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I hereby recommend that the thesis prepared under my supervision by Gilbert C. Schmidt

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THE DEGRADATION OF ARGININE BY CLOSTRIDIUM PERFRINGENS (BP6K)

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THE DEGRADATION OF ARGININE BY CLOSTRIDIUM PERFRINGENS (BP6K)

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PURPOSE

The primary purpose of this investigation was the clarification of arginine metabolism in Clostridium perfringens (BP6K). The degradation of arginine by Clostridium perfringens has been reported by Woods and Trim (1), but ammonia and carbon dioxide were the only end products identified. Urea, guanidine, citrulline, and ornithine were not attacked by the test organism and were therefore ruled out as possible intermediates.

This study is concerned with the source of the ammonia and carbon dioxide, the identification of end products other than carbon dioxide and ammonia, the identification of intermediary metabolites, and the definition of the metabolic pathway that is involved in the degradation.

The investigation had as a secondary purpose the preparation of cell-free extracts of the enzyme or enzymes that are responsible for the degradation of arginine in this organism. The attack on this phase of the problem was necessarily limited because the isolation, purification, and characterization of intracellular enzymes is an especially difficult and time consuming task.

INTRODUCTION

Amino acids are degraded by microorganisms in a great variety of ways. Both Gale (2) and Stephenson (3) have published thorough and excellent accounts of the type reactions that are involved. This discussion will, therefore, be strictly limited to information that directly pertains to the present investigation.

Woods and Trim (1) have investigated the amino acid metabolism of Clostridium perfringens (SR-9), but they were primarily concerned with the quantities of ammonia and carbon dioxide produced under varying conditions. These authors found that, of the amino acids attacked by washed cell suspensions of the test organism, arginine was peculiar in three respects:

a) No hydrogen was produced from arginine.

b) Ammonia and carbon dioxide were not produced in equimolar quantities.

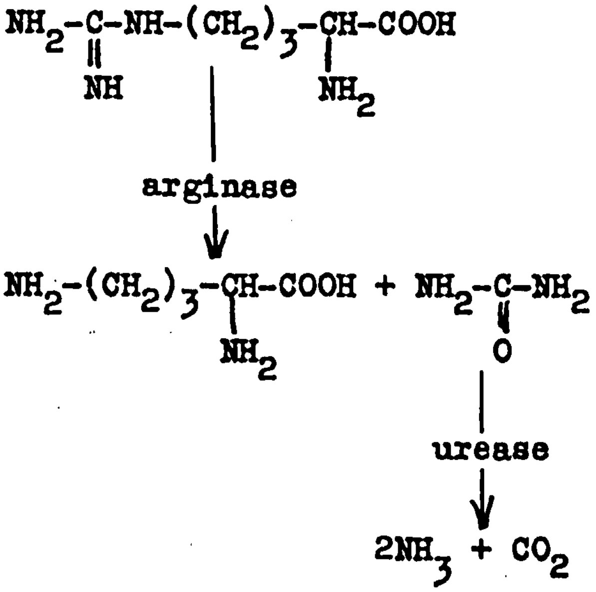
c) The quantities of ammonia and carbon dioxide finally produced varied with the age of the culture from which the washed cell suspension was prepared.

Woods and Trim employed washed cell suspensions from six hour old cultures and from ten to eleven hour old cultures. Hydrogen was not produced from arginine in either case. Ammonia and carbon dioxide were produced in both instances, but significantly smaller quantities were produced by organisms from the older cultures. With organisms from cultures of both ages, the excess of ammonia over and above one mole per mole of arginine originally present equalled the carbon dioxide produced. Woods and Trim found, for example, that organisms from six hour old cultures produced an average of 1.64 moles of ammonia and 0.59 moles of carbon dioxide per mole of arginine. Organisms from 10-11 hour old cultures produced an average of 1.31 moles of ammonia and 0.33 moles of carbon dioxide per mole of arginine. They considered this peculiar relationship between ammonia production and carbon dioxide production to be significant, but could not explain it.

The related compounds, ornithine, citrulline, urea, and guanidine were investigated in an attempt to throw light on the mechanism of the arginine breakdown, but no attack on these compounds could be detected with washed cells. No diffusible coenzyme appeared to be involved in the degradation, and changes in hydrogen ion concentration between pH 6.1 and pH 8.0 had a very small effect on either ammonia or carbon dioxide production.

The degradation of arginine by other organisms has also been reported, and appears to be identical with, or closely related to, the degradation observed by Woods and

Trim. The production of ornithine from arginine was early observed as the result of the action of mixed putrefactive organisms (4,5). It was assumed that the following mechanism was responsible for the formation of ornithine, since both urease and arginase were known to exist in animal tissues:



$$\mathrm{NH_2-\underset{\underset{NH}{\|}}{C}-NH-(CH_2)_3-\underset{\underset{NH_2}{|}}{CH}-COOH}$$

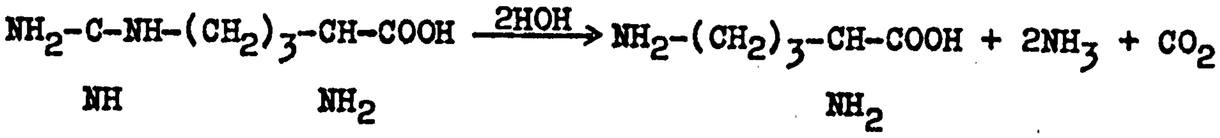
$$\Big\downarrow \text{arginase}$$

$$\mathrm{NH_2-(CH_2)_3-\underset{\underset{NH_2}{|}}{CH}-COOH + NH_2-\underset{\underset{O}{\|}}{C}-NH_2}$$

$$\Big\downarrow \text{urease}$$

$$\mathrm{2NH_3 + CO_2}$$

A later paper by Hills (6) discounted the possibility of combined arginase-urease activity in these organisms. He found that urease was absent from <u>all</u> strains of Streptococci tested, and although it was present in Staphylococci, urease activity could not account for all the ammonia and carbon dioxide that were formed from arginine. Arginine was not alpha deaminated by the organisms employed by Hills, and citrulline was not attacked. Hills therefore proposed a single step degradation of arginine to ornithine in accordance with the following equation:



$$NH_2-\underset{NH}{C}-NH-(CH_2)_3-\underset{NH_2}{CH}-COOH \xrightarrow{2HOH} NH_2-(CH_2)_3-\underset{NH_2}{CH}-COOH + 2NH_3 + CO_2$$

Hills found that ornithine was not further deaminated at either the alpha or delta linkage by gram-positive Staphylococci or Streptococci. The reductive deamination of ornithine to delta amino valerianic acid has been reported to occur with Clostridium sporogenes, however (2).

Gale (2) has stated that "arginine dihydrolase" is possessed by most Streptococci to a varying extent. He has also stated that the function of "arginine dihydrolase" is not clear, but that these organisms may depend upon the enzyme for the provision of carbon dioxide which is essential for growth. Gale (7) has also shown that with Lancefield group D Streptococci arginine can be partially replaced with ornithine, in which case cell growth and total cell production depend on carbon dioxide concentration. Niven *et al.*(8) have reported that the hydrolysis of arginine by Streptococci has been observed with all species of serological groups A to G. The viridans group was reported to lack this ability, while in the lactic group it was found to be characteristic of *Streptococcus lactis*. The absence of urease was confirmed. "Arginine dihydrolase" activity has been reported for *Proteus ichthyosmius* (9), *Streptococcus faecalis* (10), and *Pseudo-*

monas ovalis (11,12).

Horn (13) has isolated citrulline in a yield of 4.9 per cent from incubations of arginine with washed cell suspensions of Bacillus pyocyaneus. He termed the enzyme responsible for the degradation of arginine to citrulline an "arginine desimidase." No attempt was made to isolate citrulline from the reaction mixture. Ackermann (14) has reported the same results with a mixed culture and has confirmed the results obtained with Bacillus pyocyaneus. Ornithine was not determined. The presence of "arginine desimidase" in Bacillus pyocyaneus has also been confirmed by Tomota (15). Tomota also found that the enzyme was present in Salmonella enteritidis, which converts arginine or ornithine to putrescine. Citrulline and putrescine were isolated from the reaction mixtures.

Woods and Trim implied that because citrulline and ornithine were not attacked under the conditions of their experiments these compounds were not involved in the degradation of arginine by Clostridium perfringens. The remainder of their data, however, is consistent with the idea that citrulline is an intermediate and ornithine an end product. The experiments of these authors were somewhat limited, and it is quite possible that citrulline might be attacked by the test organism under different conditions. Furthermore, Woods and Trim apparently did not consider the possibility that citrulline, which is notorious for its inability to diffuse across the cell wall, failed to come in contact with the intracellular enzymes. No attempt was made to determine the presence of citrulline and ornithine in reaction mixtures by application of colorimetric methods or by isolation. For these reasons it was felt, contrary to the opinion of Woods and Trim, that neither citrulline nor ornithine could be ruled out as possible intermediates or end products.

A careful study of Hills paper showed that although ornithine was isolated from reaction mixtures no real effort was made to identify citrulline. Hills ruled out citrulline as an intermediate on the erroneous assumption that since it was not attacked by the test organism under the conditions of his experiments it could not be an intermediate. Hills further assumed that two moles of ammonia were produced from one mole of arginine, although his experimental values were always significantly lower. Ammonia production never rose above 1.81 moles per mole of arginine, and fell as low as 1.51 moles per mole. Hills' data, which purport to show that two moles of ammonia are produced from one mole of arginine, and that 0.5 moles of carbon dioxide are produced per mole of ammonia, are reproduced:

Moles NH_3 per Mole Added Arginine	Moles CO_2 per Mole NH_3
1.81	0.41
1.79	0.48

Recalculation of Hills' data to conform with the method of presentation utilized by Woods and Trim indicated that Hills probably failed to properly evaluate his data:

	Moles NH_3 per Mole Added Arginine		Moles NH_3 over and above One Mole per Mole Arginine
	1.81		0.81
	1.79		0.79
Average	1.80	Average	0.80

These observations suggested the possibility that both Hills, and Woods and Trim were dealing with the same sort of degradative mechanism. It also suggested the possibility that "arginine dihydrolase" was actually "arginine desimidase," acting in conjunction with a second enzyme to form ornithine.

Data presented in this thesis show that Clostridium perfringens completely degrades arginine to citrulline, and that citrulline is incompletely degraded to equimolar quantities of ammonia, carbon dioxide, and ornithine by the action of a second enzyme. Since the completion of this work at least three different groups of investigators (16,17, 18) have presented conclusive evidence showing that Streptococcus faecalis degrades arginine in the same way as Clostridium perfringens. It can therefore be said that the concept of "arginine dihydrolase" activity, although widely accepted in the past, appears to have been refuted.

METHODS

Maintenance of Cultures

Stock cultures of <u>Clostridium perfringens</u> and <u>Clostridium septicum</u> were maintained by serial transfers in essentially identical stock media. <u>Clostridium perfringens</u> (BP6K) was obtained from the American Type Culture Collection. <u>Clostridium septicum</u> was kindly supplied to us by Dr. L. S. McClung, Department of Bacteriology, Indiana University, Bloomington, Indiana. This particular organism which is strain number 78 of Dr. McClung's collection, possessed a very active ornithine decarboxylase but attacked neither arginine nor citrulline under the conditions of our experiments.

<u>Preparation of Dry, Solvent Extracted Beef Heart</u>. Dry, solvent extracted beef heart for use in the preparation of stock media was obtained in the following manner: Fresh beef heart was carefully freed of gross fat and was finely ground by passing through a meat chopper three times. One kilogram of ground tissue was kneaded with three liters of 95% ethanol during four hours. The alcohol was removed by decantation and by straining through cheesecloth. The alcohol extracted tissue was then extracted with 3 liters of 1:1 ethanol-ether by stirring frequently during 4 to 8 hours. The solvent was removed and the tissue was twice extracted

by stirring with 2 liter quantities of ether. After the second ether extraction the tissue was spread in a thin layer and dried at room temperature in a current of air. The dried, solvent extracted tissue was stored at room temperature in a screw-capped jar.

Preparation of Stock Medium. The stock medium on which all cultures were maintained was prepared in the following manner. Twenty grams of Proteose-Peptone (Difco) were dissolved in 1 liter of distilled water by warming over a gas flame. The solution was adjusted to pH 7.4-7.6 with sodium hydroxide, and 40 ml. quantities were transferred to 25 x 200 mm. pyrex culture tubes which contained 1 gram of solvent extracted beef heart per tube. The tubes were plugged, autoclaved at 15 poinds for 30 minutes, and stored at room temperature for as long as two weeks.

Stock Cultures. Just before inoculation, the required number of tubes of stock medium were heated in a boiling water bath for 20 minutes to expel dissolved air. The tubes were cooled to room temperature under running tap water, and 1 ml. of sterile 25% glucose was added to each tube under aseptic conditions. The tubes were inoculated at once with 2 ml. of stock culture, using aseptic technique. The inoculated tubes were incubated in a 37° C. water bath for 6 hours, then stored in the cold room (4-6° C.). Fresh transfers were made every 7 to 14 days, although cultures that had been stored for 3 months "grew out" satisfactorily after serial transfers on 2 or 3 successive days. Stock cultures of Clostridium septicum were inoculated, incubated, and stored in the same way except that 2 ml. of 25% glucose were added at the time of inoculation and the incubation period was 16-18 hours.

Preparation of Washed Cell Suspensions

Cells for the preparation of washed cell suspensions of Clostridium perfringens were grown 6 hours at 37° C. on 4.5% tryptic digest of casein and 0.6% glucose (28). Washed cell suspensions of Clostridium septicum, which were employed in ornithine decarboxylase experiments, were grown and prepared for use as outlined in the description of that assay procedure.

Tryptic Digest of Casein. Tryptic digest of casein, for use in the preparation of growth medium, was made in the following manner: 1150 grams of casein were suspended in 9 liters of distilled water which had been warmed to 50° C. The pH was adjusted to 8.0 with saturated sodium hydroxide solution. The alkali was added slowly, and the casein mixture was stirred vigorously after each addition. If this was not done, the pH of the casein mixture changed after it had apparently been adjusted. When the pH had been adjusted to 8.0, as indicated by Hydrion paper, the solution was permitted to stand for 30 minutes and more alkali was then added if necessary. The pH of the digest was finally checked with the glass electrode. 45 grams of trypsin (1:100) were suspended in enough distilled water to make a smooth flowing paste. The paste was poured into the casein mixture, 600 ml. of toluene were stirred in, and the digest was covered and placed in an incubator at 45-50° C. for 24 hours.

After incubation the toluene was removed, the digest was adjusted to pH 5.5 and boiled for 20 minutes. Approximately 4 ounces of Filter-Cel were stirred into the digest, which was then filtered through a folded filter paper containing a cone of Filter-Cel. The clear digest was stored under a layer of toluene in the cold room (4-6° C.) until used. The insoluble material that settled out on standing was not removed.

Growth Medium. Growth medium for Clostridium perfringens was prepared in the following manner: For each 1 liter of medium an amount of tryptic digest which contained 45 grams of solids was measured out, after separation from toluene in a large separatory funnel. 5.76 grams of disodium phosphate (dodecahydrate), 0.48 grams of potassium dihydrogen phosphate (anhydrous), and 0.02 grams of magnesium sulfate (heptahydrate) were dissolved in 250 ml. distilled water with the aid of heat and added to the tryptic digest of casein. Five ml. of a stock vitamin solution containing 1 mgm. of calcium d-pantothenate, 1 mgm. of pyridoxine, and 0.1 mgm. of riboflavin were added, and the solution was diluted to 1 liter. The growth medium was adjusted to pH 7.4-7.6, boiled for 10 minutes, and autoclaved at 15 pounds for 1 hour.

Growth of Cells and Preparation of Cell Suspensions. The required amount of sterile growth medium (usually 3 liters) was cooled to room temperature under running tap water. A quantity of sterile 25% glucose sufficient to make the final glucose concentration 0.625% was added aseptically. Each 3 liters of medium was then inoculated with 35-40 ml. of fresh stock culture, and incubated in a 37° C. water bath for 6 hours. After incubation, the cells were removed by centrifuging at 0° C. in an International refrigerated centrifuge until the cells were tightly packed. Thirty minutes centrifugation at 3500 R.P.M. was usually sufficient. The supernatant was poured off and discarded. The packed cells were washed into two centrifuge bottles, suspended in distilled water (100 ml. per bottle), and the bottles stoppered. The cells were washed by shaking until a smooth, homogeneous suspension was obtained, and again collected by centrifugation at 0° C. The washing operation was repeated once or twice, and the washed cells were finally suspended in 250-300 ml. of distilled water. This preparation is referred to as a "washed cell suspension" in the discussion that follows.

Total solids (usually 10-12 mgm. per ml.) and total nitrogen (usually 1.5-1.8 mgm. per ml.) were run on every washed cell suspension. Stock cultures and washed cell suspensions were checked for contamination by the Gram staining technique. These procedures are described under their respective headings. Packed washed cells and washed cell suspensions have been stored in the cold room for 12 hours before use, but either procedure results in a loss of enzyme activity. For this reason washed cells were usually suspended immediately after washing, and the washed cell suspension was used at once.

Preparation of Lyophilized Cells

For the preparation of lyophilized cells the solids from 1 liter of medium were washed in the manner previously described. The washed cells were suspended in 30 ml. of distilled water in a 100 ml. centrifuge bottle. The contents were thoroughly shaken to insure a smooth suspension and frozen in a thin layer by means of a mixture of dry ice and acetone. The frozen contents were lyophilized in the centrifuge bottle, removed, ground in an agate mortar or forced through the meshes of a tea strainer, placed in stoppered bottle, and stored in a vacuum desiccator over "Drierite." This preparation maintained activity for at least two weeks.

Preparation of Cell Extracts

The enzyme that is responsible for the conversion of arginine to citrulline was obtained in sodium chloride extracts of lyophilized cells. We have called this preparation an "arginine desimidase," to conform with the terminology that was originally employed by Horn (13). Sodium chloride extracts of lyophilized cells were prepared in the following manner:

600 mgm. of lyophilized cells were extracted with 7 ml. of 1% sodium chloride solution for 3 hours at room temperature. The debris was removed by centrifugation at 0° C. in the refrigerated centrifuge. The clear yellow extract that was obtained was used at once. An extract having apparently identical properties was also obtained by grinding lyophilized cells with aluminum oxide in an agate mortar.

Determination of Total Solids

The total solids in washed cell suspensions were determined in the following manner: Aluminum foil cups, weighing approximately 200 mgm., were made by molding "Reynolds Wrap" over a small stopper. The cups were dried for 24 hours at 105° C., tared, and stored in a desiccator. For the determination of total solids, duplicate 2 ml. aliquots of washed cell suspensions were pipetted into tared foil cups and dried to a constant weight at 105° C. Six hours drying was usually sufficient.

Determination of Total Nitrogen

The nitrogen content of cell suspensions was determined in approximately the same manner as outlined by Umbreit (29). An aliquot of washed cell suspension was diluted to contain 30-100 micrograms of nitrogen per ml. of dilution. One ml. and 0.5 ml. samples of the diluted suspension were pipetted into Pyrex test tubes graduated at 25 ml. (approximately 20 x 150 mm.), and digested in the following manner: Blanks, consisting of 1.0 ml. and 0.5 ml. of distilled water were digested in parallel with the samples.

One ml. of 5 N H_2SO_4, containing 200 mgm. of copper selenite per liter, and a few carborundum granules were added to each tube. The tubes were capped with glass caps or small funnels, placed on a 150-180° C. sandbath, and digested for 12 hours. One drop of 30% H_2O_2 was added after cooling the tubes for 1 minute, and the tubes were heated over a micro burner until dense white fumes developed. Digestion was continued for 5 minutes, the samples were cooled for 2 minutes, diluted with 5 ml. of distilled water, and Nesslerized by the addition of 5 ml. of Nessler's reagent, 7 ml. of 2 N NaOH, and distilled water to make 25 ml. The contents of the tubes were mixed, permitted to stand at least 10 minutes, transferred to colorimeter tubes, and the transmission measured in the Evelyn colorimeter using No. 515 filter.

Incubation Mixtures

The following incubation procedure was employed for all analyses except the determination of carbon dioxide. The procedure employed when carbon dioxide production was measured is included in the discussion of carbon dioxide determination.

Suitable quantities of distilled water, 0.45 M phosphate buffer of the desired pH, cell suspension (containing 10-12 mgm. solids per ml. of suspension), and substrate solution (containing 60 micromoles of L isomer per ml.) were equilibrated for 20 minutes at 37° C.

Into each sample tube to be incubated the following quantities of solutions (or multiples of them) were pipetted: 1.0 ml. of buffer, 1.0 ml. of cell suspension, and 0.5 ml. of substrate. Into each blank tube the following solutions were pipetted: 0.5 ml. of distilled water, 1.0 ml. of cell suspension, and 1.0 ml. of buffer. The tubes were transferred to a shaking water bath and incubated at 37° C. One ml. samples were withdrawn from the tubes at the desired times and pipetted into 9 ml. of 10% trichloroacetic acid to stop the reaction. Each trichloroacetic acid mixture was permitted to stand for 30 minutes at room temperature, then filtered through hard filter paper until clear. Each 1 ml. of trichloroacetic acid filtrate represented 1.2 micromoles of arginine. This filtrate was diluted, or was used directly, in the determination of ammonia, citrulline, arginine by the Sakaguchi method, and ornithine by the colorimetric ninhydrin method.

When filtrates representing higher concentrations of arginine were desired (as, for example, in chromatography) 25% or 50% solutions of trichloroacetic acid were employed and the volume was reduced accordingly.

In the determination of ornithine by enzymatic decarboxylation of reaction mixtures, the use of trichloroacetic acid was avoided. For these determinations incubations were carried out in tubes graduated at 12.5 ml. Five times the above quantities of the reaction mixture components were used, and the incubations were carried out as outlined above. At the completion of the incubation, sample and blank tubes were plunged into a boiling water bath and boiled for 10 minutes to precipitate protein. The volume was again adjusted to 12.5 ml. with distilled water, and the mixture was filtered through hard filter paper. Each 1 ml. of this solution represented 12 micromoles of arginine, and was suitable for enzymatic ornithine determinations without further dilution.

Determination of Carbon Dioxide

The enzymatic production of carbon dioxide was measured manometrically, by the direct method, with air as the gas phase. The following procedure was generally employed:

Five-tenths ml. of substrate solution (containing 8 micromoles of L isomer) and 1 ml. of 0.4 M phosphate buffer at the desired pH were pipetted into the main chamber of the Warburg vessel. One ml. of enzyme preparation (containing 12 mgm. washed cells, 24 mgm. lyophilized cells, or the NaCl extract from approximately 80 mgm. of lyophilized cells) was pipetted into the side arm. Three-tenths ml. of 6 M phosphoric acid was pipetted into a second side arm, or into a center well designed for acid tipping.

The vessels were equilibrated for 15 minutes at 37° C. and the substrate was tipped in. When the reaction had proceeded for the desired length of time the phosphoric acid was tipped in, manometer readings were taken when they had become constant, and carbon dioxide production was calculated.

The apparatus, incubation vessels, calibration procedures, and operational techniques have been thoroughly discussed by Umbreit (29) and Dixon (30).

Determination of Ammonia

Most methods for the quantitative determination of ammonia employ concentrated alkali and distillation with the aid of heat. Heat distillations could not be employed in this investigation because of the probable liberation of ammonia from accumulated intermediates and end products on heating in the presence of concentrated alkali. Pennington and Greenlee (19), Folin (20), Van Slyke (21), Thomas and Van Hauwaert (22), Falk and McGuire (23) and others have avoided the use of heat by employing an aeration technique.

Aeration appeared to be the method of choice for this investigation, but preliminary experiments disclosed a number of disadvantages. The apparatus was difficult to clean properly and required oven drying between assays. There was a tendency for alkali to carry over from reaction vessel to receiver, and antifoam agents could not be used because they, too, carried over or distilled into the receiver and interfered with Nesslerization. Blanks were variable and frequently very high, and control determinations were not sufficiently accurate or reproducible.

Very satisfactory results were obtained by diffusion in a Conway unit (24), followed by Nesslerization of an aliquot from the center well. This same technique was used by Woods and Trim (1), except that they titrated the contents of the center well. We employed Nesslerization in preference to titration because it was more rapid than titration and less susceptible to error in low nitrogen ranges.

Assay Procedure. The following procedure was employed for the determination of ammonia in reaction mixtures. The outside rim of the Conway cell was lightly greased with stopcock grease. One ml. of trichloroacetic acid filtrate of the reaction mixture, containing or diluted to contain not more than 3 micromoles of ammonia, was placed in the outside compartment of the cell. Exactly 1 ml. of 0.2 N hydrochloric acid was placed in the center well. One drop of alcoholic phenolphthalein was added to the sample in the outer compartment. One ml. of saturated aqueous sodium carbonate was placed in the outer compartment, some distance from the sample so that alkali and sample did not mix. The glass cover was placed on the cell and pressed into place. The contents of the outer compartment were thoroughly mixed by careful rotation. Ammonia was allowed to diffuse from the outer compartment into the center well by permitting the cell to stand at room temperature for 100 minutes.

After diffusion the cover of the cell was removed, and a 0.3 to 0.6 ml. aliquot was removed from the center well with a 1 ml. pipette graduated in units of 0.01 ml. The aliquot was transferred to an Evelyn tube containing 8 ml. of distilled water and 3 ml. of Nessler's reagent. When all samples had been transferred to Evelyn tubes, 5 ml. of 2 N sodium hydroxide were added to each tube, the contents were mixed by agitation, permitted to stand 15 minutes at room temperature and read in the Evelyn colorimeter with a No. 420 filter. Sample tubes were read against blanks set at 100% transmission. The ammonia content of the sample was calculated from a standard curve, prepared by Nesslerization of known amounts of standard ammonium sulfate solutions.

