~~T H E S I S~~

STUDIES ON THE AMINO ACID METABOLISM OF BACTERIA RESPONSIBLE FOR SURFACE TAINT IN BUTTER

- by -

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INTRODUCTION

Surface taint is the name given by Marker (48) to a defect in butter first observed and described by him in a lot of pasteurized Alberta creamery butter sold on the Vancouver market in 1919. The defect is characterized by a typical odour described by Thornton (71) as that of "sweaty-feet" which develops after exposure to the air on samples of butter contaminated by one or more of a group of bacteria known at present as "Surface Taint Bacteria". This group has been shown by Campbell (10) to contain among others two species of bacteria originally isolated by Hammer (31) - Proteus ichthyosmius found in a sample of evaporated milk having a fishy odour and Pseudomonas putrefaciens from so-called "putrid" butter, strains of which have more recently been isolated from samples of typical surface taint butter by Thornton et al.

The odour of surface taint has been shown, beyond doubt, to be due to bacterial activity. Little is known, however, about the chemical origin and development of the defect in butter. Present evidence indicates that the "sweaty-feet" odour of typical surface taint originates in the protein of butter. The mechanism of its elaboration is, as yet, unknown; several hypotheses concerning its formation, however, have been advanced. Outstanding among these are the indole hypothesis of Campbell (9) and the decarboxylation and deamination hypotheses of Campbell (10).

Neilson (50) has shown that the failure to detect

indole in the sera of surface taint butters is practically proof positive that indole elaboration is not essential to the production of surface taint in butter.

The investigations of Campbell (10) in which experimental evidence could not be obtained to support the hypothesis that amines are formed during the elaboration of surface taint, indicate that decarboxylative breakdown is probably not a factor to be considered in this study.

The hypothesis that the formation of hydroxy, keto and unsaturated acids from amino acids by the deaminating action of surface-taint producing bacteria was advanced by Campbell (10) as a possible explanation of the mechanism responsible for the elaboration of the "sweaty-feet" odour in surface taint butter. The object of the work reported upon herein has been to determine the validity of this hypothesis.

The early studies recorded in the literature concerning the nature of the microbial breakdown of amino acids are of little scientific value because they were carried out with mixed cultures of putrefactive microorganisms on mixtures of proteins and protein decomposition products. These studies were followed by a period of investigations employing the action of mixed cultures on pure amino acids, but the relation between the action of any one organism and the particular type of product formed could not be established. The introduction by Harden (1901) and by Ehrlich (1905) of procedures for the study of the action of pure cultures of a single microorganism on individual pure amino acids, initiated the modern

method of approach to investigations on amino acid catabolism.

The early theories of deamination of Neuberg and Langstein and of Embden postulated that the formation of alpha-hydroxy acids with the elaboration of ammonia resulted from the hydrolytic deamination of amino acids. More recent evidence, however, indicates that deamination of amino acids is not limited to one type of breakdown but may follow one of a number of courses dependent upon the structure of the amino acid, the microorganism employed and the composition and condition of the reacting medium (61). The various paths of amino acid breakdown have been brought together by Anderson (2) and his chart of their aerobic decomposition is presented in figure 1.

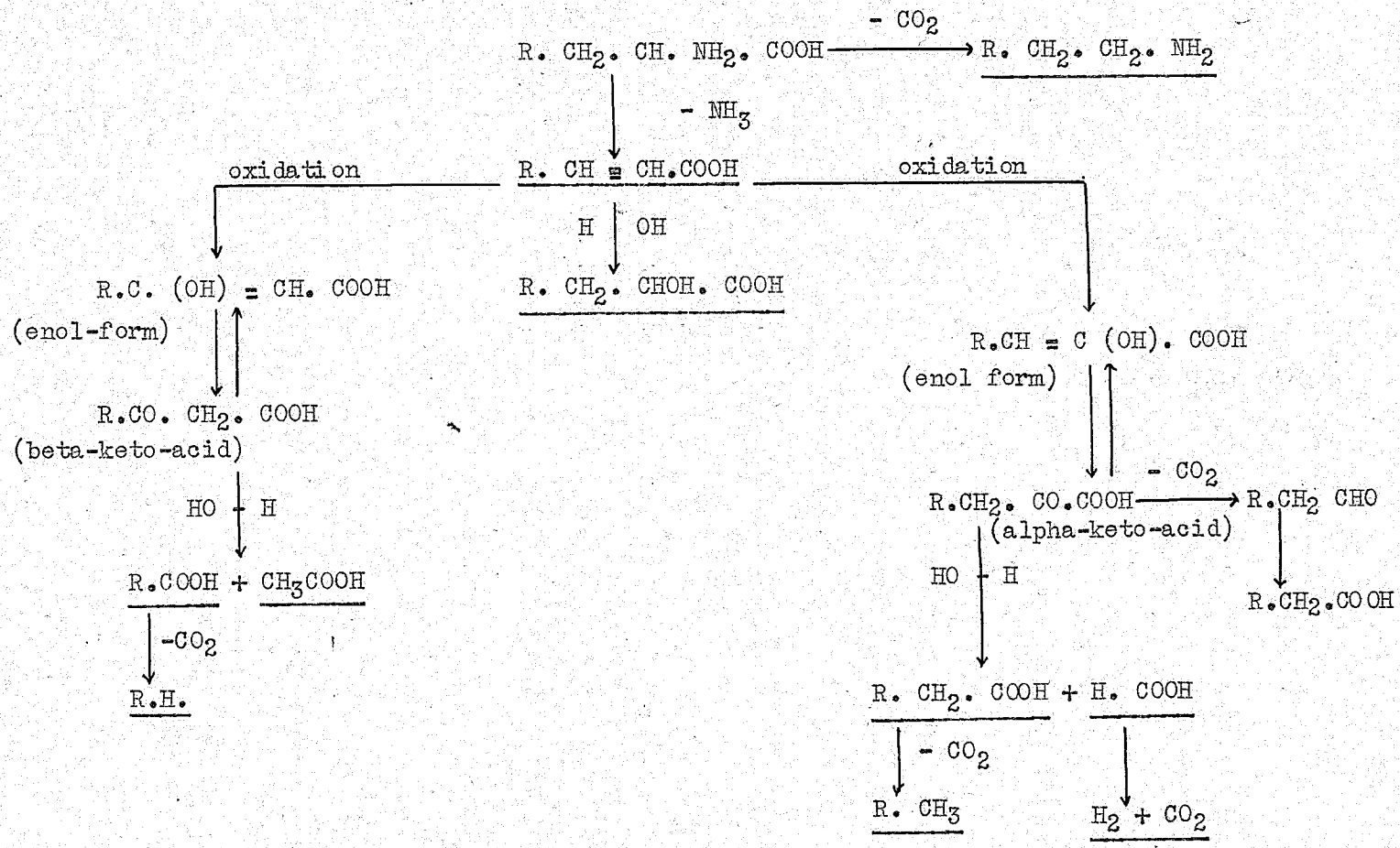
The primary aerobic breakdown of amino acids may follow one of two courses, decarboxylation or deamination. As stated earlier, Campbell (10) was unable to obtain evidence in support of the hypothesis that amines are formed during the elaboration of surface taint, indicating that decarboxylative breakdown is probably not a factor to be considered in this study.

Other studies by the same author have shown that aeration increases the development of surface taint in butter, thereby suggesting that the mechanism for the breakdown of amino acids by surface taint bacteria may be oxidative in nature.

The findings of Neilson (50), from experiments in which organic compounds that could result from the breakdown

FIGURE 1.

Aerobic Breakdown of Amino Acids (Anderson)



(The underlined compounds have actually been isolated)

of certain amino acids, were dissolved in autoclaved milk and the respective odours emitted by them determined by Thornton's test (71) add further evidence to the hypothesis that the surface taint odour is intimately related to the decomposition products of particular amino acids.

The first step in the deamination of amino acids appears to be desaturation at the alpha-beta-linkage with the formation of unsaturated acids and the liberation of ammonia. From this stage, the breakdown may proceed in one or more of three directions.

The first of these is hydrolysis giving alpha-hydroxy acids. The odours emitted by the alpha-hydroxy acids of certain amino acids are known to resemble closely those evolved from putrefactive matter and suggest their possible relationship to the surface taint odour in butter.

The second and third courses of breakdown from the unsaturated acid are oxidative in nature and result in the formation of the enol-forms of the alpha-keto and beta-keto acids respectively. Again there is the possibility that the keto acids are in some way involved in the elaboration of the surface taint odour. Decarboxylation of these keto acids results in the formation of aromatic aldehydes whereas hydrolysis causes the production of lower fatty acids. These fatty acids and aldehydes in combination with hydroxy and keto acids must also be considered as possible sources of the characteristic "sweaty-feet" odour of surface taint butter.

Further evidence in support of the deamination hypothesis is contained in the reports of Dunkley and Wolchow.

The findings of Dunkley (12) indicate that the compounds formed in the development of surface taint are acidic in nature. Wolchow (71) has suggested that the products of reductive deamination may be concerned with the elaboration of surface taint in butter.

The structure of the amino acid is one of the principal factors determining the nature of the deaminative breakdown brought about by the action of microorganisms. Anderson (2) concludes that long chain amino acids are more easily attacked than those containing ring structures, and that the ease of attack increases with the length of the chain. The findings of Neilson (50), however, show clearly that three species of surface-taint producing bacteria are able to open the iminazol ring of histidine and the pyrollidine ring of proline in contrast with their low ammonia-forming ability from the straight chain amino acids - leucine, lysine and methionine; and attack the short chain amino acids - glycine and beta alanine - to a greater degree than the above mentioned longer chain amino acids.

The position of the amino group may also play an important role not only in determining the nature of the breakdown but also the ease with which it occurs. The literature contains very little data on this point. In the same investigation (50), the above author obtained evidence that the further the amino group was from the alpha-position, the more difficult it was to deaminate by the bacterial species employed.

The presence of carbohydrates in the racting medium modifies or changes the course of amino acid catabolism. A

number of theories have been advanced to explain this observation. Kendall and his associates (39, 40 and 41) state that the presence of carbohydrate "spares" protein from attack by substituting a more readily assimilable form of food, but, as Stephenson (61) points out, this claim rests on a false comparison between the bacterial production of ammonia and the mammalian production of urea. Urea is a waste product which cannot serve as a nitrogenous food for the animal in which it was produced, therefore its rise and fall is a true measure of protein metabolism and when increased carbohydrate causes decreased production of urea, the carbohydrate is said to "spare" the protein. With bacteria, however, while ammonia is the chief nitrogenous product of the bacterial decomposition of protein, it is also an excellent nitrogen source for many species of bacteria. Therefore its disappearance from the culture media may be caused either by decreased production, or, what is more likely, by increased utilization as a nitrogen source for cell reproduction.

Raistrick (57) in an explanation of the effect of the presence of carbohydrates concludes with the belief that carbohydrate, far from having a protein-sparing effect, actually enables the bacteria to utilize more protein or protein products than they would in the absence of carbohydrate.

Waksman and Lomanitz (68) point out that a living being derives its energy from the substance which is more available to it and which may be specific for the particular organism.

The studies of a number of investigators whose findings are given in the historical section of this thesis show clearly that the addition of a specific carbohydrate to the reacting medium will change the course of amino acid breakdown and give different products. A satisfactory explanation of these results is not, as yet forthcoming.

The first part of the experimental work of this problem was devoted to an investigation of the conditions affecting the ammonia producing abilities of two species of surface-taint producing bacteria from a specific group of amino acids. These amino acids - arginine, aspartic acid, glutamic acid, histidine and proline - were chosen for a number of reasons, the more important of which are:

(1) Ammonia is readily liberated from them by species of surface taint bacteria.

(2) The decomposition products of these five amino acids are related to one another as can be seen in figure 2.

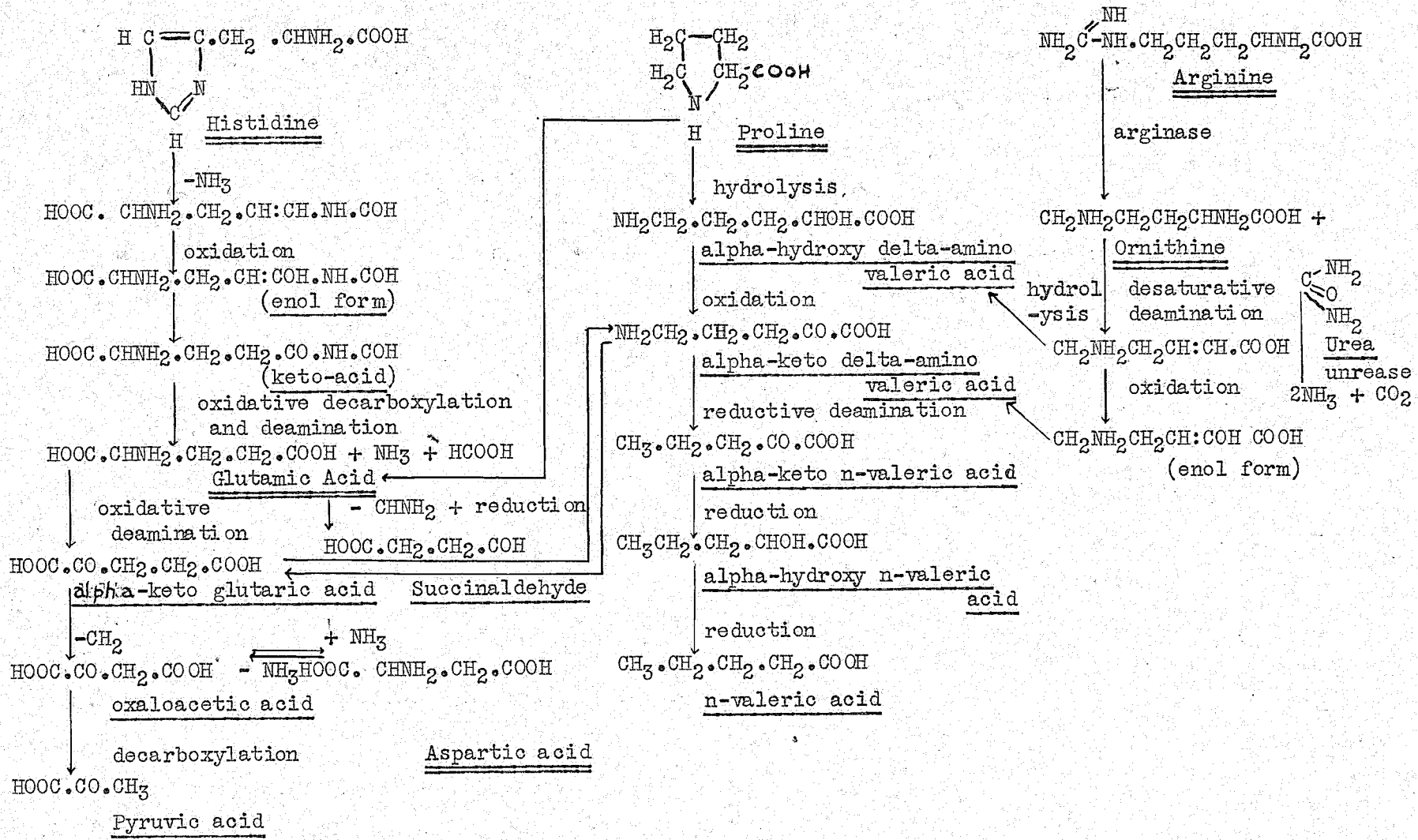
(3) Certain of these decomposition products have been shown to emit odours that closely resemble the characteristic surface taint odour (50).

From the point of view of studying the deamination of amino acids of different basic structure, the five chosen represent three structural groups.

Arginine is a complex straight chain amino acid containing four nitrogens in three different groupings. The guanido group attached to the delta carbon atom may be hydrolysed from the remaining alpha-amino valeric acid. The

FIGURE 2.

CHART OF DEAMINATION



literature contains practically no evidence for this cleavage. The usual method of decomposing arginine is through the enzyme, arginase, to give urea and ornithine (alpha, delta, diamino valeric acid). The urea may be subsequently hydrolysed by the enzyme, urease, into ammonia and carbondioxide and the ornithine deaminized at either the alpha or the delta amino group or both to give valeric acid or one of its derivatives.

The next two amino acids chosen for this study, aspartic acid and glutamic acid, are mono-amino, dicarboxylic acids, the latter containing one more methyl group in the chain between the carboxyl groups than the former.

The last two amino acids contain five-element ring structures. Histidine or alpha-amino beta-iminazol propionic acid has two nitrogens in the ring and one in the side chain. Proline, on the other hand, is not a true amino acid, as its one nitrogen is present in the ring as an imino group.

As the first part of this study developed, it soon became evident that certain of the chosen amino acids were broken down into compounds giving odours suggestive of that characteristic of surface taint. It was decided, therefore, to devote a part of the experimental study to the actual isolation and, if possible, the identification of these odour-forming compounds.

With the ultimate objective of determining the nature of the compounds responsible for the elaboration of the characteristic "sweaty-feet" odour of surface taint butter, the following approaches were made to the problem:

(1) An investigation of the conditions affecting ammonia formation from arginine, aspartic acid, glutamic acid, histidine, and proline by two species of surface-taint producing bacteria.

(2) A study of the production, isolation and identification of compounds evolving odours identical with or intimately related to the characteristic surface taint odour.

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PART I.

STUDIES ON THE DEAMINATION OF AMINO ACIDS.

HISTORICAL:

A study of the decomposition of individual amino acids by pure cultures of single strains of microorganisms has brought out many interesting facts. The composition of the medium, the conditions of growth and the type of organism employed have been shown to exert a marked influence on the activity of the enzymes formed and on the type of deamination that takes place.

Hydrolytic deamination with the formation of the hydroxy acid may result from the action of bacteria on amino acids in a medium containing alternative carbon and nitrogen sources and from the activity of yeast in a medium containing an invert sugar and no alternative nitrogen source. Schmidt, Peterson and Fred (52) isolated d-leucic acid from l-leucine employing Proteus vulgaris. Hirai (35) obtained beta-iminazol lactic acid from histidine using B. proteus. Sasaki and Otsuka (60) isolated the hydroxy acids of l-phenylalanine, l-tryptophan and l-tyrosine employing B. proteus, B. subtilis and B. coli. Hirai (35), using an alternative carbon source, glycerol, and an alternative nitrogen source, ammonium carbonate, isolated p-hydroxy beta-phenyl lactic acid from tyrosine employing Proteus vulgaris. Ehrlich and Jacobsen (19), employing Oidium lactis, obtained the hydroxy acids of l-phenylalanine, l-tryptophan and l-tyrosine. Woolf (77) isolated the hydroxy acid of aspartic acid employing B. coli

in a medium with no alternative carbon and nitrogen source and containing toluene as a bacterial growth inhibitor.

Hydrolytic deamination and decarboxylation may result in the formation of an alcohol with one less carbon atom than the corresponding amino acid. This type of breakdown is caused principally by yeasts and molds. Ehrlich (13, 14, 15, 16, 17 and 18), in a study of the decomposition of amino acids by yeasts, obtained the corresponding alcohols from l-valine, l-leucine, dl-serine, l-histidine, l-phenylalanine, l-tryptophan and l-tyrosine. Pringscheim (53), employing a number of cultures of molds and one of yeast, isolated the alcohol from l-leucine.

Reductive deamination resulting in the production of the saturated normal acid from the corresponding amino acid usually occurs in a medium containing no alternative carbon and nitrogen sources. Brasch (8), employing B. putrificus in such a medium, obtained the corresponding acids from glycine, l-alanine, dl-serine, l-aspartic acid and l-tyrosine, while Nawiasky (49), using B. proteus, and Blanchetiere (7), using B. flourescens, isolated the acid from aspartic acid. Koessler and Hanke (44) added glycerol to the medium of Brasch and isolated beta-iminazol propionic acid from histidine employing B. coli. Hopkins and Cole (36) observed that indole propionic acid was formed from tryptophan in a medium containing inorganic salts with Rochelle salts and ammonium phosphate using B. coli. Traetta-Mosca (66) has shown that p-hydroxy beta-phenyl propionic acid is formed from tyrosine in an inorganic medium containing ammonium nitrate by a

bacillus resembling B. pyocyaneus. Kiyokava (42) obtained beta-iminazol propionic acid from histidine employing Oidium lactis in an inorganic salt medium plus one percent cane sugar.

Reductive deamination and decarboxylation may result in the formation of the normal acid containing one carbon atom less than the corresponding amino acid. The literature contains only one record of this type of breakdown brought about by the action of pure cultures on amino acids. Brasch (8), employing B. putrificus isolated propionic acid from l-glutamic acid in a medium of inorganic salts.

Desaturation at the alpha-beta linkage causes the formation of the corresponding unsaturated acid with the liberation of a molecule of ammonia. Raistrick's isolation (55) of urocanic acid from l-histidine in 1917 is the first recorded instance of the bacterial conversion of an amino acid into an unsaturated acid. He employed the action of pure cultures of five species of intestinal bacteria on histidine in Ringer's solution. Hirai (35), in 1921, isolated the acrylic acid of tyrosine after twelve days incubation of B. proteus in an inorganic salt medium containing no alternative carbon and nitrogen sources. Quastel and Woolf (54) obtained fumaric acid from aspartic acid in a phosphate buffer medium containing toluene as a growth inhibitor.

The oxidative breakdown of amino acids may result in the formation of a number of acids containing a decreasing number of carbon atoms. The keto acids formed from the primary oxidation of the amino acids have not been isolated except in rare cases, but the acids from the subsequent oxidation of the

keto acids of certain amino acids have been obtained and identified. Nawiasky (49), employing B. proteus in an inorganic salt medium containing no alternative carbon and nitrogen sources, isolated acetic acid from l-alanine, isobutyric, acetic and formic acids from l-valine, acetic acid from l-aspartic acid and succinic acid from l-glutamic acid. Brasch (8) isolated formic acid from dl-serine using B. putrificus in an inorganic salt medium similar to that of Nawiasky. Raistrick (56) has shown that organisms of the coli-typhosus group in a medium of inorganic salts with no alternative carbon and nitrogen sources, rupture the iminazol ring of histidine. Hopkins and Cole (36) isolated indole acetic acid and indole from the oxidative breakdown of tryptophan by B. coli in an inorganic salt medium containing Rochelle salts and ammonium phosphate. Woods (72), Happold and Hoyle (32) and Woods (73) isolated indole, employing washed cell suspensions of B. coli on tryptophan in buffer solution. Raistrick and Clarke (57) obtained evidence for the rupture of the indole ring of tryptophan by B. pyocyaneus and B. flourescens in an inorganic salt medium. The oxidative breakdown of l-tyrosine varies with the composition of the medium. Traetta-Mosca (66) isolated p-hydroxy benzoic acid and p-cresol from tyrosine employing B. pyocyaneus in an inorganic salt medium containing ammonium nitrate. Berthelot (6) and Rhein (58 and 59) obtained evidence for the formation of phenol by B. phenologenes from tyrosine in an inorganic salt medium containing ammonium lactate and asparagin. Hirai (35) isolated p-hydroxy beta-phenyl acetic acid from tyrosine employing the same con-

ditions as for the isolation of p-hydroxy beta-phenyl acrylic acid, except using forty days' incubation in place of twelve days. Raistrick and Clarke (57) found evidence for the rupture of the ring or the removal of the hydroxy group from the ring of l-tyrosine in a medium of inorganic salts employing B. pyocyaneus and B. flourescens, but they were unable to identify the products formed.

The nature of the bacterial breakdown of amino acids may be determined by methods other than the actual isolation of decomposition products employed in the early investigations on this subject.

Bernheim, Bernheim and Webster (4) measured the oxygen uptake and carbon-dioxide output employing the Warburg respirometer. They determined the ammonia formed through the accompanying deamination by distilling it from the contents of the Warburg vessel at the completion of the experiment into a Nessler's solution. The ammonia present was then determined colorimetrically. In a study of the oxidation of certain amino acids by Proteus vulgaris, employing the washed cell technique for the preparation of their bacterial suspension, they found that, at pH 7.6, glycine was the only amino acid studied to be completely oxidized. Leucine, phenylalanine and methionine are oxidized rapidly and utilize one atom of oxygen per molecule of amino acid while serine, alanine and proline utilize three, four and seven atoms respectively. The oxidation of tyrosine and tryptophan is slower using two and three atoms of oxygen respectively. Valine, isoleucine, hydroxyproline and histidine are oxidized so slowly that no

definite uptakes of oxygen were obtained. Their work showed that only the natural isomers were oxidized except in the case of alanine and serine, both isomers of which were oxidized, and further that deamination corresponds with the oxidation except for valine.

In a later study (5) of the oxidation of thirteen amino acids by washed cell suspensions of B. pyocyaneus, they found that tryptophan and methionine were not attacked, and that there was considerable variation in the oxidation rates and the extent to which the attacked amino acids were oxidized. They showed that this organism oxidized and deaminated both isomers of alanine, serine, tyrosine and proline, but only the natural isomers of leucine, isoleucine and histidine. They suggested that the variation in the rate of oxidation of the amino acids may mean that there are separate catalysts for each amino acid and that the amino acids are attacked differently by the different bacteria, or, more probably, that the rate of formation of the enzyme-substrate complex is determined by the chemical structure of the amino acid.

Woods and Clifton (75), in the next year, employing the Warburg technique and suspensions of clostridium tatanomorphum, found that glutamate, aspartate, cysteine, cystine tyrosine and methionine yielded hydrogen and/or carbon-dioxide and were almost completely deaminated. They also observed that histidine produced more than one molecule of ammonia per amino group present and concluded that the iminazol ring had been opened.

In a study of the metabolism of the amino acids by Clostridium welchii, Woods and Trim (76) observed that this organism attacks only five of the twenty-one amino acids studied. Serine gave hydrogen, carbon-dioxide and ammonia in equimolecular proportions and required a coenzyme for its breakdown. Cystine, cysteine and threonine were attacked similarly to serine but the evidence for the need of a coenzyme factor was incomplete. Arginine was attacked to give ammonia and carbon-dioxide, the final yields of which varied with the age of the growth culture. Hydrogen was not formed and a coenzyme was not required.

A recent investigation by Neilson (50), employing the Van Slyke aeration procedure for the determination of ammonia, indicated that washed cell suspensions of Proteus ichthyosmius (Hammer) attacked beta-alanine, d-arginine, glycine and l-proline vigorously; dl-aspartic acid, l-cystine, d-glutamic acid, l-histidine, dl-isoleucine, l-leucine, d-lysine and beta-amino butyric acid less vigorously; and methionine, phenylalanine, tryptophan, tyrosine, urea, delta-amino valeric acid and epsilon-amino caproic acid only slightly. Similar suspensions of Pseudomonas putrefaciens (Hammer) attacked histidine and proline vigorously; aspartic acid and glutamic acid less vigorously; and beta-alanine, arginine, cystine, isoleucine, leucine, lysine, methionine, phenylalanine, tryptophan, tyrosine, urea, beta-amino butyric acid, delta amino valeric acid and epsilon amino caproic acid only slightly.

The mutual oxidation and reduction by pairs of amino

acids has been studied by Stickland and Woods. Stickland (63 and 64) observed that washed cell suspensions of Clostridium sporogenes activated certain amino acids as hydrogen-donators - alanine, valine and leucine, and activated others as hydrogen-acceptors - glycine, proline and hydroxyproline. The hydrogen-accepting amino acids are subjected to reduction by the hudrogen-donating amino acids which in turn are oxidized to compounds similar to those resulting from oxidative deamination. Woods (74) has observed that l-cysteine may act as hydrogen-donator in coupled reactions between amino acids induced by Clostridium sporogenes and that, in addition, it is partially deaminated in the absence of other amino acids. He also has shown that d-arginine and d-ornithine are activated as hydrogen-acceptors and are partially deaminated in the absence of hydrogen-donators. When ornithine reacts with the donator, alanine, it accepts two hydrogens and undergoes reductive deamination to delta-amino n-valeric acid.

Recent investigations employing the resting cell technique or enzymic extracts have brought forth considerable data on deamination and only the literature concerning the five amino acids studied in this problem with particular reference to their bacterial breakdown will be reviewed in detail.

The study of the deamination of aspartic acid by washed cell suspensions of E. coli carriel out by Quastal and Woolf (54) revealed that the product in the absence of any inhibitor is succinic acid, whereas, if an inhibitor such as toluene is present, the rate of deamination is not affected

in the beginning but the process does not go to completion, reaching, instead, an equilibrium mixture of aspartic acid, fumaric acid and ammonia.

In a study of the fermentation of glutamic acid by a strictly anaerobic, spore-forming bacterium, probably belonging to the genus Peotoclostridium, Barker (3) demonstrated that this acid was decomposed practically quantitatively into ammonia, carbon-dioxide, hydrogen, acetic acid and butyric acid.

Adler (1), employing a deaminase enzyme extracted from E. coli, found that glutamic acid, in the presence of coenzymes, is decomposed into ketoglutaric acid and ammonia and suggested that the breakdown was through iminoglutaric acid to ketoglutaric acid with the liberation of ammonia.

The work of Klein (43) on the oxidation ofl-aspartic and l-glutamic acids by Hemophilus parainfluenzae showed that both acids were oxidized to acetic acid, ammonia and carbon-dioxide; one molecule of aspartic acid requiring one molecule of oxygen and producing two of carbon-dioxide, and one molecule of glutamic acid requiring two and one-half molecules of oxygen and producing three of carbon-dioxide. By a study of the metabolism of possible intermediate compounds, Klein has established that the probable course of oxidation of aspartic acid by this organism proceeds through oxalacetic acid, pyruvic acid and acetaldehyde to acetic acid.

Ammonia formation from proline or from histidine in amounts greater than that given by its amino group involves opening of a five-element ring, the pyrollidine in the case

of proline and the iminazol for histidine, a feat extremely difficult to accomplish in non-biological chemistry. Investigations in biochemistry, however, have shown that certain enzymes, particularly those of bacterial origin, are capable of opening these rings preparatory to removing the nitrogen by deamination of the amino group formed.

Eldbacher and others (20, 21 and 22), in studies of the intermediate metabolism of histidine using the ensyme histidase obtained from animal liver, found that one molecule of histidine gave two of ammonia, one of glutamic acid and probably one of formic acid, the primary action of the enzyme being to open the iminazol nucleus to form an alpha, delta, diamino chain.

The investigation of Raistrick (56) into the aerobic bacterial decomposition of histidine by B. paratyphosus A and B, B. faecalis alcaligened, B. pyocyaneus and B. proteus vulgaris revealed that the first four of these organisms produced ammonia from the nitrogen in both the side chain and the iminazol nucleus, proving that they are able to open the iminazol ring; whereas the fifth organism formed ammonia only from the side chain nitrogen, showing its probable inability to split the ring.

Stickland (65), through his experiments on the coupled oxidation and reduction by pairs of amino acids, observed that washed cell suspensions of Clostridium sporogenes reduced l-proline, at the expense of the oxidation of l-alanine, to delta-amino n-valeric acid, but did not deaminate this product, whereas the alanine was deaminated during

its oxidation.

