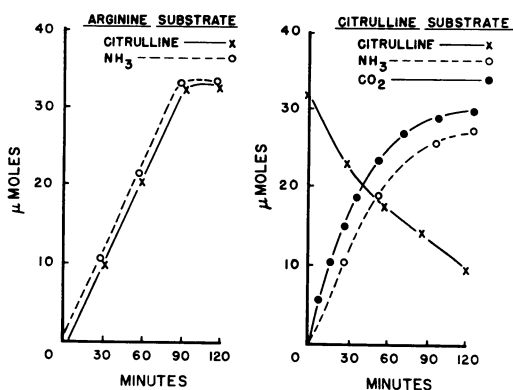

FIG. 2. *Degradation of* L-*arginine to citrulline and* NH_3, *and of* L-*citrulline to* CO_2 *and* NH_3, *by resting cells. Reaction mixtures were as indicated in Fig. 1, except that 20 μmoles of NaF was added to the flasks with* L-*arginine as substrate to inhibit citrullinase activity, and 0.2 ml of 70% perchloric acid was used to stop the reactions at the time intervals indicated.*

TABLE 1. *Enzymes of arginine catabolism[a]*

Enzyme	Specific activity[b] in extracts of		
	Cells	Spores	Germinated spores
Arginine deiminase..........	3.1	0.3	1.1
Citrullinase................	1.4	0.8	0.6
Ornithine transcarbamylase...	2.5	1.5	1.1
Carbamylphosphokinase...	0.1	0.2	0.3
Arginase...................	0	0	0
Transamidinase.............	1.0	0	0

[a] Extracts were prepared and assayed as described in Materials and Methods.

[b] Specific activities are in terms of units of enzyme per milligram of protein. A unit of enzyme is that amount producing 1 μmole of product in a given time interval—1 min for transcarbamylase, carbamylphosphokinase, and arginase, and 1 hr for the others. The carbamylphosphokinase activities were corrected for ADP independent activities.

FIG. 1. *Carbon dioxide production from* L-*arginine,* L-*citrulline, and* L-*ornithine by resting cells. The reaction mixtures in Warburg vessels contained 1 ml of 0.2* M *phosphate buffer (pH 7.0), 33 μmoles of substrate, 25 mg (dry weight) of cells, and water to 3 ml. Replicate reactions were run, and 0.2 ml of 2* M H_2SO_4 *was used to stop the reactions at the indicated intervals and to release all of the* CO_2. *See Materials and Methods for general procedures.*

kinase activity was higher in spore and germinated spore extracts than in cells; this does not often occur with catabolic enzymes. There was no arginase present, so there is apparently no direct addition of water to form urea and ornithine. However, significant transamidinase activity was detected in vegetative cells; therefore, some direct transfer of the guanidine moiety to acceptor amino acids (e.g., glycine) may occur with the formation of ornithine.

The products of arginine degradation by *C. botulinum* are given in Table 2. As indicated above, the CO_2 level is higher than could be obtained by a dihydrolase system alone, since equimolar levels of CO_2 and ornithine would be formed; there were trace amounts of volatile acids present as detected by gas chromatography. However, the carbon recovery was almost 10% too high, and, thus, there is no assurance that the presence of arginine did not stimulate some activity on endogenous substrate to yield the excess CO_2 and acids. Although catabolism of arginine-C^{14} yielded similar results, there was no assurance that CO_2 exchange was not responsible for this; our further efforts, therefore, were directed toward the fermentation of L-ornithine per se.

Fermentation of L-ornithine. Initial studies with resting cells produced in the regular growth medium demonstrated the presence of an L-ornithine decarboxylase forming CO_2 and putrescine; variable amounts of δ-aminovaleric acid, NH_3, and volatile acids were found in the reaction mixtures. Cells produced in the L-ornithine-supplemented medium degraded this substrate more rapidly and produced higher yields of volatile acids. That the products were from L-ornithine was demonstrated conclusively by fermentations of uniquely labeled substrates (Table 3). Most of the CO_2 was obviously derived from the first carbon, since the CO_2 from L-ornithine-*1*-C^{14}

TABLE 2. *Products of arginine catabolism*[a]

Product[b]	Amt (μmoles)/100 μmoles of L-arginine[c]
CO_2	91.2
NH_3	174.1
Citrulline	29.0
Ornithine	70.3
Acetate	5.6
Propionate	3.1
Butyrate	2.0
Valerate	2.5

[a] Reactions were run as indicated in Fig. 1.
[b] Carbon recovery = 109.6%; nitrogen recovery = 101.7%; redox value: 0.95.
[c] All values corrected for residual arginine.

