# ARGININE, CITRULLINE, AND ORNITHINE CATABOLISM BY *CLOSTRIDIUM BOTULINUM* TYPE 62-A

Thesis for the Degree of Ph. D.

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Brij Mohan Mitruka

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THESIS



This is to certify that the

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"Arginine, Citrulline, and Ornithine Catabolism by Clostridium botulinum Type 62-A"

presented by

Brij Mohan Mitruka

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Harold A. Sadoff
Major professor

Date August 2, 1965

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ABSTRACT

ARGININE, CITRULLINE AND ORNITHINE CATABOLISM
BY CLOSTRIDIUM BOTULINUM TYPE 62-A

by Brij Mohan Mitruka

Investigations were conducted to determine the metabolic products of arginine, citrulline and ornithine degradation by Clostridium botulinum type 62-A cells, spores and germinated spores. Since ornithine degradation was found to be unique in this organism, efforts were made to elucidate the pathway of ornithine degradation and to postulate a mechanism. An investigation was also made of some of the enzymes of vegetative cells, spores and germinated spores of C. botulinum, for eludicating a pathway for arginine and citrulline metabolism and of gaining knowledge of the metabolic potential of spores.

The major products of arginine degradation were shown to be $CO_2$, $NH_3$, ornithine and citrulline; the minor products were found to be acetic acid, propionic acid, butyric acid and valeric acid. Arginine is believed to be degraded to ornithine by a two step reaction. In the first step arginine was shown to be degraded to citrulline and $NH_3$ by an enzyme known as arginine deiminase. In the second

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step, citrulline is degraded to ornithine, $NH_3$ and $CO_2$ by citrullinase enzyme. Citrulline produced as an intermediate and ornithine produced as an end-product of arginine degradation were found to be inhibitory in the degradation of arginine.

The degradation of citrulline was found to be an exergonic reaction resulting in the generation of ATP. The major end products of citrulline were $CO_2$, ornithine and $NH_3$. A small amount of acetic, propionic, butyric and valeric acids were also found in the reaction mixture.

C. botulinum carried out the degradation of ornithine when ornithine was the only substrate in the reaction mixture. The rates of ornithine degradation by cells and cell extracts were found to be considerably lower than those for arginine or citrulline. However, with cell extracts the rate was increased by addition of the cofactors CoA, lipoic acid, ADP and $Mg^{++}$. The major products of ornithine degradation were $CO_2$, $NH_3$, putrescine, arginine, $\delta$-aminovaleric acid, acetic acid, and propionic acid. The minor products were butyric acid and valeric acid.

Extracts prepared from C. botulinum cells degraded arginine, citrulline and ornithine at a slower rate than did intact cells. The enzymes were found in the crude extracts of vegetative cells, spores and germinated spores; however the activities of arginine deiminase, citrullinase, ornithine

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transcarbamylase and transamidinase were significantly higher in vegetative cell preparations than those found in spores and germinated spores. Arginase and transaminase activities were not found in C. botulinum extracts, thus indicating that arginine is not degraded by the so-called "urea cycle" pathway.

The lower activities of the extracts of C. botulinum were shown to be due to certain cofactor requirements. Arginine deiminase was found to be stable after prolonged dialysis and no cofactors were required for the activity of this enzyme. However, addition of ADP or ATP and $Mg^{++}$ increased the activity to some extent. Ornithine present in excess inhibited the arginine deiminase activity greatly. Citrullinase enzyme required ADP, potassium phosphate and $Mg^{++}$ for the activity. Arsenate was shown to replace all these cofactors and highly increased the citrullinase activity. NaF was shown to inhibit specifically the activities of citrullinase and ornithine transcarbamylase.

Inhibitor and radioactive isotope data were consistent with enzyme assays. Thus, establishing that ornithine is slowly degraded by C. botulinum to volatile acids, $NH_3$ and $CO_2$. The mechanism for ornithine degradation was not clear, and on the basis of products formed, isotope studies, enzyme assays and fermentation inhibitors, an intermediate (x) was postulated. The possible nature of this intermediate was discussed.

# ARGININE, CITRULLINE, AND ORNITHINE CATABOLISM BY *CLOSTRIDIUM BOTULINUM* TYPE 62-A

By

Brij Mohan Mitruka

A THESIS

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# INTRODUCTION

Arginine is catabolized by various microorganisms through citrulline to ornithine, $NH_3$ and $CO_2$. Some species of the genera Streptococcus, Pseudomonas and Clostridium are known to be quite active in this breakdown. The overall reaction from arginine to ornithine was termed "arginine dihydrolase" by Hills (1940). Horn (1933) called the enzyme which carries the conversion of arginine to citrulline and ammonia, arginine desimidase. Oginsky and Gehrig (1952a) obtained arginine desimidase in cell-free extracts of Streptococcus faecalis by ultrasonic disintegration of bacterial suspensions or by water or buffer extraction of acetone treated preparations. Schmidt, Logan, and Tytell (1952) found citrulline as an intermediate and ornithine as the end product in the degradation of arginine by a washed cell suspension of Clostridium perfringens. Knivett (1953) and later Oginsky and Gehrig (1953) and Slade, Doughty and Slamp (1954) established with extracts of S. faecalis and a Pseudomonas species respectively, that, the second step of the arginine dihydrolase reaction series, namely, the breakdown of citrulline, results in the production of $NH_3$, $CO_2$, ornithine, and the esterification of inorganic phosphate into high energy phosphate.

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Jackson and Pasieka (1955) observed that degradation of arginine to ornithine by Micrococcus pyogenes var. aureus proceeds via citrulline; and the maximum activity of arginine desimidase was observed at pH 6.5. The specific activity of this enzyme within the cells was found to depend on the arginine concentration in the growth medium. Perkins and Tsuji (1962) used arginine in a complete synthetic medium for growing Clostridium botulinum and reported that arginine had completely disappeared after 2 to 3 days of incubation. They also found that increased amounts of arginine stimulated extensive sporulation. It was suggested that the sporulation induced by increased amounts of arginine could be attributed to energy yielded by the conversion of citrulline to ornithine.

The metabolic control of protein biosynthesis in Streptococcus faecalis var. liquefaciens was found to be dependent upon an inordinately large arginine requirement (Rabin and Zimmerman, 1956). Later Hartman and Zimmerman (1960) showed that the activity of the arginine dihydrolase system, by virtue of its ability to remove free arginine rapidly from the extracellular environment, regulates the extent of proteinase biosynthesis and perhaps protein synthesis generally for S. faecalis var. liquefaciens. They also found that ornithine stimulated proteinase biosynthesis by retarding the activity of arginine dihydrolase enzymes.

Although, a fermentation of ornithine has not been described, reactions are known by which an extensive

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decomposition of ornithine can occur in a system containing a suitable reducing agent and a mixture of two clostridia. Arginine can serve as a hydrogen acceptor for Clostridium sporogenes in the Stickland reaction, probably after an initial conversion to ornithine, since three moles of ammonia are formed per mole of arginine and ornithine serves as a hydrogen acceptor. An organism catalysing the Stickland reaction can reduce ornithine to δ-aminovalerate which can be fermented by an unnamed Clostridium (Hardman, Stadtman, and Szulmajster, 1958).

While the data of Perkins and Tsuji (1962) indicated that C. botulinum catabolized arginine via the dihydrolase system, the high $CO_2/NH_3$ values found by Costilow (1962) during arginine breakdown by germinating spores of this organism indicated that a different or additional system(s) was operative. The purpose of this study was to determine the patterns of arginine metabolism utilized by or present in Clostridium botulinum cells, spores and germinated spores. During these studies it was found that C. botulinum also fermented ornithine when this amino acid was used separately as a substrate. Since the fermentation of ornithine has not been reported to this date, considerable emphasis was given to this fermentation.

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## REVIEW OF LITERATURE

Metabolic pathways of arginine degradation by microorganisms: The degradation of arginine by microorganisms has been investigated by several workers. Mixed putrefective organisms employed in early investigations by Ackermann (1908) produced ornithine from arginine. Ornithine production was assumed to be the result of combined arginase-urease activity as follows:

$$\text{Arginine} \xrightarrow{\text{arginase}} \text{Ornithine} + \text{Urea}$$

$$\text{Urea} \xrightarrow{\text{urease}} NH_3 + CO_2$$

The hydrolytic cleavage of arginine to ornithine and urea, by the action of arginase is the familiar pathway in ureotelic animals (Cohen and Brown, 1960).

Hills (1940) studied the action of gram positive cocci on arginine and proposed a one step mechanism for the degradation. One mole of arginine was supposedly hydrolysed to 1 mole of ornithine with the liberation of 2 moles of ammonia and 1 mole of $CO_2$. Citrulline was not attacked and was therefore ruled out as a possible intermediate. Ornithine was isolated from the reaction mixture. Hills designated the enzyme arginine dihydrolase to distinguish it from

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$$\text{Arginine} + 2\ H_2O \xrightarrow[\text{dihydrolase}]{\text{arginine}} \text{Ornithine} + CO_2 + NH_3$$

A report by Woods and Trim (1942), who studied this system in Clostridium welchii, raised doubt as to the simplicity of the reaction; and later Sekine (1947) proposed that the degradation must follow a stepwise sequence, since he had observed both citrulline accumulation and citrulline degradation from arginine, by a strain of S. faecalis. Akamatsu and Sekine (1951) identified the two enzymes as meta-arginase and citrullinase. Knivett (1952) reported similar findings with a different strain of S. faecalis. Schmidt et al. (1952) investigated the degradation of arginine by Clostridium perfringens (BP6K). They obtained evidence that with washed cell suspensions, lyopholyzed cells, and sodium chloride extracts of lyopholyzed cells the following pathway was involved:

$$\text{Arginine} \xrightarrow{(1)} \text{Citrulline} + NH_3$$

$$\text{Citrulline} \xrightarrow{(2)} \text{Ornithine} + CO_2 + NH_3$$

However, they did not state the exact mechanism. Citrulline and ornithine were both isolated from the same reaction mixture. Oginsky and Gehrig (1952a,b) obtained similar results with the cell-free extracts of S. faecalis. They called the enzyme involved in (1) arginine desimidase, as proposed by Horn in 1933. The second (2) reaction was found

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to be present primarily in the streptococci, the pseudomonads, and the clostridia, and was carried out by an enzyme system called citrullinase, citrulline ureidase or citrulline phosphorylase. Knivett (1953) and later Oginsky and Gehrig (1953) and slade, et al. (1954) established with extracts of S. faecalis and a pseudomonas species, respectively, that the second reaction of the arginine dihyrolase reaction series was capable of generating high energy phosphate in the form of adenosine triphosphate (ATP). The phosphorolysis of citrulline to ornithine plus ATP was reported by Smith (1957) to occur in pleuropneumonia-like-organisms (PPLO).

Perkins and Tsuji (1962) reported a synthetic medium which will support spore germination, vegetative cell multiplication, toxin production and sporulation of C. botulinum strain 62A. They showed that arginine plays an important role in sporulation and that most of the arginine was broken down by a dihydrolase enzyme system through citrulline to ornithine. Furthermore, they reported that the second step in the reaction series, the degradation of citrulline, appears to be essential to sporulation. The absence of citrulline or ornithine in either growing cells or spores suggested to them that another product of the arginine dihydrolase system may be responsible for the observed stimulation of sporulation.

Schimke and Barlie (1963) presented evidence to indicate that arginine degradation by PPLO was extensive and proceeded by the following reaction series:

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$$\text{Arginine} \xrightarrow{\text{arginine deiminase}} \text{Citrulline} + NH_3$$

$$\text{Citrulline} + Pi \xrightleftharpoons{\text{ornithine transcarbamylase}} \text{Ornithine} + \text{Carbamyl phosphate}$$

$$\text{Carbamyl phosphate} + ADP \xrightleftharpoons[Mg^{++}]{\text{Carbamyl - Phosphokinase}} ATP + NH_3 + CO_2$$

They also reported that supplementation of PPLO culture broth with arginine increased the extent of PPLO growth and when the arginine content of the culture limited growth, arginine was completely converted to ornithine. Furthermore, when growth was limited by some other factor in the presence of excess arginine, citrulline was the major breakdown product. It was suggested by Schimke and Barlie (1963) that the conversion of arginine to ornithine constitutes a significant and possibly major source of ATP for this class of organism.

A new pathway of arginine metabolism of considerable interest has recently been discovered in Roche's laboratory (Thoai, Hatt, An, and Roche, 1956); viz.,

$$\text{Arginine} \longrightarrow \text{Guanido - butyramide}$$

$$\text{Guanido-butyramide} \xrightarrow{\text{Guanidobutyrate}} {}^{+}_{NH_3} \longrightarrow \gamma\text{- Aminobutyric acid}$$

They reported that an adaptive enzyme system in Streptomyces griseus apparently oxidatively decarboxylates arginine to guanido-butyramide which in turn is hydrolyzed with the formation of guanidobutyrate and $NH_3$. Of further interest,

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deguanidases in this organism act on various compounds, for example converting γ-guanidobutyrate to γ-aminobutyrate. In mammalian tissues and insects, however, the metabolic pathway was found apparently to be as follows:

Arginine ⟶ α-Keto-δ-guanidovalerate

α- Keto-δ-guanidovalerate ⟶ γ-Guanidobutyrate

γ-Guanidobutyrate was found to occur in human urine together with δ-guanido-n-valeric acid by Garcia and Couerbe (1958). Matsushiro and Nakada (1954) demonstrated arginine catabolism in a strain of Gram positive bacteria, involving oxidative deamination to α-keto-δ-guanidovaleric acid followed by oxidation of this intermediate to δ-guanidobutyric acid.

The amidine group of arginine represents but a small portion of the arginine molecule; it has, however, a high metabolic lability and has been studied extensively. Transamidation reactions in Streptomyces griseus were studied by Walker (1956). An enzyme system in this organism catalyzed reversible arginine to ornithine and canavanine to ornithine reactions. An enzyme-amidine intermediate was indicated, since formamidine was trapped by the use of hydroxylamine, and, formamidine disulfide inhibited this system. Fuld (1956) observed that arginine - glycine transamidiation was carried out in many organisms and that the reaction was reversible. By means of group transfer the high chemical potential of the amidine was conserved.

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Arginine + Glycine $\rightleftharpoons$ Ornithine + Guanidoacetic acid

In mammalian kidneys, the guanidoacetic acid, formed in this manner, is then methylated to form creatine as follows:

Guanidoacetic acid + S-Adenosylmethionine $\longrightarrow$ Creatine + S-Adenosylhomocysteine.

This system is one of the few known instances of negative feedback regulation in mammals.

In another pathway, arginine serves as the precursor of $\alpha$-aminoadipic acid, which in turn is converted by some organisms (e.g., Neurospora) to lysine.

Still another metabolic pathway of arginine was found to exist in Escherichia coli by which simple decarboxylation of arginine occurs:

Arginine $\longrightarrow$ Agmatine + $CO_2$

Melnykovych and Snell (1958) found that the growth of E. coli in chemically defined media did not induce formation of arginine decarboxylase, but the addition of a casein digest resulted in the enzyme formation. In a non-aerated culture, arginine, methionine, tyrosine and aspartic acid could replace the casein digest, and iron was found to stimulate the enzyme formation. Furthermore, they found that under aerobic conditions, the iron requirement was increased, and glutamate was also required for the enzyme synthesis. No definite role of iron for the enzymatic activity could be found by these workers.