Weil-Malherbe and Krebs (69), employing kidney tissue, studied the conversion of proline into glutamic acid and concluded that proline in the kidney is oxidized to glutamic acid which in turn may be oxidized further to alpha-keto glutaric acid with the liberation of ammonia, or may take on more ammonia to form glutamine.

Krebs (46), in his work on the oxidation of d-proline by d-amino acid oxidase, was able to isolate the 2, 4, dinitrophenylhydrazone of alpha-keto delta-amino valeric acid, thus demonstrating the opening of the ring on the carboxyl side of the nitrogen in contrast with the formation of glutamic acid when the oxidative opening of the ring occurs on the methyl group side of the nitrogen.

The decomposition of arginine, which contains one nitrogen as an alpha amino group and three nitrogens in the guanido group on the delta carbon atom, may involve a number of ensymes. Hunter and Dauphinee (38), in studies on the action of arginase extracted from liver, have found that this ensyme, at pH 8.5 and room temperature, splits arginine into urea and ornithine to the extent of 99.1 percent. The urea may subsequently be decomposed by the ensyme urease at pH 6.8 and room temperature into ammonia and carbon-dioxide.

Vovchenko (67) investigated the effect of products of arginine hydrolysis upon the action of arginase and found that the presence of ornithine and/or urea inhibit the action of the enzyme, the inhibiting effect being smaller at pH values lower than the optimal for the hydrolysis, pH 9.6, than

at higher values.

Tomota (65), from a determination of the activity of arginase extracted from a number of bacterial species, has shown that extracts from Staph. aureus, Staph. albus, Staph. citreus and Bac. subtilis possess strong arginase activity; while the one from Sarcina is fairly active and those from Streptococci, Mycobacterium phlei, Salmonella enteritidis, B. proteus, B. pyocyaneus, B. prodigiosus and Mucosus capculatus show weak and variable activity.

The results of an investigation into the decomposition of arginine by gram positive cocci in which two molecules of ammonia and one of ornithine were produced from each molecule of arginine have caused Hill (34) to conclude that the ensyme involved was not arginase but was another enzyme which he has named arginine dihydrolase. The reasons for his conclusion are: (1) the strains of streptococci used were shown not to possess urease, (2) the urease of a stock strain of Staphyloccus is insufficiently active to account for the rate of breakdonw of arginine, and (3) in a strain of Staphylococcus trained to grow on an ammonia medium, the urease activity can be varied at will between very wide limits, while the arginine enzyme varies in an inverse manner. This reciptocal relationship suggests that the enzymes may be related though not identical.

Woods (74) has observed that through the action of Clostridium sporogenes one molecule of arginine yields three molecules of ammonia either alone or as a hydrogen-acceptor coupled with alanine and similarly that ornithine gives one

molecule of ammonia and one of delta-amino valeric acid.

The literature concerning the factors influencing bacterial deamination is extensive; only those factors pertinent to this study will be reviewed, - the influence of the age of culture, aerobiosis and anaerobiosis, pH of the growth and buffer media and presense of carbohydrates in the growth and buffer media.

Stephenson and Gale have investigated the influence of these factors on the bacterial deamination of a number of amino acids by washed cell suspensions of Bacterium coli. In their first paper (62), using glycine, dl-alanine and l-glutamic acid, they found that only slight variation in the activity of the suspension occurred when the growth culture was between eight and twenty hours, that anaerobic conditions during growth inhibit the production of the deaminase for glycine and alanine but favour it for glutamic acid, and that the effect of glucose of the oxidative deamination of the three amino acids is to inhibit the formation of the enzyme during growth to the extent of ninety-five percent. Their paper (30) on dl-serine brought out that this amino acid is deaminated both aerobically and anaerobically, that both isomers are attacked though probably at different rates. The activity of serine deaminase varies markedly with the age of the growth culture, attaining its height at eleven hours and subsequently falling off, is increased by anaerobic growth conditions and, like the other deaminases, is decreased ninety-five percent or more by the presence of two percent glucose in the growth medium. The optimum pH for this

deaminase was pH 8.0. The third paper by Gale (29) on aspartic acid reports that the activity of the deaminase of this acid varies with growth conditions - aerobic conditions showing about two-thirds the activity of anaerobic conditions and two percent glucose inhibiting the activity about eighty-five percent, and with the age of the growth culture being greatest from fourteen to eighteen hours. This latter variation was explained as being caused by an alteration in the chemical constitution of the growth medium following the metabolic activity of the cells. The activity of this deaminase ranged from pH 6.0 to 8.0, being optimum at pH 7.5.

In a study of the l- and the d- amino acid oxidases extracted from kidney tissue, Krebs (45) found that the optimum pH curve of the d-amino acid deaminase shows an optimum near pH 8.8 when used for the deamination of dl-alanine and dl-aspartic acid. The deamination of l-aspartic acid shows an optimum at pH 7.4 but it still retains sixty percent of its activity at pH 6.5. Krebs also found that a final concentration of M/200 aspartic acid gave the maximum rate of deamination.

Gale and Epps (30), in an investigation of the effect of the pH of the medium during growth on the enzymic activities of E. coli and Mc. lysodeikticus, was able to divide the enzymes produced into two groups. The first group contained urease, catalase, hydrogenlyase, etc. and the second the amino acid enzymes. The decarboxylases were produced in an acid medium and the deaminases in an alkaline medium and seemed, therefore, to act as neutralization

mechanisms in an effort to bring the pH of the medium nearer neutrality.

The question of the effect of the presence of fermentable and non-fermentable carbohydrates in the growth and buffer medium has caused considerable controversy in the literature.

Kendall and his associates (39, 40 and 41) in a series of papers from 1912 to 1922 studied the production of ammonia by several bacterial species, including B. proteus, growing in protein digests and showed that this production is greatly checked and, in some cases, completely inhibited by the presence of glucose in the growth medium. They interpreted this as due to a "sparing" action exerted by the carbohydrate on the deamination of proteins, believing that in the presence of a readily available source of carbon and energy the organism decomposed less nitrogenous material.

Raistrick and Clark (57) pointed out that ammonia is not only a product of the decomposition of proteins, but also a source of nitrogen for growth, so that while, in the protein digest medium the ammonia produced is in excess of that required for cell synthesis, it may be possible that, in the presence of additional carbohydrate, this excess is used up in increased cell production. To further investigate this point, they followed the growth of B. pyocyaneus and B. flourescens in synthetic media containing tryptophan or tyrosine, with or without glycerol. They found that in the absence of glycerol there was a large amount of ammonia with very little synthesized nitrogen, but that in the presence of glycerol the

reverse was true and concluded with the belief that "carbohydrate, far from having a protein-sparing effect, actually enables the bacteria to utilize more protein or protein products than they would in the absence of carbohydrate."

Waksman and Lomanitz (68) carried out a detailed investigation of this point using species of bacteria, actinomyces and molds and came to similar conclusions but pointed out that "a living being derives its energy from a substance which is most available to it and which may be specific for a particular organism."

Nisimura (51) studied the effect of various carbohydrates on the formation of p-hydroxyphenyl lactic acid (I) by a strain of B. proteus, of p-hydroxyphenyl propionic acid (II) by another two strains of B. proteus and of tyramine (III) by a strain of B. lactis aerogenes from tyrosine in a Sasaki protein-free nutrient solution. When glucose was added to the solution, no II was formed, formation of I was distinctly decreased but no effect was found on formation of III. Addition of levulose or sucrose to the solution lowered the formation of I, II and III. The addition of lactose accelerated the formation of II by one strain of B. proteus, inhibited the formation of I by another strain of B. proteus and of III by B. lactis aerogenes. Addition of starch had no effect of the formation of I but regarded the formation of III slightly and of II distinctly. In the control experiments and those in which carbohydrates were added, the pH of the nutrient solution was generally shifted to the acid side.

Mannozzi-Toriki and Vendramini (47), in a study of

the oxidation of amino acids by Brucella abortus in Ringer-phosphate solution, found that this organism catalysed the oxidative cleavage of alanine, asparagin, glutamic acid and cysteine and that the presence of glucose had no cleavage on this cleavage.

Epps and Gale (23) compared the influence of the presence of glucose during growth on the enzymic activities of E. coli with the effect produced by fermentation acids added to the medium. They observed that the presence of glucose in the medium during growth suppresses the formation of certain enzymes, the degree of inhibition being greater than or bearing no relation to the effect produced by growth in a medium adjusted to the final pH of the glucose medium by fermentation acids; that the neutralization of fermentation acid during growth in glucose does not alter the degree of inhibition of deaminase formation produced by the glucose; and that the reduction in the activity of certain enzymes as a result of growth in glucose is not a permanent change in the enzyme constitution of the cell as it is removed immediately after growth takes place in the absence of fermentable carbohydrate.

As a part of the investigation of the mechanism for the production of indole from tryptophan by Bacterium coli, the primary step of which may be deamination, a number of workers have studied the effect of the presence of glucose in the growth and buffer medium. Happold and Hoyle (33) added glucose to the growth medium and noticed that the

inhibition of the production of the tryptophanase enzyme system does not occur from the start but shows a short latent period followed by a complete inhibition which lasts only until the fermentation of the sugar is completed. They also showed that the products of glucose fermentation have no inhibiting action.

Happold and Hoyle (32) and Fildes (26) found that washed cell suspensions of B. coli grown in a tryptophan-containing medium are twenty-five times more active producers of indole from tryptophan-phosphate-buffer solutions than similar suspensions grown in a medium completely lacking in tryptophan and that this excess activity is entirely inhibited by the presence of glucose in the tryptophan-buffer solution.

Evans, Handley and Happold (24) reported that the tryptophanase system for the production of indole does not exist as such in cells of B. coli which have been grown in a medium containing glucose, but that such cells when freed from glucose by washing will re-develop the enzyme system when left in contact with tryptophan.

The findings of Neilson (50) in a study of the relation between indole production and surface taint in butter employing washed cell suspensions of Proteus ichthyosmius confirm the observations of Evans and others. It was found that the presence of one percent glucose in the agar growth medium had no influence of the quantity of indole formed by the suspension in a glucose-free tryptophan buffer solution whereas the presence of one percent glucose-in the

tryptophan-buffer mixture exerted an inhibiting effect which was only partial when the cells were grown on a glucose-free agar but was complete when they were grown on a glucose-containing agar.

Evans, Handley and Happold (25), in trying to work out a possible mechanism for the inhibition of indole production by glucose in cultures of B. coli, studied the effect of other fermentable and non-fermentable sugars on this inhibition. Their experiments show that glycogen, starch, dextrin, sucrose, dulcitol, salicin, inulin, potassium saccharate and hexosediphosphate are not fermented in twenty-four cultures of the organism and do not affect the production of indole when tryptophan is present in the medium. On the other hand, complete inhibition of indole production is obtained with arabinose, lactose, glucose, fructose and mannitol, - sugars which are fermented by B. coli. The sugars which showed acid production without marked inhibition of indole production were rhamnose, xylose, sorbitol, galactose, d-ribose and mannose.

EXPERIMENTAL

The results of earlier studies (50) on the quantitative determination of ammonia formed from individual amino acids by surface taint bacteria gave support to the theory that deamination may be intimately concerned with the elaboration of the characteristic surface taint odour. Therefore it was decided that detailed experiments on the conditions required for ammonia formation from a number of amino acids might bring forth further evidence for the theory as well as add to our present knowledge of the microbial catabolism of amino acids.

The results referred to above showed that there was considerable ammonia formed by species of surface taint bacteria from the amino acids arginine, aspartic acid, glutamic acid, histidine and proline which are theoretically, with considerable supporting evidence in the literature, related to one another through their decomposition products.

With the object of investigating in detail the conditions of ammonia formation from the above five amino acids by two species of surface taint producing bacteria - Proteus ichthyosmius (Hammer) and Pseudomonas putrefaciens (Hammer) - the following series of experiments were carried out.

The general experimental procedure employed throughout the study was as follows:

1. The organisms were inoculated from tryptic casein digest broth onto tryptic casein digest

agar in Kolle flasks and grown for a period of 16 to 18 hours. In the case of Proteus ichthyosmius, 30° C. was employed as the temperature of incubation, and for Pseudomonas putrefaciens 23° C.

2. The bacterial cells were then washed off the agar with M/30 phosphate buffer, pH 7.4, (Sorensen's M/15 phosphate buffer diluted with an equal volume of distilled water) and centrifuged for approximately one hour in fifty or one hundred cubic centimeter centrifuge tubes.

3. The supernatant was poured off aseptically and the cells taken up and mixed thoroughly with about 30 cc of M/30 phosphate buffer and then re-centrifuged.

4. The supernatant was again poured off and the cells taken up and mixed thoroughly with about 50 cc of M/30 buffer or distilled water, depending on the nature of the experiment, (vide infra), per Kolle flask of organisms and an aliquot of 5.0 cc taken into a Hopkins vaccine tube to determine the percentage by volume of cells present. A one percent suspension was used as an inoculum.

5. The cultures were then set up in test tubes containing eight cubic centimeters of M/15 buffer, 1.0 cc of M/20 aqueous amino acid solution and 1.0 cc. of one percent washed cell suspension. In the experiments on the effect of the presence of carbohydrate, 1.0 cc of a 5% aqueous solution of the specific carbohydrate was

added and only 7.0 cc of buffer were employed in order to maintain the final volume at 10 cc. The cultures were incubated at 30° C. for the required number of days. In order to overcome the difficulties resulting from the sterilization of a mixed medium, the nitrogen source, the carbon source and the buffer were each sterilized separately and mixed just prior to inoculation.

6. Sterile equipment and materials and aseptic technique were employed throughout the above part of the procedure.

7. At the conclusion of the incubation period, the pH of the cultures was taken, when required, with a Beckman potentiometer, industrial model, and then the free ammonia determined by the Van Slyke aeration procedure using N/100 sulphuric acid.

8. The results are expressed as percent of the total nitrogen of amino acid in culture present as free ammonia and are converted from cubic centimeters of N/100 sulphuric acid to percent free ammonia by the following formula:

$$\frac{.14 \times \text{cc of N/100 } H_2SO_4 \times 100}{\text{No. of N in amino acid} \times .7} = \% \text{ free ammonia}$$

in which:

.14 = mg. of nitrogen equivalent to 1.00 cc of N/100 H_2SO_4.

.7 = mg. of nitrogen in 1.0 cc of M/20 ammonia or mono-nitrogen amino acid.

THE EFFECT OF pH ON AMMONIA FORMATION.

The literature has shown that the pH of the buffer medium containing the amino acids affects the amount of ammonia that is liberated by various species of micro-organisms. Employing the technique described above, the influence of pH on ammonia formation from d-arginine, dl-asparatic acid, d-glutamic acid, l-histidine and l-proline by Proteus ichthyosmius and Pseudomonas putrefaciens was determined. The following buffer solutions made after the manner of Clark (11) were employed:

phthalate buffer - pH 4.5, 5.0, 5.5, and 6.0.

phosphate buffer - pH 6.3, 6.8, 7.3, and 7.9.

boric acid-KCI buffer - pH 8.5, 9.0, 9.5, and 10.0.

In this particular experiment (I) the cell suspensions were, of necessity, made up in distilled water rather than phosphate buffer of pH 7.4. The cultures were incubated at 30° C. for five days after which the final pH was taken and the free ammonia determined. The results are given in tables 1 and 2, and interpreted on figures 3 to 12 inclusive.

The results of experiment I show that there is a general optimum range in pH for ammonia formation for pH 6.0 to pH 8.5. Within this general range, the different amino acids have an individual range which is sometimes narrow and at others relatively wide. Arginine under the action of Proteus ichthyosmius has a range from pH 6.3 to 8.0 with a rather sharp optimum at pH 7.4 whereas for Pseudomonas putrefaciens the range is from 6.0 to 8.8 with no definite

optimum. Aspartic acid liberates a relatively constant amount of ammonia from pH 6.0 to pH 8.5 for both species of bacteria employed.

The results for glutamic acid are similar to those for aspartic acid except that the range is narrower and further to the alkaline side of the pH scale; pH 6.5 to pH 8.3 being the range for Pseudomonas putrefaciens and pH 6.8 to pH 8.5 for Proteus ichthyosmius. Histidine, on the other hand, has both a narrow range and a sharp optimum within the range. For Proteus ichthyosmius, histidine liberates ammonia in quantity from pH 7.3 to pH 8.7 with an optimum of pH 8.0, while for Pseudomonas putrefaciens, the range is from pH 7.0 to pH 8.9 with the optimum at pH 7.6. The results obtained for proline show two different graphs for the two species of bacteria. Proteus ichthyosmius causes considerable ammonia formation from pH 7.4 to pH 8.5 with a very sharp optimum at pH 8.0. Pseudomonas putrefaciens stimulates the formation of a relatively steady quantity of ammonia from pH 6.0 to pH 8.3 without a definite optimum.

These findings are in general agreement with those of previous workers in that the range for ammonia formation is above pH 6.0 and below pH 9.0 with an optimum between pH 7.0 and pH 8.0. Within this relatively wide range, however, the optimum pH varies markedly depending on the amino acid used and the species of microorganism employed.

From these results, it was decided to use the following buffers for the different amino acids. When

employing Proteus ichthyosmius the buffers were: arginine - pH 7.5, aspartic acid - pH 6.5, glutamic acid - pH 8.0, histidine - pH 8.0 and proline - pH 8.0. When employing Pseudomonas putrefaciens the buffers were: arginine - pH 7.0, aspartic acid - pH 6.5, glutamic acid - pH 7.5, histidine - pH 7.5 and proline - pH 7.0.

In order to determine the constancy of the results recorded in tables 1 and 2, the experiment was repeated (II) after an eleven month interval employing phthalate buffer at pH 4.6, Sorensen's phosphate buffer at pH's 5.6, 6.6, and 7.6 and boric acid-KCl buffer at pH's 8.5 and 9.5. The experiment was set up in triplicate and determinations were carried out after seven, fourteen and twenty-one days incubation respectively. The results are recorded in tables 3 and 4 and shown graphically on figures 3 to 12 inclusive.

The findings of experiment II are in general agreement with those of experiment I in that the general optimum range in pH for ammonia formation from the amino acids studied varies from pH 6.0 to pH 8.5. There are, however, in some instances differences in the individual ranges of pH. The longer incubation times show that for some of the amino acids the maximum amount of ammonia is not formed after five days' incubation. The pH range of from pH 5.6 to pH 7.6 for the ammonia formed from arginine by Proteus ichthyosmius shows a shift towards the acid side. The comparatively large quantity of ammonia produced, however, increased the pH of these cultures suggesting that the true pH range is probably higher than the initial pH of the cultures indicates. An acidity

The Effect of pH on Ammonia Production by Proteus ichthyosmius and Pseudomonas putrefaciens.

Experiments I and II

Tables 1, 2, 3 and 4

Figures 3 to 12 inclusive

Experiment I - 5 days incubation - violet

Experiment II - 7 days incubation - blue

14 daysincubation - green

21 days incubation - red

TABLE I.

Proteus ichthyosmius

Effect of pH

amino acid		initial pH of culture 4.65	5.05	5.57	6.06	6.34	6.86
Arginine	1.	0.5	5.5	42.0	53.5	68.5	68.5
	2.	-	-	-	-	-	-
Aspartic acid	1.	0.5	1.0	3.5	34.5	40.0	37.0
	2.	4.40	4.92	5.38	5.89	6.34	6.86
Glutamic acid	1.	1.0	1.0	2.0	16.0	15.0	33.0
	2.	4.48	4.95	5.42	5.85	5.75	6.62
Histidine	1.					14.6	19.6
	2.					6.15	6.64
Proline	1.					14.5	18.0
	2.					6.26	6.65

		initial pH of culture 7.34	7.96	8.55	9.06	9.46	9.63
Arginine	1.	78.25	71.25	62.5	45.75	37.25	13.0
	2.	-	-	-	-	-	-
Aspartic acid	1.	35.0	36.0	36.0	29.0	25.0	17.5
	2.	7.38	7.96	8.35	8.95	9.34	9.55
Glutamic acid	1.	32.5	32.5	37.0	21.5	17.0	16.0
	2.	7.14	7.75	8.05	8.72	9.05	9.20
Histidine	1.	28.3	38.0	34.6	25.6	18.6	18.3
	2.	7.17	7.60	8.05	8.65	8.97	9.15
Proline	1.	21.0	37.0	17.5	8.0	6.0	4.5
	2.	7.18	7.86	8.20	8.85	9.16	9.34

1. percent of total nitrogen of amino acid (in culture) present as free ammonia.

2. pH of culture at time of ammonia determination.

TABLE 2.

Effect of pH — Pseudomonas putrefaciens

amino acid		initial pH of culture 4.65	5.05	5.57	6.06	6.54	7.05
Arginine	1.	0.0	0.0	1.75	6.75	10.25	10.75
	2.	4.65	5.10	5.57	6.24	6.65	7.14
Aspartic acid	1.	0.0	1.5	9.0	36.0	40.0	34.5
	2.	4.50	4.95	5.45	5.96	6.46	6.98
Glutamic acid	1.	0.5	1.0	8.0	15.0	21.0	22.5
	2.	4.55	4.95	5.44	5.80	6.43	6.95
Histidine	1.				15.3	17.3	32.0
	2.				6.15	6.53	7.06
Proline	1.	0.0	0.5	11.5	26.0	29.0	29.0
	2.	4.65	5.10	5.64	6.20	6.60	7.13

		initial pH of culture 7.60	8.25	8.42	8.85	9.25	9.57
Arginine	1.	8.0	8.0	8.5	7.5	3.25	0.5
	2.	7.72	8.30	8.38	8.78	9.15	9.45
Aspartic acid	1.	35.0	35.5	37.5	32.5	28.5	6.5
	2.	7.54	7.85	8.05	8.65	9.05	9.30
Glutamic acid	1.	23.0	21.0	12.5	10.5	4.5	3.0
	2.	7.46	7.84	8.05	8.60	9.10	9.35
Histidine	1.	40.3	32.6	25.3	31.6	24.0	13.6
	2.	7.56	7.95	8.05	8.56	9.03	9.26
Proline	1.	28.0	22.5	8.5	8.0	1.5	1.5
	2.	7.65	8.11	8.35	8.79	9.25	9.51

1. percent of total nitrogen of amino acid in culture present as free ammonia
2. pH of culture at time of ammonia determination

TABLE 3.

Effect of pH — Proteus ichthyosmius

age of culture		initial pH of cultures 4.6	5.6	6.6	7.6	8.5	9.3
Arginine							
7 days	1.	5.5	74.75	67.5	68.5	45.75	41.5
	2.	-	-	-	-	-	-
14 days	1.	10.0	84.0	84.75	70.25	45.0	31.25
	2.	4.70	6.45	7.05	8.35	8.50	9.00
21 days	1.	14.0	82.75	82.0	60.75	43.0	17.0
	2.	4.75	6.55	7.05	8.30	8.55	9.00
Aspartic Acid							
7 days	1.	0.0	48.0	44.0	45.0	48.0	29.0
	2.	-	-	-	-	-	-
14 days	1.	0.0	-	40.0	40.0	25.0	10.0
	2.	4.55	-	6.65	7.60	8.25	8.95
21 days	1.	1.0	32.0	39.0	37.0	20.0	7.0
	2.	4.60	5.95	6.70	7.70	8.40	9.05
Glutamic Acid							
7 days	1.	0.0	20.0	18.0	19.0	21.0	19.0
	2.	-	-	-	-	-	-
14 days	1.	0.0	14.0	33.0	33.0	26.0	17.0
	2.	4.55	5.60	6.65	7.60	8.25	8.95
21 days	1.	1.0	23.0	52.0	55.0	40.0	21.0
	2.	4.65	5.75	6.75	7.75	8.35	9.05
Histidine							
7 days	1.	0.3	15.6	33.3	38.3	35.3	22.0
	2.	-	-	-	-	-	-
14 days	1.	0.0	21.0	36.0	38.0	30.3	13.0
	2.	4.70	5.80	6.70	7.65	8.35	8.90
21 days	1.	1.0	25.3	35.6	34.6	24.6	8.6
	2.	4.75	6.00	6.70	7.75	8.30	8.90
Proline							
7 days	1.	0.0	15.0	19.0	35.0	32.0	15.0
	2.	-	-	-	-	-	-
14 days	1.	5.0	30.0	-	41.0	34.0	22.0
	2.	4.75	6.00	6.70	7.80	8.45	9.00
21 days	1.	2.0	49.0	59.0	56.0	30.0	18.0
	2.	4.75	6.15	6.80	7.90	8.50	9.05

TABLE 4.

Effect of pH — Pseudomonas putrefaciens

Age of culture		initial pH of cultures 4.6	5.6	6.6	7.6	8.5	9.3
Arginine							
7 days	1.	0.0	14.5	13.0	12.75	10.5	3.75
	2.	-	-	-	-	-	-
14 days	1.	0.0	35.0	36.0	32.75	17.5	1.5
	2.	4.70	6.25	6.80	7.90	8.40	9.05
21 days	1.	1.0	64.75	59.0	48.25	21.75	2.5
	2.	4.75	6.45	6.95	8.05	8.50	9.05
Aspartic Acid							
7 days	1.	0.0	42.0	38.0	43.0	41.0	27.0
	2.	-	-	-	-	-	-
14 days	1.	0.0	35.0	37.0	34.0	29.0	16.0
	2.	4.55	5.85	6.65	7.60	8.30	8.95
21 days	1.	1.0	36.0	-	31.0	23.0	8.0
	2.	4.55	5.90	-	7.55	8.35	9.00
Glutamic Acid							
7 days	1.	0.0	26.0	20.0	16.0	16.0	11.0
	2.	-	-	-	-	-	-
14 days	1.	0.0	30.0	24.0	24.0	28.0	15.0
	2.	4.60	5.95	6.65	7.60	8.20	8.95
21 days	1.	2.0	45.0	57.0	35.0	27.0	10.0
	2.	4.60	6.15	6.75	7.65	8.35	9.05
Histidine							
7 days	1.	0.0	47.6	61.6	57.3	48.3	23.3
	2.	-	-	-	-	-	-
14 days	1.	0.3	73.0	72.3	64.3	42.6	16.6
	2.	4.75	6.30	6.85	7.85	8.25	8.90
21 days	1.	0.6	77.3	74.6	61.6	44.6	9.6
	2.	4.75	6.35	6.85	7.95	8.30	8.95
Proline							
7 days	1.	0.0	45.0	39.0	32.0	15.0	3.0
	2.	-	-	-	-	-	-
14 days	1.	0.0	62.0	51.0	52.0	16.0	2.0
	2.	4.75	6.10	6.75	7.75	8.40	9.10
21 days	1.	2.0	58.0	47.0	44.0	13.0	3.0
	2.	4.75	6.15	6.75	7.80	8.45	9.10

Figure 3.

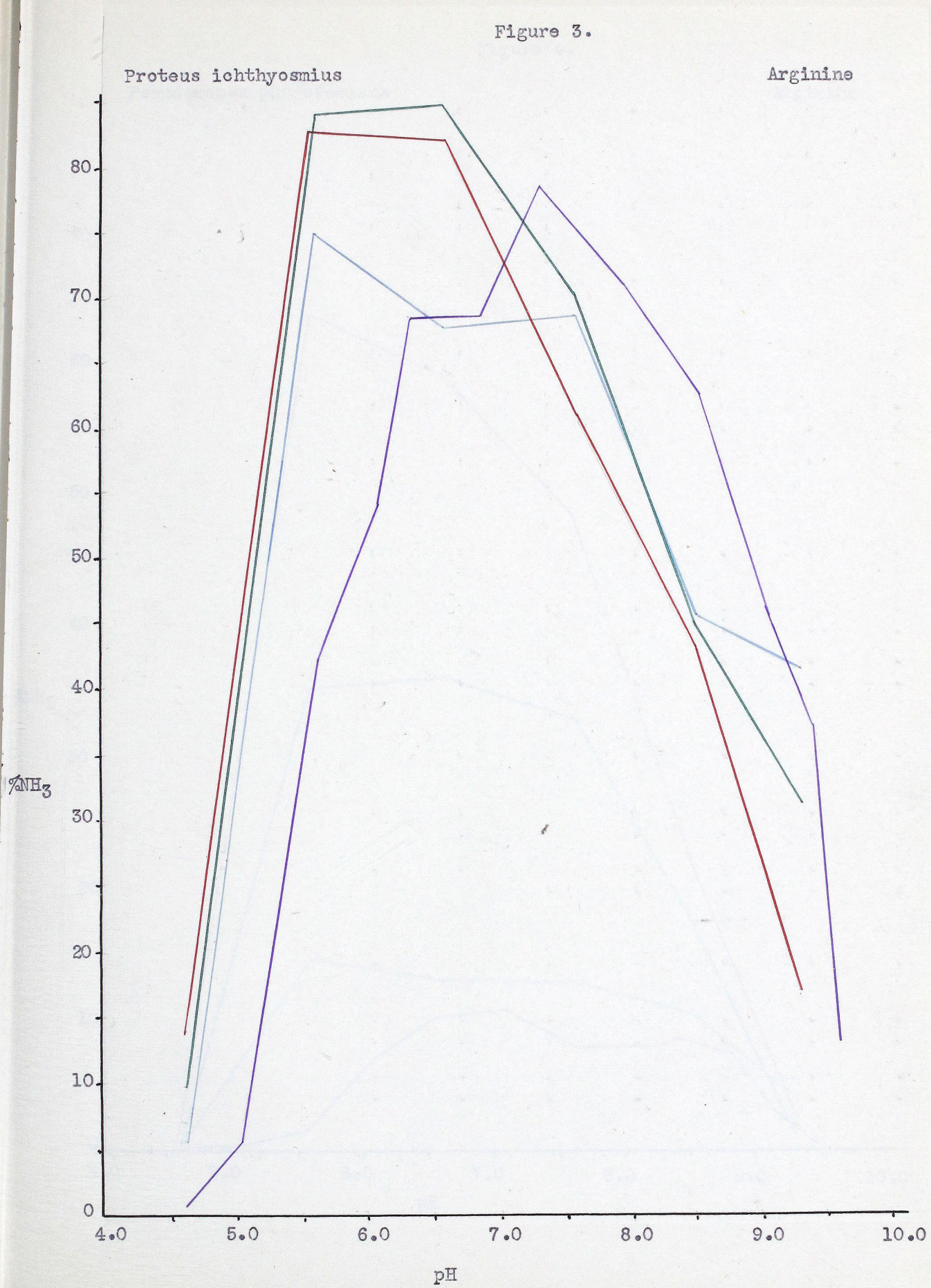

Proteus ichthyosmius
Arginine
80
70
60
50
40
%NH3
30
20
10
0
4.0
5.0
6.0
7.0
8.0
9.0
10.0
pH

Figure 4.