TABLE 3. *Distribution of isotope in the fermentation products of uniquely labeled L-ornithine*[a]

Product	Per cent of total counts/min in products from[b]		
	L-Orni-thine-C^{14} (10,200 counts per min per μmole)	1-Orni-thine-2-C^{14} (16,700 counts per min per μmole)	L-Orni-thine-*1*-C^{14} (8,800 counts per min per μmole)
CO_2	4.4	0.1	24.4
Volatile acids	13.3	18.1	4.0
δ-Aminovaleric acid	62.9	56.4	62.5
Citrulline	5.6	7.2	6.8
Putrescine	15.5	19.4	0
Totals	101.7	101.2	97.7

[a] Reactions were run as outlined in Fig. 1 by use of 28 mg (dry weight) of cells per vessel, except that 0.2 ml of 10% KOH was added to the center well to trap the CO_2. See Materials and Methods for analytical procedures.
[b] All values corrected for residual L-ornithine.

contained about 24% of the total radioactivity, whereas that from the uniformly labeled amino acid contained only about 4%; there was essentially no radioactivity in the CO_2 from the 2-C^{14} substrate. In contrast, the volatile acids, produced from the uniform and 2-C^{14} substrates, contained a much higher percentage of the label than those derived from the *1*-C^{14}. A large percentage of the C^{14} from all three substrates appeared in the δ-aminovaleric acid, indicating considerable reductive deamination. Also, there was apparently some synthesis of citrulline and considerable decarboxylation to putrescine. The recovery of radioactivity from all three substrates was very close to 100%, indicating that there were no other major products except ammonia.

A carbon and nitrogen balance of L-ornithine fermentation is presented in Table 4. L-Ornithine-C^{14} was used to permit greater sensitivity in the assays. The volatile acids found were acetate, propionate, valerate, and butyrate in order of decreasing amounts. δ-Aminovalerate was the primary reduced product, occurring in large amounts. A significant amount of the substrate was decarboxylated to putrescine, and a small amount was converted to citrulline. The carbon and nitrogen recoveries and the balanced redox indicate that L-ornithine is fermented by this organism as a single substrate. In another balance experiment run with nonradioactive substrate, in which the CO_2 was measured manometrically, the volatile acids were determined by gas chromatography, and the amino compounds were separated by column chromatography and measured

TABLE 4. *Carbon and nitrogen balances of L-ornithine fermentation*[a]

	Counts/min	Per cent of total counts/min	Amt per 100 μmoles of L-ornithine fermented		
			Product	Carbon	Nitrogen
L-Ornithine-C^{14}					
(10,200 counts per min per μmole)					
Added..............................	335,600	100			
Fermented.........................	229,200	68.4	—	500	200
Products:					
CO_2................................	10,800	4.7[b]	23.4	23.4	—
Acetate............................	14.900	6.5	16.4	32.8	—
Propionate.........................	7,300	3.2	5.3	15.9	—
Butyrate...........................	3,100	1.4	1.8	7.2	—
Valerate...........................	4,600	2.0	2.2	11.0	—
δ-Aminovalerate....................	144,000	62.8	62.0	310.0	62.0
Citrulline..........................	12,800	5.6	4.4	26.4	13.2
Putrescine.........................	35,600	15.5	19.8	79.2	39.6
NH_2................................	—	—	69.5	—	69.5
Totals in products[c]..................	233,100	101.7	—	505.9	184.3

[a] Reactions were run as indicated in Table 3.

[b] Values represent percentages of total counts per minute in L-ornithine fermented.

[c] Carbon recovery = 101.2%; nitrogen recovery = 92.2%; redox value = 1.03.