Preparation of Nessler's Reagent. The use of an alkaline solution of mercuric iodide in potassium iodide was first proposed as a reagent for the determination of ammonia by Nessler in 1856 (25). A great many variations in the reagent and in the method of preparation have been proposed since that time. The Nessler's reagent employed in this investigation was essentially the same as the reagent used by Umbreit (26). Umbreit has stated that after grinding 5 grams of KI and 2-3 grams of HgI_2 with distilled water, diluting to 500 ml. with water, and allowing to stand for several days, "the solution was filtered to remove excess mercuric iodide."

It was found that 5 grams of KI were more than sufficient to dissolve 2-3 grams of HgI_2. When the reagent was prepared as directed by Umbreit the color intensity with a given quantity of ammonia was less than when the reagent was prepared with an excess of mercuric iodide. Calculations based on the equation:

$$2KI + HgI_2 \longrightarrow K_2HgI_4$$

indicated that 5 grams of KI would dissolve 8.36 grams of HgI_2. These figures are in accord with the generally recognized fact that mercuric iodide is soluble in water on the addition of an equal weight of potassium iodide. The preparation of Nessler's reagent was therefore slightly modified so that an excess of mercuric iodide was actually used.

The Nessler's reagent used in this investigation was prepared in the following manner: Five grams of reagent grade KI and 8.5 grams of reagent grade HgI_2 were ground in a mortar with 50 ml. of distilled water. The solution and excess HgI_2 were washed into a 500 ml. volumetric flask with small portions of distilled water, made to volume with distilled water, stoppered, and allowed to stand at room temperature for 4 days. The solution was filtered free of undissolved $Hg\ I_2$, and 160 ml. of gum ghatti solution were added. The solution was diluted to 1500 ml. with distilled water, mixed by manual shaking, allowed to stand overnight, and filtered through a folded analytical filter until clear. The water clear, faintly yellow reagent was stored in glass at room temperature. No changes have been detected over a period of 18 months' storage. Umbreit has indicated that the solution is stable for years.

Difficulty was encountered in preparing a satisfactory solution of gum ghatti. Attempts to replace gum ghatti with Methocel, a cold-water-soluble methylated cellulose product, met with failure. Methocel in concentrations of 0.1%-0.01% caused a precipitation of the colored complex. A satisfactory solution of gum ghatti was obtained in the following manner:

Five grams of gum ghatti (finely powdered) were placed in a 1000 ml. round bottom flask and shaken with 300 ml. of distilled water. The flask and contents were transferred to a steam bath, a small Erlenmeyer flask was inverted in the neck of the large flask to serve as a condenser, and the gum ghatti mixture heated for 3 hours. The resulting solution, which contained appreciable amounts of undissolved material, was filtered with the aid of suction until clear. Filtration was speeded by frequent replacement of filter papers. Unless filter papers were replaced frequently the pores became clogged and filtration was nearly impossible. One hundred and sixty ml. of the resulting solution were employed as indicated.

Curve Ranges. The range over which the Beer-Lambert law was followed, at wave lengths of 515, 470, and 420 millimicrons, was determined as follows: Quantities of ammonium sulfate representing from 0-100 micrograms of nitrogen were pipetted into 25 ml. volumetric flasks, Nesslerized, made to volume with distilled water and read against the blank tube at each of the three wave lengths.

According to Gibb (27), the Beer-Lambert law can be expressed by the equation:

$$\mathrm{Log}_{10} \frac{I_o}{I} = KCL$$

where:

K is a constant for any given wave length.

L is the thickness of the absorbing medium, usually in mm.

C is the concentration of solute, usually in grams per liter.

I_o and I refer to the original and transmitted intensities, respectively, at a given wave length, after corrections for reflection losses, etc.

Gibb states that the correction for I_o and I is made by comparing the intensity (I) of a ray of light passing through a colored solution with the intensity (I_o) of the same ray passing through a colorless medium of the same refractive index and contained in a similar cell. When using the Evelyn colorimeter this correction is accounted for by the blank, since the instrument is set to transmit 100% of the incident light when the blank tube is in place. I_o is therefore 100% under these conditions, and the preceeding equation becomes:

$$\mathrm{Log}_{10} \left(\frac{100}{I}\right) = KCL$$

But:

$$\log_{10}\left(\frac{100}{I}\right) = 2 - \log_{10} \% \text{ transmission} = \text{optical density}$$

Therefore, by substitution in the second equation:

$D = KCL$, where D is optical density.

When using matched colorimeter tubes the thickness of the absorbing medium, L, is a constant so that:

$$D = K_1C$$

Solving for K_1 the above equation becomes:

$$\frac{D}{C} = K_1$$

The Beer-Lambert law is followed, therefore, when optical density divided by concentration is a constant. The actual value for the constant will vary with wave length of incident light, the units employed, and the thickness of the absorbing medium. For comparative purposes, however, these variables do not change for a given set of data.

In this series of experiments concentrations were expressed as micrograms of ammonia nitrogen per 25 ml. of solution. The solutions were compared at the stated wave length, employing matched Evelyn colorimeter tubes. The blank contained all reagents except ammonium sulfate. Blank and sample solutions were therefore of similar refractive indices and were contained in identical cells. It was therefore assumed that, for a given wave length, the Beer-Lambert law was followed when optical density divided by concentration was a constant.

The results of these determinations, which are given in Table I show that at 515 millimicrons the straight line curve range is from 10.0 through 100 micrograms of ammonia nitrogen per 25 ml. of colored solution. At 470 millimicrons it is from 5.0 through 50 micrograms, and at 420 millimicrons it is from 2.5 through 25 micrograms of ammonia nitrogen. We have employed both the No. 515 and the No. 420 filter within the preferred range, during this investigation. A typical standard curve, developed at a final volume of 16.6 ml. and using the No. 420 filter, is shown in Figure 1. The absorption spectrum of the Nessler's color is shown in Figure 2.

Table I

Curve Range for Nessler's Color, Using Different Filters

Evelyn Filter Employed	C	D	$\frac{D}{C} \times 10^4$
515	2.5	0.013	52
	5.0	0.022	48
	10.0	0.041	41
	15.0	0.061	41
	25.0	0.097	39
	35.0	0.137	39
	50.0	0.201	40
	60.0	0.237	40
	75.0	0.301	40
	85.0	0.337	40
	100.0	0.387	39
470	2.5	0.022	88
	5.0	0.041	82
	10.0	0.084	84
	15.0	0.125	83
	25.0	0.201	81
	35.0	0.288	82
	50.0	0.410	82
	60.0	0.469	78
	75.0	0.562	75
	85.0	0.638	75
	100.0	0.745	75
420	2.5	0.036	144
	5.0	0.071	142
	10.0	0.143	143
	15.0	0.215	143
	25.0	0.357	143
	35.0	0.483	138
	50.0	0.678	136
	60.0	0.796	133
	75.0	0.975	130
	85.0	1.097	129
	100.0	1.222	122

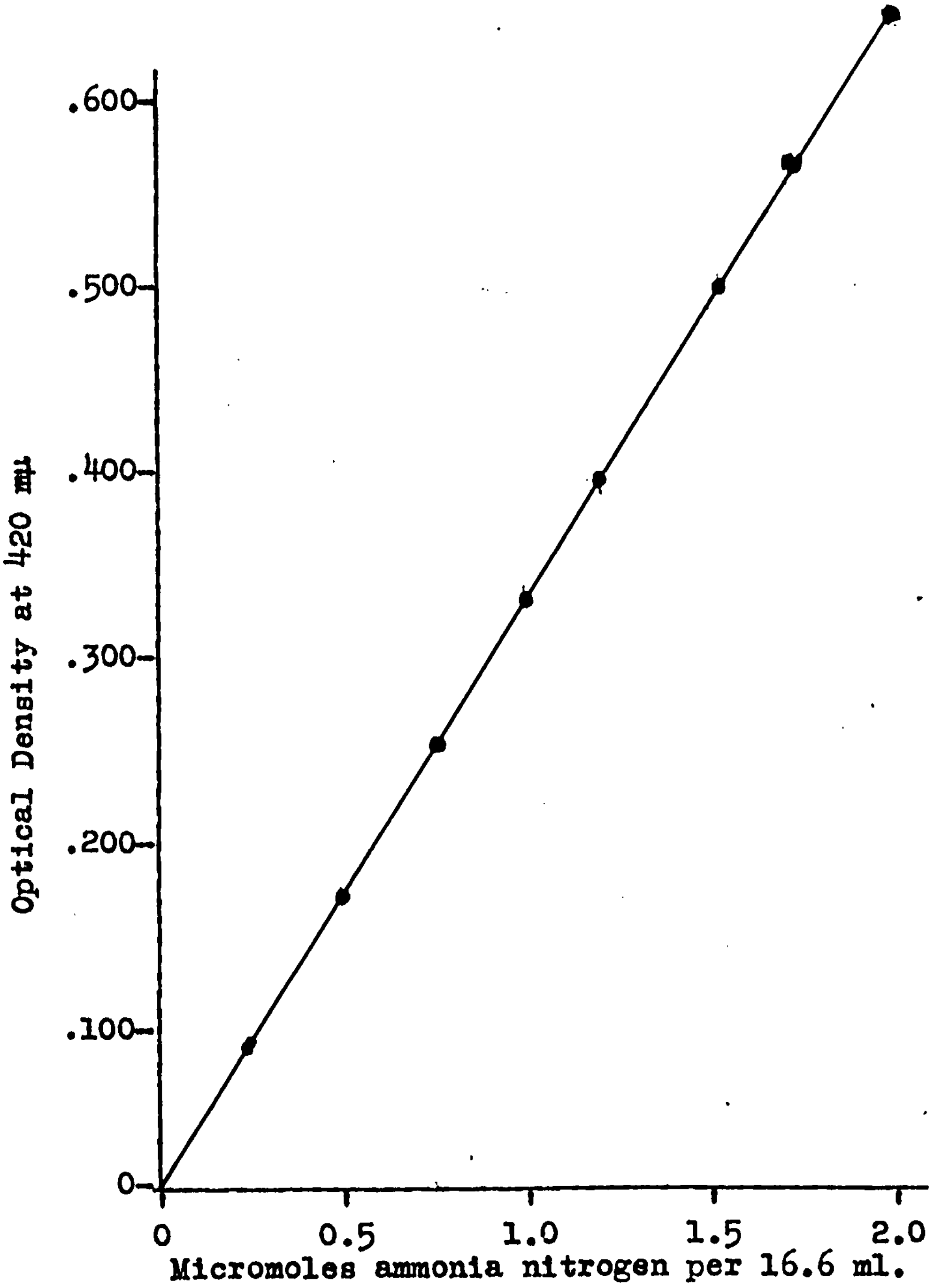


Figure 1. Standard Curve for the Determination of Ammonia by Nesslerization.

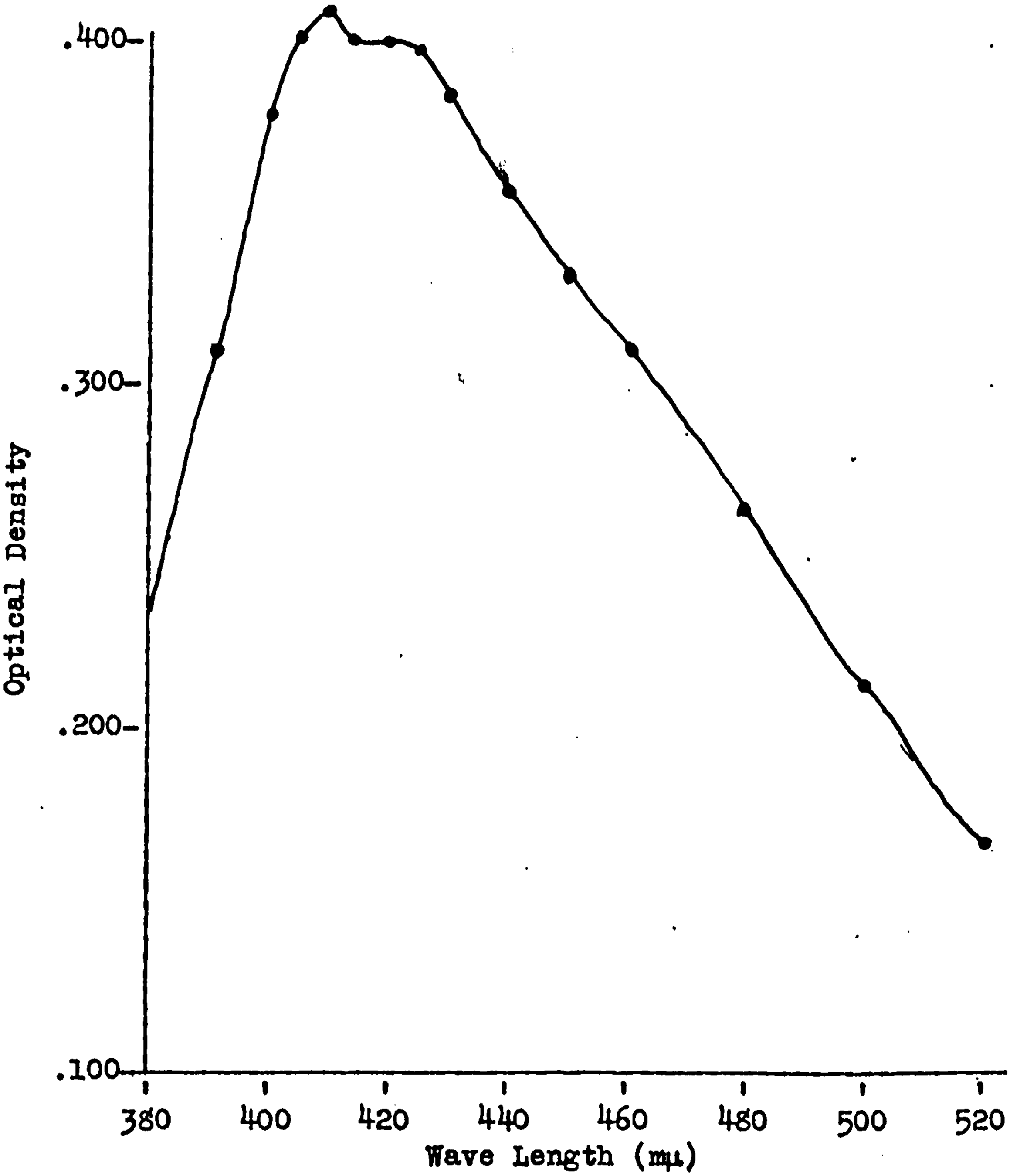


Figure 2. Absorption Spectrum of Nessler's Ammonia Color. Ammonia nitrogen concentration 50 micrograms per 25 ml. of reaction mixture. Measurements made in Coleman Jr. Model Spectrophotometer.

Stability of the Nessler's Color. The time elapsing between development and measurement of a color may be an important variable. In certain cases color intensity is a linear function of concentration only within specified time limits. The time interval between color development and color measurement must therefore be accurately controlled. Other colors increase or decrease in intensity on standing, but the linear relationship between concentration and optical density continues to hold. In these instances a specific time interval need not elapse between the development and the measurement of the color, but standards and unknowns must be run in parallel. Other colors do not change on standing for prolonged periods, and the time element is of little importance.

Nesslerization of standard ammonium sulfate solutions, which were read in the Evelyn colorimeter after standing for varying time intervals, showed that the Nessler's color is very stable. No changes in optical density occurred after the tubes had stood for 15 minutes to 5 hours. The time factor, therefore, is not critical. Table II shows the effect of prolonged standing on the Nessler's color.

Reproducibility of the Nessler's Color. The reproducibility of standard curves at either 420 millimicrons or 515 millimicrons was excellent. Optical densities obtained at varying nitrogen levels, on different days during a period

Table II

Stability of Nessler's-Ammonia Complex

Ammonia nitrogen contents are expressed in micrograms per 25 ml. of final solution. The color was developed, transferred to Evelyn tubes, and read at 5, 15, 30, 60, and 300 minutes, using the No. 515 filter. The instrument was set to read 100% transmission with a reagent blank. Values in the table are optical densities at 515 mμ.

Ammonia Nitrogen	Time between color development and reading 5	15	30	60	300
μg.			minutes		
2.5	0.013	0.013	0.013	0.013	0.013
5.0	0.022	0.022	0.022	0.022	0.024
10.0	0.041	0.041	0.041	0.041	0.041
15.0	0.056	0.061	0.061	0.061	0.066
25.0	0.092	0.097	0.097	0.097	0.097
35.0	0.125	0.137	0.137	0.137	0.137
50.0	0.194	0.201	0.201	0.201	0.201
60.0	0.227	0.237	0.237	0.237	0.237
75.0	0.292	0.301	0.301	0.301	0.301
85.0	0.337	0.337	0.337	0.337	0.337
100.0	0.387	0.387	0.387	0.387	0.387

of one year, produced standard curves that were almost exactly identical.

Accuracy of the Method. Conway and Byrne (24) have found that when employing from 14 to 140 micrograms of ammonia nitrogen, recovery by the diffusion technique was quantitative. They found a standard deviation for individual determinations of 0.06 micrograms of nitrogen at the higher level and 0.01 micrograms at the lower level. The coefficients of variation were, respectively, 0.5% and 0.7%. These calculations were based on more than 100 determinations. The accuracy of the Conway method, as employed in this investigation, was therefore limited only by pipetting errors. This error was found to be not greater than plus or minus 15 parts per thousand under the conditions of our determinations.

Attempted Nesslerization of Filtrates. During the early stages of this investigation an aeration procedure was employed for the determination of ammonia in incubation mixtures. The inconvenience of this procedure led us to consider other possibilities for the determination of ammonia. One of the possibilities considered was the direct Nesslerization of trichloroacetic acid filtrates of reaction mixtures. It was observed, however, that a great many compounds produced interfering colors on Nesslerization of samples containing ammonia.

Ornithine, arginine, glycine, diglycine, triglycine, glycine ethyl ester, glucosamine, 2-aminothiazole, guanidine, and ornithine produced a brilliant yellow color. Benzoylarginineamide, biuret base, glycinamide, chloroacetylglycylglycine, benzamide, and hippuramide produced colorless to gray solutions or precipitates. Hippurylglycine, hippuric acid, benzoylalanine, acetylglucosamine, betaine, alpha aminophenylacetic acid, acetylmethionine, norleucine, norvaline, and citrulline produced little or no interference.

The diverse nature of these compounds indicated very clearly that direct Nesslerization of any reaction mixture can be carried out only if the preparation of that reaction mixture involves a very thorough digestion, as for example, a Kjeldahl determination. The advisability of employing direct Nesslerization in Kjeldahl determinations is also open to some question.

Determination of Arginine by the Sakaguchi Method

The arginine content of reaction mixtures was determined by the colorimetric Sakaguchi method, as modified by Macpherson (31).

Assay Procedure. One to 5 ml. quantities of trichloroacetic acid filtrates of reaction mixtures (containing from 0 to 1.0 micromoles of arginine) were pipetted into 25 ml. volumetric flasks, diluted to 10 ml. with distilled water, and neutralized with 10% KOH. Two ml. of 10% KOH were added in excess of the quantity required for neutralization. The amount of alkali added was not critical, but the reaction mixture had to be strongly alkaline. Two ml. of alpha naphthol reagent (0.1% alpha naphthol in 50% ethanol, prepared fresh each day) and 1 ml. of 40% aqueous urea were added in order, mixing thoroughly after each addition. The flasks were chilled in an ice bath for exactly 5 minutes, removed from the bath, and 1 ml. of hypobromite reagent (2 gm. bromine in 100 ml. of 5% KOH, prepared fresh daily) was rapidly added to each flask while swirling to prevent local excesses of hypobromite. The flasks were permitted to stand exactly 3 minutes at room temperature, and 1 ml. of 40% aqueous urea was added to each, mixing immediately after each addition was made. One ml. of hypobromite reagent was added to each flask while swirling, the contents of the flasks were made to volume with distilled water, mixed by inversion, and transferred to Evelyn tubes. The tubes were allowed to stand exactly 15 minutes at room temperature and were read at once in the Evelyn colorimeter, uwing a No. 515 filter. A standard curve was run with each series of unknowns. Solutions were kept in an ice bath during the course of an assay and were stored in the cold room between assays.

Curve Range and Reproducibility of the Color. It became apparent, early in these experiments, that the Sakaguchi color was not nearly so reproducible as the color produced by Nesslerization of ammonia samples. Data from representative standard curves that were run during one year are shown in Table III. A typical standard curve is shown in Figure 3. The absorption spectrum of the Sakaguchi color is shown in Figure 4.

The data in Table III show that, under the conditions of these experiments, the useful curve range was 0.1-1.0 micromoles of arginine, and that the Beer-Lambert law was obeyed within this range. The color was reproducible with a given set of reagents on any one day, but curves were erratic unless the alpha naphthol and hypobromite were made fresh daily and were kept cold. The intensity of color produced by a given amount of arginine varied with each new set of reagents but always fell within the range shown in the table.

Table III

Curve Range and Reproducibility of the Sakaguchi Color

Standard Curve Run	Arginine Concentration (C)	Optical Density (D)	$\frac{D}{C} \times 10^2$
date	µM/25 ml.		
	0.1	0.090, 0.090	90, 90
	0.2	0.185, 0.185	92, 92
	0.3	0.270, 0.270	90, 90
	0.4	0.360, 0.370	90, 93
	0.5	0.445, 0.450	89, 90
3/12/51	0.6	0.525, 0.550	88, 92
	0.7	0.620, 0.620	88, 88
	0.8	0.710, 0.700	89, 88
	0.9	0.770, 0.770	85, 85
	1.0	0.825, 0.840	83, 84
	1.1	0.875, 0.875	80, 80
	1.2	0.920, 0.920	77, 77
	0.1	0.075	75
	0.2	0.150	75
	0.3	0.215	72
	0.4	0.290	73
	0.5	0.365	73
3/20/51	0.6	0.435	73
	0.7	0.505	72
	0.8	0.570	71
	0.9	0.645	72
	1.0	0.720	72
	1.1	0.770	70
	1.2	0.790	66
	0.1	0.065	65
	0.2	0.130	65
	0.3	0.195	65
	0.4	0.255	64
	0.5	0.320	64
3/23/50	0.6	0.375	63
	0.7	0.445	64
	0.8	0.510	64
	0.9	0.565	63
	1.0	0.640	64
	1.1	0.660	60
	1.2	0.680	57

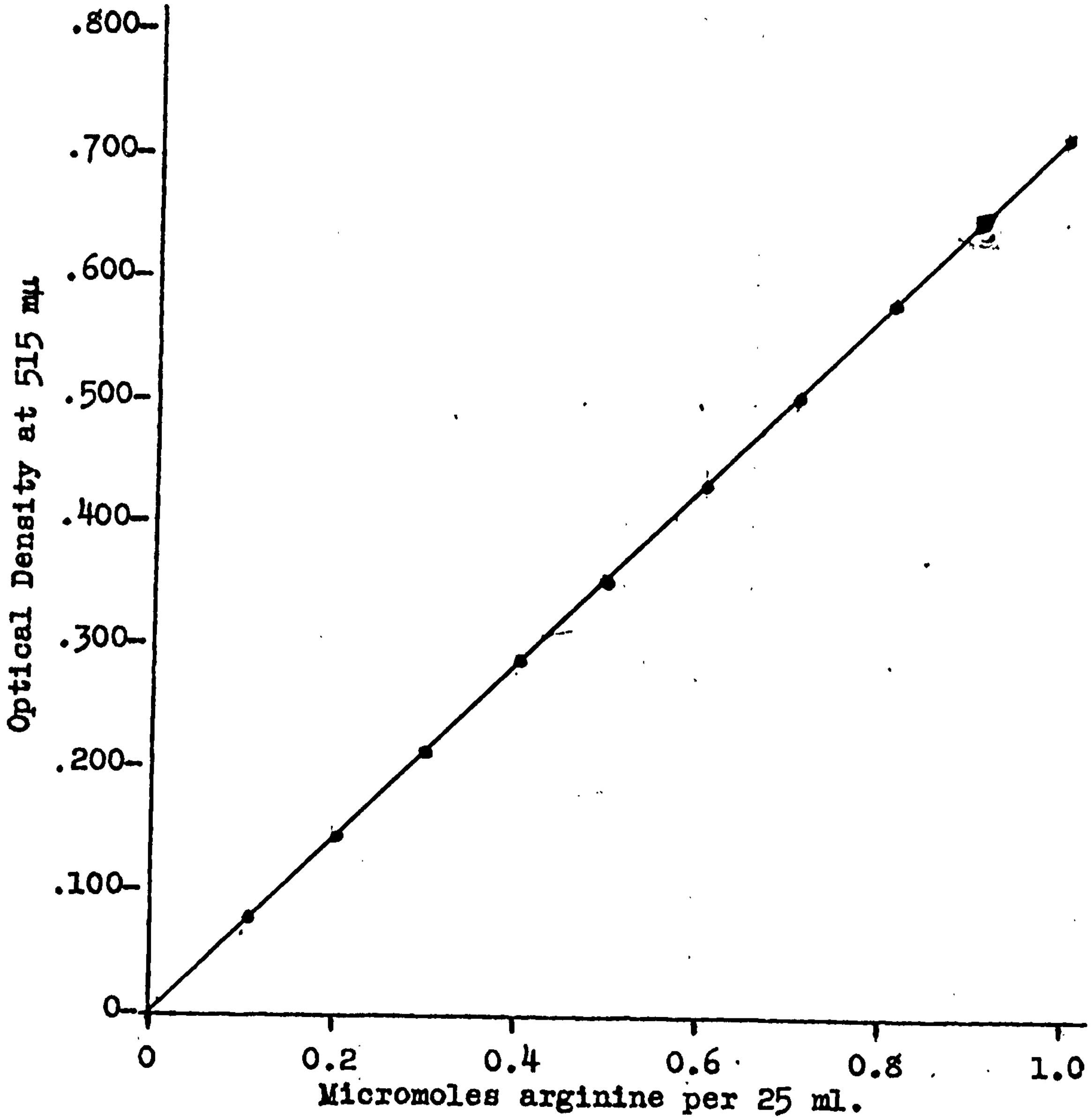


Figure 3. Standard Curve for the Determination of Arginine by the Macpherson Modification of the Colorimetric Sakaguchi Method.