Pseudomonas putrefaciens Arginine

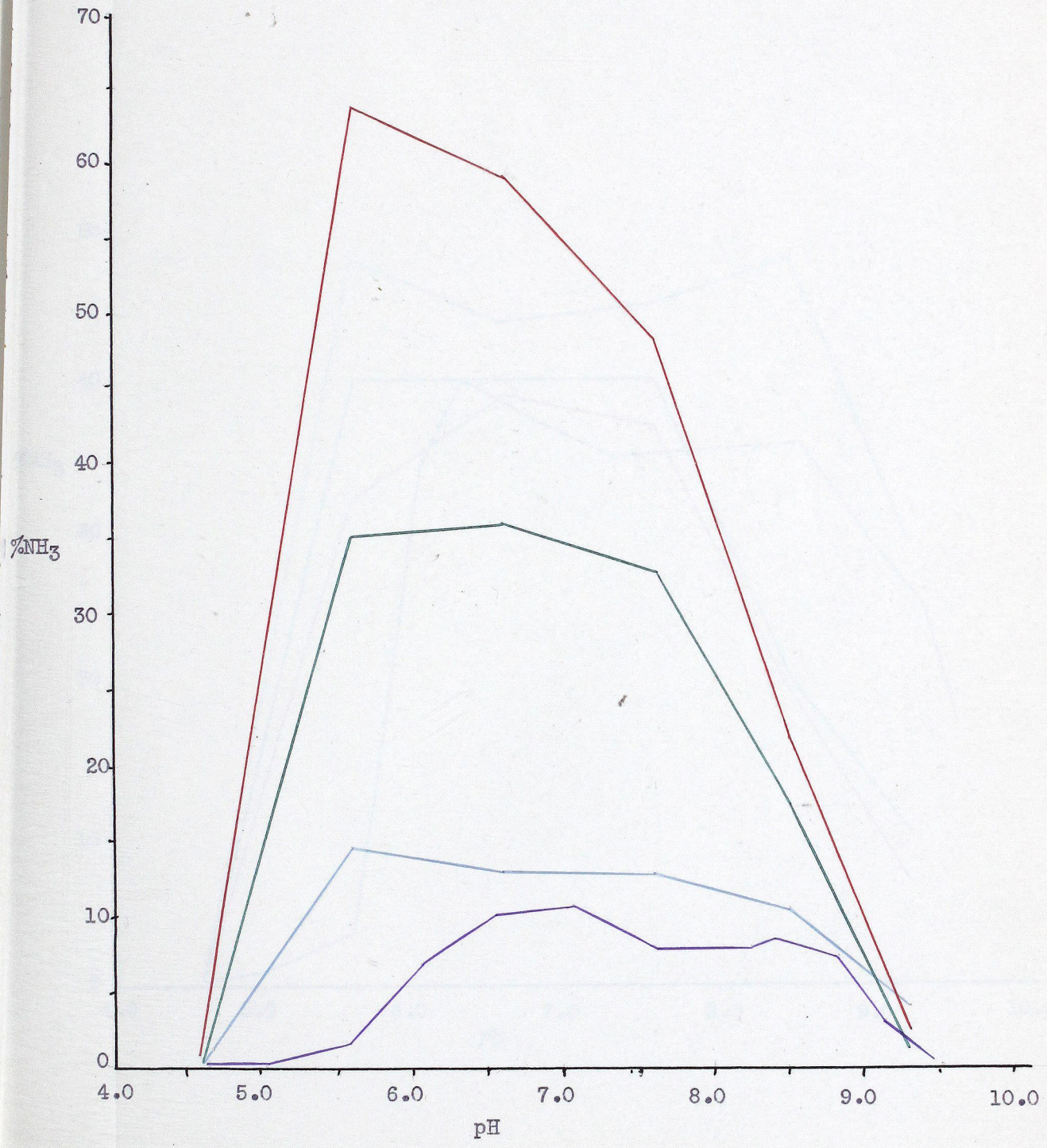


Figure 5.

Proteus ichthyosmius Aspartic Acid

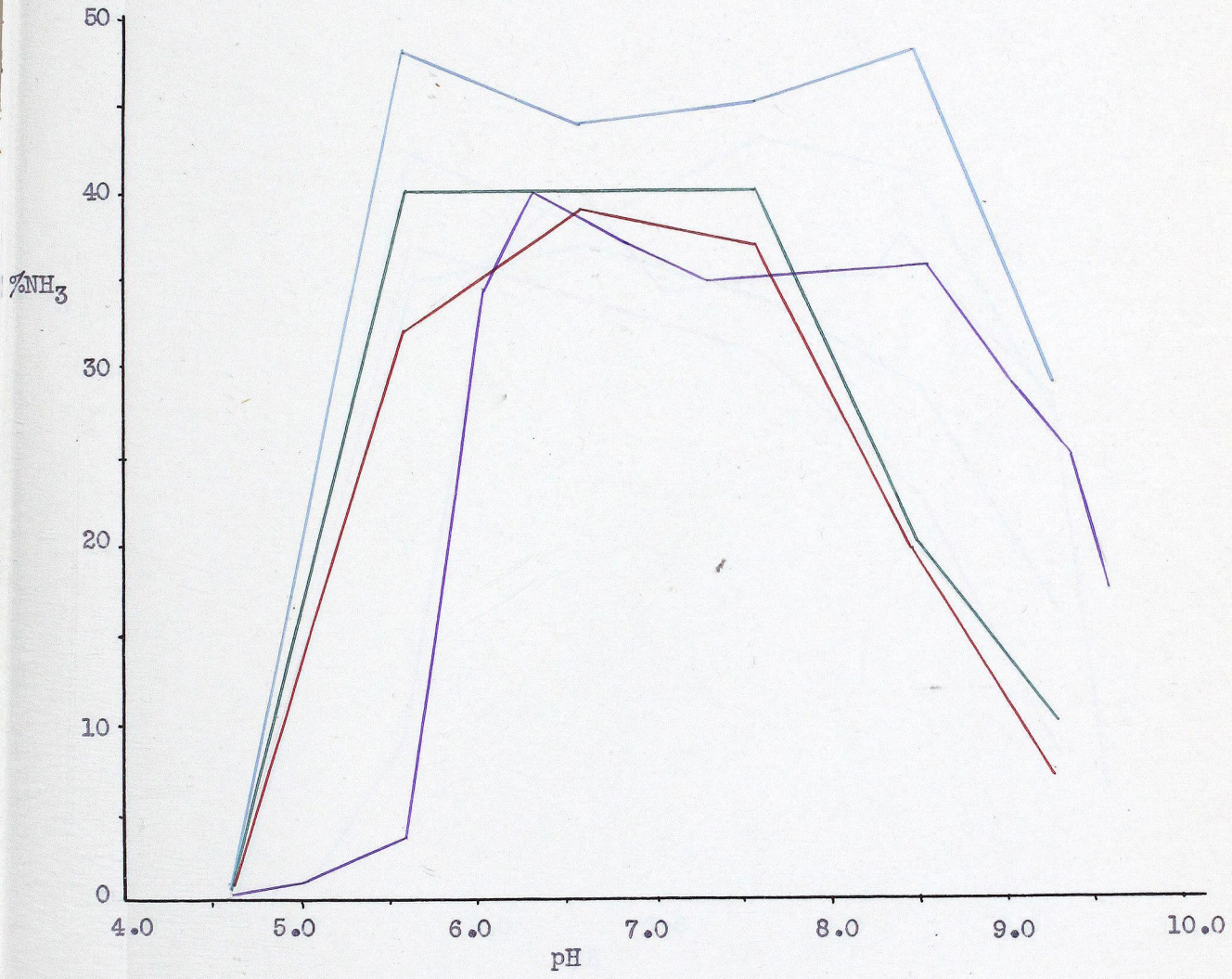


Figure 6.

Pseudomonas putrefaciens Aspartic Acid

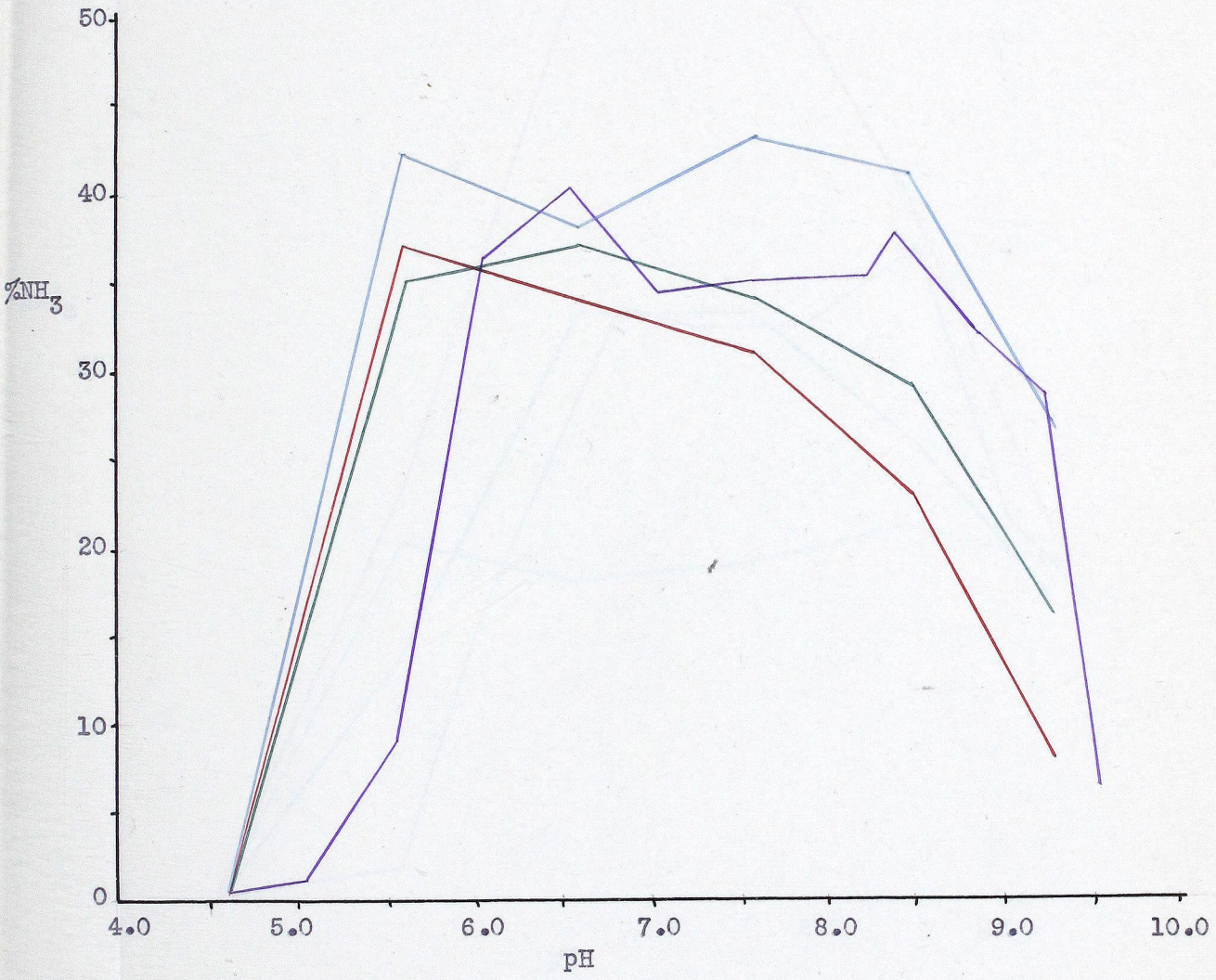


Figure 7.

Proteus ichthyosmius Glutamic Acid

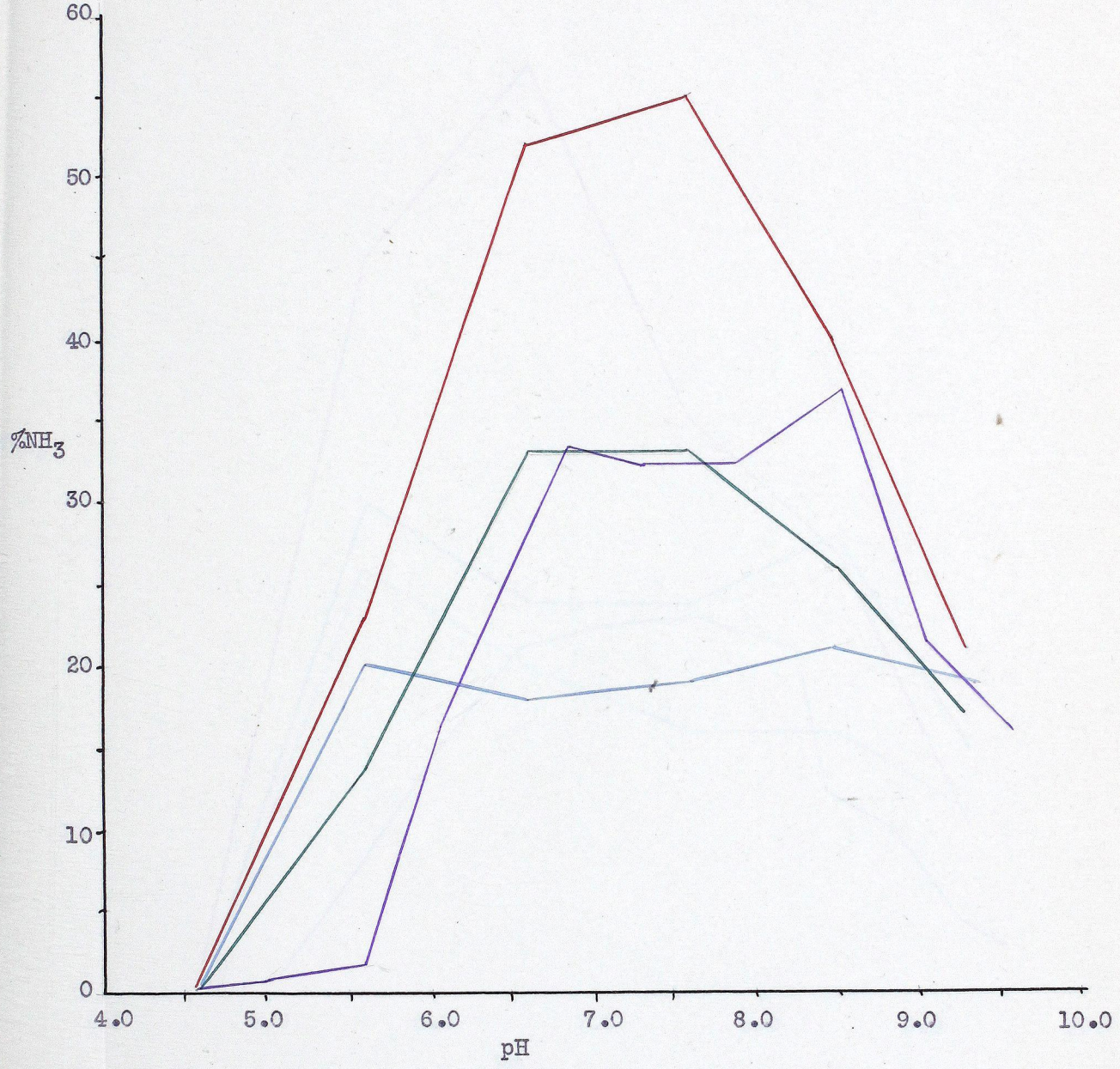


Figure 8.

Pseudomonas putrefaciens Glutamic Acid

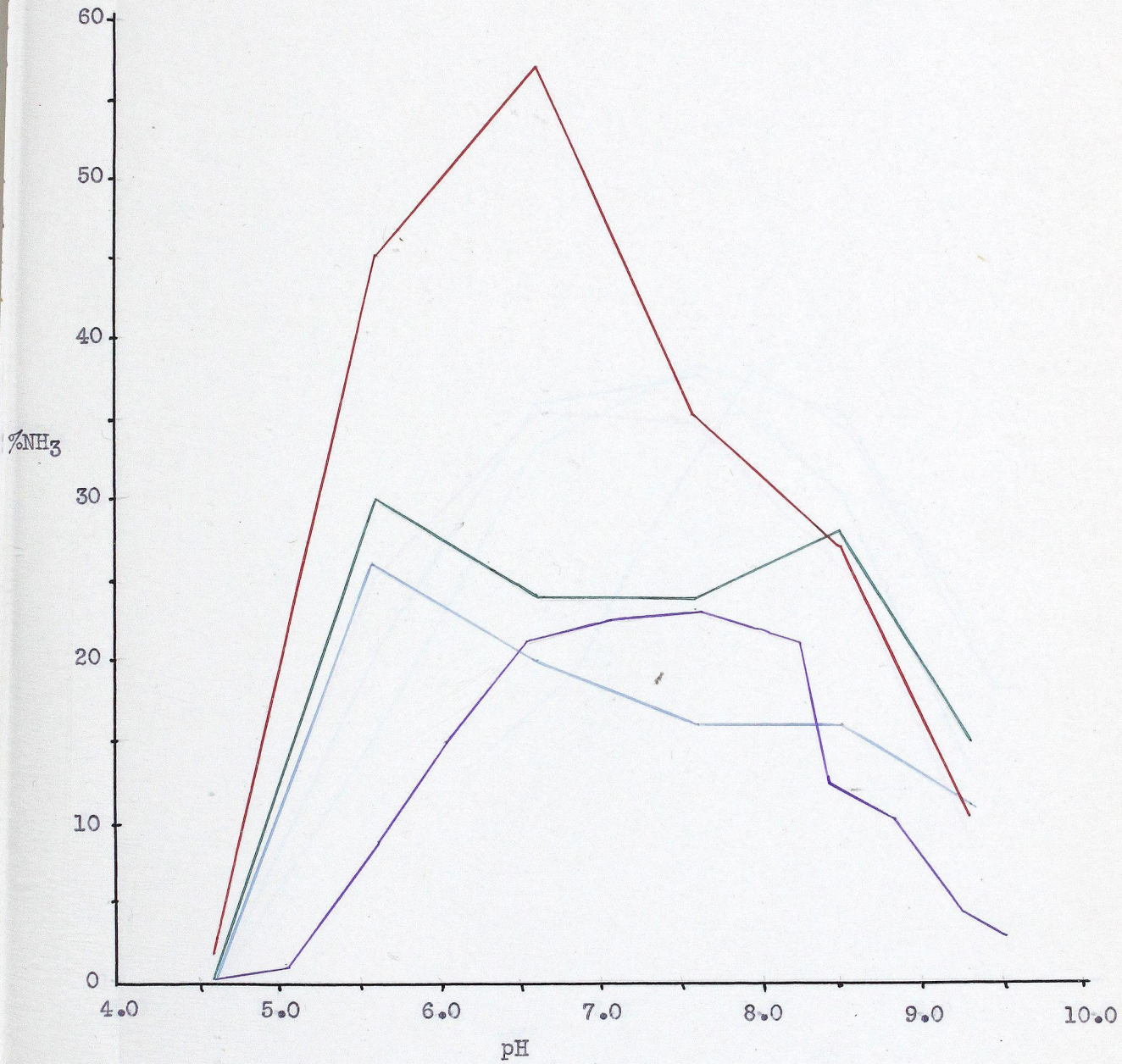


Figure 9.

Proteus ichthyosmius Histidine

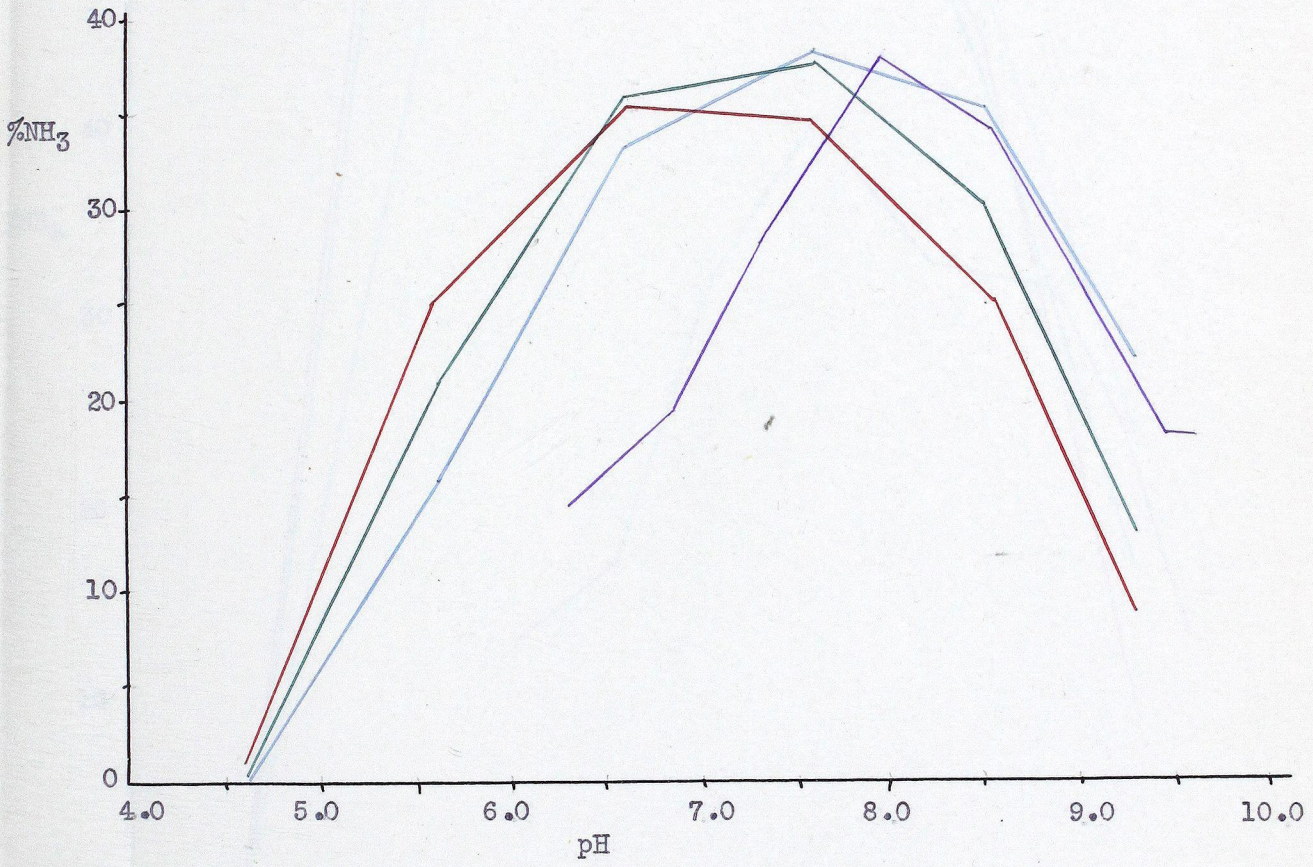


Figure 10.

Pseudomonas putrefaciens — Histidine

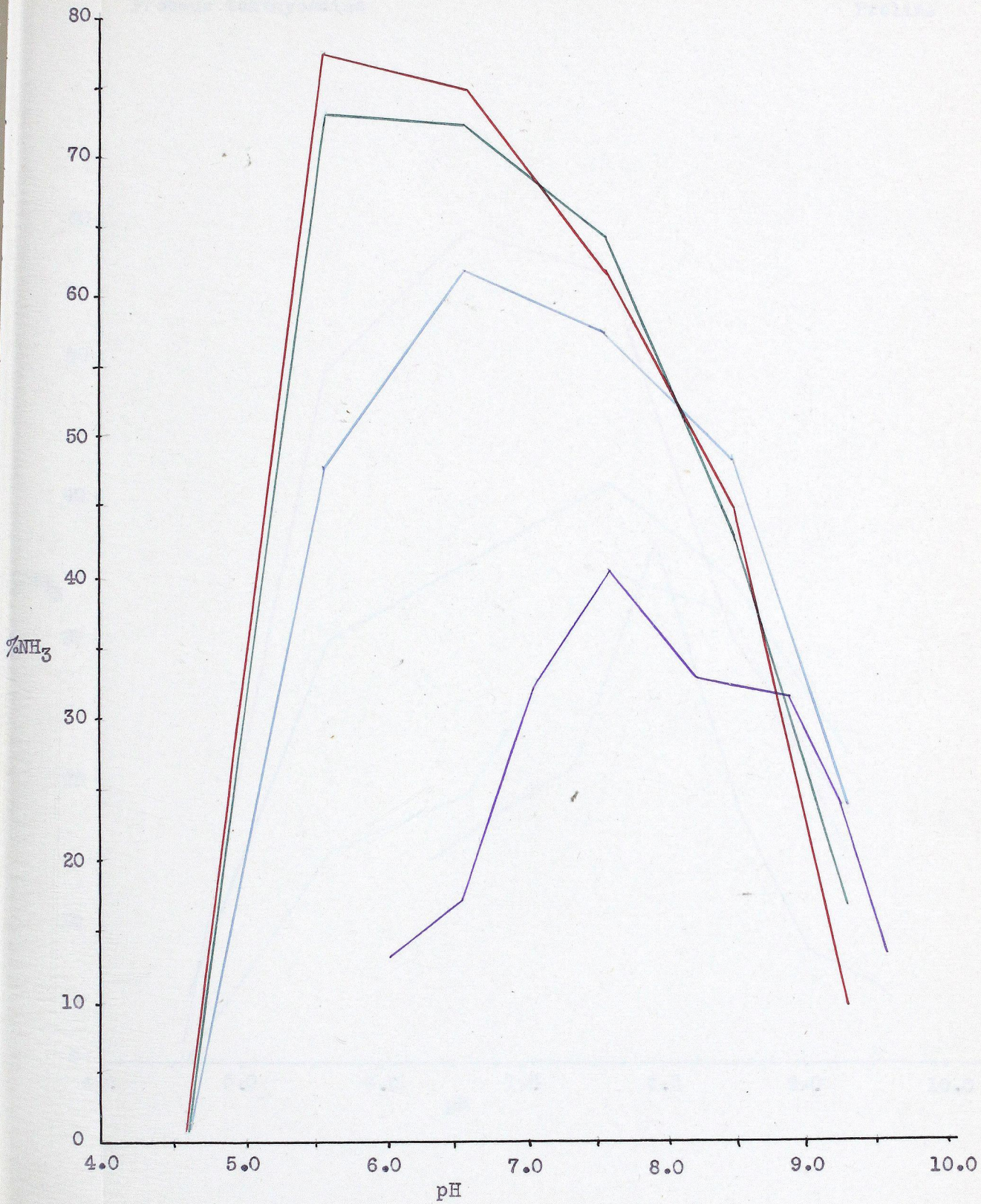


Figure 11.

Proteus ichthyosmius

Proline

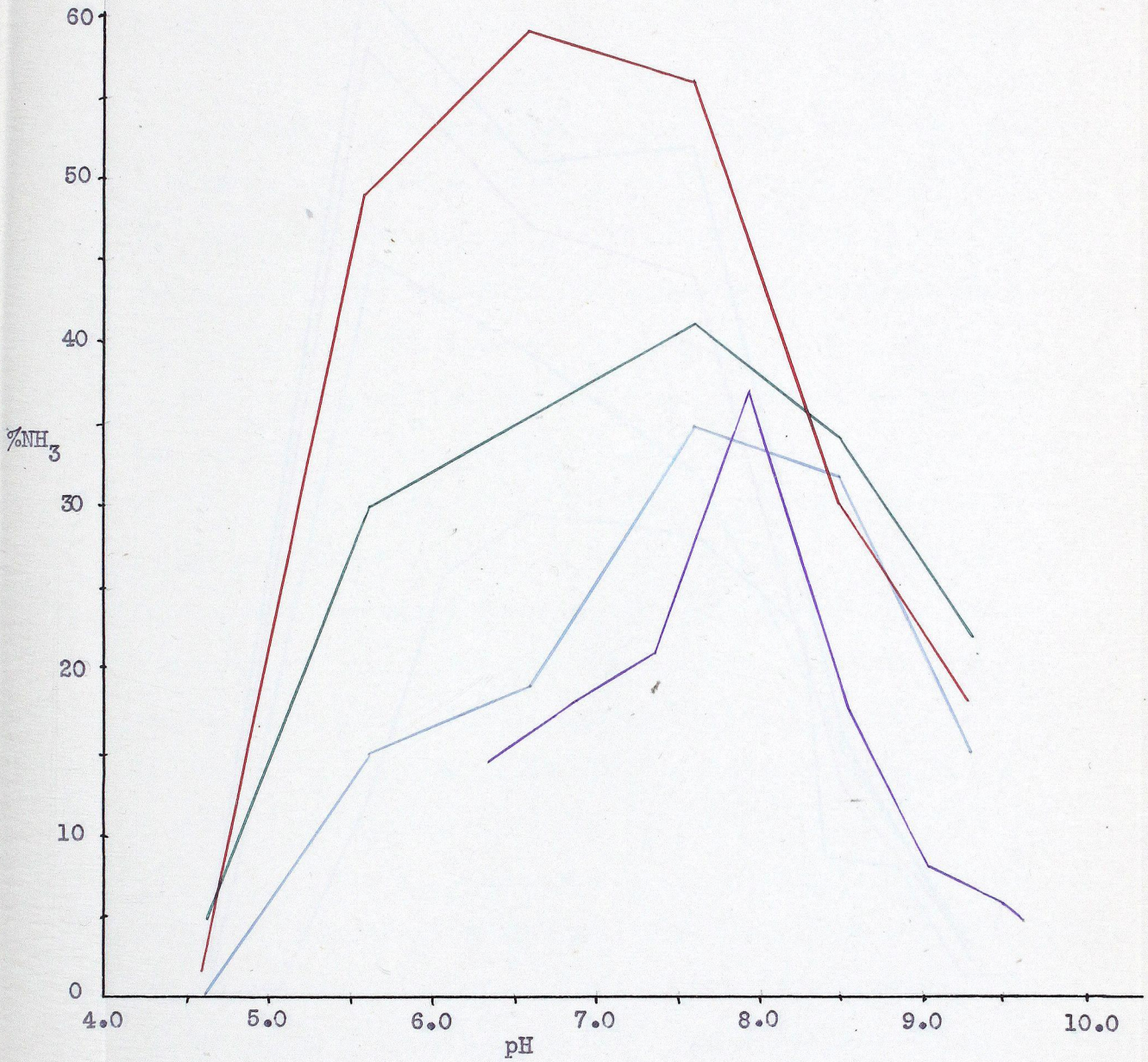
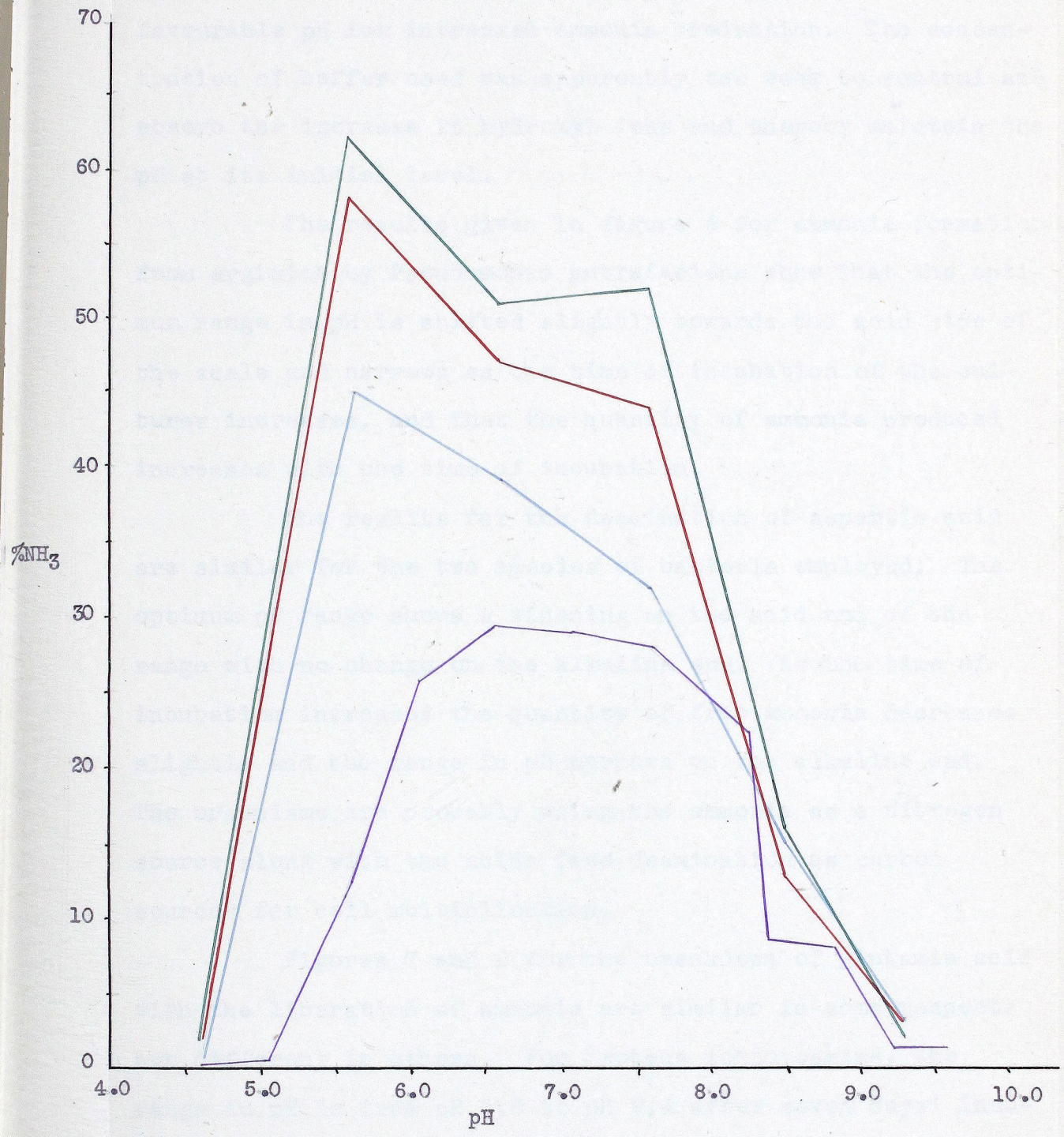


Figure 12.