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Citrulline as an intermediate in arginine metabolism: That citrulline may be an intermediate in the degradation of arginine was first indicated by Horn (1933). After incubation of arginine with Bacillus pyocyaneus (Pseudomonas aeruginosa), citrulline was isolated from the reaction mixture. Horn named the enzyme responsible, arginine desimidase. Tomota (1941) reported similar results with the same organism. Sekine (1947) and Akamatsu and Sekine (1951) found that arginine was degraded to citrulline and $NH_3$ by whole cells of S. faecalis. These authors proposed the name metaarginase for the enzyme responsible. Knivett (1952) found that when the washed suspensions of S. faecalis were incubated with arginine, complete disappearance of arginine takes place but only 70-80 percent can be accounted for by the known products of the reaction. He attempted to study the reaction using cell-free preparations or cells treated with the detergent (CTAB) or with acetone. Three preparations were obtained which attacked arginine with the liberation of one mole $NH_3$ for each mole of arginine broken down, but without production of $CO_2$ or ornithine. Citrulline was identified in the products by chromatographic techniques. The reaction was found to be inhibited by certain long chain amidines, diguanidines and substituted guanidines, including paludrine. Citrulline was identified among the products or the breakdown of arginine by intact cell suspensions, but it was attacked only very slowly by intact cells and not at all by CTAB-treated or acetone-dried cells in the absence of ATP.

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In the presence of sufficient ATP, CTAB treated cells metabolized citrulline with the production of ornithine, $NH_3$ and $CO_2$.

Oginsky and Gehrig (1952a,b) prepared cell-free extracts of S. faecalis and P. aeruginosa and reported that arginine was degraded to equimolar quantities of ammonia and citrulline. Arginine desimidase, the name proposed by Horn (1933), was adopted by Oginsky and Gehrig for the enzyme from S. faecalis. Slade (1953) has reported similar observations with the cell-free extracts of strain $D_{10}$ of S. faecalis. Schmidt et al. (1952) obtained extracts of C. perfringens (BP6K) which catalyzed the conversion of arginine to citrulline and ammonia. Roche and Lacombe (1952) found that extracts by baker's yeast also metabolize arginine to citrulline. The name arginine desiminase for the enzyme was suggested by these authors.

Lominski, Morrison and Porter (1952) obtained evidence, that arginine disappeared and citrulline accumulated in a medium in which M. pyogenes var. aureus had grown for five to seven days. The accumulation of citrulline during arginine degradation by acetone dried cells of this organism made it possible to demonstrate that citrulline is indeed an intermediate in the conversion of arginine to ornithine. Jackson and Pasieka (1955) could not detect the accumulation of citrulline with intact cells but noted a rapid degradation of arginine to ornithine. However, they reconfirmed that citrulline was the principal ninhydrin positive product of

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arginine degradation by acetone dried cells of M. pyogenes var. aureus.

Ornithine degradation: The fermentation of ornithine as a single substrate has not been reported in microorganisms. However, ornithine is known to be used in the Stickland reaction where it serves as a hydrogen acceptor. C. sporogenes, the organism studied extensively by Stickland (1935) and by Woods (1936), utilizes ornithine as a hydrogen acceptor and converts it to δ-aminovaleric acid. Dried cells of clostridium, strain HF, formed trace amounts of δ-aminovaleric acid from ornithine when incubated with molecular hydrogen and also oxidized ornithine with molecular oxygen (Stadtman, 1954). However, growth of the organism did not occur with ornithine as the only energy source. Stadtman (1954) reported that either proline or lysine must also be added to the medium. She concluded that the role of ornithine in the fermentations was at least in part that of a hydrogen donor, since, none of the reduced product, δ-aminovaleric acid, was found when ornithine plus lysine were fermented. Arginine and citrulline replaced ornithine in the fermentations by virtue of the fact that they were converted to ornithine, presumably by the arginine dihydrolase series of reactions. Woods (1936), found that arginine as well as ornithine was reduced by C. sporogenes; and, since three moles of ammonia were released per mole of arginine, he reported that it is likely that the actual

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Ornithine may be converted to citrulline by reaction with either carbamyl phosphate (prepared chemically from ammonium carbonate) or carbamyl phosphate bound to acetylglutamic acid or by reaction with carbamylglutamic acid (formed enzymatically from $NH_4^+$, $NaHCO_3$ and ATP). The thorough work of Grisolia and Cohen, (1952) has established without doubt that the δ-carbamylation of ornithine is catalyzed by N-acetylglutamic acid in the presence of ATP. In some later work Grisolia and Cohen (1953) have demonstrated that N-carbamylglutamic acid can be replaced by N-acetylglutamic acid in the formation of the labile $CO_2$-$NH_3$ complex that effects the carbamylation of the δ-amino of ornithine. Recently Grisolia, Burris and Cohen (1954) demonstrated that in the formation of the complex intermediate, the hydrogen atoms of the glutamyl portion of carbamylglutamic acid are not involved. In mammalian tissues, as demonstrated for rat liver, ammonia seems to be utilized as such in the formation of citrulline. In bacteria, represented by Lactobacillus arabinosus, the requirement for nitrogen seems to be more specific and involves glutamic and as the ammonia donor, as shown by Ory, Hood and Lyman (1954).

Slade (1953) found that neither L-glutamic acid nor carbamyl-L-glutamic acid had any effect on the rate of citrulline-ureidase reaction and, furthermore, qualitative tests for carbamyl-L-glutamate on the dialysed extracts which he prepared were negative.

The conversion of ornithine into proline and glutamic acid has been demonstrated by the findings of significant amounts of stably bound deuterium in proline and glutamic acid isolated from mice fed deutereted ornithine. The obvious structural similarity between glutamic acid, proline, hydroxyproline and ornithine has provoked much speculation and research as to their possible metabolic interrelationships. The complicated interconnections among proline, ornithine and glutamic acid have been unravelled to a considerable extent during the last few years. The brilliant work of Vogel and Davis (1952) and Vogel (1953) has shown that in E. coli the branching point in the pathways that lead to proline and to ornithine is the N-acetylation of glutamic acid. The non-acetylated part of glutamate is converted to proline through glutamic-$\gamma$-semialdehyde, cyclization of the latter to $\Delta'$ pyrroline-5-carboxylic acid, and reduction to proline. The N-acetylglutamic acid seems to be transferred intact to the corresponding semialdehyde, then to N($\alpha$)-acetylornithine and finally ornithine.

The reactions in Neurospora, seem to be different. Fincham (1953) suggested that in this organism the $\delta$-amino group of ornithine is transferred to $\alpha$-ketoglutaric acid in a transamination reaction, making ornithine the main precursor of glutamic-$\gamma$-semialdehyde and thus of proline. Vogel and Bonner (1954) have clarified the question considerably by demonstrating that in Neurospora the main route for the formation of proline is through glutamic semialdehyde

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and that exogenous ornithine may contribute to some part of the latter. It is possible that the semialdehyde, as proline precursor, does not form a common pool with the semialdehyde as ornithine precursor. The glutamate-proline-ornithine interaction in Neurospora is strikingly similar to that in mammals, but differs from that in E. coli. Especially interesting is the finding that in Neurospora no acetylated intermediates seem to participate in the reaction sequence. If the initial step from ornithine is pictured as an oxidative deamination or a transamination, one of two possible carbonyl compounds, $\alpha$-keto-$\delta$-aminovaleric acid or the $\gamma$-semialdehyde of glutamic acid, would result depending upon which of the amino groups of ornithine is lost. $\alpha$-Keto-$\delta$-aminovaleric acid is known to play a significant role in the interconversions of proline, glutamic acid and ornithine in mammalian systems.

The nature of the transamination reaction starting with ornithine has also been the subject of recent studies. Fincham (1953) prepared an extract of Neurospora crasa which was able to catalyze the transfer of the $\delta$-amino group of ornithine to $\alpha$-ketoglutaric acid with the formation of glutamic acid and glutamic-$\gamma$-semialdehyde. Evidence that this reaction is reversible was obtained in experiments in which a small amount of ornithine was formed by Neurospora extracts from glutamic semialdehyde in the presence of added glutamic acid. Since extracts of mutant strains of Neurospora require added ornithine transaminase activity, it was

concluded that there must be some mechanism of ornithine synthesis other than the reversal of the ornithine transaminase reaction.

Enzymes of arginine degradation: The enzyme system responsible for the decomposition of arginine has been investigated in some detail in S. faecalis (Knivett, 1952; Oginsky and Gehrig, 1952b; Slade, 1953); C. perfringens (Schmidt et al., 1952) and in a lesser detail in S. lactis (Korzenovsky and Werkman, 1952). In all these studies the evidence presented indicates that the total decomposition of arginine to ornithine occurs as the result of the action of at least two enzymes. The first arginine-desimidase removes the imide group to form $NH_3$ and citrulline while the second, citrulline-ureidase, catalyzes the degradation of citrulline to $NH_3$, $CO_2$ and ornithine.

Petrack, Sullivan and Ratner (1957) have partially purified arginine desiminase from S. faecalis extracts. The reaction proceeds to completion in the absence of phosphate and cannot be reversed even in the presence of ATP. A similar hydrolytic reaction converting canavanine to o-ureidohomoserine has been reported by Kihara and Snell (1957). Of particular interest, the enzyme catalyzing this reaction in S. faecalis appears to be identical with arginine desiminase.

The enzyme which catalyzes the conversion of arginine to citrulline has been referred to as arginine desimidase, (Horn, 1933) and as meta-arginase (Sekine, 1947 and

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Akamatsu and Sekine 1951). More recently Roche, and Lacombe (1952) have proposed the more appropriate name, arginine desiminase. In the reaction carried out by arginine desimidase only one of the terminal guanidine nitrogen atoms is susceptible to cleavage, in contrast to the position of the cleavage catalyzed by arginase. Petrack, et al. (1957) reported that the hydrolytic removal of $NH_3$ by arginine desiminase from arginine represents an enzyme activity quite unrelated to mechanisms concerned with the synthesis of arginine from citrulline. Isolation from crayfish muscle of a pyridoxal phosphate dependent enzyme called citrulliminase, has been recently reported. The enzyme appears to catalyze a rapid formation of arginine from citrulline and $NH_3$ (the reverse reaction) without further requirements. The properties of the two enzymes (arginine desiminase and citrulliminase) appear to be quite different. It is difficult to understand how the condensation of citrulline and $NH_3$ can proceed in the absence of an energy donor.

The enzyme system catalyzing the citrulline to $NH_3$, $CO_2$ and ornithine conversion is called "citrullinase" or "citrulline-ureidase." However, it is now known that two enzymic steps are involved in the reaction (Korzenovsky and Werkman, 1953); one being a phosphorolysis of citrulline to ornithine and carbamyl phosphate, the other being the transfer of the phosphoryl group from carbamyl phosphate to adenosine diphosphate (ADP) to form ATP. Carbamyl phosphate has been shown to serve both as a carbamyl donor in citrulline

synthesis and as a phosphate donor to ADP in the presence of extracts of S. faecalis (Jones, Spector and Lipmann, 1955). The enzymes catalyzing these reactions have been separated and partially purified. The equilibrium of the reaction Citrulline + $HPO_4^{=}$ $\rightleftharpoons$ Ornithine + $NH_2COO\ PO_3^{=}$ is far to the left. Therefore the decomposition of citrulline is dependent upon the removal of carbamyl phosphate by the following reaction:

$$NH_2COO\ PO_3^{=} + ADP \rightleftharpoons NH_3 + CO_2 + ATP$$

The equilibrium in this reaction is known to be far in the direction of ATP formation.

The steps in citrulline synthesis was further clarified by Burnett and Cohen (1957). They purified the enzyme ornithine transcarbamylase from beef liver approximately 100 fold. The equilibrium of the reaction strongly favors citrulline synthesis, the substrate specificity was reported to be high and there was found to be no cofactor requirement and also there was no indication of citrulline-phosphate formation.

Walker (1958) studied transamidation reactions in Streptomyces griseus and found that an enzyme system catalyzed reversible reactions of arginine to ornithine and canavanine to ornithine. An enzyme-amidine intermediate was indicated, since, formamidine was trapped by the use of hydroxylamine. Formamidine disulfide inhibited this system.

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## EXPERIMENTAL METHODS

Culture and cultural methods: The culture of *Clostridium botulinum* 62-A used was obtained from the American Type Culture Collection (ATCC 7948). The culture was maintained in the spore state. Spores were produced in a medium containing 4 percent Trypticase (a tryptic digest of casein BBL) and 1 ppm thiamine (pH 7.0 to 7.2), developed by Day and Costilow (1964). Vegetative cells were produced in a medium containing 4 percent Trypticase, 0.2 percent thioglycollate, and 1 ppm thiamine. For large cultures, a 12-liter flask containing 10 liters of medium was used. The flask was incubated in a 37 C water bath. Anaerobiosis was maintained by passing a slow stream of city gas first through a sterile water trap and then through the medium. The effluent gases were allowed to escape into a separate water trap flask and then into a ventilating hood. The inoculum was prepared starting with a heat shocked (80 C for 10 min) spore suspension inoculated into a tube of medium and incubated overnight (12 to 15 hr). This constituted the starting culture for a series of three transfers 3-4 hr apart, using a 10 percent inoculum each time, with the final transfer representing the inoculum for the sporulation medium. For vegetative cell production a 7-8 hr incubation period

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was satisfactory as the cells were in the logarithmic growth phase. For spore production the culture was permitted to incubate 40 to 48 hr at which time virtually all spores were found. The cells or spores were collected by centrifugation, washed three times with cold distilled water and resuspended in 0.067 M phosphate buffer (pH 7.0).

Germinated spores were obtained by allowing the spores to germinate in a solution of 4 percent Trypticase and 0.1 percent sodium bicarbonate in 0.067 M phosphate buffer (pH 7.0). The same method of attaining anaerobiosis used for sporulation was found suitable for germination and the steady flow of city gas kept the spores in constant agitation. Ten to twelve grams wet weight of spores suspended in a small volume of the germination medium were incubated 2 to 2.5 hr at 37 C.

Extracts of vegetative cells, spores and germinated spores were prepared by disruption of the cells with size No. 110 Superbrite glass beads (Minnesota Minning and Manufacturing Company, St. Paul, Minn.) in a high-speed Servall (Ivan Sorvall Inc., Norwalk, Conn.) Omnimixer. Ten to twelve grams wet weight of cells suspended in 50 ml of 0.05 M tris buffer were used in the cup along with 45 g of glass beads. The cup was chilled in an ice bath for 10 min prior to disruption of the cells and the ice bath was constantly stirred to facilitate heat transfer during the breaking. The time required for good breakage was 10 min for cells and 20-25 min for spores. The extracts so obtained were

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centrifuged at 30,000 X g for 1 hr to clarify them and dialysed against distilled water at 4 C for 24 hr. Dry weights were determined by drying 1 ml of the cell suspension to constant weight at 110 C.

Analytical methods: Carbon dioxide production was determined manometrically by the direct method using standard Warburg Techniques (Umbreit, Burris, and Stauffer, 1957). Unless otherwise noted, all components of the reaction mixture were added to the main compartment of the Warburg flask except substrate which was tipped in from a side arm after thermal equilibrium was attained. All gas not absorbed by 20 percent KOH was calculated as $H_2$.

Reaction mixtures for analysis were treated with 10 percent cold trichloroacetic acid (TCA), centrifuged, and the supernatant fluid collected and stored at 4 C for chemical and chromatographic analyses.

Free $NH_3$ was determined by the modified colorimeteric method of Johnson (1941). Samples were removed from Warburg vessels and 1 ml was placed in one side of the outer chamber of Conway plates 1 ml of 6 N NaOH in the other side, and 1 ml of 2 N $H_2SO_4$ was placed in the center well. After sealing the NaOH and sample were mixed by tipping the plates to liberate all the $NH_3$. The plates were allowed to set at room temperature for 12 hr and $NH_3$ absorbed in the standard acid was determined by Nesslerization.