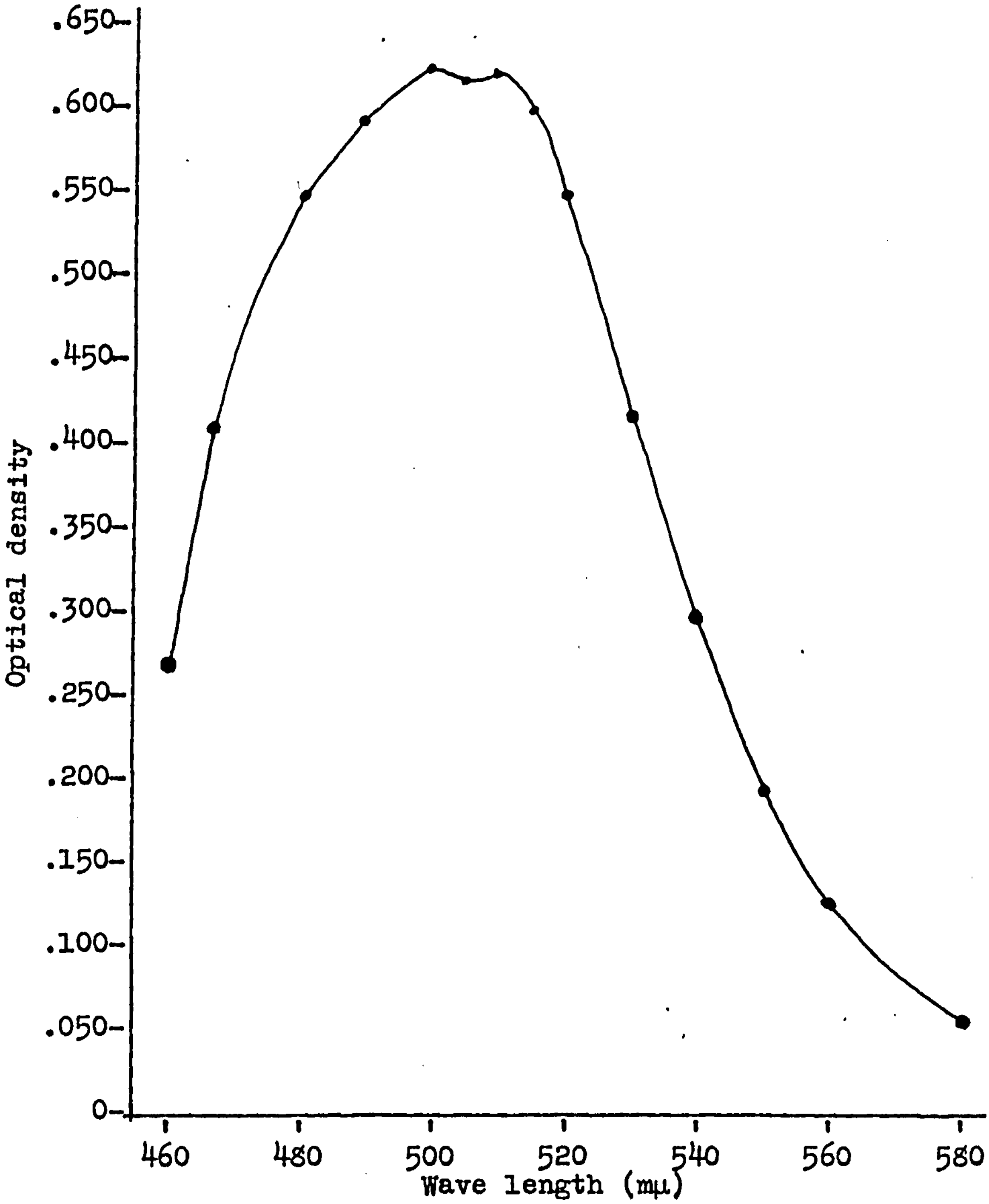


Figure 4. Absorption Spectrum of the Sakaguchi-Arginine Color

0.5 μM arginine/25 ml. Readings made in Coleman Junior Spectrophotometer.

The temperature of the reaction mixture, the method of addition of hypobromite, and hypobromite concentration were found to be important variables. These factors could be easily controlled, however, and were therefore investigated in only a qualitative way. The control of alkalinity, which might have caused a great deal of inconvenience in running large number of assays, was found to be non-critical. It was found, for example, that the addition of 2, 4, 6, and 8 ml. of 10% KOH made no difference in the standard curve that was obtained. As has been previously indicated, the reaction mixture had to be strongly alkaline. It was also found that a reasonable variation in the time interval elapsing between color development and color measurement had no effect on the standard curve. The data in Table IV show that the tubes should be permitted to stand not less than 15 minutes, and not more than 30 minutes before reading.

The difficulties that were originally encountered with the Macpherson modification of the Sakaguchi method were readily avoided by the use of fresh reagents, by the preparation of a standard curve with each series of analyses, by strict adherence to experimental conditions, and by maintenance of <u>one</u> experimental routine.

Table IV

Stability of Sakaguchi Color

Arginine contents are expressed in micromoles per 25 ml. of final solution. Color was developed, transferred to Evelyn tubes, and read at 10, 15, 30, 45, and 60 minutes, using the No. 515 filter. Instrument was set to read 100% transmission with a reagent blank. Values in the table are optical densities at 515 millimicrons.

Arginine	Time between color development and reading 10	15	30	45	60
μM			minutes		
0.1	0.108	0.102	0.102	0.102	0.102
0.2	0.208	0.208	0.208	0.181	0.174
0.3	0.284	0.284	0.284	0.260	0.252
0.4	0.367	0.367	0.367	0.328	0.310
0.5	0.432	0.432	0.432	0.398	0.367
0.6	0.552	0.523	0.523	0.482	0.456
0.7	0.585	0.602	0.602	0.585	0.552
0.8	0.678	0.678	0.678	0.638	0.602
0.9	0.745	0.745	0.745	0.721	0.699
1.0	0.824	0.824	0.824	0.770	0.745
1.1	0.854	0.886	0.886	0.854	0.854

Colorimetric Determination of Ornithine

The ornithine content of reaction mixtures was determined by a colorimetric procedure employed by Stein and Moore (32). The details of the procedure will be published by Chinard. Stein and Moore have stated that the method is specific for ornithine in the absence of proline.

Assay procedure. One-ml. quantities of trichloroacetic acid filtrates of reaction mixtures, containing or diluted to contain not more than 0.3 micromoles of ornithine, were pipetted into Folin-Wu tubes graduated at 12.5 ml. Two ml. of glacial acetic acid and 1 ml. of ninhydrin reagent (625 mgm. Pierce ninhydrin, 10 ml. 6 M phosphoric acid, and glacial acetic acid to make 25 ml.) were added. The tubes were capped with glass caps or small funnels and heated in a boiling water bath for 60 minutes. The tubes were cooled in running tap water, diluted to 12.5 ml. with glacial acetic acid, transferred to Evelyn tubes and read in the Evelyn colorimeter against a cell blank, using the No. 515 filter. A standard curve was run with each series of assays.

Curve Range and Reproducibility of Color. The range over which Beer's-Lambert's law was followed was determined as described under the determination of ammonia. The useful curve range, employing the Evelyn colorimeter and the No. 515 filter, was found to be from 0 to 0.3 micromoles of ornithine per 12.5 ml. of reaction mixture. The color did not fade on standing 90 minutes at room temperature, and standard curves were easily reproducible. A slight decrease in color production was noted after ninhydrin solutions had stood at room temperature for more than one week. A typical standard curve is shown in Figure 5, and the absorption spectrum of the ornithine color is shown in Figure 6.

Determination of Ornithine by Enzymatic Decarboxylation

The ornithine content of reaction mixtures was measured enzymatically, employing the usual Warburg techniques (33), and Clostridium septicum suspensions as a source of ornithine decarboxylase.

Growth of the Test Organism. The methods employed in the maintenance of stock cultures of the test organism have been described under "Maintenance of Cultures." Cells for the preparation of washed cell suspensions were grown in the following manner: Enough tryptic digest of casein to yield 24 grams total solids was transferred to a 1 liter Pyrex bottle, diluted to 800 ml. with distilled water, adjusted to pH 7.0, and 3 grams of solvent extracted beef heart were added. The bottles were plugged and autoclaved for 1 hour at 15 pounds. A small flask containing 16 grams of glucose in approximately 30 ml. of distilled water was autoclaved at the same time. The sterile medium and glucose

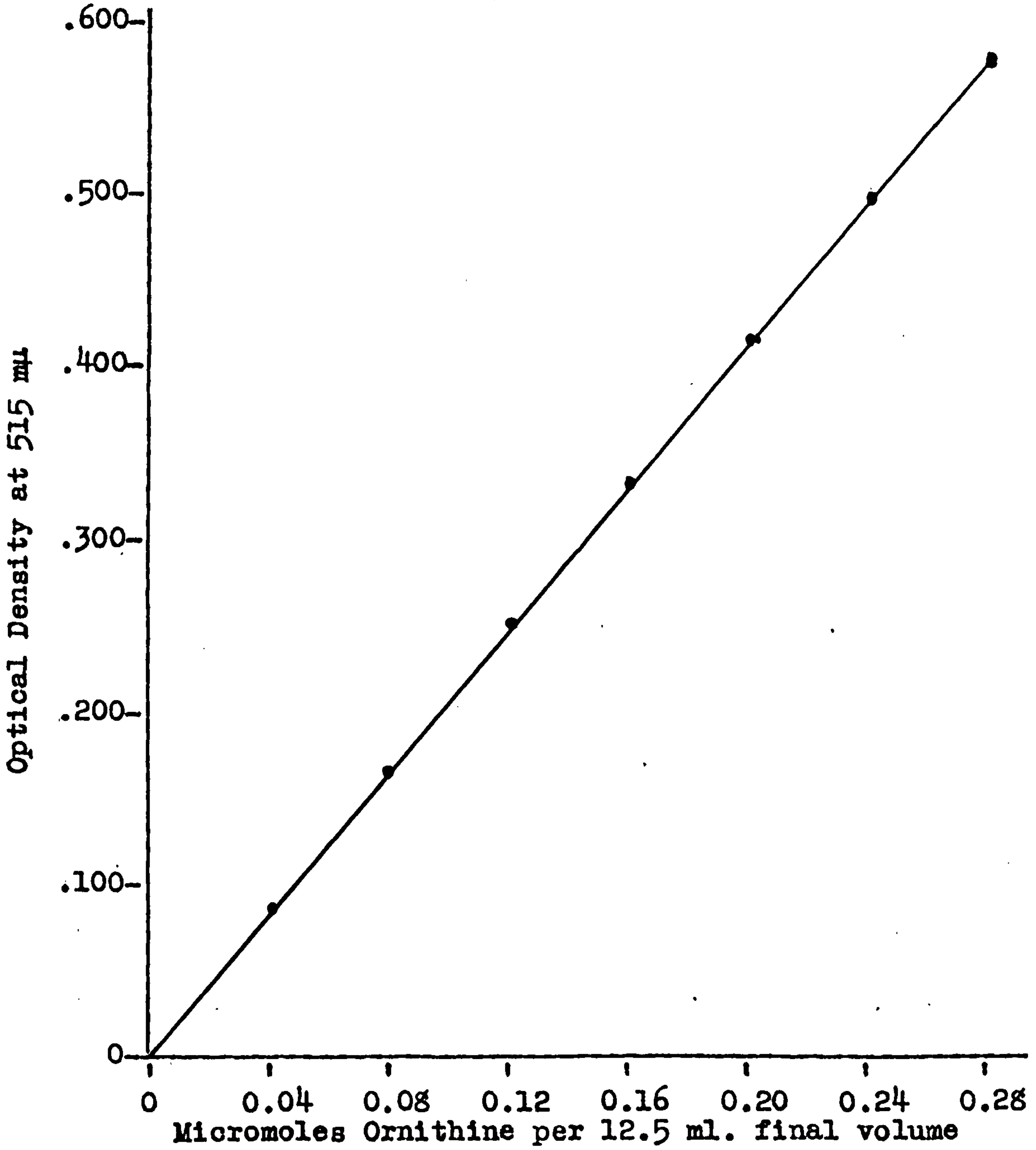


Figure 5. Standard Ornithine-Ninhydrin Curve Produced at pH 1.

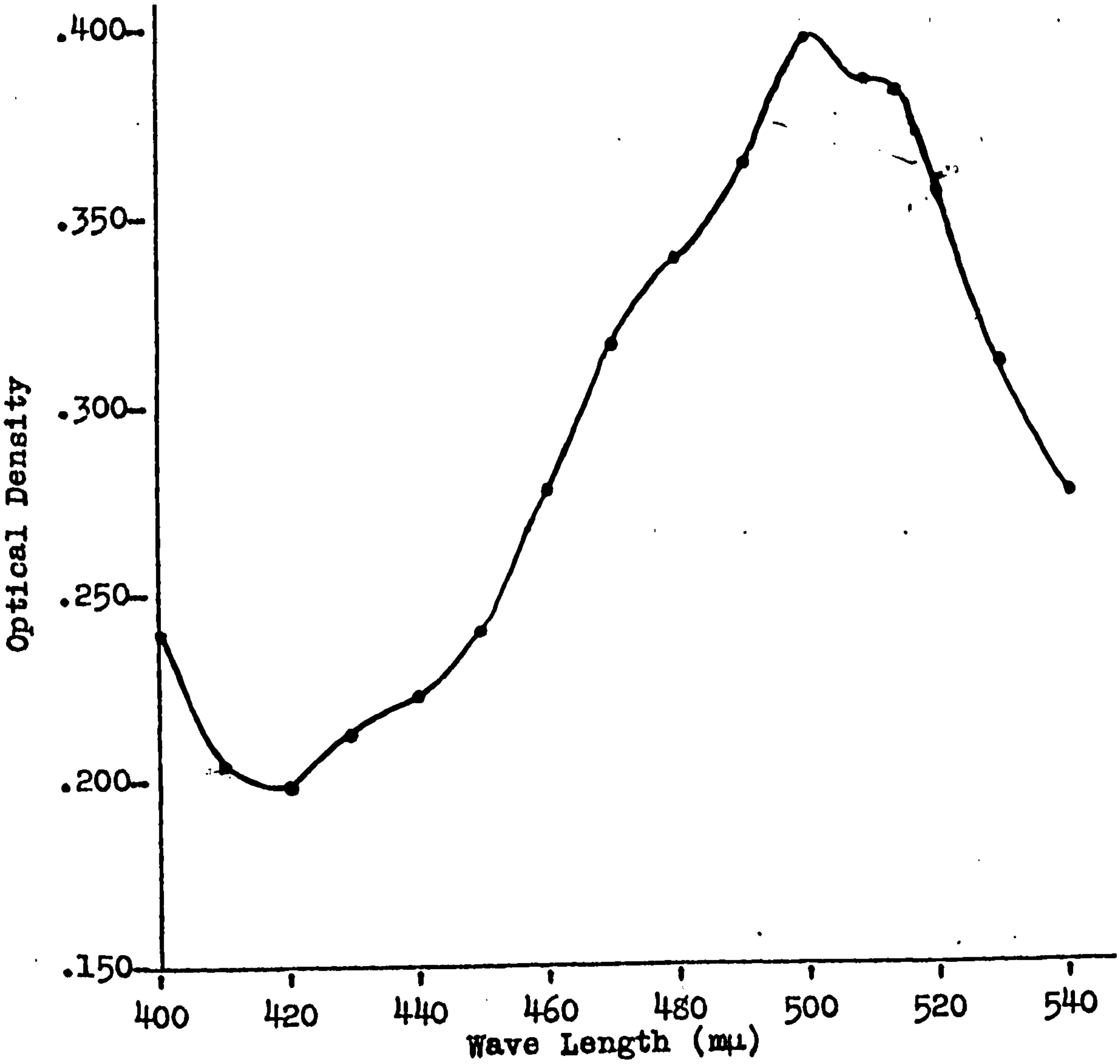


Figure 6. Absorption Spectrum Ornithine-Ninhydrin Color Produced at pH 1

Ornithine concentration 0.2 micromoles per 12.5 ml. final volume reaction mixture. Readings made in the Coleman Jr. Model Spectrophotometer.

were cooled to room temperature under running tap water, the glucose was aseptically added to the medium, and the bottle was immediately inoculated with 40 ml. of a fresh stock culture of Clostridium septicum (McClung's strain No. 78). The cells were then grown at 37° C. (water bath incubator) for 16-18 hours.

Preparation of Cell Suspensions. The cells from 800 ml. of 16-18 hour culture were removed by centrifugation at 0° C. in an International refrigerated centrifuge, washed once with distilled water, and finally suspended in 10 ml. of distilled water. The cell suspension was stored at 0°-4° C. for not more than 12 hours. Three-tenths ml. of this suspension were employed in each assay.

Assay Procedure. Incubations were carried out, and heat-deproteinized filtrates were prepared as outlined under "Incubation Mixtures." The production of carbon dioxide from a known quantity of ornithine on decarboxylation with Clostridium septicum was determined in the following manner: Five-tenths ml. of standard ornithine solution (containing 10 micromoles of L isomer) and 0.5 ml. of distilled water were pipetted into the center compartment of separate Warburg vessels. Five-tenths ml. of phosphate buffer (0.2 M, pH 6.0) were then added. Three-tenths ml. of Clostridium septicum suspension were pipetted into one side arm of each vessel, and 0.3 ml. of 6 M H_3PO_4 were pipetted into the other side arm of each vessel. The vessels were equilibrated at 37° C. for 15 minutes, the cell suspension was tipped in, and the vessels were shaken at 37° C. until carbon dioxide ceased to be evolved. The acid was then tipped in, and the manometers were read after they became constant. The reading of the ornithine standard was corrected for the blank, and microliters of CO_2 per micromole of ornithine was calculated.

The production of carbon dioxide from deproteinized reaction mixtures was determined in parallel runs. Reaction mixture filtrates and filtrates of reaction mixture blanks replaced the ornithine standard and distilled water, respectively. The unknowns were corrected for their respective blanks, and the production of CO_2 was calculated in microliters. The ornithine content of the unknowns was calculated by simple proportion:

$$\frac{\mu l.\ CO_2 \text{ produced by unknown}}{\mu l.\ CO_2/\text{mole ornithine (std.)}} = \mu M \text{ of ornithine in unknown}$$

<u>Specificity of the Method</u>. Although no detailed studies of ornithine decarboxylase specificity were made, it was established that neither arginine nor citrulline were attacked under the conditions of the assay. The procedure was therefore sufficiently specific for the purpose at hand, the determination of ornithine in the presence of citrulline and arginine.

Reproducibility and Accuracy of the Method. No detailed investigation of reproducibility or accuracy was made. The production of carbon dioxide from a given quantity of ornithine standard did not vary more than plus or minus 5% during ten assays, and all values were within 95% of the calculated values.

Determination of Citrulline

The citrulline content of trichloroacetic acid filtrates of reaction mixtures was determined by the colorimetric diacetyl reaction of Fearon (34), as modified by Kawerau (35).

Assay Procedure. One to seven ml. of trichloroacetic acid filtrates, containing 0.1 to 0.5 micromoles of citrulline, were pipetted into a Folin-Wu tube graduated at 12.5 ml. Two ml. of diacetyl reagent (1 gm. diacetyl monoxime in 100 ml. 5% acetic acid) and 3 ml. of either oxidizing mixture A or oxidizing mixture B were added. (Oxidizing mixture A was 1 vol. concentrated H_2SO_4 and 3 vol. sirupy H_3PO_4, using only the clear supernatant. Oxidizing mixture B was 10 gm. As_2O_3 in 100 ml. concentrated HCl.) The contents of the tubes were diluted to 12.5 ml., mixed by inversion, and the tubes were heated at 100^o in a deep water bath for exactly 20 minutes. The tubes were cooled to room temperature under running tap water, the volume was again adjusted to 12.5 ml. with distilled water, and the color was read in the Coleman Jr. Model Spectrophotometer at 490 millimicrons. Readings were made within 10 minutes of the time the tubes were removed from the cooling bath.

Curve Range. Employing a final volume of 12.5 ml., and reading the color at 490 millimicrons in the Coleman, the Beer-Lambert law was followed within the range 0 to 0.5 micromoles of citrulline. The sensitivity of the method fell off rapidly on either side of the absorption peak, the sensitivity at 515 millimicrons being less than half the sensitivity at 490 millimicrons. The absorption spectrum of the diacetyl color, as determined in the Coleman, is shown in Figure 8.

Reproducibility of Standard Curves. Standard curves were found to vary as much as plus or minus 10% from the mean, but triplicate samples of citrulline developed at the same time produced identical color intensities. The difficulty in reproducing standard curves appeared to be caused by variations in heating time and by differences in exposure to light during the color development. The critical nature of the heating period is shown in Table V. Satisfactory results were obtained by using a deep water bath (granite pitcher), by adherence to a set routine, by running duplicate analyses of unknowns, and by development of a standard curve with each series of unknowns.

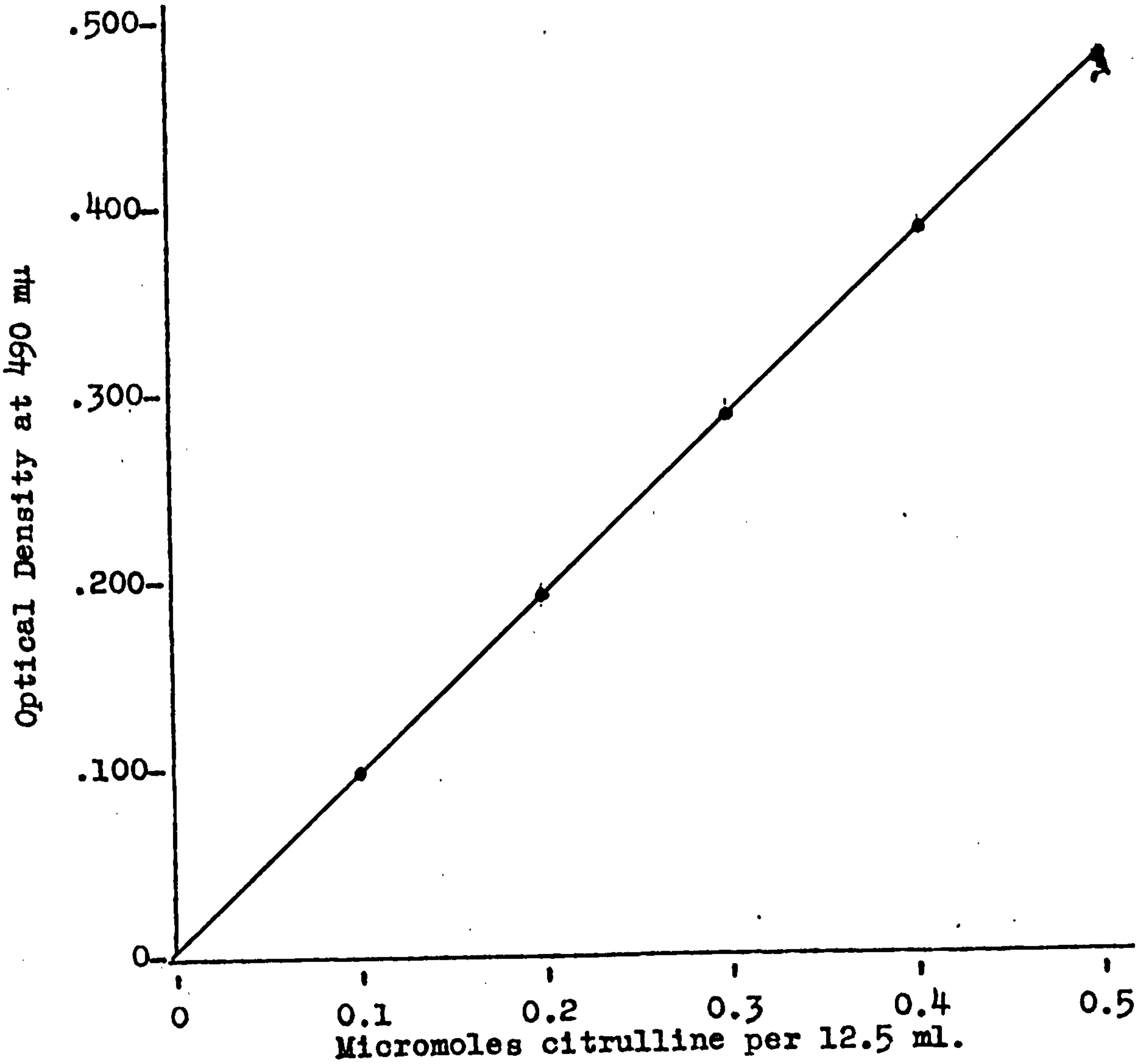


Figure 7. Standard Curve, Citrulline-Diacetyl Color

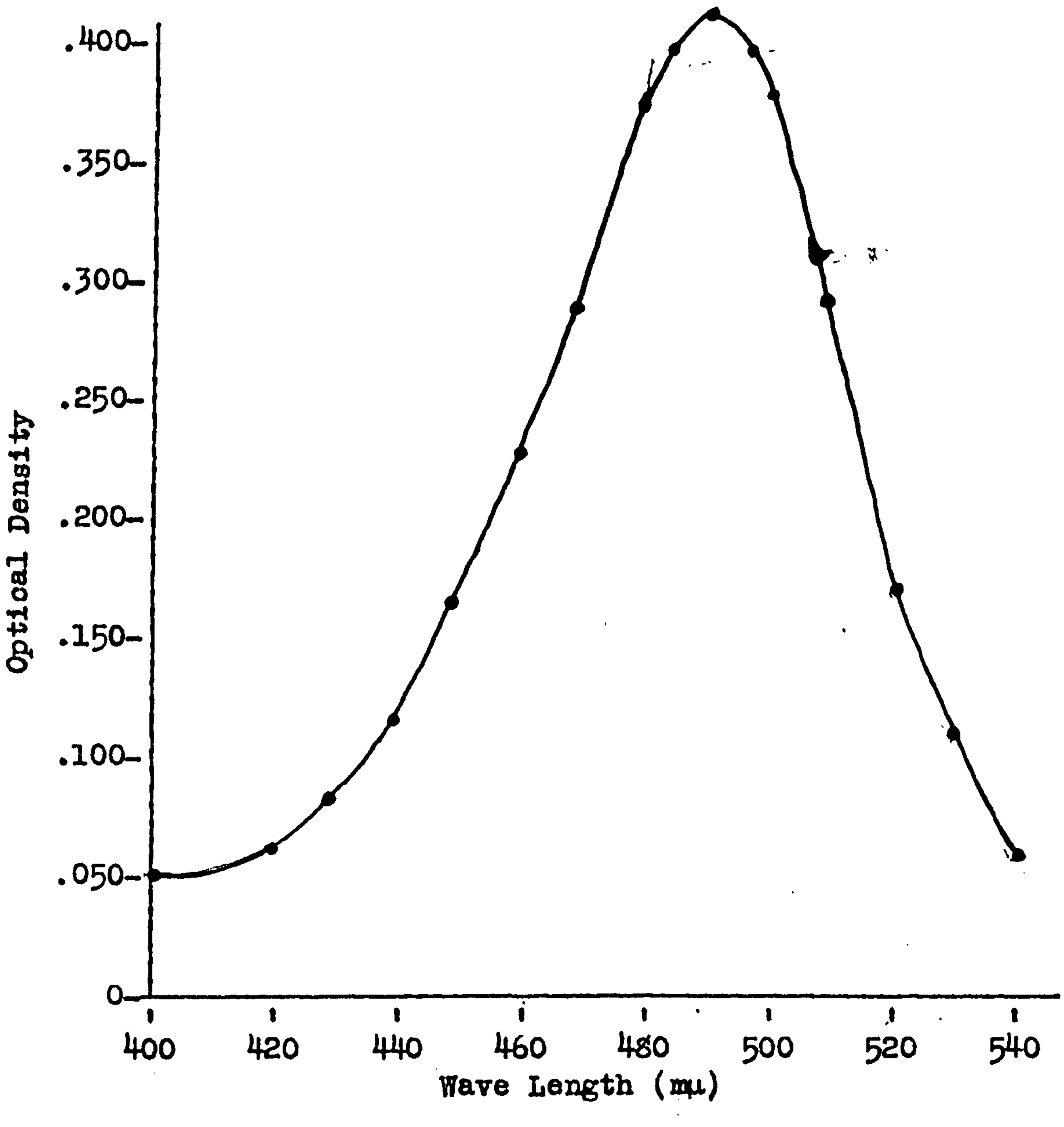


Figure 8. Absorption Spectrum of Citrulline-Diacetyl Color

Citrulline concentration 0.5 micromole per 12.5 ml. of reaction mixture. Readings made in Coleman Jr. Model Spectrophotometer.