Pseudomonas putrefaciens Proline



between pH 5.0 and pH 5.5 permits the formation of small amounts of ammonia, as seen from the previous results. This ammonia will raise the pH of the reacting medium to a more favourable pH for increased ammonia production. The concentration of buffer used was apparently too weak to control and absorb the increase in hydroxyl ions and thereby maintain the pH at its initial level.

The results given in figure 4 for ammonia formation from arginine by Pseudomonas putrefaciens show that the optimum range in pH is shifted slightly towards the acid side of the scale and narrows as the time of incubation of the cultures increases, and that the quantity of ammonia produced increases with the time of incubation.

The results for the deamination of aspartic acid are similar for the two species of bacteria employed. The optimum pH range shows a widening on the acid end of the range with no change on the alkaline end. As the time of incubation increases the quantity of free ammonia decreases slightly and the range in pH narrows on the alkaline end. The organisms are probably using the ammonia as a nitrogen source along with the acids from deamination as carbon sources for cell multiplication.

Figures 7 and 8 for the breakdown of glutamic acid with the liberation of ammonia are similar in some respects but different in others. For Proteus ichthyosmius, the range in pH is from pH 5.6 to pH 9.4 after seven days' incubation and narrows to a range from pH 6.6 to pH 7.6 after fourteen and twenty-one days' incubation. The quantity of

ammonia increases regularly with increased incubation time. For Pseudomonas putrefaciens, the range in pH after seven and fourteen days' incubation is from pH 5.6 to pH 8.5 with an optimum at pH 5.6 after the seven-day incubation. After twenty-one days' incubation, however, the range has narrowed down to a single optimum of pH 6.6. The final quantity of ammonia formed by Pseudomonas is the same as for Proteus but the rate of formation seems to be slower between seven and fourteen days than it is in the first seven and the last seven days of incubation.

The curves given on figures 9 and 10 for ammonia formation from histidine by the two bacterial species are entirely different. Those for Proteus ichthyosmius from experiment II show a wider range in pH compared with that found in experiment I. The optimum range is from pH 6.6. to pH 8.8 with a slight change towards acidity as the time of incubation increases, whereas previously the range was narrower and definitely on the alkaline side of the pH scale. The quantity of ammonia remains constant as the time of incubation increases showing that the maximum amount has been formed after seven days' incubation. The curves for Pseudomonas putrefaciens show a more pronounced shift to the acid side of the pH scale for optimum ammonia formation from histidine. While the optimum from experiment I was pH 7.6, that from experiment II is pH 6.6 after seven days' incubation and between pH 5.6 and pH 6.6. after fourteen days, bringing the pH nearer to the seven-day optimum. The increase in ammonia, however, causes a shift in pH from pH 5.6 to pH 6.3. The

maximum quantity of ammonia is not formed after seven days but increases until fourteen days. The total quantity for Pseudonomas putrefaciens is about twice that for Proteus ichthyosmius.

As in the case of histidine, the optimum pH range for the formation of ammonia from proline by the two bacterial species are on different sides of the neutral point of the pH scale. Proteus ichthyosmius breaks down proline best between pH 7.0 and pH 8.0 except after twenty-one days' incubation when the optimum shifts to between pH 6.6 and pH 7.6. On the other hand, Pseudomonas putrefaciens prefers a distinctly acid hydrogen-ion concentration of from pH 5.6 to pH 6.6. The final pH for both groups of proline cultures is raised from pH 5.6 to pH 6.1.

In general, where the shift in the optimum pH range is towards a more acid pH, as the time of incubation increases, there has been an increase in the initial pH of the buffer, particularly at pH 5.6, caused by the relatively large quantities of ammonia formed during the early days of incubation.

The Effect of the Age of Culture on Ammonia Formation.

With the object of determining the effect of the age of the growth culture upon the subsequent ammonia formation from the five amino acids in buffer cultures, an experiment (III) employing Proteus ichthyosmius was carried out. Six Kolle flasks were inoculated at the same time. One flask was removed from the incubator at the end of ten, twelve,

The Effect of the Age of the Growth Culture on Subsequent Ammonia Production by Proteus ichthyosmius.

Experiment III.

Table 5.

Figure 13.

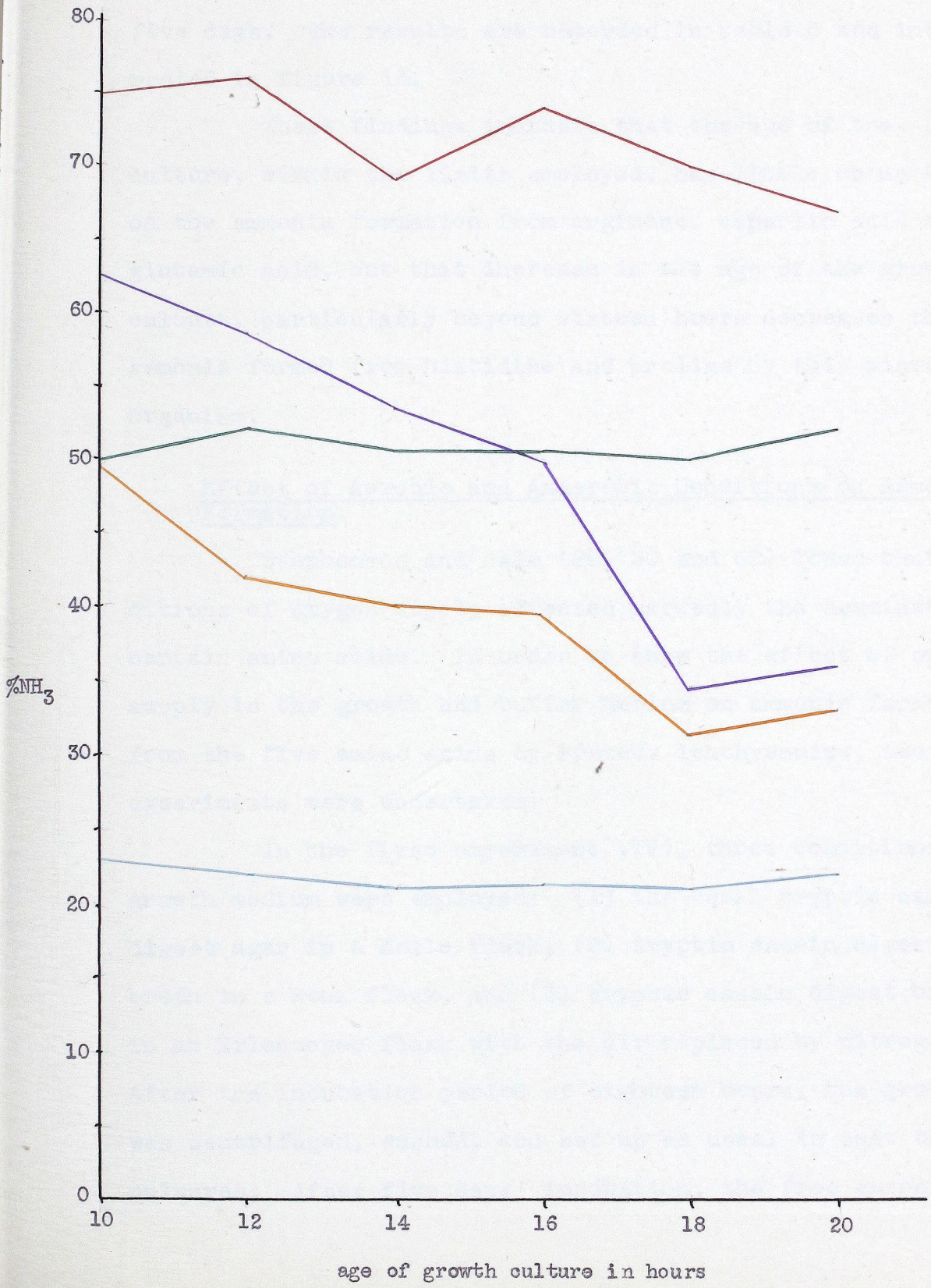
Arginine	-	red
Aspartic Acid	-	green
Glutamic Acid	-	blue
Histidine	-	violet
Proline	-	orange

TABLE 5.

Effect of Age of Culture — Proteus ichthyosmius

amino acid	age of culture in hours					
	10	12	14	16	18	20
Arginine	74.75	76.25	69.0	74.0	69.75	67.0
Aspartic acid	50.0	52.0	50.5	50.5	50.5	51.0
Glutamic acid	23.0	22.0	21.0	21.5	21.0	22.0
Histidine	62.6	58.5	53.5	50.0	34.6	36.0
Proline	49.5	42.0	40.0	39.5	31.5	33.5

Figure 13.


80
70
60
50
40
30
20
10
0
%NH3
10
12
14
16
18
20
age of growth culture in hours

fourteen, sixteen, eighteen and twenty hours incubation respectively, the contents washed and centrifuged as usual and the buffer cultures, thus prepared, incubated at 30° C. for five days. The results are recorded in table 5 and interpreted in figure 13.

These findings indicate that the age of the growth culture, within the limits employed, has little or no effect on the ammonia formation from arginine, aspartic acid and glutamic acid, but that increase in the age of the growth culture, particularly beyond sixteen hours decreases the ammonia formed from histidine and proline by this microorganism.

Effect of Aerobic and Anaerobic Conditions on Ammonia Formation.

Stephenson and Gale (28, 30 and 62) found that conditions of oxygen supply affected markedly the deamination of certain amino acids. In order to show the effect of oxygen supply in the growth and buffer medium on ammonia formation from the five amino acids by Proteus ichthyosmius, two experiments were undertaken.

In the first experiment (IV), three conditions of growth medium were employed: (1) the usual tryptic casein digest agar in a Kolle flask, (2) tryptic casein digest broth in a Roux flask, and (3) tryptic casein digest broth in an Erlenmeyer flask with the air replaced by nitrogen. After the incubation period of eighteen hours, the growth was centrifuged, washed, and set up as usual in test tube cultures. After five days' incubation, the free ammonia of

the buffer cultures was determined. The results are given in table 6 and figure 14.

In general, the growth on agar in Kolle flasks gave the greatest subsequent ammonia formation. For the amino acids, - arginine, aspartic acid and proline, - growth on agar gave highest ammonia formation, growth aerobically in broth intermediate results, and growth anaerobically in the presence of nitrogen the lowest ammonia formation. For glutamic acid, the aerobic broth procedure gave slightly higher results than the agar and the anaerobic broth method slightly lower than the agar. For histidine, the agar grown culture and the anaerobic broth grown culture were practically the same and gave slightly lower results than the aerobic broth. The outstanding finding of this experiment is that, for proline, the agar procedure for growing the cells gives about twice the ammonia formation as the anaerobic broth procedure for growth, suggesting that oxidation may be a factor in the opening of the pyrollidine ring prior to the liberation of ammonia.

In the second experiment (V), of this group, the cells were grown as usual on agar in a Kolle flask and set up in buffer cultures in two ways: (1) aerobically in fifty cubic centimeter centrifuge tubes which were shaken at daily intervals and (2) anaerobically in test tubes with the air replaced by nitrogen. After five days' incubation the free ammonia was measured and is shown in table 6 with graphic interpretation in figure 15. The cultures provided with the increased oxygen supply in the buffer medium showed only

The Effect of Aerobic and Anaerobic Conditions on Ammonia Production by Proteus ichthyosmius.

Experiment IV and V

Table 6

Figure 14 (Experiment IV)

Agar - Aerobic - A

Broth - Aerobic - B

Broth - Anaerobic - C

Figure 15 (Experiment V)

Aerobic - A

Anaerobic - B

Arginine - red

Aspartic Acid - green

Glutamic Acid - blue

Histidine - violet

Proline - orange

TABLE 6.

Effect of Oxygen Supply in the Growth and Buffer Media.

Growth Conditions	agar-aerobic	broth-aerobic	broth-anaerobic	agar-aerobic	agar-aerobic
Buffer Conditions	aerobic	aerobic	aerobic	aerobic	anaerobic
arginine	75.75	67.25	61.75	68.75	66.0
aspartic acid	50.0	43.0	39.0	45.0	42.0
glutamic acid	21.0	25.0	15.5	27.5	26.0
histidine	37.6	40.8	38.3	34.0	33.3
proline	47.0	41.0	24.0	34.5	17.5

Figure 14.

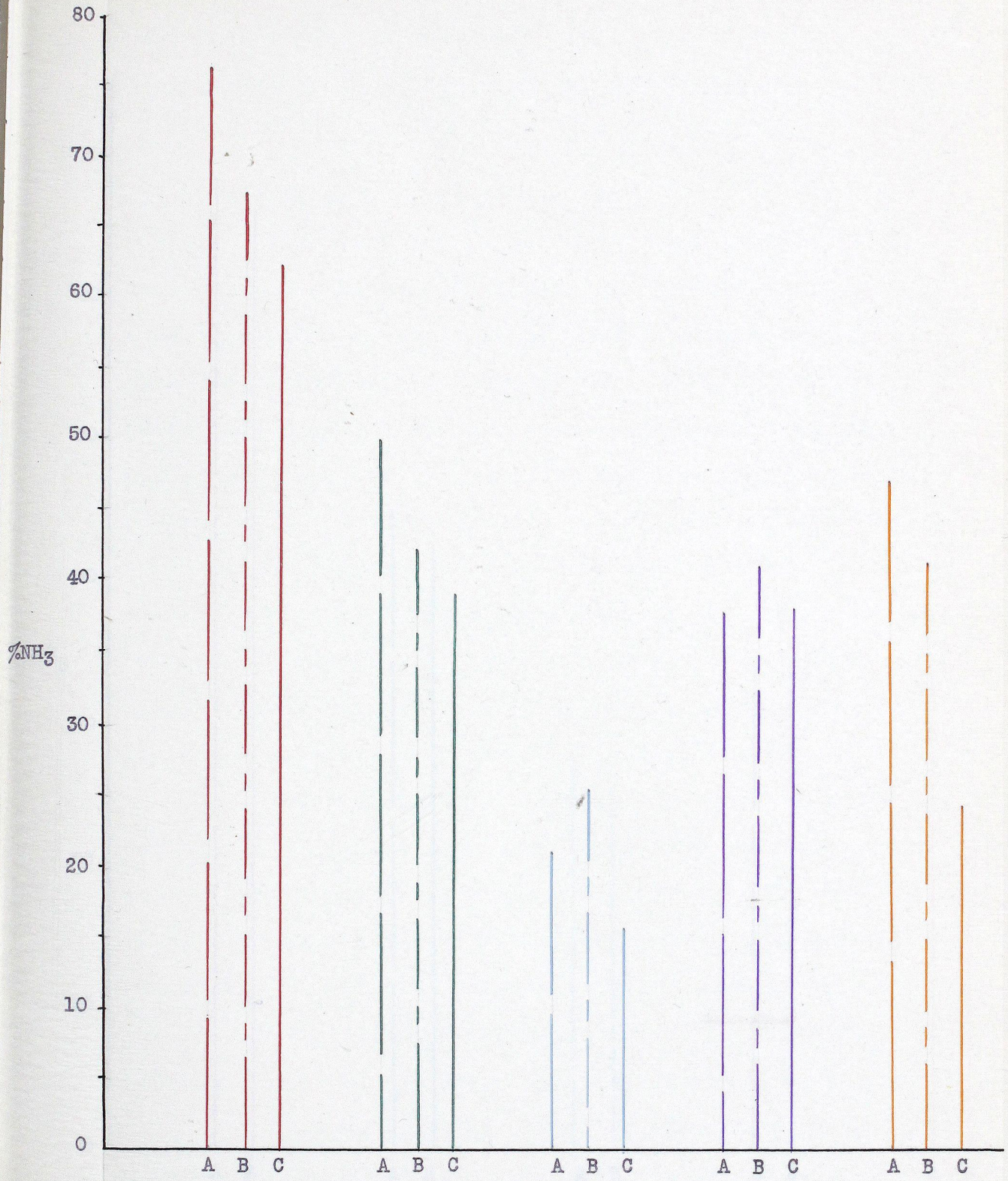
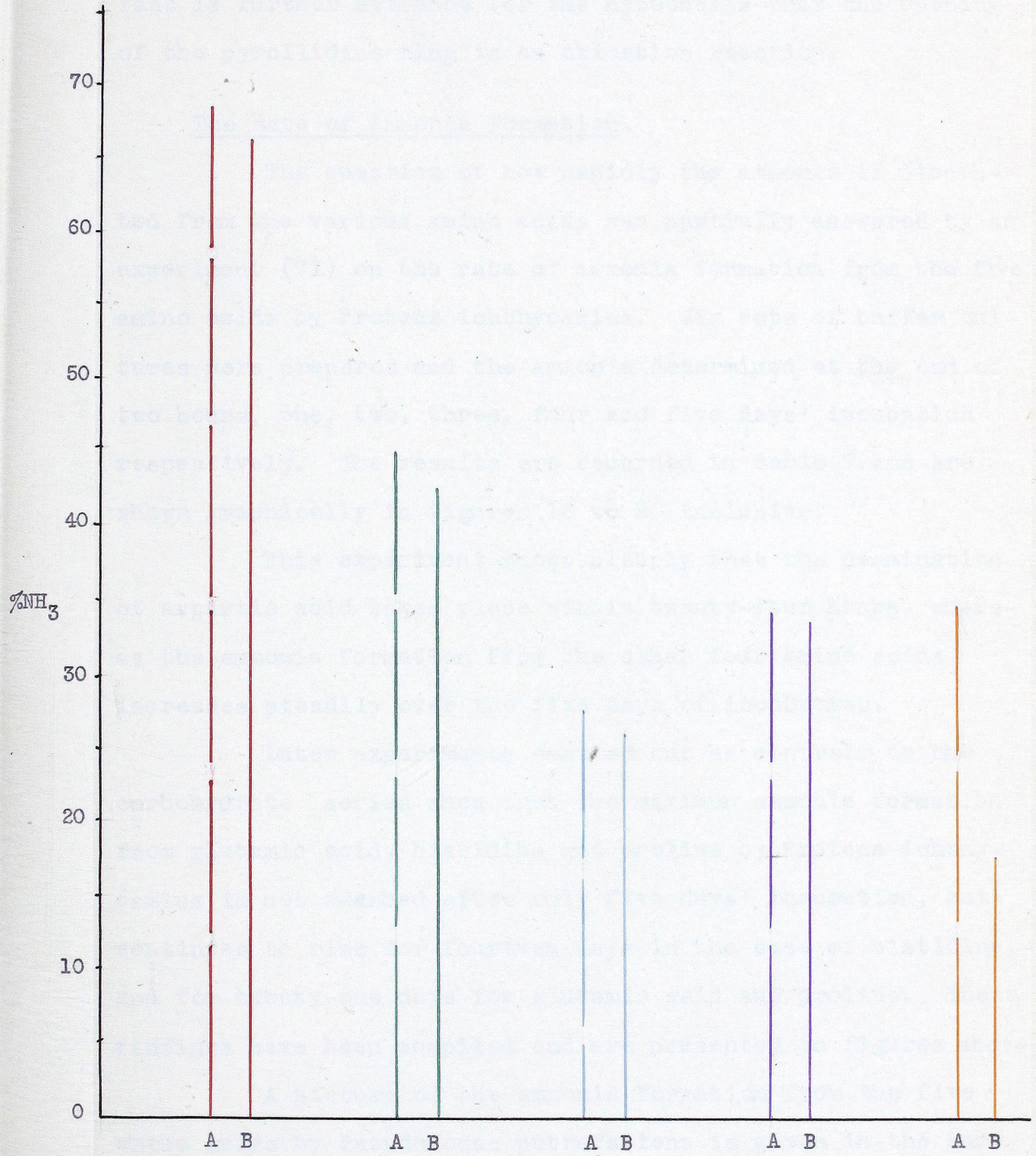


Figure 15.



slight increase in ammonia formation over the anaerobic buffer cultures except in the case of proline which formed only half the ammonia anaerobically that it did aerobically. This fact is further evidence for the hypothesis that the opening of the pyrollidine ring is an oxidation reaction.

The Rate of Ammonia Formation.

The question of how rapidly the ammonia is liberated from the various amino acids was partially answered by an experiment (VI) on the rate of ammonia formation from the five amino acids by Proteus ichthyosmius. Six sets of buffer cultures were prepared and the ammonia determined at the end of two hours, one, two, three, four and five days' incubation respectively. The results are recorded in table 7 and are shown graphically in figures 16 to 20 inclusive.

This experiment shows clearly that the deamination of aspartic acid takes place within twenty-four hours, whereas the ammonia formation from the other four amino acids increases steadily over the five days of incubation.

Later experiments carried out as controls in the carbohydrate series show that the maximum ammonia formation from glutamic acid, histidine and proline by Proteus ichthyosmius is not reached after only five days' incubation, but continues to rise for fourteen days in the case of histidine, and for twenty-one days for glutamic acid and proline. These findings have been compiled and are presented in figures above.

A picture of the ammonia formation from the five amino acids by Pseudomonas putrefaciens is given in the same

The Rate of Ammonia Production by Proteus ichthyosmius and Pseudomonas putrefaciens.

Experiment VI

Table 7

Figures 16 to 20 inclusive

Proteus ichthyosmius - blue
(Experiment VI. - June 1942)

Proteus ichthyosmius - orange
(Experiment X. - October 1942)

Proteus ichthyosmius - green
(Experiment XII - June 1943)

Pseudomonas putrefaciens - violet
(Experiment XI - January 1943)

Pseudomonas putrefaciens - red
(Experiment XIII - July 1943)

TABLE 7.

Rate of Ammonia Production — Proteus ichthyosmius

amino acid	age of buffer culture in days: 2 hrs.	1	2	3	4	5
Arginine	1.5	33.75	56.0	62.0	72.0	83.0
Aspartic acid	3.0	49.5	50.5	48.0	49.5	50.0
Glutamic acid	2.0	4.0	9.5	18.0	26.0	26.0
Histidine	0.6	4.3	12.0	27.6	39.3	43.0
Proline	3.0	4.0	8.5	14.5	29.0	52.0

Figure 16.

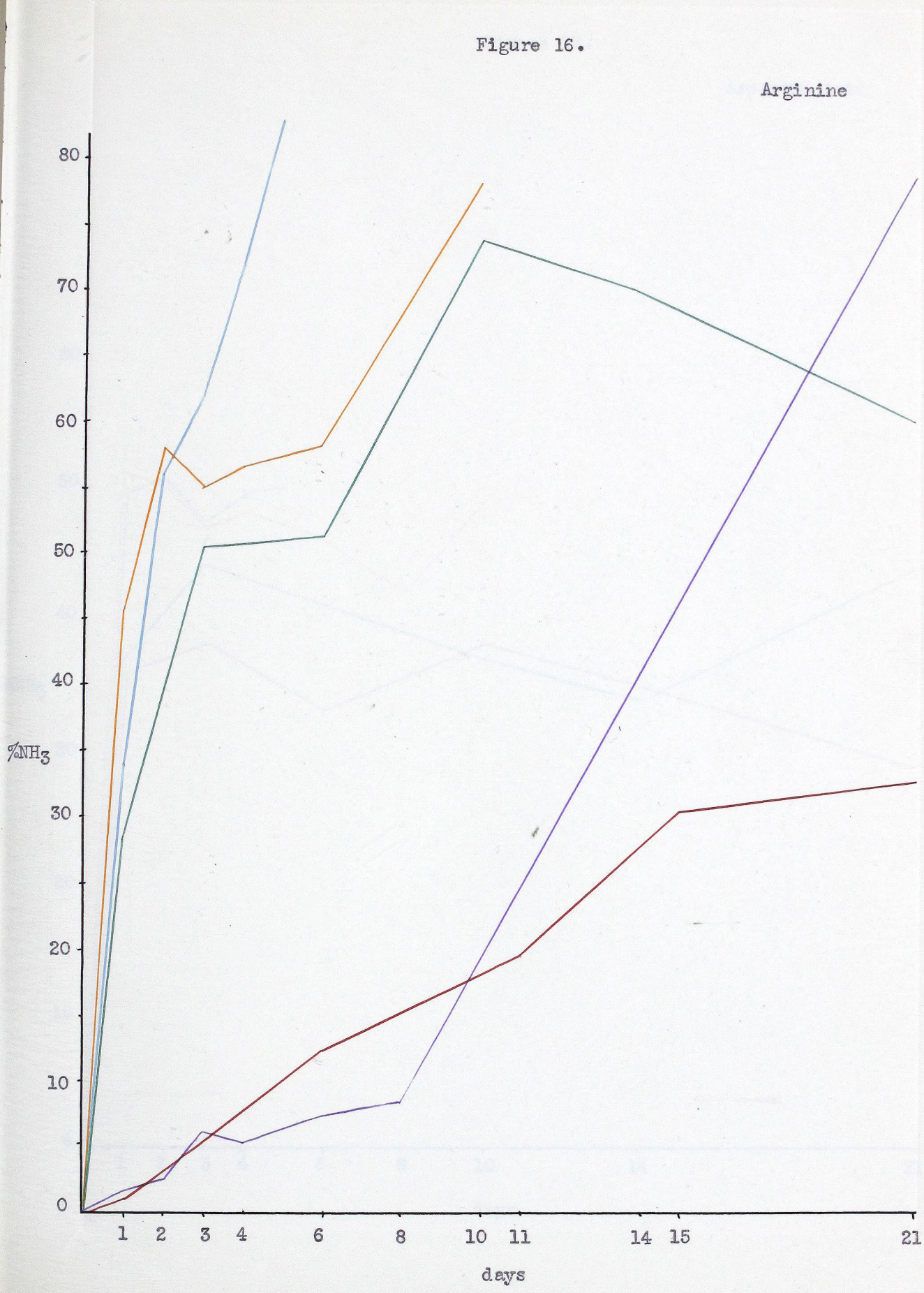


Figure 17.

Aspartic Acid

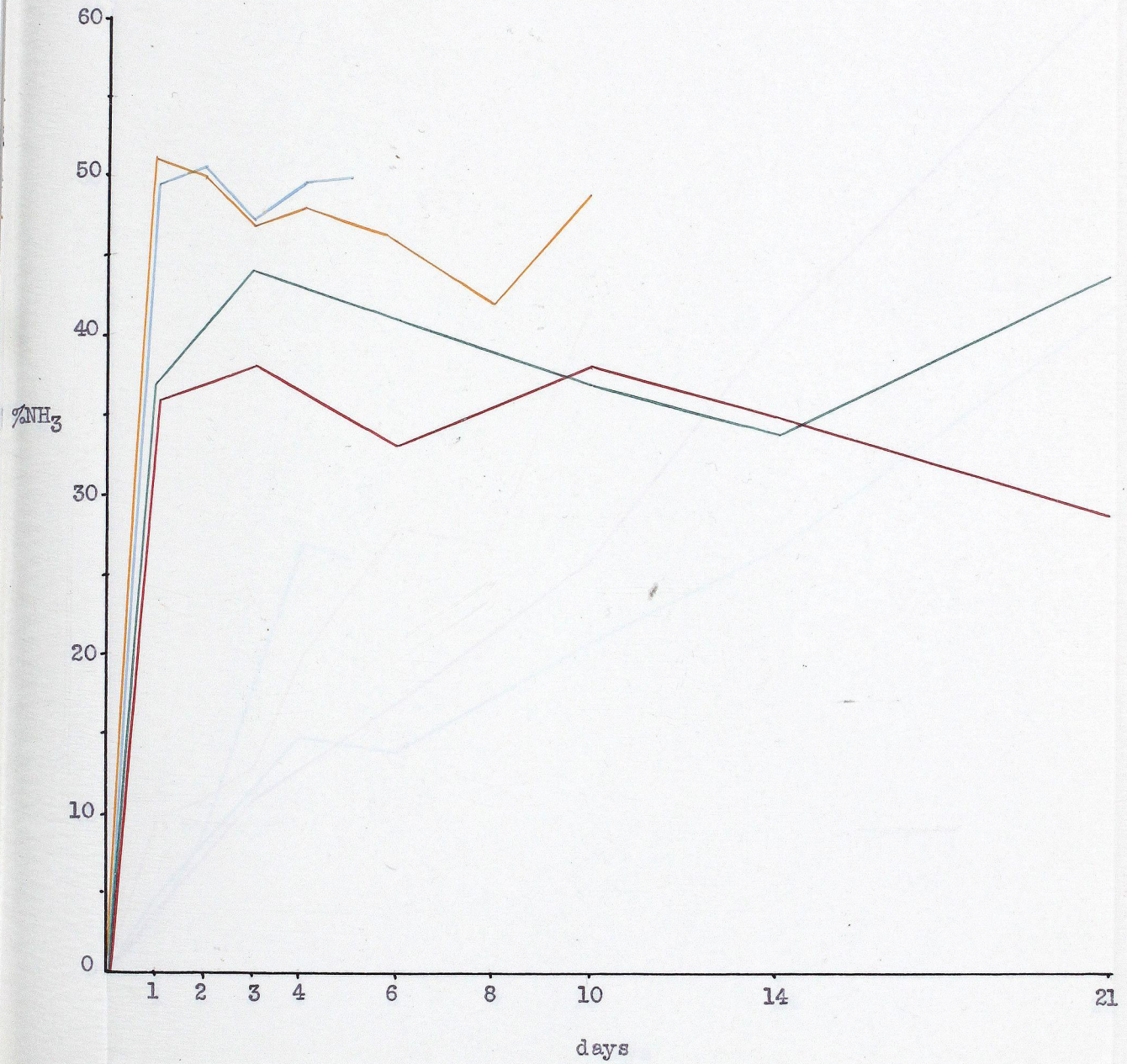


Figure 18.