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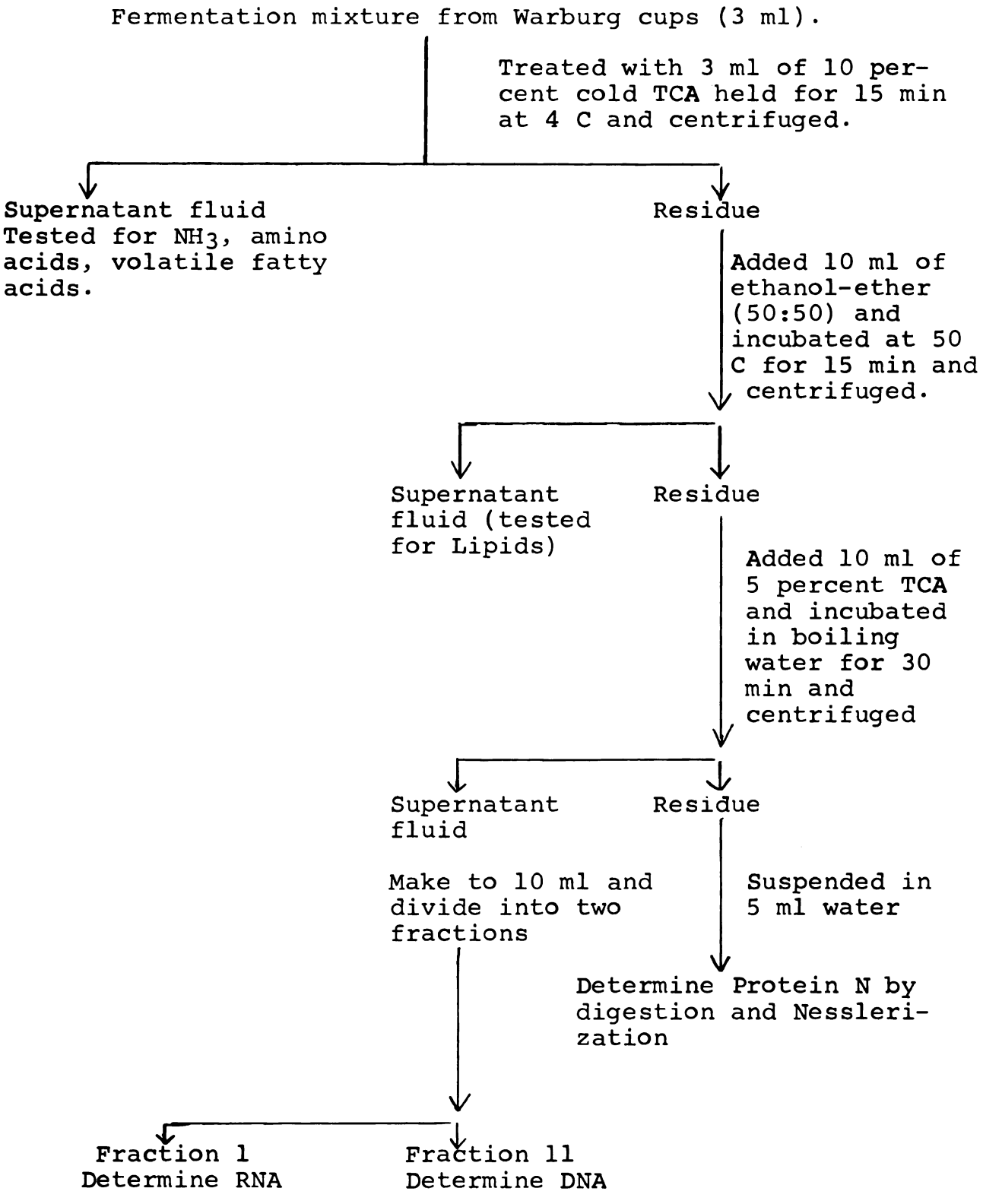
Cells were fractionated to estimate the incorporation of amino acids into proteins, nucleic acids, and lipids according to the following scheme:



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The nucleic acids, ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) from the cells and reaction mixtures, were determined by chemical tests. The cells were extracted with hot TCA to Solublize the nucleic acid component (Fitz-James, 1955; Schneider, 1945). RNA was then determined by the orcinol reaction (Schneider, 1945) and DNA was determined by the Keck modification of the Corriotti indole method (Keck, 1956). Proteins were estimated by the Folin-Ciocalteu method of Lowry, Rosebrough, Farr, and Randall (1951). Crystalline egg albumen was used as the standard.

Total lipids were determined by evaporating the solvents in hood and weighing the lipid fraction. Absorbancy at 280 mμ and 260 mμ was also determined using a Beckman DU spectrophotometer. Where radioactive amino acids were fermented, a similar scheme was followed and instead of chemical tests at each step, duplicate samples of 1 ml each fraction were placed in 20 ml radioactive isotope counting bottles and counted using a Tricarb Scintillation Spectrometer with control model 314-DC (Packard Instrument Company Inc., LaGrange, Illinois).

Arginine-guanidino-$C^{14}$, arginine-U-$C^{14}$, citrulline-ureido-$C^{14}$, and ornithine-2-$C^{14}$ were mixed with cold substrate and metabolized by resting cells in Warburg vessels. The activity of radioactive substrate did not exceed 0.1 μc per vessel except where otherwise noted. Durham tubes cut in half and containing 0.2 ml of 20 percent KOH were placed

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in the center well of the Warburg flask to trap $CO_2$. After the reaction was stopped with 2 M $H_2SO_4$ the tube was removed with forceps and the contents transferred to a 20 ml sample vial with a bulb pipette. The tube and pipette were successively washed with measured quantities of distilled water and added to the radioactive sample. Distilled water was added to 1.0 ml. Samples were taken from the fermentation mixture, treated with cold TCA and centrifuged. After separation of amino acids in the supernate on a Dowex-50 columns, and fraction of the cells carried out as described earlier, 1.0 ml samples were placed in vials and the radioactivity determined. Fifteen ml of scintillation solution as described by Gordon and Wolfe (1960) were transferred by means of a syringe into the 20 ml vial containing 1 ml of diluted sample. The vial was closed and vigorously shaken to mix the contents. The disintegrations were counted with a Tricarb Scintillation Spectrometer. Benzoic acid-$C^{14}$ was used as a counting standard.

Chromatographic techniques were employed for qualitative and quantitative determinations of the products of arginine, citrulline, and ornithine fermentations. One dimensional, ascending paper chromatography was used to identify arginine, citrulline, ornithine, $\delta$-aminovaleric acid, and putrescine by a method described by Stepka (1957). The solvent systems used for arginine, citrulline, ornithine, and $\delta$-aminovaleric acid were: butanol, acetic acid, water (4:1:5); methanol, pyridine, water (7:2:1); methanol,

triethylamine, water (8.5:0.4:1.1). Rf values for each amino acid were compared with standards. Putrescine was identified by using a descending paper chromatography method described by Herbst, Keister, and Weaver (1958). The solvent used for this was methyl cellosolve, propionic acid and water (7.5:1.5:1.5) saturated with NaCl. The developing reagent was 0.1 percent ninhydrin in n-butanol containing 1 percent glacial acetic acid or in anhydrous acetone. The spots were developed by brief heating at 105 C.

Paper chromatography was also used for the identification of short chain volatile fatty acids according to the method of Kennedy and Barker (1951). The volatile acids were first converted to the non-volatile ammonium salts. After the acids had been eluted from a celite column, 1.0 ml of concentrated $NH_4OH$ was added to each sample. The solvent was evaporated on a steam bath and the aqueous portion was used to spot the chromatogram. A volume of 0.01 ml was applied at the origin of a 45 x 25 cm piece of Whatman No. 1 chromatographic grade filter paper. The paper was made into a cylinder, stapled and placed in a battery jar containing 200 ml of a solution of ethanol and concentrated $NH_4OH$ in a ratio of 100 to 1. This was allowed to develop at room temperature for 6-8 hr. The paper was then dried at 100 C for 5 min and sprayed with a solution of 50 mg of bromophenol blue and 211 mg of citric acid in 100 ml of distilled water. Samples of known acids were run in the same manner.

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Arginine, citrulline, ornithine, putrescine, and δ-aminovaleric acid from the fermentation mixture were separated and determined quantitatively using a method described by Moore and Stein (1954) with the following modifications. A sulfonated polystyrene, cross-linked with 4 percent divinyl benzene (Dowex-50 x 4) 400 mesh resin, was used as adsorbent. The suspended resin was transferred to a 150 x 0.9 cm chromatographic tube and a filter paper disc placed on top to prevent channels. The reaction mixture (2 ml), adjusted to pH 2.0 to 2.5 was added to the surface of the resin column with a bent-tip pipette. Three, 0.3 ml aliquots of buffer (pH 2.2) were used to wash in the sample. The gradient elution was set up and the effluent was collected using a fraction collector. Water from a constant temperature bath was circulated through the jackets of the columns. Citrulline was eluted at pH 3.1 and 50 C. Ornithine was eluted next and finally the pH was raised to 5.1 and the temperature to 75 C in order to elute arginine.

Putrescine was eluted with 2.5 N HCl using additional columns (0.6 x 8 cm), as described by Tabor and Rosenthal (1963). Gradient elution was carried out with 2.5 N HCl; 300 ml of $H_2O$ were in the mixing vessel. The flow rate was maintained at approximately 20 ml/hr.

δ-aminovaleric acid was isolated on Dowex-50 resin in the hydrogen form by elution with 1.5 N HCl as described by Greenberg and Rothstein (1957).

The fractions collected from the columns were analysed spectrophotometrically by using an Auto Analyzer (Technicon Instrument Co., Chauncy, New York) according to the procedure described by Piez and Morris (1960). The ninhydrin reagent for the color development was prepared by a method described by Duggan (1957).

In the experiments using radioactive amino acids the volatile fatty acids were separated on a column of celite (Johns Manville Products, Detroit, Michigan), containing 3-(4-anilino, 1-naphthylazo) 2,7-naphthylene disulfonic acid mono ammonium salt as an internal indicator. The column was prepared as described by Wiseman and Irvin (1957). The developing solvents consisted of various percentages by volume of acetone in Skellysolve B (Skelly Oil Company, St. Louis, Missouri). The lower concentrations of acetone eluted the higher molecular weight organic acids. The resulting eluates were dried on a steam bath and 1 ml samples were used for counting radioactivity as described above. Standards of known amounts of acetic, propionic, butyric, valeric and hexanoic acids were placed on the column and eluted in the same way with 98.5 percent recovery. These techniques were also used with fermentation mixtures of arginine, citrulline, and ornithine (non-radioactive), in order to check the results obtained with gas chromatography techniques. In the case where non-radioactive amino acids were fermented the eluates from the celite columns were titrated with 0.05 N

$Ba(OH)_2$. No significant difference in the amounts of volatile acids as determined by gas chromatography or by celite columns were found.

Gas chromatographic techniques were used to analyze short chain volatile fatty acids in the fermentation mixtures. The instrument used was a Model A-600-B, "HY-FI" Gas Chromatographer, with a hydrogen flame ionization detector (Wilkens' Instrument and Research Inc., Walnut Creek, California). The columns used were 9 ft Carbowax, 20 M TCA 60/80 HMDS, 4.2 g. Steam saturated $N_2$ was maintained at 12 psi, with a flow rate of 25 ml/min. Five microliter samples were injected onto the column by a micropipette. Standard graphs were prepared by injecting known amounts of volatile acids onto the columns prior to the test runs.

Arginine was determined by a modified method of VanPilsum, Martin, Kito, and Hess (1956). Five-hundredths ml of alkaline $\alpha$-naphthol, thymine mixture (1:1) was pipetted into a 3 ml cuvette containing an aliquot of fermentation mixture. After mixing, 0.2 ml of a 2 percent NaOCl solution was added with immediate mixing. Exactly 1 min later, 0.2 ml of a 2 percent $Na_2S_2O_3$ solution was added with immediate mixing. The absorbancy was read at 515 mμ using a Beckman DU spectrophotometer. The Sakaguchi color was found to be stable in the cold for several hours. An arginine standard curve was prepared with solutions containing 5-50 μg of arginine per ml.

Citrulline was determined using the method of Archibald (1944) modified by Spector and Jones (1963), based on the formation in the dark of a colored reaction product with diacetyl monoxime in acid solution. The color developed was read at 490 mμ using a Beckman DU spectrophotometer. A standard curve was prepared with solutions containing 10 to 100 μg of citrulline per ml.

Ornithine was determined using a method described by Chinard (1952). Color development was followed at 515 mμ using a Beckman DU spectrophotometer.

Enzyme assays: Arginase was assayed according to the method of Greenberg (1955) with some modifications. The activity was increased by pre-incubation in a final concentration of 0.05 M $MnSO_4$ for 5 min at 55 C. Incubations with the substrate (arginine) were for 10 min in 1.0 ml containing 0.1 to 0.5 ml of $MnSO_4$ treated extracts. The reaction was stopped by the addition of 15 percent perchloric acid, and the mixture was centrifuged. An aliquot of the supernatant fluid was used for urea determinations.

Urea was determined by the colorimetric method of Brown and Cohen (1959). A 2 ml sample was treated with 1.5 ml of an acid mixture ($H_2SO_4$, 1 volume; sirupy $H_3PO_4$, 3 volumes; water, 1 volume) and 0.4 ml of 4 percent α-isonitrosopropiophenone. The assay tubes were shaken thoroughly, stoppered and boiled in the dark for 60 min. After they have cooled at room temperature for 15 min the color which

developed was read at 540 mμ using a Spectronic-20 colorimeter (Bausch and Lomb Optical Company, Rochester, New York). A urea standard curve was prepared with samples containing 0 to 150 μg of urea per ml.

Arginine deiminase was assayed by a method described by Oginsky (1955). Four-tenths ml of 0.1 M arginine, pH 6.5, was mixed in a test tube with 1 ml of 0.2 M phosphate buffer, pH 6.5, 0.2 ml of dialysed extract and water to make 3 ml. The mixture was incubated at 37 C and the reaction stopped at 15 min intervals with 70 percent perchloric acid. Replicate tubes were prepared. The contents of the reaction mixtures were centrifuged and the supernatant fluid was assayed for citrulline and $NH_3$ according to the methods described earlier. Citrullinase in dialysed extracts does not interfere with the deiminase assay.

Citrullinase was assayed by measuring the enzymatic breakdown of citrulline using standard manometric techniques for the determination of $CO_2$ (Oginsky, 1955). The following were placed in the main compartment of a double side arm Warburg vessel: 1.0 ml of 0.5 M acetate buffer, pH 5.8; 0.5 ml of 0.01 M ADP, pH 5.5-6.0; 0.1 ml of 0.1 M $MgCl_2$; 0.5 ml of 0.1 M phosphate buffer, pH 5.8; and 0.5 ml of extract. Two-tenths ml of 0.1 M L-citrulline, pH 5.5-6.0, was placed in one side arm of the flask and 0.2 ml of 2.0 M $H_2SO_4$ was placed in the other side arm. The flask was flushed with $N_2$ gas for 10 min and incubated at 37 C. Citrulline was tipped in at 0 time and the $CO_2$ released was measured at 5 min

intervals. $H_2SO_4$ was tipped in at the end of the experiment to release the bound $CO_2$.

Ornithine transcarbamylase (OTC) was assayed according to the method described by Jones (1962). An assay mixture was prepared from equal volumes of M tris buffer, (pH 8.5), 0.1 M L-ornithine hydrochloride, and 0.1 M dilithium carbamyl phosphate. The total volume was 0.5 ml, which consisted of 0.15 ml of the assay mixture, water and aliquots of the enzyme. This was incubated at 37 C for 15 min. A vessel containing the assay mixture but no enzyme was prepared to correct for the urea in carbamyl phosphate solutions as well as for the small amount of non-enzymatic synthesis of citrulline during the incubation at 37 C. The reaction was stopped by the addition of 1 ml of 5 percent TCA and the protein was removed by centrifugation. Aliquots of the reaction mixture were analyzed for citrulline by the method described previously.

The method of Jones (1962) was used to assay carbamyl phosphokinase activity in C. botulinum extracts. The assay mixture was made with 1 part 1 M acetate buffer (pH 5.5), 0.1 part 0.4 M $MgCl_2$, 0.4 part 0.1 M ADP(pH 7.0) and 0.4 part 0.1 M dilithium carbamyl phosphate. The reaction mixture consisted of 0.5 ml total volume of which 0.2 ml was the assay mixture and the rest was either water or enzyme. This mixture was incubated for 10 min at 37 C. A zero time blank was prepared with each test. After incubation an aliquot

equal to 1/5 of the reaction mixture was used for the determination of the sum of carbamyl phosphate and orthophosphate. The assay samples, a water blank and phosphate standards were then allowed to stand with 0.1 N KOH for 10 min at room temperature to decompose the carbamyl phosphate to orthophosphate. Next the reagents for the Fiske-Subbarow orthophosphate determination (Leloir and Cardini, 1957) were added and the samples were brought to a volume of 10 ml. After 20 min the color which developed was read at 660 mμ using a Spectronic-20 Colorimeter. The ATP formed or the carbamyl phosphate utilized were calculated by the difference between the zero time tube and the incubated sample.