TABLE 5. *Fermentation of L-ornithine by cell extracts*[a]

Cofactors added	Per cent of counts/min from L-ornithine-C^{14} in		
	CO_2	Volatile acids	Cations
ADP......................	6.8	1.7	89.8
ADP, CoA, lipoic acid.....	8.2	10.3	78.7

[a] Reaction mixtures in Warburg vessels contained 33 μmoles of L-ornithine-C^{14}, 1 ml of 0.2 M phosphate buffer (pH 7.0), 25.6 mg of extract protein, 1 μmole of $MgCl_2$, 10 μmoles of ADP, and, where indicated, 1 μmole of CoA and 1 μmole of lipoic acid. The center well contained 0.2 ml of 10% KOH. The total liquid volume was 3 ml. The atmosphere was helium. After 2 hr at 37 C, 0.2 ml of 2 M H_2SO_4 was tipped in from a side arm to stop the reaction and release all the CO_2. See Materials and Methods for assay procedures.

colorimetrically, similar results were obtained; however, the carbon recovery was about 7% too high, and the redox was only about 0.88. This is believed to have resulted from errors in determining the various products, many of which were in very small amounts.

L-Ornithine was also fermented by crude cell extracts (Table 5). The cofactor requirements were not studied extensively, but the addition of coenzyme A (CoA) or lipoic acid, along with adenosine diphosphate (ADP), greatly stimulated

the production of volatile acids. Carbon dioxide production is not a good measure of L-ornithine fermentation by this organism because of the active decarboxylase present.

DISCUSSION

C. botulinum 62-A catabolizes L-arginine primarily via citrulline to ornithine, CO_2, and NH_3. This confirms the proposals of Perkins and Tsuji (19). The system is apparently the same as reported for *C. perfringens* (22), *Streptococcus lactis* (15), and *S. faecalis* (24).

Previous data (3) indicated that the spores of *C. botulinum* contain the enzymes for arginine degradation; this was confirmed in the present study. Thus, there is further substantiation of the previous conclusion (23) that spores contain low levels of essentially all of the catabolic enzymes found in corresponding cells.

The fermentation of L-ornithine appears quite complex. If the balance data are corrected for the putrescine formed by decarboxylation, and for citrulline which could be produced by the citrullinases with an appropriate energy source, the yields of products in micromoles per 100 μmoles of L-ornithine fermented are as follows: CO_2, 10.4; acetate, 21.4; propionate, 6.9; butyrate, 2.3; valerate, 2.9; δ-aminovalerate, 81; and NH_3, 100. The carbon and nitrogen recoveries calculated for these products are 101 and 91%, respectively, and the redox index is 1.08. Thus, the fermentation is in reasonable balance, although the nitro-

gen recovery is too low and the reduced product is somewhat high. These variations are probably within the range of experimental error.

It is obvious that a great variety of reactions would be required to produce the products found. Stadtman and White (26) showed the production of considerable acetate and butyrate from ornithine by *Clostridium* HF, but this organism would not ferment the ornithine as a single substrate. The catabolism appeared to be mostly oxidative in nature, and no δ-aminovalerate was produced. In contrast, *C. botulinum* reductively deaminates L-ornithine, and the resulting δ-aminovalerate is the primary reduced product. Most of the CO_2 is derived from carbon 1 as shown by the fermentation of L-ornithine-1-C^{14}, and only a trace of the 1-C^{14} was in the volatile acids. Thus, oxidative decarboxylation must occur. One might expect this to be of an α-keto acid which could yield γ-aminobutyrate. Landgrebe and Weaver (Bacteriol. Proc., p. 103, 1966) reported that *C. botulinum* deaminates arginine, citrulline, and ornithine as determined by the production of α-keto acids and NH_3 from these substrates by resting cells. γ-Aminobutyrate could be fermented to acetate and butyrate in a manner similar to that proposed by Hardman and Stadtman (8, 10, 11) for *C. aminobutylicum*. The other volatile acids could be derived from a fermentation similar to that shown for *C. aminovalericum* by Hardman and Stadtman (9) which ferments δ-aminovalerate with the production of acetate, propionate, and valerate. The reductive deamination of L-ornithine provides such an excellent electron acceptor that the yields of the various products could vary widely, and no stoichiometric reaction for the complete fermentation is possible. Determinations of the reaction sequences involved in this fermentation are under investigation.

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