Glutamic Acid

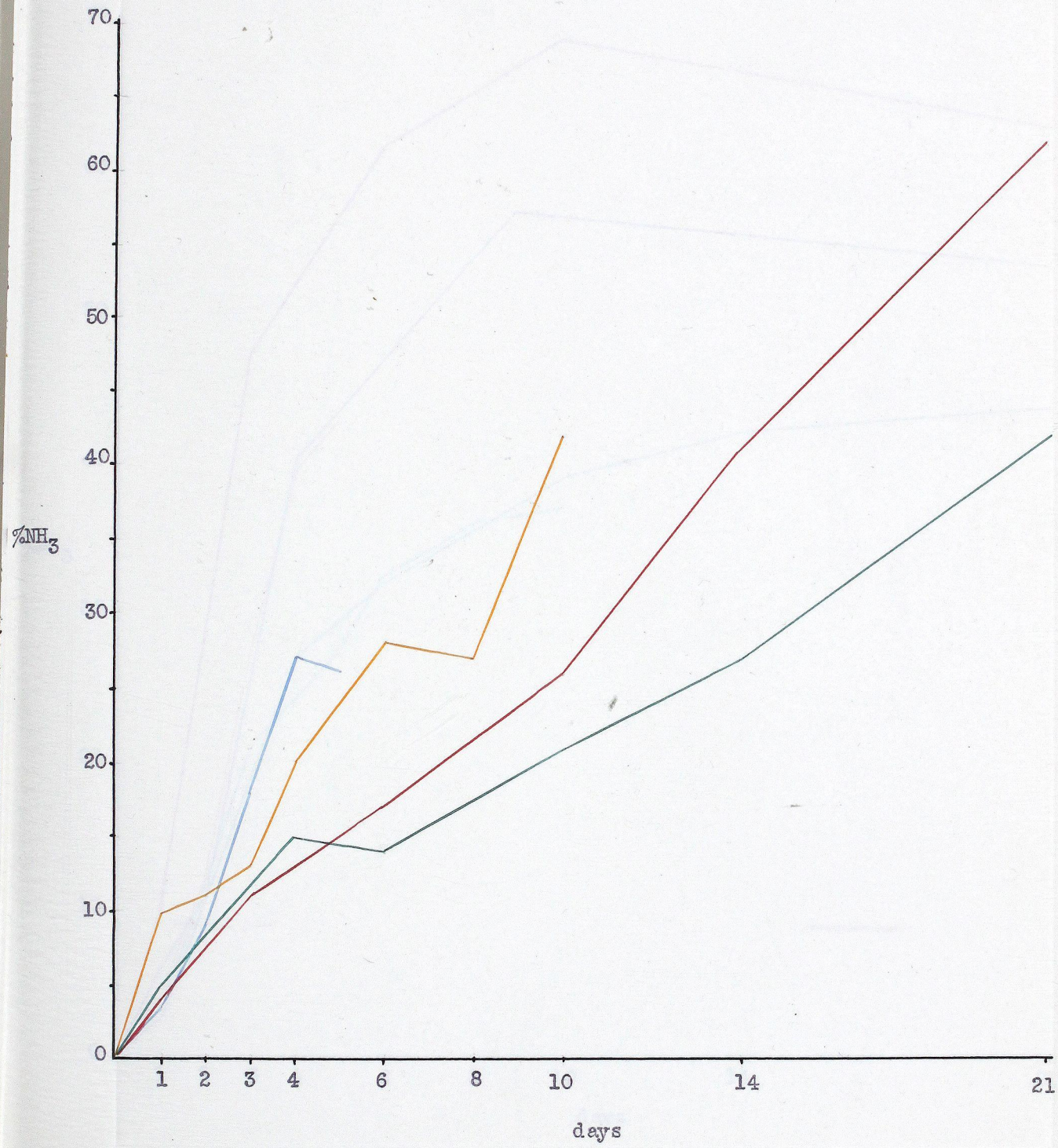


Figure 19.

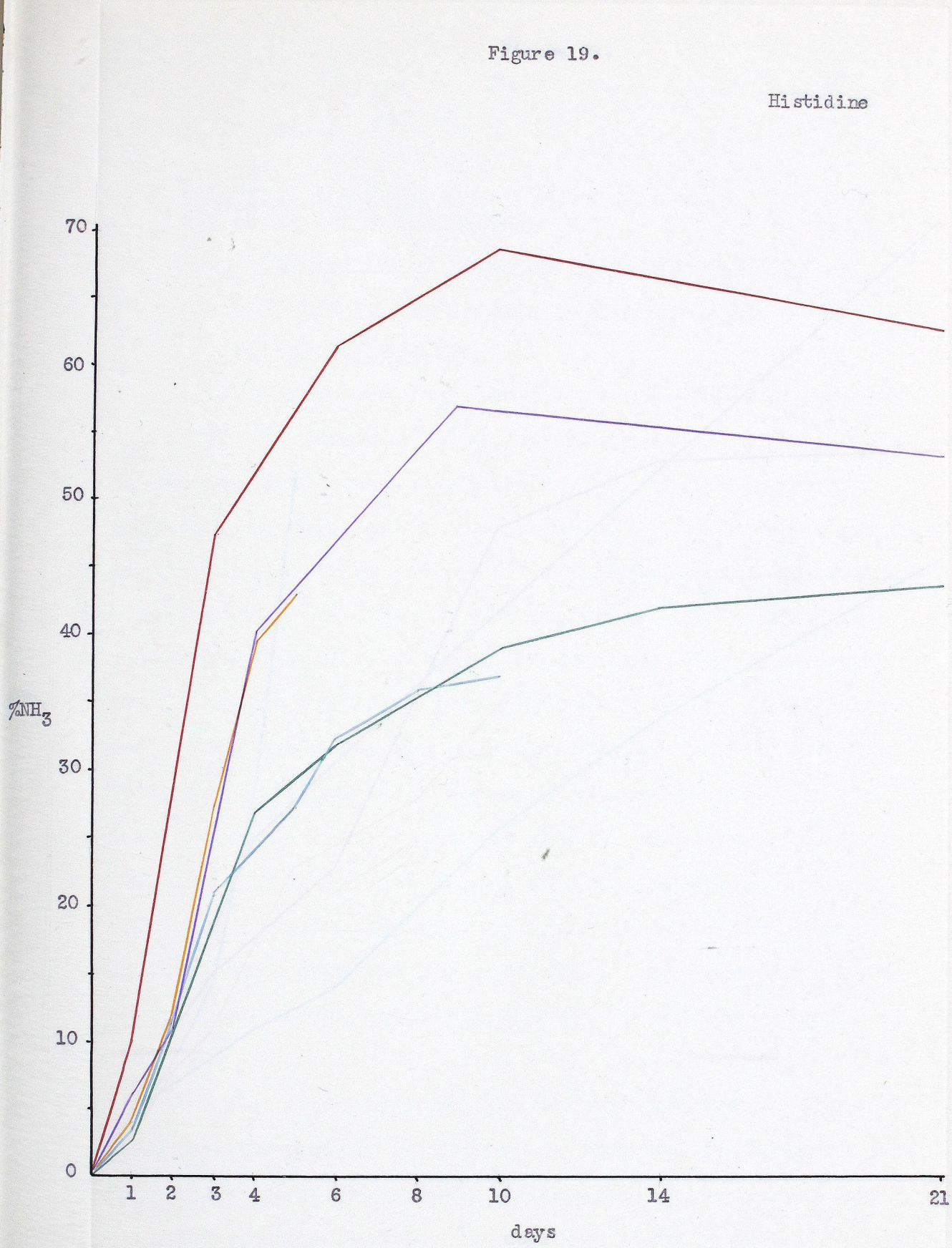
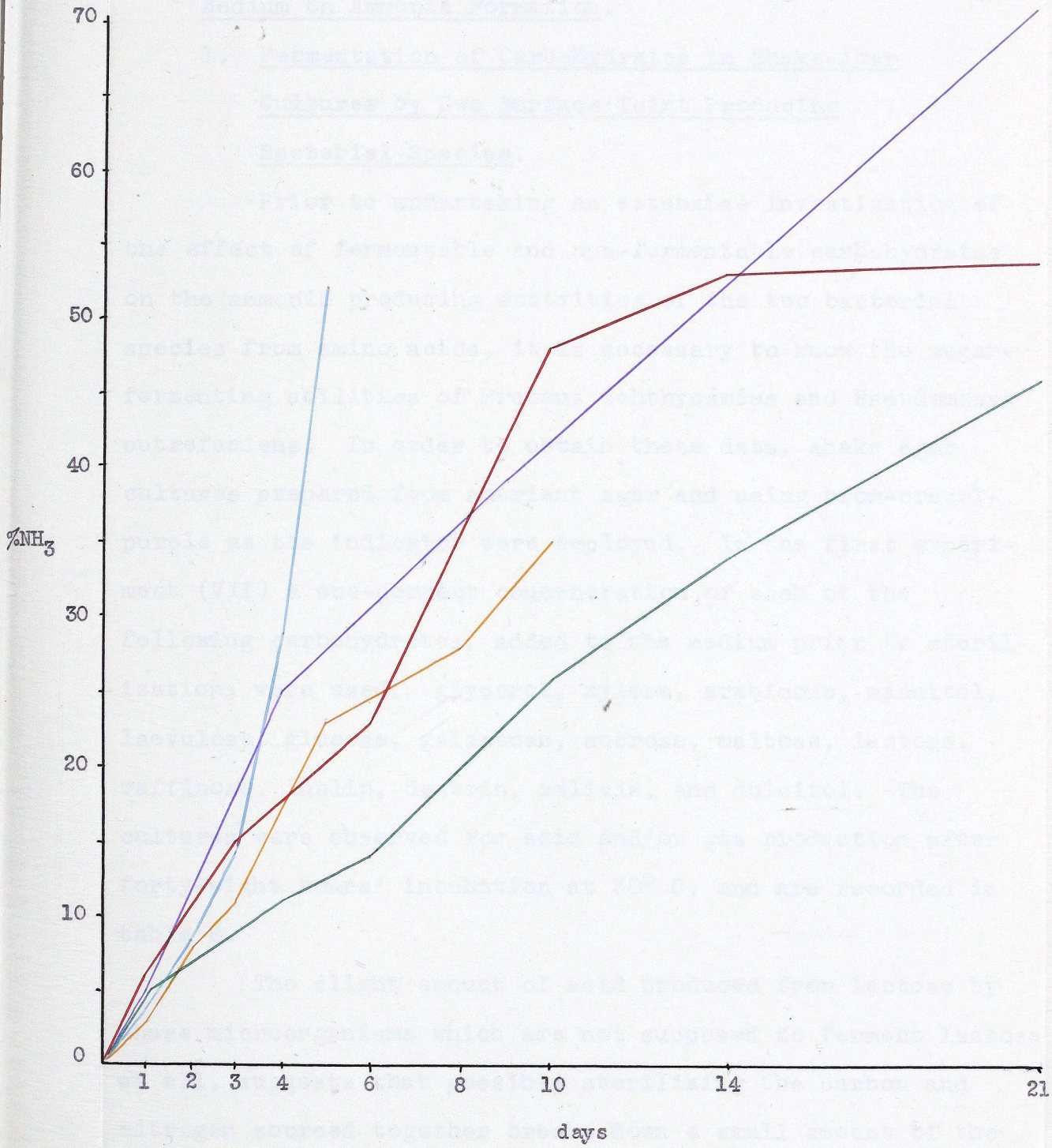


Figure 20.

Proline



series of figures compiled for Proteus ichthyosmius above.

These figures will be discussed in the section titled "Discussion."

Effect of the Presence of Carbohydrates in the Medium on Ammonia Formation.

1. Fermentation of Carbohydrates in Shake-Agar Cultures by Two Surface Taint Producing Bacterial Species.

Prior to undertaking an extensive investigation of the effect of fermentable and non-fermentable carbohydrates on the ammonia producing activities of the two bacterial species from amino acids, it is necessary to know the sugar-fermenting abilities of Proteus ichthyosmius and Pseudomonas putrefaciens. In order to obtain these data, shake agar cultures prepared from nutrient agar and using brom-cresol-purple as the indicator were employed. In the first experiment (VII) a one-percent concentration of each of the following carbohydrates, added to the medium prior to sterilization, were used: glycerol, xylose, arabinose, mannitol, laevulose, glucose, galactose, sucrose, maltose, lactose, raffinose, inulin, dextrin, salicin, and dulcitol. The cultures were observed for acid and/or gas production after forty-eight hours' incubation at 30° C. and are recorded in table 8.

The slight amount of acid produced from lactose by these microorganisms which are not supposed to ferment lactose at all, suggests that possibly sterilizing the carbon and nitrogen sourced together breaks down a small amount of the

TABLE 8.

Shake-Agar Sugar Fermentations

(Carbon and Nitrogen Sources Sterilized Together)

Carbohydrate	Proteus ichthyosmius			Pseudomonas putrefaciens		
	acid	alkaline	gas	acid	alkaline	gas
glycerol	++	0	++	+++	0	+
xylose	0	0	0	0	0	0
arabinose	+	0	+	0	surface	0
mannitol	+++	0	+++	+++	0	++
laevulose	++++	0	++	+++	0	++
glucose	++++	0	+++	+++	0	+++
galactose	+++	0	++	+++	0	++
sucrose	++++	0	+++	+++	0	++
maltose	++++	0	+++-	+++	0	++
lactose	trace	surface	+	++	surface	+
raffinose	trace	surface	+	+++	0	+
inulin	0	surface	trace	++	surface	trace
dextrin	++++	0	+	++	surface	trace
salicin	++++	0	++	++++	0	++
dulcitol	0	surface	trace	+	surface	trace

lactose into its component sugars - glucose and galactose - both of which are readily fermented. In order to eliminate this possibility, the experiment (VIII) was repeated employing the technique of sterilizing the carbohydrate source separately and adding it to the medium aseptically just prior to inoculation. The cultures were incubated at 30° C. and observed at twenty-four, forty-eight and ninety-six hour intervals for acidity, alkalinity and gas production. The findings are given in tables 9 and 10.

This experiment shows that Proteus ichthyosmius produces acid and gas from glycerol, mannitol, glucose, galactose, sucrose, maltose, dextrin and salicin and has no immediate action but goes slowly alkaline in xylose, lactose, dulcitol and the water control; whereas Pseudomonas putrefaciens produces acid and gas from xylose, mannitol, glucose, maltose and salicin, slowly produces acid with no gas from glycerol, and has no immediate reaction but slowly goes alkaline in lactose, dextrin and the water control. The differentiating sugars for these microorganisms are xylose and dextrin: Proteus ichthyosmius ferments dextrin but not xylose while Pseudomonas putrefaciens ferments xylose but not dextrin.

TABLE 9.

Shake-Agar Sugar Fermentations

(Carbon and Nitrogen Sources Sterilized Separately)

Proteus ichthyosmius

Carbohydrate	Acid			Alkaline			Gas		
	24hr.	48hr.	96hr.	24hr.	48hr.	96hr.	24hr.	48hr.	96hr.
glycerol	++	++	+++	0	0	0	++	++	++
xylose	0	0	0	0	++++	++++	0	0	0
mannitol	++++	++++	++++	sur.	sur.	sur.	+++	+++	+++
glucose	++++	++++	++++	0	0	0	++	++	++
galactose	++++	++++	++++	0	0	0	++	++	++
sucrose	++++	++++	++++	0	0	0	+++	+++	+++
maltose	++++	++++	++++	0	0	0	++	++	++
lactose	0	0	0	0	++++	++++	0	0	0
dextrin	++++	++++	++++	0	0	0	++	++	++
salicin	++++	++++	++++	0	0	0	++	++	++
dulcitol	0	0	0	sur.	++++	++++	0	0	0
water (K)	0	0	0	sur.	++++	++++	0	0	0

sur. - surface

TABLE 10.

Shake-Agar Sugar Fermentations

(Carbon and Nitrogen Sources Sterilized Separately)

Pseudomonas putrefaciens

Carbohydrate	Acid			Alkaline			Gas		
	24hr.	48hr.	96hr.	24hr.	48hr.	96hr.	24hr.	48hr.	96hr.
glycerol	trace	+	++	0	0	sur.	0	trace	trace
xylose	++	++	+++	0	sur.	sur.	trace	+	+
mannitol	+++	+++	+++	0	sur.	sur.	+++	+++	+++
glucose	+++	+++	+++	sur.	sur.	sur. +	+++	+++	+++
galactose	+++	+++	+++	sur.	sur.	sur. +	++	++	++
sucrose	+++	+++	+++	sur.	sur.	sur. +	++	++	++
maltose	+++	+++	+++	sur.	sur.	sur. +	++	++	++
lactose	trace	0	0	0	0	+	0	0	0
dextrin	trace	trace	0	sur.	sur.	sur. ++	trace	trace	trace
salicin	+++	+++	+++	0	0	0	++	+++	+++
dulcitol	0	0	0	sur.	+++	+++	0	0	0
water (K)	0	0	0	sur.	+++	+++	0	0	0

sur. - surface

2. Effect of the Presence of Carbohydrates in the Growth and Buffer Media on Ammonia Formation.

The literature contains considerable data and a number of theories on the effect of fermentable and non-fermentable carbohydrates in the growth and buffer media on the subsequent activity of ammonia producing enzymes from various species of microorganisms. With the object of investigating the effect of a number of carbohydrates on the ammonia formation from arginine, aspartic acid, glutamic acid, histidine and proline by Proteus ichthyosmius and Pseudomonas putrefaciens a series of experiments was undertaken.

The object of the first experiment (IX) in this series was to determine the effect of the presence of one percent glucose in the growth medium on ammonia formation from arginine, aspartic acid and glutamic acid in buffer media containing glucose, sucrose, maltose, lactose, glycerol, mannitol or water (control) respectively by Proteus ichthyosmius. The cultures were prepared as outlined in the general procedure and incubated at 30° C. The ammonia from aspartic acid was determined after twenty-four hours' incubation, from glutamic acid after four days and from arginine after five days. The

results are shown in table II and figures 21, 22 and 23.

The glucose growth agar flask was incubated for forty-two hours before harvesting the bacterial cells and the growth was still only about twenty-five percent of that of the eighteen-hour non-glucose agar flask. In general, the presence of glucose in the growth medium only slightly decreases the quantity of free ammonia in the tubes. The amount of free ammonia from the two sugars fermented most rapidly by Proteus ichthyosmius is more than twice as great employing the cells grown on glucose free agar as that using the cells grown on glucose containing agar. The volume of free ammonia from arginine is greatest in the water control and the lactose containing cultures, less in the maltose, glycerol and mannitol containing cultures and least in the glucose and sucrose containing cultures. The quantity of ammonia from aspartic acid in the cultures containing glucose and sucrose is the same for the cells grown on the two types of agar whereas in the culture containing lactose, it is about twenty-five percent greater employing cells from glucose free agar than cells from glucose containing agar. In addition, there is no marked difference in the amount of ammonia present in the cultures containing the various carbohydrates. The cultures containing lactose and the water control show a small quantity of ammonia that is slightly greater from cells grown on glucose free agar than those from glucose containing agar,

The Effect of the Presence of Carbohydrates in the Growth and Buffer Media on Ammonia Production by Proteus ichthyosmius.

Experiment - IX.

Table - 11.

Figures - 21, 22 and 23.

Growth Medium:

Glucose-Free Medium - straight line

Glucose-Containing Medium - broken line.

Buffer Medium:

Water (control) - red

Glucose - violet

Sucrose - green

Maltose - brown

Lactose - orange

Glycerol - blue

Mannitol - black

TABLE 11.

Effect of the Presence of Carbohydrates in the Growth and Buffer Media

Growth Medium	Buffer Medium	Arginine	Aspartic Acid	Glutamic Acid
no glucose	no sugar	62.5	38.5	17.0
glucose	no sugar	54.0	34.5	10.5
no glucose	glucose	28.75	35.5	2.5
glucose	glucose	10.5	36.0	0.0
no glucose	sucrose	32.5	39.5	2.0
glucose	sucrose	13.0	40.0	0.0
no glucose	maltose	39.0	41.0	2.5
glucose	maltose	34.0	33.0	0.0
no glucose	lactose	59.25	43.5	9.0
glucose	lactose	54.5	31.0	5.0
no glucose	glycerol	46.0	44.5	5.0
glucose	glycerol	40.5	39.0	0.0
no glucose	mannitol	36.25	37.0	4.0
glucose	mannitol	31.25	29.0	0.0

Figure 21.

Arginine

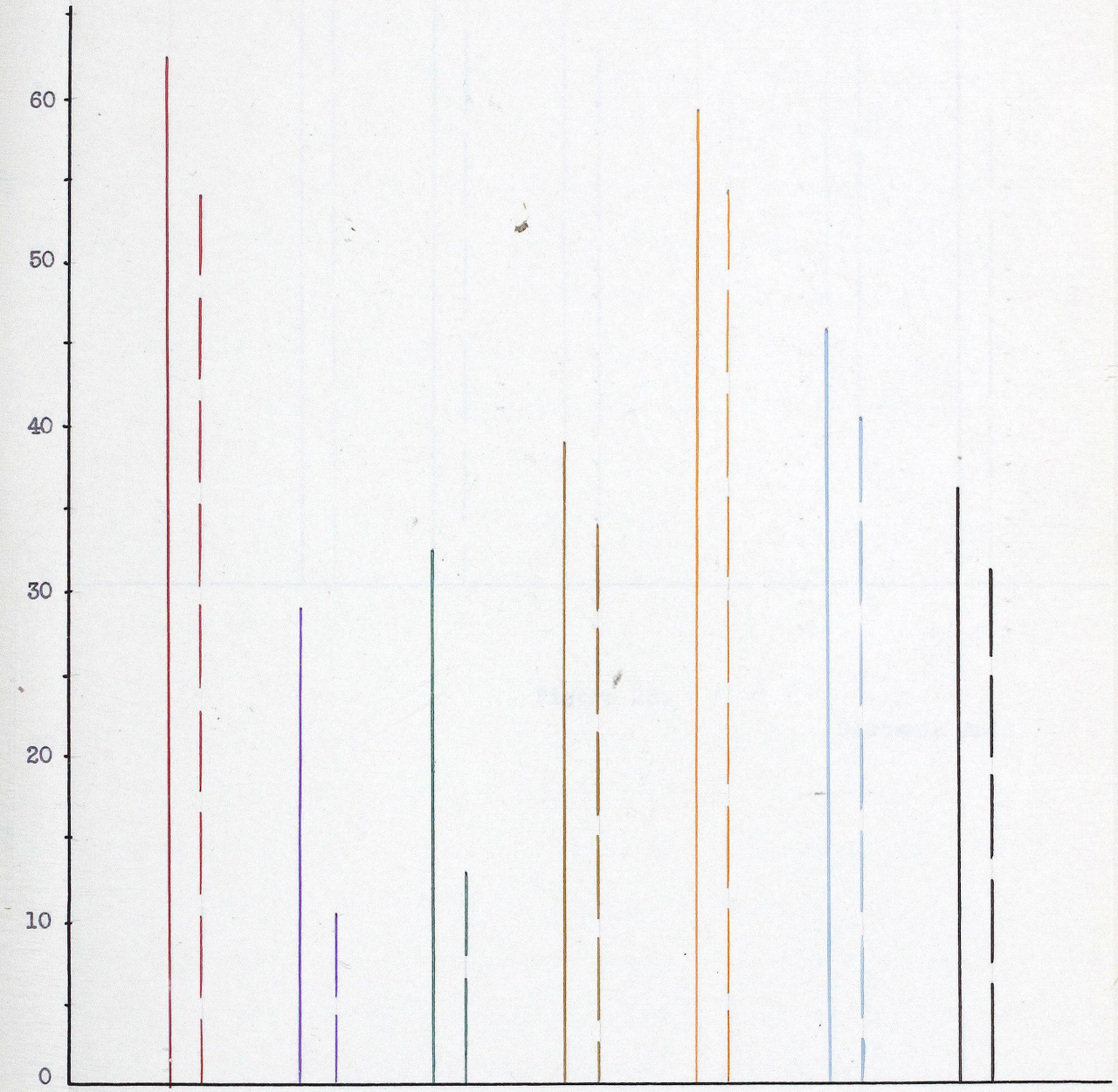


Figure 22.

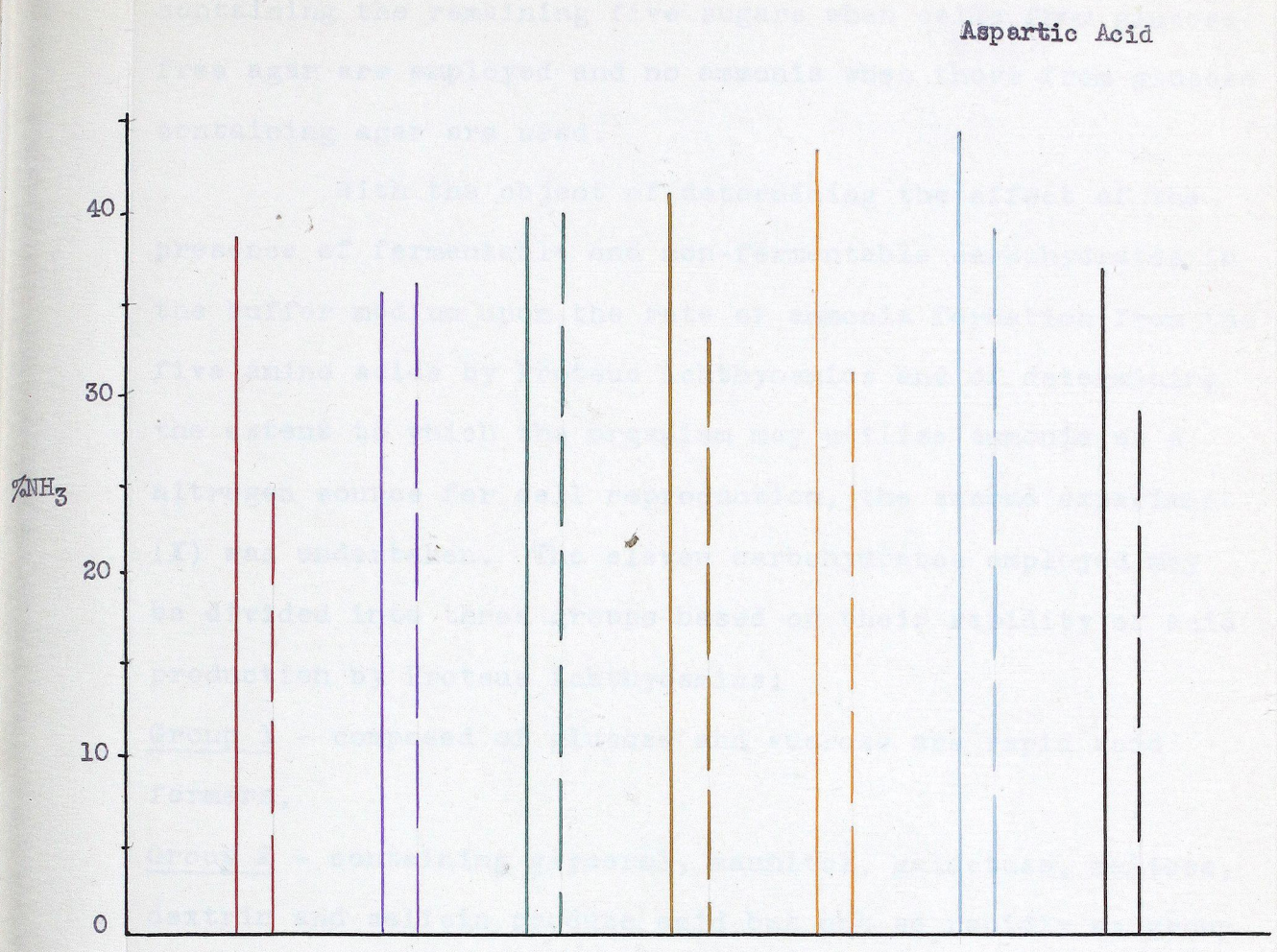
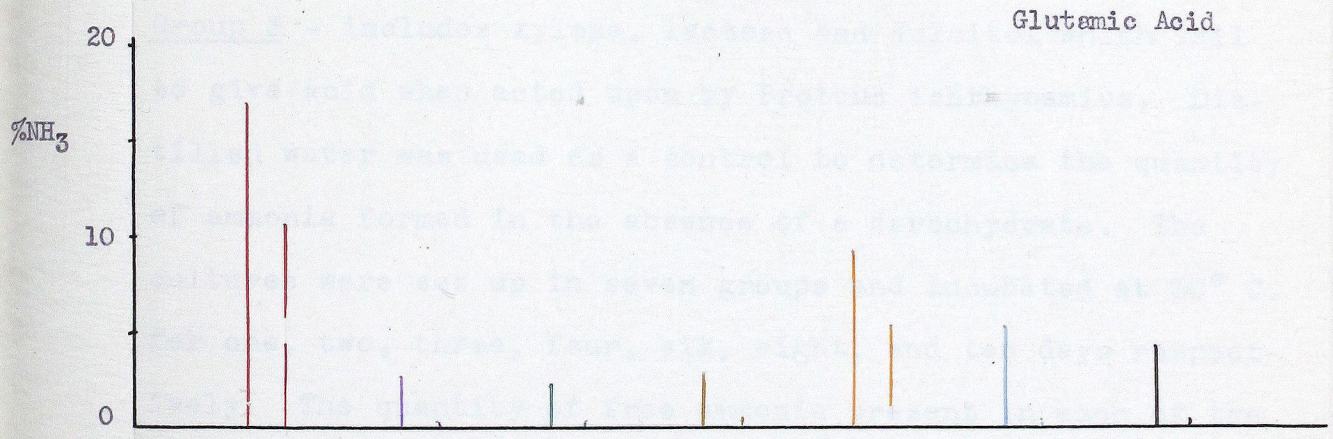


Figure 23.



while there is only a slight amount of ammonia from cultures containing the remaining five sugars when cells from glucose free agar are employed and no ammonia when those from glucose containing agar are used.