Transamidinase activity in C. botulinum extracts was assayed by the method described by Ratner (1962). Reaction mixtures contained 0.15 ml of 0.1 M L-arginine, 0.25 ml of 0.1 M glycine, 1.0 ml of 1 M potassium phosphate (pH 7.5), diluted enzyme, and water to 1.5 ml. After 20 min at 38 C the reaction was stopped with 2.0 ml of 8.3 percent TCA. A zero time blank was always included. A control without glycine was also incubated to correct for arginase activity, if present, in the extract. An aliquot of the reaction mixture was used for ornithine determination by the method described previously.

## RESULTS

$CO_2$ production from arginine, citrulline and ornithine by resting cells of C. botulinum: A resting cell suspension of C. botulinum when allowed to act on arginine, citrulline or ornithine gave rates of $CO_2$ evolution as shown in Fig. 1. The rate of gas production was highest with arginine, intermediate with citrulline and lowest with ornithine. The constant gas (calculated as $CO_2$) rates were 15.0, 13.7 and 4.1 μliters/hr/mg cells (dry wt) for arginine, citrulline and ornithine respectively. These results indicate that arginine is a preferred substrate for degradation by C. botulinum over citrulline or ornithine. Furthermore, citrulline and ornithine acted as inhibitors of arginine degradation by resting cells (Fig. 2).

The degradation of arginine by intact cells of C. botulinum was found to proceed via citrulline as shown in Fig. 3. In the first step of the degradation, arginine is converted to citrulline and $NH_3$. Citrulline was accumulated at the identical rate as $NH_3$ in the presence of NaF. NaF is known not to interfere with arginine degradation to citrulline (Ratner 1962). In the second step, the disappearance of citrulline and the production of equimolar levels of $CO_2$ and $NH_3$ are shown. Citrulline was found not to be completely

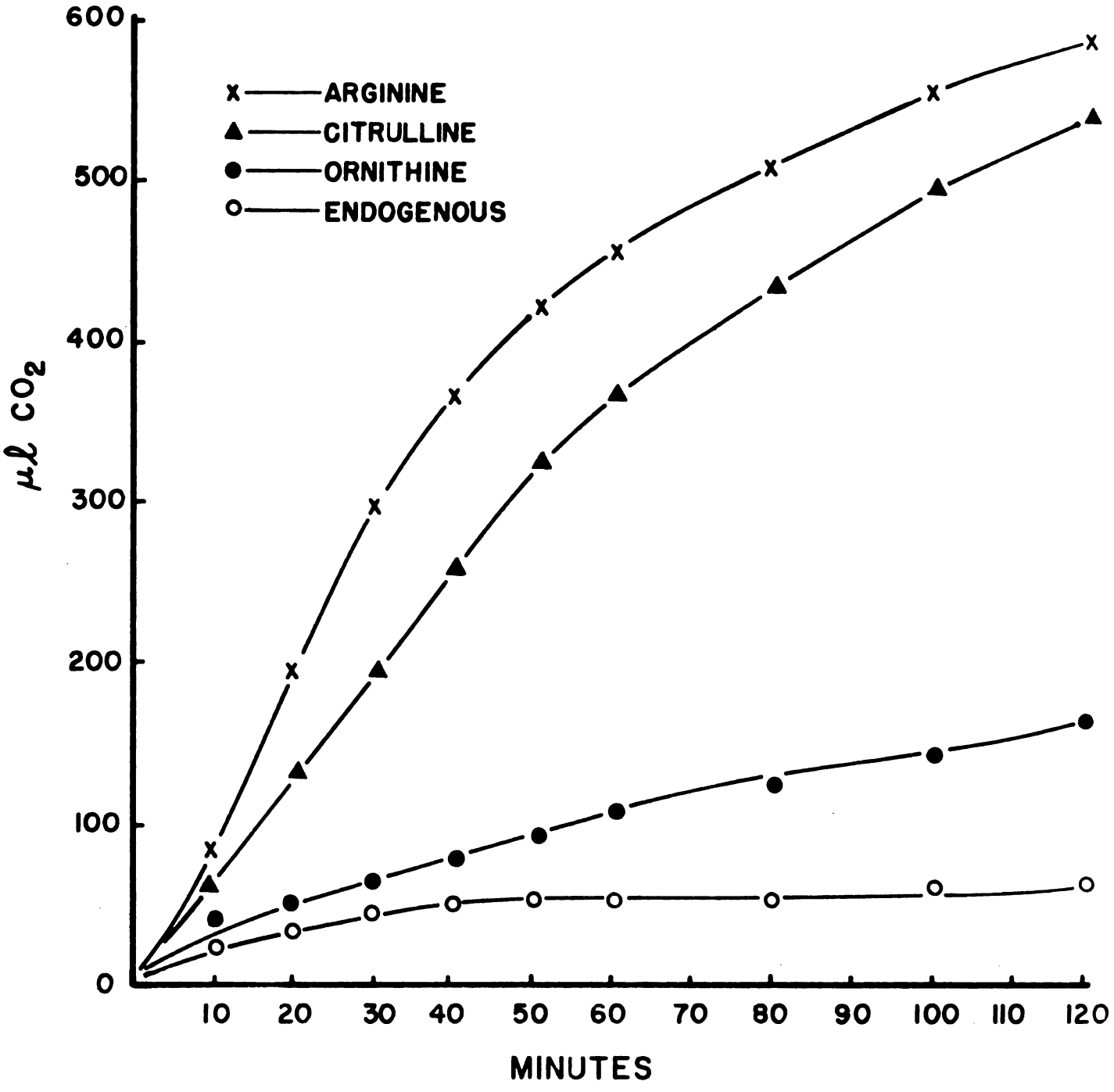



Figure 1. Degradation of arginine, citrulline, and ornithine by vegetative cell suspensions of C. botulinum-62 A. The reaction mixture in Warburg cups contained 1 ml of 0.2 M phosphate buffer pH 7.0, 33 μmoles substrate, 25 mg (dry wt.) cells and water to make 3 ml; 0.2 ml of 2 M $H_2SO_4$ was tipped into individual vessels at the intervals indicated to determine the total $CO_2$ produced. The reaction temperature was 37 C, and the gas phase was helium.

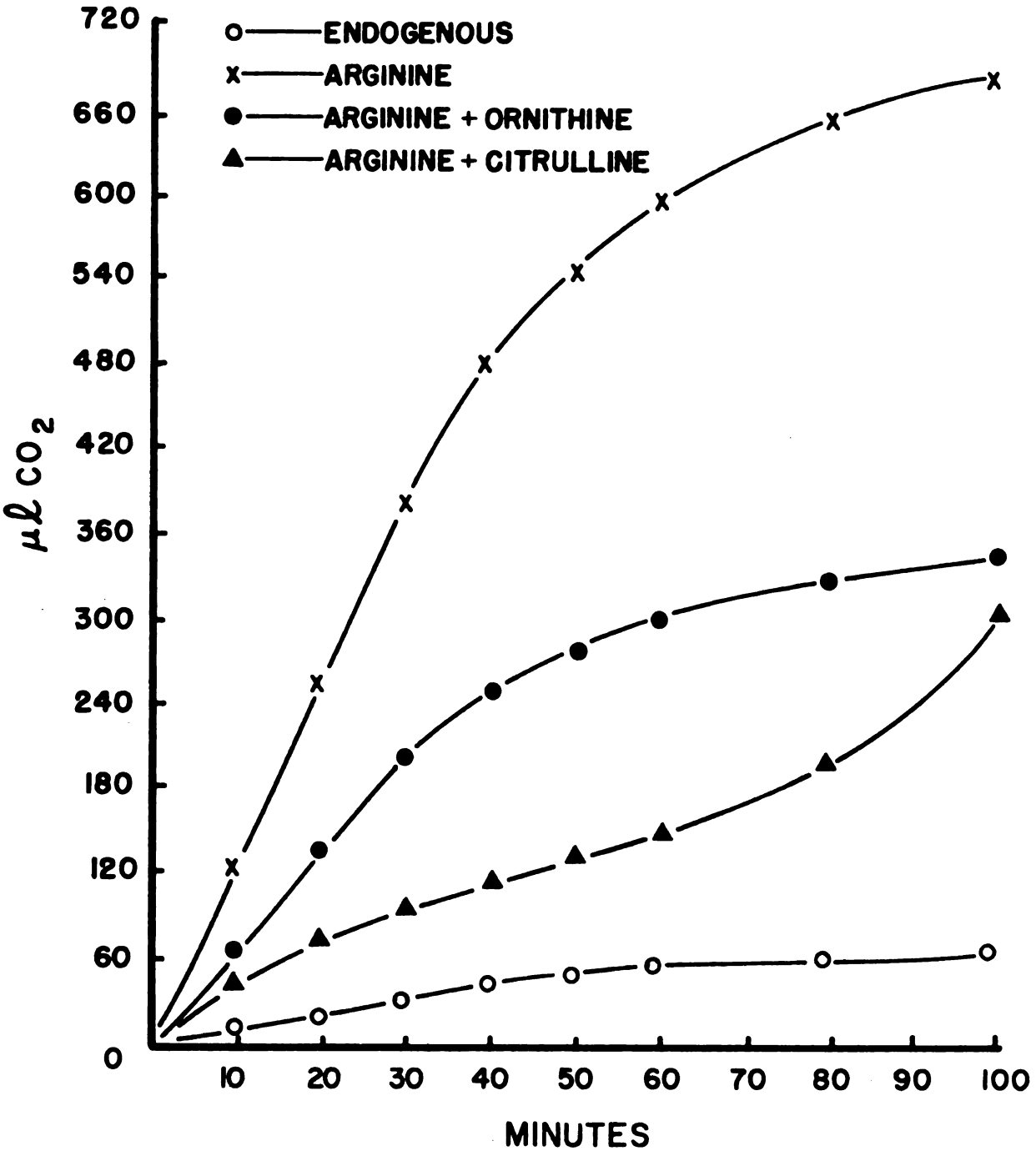
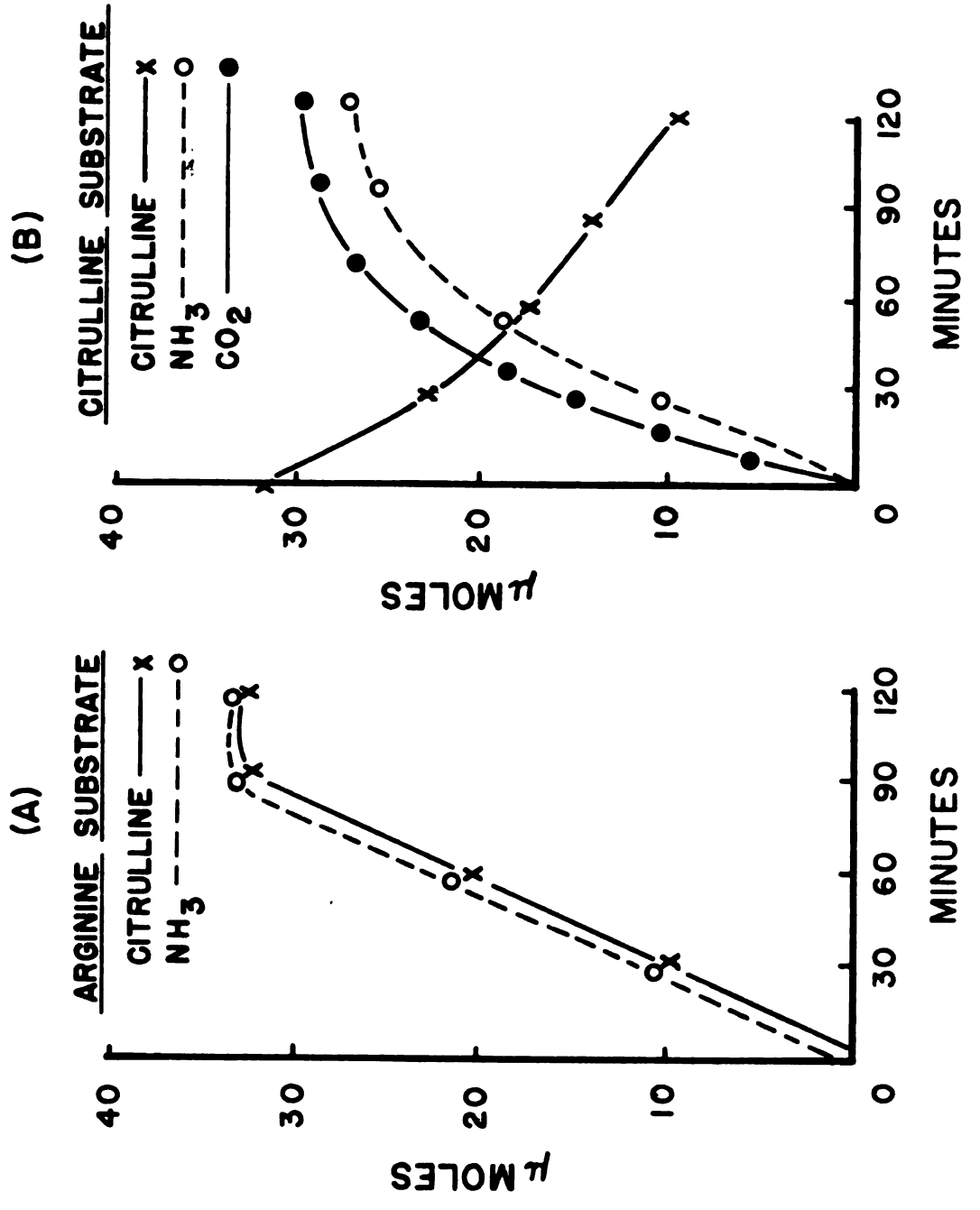



Figure 2. Effect of citrulline and ornithine on the degradation of arginine by resting cell suspension of C. botulinum. The reaction conditions were the same as described in Figure 1. Where mixed substrates were present 16.5 μmoles of each were used.

Figure 3. Arginine breakdown by *C. koseanum* via citrulline (A) the reaction mixture in 4-dram vessels contained 1 ml of 0.2 M phosphate buffer, 40 μmoles arginine and 30 mg (dry wt.) of cells. The temperature was 37 C and the gas phase was helium. Reactions were stopped with 0.2 ml of 70% perchloric acid in 4 flasks at the intervals indicated. These 4 flasks contained $2 \times 10^{-2}$ M $NaF$, which inhibited citrullinase activity but did not interfere with arginine deiminase activity. (B) Reaction mixtures were as in (A) except they contained 32 μmoles of citrulline, and no arginine or $NaF$. All the data were corrected for parallel series without substrate.

Figure 3. Arginine breakdown by C. botulinum via citrulline. (A) The reaction mixture in Warburg vessels contained 1 ml of 0.2 M phosphate buffer, 40 μmoles arginine and 30 mg (dry wt.) of cells. The temperature was 37 C and the gas phase was helium. Reactions were stopped with 0.2 ml of 70% perchloric acid in 4 flasks at the intervals indicated. These 4 flasks contained $2 \times 10^{-2}$ M NaF, which inhibited citrullinase activity but did not interfere with arginine deiminase activity. (B) Reaction mixtures were as in (A) except they contained 32 μmoles of citrulline, and no arginine or NaF. All the data were corrected for parallel series without substrate.