Table V

Effect of Duration of Heating on Intensity of Citrulline Diacetyl Color

0.5 micromoles citrulline per 12.5 ml. Color developed by method of Kawerau (35). Readings made in Coleman Jr. Model Spectrophotometer at 490 millimicrons. Color read within 10 minutes of development.

Duration of Heating	Optical Densities Obtained on Repeated Runs			
10	0.375	0.355	0.385	0.340
20	0.470	0.440	0.480	0.420
30	0.420	0.395	0.435	0.380
40	0.210	0.180	0.210	0.175

Precipitation of Arginine Flavianate

Precipitation of arginine flavianate from reaction mixtures was carried our in essentially the manner described by Vickery (36). A volume of enzyme reaction mixture representing approximately 1 millimole of arginine (usually 100 ml.) was treated with enough 50% trichloroacetic acid to give a final concentration of 10%. The precipitated protein was removed by filtration and the trichloroacetic acid was removed by extraction with ether. The ether-extracted filtrate was evaporated to 50 ml. on the steam bath and filtered. If the filtrate was not weakly acid to Congo red paper a drop or two of hydrochloric acid was added, and the solution was precipitated as described below. A failure to precipitate arginine flavianate under these conditions indicated that the enzymatic degradation of arginine was at least 95% complete, since 50 micromoles of arginine in a final volume of 50 ml. produced a nearly quantitative yield of arginine flavianate, as indicated below.

Sensitivity of Flavianate Precipitation. The sensitivity of arginine flavianate precipitation was demonstrated by precipitation of known quantities of arginine with flavianic acid in a final volume of 50 ml. in the following manner:

Quantities of standard arginine solution representing 7.9, 79.0, 197.5, 395.0, 592.5, and 790.0 micromoles of arginine were pipetted into 100 ml. beakers, diluted to 50 ml. with distilled water, and acidified to Congo red by the addition of HCl. 1.3 grams of flavianic acid (ca. 5 mole/mole of arginine at highest arginine level) were added, and the beakers swirled to dissolve the flavianic acid.

The beakers were scratched to start precipitation if necessary, covered, and set aside in the cold room for four days. The precipitated arginine diflavianate was filtered off on a coarse sintered glass funnel or crucible and washed three times with a total volume of 30 ml. saturated aqueous flavianic acid. The crucible was attached to a small suction flask and the precipitated flavianate was stirred with a little hot distilled water in the crucible. 5 N NH_4OH was added from a 1 ml. pipette graduated at 0.1 ml. intervals until all the precipitate dissolved. The minimal amount of ammonia was used, the solution barely having an odor of ammonia.

The solution was drawn into the flask by suction and the filter washed with water until all the flavianate was washed out of the pores. The clear filtrate was washed back into the precipitation beaker and any crystals that escaped transfer were dissolved. The total volume of solution at this point was from 40-50 ml. The solution was carefully brought to a boil and 1 N H_2SO_4, slightly in excess of the amount of ammonia used to effect solution, was added. The beakers were scratched to start precipitation and allowed to stand at room temperature until precipitate ceased to form. The beakers were then transferred to the cold room and allowed to remain for at least 24 hours.

The precipitated arginine monoflavianate was filtered on a weighed micro-Gooch crucible, washed with a little cold ethanol, dried at 110° C. for a few hours, cooled in a desiccator, and weighed without undue exposure to air. Recoveries, calculated as percentage of theoretical, are shown in Table VI.

Table VI

Sensitivity of Arginine Flavianate Precipitation

Final molarities of arginine range from 0.000158 to 0.01580. Final volume at time of precipitation 50 ml. Theoretical weight of flavianate, expressed in mgm., equals millimoles arginine times 488.4; where 488.4 is the molecular weight of arginine monoflavianate.

Final Molarity Arginine	Arginine Present	Weight of Arginine Monoflavianate Theoretical	Actual	Recovery
	μM	mgm.	mgm.	%
0.01580	790.0	385.9	376.7	97.6
0.01185	592.5	289.4	277.4	96.6
0.00790	395.0	193.0	186.8	96.8
0.00395	197.5	96.5	90.2	93.5
0.00158	79.0	38.6	32.6	84.5
0.00016	7.9	3.9	Few crystals only	

Precipitation of Dixanthydryl Urea

The procedure of Graff (37) was employed in attempts to precipitate dixanthydryl urea from reaction mixtures.

<u>Reagents</u>. The following reagents were required for the precipitation of dixanthydryl urea:

1. Glacial acetic acid
2. Xanthydrol (Eastman Kodak Co.)
3. Dioxane
4. Solution of xanthydrol in methanol, approximately 10%. Suspend 10 grams xanthydrol in 100 ml. absolute methanol. Shake vigorously. After 2 days filter through dry paper into a narrow mouth amber bottle. Keep well stoppered.
5. Tanret's reagent. Suspend 1.35 grams $HgCl_2$ in 25 ml. of distilled water. Dissolve 3.32 grams KI in 25 ml. distilled water. Mix the two solutions and dilute to 60 ml. Add 20 ml. of glacial acetic acid.

<u>Procedure</u>. Five ml. of enzyme reaction mixture, representing 60 micromoles of arginine, were acidified with glacial acetic acid and the protein precipitated by the addition of 1 ml. of Tanret's reagent. The mixture was diluted to 10 ml. and the protein filtered off. Five ml. of the

filtrate (representing 30 micromoles arginine) were transferred to a test tube and 5 ml. of glacial acetic acid were added. Five-tenths ml. of methanolic xanthydrol were added, the tube was stoppered and shaken vigorously for about 10 minutes, then set aside for 1-2 hours. Dixanthydryl urea, if present, separated in loose clumps. The collected crystals may be recrystallized from a mixture of 2 parts dioxane and 1 part water.

Determination of Urea with Urease

The urea content of reaction mixtures was determined enzymatically by the method of Conway (38), employing Nesslerization of an aliquot from the center well, as described under "Determination of Ammonia."

Reagents. The following reagents were required for the estimation of urea in deproteinized reaction mixture filtrates.

1. Phosphate Solution. The phosphate solution for the preparation of urease was prepared in the following manner: 69 grams NaH_2PO_4 and 179 grams Na_2HPO_4 were dissolved in 100 ml. distilled water and stored in the refrigerator to prevent the formation of mold.

2. Permutit. Permutit for use in the preparation of urease was prepared in the following manner: 22 grams of finely powdered permutit were thoroughly washed with 2% acetic acid. The acid treated permutit was then washed twice with distilled water.

3. Stock Glycerol Extract of Urease. 22 grams of washed, acid treated permutit were mixed with 45 grams of finely powdered jack bean meal and 15 ml. of distilled water. The mixture was shaken for 30-45 minutes, 225 ml. of glycerol were added, and the whole was mixed by repeated inversion. The extract was filtered and the filtrate collected and stored in the cold room.

4. Diluted Urease for Use. The glycerol extract was diluted for use by the addition of 1 volume of phosphate solution and 8 volumes of distilled water to 1 volume of extract. The diluted extract was prepared fresh for each series of assays.

5. Other Reagents. The other reagents were prepared as described under "Determination of Ammonia."

Procedure. One ml. of reaction mixture filtrate, prepared as described under "Determination of Ornithine by Enzymatic Decarboxylation," and representing 12 micromoles of arginine, was diluted to yield not more than 6 micromoles of ammonia per ml. of diluted filtrate.

Five-tenths ml. of diluted filtrate were placed in the outer chamber of the Conway unit, and 0.5 ml. of urease-phosphate was added. One ml. of 0.2 N HCl was pipetted into the center well, the cover of the vessel was pressed into place, the fluid in the outer chamber was mixed by rotation, and the vessel was set aside for 20 minutes at room temperature. Two blanks, one in which 0.5 ml. of distilled water replaced the urease solution and one in which 0.5 ml. of distilled water replaced the sample, were set up at the same time in order to determine the preformed ammonia and ammonia from the urease preparation.

After incubation, the vessels were slightly tilted, 1 drop of phenolphthalein and 1 ml. of saturated potassium carbonate were added, and the cover was quickly replaced and pressed into position. The vessels were permitted to stand at room temperature for 100 minutes, the covers were removed, and an aliquot from the center well of each vessel was Nesslerized as described under "Determination of Ammonia." The difference between ammonia production in the presence of urease and ammonia production in the absence of urease (preformed ammonia) represented the urea content of the reaction mixture, after correction for dilution.

Determination of Arginine with Arginase

The arginine content of reaction mixtures was determined enzymatically by modifications of the Graff (37) and Hunter (39) procedures. The urea liberated on treatment with arginase was estimated by both xanthydrol precipitation and by treatment with urease in the Conway apparatus.

Preparation of Arginase. Fifty grams of fresh beef liver were ground in a mortar with a little sand and 50 ml. of 75% glycerol. The mixture was transferred to a centrifuge bottle and shaken for 10-15 minutes, then placed in a 60-65° water bath until the contents of the bottle had reached 58° C. The temperature was held at 58° C. for 5 minutes, the suspension was cooled under the tap, centrifuged in the refrigerated centrifuge at 0° C., and the supernatant stored in the ice box for 12 hours. The preparation was adjusted to pH 7 with NaOH after standing in the ice box.

Incubation Procedure. Five ml. of reaction mixture, containing 12 micromoles of arginine per ml., and deproteinized by heating as described under "Determination of Ornithine by Decarboxylation," were pipetted into each of two 8 inch culture tubes. One ml. of arginase was added to one tube and 1 ml. of distilled water was added to the other tube, a few drops of toluene were added to each, the tubes were stoppered and incubated in a 37° C. water bath for 24 hours.

Analysis by Urease Method. Two ml. aliquots were removed from each tube and diluted to 10 ml. with distilled water. Two samples of 0.5 ml. each were removed from each dilution and pipetted into the outer chambers of four separate Conway vessels. One ml. of 0.2 N hydrochloric acid was pipetted into the center well of each vessel. Five-tenths ml. of distilled water was added to the outer chamber of one vessel containing arginase (A) and one vessel containing no arginase (B). Five-tenths ml. of urease solution was added to the outer chamber of the other vessel containing arginase (C) and to the other vessel containing no arginase (D). The vessels were then incubated and analyzed for ammonia in the manner described under "Determination of Urea with Urease."

The ammonia found in vessel (C) represented preformed ammonia, ammonia from the arginase preparation, ammonia from the urease preparation, ammonia from preformed urea, and ammonia that resulted from the decomposition of arginine to urea on treatment with arginase. The ammonia found in vessel (A) was preformed ammonia, plus ammonia arising from the arginase preparation itself. The ammonia found in vessel (D) represented preformed ammonia, ammonia from the urease preparation, and ammonia from the action of urease on any preformed urea that was present. The ammonia found in vessel (B) represented only preformed ammonia.

The sum of ammonia production in (A) and (D), minus the ammonia production in (B) therefore represented preformed ammonia plus ammonia from the arginase preparation plus ammonia from the urease preparation plus ammonia from any preformed urea that might have been present. Ammonia production in (A) plus that in (D), minus that in (B) was therefore the blank. Ammonia production in (C) minus the blank represented ammonia produced as a result of the action of arginase on arginine, and was therefore the desired analytical figure.

Analysis of Xanthydrol Precipitation. For the analysis of arginase reaction mixtures by the xanthydrol precipitation method, the following procedure was used: A 5 ml. sample was removed from the incubation tube that contained arginase, and a 5 ml. sample was removed from the tube that contained no arginase. The first sample represented preformed urea plus urea produced as a result of the action of arginase on arginine, the second sample represented preformed urea only. The two samples were treated as described under "Precipitation of Dixanthydryl Urea," and the precipitates of dixanthydryl urea were weighed. The difference in the two weights represented dixanthydryl urea produced as a result of the action of arginase on arginine, and therefore represented the arginine that was present.

Paper Chromatography of Reaction Mixtures

Ascending paper chromatograms were employed to demonstrate the absence of arginine, and for the tentative identification of the suspected reaction products, citrulline and ornithine. The following procedure was employed.

Untreated Whatman No. 1 or No. 2 filter paper was cut into 22 cm. squares, and a base line was drawn with a pencil 1 cm. from one edge of the paper. The paper was rolled into a cylinder, with the base line visible, and the edges of the cylinder were sewed together with thread. These paper cylinders were chromatograms.

Aqueous solutions of arginine, citrulline, and ornithine were prepared to contain 12 micromoles of the desired amino acid per ml., and trichloroacetic acid in a final concentration of 10%.

Water miscible solvents containing ethanol and water, or methanol and water, with added triethylamine or pyridine, were prepared in various alcohol concentrations and stored in tightly stoppered bottles in the constant temperature room (25° C.).

Chromatography of the amino acid solutions was carried out in the following manner: Small spots of the amino acid solutions were placed at intervals along the base line and the papers were air dried. The location and identification of each amino acid was indicated by a pencil dot and number placed on the chromatogram prior to addition of the amino acids. The desired solvent mixture was placed in a Petri dish, the dish was covered with a 6 inch x 24 inch Pyrex cylinder, and the vapor under the Pyrex cylinder was allowed to reach equilibrium with the solvent phase.

The dried chromatogram was set in the solvent, base line down, the Pyrex cylinder was replaced, and the solvent was allowed to ascend to within 3 cm. of the top edge of the chromatogram. The time required for ascension varied with the solvent mixture that was used, but was usually about 6 hours. The chromatogram was removed, the solvent front was quickly marked with a series of pinholes or with a pencil, the threads were cut, and the chromatogram was suspended by means of a clamp and air dried. The air dried chromatogram was dipped in 0.2% ninhydrin in 90% isopropanol, dried in an oven at 105° C. to develop the spots, and the spots outlined with a pencil. The Rf values of the various amino acids were then calculated.

Choice of Solvent Mixtures. Solvent mixtures for use in demonstrating the absence of arginine and the presence of both citrulline and ornithine were chosen on the basis of the results shown in Figure 9 and Figure 10. On the basis of these two diagrams, the solvents considered to give the best separation of citrulline, ornithine, and arginine were 85% methanol, 80% methanol, 80% ethanol, and 70% ethanol, all having triethylamine present in a final concentration of 4%. 80% isopropanol was also used, but movement of all three amino acids was slight. 70% methanol, containing pyridine in a final concentration of 20% was the most effective solvent for the separation of ornithine and citrulline, but was used only when arginine was known to be absent since it did not separate ornithine and arginine.

Procedure for Identification of Unknowns. The procedure for the identification of reaction mixture products was identical with that outlined above, except that a spot of trichloroacetic acid filtrate of the reaction mixture was added in addition to the others.

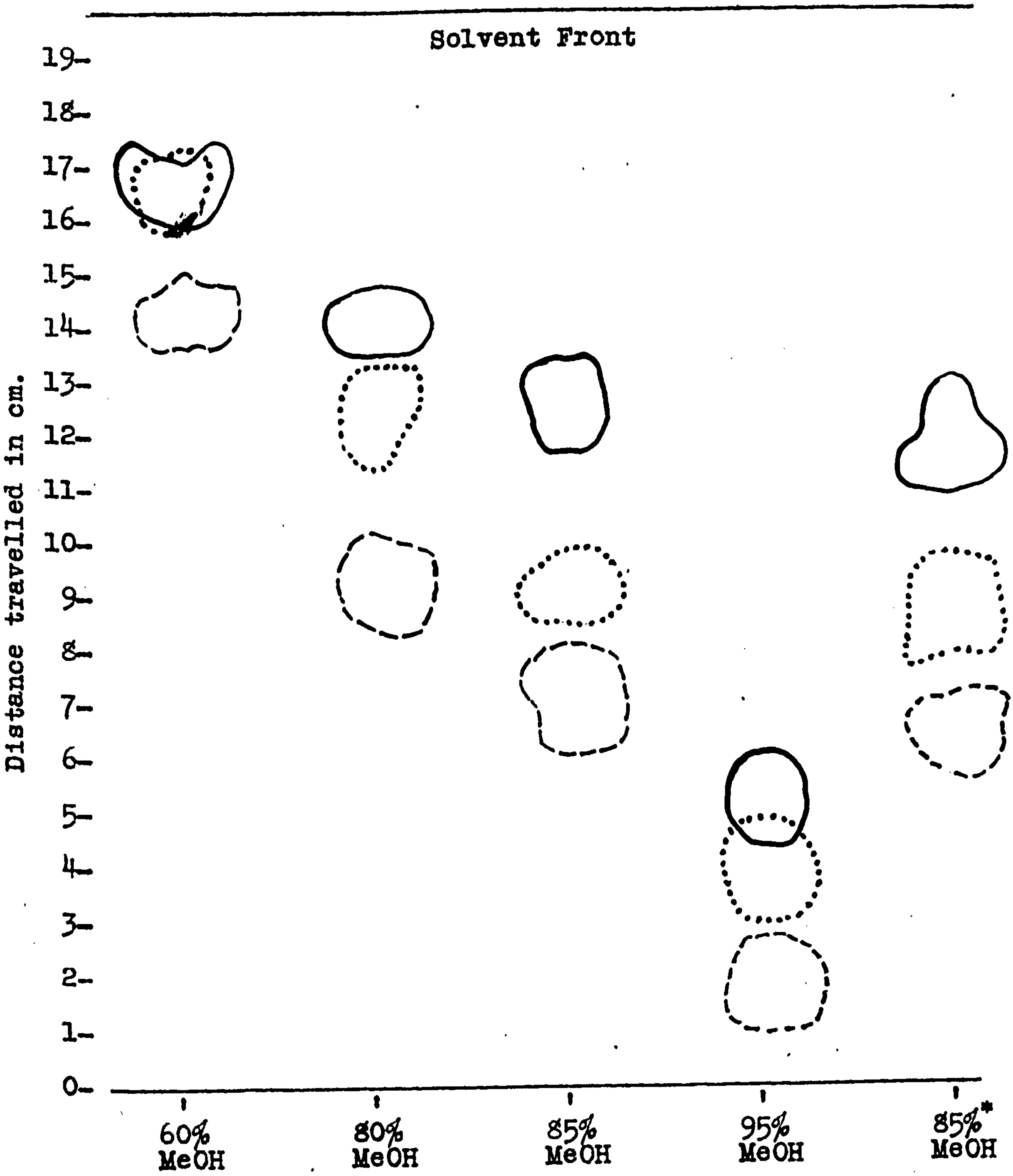


Figure 9. One Dimensional Ascending Chromatograms Employing Methanol

All solvents contain 4% triethylamine plus H_2O to make 100%.
Citrulline ▬▬ , ornithine ...., arginine — — —.
* Whatman No. 1 paper used instead of Whatman No. 2 paper.

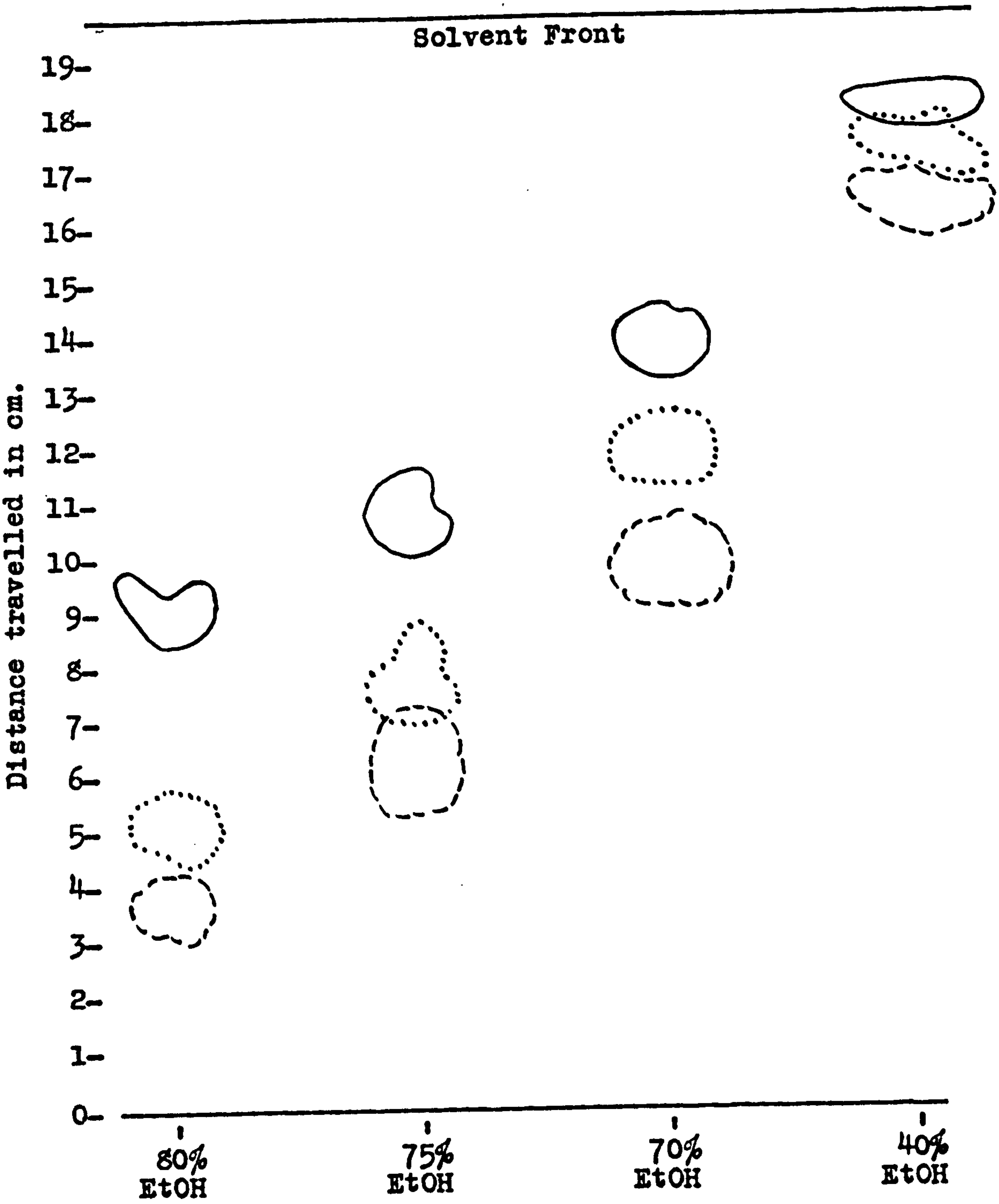


Figure 10. One Dimensional Ascending Chromatograms Employing Ethanol.

All solvents contain 4% triethylamine plus H_2O to make 100%
Watman No. 2 paper employed.
Citrulline——, ornithine....., arginine-----.

Determination of Alpha Amino Nitrogen and Carboxyl Carbon Dioxide by the Gasometric Ninhydrin Method

Arginine, and other amino acids, when boiled with an excess of ninhydrin, evolve 1 mole of carbon dioxide from the carboxyl group. The reaction, which was discovered by Ruhemann (40), is specific for free amino acids, since it requires the presence of the unconjugated carboxyl group and the alpha amino group. The method was employed in this investigation to determine whether or not the carboxyl-alpha amino relationship of arginine was disturbed during the degradation of arginine by *Clostridium perfringens*, thereby establishing the source of ammonia and carbon dioxide known to be produced during the enzymatic reaction. The procedure employed was a combination of two published methods (41,42).