With the object of determining the effect of the presence of fermentable and non-fermentable carbohydrates in the buffer medium upon the rate of ammonia formation from the five amino acids by Proteus ichthyosmius and of determining the extent to which the organism may utilize ammonia as a nitrogen source for cell reproduction, the second experiment (X) was undertaken. The eleven carbohydrates employed may be divided into three groups based on their rapidity of acid production by Proteus ichthyosmius:

Group 1 - composed of glucose and sucrose are rapid acid formers,

Group 2 - containing glycerol, mannitol, galactose, maltose, dextrin and salicin produce acid but not so rapidly as group one - acid production from glycerol being much slower than that from the other carbohydrates in this group,

Group 3 - includes xylose, lactose and dulcitol which fail to give acid when acted upon by Proteus ichthyosmius. Distilled water was used as a control to determine the quantity of ammonia formed in the absence of a carbohydrate. The cultures were set up in seven groups and incubated at 30° C. for one, two, three, four, six, eight, and ten days respectively. The quantity of free ammonia present in each of the cultures after each period of incubation is recorded in

The Effect of the Presence of Carbohydrate in the Buffer Medium on the Rate of Ammonia Production by Proteus ichthyosmius.

Experiment X.

Tables 12 to 16 inclusive

Figures 24 to 28 inclusive

Carbohydrates:

Water (contol)	- red
Glycerol	- blue
Xylose	- green
Mannitol	- broken blue
Glucose	- violet
Galactose	- broken green
Sucrose	- broken violet
Maltose	- broken orange
Lactose	- orange
Dextrin	- brown
Salicin	- broken brown
Dulcitol	- broken red

TABLE 12.

Effect of the Presence of Carbohydrates

Proteus ichthyosmius Arginine

carbohydrate	age of culture in days 1	2	3	4	6	8	10
glycerol	49.0	49.0	46.75	46.75	46.5	40.0	41.5
xylose	36.5	47.5	51.0	49.25	56.0	71.0	79.5
mannitol	36.0	38.75	36.75	36.75	34.5	-	36.75
glucose	5.25	8.5	12.75	15.0	20.75	-	24.25
galactose	41.0	39.0	35.75	39.5	31.5	-	36.0
sucrose	12.0	18.0	22.5	24.5	24.0	-	25.75
maltose	40.0	38.25	35.0	38.5	33.5	-	37.75
lactose	49.5	51.75	54.25	61.5	59.5	-	74.0
dextrin	36.0	35.25	35.5	35.5	32.25	-	36.25
salicin	42.0	42.25	40.75	41.5	39.5	-	43.25
dulcitol	47.0	56.25	59.0	62.0	64.5	-	77.0
water	45.5	58.0	57.0	56.5	58.25	-	78.5

TABLE 13.

Effect of the Presence of Carbohydrates

Proteus ichthyosmius — Aspartic Acid

carbohydrate	age of culture in days 1	2	3	4	6	8	10
glycerol	45	45	43	43	42	43	39
xylose	49	47	47	49	46	48	48
mannitol	46	43	42	44	34	35	39
glucose	42	42	39	41	34	-	39
galactose	43	44	42	41	31	33	42
sucrose	44	45	42	43	33	33	42
maltose	40	38	32	37	27	28	33
lactose	49	48	41	44	37	41	48
dextrin	40	37	35	34	30	32	39
salicin	44	46	47	46	44	44	50
dulcitol	52	48	47	50	47	49	51
water	51	50	47	48	46	42	49

TABLE 14.

Effect of the Presence of Carbohydrates

Proteus ichthyosmius — Glutamic Acid

carbohydrate	age of culture in days 1	2	3	4	6	8	10
glycerol	5	6	6	4	1	0	0
xylose	8	8	9	13	17	21	33
mannitol	1	0	0	0	0	0	0
glucose	1	1	0	0	0	0	0
galactose	0	0	0	0	0	0	0
sucrose	2	0	0	0	0	0	0
maltose	1	0	0	0	0	0	0
lactose	8	10	10	12	17	18	21
dextrin	1	0	0	0	0	0	0
salicin	3	1	0	0	0	0	0
dulcitol	10	-	13	16	26	32	37
water	10	11	13	20	28	27	42

TABLE 15.

Effect of the Presence of Carbohydrates

Proteus ichthyosmius — Histidine

carbohydrate	age of culture in days						
	1	2	3	4	6	8	10
glycerol	3.6	8.0	13.3	21.3	21.3	20.6	22.0
xylose	3.3	9.3	29.3	31.3	32.6	33.0	35.0
mannitol	0.6	1.6	0.6	4.6	4.0	9.3	8.6
glucose	0	0	0	0.3	0.3	0.3	0.6
galactose	1.3	1.6	2.0	2.3	2.6	3.3	3.6
sucrose	0	0	0	0.6	0.6	0.3	0.3
maltose	0.6	0.6	1.3	1.3	2.0	3.0	4.0
lactose	2.6	9.3	12.6	19.3	22.6	24.6	26.6
dextrin	0	0.3	0.6	1.3	1.3	2.0	2.6
salicin	2.3	4.6	5.0	6.0	1.0	5.0	5.3
dulcitol	3.3	13.6	21.3	31.0	35.0	36.0	40.6
water	2.6	12.0	20.6	27.3	32.3	35.6	37.0

TABLE 16.

Effect of the Presence of Carbohydrates

Proteus ichthyosmius Proline

carbohydrate	age of culture in days 1	2	3	4	6	8	10
glycerol	1	2	3	0	0	0	0
xylose	2	6	9	11	19	25	31
mannitol	0	0	0	0	-	0	0
glucose	0	0	0	0	-	0	0
galactose	0	0	0	0	-	0	0
sucrose	0	0	0	2	0	0	0
maltose	0	0	0	0	-	0	0
lactose	2	4	8	9	19	10	14
dextrin	0	0	0	0	-	0	0
salicin	0	0	0	0	-	0	-
dulcitol	2	7	12	21	28	31	-
water	3	8	11	23	18	28	35

Figure 24.

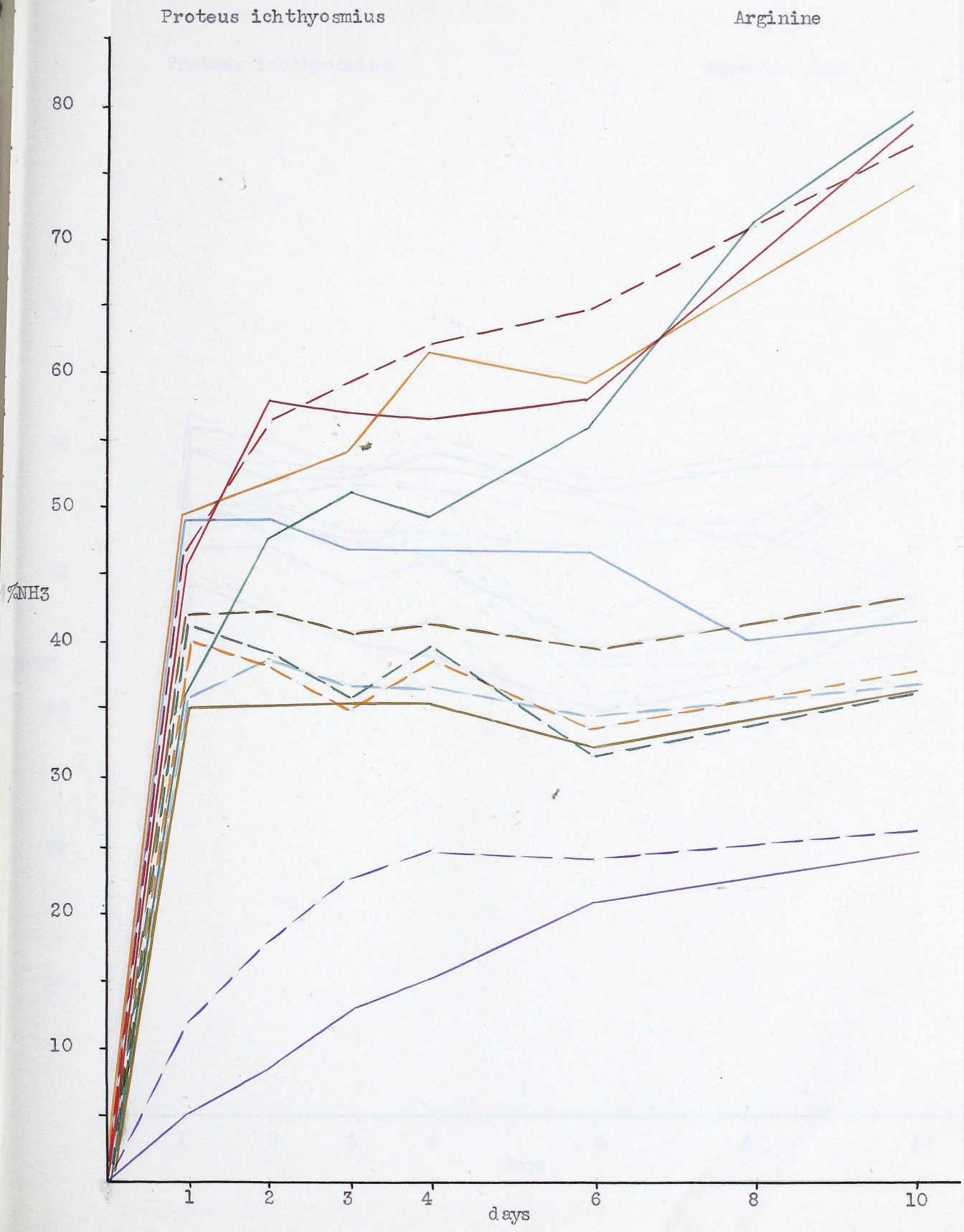

Proteus ichthyosmius
Arginine
80
70
60
50
%NH3
40
30
20
10
1
2
3
4
6
8
10
days

Figure 25

Proteus ichthyosmius

Aspartic Acid

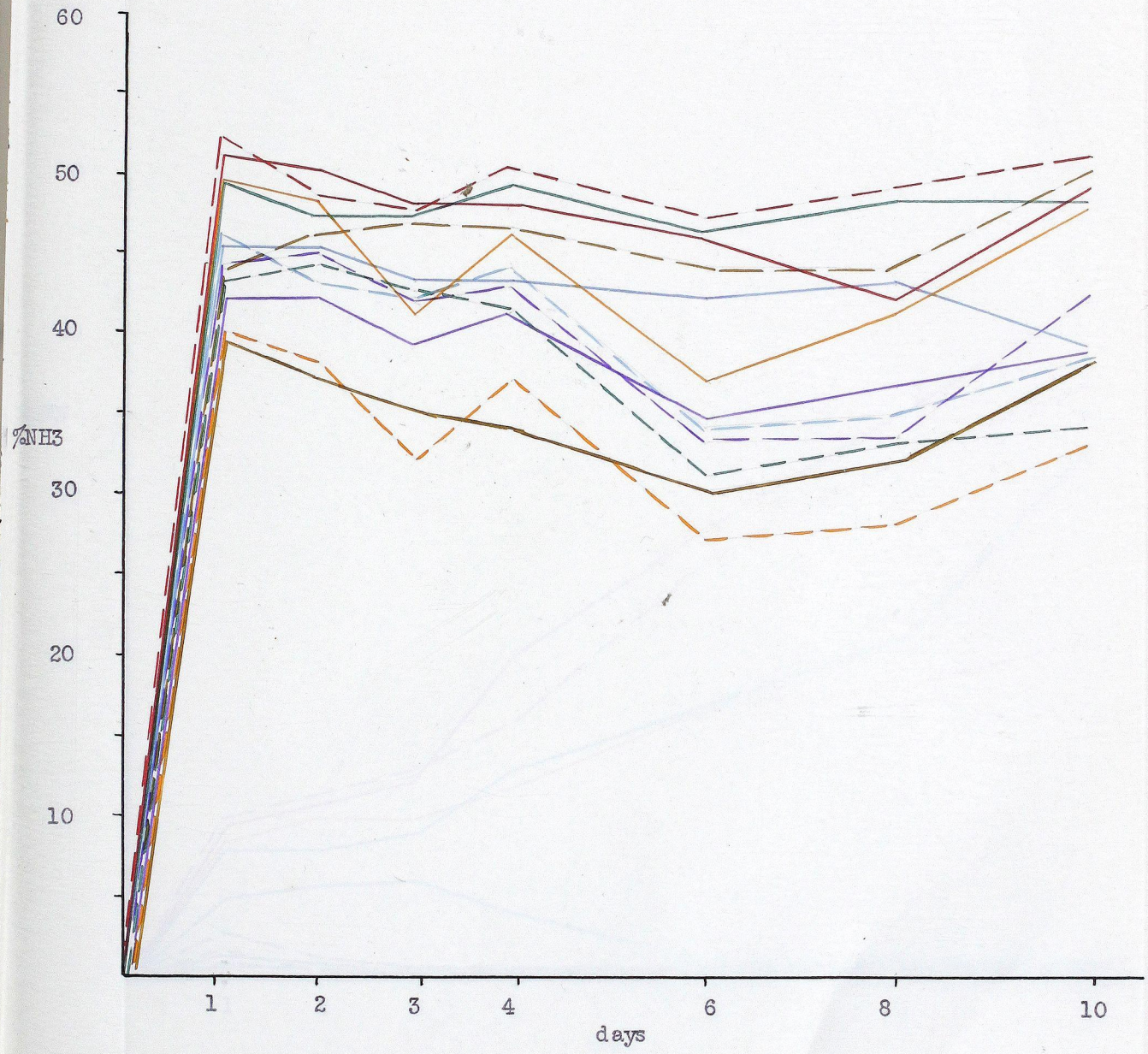


Figure 26

Proteus ichthyosmius

Glutamic Acid

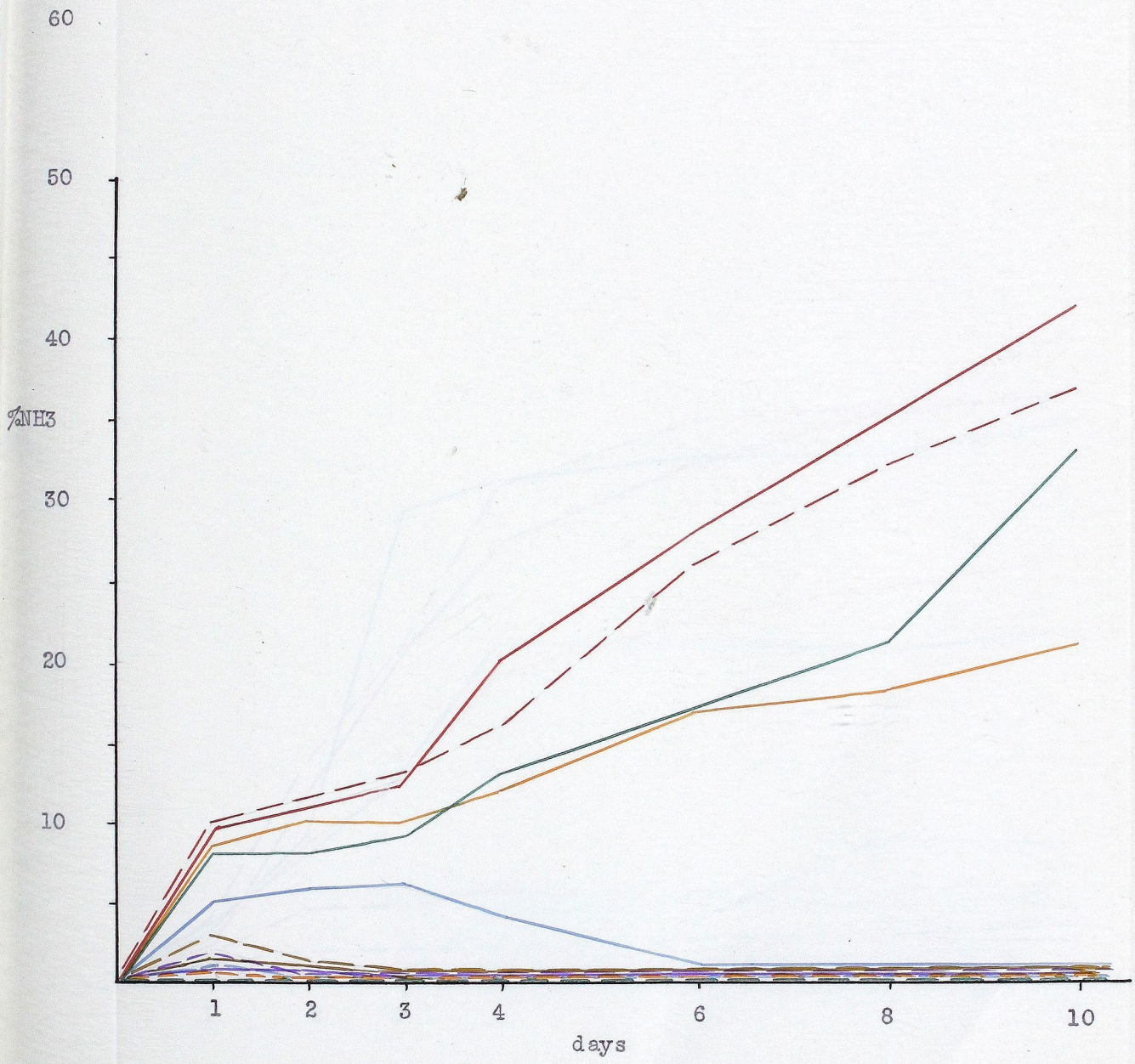


Figure 27

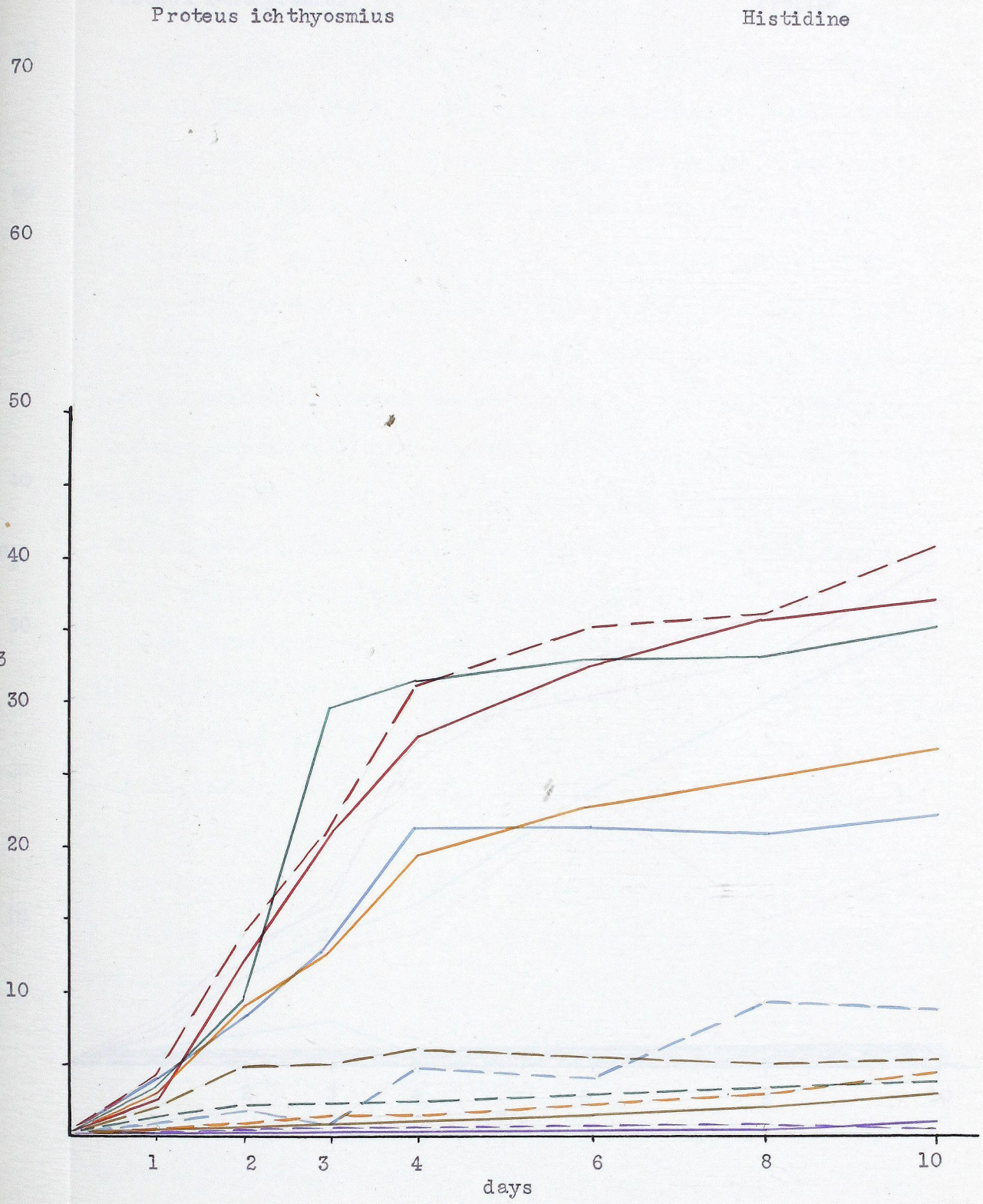
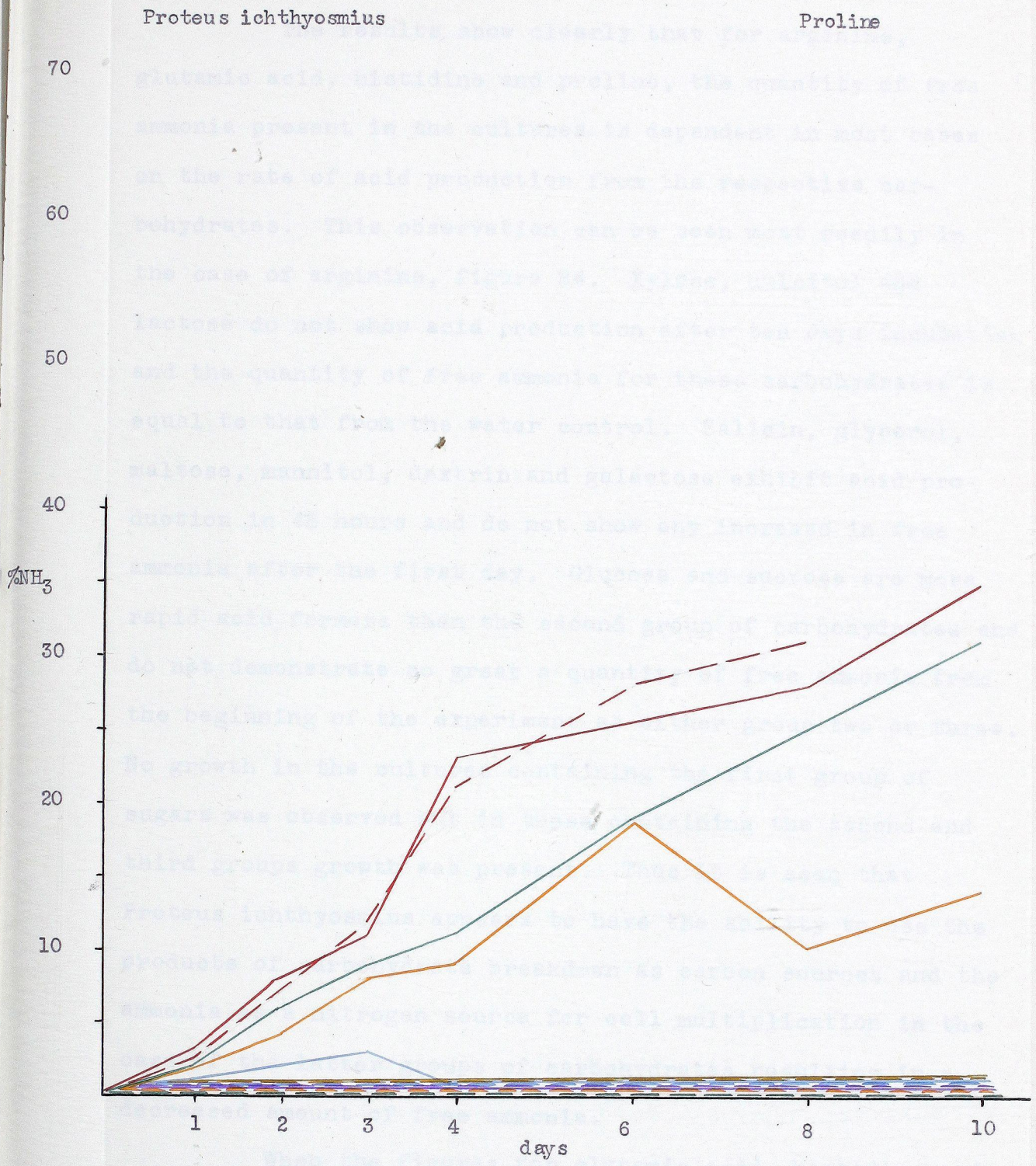

Proteus ichthyosmius
Histidine
70
60
50
40
%NH3
30
20
10
1
2
3
4
6
8
10
days

Figure 28


Proteus ichthyosmius
Proline
70
60
50
40
%NH3
30
20
10
1
2
3
4
6
8
10
days

tables 12 to 16 inclusive and interpreted on figures 24 to 28 inclusive.

The results show clearly that for arginine, glutamic acid, histidine and proline, the quantity of free ammonia present in the cultures is dependent in most cases on the rate of acid production from the respective carbohydrates. This observation can be seen most readily in the case of arginine, figure 24. Xylose, dulcitol and lactose do not show acid production after ten days incubation and the quantity of free ammonia for these carbohydrates is equal to that from the water control. Salicin, glycerol, maltose, mannitol, dextrin and galactose exhibit acid production in 48 hours and do not show any increase in free ammonia after the first day. Glucose and sucrose are more rapid acid formers than the second group of carbohydrates and do not demonstrate so great a quantity of free ammonia from the beginning of the experiment as either group two or three. No growth in the cultures containing the first group of sugars was observed but in those containing the second and third groups growth was present. Thus it is seen that Proteus ichthyosmius appears to have the ability to use the products of carbohydrate breakdown as carbon sources and the ammonia as a nitrogen source for cell multiplication in the case of the latter groups of carbohydrates resulting in a decreased amount of free ammonia.

When the figures for glutamic acid, histidine and proline (figures 26, 27 and 28) are considered the results

obtained are, on the whole, similar to those recorded in the case of arginine. Inspection of these figures shows that the total amount of ammonia formed is considerably less but that the influence of the specific carbohydrates on the relative amount of ammonia formed is unchanged, practically no ammonia being formed in the cultures containing the first and second groups of carbohydrates.

A striking difference between arginine on the one hand and glutamic acid, histidine and proline on the other hand is to be seen when the amounts of ammonia formed during the first twenty-four hours incubation are compared. This difference is undoubtedly related to the chemical structure of the amino acids and to the mechanism of breakdown possessed by Proteus ichthyosmius. This interesting relationship will be considered in the final discussion and will be compared and contrasted with that of Pseudomonas putrefaciens.

In the case of aspartic acid, a picture distinct from that obtained for the other four amino acids employed is to be seen, figure 25. It will be noted that for this amino acid, ammonia production occurs rapidly, deamination of the natural isomer of dl-aspartic acid being complete within twenty-four hours' incubation; whereas in the case of the other four amino acids, ammonia formation takes place at the same time as acid production, a postulate which has been confirmed by later work, (vide experiment XIII). As the time of incubation increases, in the case of aspartic acid, the

level of free ammonia in the cultures containing the fermented carbohydrates decreases only slightly, with a slight increase in the number of bacterial cells, while that in the cultures of the non-acid producing group remains practically unchanged. In the presence of ample quantities of carbon and nitrogen sources in the cultures, one would expect more active cell multiplication with a correspondingly greater decrease in the free ammonia present. This lack of activity suggests that possibly the conditions were not optimum for the growth of the organism.

The experiment was repeated, (XI), employing Pseudomonas putrefaciens in place of Proteus ichthyosmius, and, in order to determine if pH was the limiting factor encountered above, brom-cresol-purple indicator was added to the set of cultures to be left for the longest period of incubation. A control set of cultures containing carbohydrate without an amino acid was set up to determine the changes in pH of the cultures in the absence of cell growth. The cultures were incubated at 30° C. After twenty-four hours' incubation, the cultures containing fermentable sugars showed marked acid production and it was decided to determine electrometrically the final pH of the cultures prior to the determination of the ammonia content. Because of unusual weather conditions, the University was closed for a number of days preventing the carrying out of determinations at all intervals employed in experiment X, one set of cultures of necessity was not completed until an interval of twenty-four

days' incubation had elapsed. It was not possible to complete the work using aspartic and glutamic acids, but the results for the early days of incubation indicate that in the presence of certain carbohydrates there is marked acid production and the pH of these cultures is reduced below the level required for bacterial activity. These results also show that aspartic acid is deaminated more slowly by Pseudomonas putrefaciens than by Proteus ichthyosmius, while glutamic acid is deaminated at about the same rate by both species of bacteria. The results obtained for arginine, histidine, proline and the water control are given in tables 17 to 20 inclusive and figures 29 to 35 inclusive.

The unavoidable delay until the last set of cultures was twenty-four days old when the ammonia content was determined shows that in the case of arginine and proline the ammonia formation by Pseudomonas putrefaciens is not at its maximum at ten days incubation.

The carbohydrates are divided into three groups based on their acid production by Pseudomonas putrefaciens. The first group including lactose, dextrin and dulcitol does not produce acid in shake-agar cultures, the second contains glycerol which produces acid slowly, while the third group made up of xylose, glucose, mannitol, galactose, maltose, sucrose and salicin shows rapid acid production. The pH determinations on the control series containing carbohydrates only, shown in figure 35, also clearly divide the carbohydrates into the same three groups. The pH recording for

The Effect of the Presence of Carbohydrate in the Buffer Medium on the Rate of Ammonia Formation by Pseudomonas putrefaciens.

Experiment XI.

Tables 17 to 20 inclusive

Figures 29 to 35 inclusive

Carbohydrates

Water (control)	- red
Glycerol	- blue
Xylose	- green
Mannitol	-broken blue
Glucose	- violet
Galactose	- broken green
Sucrose	- broken violet
Maltose	- broken orange
Lactose	- orange
Dextrin	- brown
Salicin	- broken brown
Dulcitol	- broken red

TABLE 17.