(A)
ARGININE SUBSTRATE
CITRULLINE ——x
NH3 - - - - o
μ MOLES
40
30
20
10
0
30
60
90
120
MINUTES
(B)
CITRULLINE SUBSTRATE
CITRULLINE ——x
NH3 - - - - o
CO2 ——●
μ MOLES
40
30
20
10
0
30
60
90
120
MINUTES

degraded. The accumulation of citrulline during arginine degradation in the absence of NaF was observed even when a small amount (10 μmoles) of arginine was used as a substrate. This indicates that neither destruction of enzyme nor accumulation of toxic end-products was responsible for the incomplete breakdown of citrulline.

The maximum gas evolution from citrulline was observed at pH 5.8 (Fig. 4). However, there was no significant difference in the rates of gas production over the pH range tested (pH 5.8-pH 7.0).

Products produced: Products of arginine, citrulline and ornithine degradations are shown in Table 1. The major products of arginine degradation were $CO_2$, $NH_3$, citrulline and ornithine; and the minor products were acetic, propionic, butyric and valeric acids. The degradation activities of the cells varied to some extent between experiments due to the differences in time of harvesting, exposure to oxygen and the amount of cells used for the degradation. Nonetheless, degradation of arginine by intact cells of C. botulinum was carried out basically following the same patterns as in Clostridium perfringens as reported by Schmidt et al. (1952). The results obtained with C. botulinum are consistent with the idea that the imido group of arginine is removed to form 1 mole of citrulline and 1 mole of ammonia, and the citrulline is partially degraded to equimolar quantities of $NH_3$, and $CO_2$ and ornithine. However, the appearance of volatile

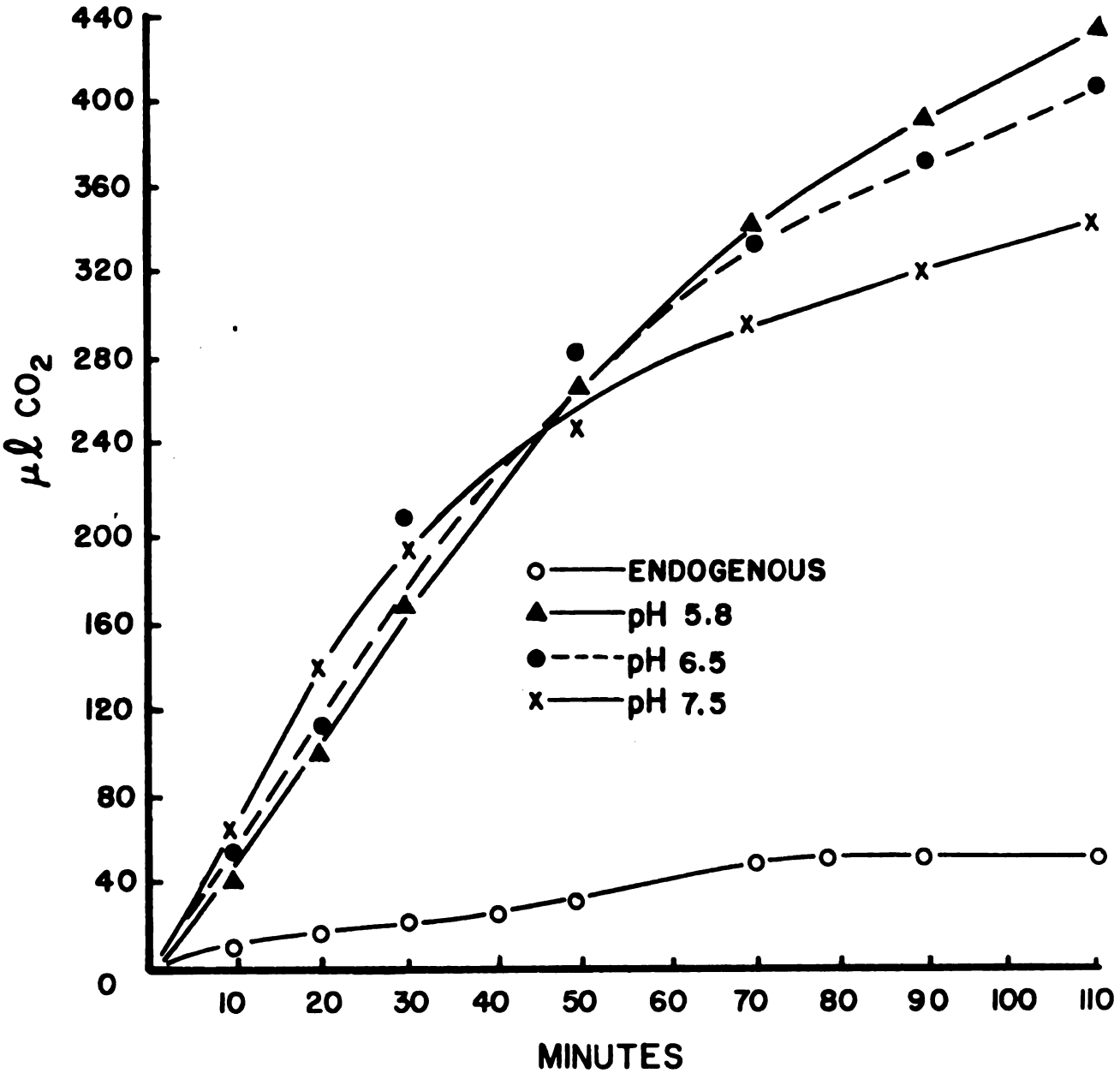



Figure 4. Effects of pH on citrulline degradation by vegetative cell suspension of C. botulinum. Reactions were run as in Figure 1 at the pH levels indicated.

Table 1. Products of arginine, citrulline and ornithine degradation by C. botulinum resting cells.

| Substrate | Arginine | Citrulline | Ornithine |
|---|---|---|---|
| Initial concentration (μmoles) | 33 | 33 | 33 |
| μmoles undegraded | 3.68 | 13.33 | 19.91 |
| μmoles degraded[1] | 29.32 | 19.67 | 13.09 |
| Products produced in μmoles[2] | | | |
| $CO_2$ | 26.83 | 21.65 | 7.16 |
| $NH_3$ | 51.20 | 19.10 | 6.00 |
| Arginine | - | 0 | 2.67 |
| Citrulline | 8.53 | - | 0 |
| Ornithine | 20.68 | 18.09 | - |
| Acetic acid | 1.64 | 2.56 | 3.24 |
| Propionic acid | 0.90 | 1.38 | 1.56 |
| Butyric acid | 0.60 | 0.54 | 0.72 |
| Valeric acid | 0.72 | 0.60 | 0.54 |
| Putrescine | 0 | 0 | 3.98 |
| δ-aminovaleric acid | 0 | 0 | 2.80 |

The Warburg flasks contained 1 ml of 0.2 M phosphate buffer pH 7.0, 25 mg cell suspension, and 33 μmoles substrate in a total volume of 3 ml. The temperature was 37 C, and the gas phase helium. The reaction was run for 2 hr and 0.2 ml of 2 M $H_2SO_4$ tipped at the end of the reaction. The flask contents were centrifuged and assayed for the products.

[1]Substrate degraded was calculated on the basis initial substrate added minus the substrate remaining in the reaction mixture at the end of reaction.

[2]All the values are corrected for endogenous activities.

acids during the degradation of all these substrates indicates that there is some fermentation of the ornithine formed.

The ratio of $NH_3$ and $CO_2$ was found to be close to 2:1 from arginine degradation and 1:1 from citrulline and ornithine degradation. Table 2 shows the comparison of $NH_3$, $CO_2$ and citrulline production from arginine when degraded for prolonged times. It is apparent from this Table that $CO_2$ production (e.g. in 240 min, 92 μmoles $CO_2$) was higher than the theoretical value (60 μmoles) for the degradation of arginine by the arginine dihydralase system alone, since there were 39 μmoles of citrulline accumulated. The theoretical value for $NH_3$ for conversion of arginine to citrulline is 1:1 and of citrulline to ornithine is 1:1. Thus, the $NH_3$ measured (167 μmoles) is quite close to the theoretical of 161. Thus, these data indicate that the degradation of ornithine involves little deamination. This excess of $CO_2$ may have come from ornithine produced in the reaction mixture of arginine degradation.

In order to make sure if essentially all of the products of arginine, citrulline and ornithine degradations were accounted for in the reaction mixtures, carbon balances and nitrogen balances were determined. Table 3 shows the carbon balance of arginine degradation. It is seen from this Table that 109 percent of the carbon was recovered. This high percent recovery of carbon may easily be accounted for by the errors in determining the many products. Arginine is a reduced substrate with an oxidation value for 100 μmoles of

Table 2. Comparison of the amounts of $NH_3$, $CO_2$ and citrulline formed from 100 μmoles of arginine by C. botulinum cells.

| Experiment No. | Time | Total μmoles/100 μmoles arginine | | | $CO_2$/Arginine | $NH_3$/Arginine | $NH_3/CO_2$ |
|---|---|---|---|---|---|---|---|
| | | $CO_2$ | $NH_3$ | Citrulline | | | |
| 1 | 240 min | 92.07 | 166.77 | 38.97 | 0.92 | 1.67 | 1.81 |
| 2 | 180 min | 83.34 | 162.54 | 34.92 | 0.83 | 1.62 | 1.95 |
| 3 | 120 min | 80.49 | 153.60 | 31.59 | 0.80 | 1.54 | 1.91 |

The Warburg flasks contained 1.0 ml of 0.2 M phosphate buffer pH 7.0, 25 mg dry wt of cells, and 33 μmoles arginine in a total volume of 3.0 ml. The temperature was 37 C, and the gas phase helium. 0.2 ml of 2 M $H_2SO_4$ was added at the end of the reaction interval, flask contents centrifuged, and the supernatant fluid assayed for citrulline and $NH_3$. All the data are averages of duplicate flasks.

Table 3. Carbon balance of arginine degradation by C. botulinum.

| Material | µg | Percent of arginine degraded | µmoles per 100 µmoles | µmoles carbon | Oxid. value | Oxid. product | Reduced product |
|---|---|---|---|---|---|---|---|
| Arginine added | 5775.00 | | | | | | |
| Arginine undegraded | 644.00 | | | | | | |
| Arginine degraded | 5131.00 | 88.85 | 100 | 600 | -5 | | |
| $CO_2$ | 1180.52 | 23.01 | 91.22 | 91.22 | +2 | 182.44 | - |
| Citrulline | 1484.22 | 28.93 | 29.00 | 174.00 | -3.5 | - | 101.50 |
| Ornithine | 2729.76 | 53.20 | 70.31 | 351.55 | -4 | - | 281.24 |
| Acetic acid | 98.40 | 1.92 | 5.58 | 11.16 | 0 | - | 0 |
| Propionic acid | 66.60 | 1.30 | 3.06 | 9.18 | -1 | - | 3.06 |
| Butyric acid | 52.80 | 1.03 | 2.04 | 8.16 | -2 | - | 4.08 |
| Valeric acid | 73.44 | 1.43 | 2.45 | 12.25 | -3 | - | 7.35 |
| $NH_3$ | 870.40 | 16.96 | 174.08 | 0 | -1.5 | - | 261.08 |

Total C recovered = 657.52 Oxidation value of 100 µmoles of arginine = -500.00

Percent C recovered = 109.59 Net oxidation value of products = -475.87

The experimental conditions were the same as described in Table 1.

-500, therefore, a number of reduced products were expected. The results obtained were within the range of experimental error as a net oxidation value for the products was found to be -476. The higher oxidation value from products could be due to the production of traces of $H_2$ since Costilow (1962) has shown that the germinating spores of C. bolutlinum have the capacity to produce $H_2$ with some substrates. However, no $H_2$ production was observed in these studies with resting cell suspensions.

A nitrogen balance (Table 4) demonstrates that the citrulline, ornithine and $NH_3$ determinations were reasonably accurate since they accounted for all of the nitrogen from arginine.

The carbon and nitrogen balances of citrulline degradation are shown in Table 5 and Table 6 respectively. The results obtained with citrulline were in close agreement with the theoretical values. However, the total oxidation value of the products was higher (-315) than that expected (-350). This may be due, either small experimental errors in the determinations of various products or the presence of some reduced product not determined.

The nitrogen balance for citrulline degradation shows that 96 percent of the nitrogen was recovered in the products (Table 6). The percent error was not significant.

The carbon balance of ornithine degradation is shown in Table 7. The carbon recovery and net oxidation value calculated for them are within experimental error since

Table 4. Nitrogen balance of arginine degradation by C. bolulinum.

| Material | µg nitrogen | Percent of arginine degraded | µmoles nitrogen |
|---|---|---|---|
| Arginine added | 1848.00 | | |
| Arginine undegraded | 206.08 | | |
| Arginine degraded | 1641.92 | 88.85 | 117.28 |
| $NH_3$ | 716.80 | 43.66 | 51.20 |
| Citrulline | 358.26 | 21.82 | 25.59 |
| Ornithine | 573.44 | 34.92 | 40.96 |
| Nitrogen incorporated into cell | 21.56 | 1.31 | 1.54 |
| TOTAL N | 1670.06 | - | 119.29 |

Percent nitrogen recovered = $\frac{119.29}{117.28}$ = 101.71

The experimental conditions were the same as described in Table 1.

Table 5. Carbon balance of citrulline degradation by C. botulinum.

| Material | µg | Percent of citrulline degraded | µmoles per 100 µmoles | µmoles carbon | Oxid. value | Oxid. product | Reduced product |
|---|---|---|---|---|---|---|---|
| Citrulline added | 5742.00 | | | | | | |
| Citrulline undegraded | 2319.42 | | | | | | |
| Citrulline degraded | 3422.58 | 59.61 | 100 | 600 | -3.5 | | |
| $CO_2$ | 952.60 | 27.83 | 109.98 | 109.98 | +2 | 219.96 | - |
| Ornithine | 2387.88 | 69.77 | 91.90 | 459.50 | -4 | - | 367.60 |
| Acetic acid | 153.60 | 4.49 | 13.00 | 26.00 | 0 | - | 0 |
| Propionic acid | 102.12 | 2.98 | 7.00 | 21.00 | -1 | - | 7.00 |
| Butyric acid | 47.52 | 1.39 | 2.74 | 10.96 | -2 | - | 5.48 |
| Valeric acid | 61.20 | 1.79 | 3.04 | 15.20 | -3 | - | 9.12 |
| $NH_3$ | 324.70 | 9.49 | 97.02 | 0 | -1.5 | - | 145.52 |

Total C recovered = 642.64 Oxidation value of 100 µmoles of citrulline = -350.00

Percent C recovered = 107.11 Net oxidation value of products = -314.76

The experimental conditions were the same as described in Table 1.

Table 6. Nitrogen balance of citrulline degradation by *C. botulinum*

| Material | µg nitrogen | Percent of citrulline degraded | µmoles nitrogen |
|---|---|---|---|
| Citrulline added | 1386.00 | | |
| Citrulline undegraded | 559.86 | | |
| Citrulline degraded | 826.14 | 59.61 | 59.01 |
| $NH_3$ | 267.40 | 32.37 | 19.10 |
| Ornithine | 506.52 | 61.31 | 36.18 |
| Nitrogen incorporated into cells | 20.16 | 2.44 | 1.44 |
| TOTAL N | 794.08 | - | 56.72 |

Percent nitrogen recovered = $\frac{56.72}{59.01}$ = 96.12

The experimental conditions were the same as described in Table 1.