Apparatus and Reagents

1. Van Slyke-Neill manometric assembly (42) and Pyrex reaction vessels (41).
2. Ninhydrin (Pierce Co.)
3. Citrate buffer, pH 2.5; prepared in the following manner: 2.06 grams trisodium citrate and 19.15 grams citric acid were ground to fine powders, mixed, and ground again.
4. Lubricant, "Nevastane XX" (E. Machlett and Sons, N. Y.)

5. Approximately 0.5 N NaOH of minimal CO_2 content, in nearly saturated sodium chloride; prepared by dilution of saturated, aqueous, CO_2-free NaOH with 25% aqueous, CO_2-free NaCl solution.
6. Approximately 2 N lactic acid in nearly saturated NaCl; prepared by diluting 1 vol. concentrated lactic acid (sp. gr. 1.20) with 5 vol. 25% NaCl solution.

Range. For submicroanalyses, reading at the 0.5 ml. level, 3 to 15 micromoles of carbon dioxide gave a pCO_2 of 100 to 500 mm. For microanalyses, reading at the 2 ml. level, 10 to 50 micromoles of carbon dioxide gave a pCO_2 of 100 to 500 mm. of mercury.

Preparation of Perfringens Reaction Mixture for Analysis. The reaction mixture was deproteinized by boiling, in the manner outlined under "Determination of Ornithine by Decarboxylation." One or two ml. of the deproteinized filtrate were transferred to an all glass reaction vessel, a drop of bromphenol blue was added, and the pH was adjusted to 3 or 4 by the addition of acid. Fifty mgm. of citrate buffer were added to each tube.

Removal of Preformed Carbon Dioxide. Carbon dioxide, preformed in the degradation of arginine by *Clostridium perfringens* was removed in the following manner: A few carborundum granules were added as an anti-bumping agent, and a drop of caprylic alcohol was added as an anti-foam agent. The reaction mixture was heated to boiling over a microburner and boiled for exactly one minute. The vessel was set in an ice bath and cooled to room temperature (below 25° C., 2 minutes cooling required).

Addition of Ninhydrin. The ground glass surface of the reaction vessel was dried with a clean cloth, 100 mgm. of ninhydrin were added through a funnel, and the greased stopper was set in place with the opening through the side arm open, and the vessel was immediately evacuated to 30 mm. pressure or less. After evacuation was complete the vessel was sealed by turning the stopper, and set aside for boiling. Undue effervescence of the solution was controlled by tilting and shaking during the evacuation and by controlling the vacuum with the stopcock of the vessel.

Heating. Reaction vessels, prepared and evacuated in the manner just described, were immersed in an actively boiling water bath and boiled for 30 minutes, shaking gently during the heating period. The tubes were removed from the bath, the stoppers were turned 5 to 10 degrees to insure a complete seal, and set aside for analysis.

Absorption of Carbon Dioxide. The chamber of the Van Slyke apparatus was charged with 2 ml. of alkali-sodium chloride, and the gases extracted and ejected in the usual manner (41). The glass plug was removed from the all-glass reaction vessel, and the side arm was evacuated on the vacuum pump, to remove any carbon dioxide that might have evolved from the rubber connection and collected in the side arm. The tube was then pinched flat and attached to the side arm of the Van Slyke apparatus and the reaction tube warmed to 38° C. The mercury was lowered about half way in the chamber, the all-glass reaction vessel was opened to the Van Slyke chamber, and the carbon dioxide was transferred to the alkali by raising and lowering the mercury six times. The reaction vessel was swirled during each lowering of the mercury to insure complete transfer of the carbon dioxide. If the tubes were not warmed to 38° C. 10-15 excursions were required to effect complete absorption of carbon dioxide. After the last upward trip, the mercury was lowered to the middle of the chamber, the stopcock at the top of the chamber and that leading to the levelling bulb were closed, the reaction vessel was removed, and the side arm of the Van Slyke apparatus was sealed with mercury. The mercury was lowered to the 50 ml. mark, the chamber was shaken for 3 minutes to extract gases, the gases were ejected from the chamber through the upper stopcock, and the chamber was sealed with mercury.

Liberation of Carbon Dioxide. The technique of carbon dioxide liberation was that previously described (41), except that the lactic acid was saturated with sodium chloride, a 3-minute shaking period was employed, and the carbon dioxide was not reabsorbed.

One ml. of 2 N lactic acid was introduced to the chamber through a mercury seal, the chamber was sealed with mercury, and the mercury in the chamber was lowered to the 50 ml. mark. The chamber was shaken for 3 minutes, the meniscus of the mercury was raised to the 2 ml. or 0.5 ml. mark (depending on the amount of carbon dioxide present), and the manometer pressure was read. This reading was p_S.

Blanks. Blanks were carried through the entire procedure, and the pressure exerted by blank carbon dioxide was determined in the manner outlined, the only difference being that blank tubes contained no arginine during the incubation with *Clostridium perfringens*. The pressure exerted by the blank (p_B) was subtracted from the pressure exerted by the sample (p_S) to give the corrected sample reading (p_C).

Calculations. The number of micromoles of carbon dioxide liberated by the action of ninhydrin, and therefore the number of micromoles of amino acid remaining, was calculated from the following formula:

$$\text{Micromoles } CO_2 = \frac{p_C \times F \times 1000}{12}$$

where F is the Van Slyke factor (41) for converting pCO_2 to milligrams of carbon. F varies with the level at which readings are made, so that the correction differs depending on whether readings are made at 2 ml. or 0.5 ml.

Recovery from Known Arginine Solutions. The reliability of the method was checked by analysis of standard arginine solutions covering the expected range of carbon dioxide production. The results of these determinations, which are shown in Table VII, indicate satisfactory recoveries of ninhydrin carbon dioxide in the range 5 to 50 micromoles of arginine by this method.

Table VI

Recovery of Carbon Dioxide from Standard Arginine Solutions by the Gasometric Ninhydrin Procedure

Volume reaction mixture 1-2 ml. Reaction carried out in citrate buffer at pH 2.5. Heating time 30 minutes. 100 mgm. ninhydrin per tube. Carbon dioxide determined in Van Slyke-Neill apparatus by absorption in alkali and liberation with lactic acid. Carbon dioxide pressure was read without reabsorption in alkali. Readings made to 2 ml. or 0.5 ml. level, depending on the amount of CO_2.

Arginine Used	CO_2 Recovered	CO_2 Recovery
µM	µM	%
50.00	50.00	100.0
45.00	44.99	100.0
30.00	29.99	100.0
30.00	29.80	99.3
20.00	19.98	99.9
10.00	10.13	101.3
8.00	7.75	96.9
6.00	5.92	98.7
6.00	6.00	100.0
6.00	6.03	100.5
5.00	4.88	97.7

EXPERIMENTAL PROCEDURE AND RESULTS

Production of Ammonia and Carbon Dioxide from Arginine

The production of ammonia and carbon dioxide from arginine was found to occur with both washed cell suspensions and suspensions of lyophilized cells. Carbon dioxide production was measured in the Warburg apparatus, and ammonia production was measured by the Conway microdiffusion technique, as described under "Methods."

Overall Production of NH_3 and CO_2. The overall production of NH_3 and CO_2 was determined on reaction mixtures incubated for 3 hours at 37° C. in the presence of pH 7.2 phosphate buffer. The results of these determinations, which are shown in Table VIII, indicate that the excess of NH_3 over one mole per mole of arginine equals the total molar quantity of CO_2 produced. The results of these experiments, which were carried out under essentially the same conditions as were employed by Woods and Trim (1), confirm the findings of these authors insofar as the relative production of carbon dioxide and ammonia is concerned.

Disappearance of Arginine. The complete disappearance of arginine from three-hour incubation mixtures was demonstrated by negative Sakaguchi reactions, by failure to precipitate arginine monoflavianate, by failure to produce

Table VIII

Production of Ammonia and Carbon Dioxide from Arginine by *Clostridium perfringens* (BP6K)

Incubations 90 min. at 37° C. in phosphate buffer pH 7.2 and final molarity of 0.18. Final substrate concentration 3.2 micromoles of L(+) isomer per ml. Warburg flasks acid tipped at the end of the reaction.

Number of experiments	Moles of product per mole of arginine originally present (Range)	Moles of product per mole of arginine originally present (Average)
	CARBON DIOXIDE	
7*	0.41 - 0.51	0.46
3**	0.54 - 0.63	0.58
	AMMONIA	
7*	1.41 - 1.56	1.47
3**	1.55 - 1.70	1.62

* Final cell concentration 9.6 mgm. per ml. when lyophilized cells were used.

** Final cell concentration 4.84 mgm. per ml. when washed cells were used.

urea on treatment with arginase, and by failure to demonstrate an arginine spot on paper chromatograms. Although no arginine spot was present on chromatograms, the presence of two new spots was noted. The following experimental procedure was employed in this series of experiments:

One hundred ml. of reaction mixture (pH 7.2, phosphate buffer 0.18 M final concentration, 6 mgm. washed cells per ml., 12 micromoles arginine per ml.) were incubated at 37° C. for 3 hours. This was the "Primary Mixture." Blank mixtures, one in which water replaced the cell suspension ("Arginine Blank"), and one in which no arginine was present ("Cell Blank") were incubated in parallel with the "Primary Mixture."

At the completion of the incubation a 1 ml. aliquot was removed from each flask, pipetted into test tubes containing 9 ml. of 10% trichloroacetic acid (TCA), and the precipitated protein filter off. The resulting TCA filtrates were used for the control determination of ammonia and for demonstrating disappearance of the Sakaguchi color. A 1 ml. aliquot was removed from each of the three original reaction mixtures and the protein precipitated with 5 drops of 50% TCA. The protein was removed by centrifugation and the clear supernatants were used for demonstrating the disappearance of arginine by paper chromatography. A 10 ml. aliquot was removed from each of the incubation mixtures, deproteinized by boiling, and the filtrates used to demonstrate the disappearance of arginine by the arginase method. The remainder of each incubation mixture was treated with enough 50% TCA to give a final concentration of 10%, the precipitated protein was filtered off, and the filtrates were employed in flavianate precipitations.

In the control determination of NH_3, no NH_3 was produced by either of the two blanks. NH_3 production in the "Primary Mixture" represented 1.5 moles per mole of arginine. In Sakaguchi determinations, the "Arginine Blank" showed a residual arginine content of 0.98 moles per mole of arginine originally present. The "Primary Mixture" showed no residual arginine.

Paper chromatograms employing 85% methanol, 80% methanol, 80% ethanol, and 70% ethanol gave the following results: The "Arginine Blank" produced a single spot, corresponding in each instance to a spot produced by a standard arginine solution. The "Primary Mixture" produced <u>no</u> spot corresponding to arginine, but produced two definite, unidentified spots with each of the solvent mixtures used. The unidentified spots were later identified as citrulline and ornithine. The "Primary Mixture" also produced several very faint spots that corresponded, in each case, to spots produced by the "Cell Blank." These faint spots were, therefore, apparently due to products of cell lysis.

Analysis by the arginase method produced the following results: Treatment of the "Primary Mixture" with arginase and urease produced no more ammonia than was found in the control. Treatment of the "Primary Mixture" with urease, followed by the addition of xanthydrol reagent, produced no dixanthydryl urea. Treatment of the "Arginine Blank" with arginase plus urease yielded 0.9 moles of ammonia per mole of arginine, over and above the ammonia found in the control. Treatment of the "Arginine Blank" with urease, followed by the addition of xanthydrol reagent, produced a precipitate of dixanthydryl urea. The precipitate was not weighed.

On precipitation of the three reaction mixtures with flavianic acid a precipitate was obtained with the "Arginine Blank" only. The precipitate was filtered off, recrystallized, dried and weighed. 465 mgm. of arginine monoflavianate, corresponding to an overall yield of 90% of theoretical, were obtained.

Effect of pH Changes. The pH optimum for the production of NH_3 from arginine by washed cell suspensions was determined in the following manner: Ammonia determinations were carried out after incubating reaction mixtures at pH 5.5, pH 6.3, pH 7.2, and pH 7.8 for 3 hours. Similar incubations were set up in the Warburg apparatus, the flasks were acid tipped after incubating for 3 hours, and the carbon dioxide production was determined. Curves drawn from the data so obtained are shown in Figure 11. The curves show that, in the pH range 5.5 to 7.2, the overall production of both ammonia and carbon dioxide was within 90% of the maximum obtained at pH 6.3. Considerably less ammonia and carbon dioxide were produced at pH 7.8.

The effect of pH changes on the rate of ammonia production from arginine was determined in the same way, except that samples were withdrawn at 10 minute intervals for a period of 1 hour. The TCA filtrates were analyzed for ammonia, and ammonia production was plotted as a function of time at each pH level. These curves are shown in Figure 12. Within the pH range 5.5 to 7.2, the rate of formation of ammonia from arginine never fell below 80% of the maximum obtained at pH 6.3. The rate decreased significantly at pH 7.8. These findings are in good agreement with the results reported by Woods and Trim (1).

Inhibition by Borate and Phthalate. An apparent inhibition of ammonia production was noted when borate and phthalate buffers were employed. Further investigation showed that ammonia production did not exceed one mole per mole of arginine. Duplicate incubations were carried out at pH 6.2, one tube containing phosphate buffer and the other containing phthalate buffer. Duplicate incubations were also

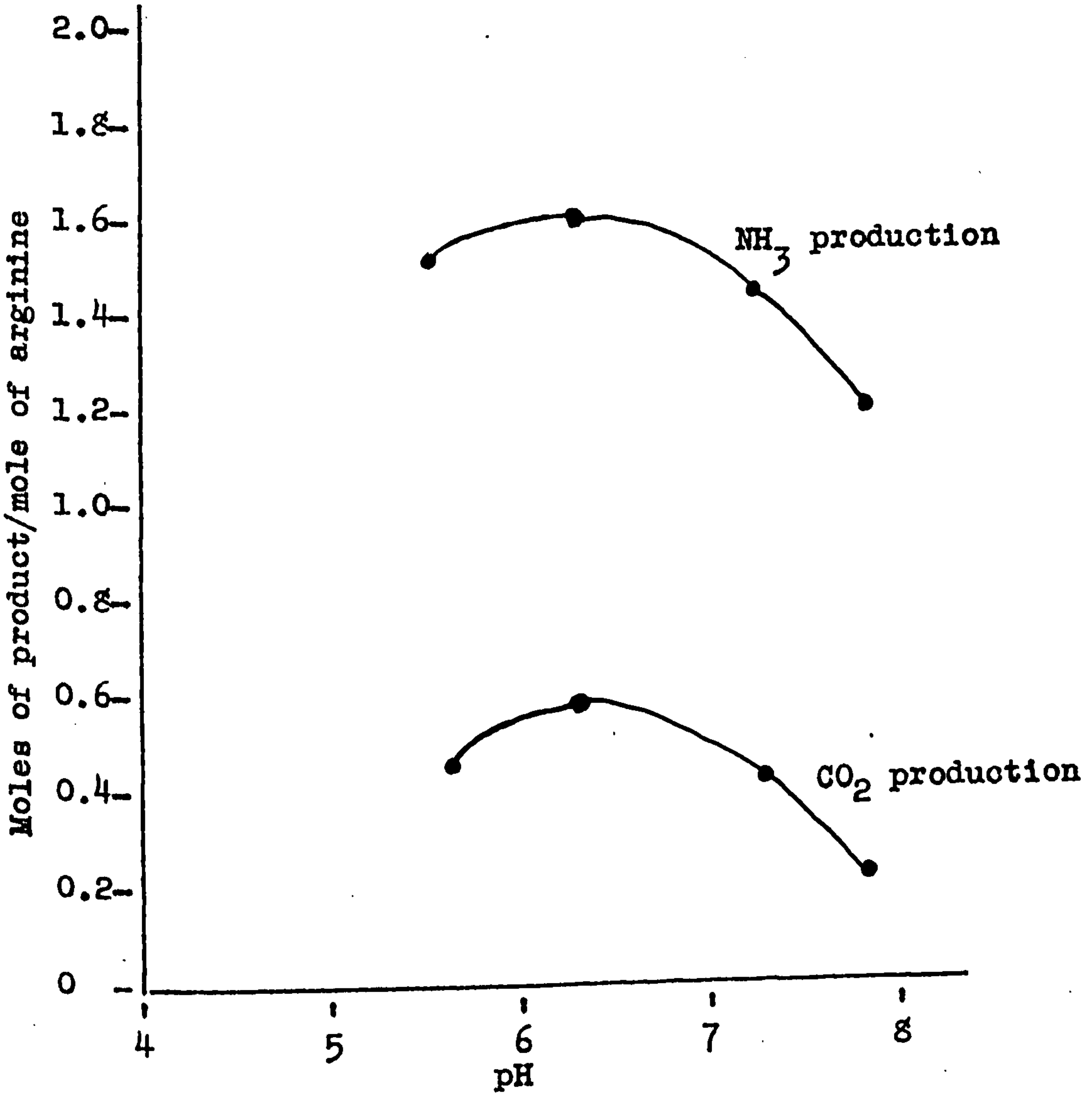


Figure 11. pH Optima for the Overall Production of Ammonia and Carbon Dioxide

Incubations at 37° C. in phosphate buffer, final concentration 0.18 M. Final substrate concentration 12 micromoles per ml. Final cell concentration 6 mgm. per ml. Duration of incubation 3 hours.

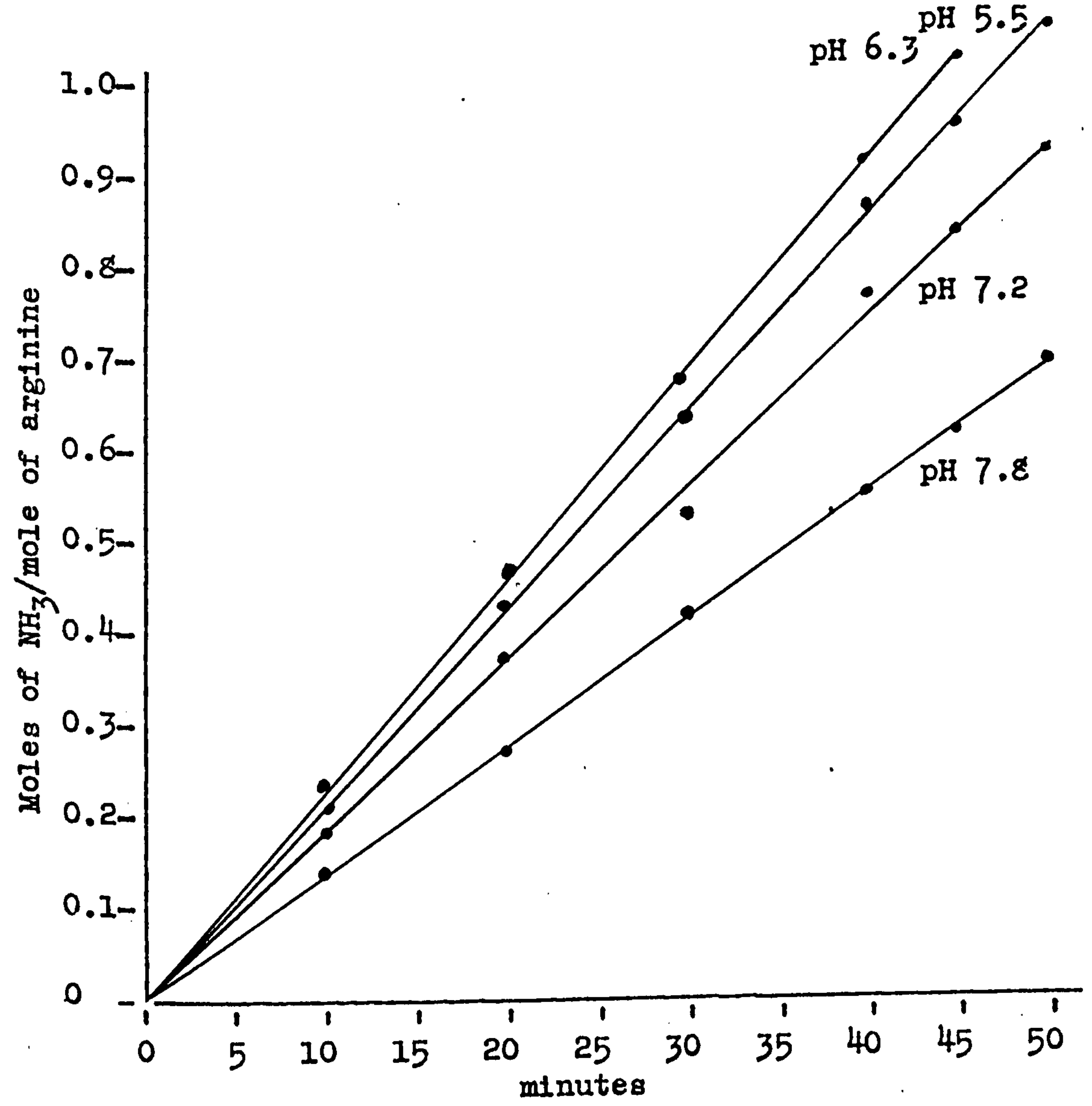


Figure 12. Effect of pH Changes on Rate of Formation of Ammonia.

Incubations at 37° C. in phosphate buffer, final concentration 0.18 M. Final substrate concentration 12 micromoles per ml. Final cell concentration 6 mgm. per ml. of reaction mixture.

carried out at pH 7.8, one tube containing phosphate buffer and the other tube containing borate buffer. Substrate, cell, and buffer concentrations were identical in all cases and corresponded to incubation mixtures previously described. Samples were removed after incubating for 1 hour and after incubating for 3 hours. TCA filtrates were prepared and analyzed for ammonia content. The results are shown in Table IX.

Table IX

Inhibition of Ammonia Production from Arginine in the Presence of Borate and Phthalate Buffers

Incubation Mixtures: Borate, phthalate, or phosphate buffer of the desired pH and 0.18 M final concentration. Final cell concentration 4.2 micromoles per ml. Final substrate concentration 12 micromoles per ml.

Composition and pH of Buffer	Moles of Ammonia Produced per Mole of Arginine Originally Present	
	1 hour	3 hours
Phosphate, pH 6.2	0.72	1.68
Phthalate, pH 6.2	0.61	0.98
Phosphate, pH 7.8	0.70	1.45
Borate, pH 7.8	0.58	1.02

These figures show that both borate and phthalate decreased production of ammonia. The extent of inhibition appears to be the same in both buffers, and in neither case does ammonia production exceed one mole per mole of arginine. This observation suggested the presence of two enzymes, one of which was responsible for the production of one mole of NH_3 from arginine and was not inhibited by either borate or phthalate. The second enzyme appeared to be responsible for the fractional mole of ammonia and was inhibited by both borate and phthalate, but not by phosphate.

Effect of Cell Aging. Woods and Trim (1) reported that the production of ammonia and carbon dioxide from arginine decreased as the growth period of the cells increased. Although no such studies were included in this investigation, a considerable amount of work was done with old cells during the early stages of the investigation. The cells employed at that time were obtained from Lederle laboratory and had been grown for 16-18 hours. They were shipped as a sludge obtained from the Sharples centrifuge, and were often stored at 4° C. for a week before they were washed and lyophilized. In no instance did these cells produce more than 1 mole of ammonia per mole of arginine, and in no instance was the production of carbon dioxide from arginine demonstrated. Furthermore, studies of the effect of phthalate and borate buffers showed no inhibition with respect to phosphate. These facts suggested that the Lederle cells contained the enzyme responsible for the production of one mole of ammonia, but did not contain a second enzyme, which was assumed to be responsible for the fractional mole of ammonia. They also suggested that carbon dioxide production and the production of the fractional mole of ammonia were closely related.

Source of Ammonia and Carbon Dioxide

The ammonia and carbon dioxide produced from arginine on incubation with washed cell suspensions did not arise from the alpha amino and carboxyl groups of arginine. Aliquots of incubation mixtures were removed at intervals for a period of 3 hours, deproteinized by boiling, and the deproteinized filtrates analyzed by the gasometric ninhydrin method. The results of two such determinations are shown in Table X. A loss of either carboxyl CO_2 or alpha amino NH_3 during the course of the enzymatic reaction would have resulted in a progressive decrease of ninhydrin CO_2. That no such decrease occurred is apparent from the figures in Table X. Control determinations of ammonia production and Sakaguchi disappearance indicated that the enzymatic reaction had proceeded in a normal manner. It was therefore concluded that the ammonia and carbon dioxide produced from arginine by _Cl. perfringens_ arose solely from the guanido group of the arginine molecule.

Table X

Analysis of Reaction Mixtures by the Gasometric Ninhydrin Method

Incubation mixtures contained 0.18 M phosphate buffer, pH 7.2, 12 micromoles arginine per ml., and 3.9 mgm. cells per ml. Cell blank was identical except that no arginine was present. Aliquots removed at 15 minute intervals, deproteinized by heating, and 2 ml. of deproteinized filtrate analyzed by the gasometric ninhydrin method.

Duration of Incubation	Arginine Represented by Aliquot Analyzed	Ninhydrin CO_2 Recovered
min.	µM	µM
0	24	23.5, 24.0
15	24	23.6, 24.0
30	24	24.0, 23.9
45	24	23.1
60	24	23.8, 25.5
75	24	25.0, 23.1
90	24	25.0, 23.5
105	24	24.5
120	24	24.6, 24.2
135	24	23.5
150	24	23.4, 23.3
165	24	23.5
180	24	23.5, 24.2

The inter-relationships of ammonia formation, carbon dioxide production, and disappearance of the guanido grouping were established by ammonia, carbon dioxide, and Sakaguchi analyses during the course of the enzyme reaction. The carbon dioxide produced at different times during the enzyme reaction was measured by successively acid tipping a series of Warburg incubations at appropriate time intervals. Incubations for ammonia and Sakaguchi analyses were set up at the same time, and under the same conditions as the Warburg incubations. Aliquots were removed from these incubation mixtures at appropriate time intervals, pipetted into TCA to stop the reaction, and the TCA filtrates analyzed by the Conway and Sakaguchi methods.