Effect of the Presence of Carbohydrates

Pseudomonas putrefaciens — Arginine

CARBOHYDRATE		age of culture in days: 1	2	3	4	6	8	24
glycerol	1.	0.25	0.5	0.5	1.0	0.25	1.0	44.25
	2.	6.90	6.80	6.65	6.35	6.30	6.25	7.05
xylose	1.	0.25	0	0	0.25	0.25	4.25	45.75
	2.	6.95	5.75	5.40	5.45	5.60	6.30	7.05
mannitol	1.	0.25	0.5	0.75	0.75	1.5	11.25	42.25
	2.	6.40	5.55	5.45	5.30	6.05	6.45	7.05
glucose	1.	1.0	0.75	0.75	1.0	0.5	6.25	54.25
	2.	5.75	5.05	5.10	5.00	5.10	5.95	7.15
galactose	1.	0.75	1.0	1.0	0.25	0.5	1.0	48.5
	2.	5.80	5.25	5.55	5.20	5.25	5.80	7.15
sucrose	1.	1.5	1.5	1.25	0.75	0.5	3.0	40.5
	2.	5.80	5.45	5.45	5.50	5.75	5.50	7.05
maltose	1.	0.5	0.5	0.25	0.5	0	5.75	45.5
	2.	6.90	6.00	5.50	5.15	5.25	6.20	7.10
lactose	1.	1.0	3.0	2.75	6.25	6.5	15.25	42.75
	2.	6.95	6.95	7.10	6.90	6.85	6.80	6.05
dextrin	1.	1.25	3.0	5.0	6.75	9.25	13.0	50.5
	2.	6.95	6.95	6.95	6.90	7.05	6.95	7.15
salacin	1.	0.25	0.75	0.75	1.25	1.25	0.75	2.25
	2.	6.55	5.40	5.30	5.20	5.40	5.10	5.15
dulcitol	1.	1.5	3.25	1.0	6.0	6.5	11.0	84.5
	2.	7.00	7.00	7.05	6.95	7.05	7.05	7.55
water	1.	1.75	2.75	6.25	5.5	7.5	8.5	78.75
	2.	7.00	7.00	7.05	7.00	7.05	7.05	7.55

TABLE 18.

Effect of the Presence of Carbohydrates

Pseudomonas putrefaciens — Histidine

Carbohydrate		age of culture in days 1	2	4	9	24
glycerol	1.	3.0	4.3	10.6	20.6	37.3
	2.	7.10	6.75	6.60	6.20	6.55
xylose	1.	3.0	3.3	4.3	8.3	10.6
	2.	7.10	6.05	5.45	5.25	5.30
mannitol	1.	2.0	3.6	4.3	6.6	16.6
	2.	6.50	5.65	5.60	5.20	6.10
glucose	1.	0.3	1.0	1.6	3.3	16.0
	2.	5.65	4.90	5.10	5.25	6.25
galactose	1.	-	1.3	1.6	2.6	4.3
	2.	5.80	5.05	5.30	5.30	5.15
sucrose	1.	-	2.0	4.0	8.6	20.3
	2.	5.90	5.40	5.65	5.45	6.00
maltose	1.	-	8.0	7.3	13.3	13.0
	2.	7.05	5.80	5.30	4.90	4.90
lactose	1.	3.0	8.3	14.6	45.0	54.6
	2.	7.15	6.95	7.20	7.05	6.05
dextrin	1.	4.6	11.0	30.6	50.0	49.0
	2.	7.15	6.95	7.20	7.20	6.95
salicin	1.	2.0	3.3	5.0	6.0	8.3
	2.	6.75	5.85	5.50	5.55	5.40
dulcitol	1.	5.6	12.0	37.3	51.0	55.6
	2.	7.25	7.10	7.40	7.55	7.90
water	1.	6.0	10.6	39.6	57.0	53.6
	2.	7.25	7.05	7.45	7.70	7.90

TABLE 19.

Effect of the Presence of Carbohydrates

Pseudomonas putrefaciens Proline

Carbohydrate		age of culture in days 1	2	4	9	24
glycerol	1.	1	0	1	6	32
	2.	6.85	6.55	6.55	6.05	6.50
xylose	1.	-	0	0	1	2
	2.	6.80	5.95	5.55	5.05	5.00
mannitol	1.	-	0	0	2	5
	2.	6.65	6.05	5.65	5.35	5.05
glucose	1.	-	0	0	1	5
	2.	6.05	5.25	5.20	5.20	5.00
galactose	1.	-	0	0	-	9
	2.	6.05	5.35	5.40	5.20	6.25
sucrose	1.	-	0	0	-	5
	2.	6.15	5.65	5.55	5.75	5.75
maltose	1.	-	0	0	-	2
	2.	6.80	6.15	5.25	4.85	4.80
lactose	1.	3	10	11	21	40
	2.	6.95	6.65	6.95	6.55	5.80
dextrin	1.	4	8	8	16	17
	2.	6.85	7.35	6.95	6.80	6.85
salicin	1.	0	0	0	-	3
	2.	6.65	5.90	5.60	5.35	5.35
dulcitol	1.	7	14	20	4	70
	2.	6.95	6.80	7.15	7.45	7.05
water	1.	5	12	25	39	71
	2.	6.95	6.80	7.15	7.10	7.05

TABLE 20.

Fermentation of Carbohydrates in the Absence of a Nitrogen Source.

Pseudomonas putrefaciens — Water (control)

Carbohydrate	age of culture in days			
	1	2	4	9
glycerol	7.40	7.10	7.25	6.70
xylose	7.25	6.65	6.55	5.15
mannitol	7.20	6.80	6.85	5.60
glucose	6.60	5.95	5.65	5.30
galactose	6.60	5.90	5.85	5.05
sucrose	6.65	5.95	6.05	5.65
maltose	7.35	6.85	6.85	5.95
lactose	7.40	7.20	7.45	7.20
dextrin	7.40	7.15	7.40	7.25
salicin	7.10	6.55	6.45	5.50
dulcitol	7.40	7.30	7.65	7.05
water	7.40	7.35	7.60	7.45

(figures express final pH of cultures)

Figure 29

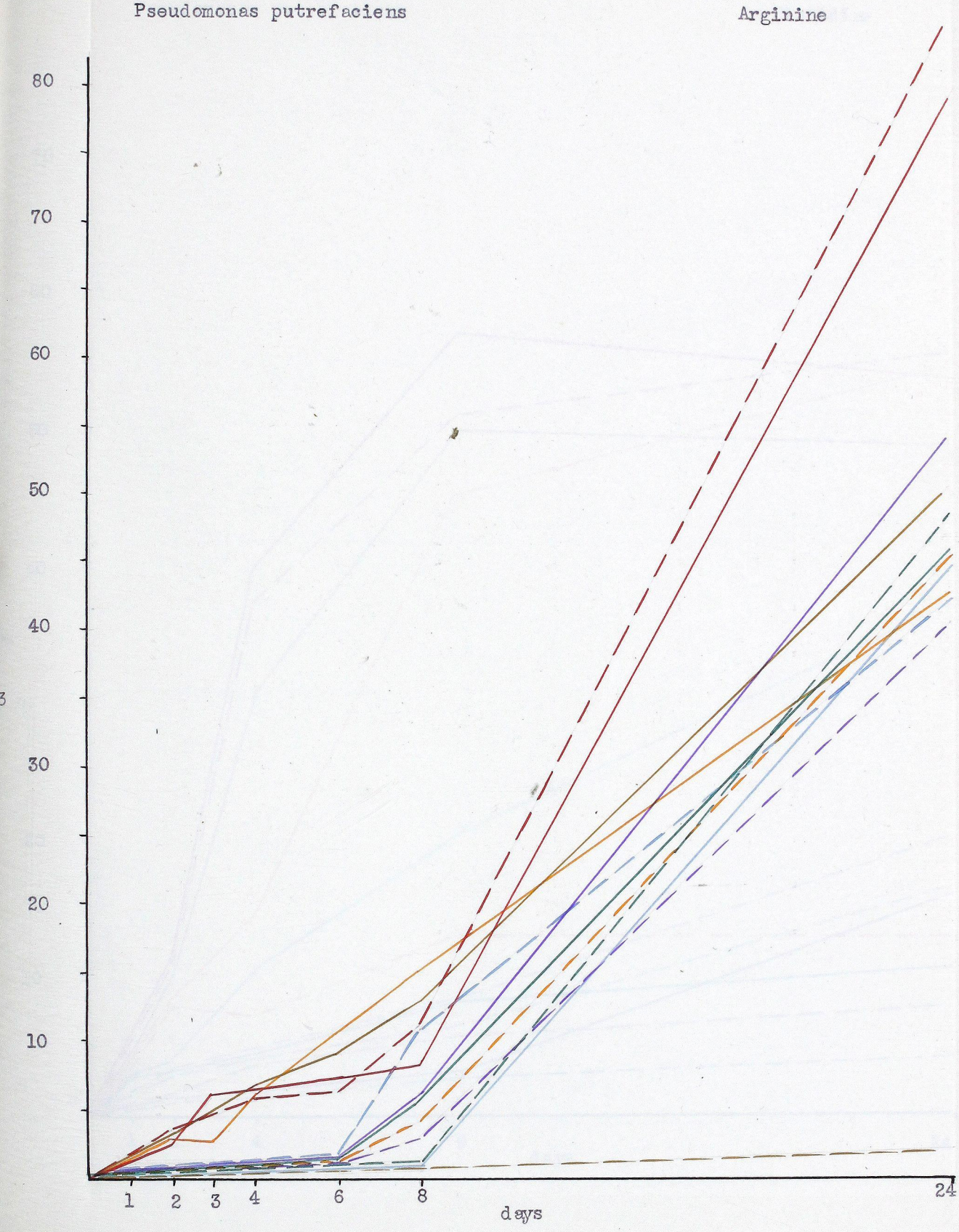

Pseudomonas putrefaciens
Arginine
80
70
60
50
40
%NH3
30
20
10
1
2
3
4
6
8
24
days

Figure 30

Pseudomonas putrefaciens

Histidine

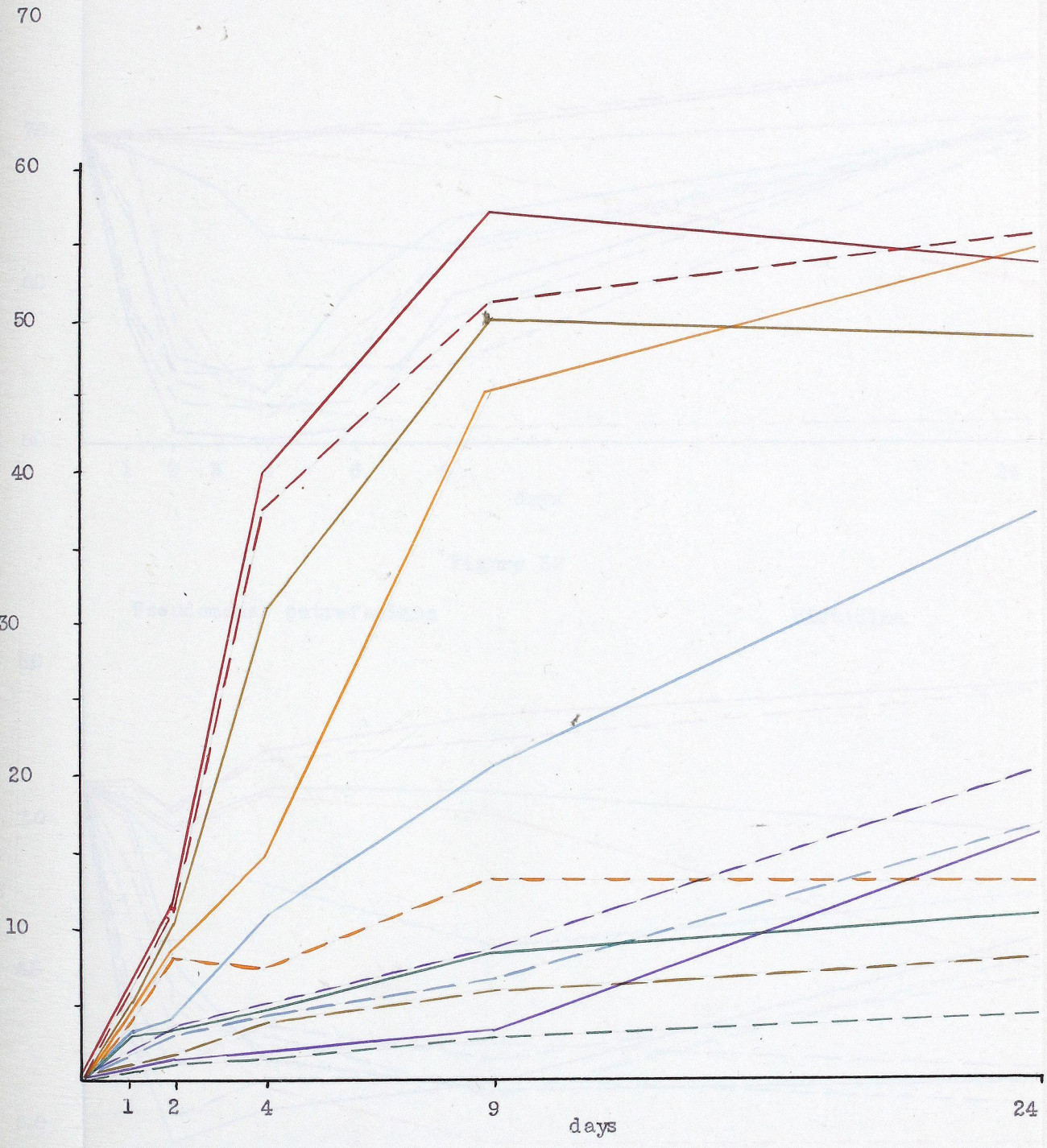


Figure 31

Pseudomonas putrefaciens Arginine

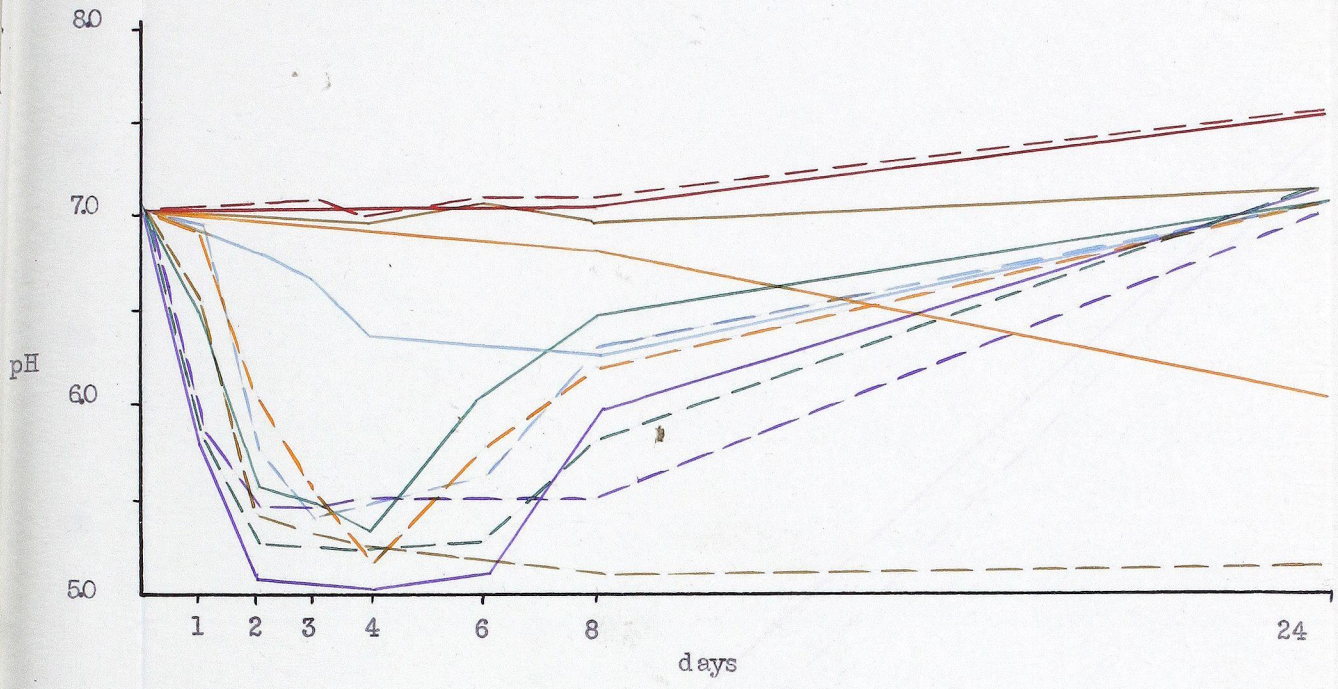


Figure 32

Pseudomonas putrefaciens Histidine

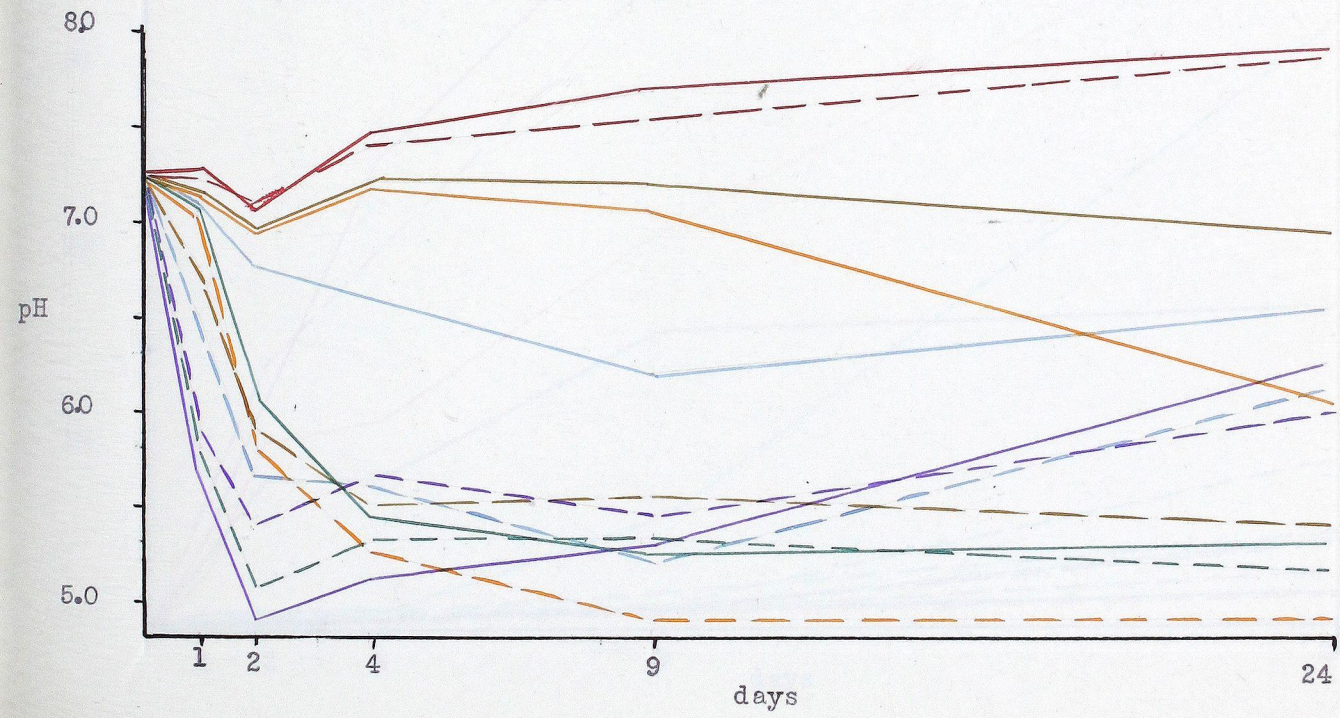


Figure 33

Pseudomonas putrefaciens

Proline

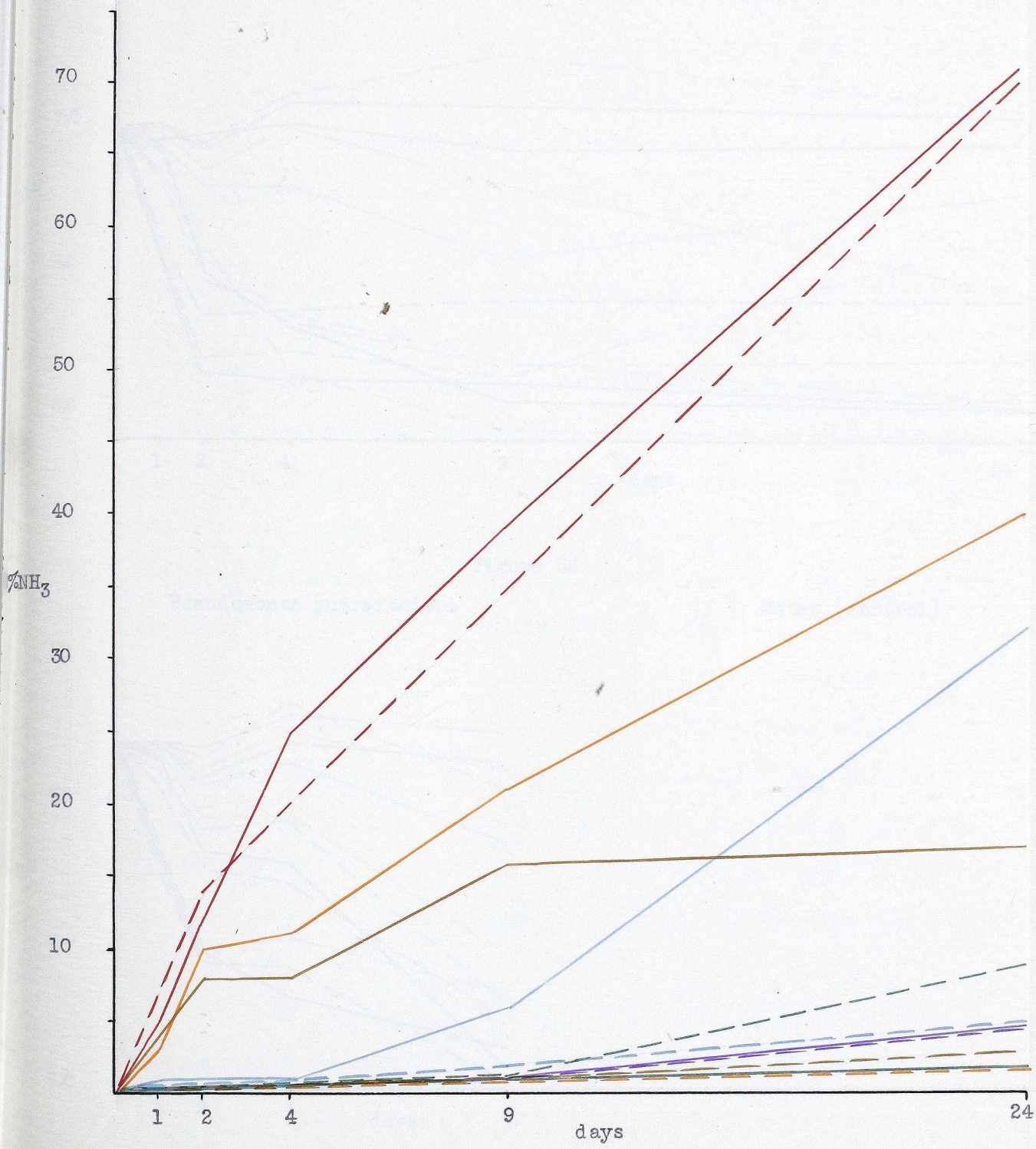


Figure 34

Pseudomonas putrefaciens — Proline

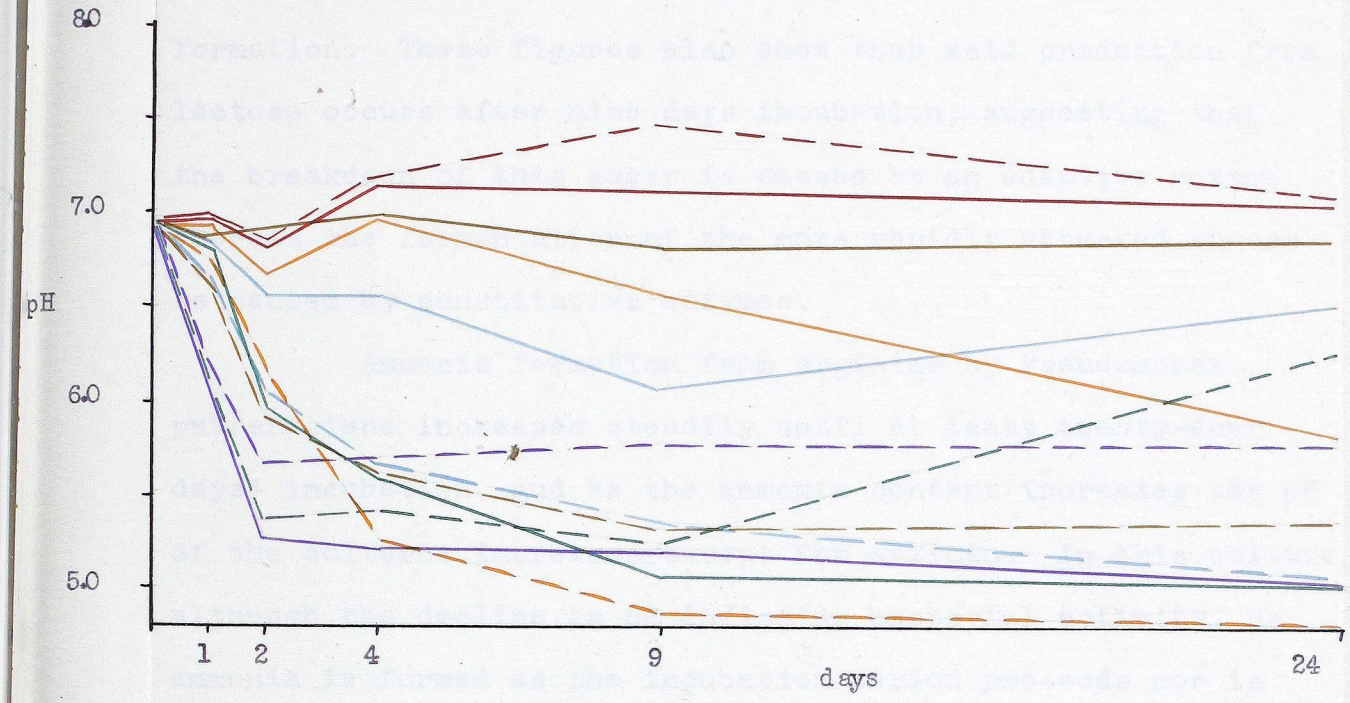
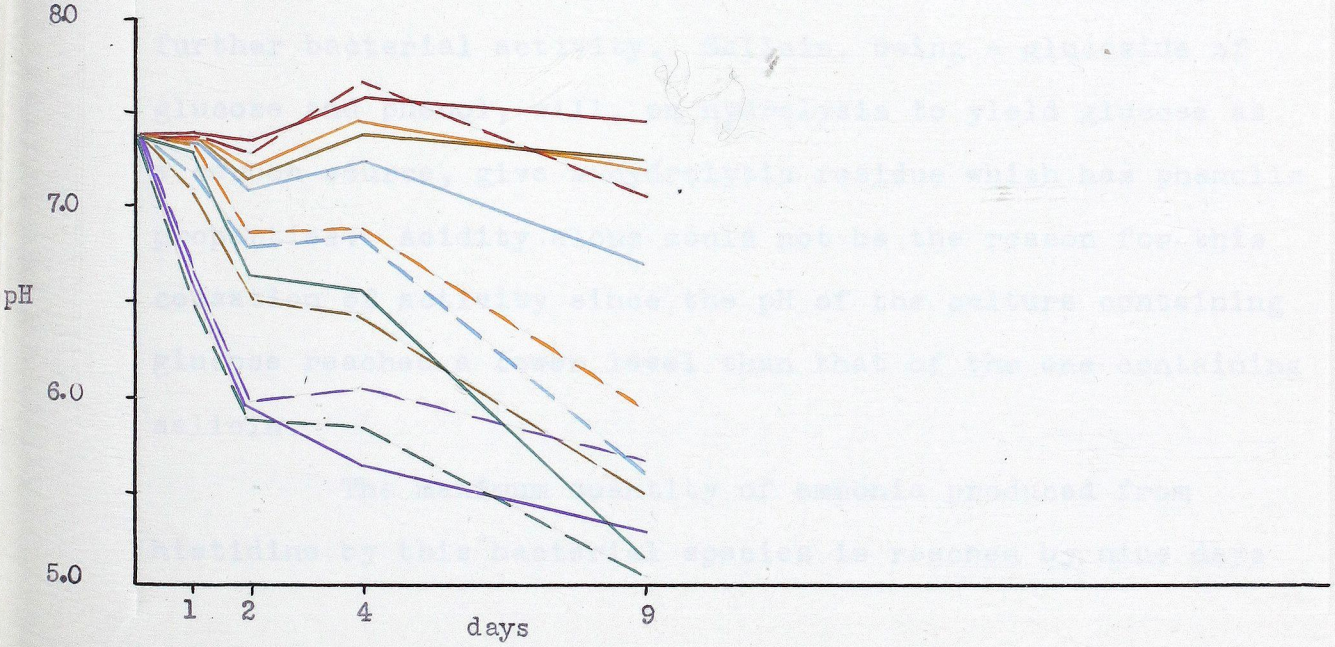


Figure 35

Pseudomonas putrefaciens — Water (control)



for the first nine days of incubation, as seen in figure 31, 32, and 34, show that the carbohydrates are fermented in a similar manner for the three amino acids, indicating that the process of fermentation is independent of that of ammonia formation. These figures also show that acid production from lactose occurs after nine days incubation, suggesting that the breakdown of this sugar is caused by an adaptive enzyme whereas the fermentation of the more rapidly attacked sugars is caused by constitutive enzymes.

Ammonia formation from arginine by Pseudomonas putrefaciens increases steadily until at least twenty-four days' incubation, and as the ammonia content increases the pH of the cultures increases except for salicin. In this culture, although the decline in pH indicates bacterial activity, no ammonia is formed as the incubation period proceeds nor is there a subsequent increase in pH. It is possible that the failure to form ammonia in the case of salicin is due to the elaboration of toxic products of fermentation preventing further bacterial activity. Salicin, being a glucoside of glucose and phenol, will, on hydrolysis to yield glucose as a carbon source, give a hydrolytic residue which has phenolic properties. Acidity alone could not be the reason for this cessation of activity since the pH of the culture containing glucose reached a lower level than that of the one containing salicin.

The maximum quantity of ammonia produced from histidine by this bacterial species is reached by nine days

incubation in the cultures containing the non-acid producing carbohydrates. In the cultures containing glycerol, sucrose, mannitol and glucose, the ammonia content increases up to twenty-four days incubation with a corresponding increase in pH, as may be seen in figure 32. The cultures containing maltose, xylose, salicin and galactose, on the other hand, do not increase in pH or ammonia content after nine days incubation indicating that activity has ceased in these tubes at nine days.

The results obtained with proline show that when the pH reaches too low a level, as in the cultures containing salicin, mannitol, glucose, xylose, sucrose and maltose, activity decrease markedly and the bacteria cannot produce ammonia to overcome the unfavourable acidity as they do in the cultures containing galactose. The culture containing salicin did not reach as low a pH as that containing galactose, but it did not show an increase in ammonia suggesting, again, that there is something among the breakdown products of salicin that is not present in those of galactose and that is capable of preventing further bacterial activity.