Table 7. Carbon balance of ornithine degradation by C. botulinum.

| Material | µg | Percent of ornithine degraded | µmoles per 100 µmoles | µmoles carbon | Oxid. value | Oxid. product | Reduced product |
|---|---|---|---|---|---|---|---|
| Ornithine added | 4356.00 | | | | | | |
| Ornithine undegraded | 2628.12 | | | | | | |
| Ornithine degraded | 1727.88 | 39.67 | 100 | 500 | -4 | | |
| $CO_2$ | 315.04 | 18.23 | 54.70 | 54.70 | +2 | 109.40 | |
| Arginine | 724.25 | 4.19 | 20.40 | 122.40 | -5 | - | 102.00 |
| Acetic acid | 194.40 | 11.25 | 24.75 | 49.50 | 0 | - | - |
| Propionic acid | 115.44 | 6.68 | 11.92 | 35.76 | -1 | - | 11.92 |
| Butyric acid | 63.36 | 3.67 | 5.50 | 22.00 | -2 | - | 11.00 |
| Valeric acid | 55.08 | 3.19 | 4.12 | 20.60 | -3 | - | 12.36 |
| Putrescine | 350.24 | 20.27 | 30.41 | 121.64 | -6 | - | 182.46 |
| δ-aminovaleric acid | 327.60 | 18.96 | 21.39 | 106.95 | -3.5 | - | 74.86 |
| $NH_3$ | 102.0 | 5.90 | 45.84 | 0 | -1.5 | - | 68.76 |

Total C recovered = 533.55

Percent C recovered = 106.71

Oxidation value of 100 µmoles of ornithine = -400.00

Net oxidation value of products = -353.96

The experimental conditions were the same as described in Table 1.

there were small amounts of many compounds present. The nitrogen balance of ornithine products is shown in Table 8 and is in close agreement with the carbon balance for this fermentation.

With the principal products from arginine, citrulline, and ornithine established, efforts were then made to determine the metabolic routes of the catabolism of these amino acids. The approaches involved, the use of $C^{14}$ labelled substrates, the direct assay of individual enzymes, and the use of inhibitors. The results of distribution of $C^{14}$ in the degradation products of labelled arginine, citrulline, and ornithine are shown in Table 9. The $CO_2$ derived from the degradation of arginine-guanid.-$C^{14}$ had a specific activity (Total CPM in $CO_2 / 2.22 \times 10^6$ x µmoles $CO_2$ produced) of $0.74 \times 10^{-3}$ µc/µmoles, which was one-half of the specific activity of the labelled compound. This indicates that 50 percent of the $CO_2$ was derived from the guanido carbon of arginine. As an internal control, the specific activity of $CO_2$ derived from uniformly labelled arginine was identical to the specific activity of that compound. Radioactive agmatine could not be detected in experiments employing either arginine-U-$C^{14}$ or arginine-guanid.-$C^{14}$, thus excluding the decarboxylation of arginine as a source of $CO_2$. The presence of labelled putrescine provides evidence for the decarboxylation of ornithine as an additional source of $CO_2$.

Table 8. Nitrogen balance of ornithine degradation by C. botulinum.

| Material | μgrams nitrogen | Percent of ornithine degraded | μmoles nitrogen |
|---|---|---|---|
| Ornithine added | 924.00 | | |
| Ornithine undegraded | 557.48 | | |
| Ornithine degraded | 366.52 | 39.67 | 26.18 |
| $NH_3$ | 84.00 | 22.92 | 6.00 |
| Arginine | 149.52 | 40.79 | 10.68 |
| Putrescine | 111.44 | 30.40 | 7.96 |
| δ-aminovaleric acid | 39.20 | 10.70 | 2.80 |
| Nitrogen incorporated into cells | 10.08 | 2.75 | 0.72 |
| TOTAL N | 394.24 | - | 28.16 |

Percent nitrogen recovered = $\frac{28.16}{26.18}$ = 107.56

The experimental conditions were the same as described in Table 1.

Table 9. Distribution of $C^{14}$ in the degradation products of labelled arginine, citrulline and ornithine.

| Substrate | Arginine-U-$C^{14}$ | Arginine-Guanid.-$C^{14}$ | Citrulline-Ureido-$C^{14}$ | Ornithine-2-$C^{14}$ |
|---|---|---|---|---|
| Initial CPM[1] | 1450800 | 156200 | 58200 | 59900 |
| | Percent Counts in Products[2] | | | |
| Reaction Mixture: | | | | |
| $CO_2$ | 9.98 | 48.85 | 80.50 | 0.31 |
| Volatile acids | 5.28 | 2.97 | 19.75 | 26.02 |
| Arginine | - | - | 0 | 8.00 |
| Citrulline | 30.82 | 48.48 | - | 0 |
| Ornithine | 55.47 | 0 | 0 | - |
| Putrescine | 0 | 0 | 0 | 30.02 |
| δ-aminovaleric acid | 0 | 0 | 0 | 38.04 |
| Cells: | 1.97 | 1.68 | 2.47 | 2.75 |
| Lipids | 1.24 | 0.87 | 1.74 | 0.66 |

Table 9. Continued

| Substrate | Arginine-U-$C^{14}$ | Arginine-Guanid.-$C^{14}$ | Citrulline-Ureido-$C^{14}$ | Ornithine-2-$C^{14}$ |
|---|---|---|---|---|
| Initial CPM[1] | 1450800 | 156200 | 58200 | 59900 |
| | Percent Counts in Products[2] | | | |
| Proteins | 0.05 | 0.23 | 0.20 | 0.28 |
| Nucleic acid | 0.68 | 0.58 | 0.53 | 1.81 |
| Total Final CPM | 1292100 | 137500 | 60200 | 60500 |
| Percent Recovered | 99.05 | 98.00 | 103.56 | 100.93 |

[1] Counts per minute.

[2] (CPM Products/CPM Substrate degraded) x 100.

The experimental conditions were the same as described in Table 1, except that 0.1 μc of the radioactive substrate was mixed with cold substrate (0.066 M) and 0.5 ml of this was used as the substrate in a Warburg cup. The radioactive counts were measured by a method as described in Experimental Methods.

Of particular interest is the fact that about 26 percent of the isotope in labelled ornithine was found in volatile acids indicating that a true fermentation of this substrate may be occurring. Table 9 also shows that a small percent of carbon from arginine, citrulline and ornithine was incorporated into cell material.

The results obtained with isotopic studies were found to be in general agreement with those of carbon balances. However, some variations in results were also observed. For example, in ornithine degradation, the carbon balance shows that the putrescine produced was 20 percent of ornithine degraded whereas from isotope studies 15 percent of total final counts (30 percent of that degraded) were found in the putrescine. These variations in results are understandable in light of the view that each time these degradation experiments are run there are variable conditions, which causes the variations in the results, such as time of harvest of cells, time of analysis of reaction products, and length of exposure to air.

Activities of cell free extracts: Cell free extracts prepared from cells of C. botulinum were dialysed against distilled water for 24 hr at 4 C. Arginine degradation by cell free extracts required no cofactor. However, additions of ATP and $Mg^{++}$ were shown to increase the initial rate of production of $CO_2$ (Fig. 5). This is probably due to an increase in citrullinase activity, since citrulline to

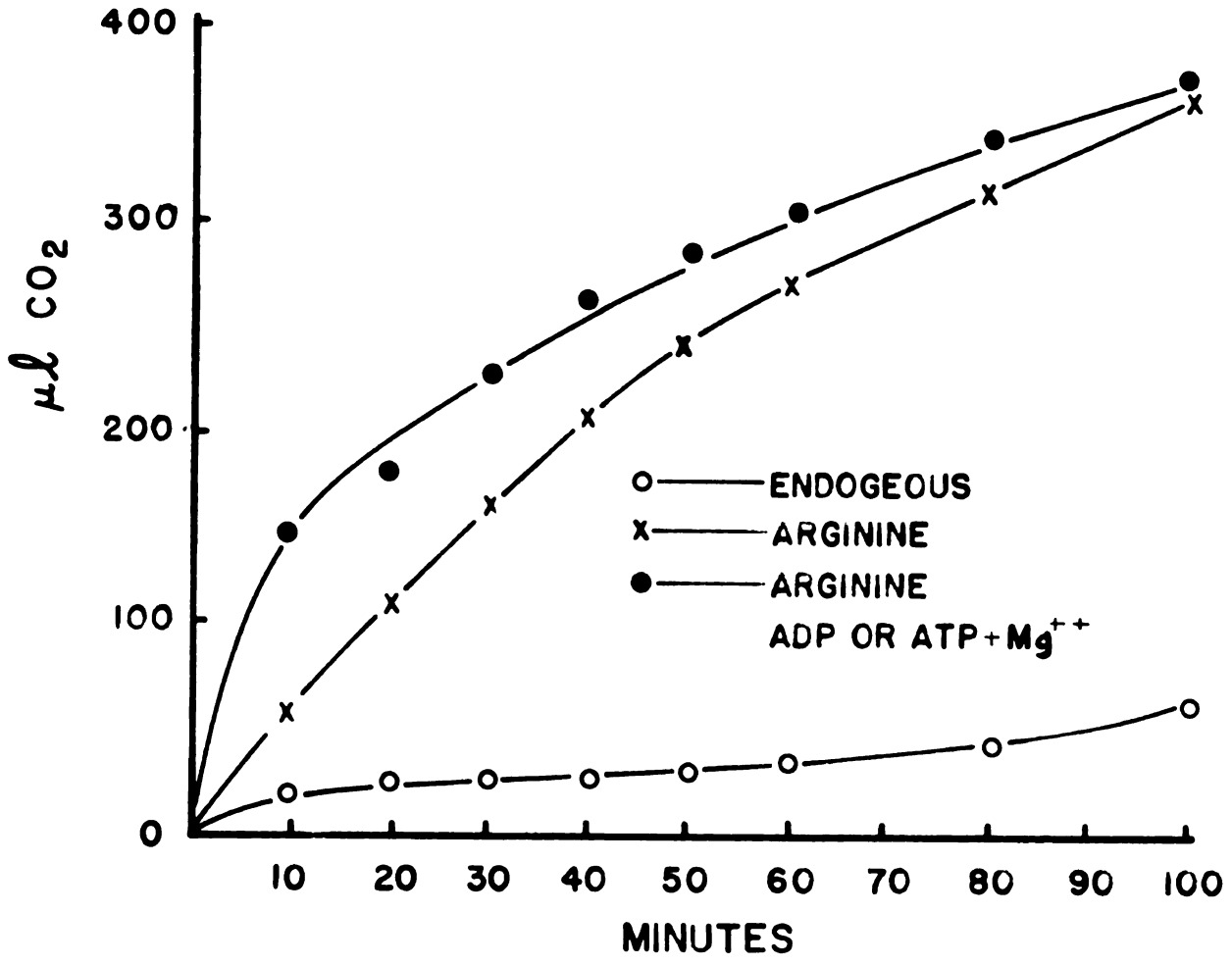



Figure 5. Effect of ADP or ATP and $Mg^{++}$ on the breakdown of arginine by cell extracts of C. botulinum. Reaction vessels contained 1 ml of 0.2 M phosphate buffer pH 7.0; 1 ml dialysed extract (22.2 mg protein), and, where indicated 0.1 ml of 0.1 M ADP or ATP and 0.1 ml of 0.01 M $MgCl_2$. A side arm contained 0.2 ml of 2 M $H_2SO_4$ which was tipped at the time intervals indicated. Total volume was brought to 3 ml with water. The reaction was run for 100 min at 37 C with helium as the gas phase.

ornithine degradation produces $CO_2$ in the second step of arginine degradation. Furthermore, cofactors such as ADP, $Mg^{++}$ and phosphate were found to be essential for the citrullinase activity as shown in Fig. 6. This figure also shows, that, the citrullinase activity of C. botulinum was further increased by replacing ADP, $Mg^{++}$ and phosphate with arsenate. Arsenolysis of many enzymes (e.g. acyl enzyme and phosphorylase) is known and the arsenate-enzyme-substrate complex is much more unstable in water than the carbonyl phosphate group and thus rapidly undergoes hydrolysis to ornithine, $CO_2$ and arsenate. This rapid turnover mechanism may explain the greater activity of citullinase with arsenate than with phosphate, $Mg^{++}$ and ADP together.

The degradation of ornithine can be significantly increased by the addition of coenzyme A (CoA), lipoic acid, ADP and $Mg^{++}$, as shown in Fig. 7. The requirements for CoA and lipoic acid might be expected from the products of ornithine degradation since the production of volatile acids from amino acids usually involves CoA activated intermediates and the production of ATP.

Extracts from spores and germinated spores of C. botulinum were prepared and treated in the same manner as the vegetative cell extracts in order to gain more knowledge on arginine, citrulline and ornithine degradation activities in these phases of the life cycle of C. botulinum. The activities of spore and germinated spore extracts as measured by $CO_2$ and $NH_3$ production were compared with the vegetative

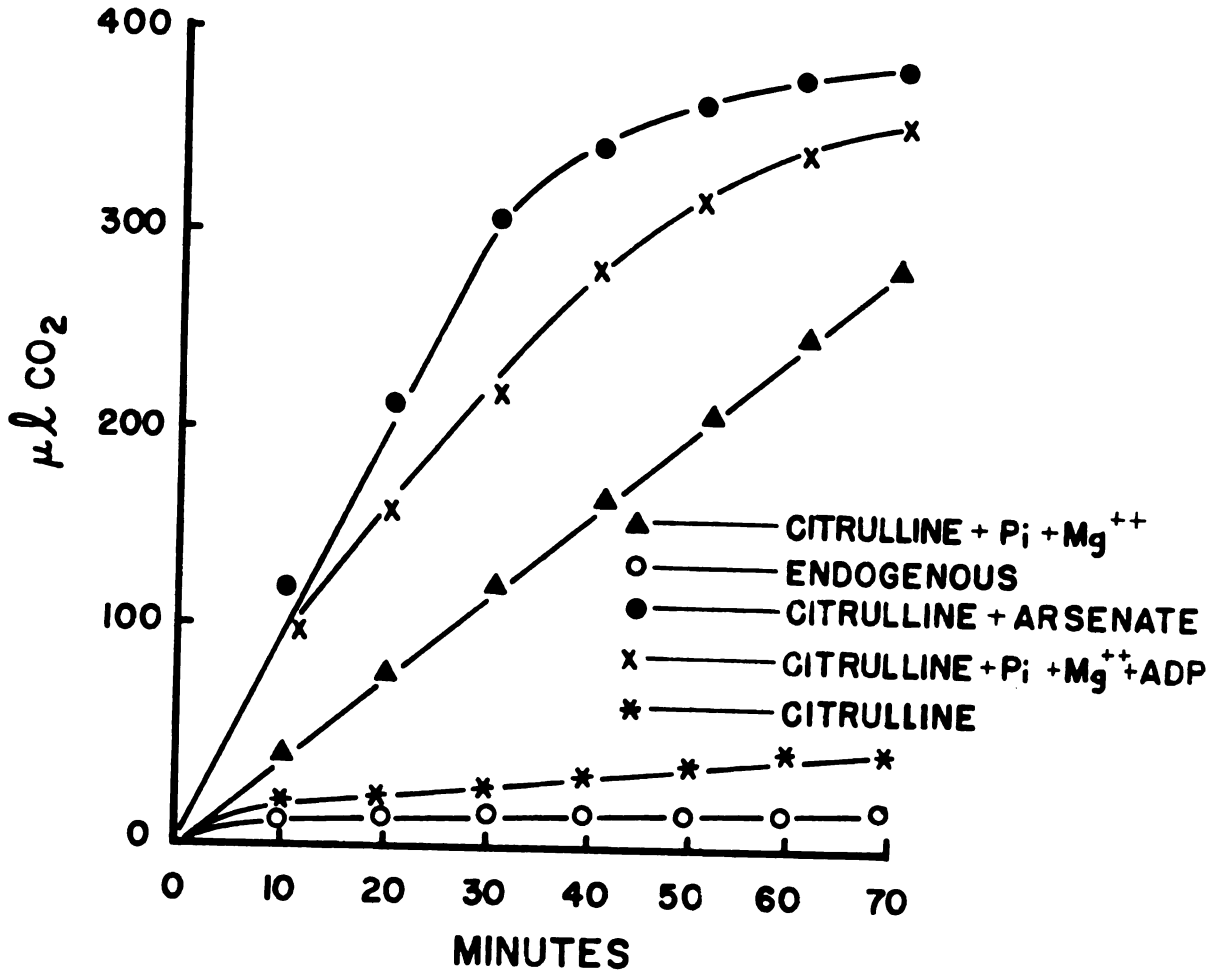



Figure 6. Effect of arsenate, ADP, $Mg^{++}$, and inorganic phosphate (Pi), on citrullinase activity of extracts of vegetative cells of C. botulinum. The reaction mixture in a Warburg vessel contained, 0.2 ml of 0.1 M citrulline; 1 ml of 0.5 M acetate buffer (pH 5.8); 0.1 ml of 0.1 M $MgCl_2$; 0.5 ml of 0.1 M potassium phosphate (pH 5.8); 0.1 ml of 0.01 M ADP (pH 5.8); and 0.5 ml of enzyme preparation (vegetative cell, 11.1 mg protein; spore, 9.5 mg protein; germinated spore, 7.5 mg protein). Where indicated, 0.5 ml of 0.1 M arsenate (pH 5.8) was used instead of ADP, $MgCl_2$, and Pi. Reactions were stopped with 0.2 ml of 2 M $H_2SO_4$ at the intervals indicated. The reaction was run for 70 min at 37 C with $N_2$ as the gas phase.