The results of four different incubations, employing washed cell suspensions from four different growths of the test organism, are shown in Figures 13, 14, 15, and 16. The data show that, although 1 mole of the ammonia finally produced in the degradation of arginine can be accounted for on the basis of disappearance of the Sakaguchi color, all the ammonia produced at a given time during the degradation can not be accounted for solely on the basis of disappearance of the Sakaguchi color. It is also apparent from the curves that the difference between ammonia production and Sakaguchi disappearance, at any time during the reaction, equals the carbon dioxide produced at the same time. These

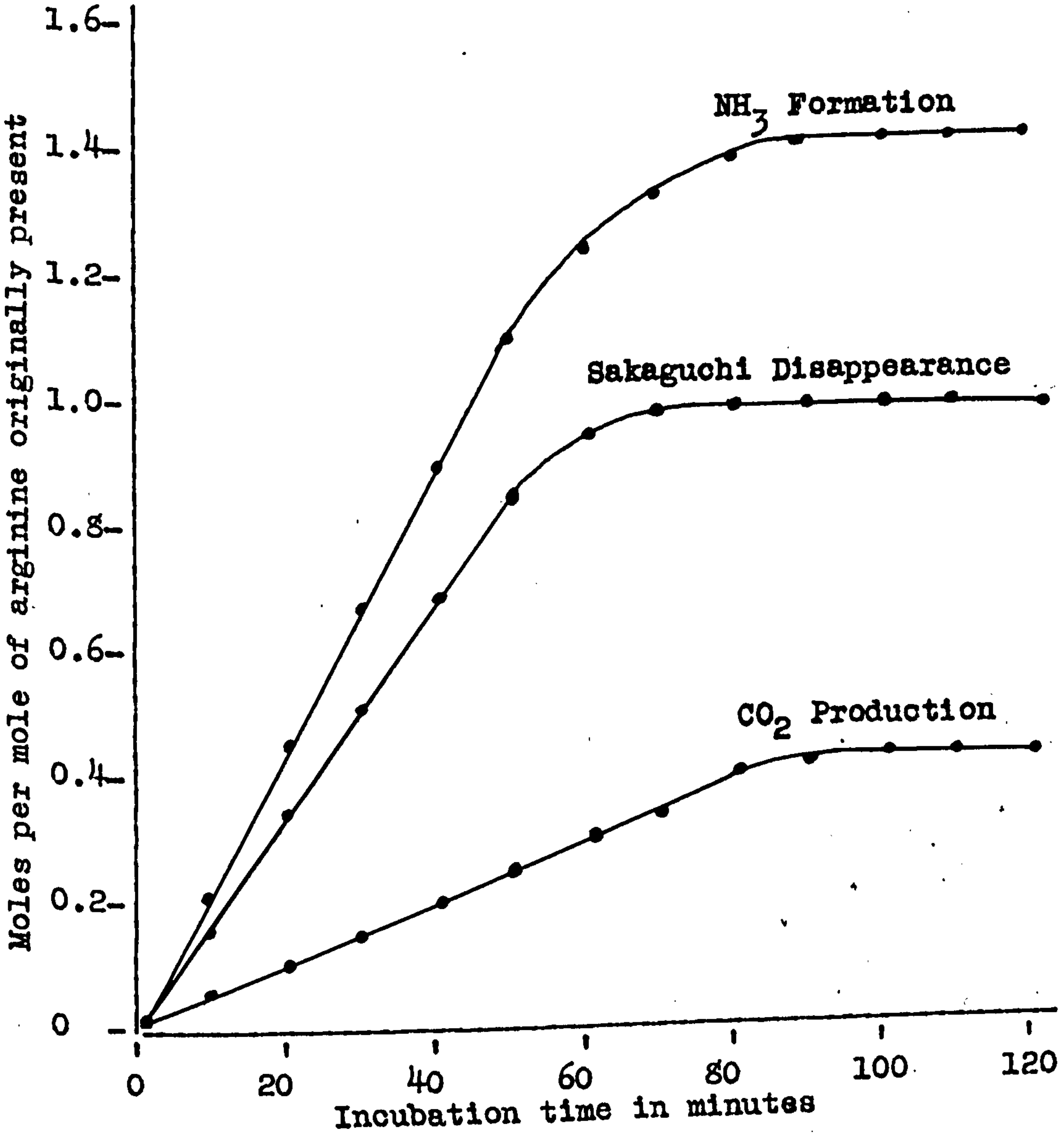


Figure 13. Changes during the Incubation of Arginine with Washed Cell Suspensions of <u>Clostridium perfringens</u>.

Incubation temperature 38° C., phosphate buffer, pH 7.2, final concentration 0.18 M. Final cell concentration 3.4 mgm. per ml. Final arginine concentration 12 micromoles per ml.

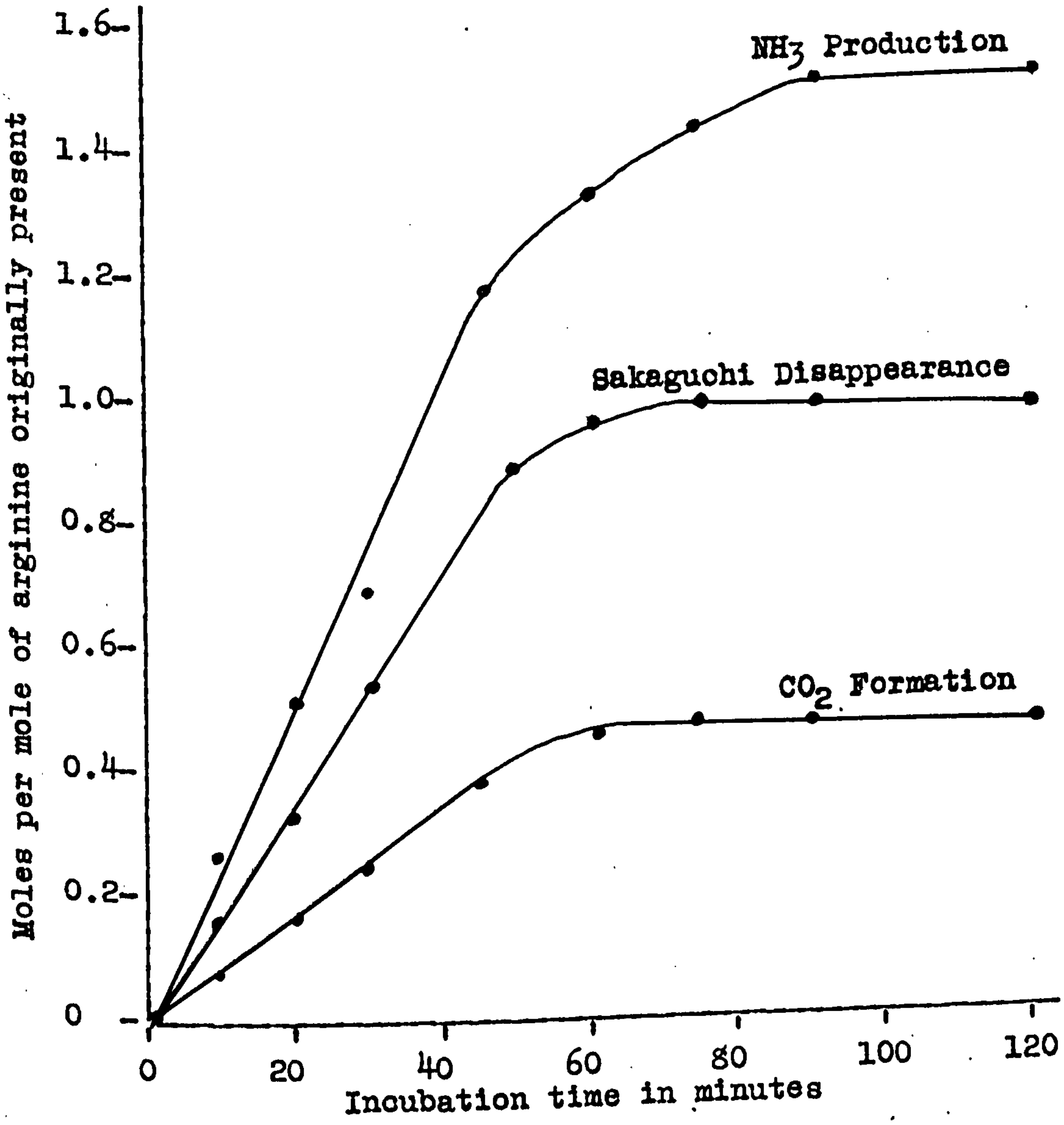


Figure 14. Changes during the Incubation of Arginine with Washed Cell Suspensions of <u>Clostridium perfringens</u>

Incubation temperature 38° C., phosphate buffer, pH 7.2, final concentration 0.18 M. Final arginine concentration 12 micromoles per ml. Final cell concentration 3.5 mgm. per ml.

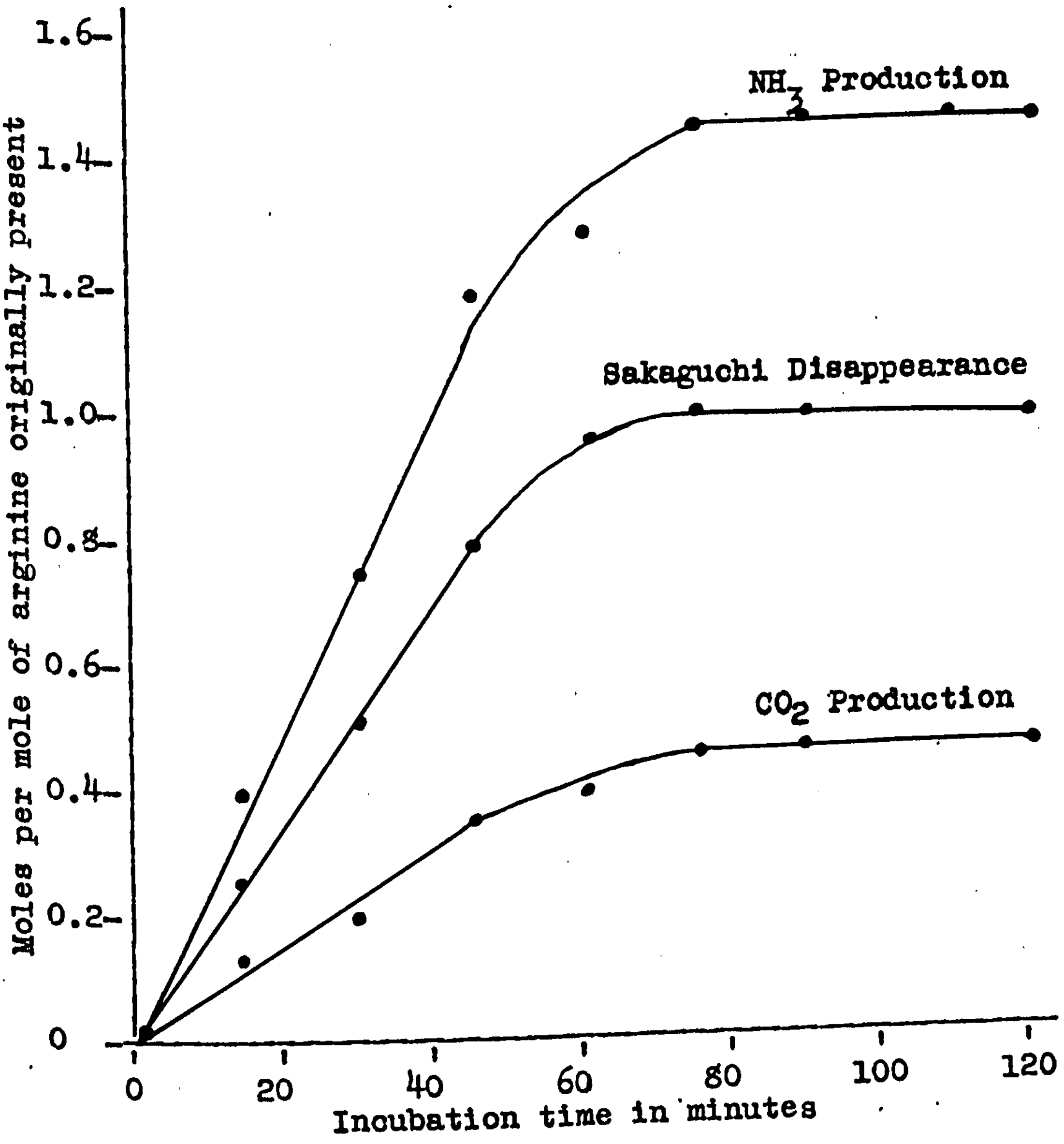


Figure 15. Changes during the Incubation of Arginine with Washed Cell Suspensions of Clostridium perfringens

Incubation temperature 38° C., phosphate buffer, pH 7.2, final concentration 0.18 M. Final arginine concentration 12 micromoles per ml. Final cell concentration 3.8 mgm. per ml.

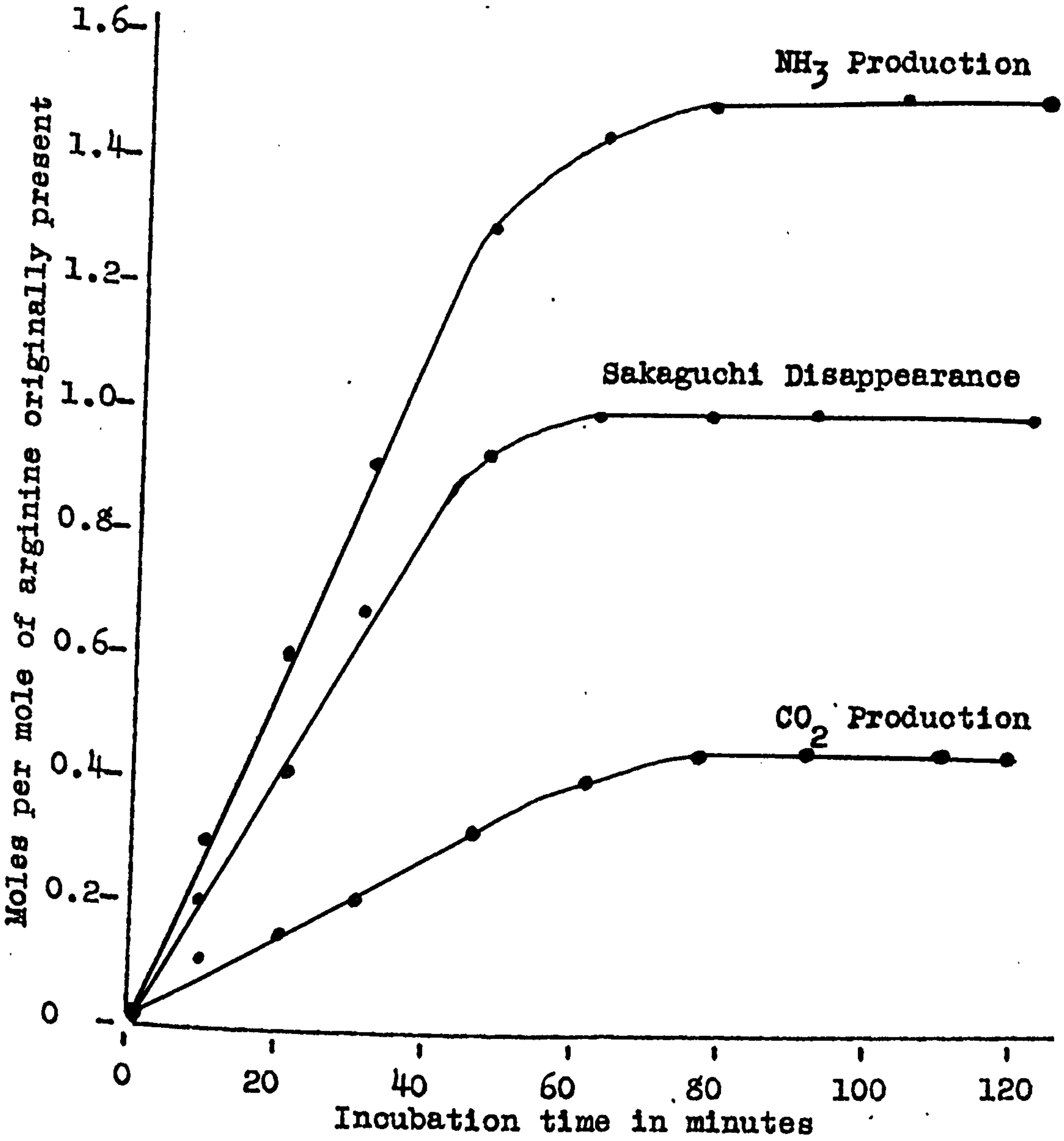


Figure 16. Changes during the Incubation of Arginine with Washed Cell Suspensions of Clostridium perfringens

Incubation temperature 38° C., phosphate buffer, pH 7.2, final concentration 0.18 M. Final arginine concentration 12 micromoles per ml. Final cell concentration 4.1 mgm. per ml.

results indicate the possibility that ammonia is removed from the arginine molecule, with consequent loss of the Sakaguchi color and formation of an intermediate, but without the formation of carbon dioxide. The intermediate is then decomposed with the formation of equimolar quantities of ammonia, carbon dioxide, and an unidentified end product, thereby accounting for the fact that the difference between total ammonia production and Sakaguchi disappearance equals carbon dioxide formation. Since two moles of ammonia and 1 mole of carbon dioxide are never produced, the accumulation of the intermediate must be assumed.

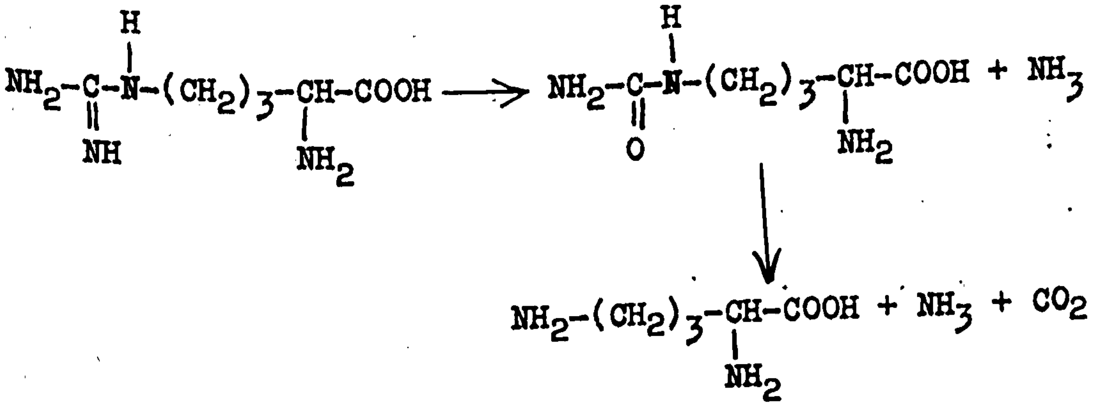
The curves also indicate that a single step breakdown of arginine to ornithine, such as has been postulated by Hills (6), does not occur in this instance. In a single step breakdown to ornithine, the ammonia:carbon dioxide ratio should be 2:1 at all times, the ammonia:Sakaguchi disappearance should be 2:1 at all times, and the carbon dioxide: Sakaguchi disappearance ratio should be 1:1 at all times. These relationships obviously do not hold.

If urea were an intermediate the ammonia:carbon dioxide ratio would be 2:1, the ammonia:Sakaguchi disappearance ratio would be 2:1, and the carbon dioxide:Sakaguchi disappearance ratio would be 1:1. Figures 13, 14, 15, and 16 indicate that these relationships do not hold. Urea was not attacked by washed cell suspensions in the pH range 6.0

to 7.2, no dixanthydryl urea could be precipitated from reaction mixtures, and no additional ammonia or carbon dioxide were produced on treatment of arginine incubation mixtures with urease. For these reasons urea was ruled out as a possible intermediate in the degradation of arginine.

Course of the Reaction

The results of the preceeding experiments are consistent with the idea that the imido group of arginine is removed to form one mole of citrulline and one mole of ammonia, but no carbon dioxide. In a second reaction the citrulline is partially degraded to equimolar quantities of ammonia, carbon dioxide, and ornithine:



$$NH_2\text{-}\underset{\underset{NH}{\|}}{C}\text{-}\overset{\overset{H}{|}}{N}\text{-}(CH_2)_3\text{-}\underset{\underset{NH_2}{|}}{CH}\text{-}COOH \longrightarrow NH_2\text{-}\underset{\underset{O}{\|}}{C}\text{-}\overset{\overset{H}{|}}{N}\text{-}(CH_2)_3\text{-}\underset{\underset{NH_2}{|}}{CH}\text{-}COOH + NH_3$$

$$\downarrow$$

$$NH_2\text{-}(CH_2)_3\text{-}\underset{\underset{NH_2}{|}}{CH}\text{-}COOH + NH_3 + CO_2$$

<u>Colorimetric Identification of Citrulline and Ornithine</u>. Presumptive evidence for the presence of citrulline and ornithine in reaction mixtures was obtained by

colorimetric methods. TCA filtrates of reaction mixtures were observed to produce a red color on treatment with ninhydrin at pH 1. Stein and Moore (32) have stated that the color is specific for ornithine in the absence of proline, which interferes. TCA filtrates of reaction mixtures were also observed to produce a color on treatment with diacetyl monoxime under the conditions specified by Kawerau (35). The identity of the diacetyl absorption spectra of a known citrulline sample and a reaction mixture aliquot are shown in Figure 17. Neither the ornithine color nor the citrulline color was produced by cell blanks, arginine, ammonia, TCA, or phosphate buffer.

Enzymatic Identification of Ornithine. The presence of ornithine in reaction mixtures was demonstrated by application of the ornithine decarboxylase method to reaction mixture filtrates. Fifteen ml. of Clostridium perfringens reaction mixture, containing 12 micromoles of arginine and 6 mgm. of washed cells per ml., and buffered at pH 6.0 in 0.18 M phosphate buffer, were incubated in 150 ml. Warburg cups for 120 minutes, and the production of carbon dioxide was measured. A 1 ml. aliquot was removed, pipetted into TCA, and ammonia production determined in the Conway apparatus. A 10 ml. aliquot was removed, deproteinized by boiling, and assayed for ornithine content by the ornithine decarboxylase method. The results of four determinations, employing

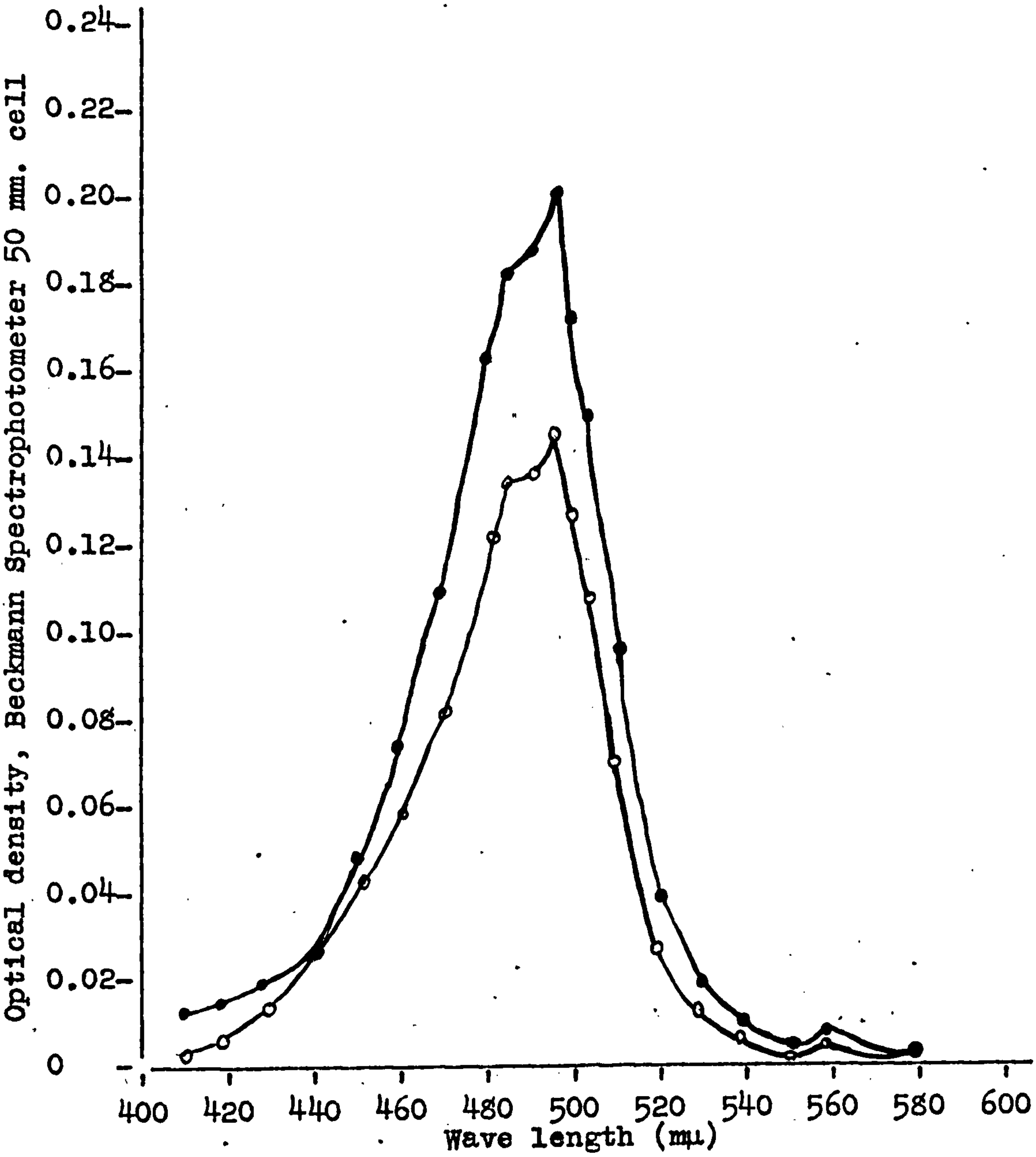


Figure 17. Absorption spectra of diacetyl colors with known citrulline solution (upper curve) and trichloroacetic acid filtrate of reaction mixture (lower curve). Citrulline concentration 0.29 micromoles per ml. of diacetyl reaction solution (upper curve).

washed cells from different growths of Clostridium perfringens are shown in Table XI. The data clearly indicate the presence of ornithine, and are consistent with the proposed mechanism in that carbon dioxide and ornithine are produced from arginine in equimolar quantities. Furthermore, the ammonia production over one mole per mole of arginine parallels carbon dioxide and ornithine production, which is also in accord with the proposed mechanism.