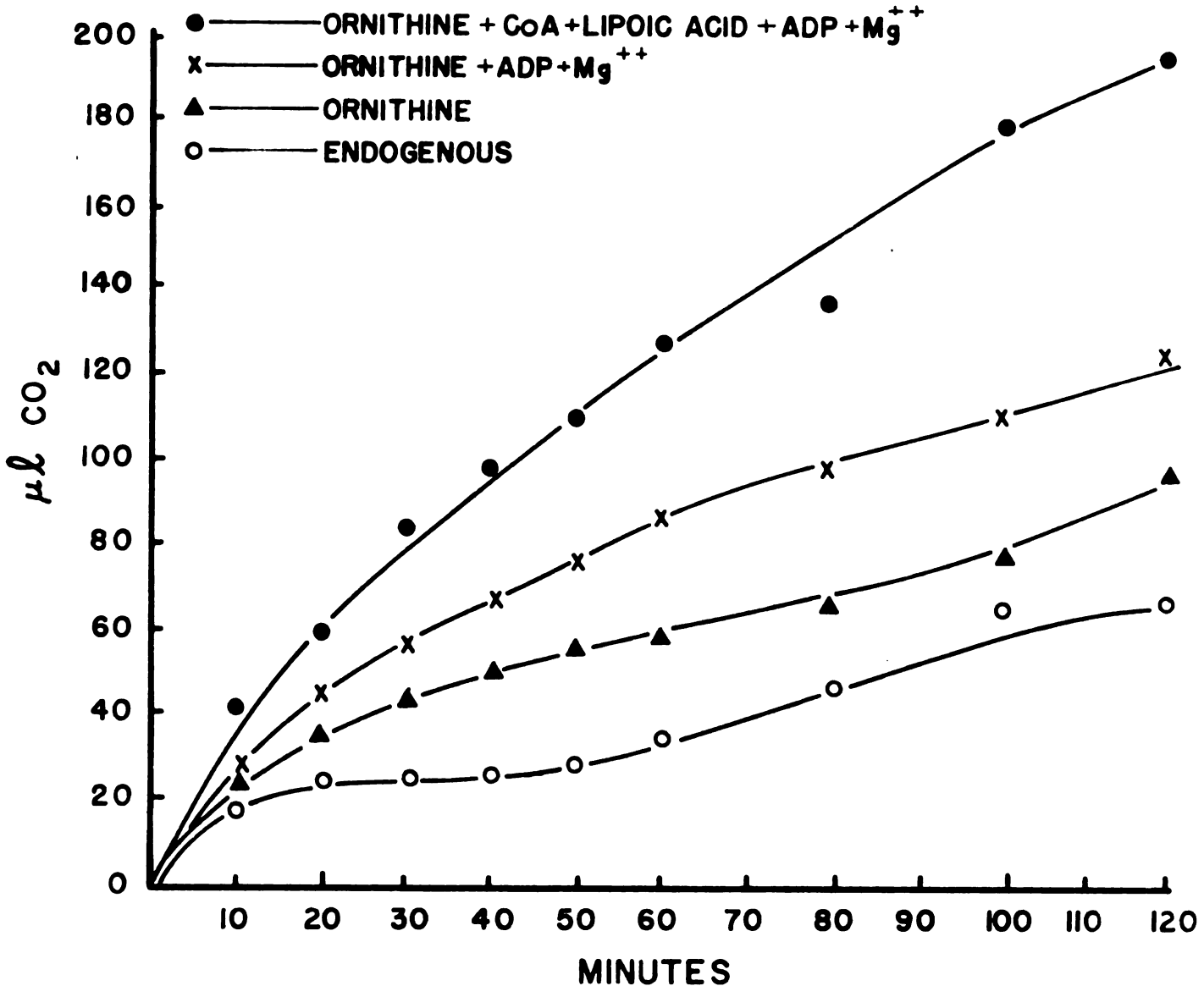



Figure 7. Effect of CoA, ADP, lipoic acid, and $Mg^{++}$ on ornithine degradation by cell extracts of C. botulinum. Each Warburg vessel contained 33 μmoles ornithine, dialysed cell extract (22.2 mg protein), 1 ml of 0.2 M phosphate buffer (pH 7.0), and 0.2 M $H_2SO_4$ in the side arm. Total volume was brought to 3 ml with water. Where indicated, 0.1 ml of $1.6 \times 10^{-4}$ M CoA, 0.1 ml of $6 \times 10^{-4}$ M lipoic acid, 0.1 ml of 0.1 M ADP and 0.1 ml of 0.1 M $MgCl_2$ were added.

cell extract activities, (Table 10). The activities in spore and germinated spore extracts were much lower than that in cell extracts: However, $NH_3/CO_2$ ratios were found to be essentially the same. The low activity of spores and germinated spores was expected because of the fact that bacterial spores in general show a very low metabolic activity.

As mentioned earlier, one of the approaches used for the determination of metabolic routes of amino acid degradation, was the direct assay of individual enzymes. Some of the enzymes of arginine, citrulline and ornithine degradation were studied and their relative activities are summarized in Table 11. There was no arginase activity found in vegetative cells, spores and germinated spores of C. botulinum. Thus, the addition of water to form ornithine and urea (Greenberg, 1955) does not apparently occur in C. botulinum.

Arginine deiminase is known to catalyze the breakdown of arginine to citrulline and $NH_3$. (Schmidt et al., 1952; Oginsky and Gehrig, 1952a,b). It was found to be present in extracts of all three life stages studied, although with much lower activities in extracts of spores and germinated spores than in those of cells. The enzyme had no cofactor requirement, and produced citrulline and $NH_3$ from arginine (Fig. 8). It is shown here, that, under the experimental conditions used, the ratio of $NH_3$ to citrulline was essentially 1:1, with the cells, spores and germinated spores. It is also shown in Fig. 8, that, the rate of production of citrulline and $NH_3$ was much higher with vegetative cell

Table 10. $CO_2$ and $NH_3$ production from arginine, citrulline and ornithine by C. botulinum.

| Substrate | Vegetative Cell Extract | | | Spore Extract | | | Germinated Spore Extract | | |
|---|---|---|---|---|---|---|---|---|---|
| | μmoles | | $NH_3/CO_2$ | μmoles | | $NH_3/CO_2$ | μmoles | | $NH_3/CO_2$ |
| | $CO_2$ | $NH_3$ | | $CO_2$ | $NH_3$ | | $CO_2$ | $NH_3$ | |
| L-Arginine 0.033 M | 26.83 | 51.20 | 1.91 | 3.68 | 7.20 | 1.96 | 4.62 | 11.73 | 2.52 |
| L-Citrulline 0.033 M | 21.65 | 19.10 | 0.88 | 13.62 | 12.80 | 0.94 | 14.51 | 14.82 | 1.02 |
| L-Ornithine 0.033 M | 7.16 | 6.00 | 0.84 | 0.92 | 0.71 | 0.77 | 1.10 | 1.32 | 1.31 |

The Warburg flasks contained 1.0 ml of 0.2 M phosphate buffer pH 7.0 with arginine or ornithine substrate and 0.2 M phosphate buffer (pH 5.8) with citrulline as a substrate. Extracts used were: vegetative cell, 22.2 mg protein; spore extract, 19.0 mg protein; germinated spore extract 15.0 mg protein. The total volume in each cup was made to 3.0 ml with $H_2O$. 0.2 ml of 2 M $H_2SO_4$ was tipped in at the end of the experiment. Reactions were run for 2 hr at 37 C with helium as the gas phase. At the end of the reaction, flask contents was centrifuged and the supernatant fluids assayed for $NH_3$.

Table 11. Comparative activities of enzymes in extracts of vegetative cells, spores and germinated spores of C. botulinum.

| Enzyme Systems | Specific activity in Extracts[1] | | |
|---|---|---|---|
| | Vegetative cells | Spores | Germinated spores |
| Arginase[2] | 0 | 0 | 0 |
| Arginine deiminase[3] | 3.09 | 0.29 | 1.10 |
| Citrullinase[4] | 1.38 | 0.78 | 0.61 |
| Ornithine trans-carbamylase[5] | 37.07 | 22.74 | 16.38 |
| Carbamylphosphokinase[6] | 1.17 | 6.79 | 8.67 |
| Transamidinase[7] | 1.00 | 0 | 0 |

[1]Specific activity is unit enzyme per mg protein.

[2]A unit of arginase activity is that amount which will liberate 1 μmole urea in 1 min at 25C and pH 9.5 and with a substrate concentration of 0.285 M arginine.

[3]A unit of arginine deiminase is that amount of enzyme which catalyzes the production of 1 μmole product per hr under the assay conditions.

[4]A unit of citrullinase is that amount which catalyzes the production of 1 μmole $CO_2$ per hr.

[5]A unit of ornithine transcarbamylase is defined as that amount of enzyme required to form 1 μmole citrulline in 15 min.

[6]A unit of carbamyl phosphokinase is that amount of enzyme required to form 1 μmole ATP in 10 min.

[7]A unit of transamidinase enzyme catalyzes the formation of 1 μmole of product per hr at 38 C.

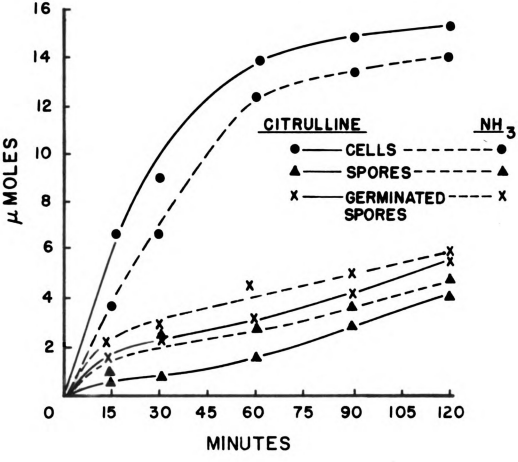



Figure 8. Arginine deiminase activity in dialysed extracts (24 hours) of C. botulinum vegetative cells, spores, and germinated spores. Assay mixture in a test tube contained 0.4 ml of 0.1 M L-arginine hydrochloride pH 6.5, 1 ml of 0.2 M phosphate buffer pH 6.5, and 0.2 ml extract, (4.4 mg protein from vegetative cells; 3.8 mg protein from spores; 3.6 mg protein from germinated spores), water to 3 ml, and incubated at 37 C. The reaction was stopped in replicate tubes at 15 min interval with 0.2 ml of 70 per cent perchloric acid and after centrifugation, aliquots of supernatant fluid were assayed for citrulline and ammonia.

extracts than with extracts of spores or germinated spores indicating the relative concentrations of the enzyme present in the preparations. The activity of arginine deiminase was inhibited by excess ornithine in the assay mixture. The reason for this inhibition is not known.

The activity of citrullinase was shown to be lower in spores and germinated spores than in vegetative cells as measured by $CO_2$ production (Table 11, Fig. 9). Citrullinase was found to require ADP, phosphate and $Mg^{++}$ as cofactors. The exact nature of the citrullinase enzyme is not known as yet but it has been noted by many workers in this field that it is a complex enzyme.

Ornithine transcarbanylase (OTC) has been reported to catalyze the following reaction in S. faecalis and S. lactis:

Ornithine + Carbamyl phosphate $\rightleftharpoons$ Citrulline + $HPO_4^{=}$ + $H^+$

(Jones, 1962). It was present in extracts of cells, spores and germinated spores of C. botulinum, but at much lower levels in extracts of the latter two forms. The enzyme was found to be specific for ornithine and was strongly inhibited by NaF (Table 12). It is seen from this table, that, a 99 percent inhibition of OTC activity of vegetative cell was obtained with $10^{-2}$ M NaF. The percent inhibition was less for spores and germinated spores than for vegetative cells. Orthophosphate was found to competitively inhibit OTC in the synthesis of citrulline from ornithine and carbamyl phosphate. These results suggest that OTC found in C. botulinum may be

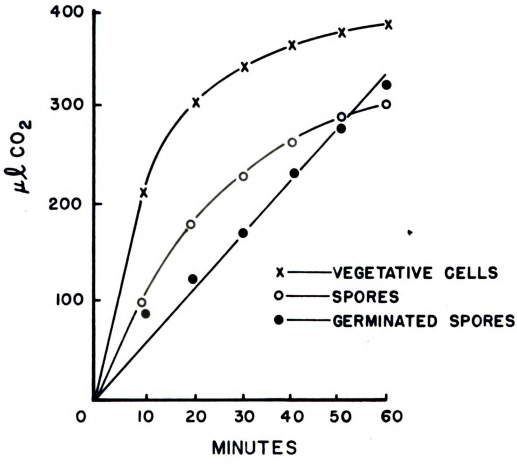



Figure 9. Citrullinase activity in C. botulinum extracts prepared from vegetative cells, spores, and germinated spores. The reaction conditions were the same as described in Figure 6.

Table 12. Effect of NaF on ornithine transcarbamylase activity of vegetative cells, spores and germinated spores of C. botulinum.

| Addition | Vegetative Cell | | Spores | | Germinated Spores | |
|---|---|---|---|---|---|---|
| | Activity[1] | Percent Inhibition | Activity | Percent Inhibition | Activity | Percent Inhibition |
| Control | 37.07 | - | 22.74 | - | 16.38 | - |
| NaF 2 x $10^{-3}$ M | 19.65 | 47 | 14.33 | 37 | 10.5 | 42 |
| NaF 1 x $10^{-2}$ M | 0.37 | 99 | 7.96 | 65 | 3.93 | 76 |

[1] µmoles citrulline per hr per mg protein.

The reaction mixture contained 1 M tris buffer pH 8.5, 0.1 M dilithium carbamyl-P, 0.1 M L-ornithine hydrochloride per ml. The protein concentrations of extracts were: vegetative cell, 2.2 mg; spore extract, 1.90 mg; germinated spores, 0.55 mg. The reactions were run at 37 C and were stopped by 5 percent TCA. A 0.5 ml aliquot of the reaction mixture analyzed for citrulline.

useful for the synthesis of arginine via citrulline from ornithine and carbamyl phosphate. Ornithine metabolism in these studies has shown that arginine was synthesized by C. botulinum under suitable conditions. However, citrulline was not found in the reaction mixture of ornithine metabolism.

The enzyme carbanyl phosphokinase (CP) is known to catalyze the following reaction:

$$NH_2\text{-}COO^- + ATP \rightleftharpoons NH_2\text{-}\overset{\overset{O}{\|}}{C}\text{-}O\text{-}P_3^{=} + ADP$$

as reported by Jones (1962). The enzyme was found to be specific for ammonium carbamate. In C. botulinum the carbamyl phosphate was formed from ATP and ammonium bicarbonate in citrulline biosynthesis in the presence of ornithine and OTC.

The relative activity of CP was found to be greater in spores and germinated spores than in vegetative cell preparations. This may be due to the fact that the CP activity was measured by the loss of alkali-labile phosphate in the presence of ADP and $Mg^{++}$. In spores and germinated spores the loss of alkali-labile phosphate could also be accounted for by the activity of other enzymes, e.g. pyrophosphatase, found in relatively large amounts in spores and germinated spores of C. botulinum. This was supported by the fact that considerable amount of CP activity was found in spores and germinated spores even when ADP and $Mg^{++}$ were excluded in measuring the loss of alkali-labile phosphate.