Chromatographic Identification of Citrulline and Ornithine. Additional evidence for the presence of citrulline and ornithine in 120 minute reaction mixtures was obtained by paper chromatography of TCA filtrates of reaction mixtures in parallel with known citrulline, ornithine, and arginine samples. Representative chromatograms, employing different solvent mixtures, are shown in Figures 18, 19, 20, 21, 22, and 23. The Rf values obtained in each of the six solvent systems are tabulated in Table XII.

The presence of citrulline and ornithine spots in reaction mixture aliquots is apparent. The complete disappearance of arginine is indicated by the absence of a corresponding spot in reaction mixture chromatograms. No other compounds that produce the characteristic purple ninhydrin color were present, except for several very faint spots which were also present in cell blanks and were therefore assumed to be products of cell lysis.

Table XI

The Ornithine Content of Reaction Mixtures, Determined by Decarboxylation of Ornithine with Clostridium septicum

Incubation of arginine with Clostridium perfringens for 120 minutes at 37° C. Buffer pH 6.0 phosphate, final concentration 0.18 M. Final cell concentration 6 mgm. per ml. Final arginine concentration 12 micromoles per ml. Incubation with arginine carried out in 150 ml. Warburg cups, employing 15 ml. of reaction mixture, and CO_2 production measured directly. Ammonia production determined on TCA filtrate of a 1 ml. aliquot. Ornithine content determined on 1 ml. of reaction mixture filtrate that had been deproteinized by boiling.

Reaction Mixture	Moles CO_2/ Mole Arginine	Moles NH_3/ Mole Arginine	Moles Ornithine per Mole Arginine
No.			
1	0.88	1.81	0.73
2	0.88	1.85	0.89
3	0.48	1.50	0.52
4*	0.56	1.62	0.60

* 10 mgm. lyophilized Clostridium perfringens instead of washed cells.

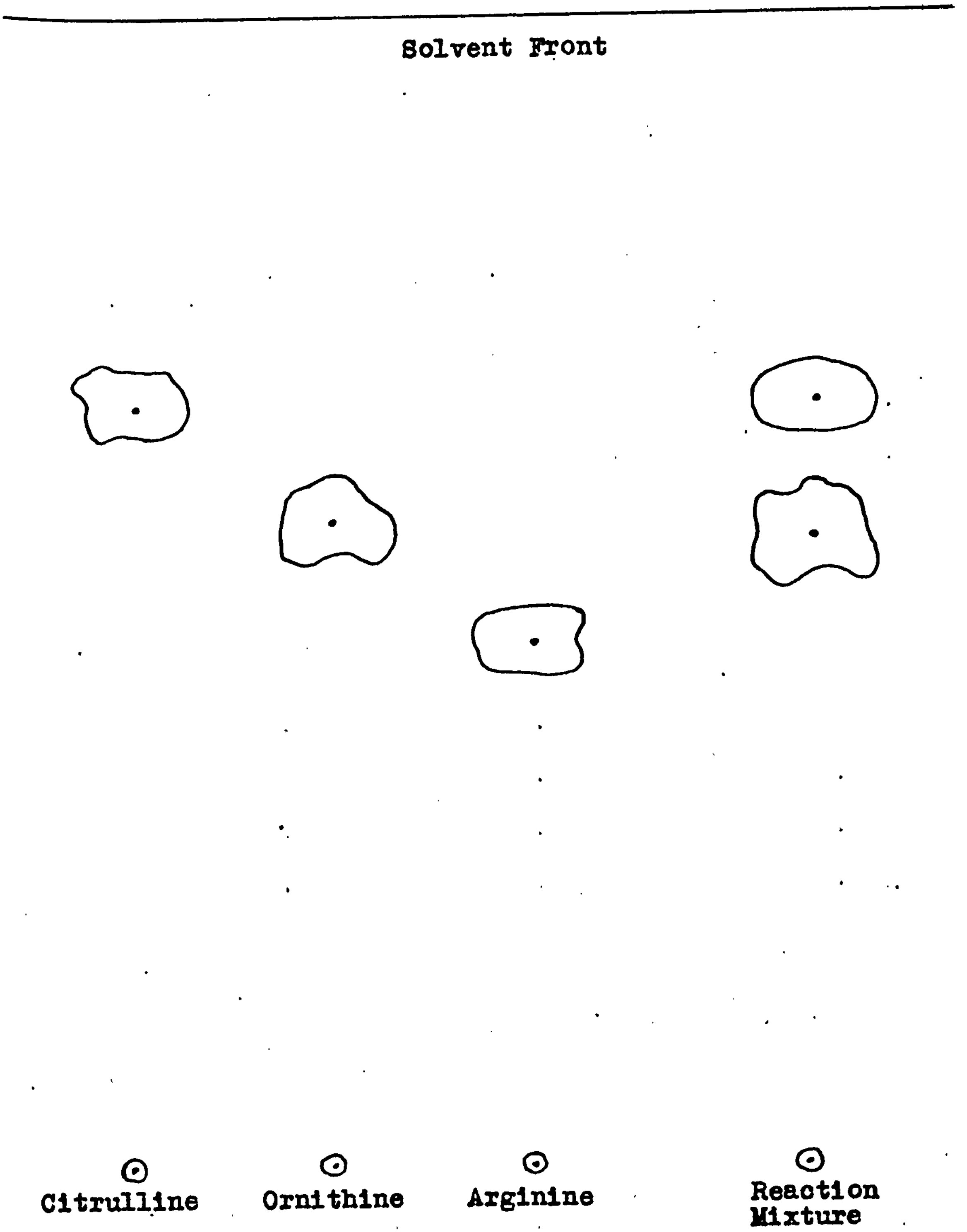


Figure 18. One Dimension Ascending Chromatogram of Reaction Mixture
Solvent: 70% ethanol containing 4% triethylamine

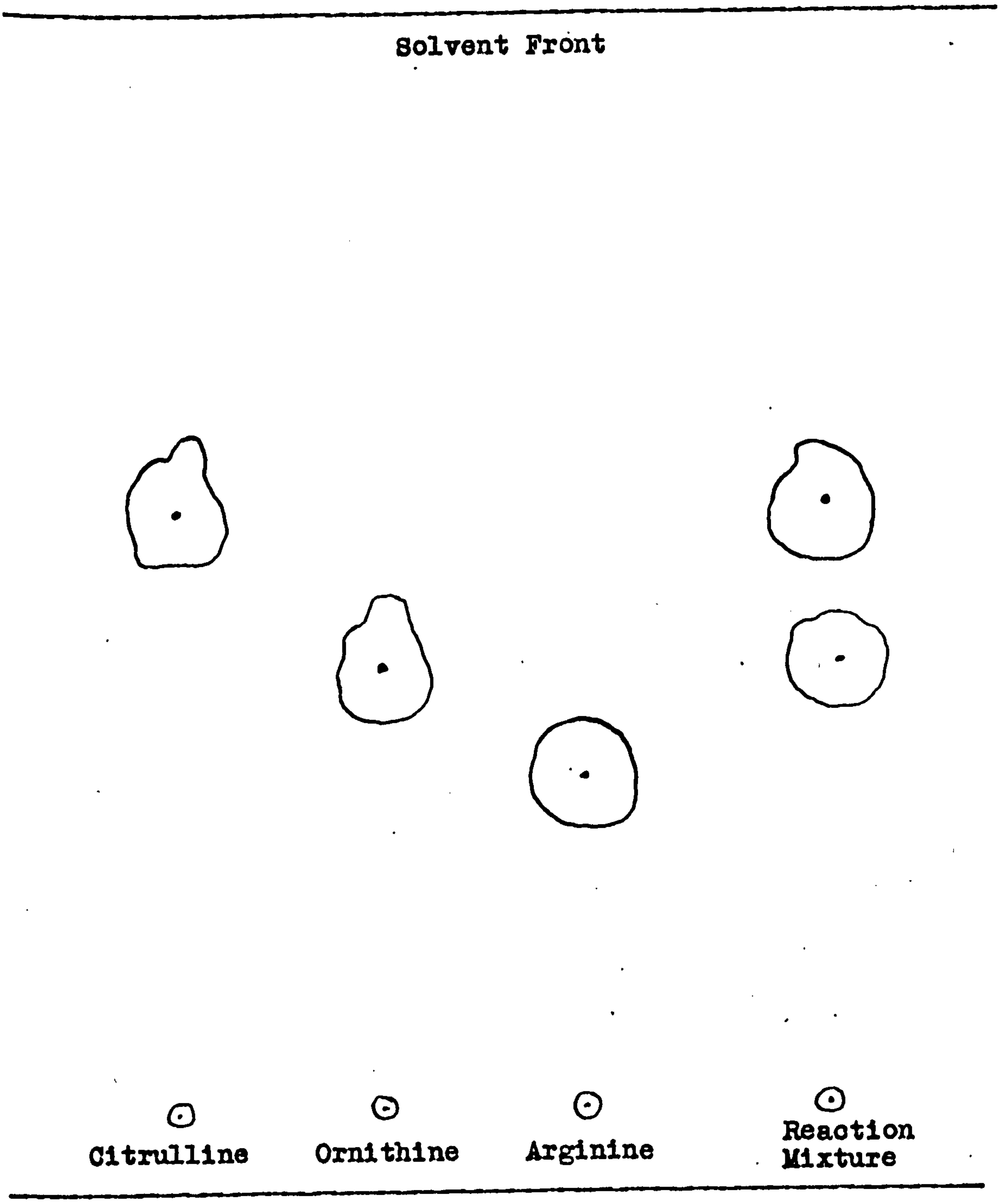


Figure 19. One Dimension Ascending Chromatogram of Reaction Mixture
Solvent: 76% ethanol containing 4% triethylamine

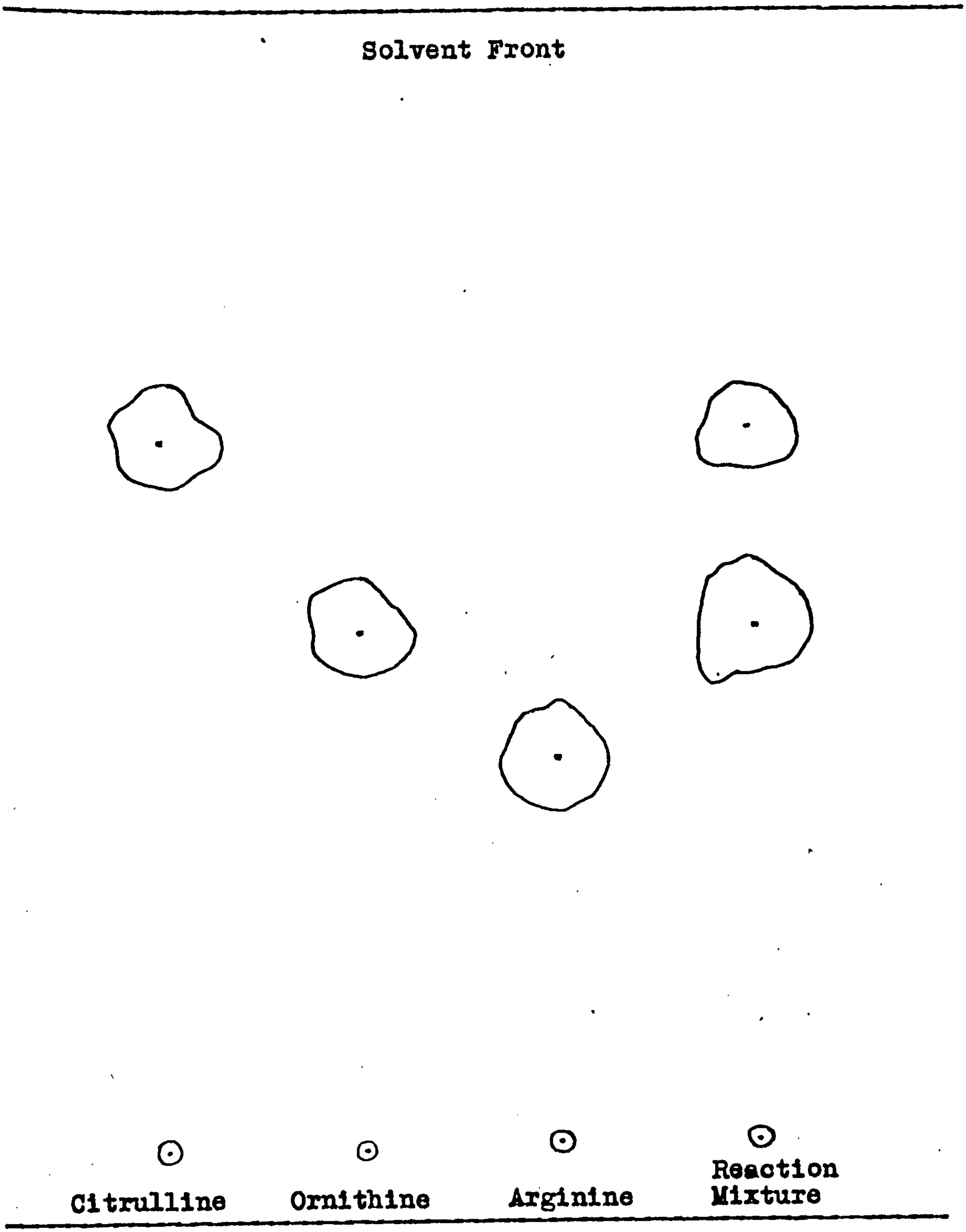


Figure 20. One Dimension Ascending Chromatogram of Reaction Mixture
Solvent: 85% methanol containing 4% triethylamine

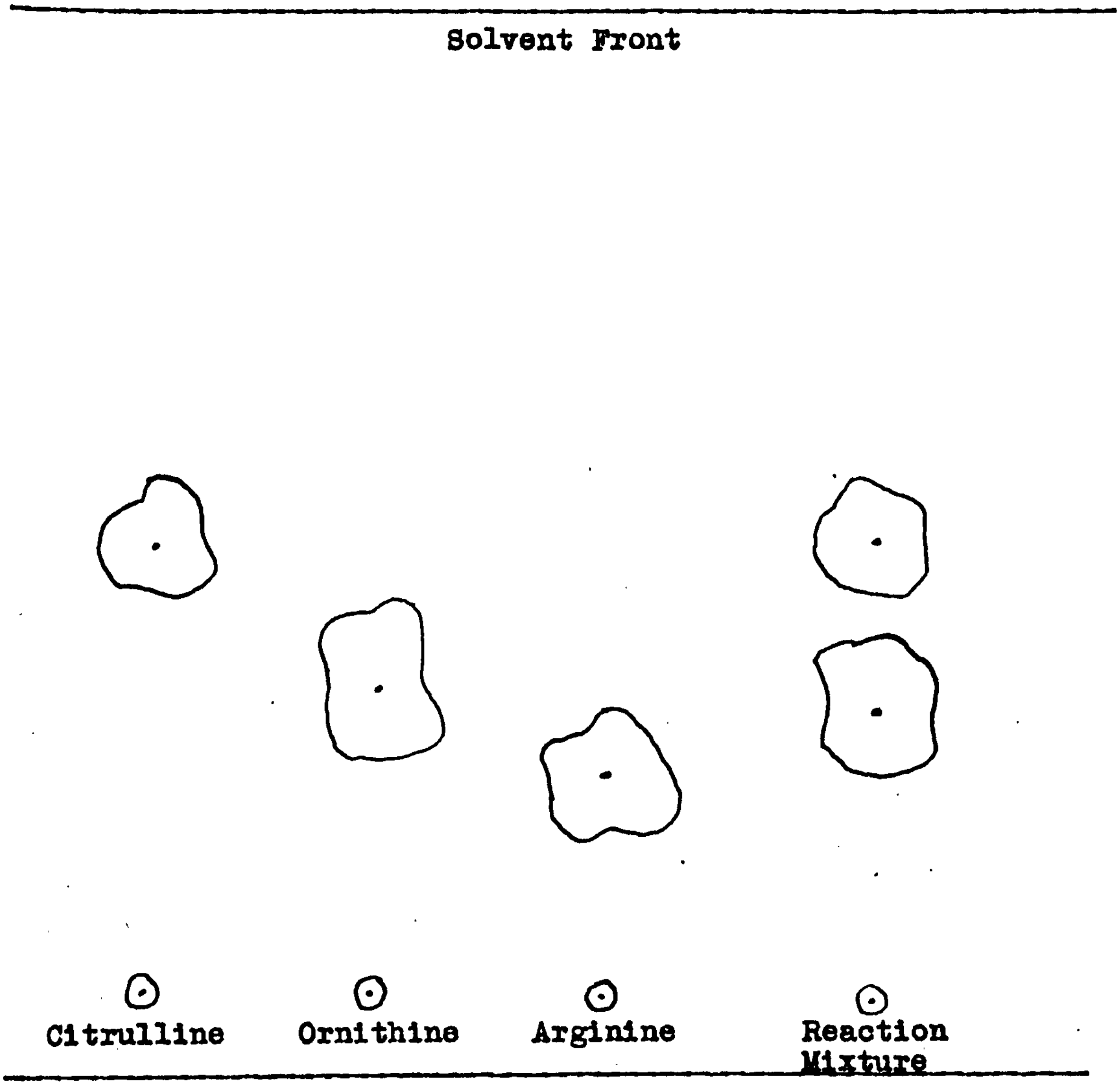


Figure 21. One Dimension Ascending Chromatogram of Reaction Mixture
Solvent: 80% ethanol containing 4% Triethylamine

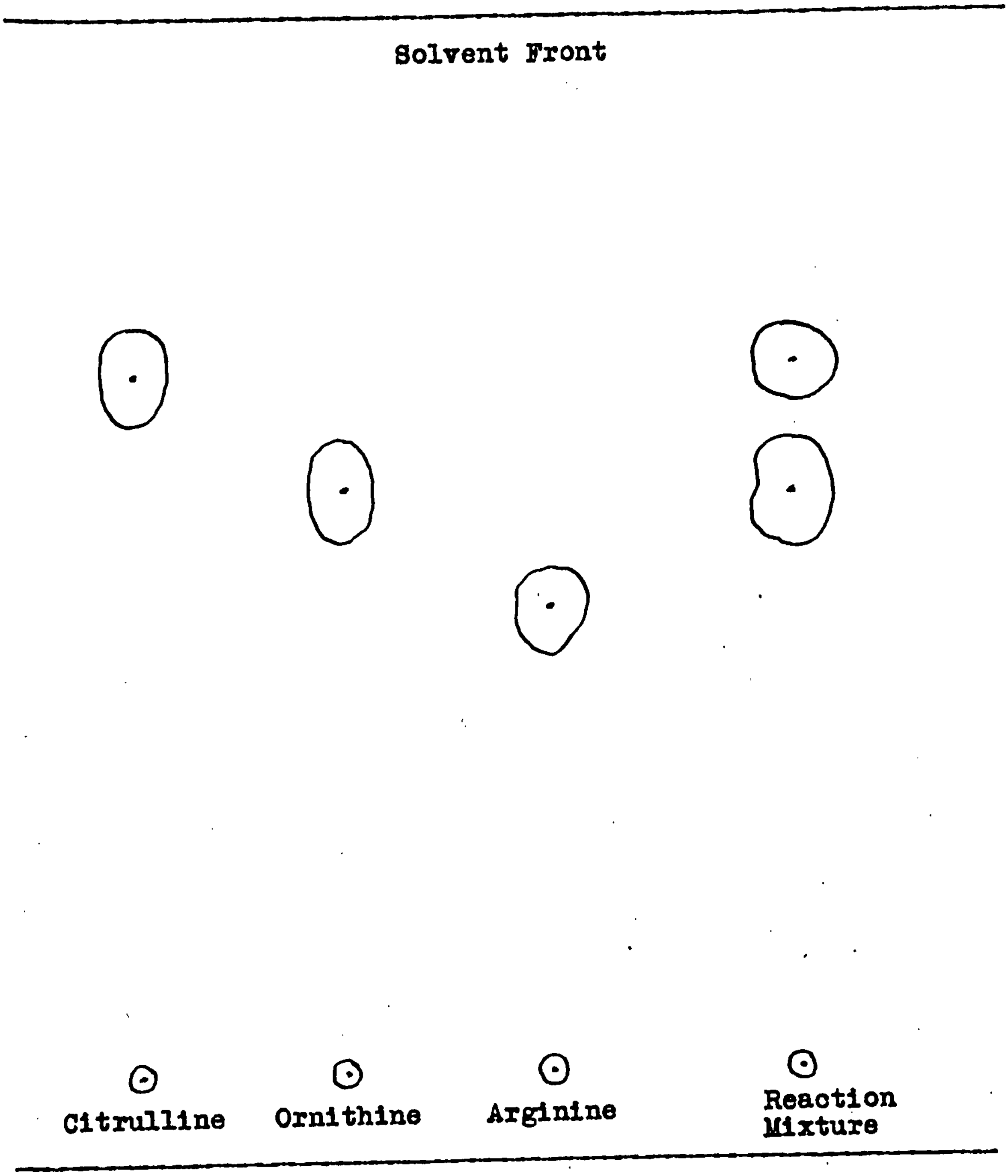


Figure 22. One Dimension Ascending Chromatogram of Reaction Mixture
Solvent: 80% methanol containing 4% triethylamine

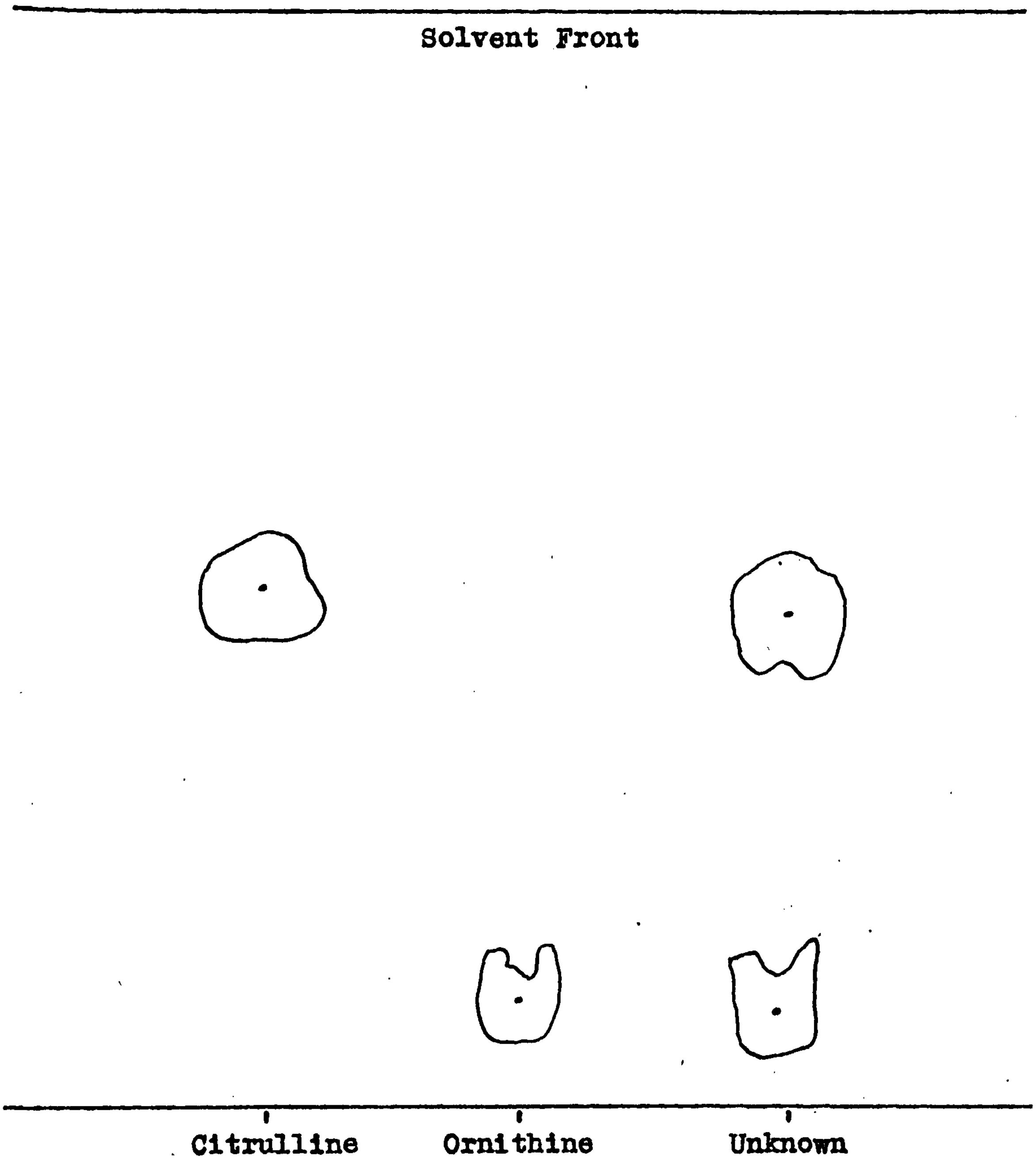


Figure 23. One Dimension Ascending Chromatogram of Reaction Mixture
Solvent: 70% methanol containing 20% pyridine

Table XII

Ascending Paper Chromatography of Reaction Mixtures

Solvents: (1) 85% methanol, (2) 80% methanol, (3) 80% ethanol, (4) 70% ethanol, (5) 70% methanol, (6) 80% isopropanol, and (7) 76% ethanol. All solvent mixtures contain 4% triethylamine and distilled water to make 100% by volume except (5), which contains 20% pyridine and distilled water to make 100%.

	Rf Values Obtained						
Solvent	1	2	3	4	5	6	7
Citrulline	0.63	0.66	0.46	0.65	0.48	0.14	0.55
Reaction Mixture	0.64	0.66	0.47	0.66	0.50	0.15	0.55
Ornithine	0.45	0.56	0.31	0.56	0.15	0.08	0.41
Reaction Mixture	0.46	0.55	0.30	0.54	0.12	0.07	0.41
Arginine	0.34	0.44	0.23	0.45	--	--	0.36

Isolation of Citrulline and Ornithine. The presence of both ditrulline and ornithine in reaction mixtures was definitely established by isolation of these two compounds from the same reaction mixture. Citrulline was isolated as the free base, and ornithine as the dibenzoyl derivative, by the following procedure:

900 ml. of incubation mixture, containing 21.6 millimoles of L(+) arginine monohydrochloride and washed cells from 3 liters of medium, were incubated at 37° C. in a water bath for 4 hours without buffer. The final cell concentration was 3.6 mgm. per ml. of reaction mixture. The use of buffer was avoided to facilitate isolation of the desired compounds. Control determinations showed 1.3 moles of ammonia per mole of arginine originally present, complete disappearance of the Sakaguchi color, the absence of an arginine spot and the presence of citrulline and ornithine spots on paper chromatograms, a positive ninhydrin color at pH 1 corresponding to 0.3 moles of ornithine per mole of arginine originally present, and a positive diacetyl color corresponding to 0.7 moles of citrulline per mole of arginine originally present. The pH of the reaction mixture rose to approximately 9, and citrulline accumulation appeared to be favored at the expense of ornithine formation as a result of omitting buffer from the reaction mixture. Except for

this apparent change in the relative amounts of citrulline and ornithine at the completion of the reaction, the reaction appeared to follow the usual course.

Protein was precipitated from the reaction mixture by the addition of 100 grams of TCA dissolved in 100 ml. of distilled water. The reaction mixture was permitted to stand overnight in the cold room, the protein was filtered off, and the filtrate was diluted to 3 liters with distilled water. The diluted filtrate was passed over a 300 gram column of IR-100, analytical grade, (contained in a 3 cm. x 140 cm. glass column, and operating in the hydrogen cycle) at a flow rate of 3 ml. per minute. The column was flushed with 6 liters of distilled water. The effluent and washings contained no citrulline or ornithine and were discarded.

Citrulline was eluted with 5% aqueous pyridine at a flow rate of 2 to 3 ml. per minute. The extent of elution was determined by the colorimetric diacetyl reaction. The first 3 liters of eluate, containing 1.5 grams of citrulline, were reserved for the isolation of citrulline. The column was then washed free of citrulline with 5% aqueous pyridine, as determined by a negative diacetyl reaction with 5 ml. of effluent. Approximately 9 liters of pyridine solution were required. The column was flushed with distilled water until the effluent was free of the odor of pyridine. Ornithine was then eluted with 4% sulfuric acid until 5 ml. of effluent gave a negative ninhydrin reaction for ornithine. The column was then flushed free of sulfuric acid with distilled water, and was thus regenerated for further use.

Pyridine was removed from the citrulline fraction by concentration under reduced pressure to 100 ml. The flask was rinsed with distilled water and the combined concentrate and washings (pH 1 to 2) treated with IR-4B, analytical grade, until the pH rose to 6 or 7. The odor of pyridine was pronounced, indicating the decomposition of pyridine salts by IR-4B. The resin was filtered off and washed with approximately 600 ml. of distilled water. Pyridine was removed from the filtrate and washings by concentrating under reduced pressure to 25 ml. The treatment with the resin and the vacuum concentration were repeated to remove the last traces of pyridine.

The concentrate (10 ml., pH 6 to 7) was decolorized with 1 gram of Norite, filtered, and evaporated to about 3 ml. on the steam bath. Citrulline was precipitated by the addition of absolute ethanol, air dried, and weighed (900 mgm., M. P. 204 to 209^{o} corrected).

The impure citrulline was dissolved in 3 ml. of distilled water, absolute ethanol was added dropwise to the first signs of turbidity, and the solution permitted to crystallize in the cold room overnight. Optically active citrulline precipitated as light, colorless, crystalline platelets which gave the analyses shown in Table XIII after washing with ice cold, absolute ethanol and after air drying. Paper chromatograms of the isolated product and a known citrulline sample are shown in figure 24.

Sulfuric acid was removed from the ornithine fraction by treatment with IR-4B. The resin was filtered off, thoroughly washed, and the combined filtrate and washings decolorized with Norite. The solution was concentrated under reduced pressure to approximately 500 ml., again decolorized with Norite, then treated with saturated sodium hydroxide until strongly alkaline to phenolphthalein. The alkaline solution was concentrated under reduced pressure to 100 ml. to remove ammonia and traces of pyridine, and then benzoylated in the usual manner.

The impure dibenzoyl ornithine, which precipitated on acidification of the benzoylation mixture, was filtered, thoroughly washed with distilled water, air dried, and repeatedly extracted with small portions of anhydrous ether. The residue from the ether extraction was dissolved in the smallest possible quantity of warm ethanol, cooled to room temperature, and water added dropwise until a faint permanent turbidity resulted. The precipitate, which formed on standing in the cold room overnight was recrystallized from ethanol-water in the manner just described. The pure

Table XIII

Analysis of Isolated Citrulline

	Values Found for Isolated Sample	Reported or Theoretical Values for Citrulline
Melting Point	225° - 226° with gas evolution (corrected)	220° - 226° (43,44,45)
Specific Rotation	23°*	17.9°* (46) 22.3°-24.2°** (47)
Nitrogen	23.97%***	23.99%
Carbon	41.21%****	41.13%
Hydrogen	7.58%****	7.48%

* In 1 N HCl.
** In acid.
*** Kjeldahl determinations run by Mrs. Elizabeth Titus.
**** Results of analyses by Galbraith Laboratories, Knoxville, Tennessee.

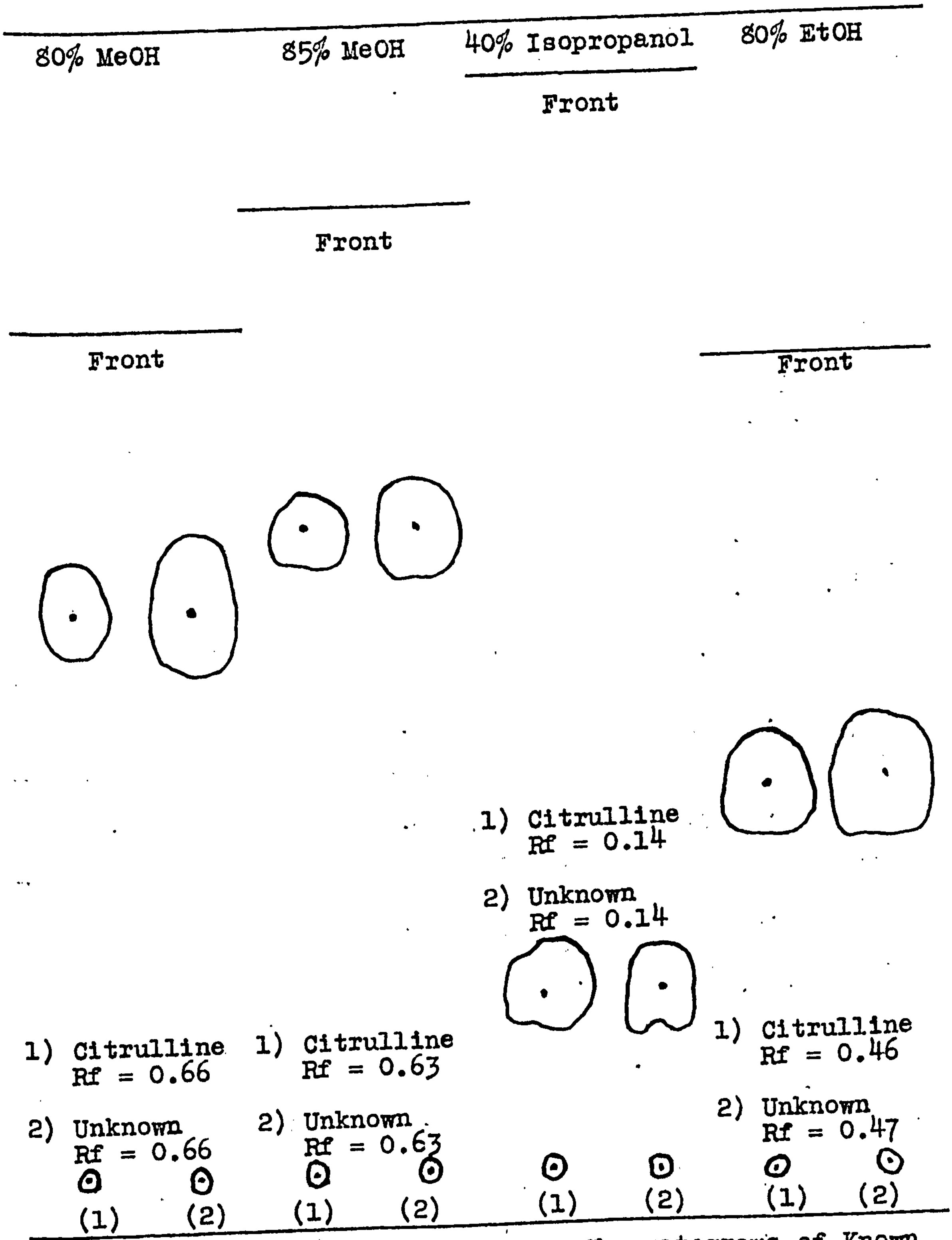


Figure 24. One Dimension Ascending Chromatograms of Known Citrulline Sample and Isolated Product. All solvents contain 4% triethylamine.

dibenzoyl ornithine was collected on a Hirsch funnel, air dried and analyzed. The results of the analyses are shown in Table XIV.

Absence of Other End Products. The absence of end products other than citrulline, ornithine, ammonia, and carbon dioxide was established by balance sheet studies. Reaction mixtures containing 12 micromoles of arginine and 4 mgm. washed cells per ml., in 0.18 M phosphate buffer of the desired pH, were incubated at 37° C. Aliquots were removed at 30 minute intervals for a period of 3 hours, pipetted into 9 volumes of 10% TCA to stop the enzyme reaction, and filtrates prepared. Blanks containing all reaction mixture components except arginine were incubated and precipitated in parallel with the sample tubes. The TCA filtrates were used in the determination of arginine disappearance by the Sakaguchi method, ammonia formation by Conway diffusion, ornithine content by the colorimetric ninhydrin method at pH 1, and citrulline content by the colorimetric diacetyl reaction.

Reaction mixtures for the determination of carbon dioxide production were incubated at the same time, and under the same conditions, in a series of Warburg vessels. The vessels were acid tipped, in succession, at 30 minute intervals, to stop the enzyme reaction and liberate bound carbon dioxide.

Table XIV

Analysis of Isolated Dibenzoyl Ornithine

	Values Found for Isolated Sample	Reported or Theoretical Values for Dibenzoyl Ornithine
Melting Point	184° - 186° (corrected)	183° - 185° (43)
Nitrogen	8.35%*	8.23%
Carbon	67.01%**	67.05%
Hydrogen	6.15%**	5.92%

* Kjeldahl determinations run by Mrs. Elizabeth Titus.

** Results of analyses by Galbraith Laboratories, Knoxville, Tennessee.

The results of balance sheet studies carried out at pH 5.5, 6.3, 7.2, and 7.8 are shown in Tables XV, XVI, XVII, and XVIII. The agreement between observed arginine disappearance and that calculated from observed ornithine and observed citrulline contents is good. There is also good agreement between observed ammonia production and that calculated from observed citrulline and ornithine values. Ornithine and carbon dioxide production are identical in nearly every instance.

These findings are in accord with the proposed mechanism, and indicate that ammonia, carbon dioxide, citrulline, and ornithine are the only end products formed. They do not preclude the possibility that additional intermediary compounds are involved in the degradation.

Conversion of Citrulline to Ornithine

If citrulline is an intermediate in the degradation of arginine by Clostridium perfringens, one would expect to demonstrate the breakdown of citrulline by the test organism. It has been found, contrary to the work of Woods and Trim (1), that citrulline is attacked by both washed cell suspensions and by suspensions of lyophilized cells. Equimolar quantities of ammonia, carbon dioxide, and ornithine are produced in both instances. The results of these studies

Table XV

Balance Sheet for the Degradation of Arginine by <u>Clostridium perfringens</u>

Reaction carried out at 37° C. in pH 6.3 phosphate buffer, final concentration 0.18 M. Final substrate concentration 12 micromoles per ml. Final cell concentration 4 mgm. per ml. All values in the table are moles per mole of arginine originally present.

Time	Arginine Disappearance		CO_2	Ornithine	Citrulline	- - - Ammonia - - - -	
	Observed	Calculated*	- - - - - - Observed		- - - - - -	Observed	Calculated**
min.							
30	0.39	0.45	0.17	0.23	0.22	0.67	0.68
60	0.81	0.89	0.41	0.52	0.37	1.32	1.41
90	1.00	1.01	0.58	0.59	0.42	1.57	1.60
120	1.00	1.04	0.58	0.62	0.42	1.57	1.66
180	1.00	1.04	0.58	0.62	0.42	1.57	1.66

* Observed ornithine plus observed citrulline.
** 2 x observed ornithine plus citrulline.

Table XVI

Balance Sheet for the Degradation of Arginine by Clostridium perfringens

Reaction carried out at 37° C. in pH 5.5 phosphate buffer, final concentration 0.18 M. Final substrate concentration 12 micromoles per ml. Final cell concentration 4 mgm. per ml. All values in the table are moles per mole of arginine originally present.

Time	Arginine Disappearance		CO_2	Ornithine	Citrulline	Ammonia	
	Observed	Calculated*	Observed			Observed	Calculated**
min.							
30	0.41	0.44	0.24	0.25	0.19	0.60	0.69
60	0.87	0.86	0.48	0.50	0.36	1.34	1.36
90	1.00	1.01	0.62	0.64	0.37	1.63	1.65
120	1.00	1.01	0.62	0.64	0.37	1.63	1.65
180	1.00	1.01	0.62	0.64	0.37	1.63	1.65

* Observed ornithine plus observed citrulline.

** 2 x observed ornithine plus citrulline.

Table XVII

Balance Sheet for the Degradation of Arginine by *Clostridium perfringens*

Reaction carried out at 37° C. in pH 7.8 phosphate buffer, final concentration 0.18 M. Final substrate concentration 12 micromoles per ml. Final cell concentration 4 mgm. per ml. All values in the table are moles per mole of arginine originally present.

Time	Arginine Disappearance		CO_2	Ornithine	Citrulline	Ammonia	
	Observed	Calculated*	Observed			Observed	Calculated**
min.							
30	0.27	0.29	0.13	0.11	0.18	0.35	0.40
60	0.53	0.54	0.25	0.25	0.29	0.81	0.79
90	0.76	0.85	0.36	0.40	0.45	1.16	1.25
120	0.98	0.93	0.38	0.40	0.53	1.40	1.33
180	0.98	0.98	0.38	0.40	0.58	1.40	1.38

* Observed ornithine plus observed citrulline.

** 2 x observed ornithine plus citrulline.

Table XVIII

Balance Sheet for the Degradation of Arginine by _Clostridium_ _perfringens_

Reaction carried out at 37° C. in pH 7.2 phosphate buffer, final concentration 0.18 M. Final substrate concentration 12 micromoles per ml. Final cell concentration 4 mgm. per ml. All values in the table are moles per mole of arginine originally present.

Time	Arginine Disappearance		CO_2	Ornithine	Citrulline	- - - Ammonia - - - -	
	Observed	Calculated*	- - - - - - Observed		- - - - - -	Observed	Calculated**
min.							
30	0.44	0.44	0.22	0.24	0.20	0.63	0.68
60	1.73	0.78	0.38	0.43	0.34	1.21	1.20
90	1.00	0.96	0.46	0.54	0.41	1.43	1.49
120	1.00	1.05	0.60	0.59	0.46	1.60	1.64
180	1.00	1.05	0.60	0.59	0.46	1.61	1.64

* Observed ornithine plus observed citrulline.
** 2 x observed ornithine plus citrulline.

are shown in Table XIX. The data also indicate that pH is an important variable in the attack on citrulline, since degradation is not significant at pH 7.2, but is approximately 50% complete at pH 6.3.

Separation of Arginine Desimidase

The two step nature of the arginine degradation was established by the preparation of the arginine to citrulline enzyme (arginine desimidase) free of citrulline to ornithine activity. The following procedure was employed in the extraction of the enzyme from the cell:

600 mgm. of lyophilized cells from a 6-hour culture were extracted with 7 ml. of 1% NaCl for 3 hours at room temperature. The debris was removed by centrifugation at 0° C. in an International refrigerated centrifuge, and a clear, yellow extract was obtained. The extract has also been prepared by grinding lyophilized cells with aluminum oxide and water. Neither preparation was found to attack citrulline within the pH range 5.5 to 7.8 in phosphate buffer. Preliminary studies with the sodium chloride extract indicated that pH is a critical factor in the attack of arginine by the extract. Incubations at pH 7.2, 6.8, and 5.5 gave the following values for ammonia production after 3-hour incubation at 37° C., 0.06 mole/mole, 0.64 mole/mole,

Table XIX

Degradation of Citrulline by *Clostridium perfringens*

Incubations 3 hours at 37° C. Final buffer concentration 0.18 M. Final substrate concentration 24 micromoles DL-citrulline per ml. Final cell concentration 6 mgm. per ml. when washed cell suspensions were used; 12 mgm. per ml. when suspensions of lyophilized cells were used. All calculations are based on the concentration of the L(+) isomer of citrulline. Analytical values are expressed as moles of product per mole of citrulline originally present.

pH of the Reaction Mixture	NH_3 Formed	CO_2 Formed	Ornithine Formed
	moles	moles	moles
Incubations with washed cell suspensions			
7.2	0.07	0.08	0.07
6.3	0.31	0.31	0.34
5.5	0.41	0.43	0.46
Incubations with suspensions of lyophilized cells			
7.2	0.16	0.16	—
6.3	0.46	0.50	—
5.5	0.57	0.51	0.58

and 0.84 mole/mole of arginine respectively. For this reason balance sheet studies employing the sodium chloride extract were conducted at pH 5.5

The preparation was tested as follows: incubation mixtures containing 8 micromoles of arginine per ml. of reaction mixture, buffer, and extract were maintained in Warburg vessels at 37° C. until the evolution of carbon dioxide had ceased. Aliquots were removed, pipetted into 10% trichloroacetic acid, and filtrates prepared. The determination of ammonia, residual arginine, citrulline, and ornithine were carried out on the filtrates. Control determinations with the lyophilized cells from which the extract was prepared were carried out in the presence of both arginine and citrulline as substrates. A control with the extract and citrulline substrate was also run. The results of these determinations are given in Table XX.

The data indicate that the first step in the degradation of arginine by <u>Clostridium perfringens</u> (BP6K) results in the formation of equimolar amounts of citrulline and ammonia but no carbon dioxide. Attempts to obtain the enzyme responsible for the conversion of citrulline to ornithine have been unsuccessful. The mechanism whereby arginine is degraded to citrulline can not be established from presently available data. The hydrolytic removal of the imido group of arginine, followed by tautomerization of the intermediate

Table XX

Comparison of the Effects of "Arginine Desimidase" and Lyophilized Cells on Arginine and Citrulline

Incubations 2 hours at 37° C. in pH 5.5 phosphate buffer, final concentration 0.18 M. Arginine and citrulline concentrations 3.2 micromoles of L(+) isomer per ml. of reaction mixture. Cell extract or suspension 1.0 ml. Final volume reaction mixture 2.5 ml.

	NH_3 Formed	CO_2 Formed	Residual Substrate	Citrulline Formed	Ornithine Formed
	--Mole/mole of substrate originally present--				
			Arginine Substrate		
Desimidase Preparation	0.93	0	0.10	0.89	0
Lyophilized Cells	1.46	0.43	0	0.43	0.51
			Citrulline Substrate		
Desimidase Preparation	0	0	----	----	0
Lyophilized Cells	0.60	0.62	----	----	0.65

hydroxyl compound to citrulline, has been postulated for a similar degradation by Streptococcus faecalis (17).

SUMMARY AND CONCLUSIONS

In the overall production of ammonia and carbon dioxide from arginine, the excess of ammonia over one mole per mole of substrate was equal to the total quantity of carbon dioxide produced. The disappearance of arginine from overall reaction mixtures was demonstrated by disappearance of the Sakaguchi color, by absence of an arginine spot on paper chromatograms, by failure to precipitate arginine flavianate, and by failure to obtain urea on treatment with arginase.

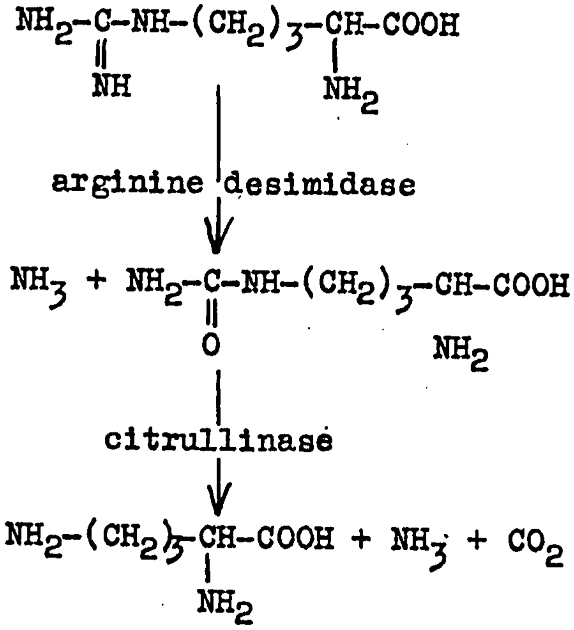
All the ammonia and carbon dioxide produced from arginine arose from the guanido grouping of the molecule. This fact was demonstrated by application of the gasometric ninhydrin procedure to reaction mixture aliquots removed during the course of the enzyme reaction. The possibility of a single step breakdown of arginine to ornithine, such as has been postulated by Hills was ruled out on the basis of ammonia production:carbon dioxide production:Sakaguchi disappearance figures. Urea was ruled out as a possible intermediate on the basis of ammonia:carbon dioxide ratios, failure to precipitate dixanthydryl urea, failure to demonstrate ammonia or carbon dioxide production on treatment of reaction mixtures with urease, and failure to demonstrate attack on urea with the test organism.

The presence of citrulline was demonstrated by colorimetric, chromatographic, and isolation procedures. Optically active citrulline was isolated from reaction mixtures as the free base. The presence of ornithine was established by colorimetric, enzymatic, chromatographic, and isolation procedures. Citrulline was attacked by the test organism, but ornithine was not.

The absence of end products other than citrulline, ornithine, carbon dioxide, and ammonia was demonstrated by balance sheet studies. All the arginine originally present in reaction mixtures could be accounted for _at any time during the reaction_ on the basis of these end products plus residual arginine.

That the degradation involves at least two enzymes was indicated by complete inhibition of carbon dioxide formation, and the production of only one mole of ammonia when borate or phthalate buffers were employed. The fact that old cells produced only one mole of ammonia per mole of arginine, and no carbon dioxide was an additional indication. The two step nature of the degradation was definitely established by the accumulation of citrulline and by the preparation of "arginine desimidase," free of "citrullinase" activity. Arginine desimidase was found to degrade arginine to citrulline with the formation of one mole of ammonia. Washed cell suspensions were found to degrade citrulline to equimolar quantities of ammonia, carbon dioxide, and ornithine. Attempts to obtain this enzyme in the cell free state were unsuccessful.

In summary, the degradation of arginine by <u>Clostridium</u> <u>perfringens</u> (BP6K) has been investigated. Evidence obtained with washed cell suspensions, suspensions of lyophilized cells, and NaCl extracts of lyophilized cells is consistent with the idea that the following metabolic pathway is involved:



$$NH_2\text{-}\underset{\underset{NH}{\|}}{C}\text{-}NH\text{-}(CH_2)_3\text{-}\underset{\underset{NH_2}{|}}{CH}\text{-}COOH$$

$$\Big\downarrow\ \text{arginine desimidase}$$

$$NH_3 + NH_2\text{-}\underset{\underset{O}{\|}}{C}\text{-}NH\text{-}(CH_2)_3\text{-}\underset{NH_2}{CH}\text{-}COOH$$

$$\Big\downarrow\ \text{citrullinase}$$

$$NH_2\text{-}(CH_2)_3\text{-}\underset{\underset{NH_2}{|}}{CH}\text{-}COOH + NH_3 + CO_2$$

SUGGESTIONS FOR FUTURE WORK

The preparation and purification of "arginine desimidase" should be investigated with the purpose of obtaining a pure enzyme in reasonable yields. The enzyme could then be characterized with respect to pH optima, inhibition, specificity toward synthetic substrates, heat stability, activation, etc. The characterization of one enzyme in the presence of the intact cell, or in the presence of all the products of cell lysis has little meaning because of the numerous indeterminate factors that are involved. Purification should, therefore, precede characterization studies. Purification can not be achieved unless reasonable yields of the crude product can be obtained. Consequently a quantitative investigation of the extraction of the enzyme from the cell must first be carried out.

The preparation of pure arginine desimidase should lead to the development of a specific, rapid, enzymatic method for the determination of arginine. This method would possess an advantage over the use of decarboxylase, since ammonia production would be utilized as a measure of arginine content and the use of the Warburg would be unnecessary.

The development of a practical method for the production of optically active citrulline appears to be feasible.

The method might be developed by refinement of the isolation techniques outlined in this thesis so that yields would be increased to a practical level. However, the use of a pure arginine desimidase preparation appears to be a more practical approach for the following reasons: The necessity for separating citrulline from ornithine would be obviated. Much higher substrate concentrations could probably be employed, thereby diminishing reaction mixture volumes and greatly decreasing the amount of protein to be removed. This might open the way for a direct alcohol precipitation of citrulline from reaction mixtures.

Attempts to obtain citrullinase activity in cell extracts were not successful. The reasons for failure to demonstrate activity in the absence of the intact cell are not clear. Practically nothing is known about citrullinase at this time and the need for additional investigation is obvious.

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