The activity of transamidinase was found only in vegetative cells of C. botulinum. The enzyme catalyzes the following reaction:

Arginine + Glycine $\rightleftharpoons$ Guanidinoacetate + Ornithine

(Ratner, 1962). The enzyme is known to handle a number of amidine donors and acceptors. The presence of the enzyme in C. botulinum vegetative cell preparations indicated that in the presence of a suitable amino acid, the guanidino group of arginine can be transferred directly and vice versa. This also indicated that if a compound containing a guanidino group was present in the ornithine reaction mixture, ornithine could act as the acceptor of the guanidino group to synthesize arginine. Arginine found as a product of ornithine degradation may have come partially from such a reaction.

## DISCUSSION

The lines of evidence presented in these studies show that arginine is catabolized by C. botulinum primarily to citrulline, ornithine, $CO_2$ and $NH_3$ by the following reactions:

$$\text{Arginine} \longrightarrow \text{Citrulline} + NH_3 \quad (1)$$

$$\text{Citrulline} \longrightarrow \text{Ornithine} + NH_3 + CO_2 \quad (2)$$

The first reaction (1) was shown to be catalyzed by arginine deiminase, and the second reaction (2) by citrullinase. This pathway of arginine degradation found in C. botulinum is known to occur in many organisms. It was demonstrated by Schmidt et al. (1952) in C. perfringens; Korzenovsky and Werkman (1953) in S. lactis; and Slade (1953) in S. faecalis. However, none of these workers reported further degradation of the ornithine produced as noted in these studies with C. botulinum.

The presence of citrulline or ornithine significantly inhibited gas production from arginine. These results were in close agreement with those reported by Hartman and Zimmerman (1960), who found that ornithine lowered the rate of arginine degradation by Streptococcus faecalis var. liquefaciens by retarding the arginine dihydrolase system. In C. botulinum an excess of ornithine inhibition may be due

to the inhibition of the first step of citrullinase reaction:

$$\text{L-Citrulline} + HPO_4^{=} \rightleftharpoons \text{L-Ornithine} + NH_2\ COO\ PO_3^{=}.$$

The equilibrium in this reaction is in favor of citrulline production. Therefore the decomposition of citrulline is dependent upon the removal of carbamyl phosphate, the source of $CO_2$ and $NH_3$ in citrulline degradation. If carbamyl phosphate is not removed rapidly and exogenous ornithine is added, reversion of the above reaction occurs. It is not apparent why the presence of citrulline inhibits gas production from arginine, since $CO_2$ is produced from citrulline.

The guanidino group of arginine is attacked by C. botulinum and most of the $CO_2$ and $NH_3$ come from this group. However, other carbons of arginine do contribute a small amount of $CO_2$ as was indicated from the $NH_3$: $CO_2$ ratio and by the distribution of $C^{14}$ in the degradation products of labelled arginine. Upon the degradation of arginine-guanid.-$C^{14}$ to citrulline and products, 48 percent of the radioactivity was found in citrulline. It was expected, therefore, that 52 percent of the radioactivity would be found in $CO_2$. Only 49 percent of the radioactivity was in the $CO_2$, the remainder being incorporated into volatile acids. Approximately 3 percent of the $CO_2$ was apparently participating in the synthesis of volatile acids. Of the citrulline degraded, 80 percent of ureido-$C^{14}$ was found in $CO_2$ and the remainder primarily in volatile acids. Thus, it appears that there is a fixation of $CO_2$ by these cells.

Citrulline was shown to be an intermediate in arginine degradation by C. botulinum since it accumulated in stoichometric amount when NaF was used to inhibit its degradation. When used as a substrate, it was degraded according to the following reactions:

$$\text{Citrulline} + \text{ADP} + Mg^{++} + \text{Pi} \longrightarrow \text{Ornithine} + CO_2 + NH_3 + \text{ATP} \quad (3)$$

$$\text{Citrulline} + ASO_4^{=} \longrightarrow \text{Ornithine} + CO_2 + NH_3 + ASO_4^{=} \quad (4)$$

Cleavage of the ureido group of citrulline has been demonstrated with extracts of S. faecalis (Slade, 1953 and Akumatsu and Sekine, 1951) and S. lactis (Korzenovsky and Werkman, 1953) and with a cell suspension of C. perfringens (Schmidt et al., 1952). With S. faecalis and S. lactis the reaction requires the presence of orthophosphate and a phosphate acceptor such as adenosine-5-phosphate or ADP. In C. botulinum citrulline degradation is believed to be an exergonic reaction in which ATP is generated from ADP, phosphate and $Mg^{++}$ [reaction (3)]. This was shown by the experiments in which ADP, phosphate and $Mg^{++}$ were required for the activity of citrullinase. The enzyme system catalysing reaction (3) is believed to function in two steps:

$$\text{L-Citrulline} + HPO_4^{=} \rightleftharpoons \text{L-Ornithine} + NH_2\ COOPO_3^{=} \quad (5)$$

$$NH_2COOPO_3^{=} + \text{ADP} \rightleftharpoons NH_3 + CO_2 + \text{ATP} \quad (6)$$

one being a phosphorolysis of citrulline [reaction (5)] to ornithine and carbamyl phosphate, the other being the transfer of the phosphoryl group from carbamyl phosphate to ADP to form ATP [reaction (6)]. Carbamyl phosphate has been shown to serve both as a carbamyl donor in citrulline synthesis [the reverse of the reaction (5)] and as a phosphate donor [reaction (6)] in the presence of extracts of S. faecalis (Jones, et al. 1955).

Arsenate was shown to replace phosphate in the citrulline reaction using cell free extracts of C. botulinum [reaction (4)]. Under such conditions phosphate acceptor and divalent ions were not required and the products formed were the same as in the presence of phosphate, except, that ATP was not formed. These results are in close agreement with those reported for S. faecalis (Slade, 1953) and for Pseudomonas areuginosa (Slade et al., 1954).

C. botulinum degrades ornithine by a different and unique pathway(s) to a number of products. There has been no report that ornithine as a separate substrate is degraded by microorganisms. However, the reactions are known by which an extensive degradation of ornithine can occur in a system containing a suitable reducing agent and a mixture of two clostridia (Barker, 1961).

In light of the products produced and the enzymes found in C. botulinum cell preparations, it is thought that there is more than one pathway of ornithine degradation present in C. botulinum. The conversion of ornithine into

putrescine and $CO_2$ indicate that ornithine is partially degraded by the following decarboxylation reaction:

$$\text{Ornithine} \longrightarrow CO_2 + \text{Putrescine} \qquad (7)$$

Ornithine decarboxylase has been prepared from C. septicum by Gale (1945). In C. botulinum some of the $CO_2$ produced during the degradation of arginine and citrulline undoubtedly resulted from this reaction (7). $CO_2$ has been reported to be important in the initiation of growth of C. septicum (Gale, 1945).

The other products of ornithine degradation, such as δ-aminovaleric acid, volatile acids, and $NH_3$ indicate that ornithine may partially be degraded by other pathways. It is possible that the following reaction, with some unknown intermediate compound(s) may occur in C. botulinum.

$$\text{Ornithine} \longrightarrow (x)\ ? \longrightarrow \delta\text{-Aminovalerate} + NH_3 \qquad (8)$$

Since the degradation of ornithine to δ-aminovalerate is a reductive deamination reaction, it is required to have some compound which could act as a $H^+$ donor. Organisms which carry out the Stickland reaction can reduce ornithine to δ-aminovalerate. Clostridium sporogenes, the organism studied extensively by Stickland (1935) and by Woods (1936) utilizes ornithine in this manner. However, Stadtman (1954) found that dried cells of Clostridium (strain HF), formed trace amounts of δ-aminovaleric acid from ornithine when incubated with molecular hydrogen and also oxidized ornithine with molecular oxygen. However, growth did not occur with

ornithine as the sole source of energy; either proline or lysine was required in the medium. The role of ornithine in fermentation appeared to be, at least in part, that of a hydrogen donor; since none of the reduced product, δ-amino-valeric acid, was found when ornithine plus lysine were fermented.

If the initial step from ornithine is pictured as an oxidative deamination or a transamination, one of the three possible carbonyl compounds (α-keto-δ-aminovaleric acid, the γ-semiladehyde of glutamic acid, or β-keto-δ-aminovaleric acid) would result depending upon which of the amino groups of ornithine is lost. The first two of these compounds are known as possible intermediates of glutamate, ornithine and proline interconversions by microorganisms (Vogel, 1955). The third compound (β-keto-δ-aminovalerate) could be a common intermediate (x) in the following series of reactions of ornithine degradation, written on the basis of products obtained in the reaction mixture:

$$\text{Ornithine} \longrightarrow (x) \xrightarrow{+\ 2[H]} \delta\text{-Aminovaleric acid} + NH_3 \qquad (8)$$

$$(x) \xrightarrow{+\ \text{CoA} + \text{Lipoic acid} + \text{ADP} + Mg^{++}} NH_3 + CO_2 + \text{ATP} + C_1\text{-}C_5 \text{ Volatile acids} \qquad (9)$$

The presence of compound (x) is postulated, because of the products $CO_2$, $NH_3$, δ-aminovaleric acid, acetic, propionic, butyric and valeric acids in the reaction mixture. Of course, there would undoubtedly be a number of intermediates

involved, but a common compound such as $\beta$-keto-$\delta$-amino-valeric acid might easily be converted to the variety of products produced. Thus, the volatile acids could be produced by $\beta$-oxidation [reaction (9)] involving CoA, lipoic acid, ADP, and $Mg^{++}$ as cofactors; and by reduction, dehydration and a second reduction $\delta$-aminovalerate may be produced, [reaction (8)]. However, compounds such as $\gamma$-semialdehyde of glutamic acid may be produced upon $\delta$-transamination. Semialdehyde production is well known in the metabolic interrelations of ornithine, glutamate and proline (Vogel, 1955); and proline in the presence of some hydrogen donor may be reduced to $\delta$-aminovaleric acid. However, no transaminase activity was found in the crude extracts of C. botulinum.

The postulated intermediate (x) responsible for the partial degradation or ornithine could not be detected in reaction mixtures with cell extracts. This may be due to several reasons: e.g., (1) the amount present being too small to be determined, or (2) it may be a very unstable compound which is rapidly degraded thus not allowing its determination. Any of these reasons or any of a number of others may account for the inability to determine the nature of the intermediate (x). Many procedures were tried to accumulate this intermediate; for example, by the use of inhibitors and $C^{14}$ labelled substrate, but none was suitable to identify it. It is felt that if selective mutants are used, they may prove to be helpful in elucidating the

mechanism of ornithine degradation. No attempt was made to isolate such mutants during this study.

Among the products of ornithine metabolism, arginine is one of the major products found in the reaction mixture. The synthesis of arginine from ornithine may have occurred by the following reactions:

$$CO_2 + NH_3 + ATP \longrightarrow \text{Carbamyl phosphate} \quad (10)$$

$$\text{Carbamyl phosphate} + \text{Ornithine} \longrightarrow \text{Citrulline} \quad (11)$$

$$\text{Citrulline} + NH_3 \longrightarrow \text{Arginine} \quad (12)$$

The enzymes carbamyl phosphokinase and ornithine transcarbamylase which, catalyze reactions (10) and (11) respectively were found in C. botulinum. The synthesis of arginine by this pathway is known as the "ornithine cycle" and is found commonly in the mammalian liver. Srb and Horowitz (1944) described an ornithine, citrulline and arginine sequence in a mutant strain of Neurospora. Evidence is also available that arginine is synthesized from ornithine by reactions (10) (11) and (12) in Penicillium (Bonner, 1946); lactic acid bacteria (Volcani and Snell, 1948); Escherichia coli (Abelson, Bolton and Aldous, 1952); Tetrahymena galii, (Wu and Hogg, 1952) and other organisms, some of which lack arginase. Wherever it has been investigated, citrulline invariably appears to lie in the pathway of arginine synthesis from ornithine. However, citrulline was not found as a product of ornithine catabolism. No explanation for this is evident at this time.

Arginine may also have been synthesized from ornithine, at least partially by the following reaction:

$$\text{Ornithine} + \text{Guanidinoacetate} \longrightarrow \text{Arginine} + \text{Glycine} \qquad (13)$$

This reaction is catalyzed by transamidinase and this enzyme was found to be present in extracts of C. botulinum cells. Transamidinase was found to be a non-specific enzyme in that it handles guanidino group transfer for a variety of donors and acceptors. This could mean that the presence of the enzyme transamidinase in C. botulinum vegetative cells may be one of the regulatory mechanism for arginine catabolism and for maintaining a minimal effective concentration in the cell.

It is very hard to visualize the stoichometric relations of ornithine degradation and the products produced because of the variable nature of the products as a result of many pathways involved. However, it is beyond any doubt that ornithine is being degraded by C. botulinum and most of the products produced have been identified. Since this is an anaerobic system and involves oxidation and reduction systems, it must be considered as a fermentation. Further studies on ornithine degradation may reveal the mechanism(s) involved. Ornithine may be a source of energy for the cell and/or it may serve as an important source of carbon for cell material.

This study further supports the conclusions of Simmons and Costilow (1962) that spores of C. botulinum contain low levels of most of the catabolic enzymes found in cells. All of the enzymes of the arginine dihydralase system were found in extracts of spores and germinated spores.

## SUMMARY

This investigation was carried out in order to gain knowledge of the catabolism of arginine, citrulline and ornithine by C. botulinum 62-A. Emphasis was given to the elucidation of pathways for arginine, citrulline and ornithine degradation. A comparison was also made of the relative activities of arginine dihydrolase enzymes from vegetative cells, spores and germinated spores of C. botulinum.

Manometric studies showed that $CO_2$ production was highest with arginine, intermediate with citrulline and lowest with ornithine. Ornithine and citrulline significantly inhibited gas production from arginine. Carbon and nitrogen balances demonstrated that $CO_2$, $NH_3$, citrulline and ornithine were major products of arginine degradation while acetic, propionic, butyric and valeric acids comprised the minor products. The major products from citrulline were $CO_2$, $NH_3$, and ornithine. Small amounts of acetic, propionic, butyric and valeric acids were found as minor products of citrulline degradation. The degradation of ornithine gave a number of products the major ones being $CO_2$, $NH_3$, putrescine, $\delta$-aminovaleric acid, acetic acid, propionic acid, and arginine. The minor products were butyric and valeric acids.

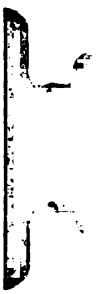
The use of radioactive substrates demonstrated that these products were truly derived from the substrates added.

Results obtained with cell-free extracts demonstrated that no cofactors were required for arginine degradation; however, additions of ADP or ATP increased the rate of arginine degradation. For citrulline degradation, ADP, $Mg^{++}$ and Pi were required as cofactors and arsenate replaced all of these cofactors and increased the rate of citrulline degradation. The cofactors required for ornithine degradation were CoA, lipoic acid, ADP and $Mg^{++}$.

The enzyme assays of cell-free extract preparations of C. botulinum revealed that the following enzymes were important in carrying out the degradation of arginine, citrulline and ornithine: arginine deiminase, citrullinase, ornithine transcarbamylase, carbamyl phosphokinase, and transamidinase. The levels of these enzymes were significantly higher in vegetative cell preparations than in those of spores or germinated spores. Only the activity of carbamyl phosphokinase was found to be higher in spores and germinated spores than in vegetative cells and this may not be a true picture since the assay was not completely specific. The use of NaF as an inhibitor showed that it specifically inhibited the activities of citrullinase and ornithine transcarbamylase.

The action of inhibitors, radioactive isotope studies and enzyme assays gave ample evidence for the catabolism of arginine via citrulline to ornithine by the well

known arginine dihydrolase system. The degradation of citrulline was found to be an exergonic reaction resulting in generation of ATP. On the basis of results obtained with the degradation of ornithine, a pathway was postulated with an unknown common intermediate (x). It is hoped that further studies on ornithine degradation may clarify the identity of this postulated intermediate.



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