The question that arises from these results is that, if the pH could be controlled by increasing the buffering power of the cultures, would ammonia be formed in the presence of acid-forming carbohydrates? In an attempt to obtain data on this aspect of the problem, experiments employing five key carbohydrates in the presence of varying buffer concentrations were undertaken employing Proteus ichthyosmius and Pseudomonas

putrefaciens. The carbohydrates selected were glucose -- from which both species of bacteria produce acid, glycerol -- showing slow acid formation by both species, lactose -- from which no acid is formed by either species, xylose -- from which Pseudomonas putrefaciens produces acid but Proteus ichthyosmius does not, and dextrin -- from which Proteus ichthyosmius produces acid but Pseudomonas putrefaciens does not. A control of distilled water in place of the carbohydrate was used to determine the ammonia formation in the absence of carbohydrate.

In the first experiment, the action of Proteus ichthyosmius on arginine in the presence of three buffer concentrations was studied. The first set of cultures had a final buffer concentration the same as that used in previous experiments, M/20, the second had double the buffer concentration, M/10, and the third had four times the buffer concentration, M/5. Each set of cultures was prepared in six groups and incubated for one, three, six, ten, fourteen, and twenty-one days respectively at 30° C. The final pH of the cultures and the free ammonia content are given in table 21 and figures 36 to 39 inclusive. For the sake of clarity in presentation, the results obtained when M/10 buffer was employed are not portrayed. These results fell between those found for the lower and higher buffer concentrations.

The results of this experiment show that an increase of four times the buffer concentration controls the pH of the medium and prevents the fermentation acids from lowering the

pH to a level unfavourable to ammonia formation. This fact is brought out most clearly in the cultures containing glucose. In the presence of M/20 buffer concentration, the pH of this culture drops to pH 5.3 and does not rise to any considerable extent with the result that the quantity of free ammonia is small. When, however, the buffer concentration is increased, the pH of the glucose cultures drops only to pH 6.5 and the quantity of ammonia increases to the same amount as that in the cultures containing dextrin and glycerol, the other carbohydrates from which this organism produces acid. In the cultures containing dextrin, in the presence of M/20 buffer, the pH does not drop below pH 5.5 and because of the greater ammonia formation and the slower rate of acid production during the first three days of incubation, as compared with the glucose containing cultures, the bacteria remain active and continue to produce ammonia and, as a result, raise the pH of the medium practically to its original hydrogenion concentration - pH 7.6. Whether the pH is raised directly by the increased ammonia, or indirectly by the bacteria using the fermentation acids as carbon sources with the ammonia as a nitrogen source for cell multiplication, is a question which cannot be answered at present.

Another interesting observation from figures 38 and 39 depicting the pH changes by Proteus ichthyosmius is that, in the presence of arginine, lactose is broken down with considerable acid production after ten days' incubation. The specific influence of arginine on acid production from

lactose is apparent when the action of this organism on lactose in the presence of other amino acids is considered, (vide infra).

In order to obtain more data on the influence of buffer concentration on ammonia formation, experiment XII was extended to include the action of Proteus ichthyosmius on aspartic acid, glutamic acid, histidine and proline, and of Pseudomonas putrefaciens on the same five amino acids in the presence of M/20 and M/5 buffer concentrations employing each of the five carbohydrates used above. The results of this experiment (XIII) are recorded in tables 22 to 30 inclusive and interpreted in figures 40 to 75 inclusive.

In general, the findings of this experiment show that increasing the concentration of the buffer prevents the lowering of the pH of the cultures by the fermentation acids to a level that is unfavourable and, in some cases, fatal to further bacterial activity. This control of the pH of the culture medium permits the bacteria to continue to elaborate ammonia and, in the presence of available carbohydrate, to use the ammonia for cell multiplication.

A more detailed examination of the results brings forth a number of interesting observations, for some of which no adequate explanation can be advanced.

Increasing the buffer concentration markedly decreases the quantity of ammonia formed by Pseudomonas putrefaciens from arginine in the presence of the non-acid producing carbohydrates -- lactose and dextrin -- and of the water

The Effect of Increasing the Buffer Concentration in the Presence of Carbohydrates on Ammonia Formation by Proteus ichthyosmius and Pseudomonas putrefaciens.

Experiment	XII and XIII
Tables	21 to 30 inclusive
Figures	36 to 75 inclusive

Carbohydrates

Water (control)	- red
Glycerol	- blue
Xylose	- green
Glucose	- violet
Lactose	- orange
Dextrin	- brown

TABLE 21.

Proteus ichthyosmius — Arginine

Buffer Concentration M/20

carbohydrate		age of culture in days 1	3	6	10	14	21
glycerol	1.	24.5	40.75	40.75	33.7	34.0	32.0
	2.	7.65	7.45	7.30	6.90	7.15	7.25
xylose	1.	22.5	47.25	49.5	53.75	77.25	47.0
	2.	7.65	7.90	7.95	8.00	8.10	8.00
glucose	1.	1.5	11.5	16.5	20.0	20.5	16.5
	2.	5.85	5.40	5.30	5.40	5.65	5.40
lactose	1.	32.5	49.0	54.75	59.75	54.25	39.75
	2.	7.75	7.90	7.75	7.65	7.35	6.15
dextrin	1.	29.5	30.75	25.5	30.0	32.5	41.0
	2.	5.90	5.55	6.25	6.70	7.05	7.30
water	1.	28.5	50.25	51.25	74.00	69.75	60.25
	2.	7.75	7.85	7.90	8.15	8.20	8.20

Buffer Concentration M/5

Carbohydrate		age of culture in days 1	3	6	10	14	21
glycerol	1.	31.0	38.0	36.25	30.5	27.5	40.75
	2.	7.55	7.55	7.35	7.20	7.15	7.25
xylose	1.	31.5	42.75	48.75	54.25	74.25	64.25
	2.	7.55	7.65	7.75	7.75	7.85	7.75
glucose	1.	27.0	28.0	39.5	30.75	41.5	43.75
	2.	6.50	6.75	7.05	7.15	7.25	7.25
lactose	1.	37.5	46.25	55.25	61.5	60.0	53.0
	2.	7.65	7.65	7.65	7.65	7.50	6.85
dextrin	1.	19.0	31.25	28.75	27.75	32.0	36.5
	2.	6.70	6.75	7.05	7.25	7.10	7.30
water	1.	22.75	51.75	59.0	56.5	80.5	80.0
	2.	7.65	7.70	7.75	7.75	7.75	7.90

1. percent of total nitrogen of amino acid in culture present as free ammonia.
2. pH of culture at time of ammonia determination.

TABLE 22.

Proteus ichthyosmius — Aspartic Acid

Buffer Concentration M/20

carbohydrate		age of culture in days 1	3	6	10	14	21
glycerol	1.	38	35	31	25	23	21
	2.	7.15	7.00	6.90	6.65	6.50	6.35
xylose	1.	40	37	37	35	38	39
	2.	7.25	7.25	7.35	7.45	7.55	7.60
glucose	1.	24	28	24	18	24	23
	2.	5.90	5.45	5.45	5.40	5.40	5.50
lactose	1.	40	36	36	31	34	29
	2.	7.20	7.25	7.35	7.25	7.40	7.35
dextrin	1.	9	19	16	8	15	22
	2.	5.90	5.60	5.45	5.55	5.30	5.50
water	1.	37	44	41	24	34	44
	2.	7.25	7.35	7.35	7.50	7.60	7.60

Buffer Concentration M/5

carbohydrate		age of culture in days 1	3	6	10	14	21
glycerol	1.	31	38	31	12	14	26
	2.	7.40	7.35	7.35	7.25	7.15	7.20
xylose	1.	21	41	40	22	31	39
	2.	7.45	7.45	7.50	7.50	7.55	7.55
glucose	1.	16	9	8	8	6	8
	2.	6.95	6.75	6.70	6.65	6.70	6.75
lactose	1.	30	34	33	24	20	21
	2.	7.45	7.45	7.45	7.45	7.45	7.40
dextrin	1.	5	3	2	3	3	1
	2.	6.95	6.75	6.85	6.80	6.95	6.95
water	1.	31	30	34	33	25	32
	2.	7.45	7.45	7.45	7.50	7.55	7.55

1. percent of total nitrogen of amino acid in culture present as free ammonia.
2. pH of culture at time of ammonia determination.

TABLE 23.

Proteus ichthyosmius — Glutamic Acid

Buffer Concentration M/20

carbohydrate		age of culture in days 1	4	6	10	14	21
glycerol	1.	3	4	4	1	0	1
	2.	7.25	7.15	7.15	6.90	6.75	6.55
xylose	1.	4	11	15	26	25	41
	2.	7.25	7.25	7.30	7.45	7.50	7.60
glucose	1.	2	2	2	1	0	1
	2.	5.90	5.40	5.40	5.40	5.40	5.45
lactose	1.	4	10	10	11	9	11
	2.	7.30	7.30	7.30	7.25	7.20	7.25
dextrin	1.	2	1	2	1	0	0
	2.	6.60	5.75	5.80	5.95	5.95	5.85
water	1.	5	15	14	21	27	42
	2.	7.25	7.30	7.35	7.40	7.45	7.65

Buffer Concentration M/5

carbohydrate		age of culture in days 1	4	6	10	14	21
glycerol	1.	2	3	3	1	0	0
	2.	7.45	7.40	7.40	7.40	7.35	7.30
xylose	1.	4	8	8	14	7	26
	2.	7.45	7.45	7.45	7.45	7.55	7.55
glucose	1.	1	1	2	0	0	1
	2.	7.05	6.70	6.75	6.65	6.75	6.75
lactose	1.	4	5	6	2	1	1
	2.	7.45	7.45	7.45	7.45	7.40	7.35
dextrin	1.	1	1	1	1	0	2
	2.	7.35	6.85	6.75	6.95	6.95	7.05
water	1.	4	10	11	16	22	37
	2.	7.45	7.50	7.45	7.50	7.55	7.60

1. percent of total nitrogen of amino acid in culture present as free ammonia.
2. pH of culture at time of ammonia determination.

TABLE 24.

Proteus ichthyosmius — Histidine

Buffer Concentration M/20

carbohydrate		age of culture in days 1	4	6	10	14	21
glycerol	1.	2.0	12.0	21.6	17.0	20.0	14.6
	2.	7.30	7.10	7.05	6.75	6.55	6.25
xylose	1.	2.3	10.3	29.3	29.6	41.0	37.0
	2.	7.35	7.35	7.35	7.45	7.60	7.55
glucose	1.	.6	.3	1.3	.6	1.0	6.6
	2.	6.15	5.55	5.45	5.40	5.55	5.45
lactose	1.	2.3	7.6	19.6	26.6	32.0	25.3
	2.	7.30	7.35	7.35	7.30	7.35	7.35
dextrin	1.	0.0	1.0	3.6	1.3	2.0	4.6
	2.	6.35	5.60	6.05	5.65	5.60	5.85
water	1.	2.6	26.6	32.0	39.0	42.0	43.6
	2.	7.30	7.40	7.35	7.45	7.60	7.65

Buffer Concentration M/5

carbohydrate		age of culture in days 1	4	6	10	14	21
glycerol	1.	2.0	21.0	26.0	23.6	20.0	16.3
	2.	7.45	7.40	7.40	7.25	7.15	7.05
xylose	1.	2.0	21.3	31.6	35.0	37.0	38.3
	2.	7.45	7.45	7.50	7.50	7.55	7.55
glucose	1.	.3	3.3	3.6	10.6	8.6	9.0
	2.	7.05	6.75	6.75	6.75	6.85	6.80
lactose	1.	3.6	18.3	14.3	20.3	28.0	22.0
	2.	7.45	7.45	7.45	7.45	7.45	7.35
dextrin	1.	.3	7.3	12.0	12.0	17.6	18.6
	2.	6.95	6.80	6.90	6.80	6.95	7.05
water	1.	3.6	25.6	27.6	30.0	38.3	37.6
	2.	7.45	7.50	7.50	7.45	7.55	7.55

1. percent of total nitrogen of amino acid in culture present as free ammonia.
2. pH of culture at time of ammonia determination.

TABLE 25.

Proteus ichthyosmius — Proline

Buffer Concentration M/20

carbohydrate		age of culture in days 1	4	6	10	14	21
glycerol	1.	2	3	2	1	0	1
	2.	7.55	7.35	7.25	7.10	6.90	6.35
xylose	1.	4	7	8	17	31	47
	2.	7.60	7.55	7.60	7.60	7.70	7.75
glucose	1.	1.	1	2	1	1	1
	2.	6.70	5.85	6.00	5.95	6.05	5.60
lactose	1.	3	7	8	9	4	2
	2.	7.60	7.45	7.55	7.45	7.45	7.25
dextrin	1.	2	1	1	1	0	1
	2.	6.95	6.45	6.25	6.45	6.45	6.30
water	1.	5	11	14	26	34	46
	2.	7.65	7.55	7.65	7.65	7.70	7.75

Buffer Concentration M/5

carbohydrate		age of culture in days 1	4	6	10	14	21
glycerol	1.	1	3	2	2	0	1
	2.	7.55	7.45	7.45	7.45	7.35	7.20
xylose	1.	3	5	8	16	32	44
	2.	7.55	7.55	7.55	7.55	7.65	7.60
glucose	1.	1	1	2	0	0	1
	2.	7.25	7.00	6.95	6.75	6.85	6.85
lactose	1.	3	5	8	3	1	1
	2.	7.55	7.50	7.50	7.45	7.45	7.25
dextrin	1.	1	1	2	1	1	2
	2.	7.35	7.15	7.05	6.96	7.15	7.05
water	1.	4	9	14	16	40	43
	2.	7.55	7.55	7.55	7.50	7.55	7.60

1. percent of total nitrogen of amino acid in culture present as free ammonia
2. pH of culture at time of ammonia determination.

TABLE 26.

Pseudomonas putrefaciens — Arginine

Buffer Concentration M/20

Carbohydrate		age of culture in days 1	3	6	11	15	21
glycerol	1.	0.25	1.5	2.75	3.5	1.25	8.5
	2.	7.50	6.95	6.65	6.95	6.95	7.00
xylose	1.	0.25	0.25	0.0	2.25	1.75	3.25
	2.	7.40	5.35	5.85	6.60	6.65	6.60
glucose	1.	1.0	1.5	2.25	1.25	4.5	8.25
	2.	6.05	5.10	5.10	6.55	6.70	6.70
lactose	1.	1.0	2.0	6.5	18.75	30.5	30.75
	2.	7.50	7.45	7.45	7.15	6.75	5.90
dextrin	1.	1.25	2.0	4.5	7.75	32.5	34.5
	2.	7.45	7.50	7.25	7.30	7.60	7.40
water	1.	1.0	5.5	12.5	19.5	30.5	32.75
	2.	7.55	7.60	7.65	7.65	7.85	7.90

Buffer Concentration M/5

Carbohydrate		age of culture in days 1	3	6	11	15	21
glycerol	1.	0.0	0.5	1.0	0.75	2.5	3.25
	2.	7.50	7.40	7.25	7.15	7.05	7.15
xylose	1.	0.25	0.0	0.25	1.00	1.0	2.0
	2.	7.50	7.00	6.90	7.05	7.15	7.25
glucose	1.	0.0	1.25	2.0	4.75	4.5	4.5
	2.	7.15	6.80	7.00	7.10	7.15	7.15
lactose	1.	0.5	1.5	1.5	4.25	9.5	14.75
	2.	7.50	7.50	7.40	7.45	7.30	7.15
dextrin	1.	0.5	1.0	1.25	3.75	6.25	6.25
	2.	7.45	7.50	7.45	7.45	7.50	7.50
water	1.	0.25	3.0	3.25	4.0	4.25	11.5
	2.	7.50	7.55	7.55	7.55	7.60	7.60

1. percent of total nitrogen of amino acid in culture present as free ammonia.
2. pH of culture at time of ammonia determination

TABLE 27.

Pseudomonas putrefaciens — Aspartic Acid

Buffer Concentration M/20

carbohydrate		age of culture in days 1	3	6	10	14	21
glycerol	1.	37	33	31	35	38	28
	2.	7.20	6.90	6.55	6.45	6.45	6.25
xylose	1.	26	30	26	34	38	23
	2.	7.20	6.00	5.45	5.10	5.05	5.55
glucose	1.	29	26	24	34	38	24
	2.	6.45	5.35	5.30	5.25	4.95	5.20
lactose	1.	45	35	31	33	38	27
	2.	7.25	7.20	7.15	7.15	6.85	6.80
dextrin	1.	33	33	18	14	15	10
	2.	7.25	7.20	7.30	7.30	7.20	7.20
water	1.	36	38	33	38	35	29
	2.	7.35	7.30	7.50	7.55	7.55	7.65

Buffer Concentration M/5

carbohydrate		age of culture in days 1	3	6	10	14	21
glycerol	1.	36	32	25	-	17	14
	2.	7.50	7.35	7.15	7.05	6.90	7.05
Xylose	1.	18	22	14	-	17	14
	2.	7.50	7.05	6.75	6.70	6.70	6.85
glucose	1.	21	15	11	18	23	15
	2.	7.05	6.70	6.75	6.85	6.80	6.95
lactose	1.	36	38	18	29	29	20
	2.	7.50	7.45	7.45	7.35	7.25	7.25
dextrin	1.	24	30	12	10	9	6
	2.	7.45	7.45	7.45	7.40	7.40	7.35
water	1.	1	28	24	38	35	40
	2.	7.50	7.45	7.50	7.55	7.55	7.55

1. percent of total nitrogen of amino acid in culture present as free ammonia
2. pH of culture at time of ammonia determination

TABLE 28.

Pseudomonas putrefaciens — Glutamic Acid

Buffer Concentration M/20

Carbohydrate		age of culture in days 1	3	6	10	14	21
glycerol	1.	1	3	3	6	9	13
	2.	7.25	7.00	6.55	6.30	6.25	6.15
xylose	1.	1	2	1	2	0	3
	2.	7.25	5.55	5.25	5.25	5.15	5.05
glucose	1.	1	4	4	4	6	24
	2.	6.30	5.10	5.25	5.25	5.15	6.10
lactose	1.	3	6	9	15	27	37
	2.	7.25	7.20	7.20	6.90	6.80	6.55
dextrin	1.	2	5	3	11	19	24
	2.	7.25	7.20	7.10	7.15	7.10	7.15
water	1.	4	11	17	26	41	62
	2.	7,30	7.30	7,40	7.55	7.50	7,85

Buffer Concentration M/5

carbohydrate		age of culture in days 1	3	6	10	14	21
glycerol	1.	1	3	0	1	2	0
	2.	7.50	7.45	7.25	7.00	6.95	6.95
xylose	1.	2	2	1	4	7	20
	2.	7.45	6.95	6.75	6.75	6.70	6.85
glucose	1.	1	3	2	14	19	27
	2.	7.15	6.70	6.70	6.75	6.85	6.90
lactose	1.	3	8	5	14	18	39
	2.	7.50	7.40	7.45	7.35	7.20	7.15
dextrin	1.	2	5	5	10	18	32
	2.	7.45	7.45	7.45	7.40	7.35	7.45
water	1.	4	11	12	31	37	62
	2.	7.50	7.45	7.50	7.55	7.50	7.65

1. percent of total nitrogen of amino acid in culture present as free ammonia.
2. pH of culture at time of ammonia determination.

TABLE 29.

Pseudomonas putrefaciens — Histidine

Buffer Concentration M/20

carbohydrate		age of culture in days 1	3	6	10	14	20
glycerol	1.	6.6	25.6	29.6	36.0	41.3	46.6
	2.	7.30	6.95	6.40	6.35	6.30	6.70
xylose	1.	6.6	6.6	5.6	7.6	8.3	8.0
	2.	7.25	5.30	5.40	5.55	5.25	5.25
glucose	1.	3.6	4.6	3.3	7.3	4.6	5.6
	2.	5.95	5.15	5.15	5.50	5.20	5.35
lactose	1.	8.3	29.0	42.3	57.6	65.0	65.3
	2.	7.35	7.30	7.25	7.20	6.95	6.65
dextrin	1.	7.3	36.6	49.3	54.3	57.3	58.3
	2.	7.35	7.35	7.15	7.25	7.20	7.05
water	1.	10.0	47.3	61.3	68.6	69.6	62.6
	2.	7.40	7.45	7.65	7.80	7.90	8.10

Buffer Concentration M/5

carbohydrate		age of culture in days 1	3	6	10	14	21
glycerol	1.	6.0	26.6	40.6	43.6	46.3	41.0
	2.	7.50	7.35	7.05	6.90	6.85	7.00
xylose	1.	7.0	6.3	19.6	31.3	39.3	40.6
	2.	7.45	6.65	6.70	6.80	6.85	6.95
glucose	1.	1.6	5.0	11.0	25.6	32.0	40.6
	2.	6.95	6.65	6.75	6.85	6.85	7.05
lactose	1.	7.3	23.0	36.6	56.6	54.6	50.3
	2.	7.50	7.45	7.45	7.45	7.25	7.20
dextrin	1.	6.6	31.6	39.6	53.0	50.6	44.0
	2.	7.50	7.50	7.45	7.40	7.30	7.35
water	1.	9.6	50.0	48.0	65.0	67.3	54.3
	2.	7.50	7.50	7.55	7.60	7.65	7.75

1. percent of total nitrogen of amino acid in culture present as free ammonia.
2. pH of culture at time of ammonia determination.

TABLE 30.

Pseudomonas putrefaciens — Proline

Buffer Concentration M/20

carbohydrate		age of culture in days 1	3	6	10	14	21
glycerol	1.	2	1	1	1	1	8
	2.	7.55	7.10	6.50	6.35	6.35	6.95
xylose	1.	1	1	0	1	1	2
	2.	7.25	6.30	5.45	5.05	5.40	5.20
glucose	1.	0	1	0	2	2	0
	2.	6.80	5.90	5.55	5.05	5.85	6.25
lactose	1.	6	11	14	33	30	37
	2.	7.55	7.45	7.30	7.05	6.80	6.55
dextrin	1.	4	9	11	34	25	35
	2.	7.55	7.40	7.30	7.30	7.05	7.15
water	1.	6	15	23	48	53	54
	2.	7.65	7.60	7.65	7.65	7.70	7.95

Buffer Concentration M/5

carbohydrate		age of culture in days 1	3	6	10	14	21
glycerol	1.	2	2	0	1	0	1
	2.	7.55	7.45	7.25	7.05	7.00	7.00
xylose	1.	1	1	1	1	0	7
	2.	7.50	7.15	7.00	6.80	6.65	6.95
glucose	1.	0	1	0	2	4	9
	2.	7.35	7.05	6.90	6.80	6.85	7.05
lactose	1.	4	9	19	20	21	29
	2.	7.55	7.50	7.45	7.30	7.20	7.05
dextrin	1.	3	10	12	24	20	18
	2.	7.55	7.50	7.45	7.45	7.35	7.40
water	1.	4	10	20	32	35	43
	2.	7.55	7.55	7.55	7.55	7.55	7.65

1. percent of total nitrogen of amino acid in culture present as free ammonia.
2. pH of culture at time of ammonia determination

Figure 36

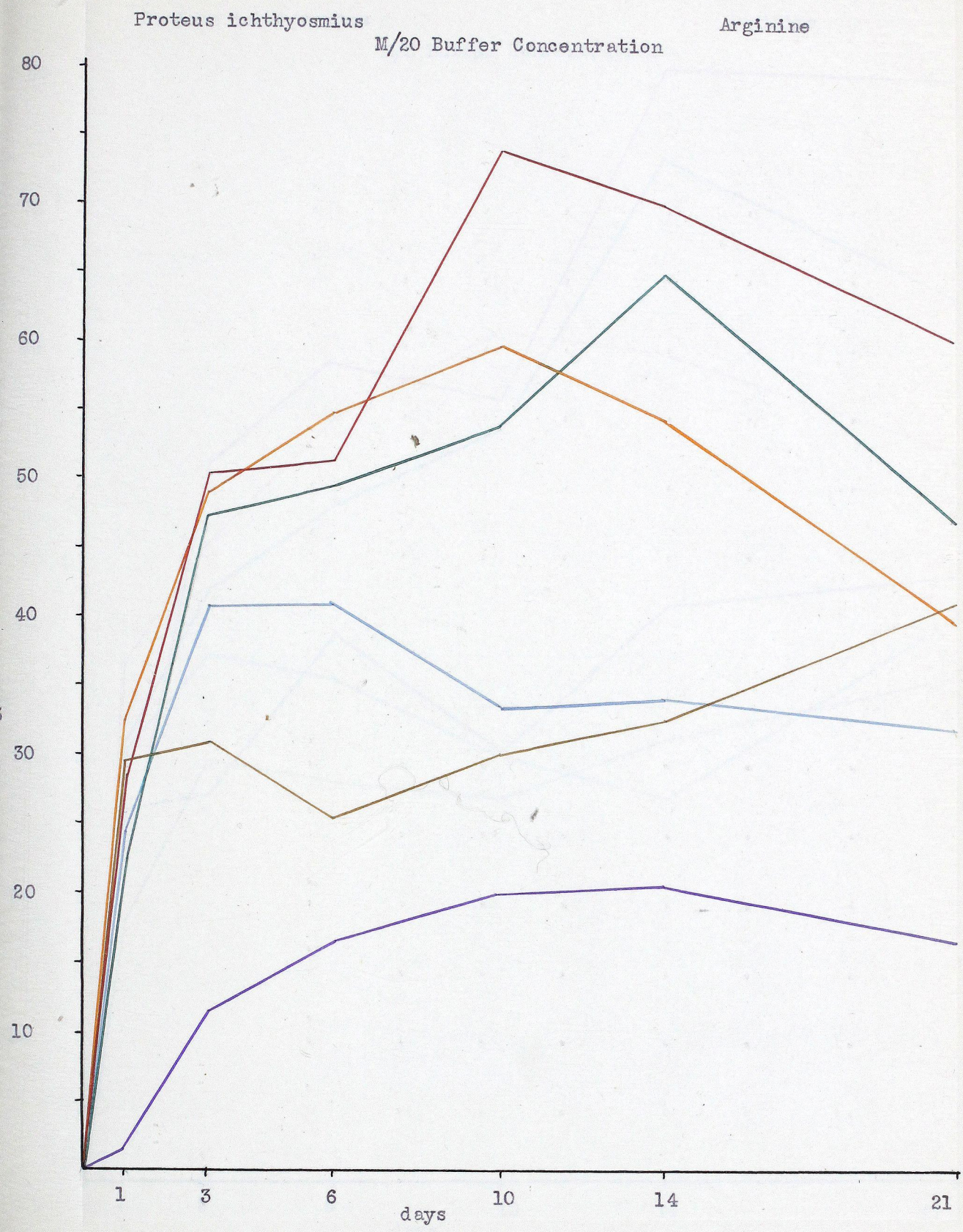

Proteus ichthyosmius
Arginine
M/20 Buffer Concentration
80
70
60
50
40
30
20
10
%NH3
1
3
6
10
14
21
days

Figure 37

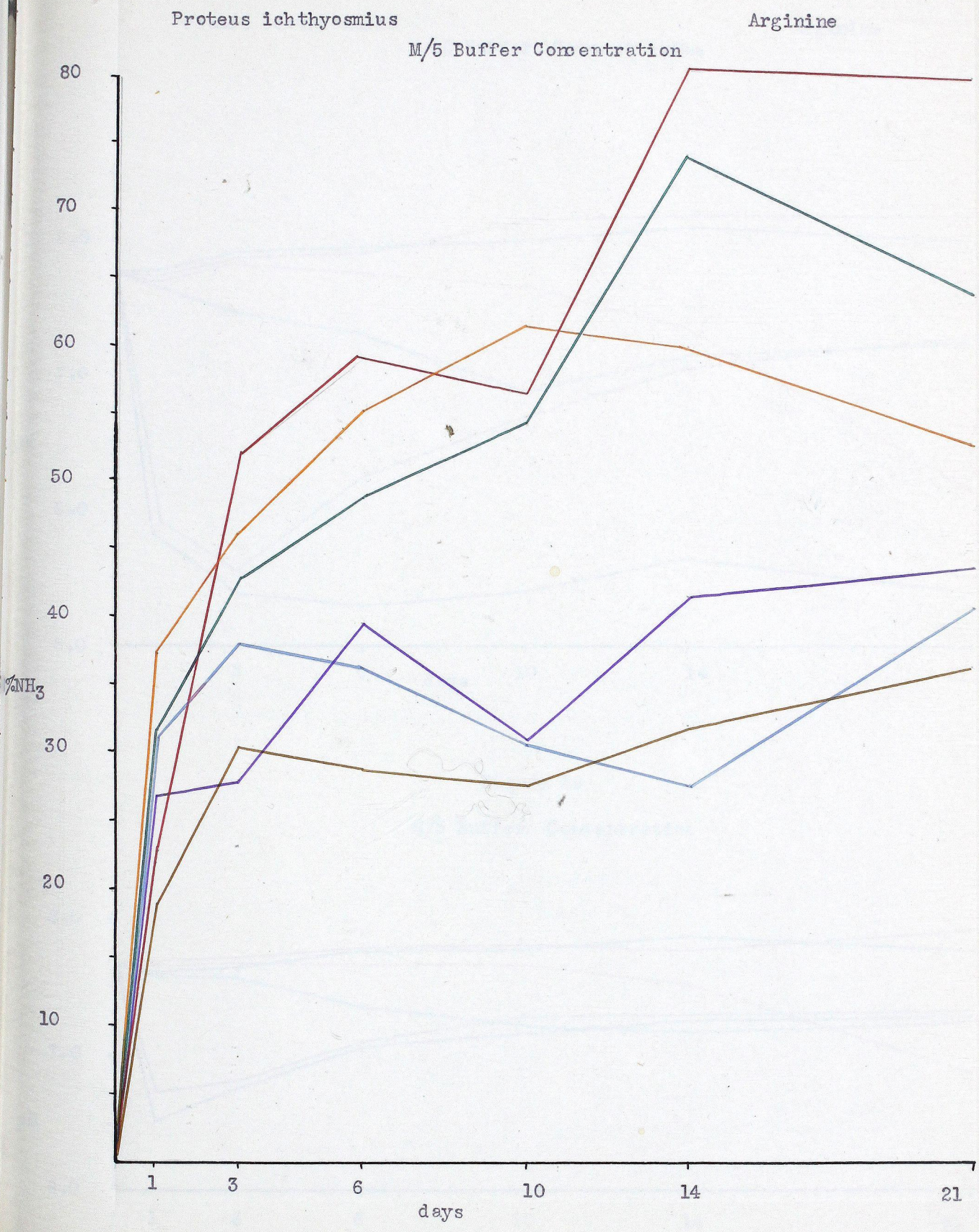

Proteus ichthyosmius
Arginine
M/5 Buffer Concentration
80
70
60
50
40
%NH3
30
20
10
1
3
6
10
14
21
days

Figure 38

Proteus ichthyosmius Arginine

M/20 Buffer Concentration

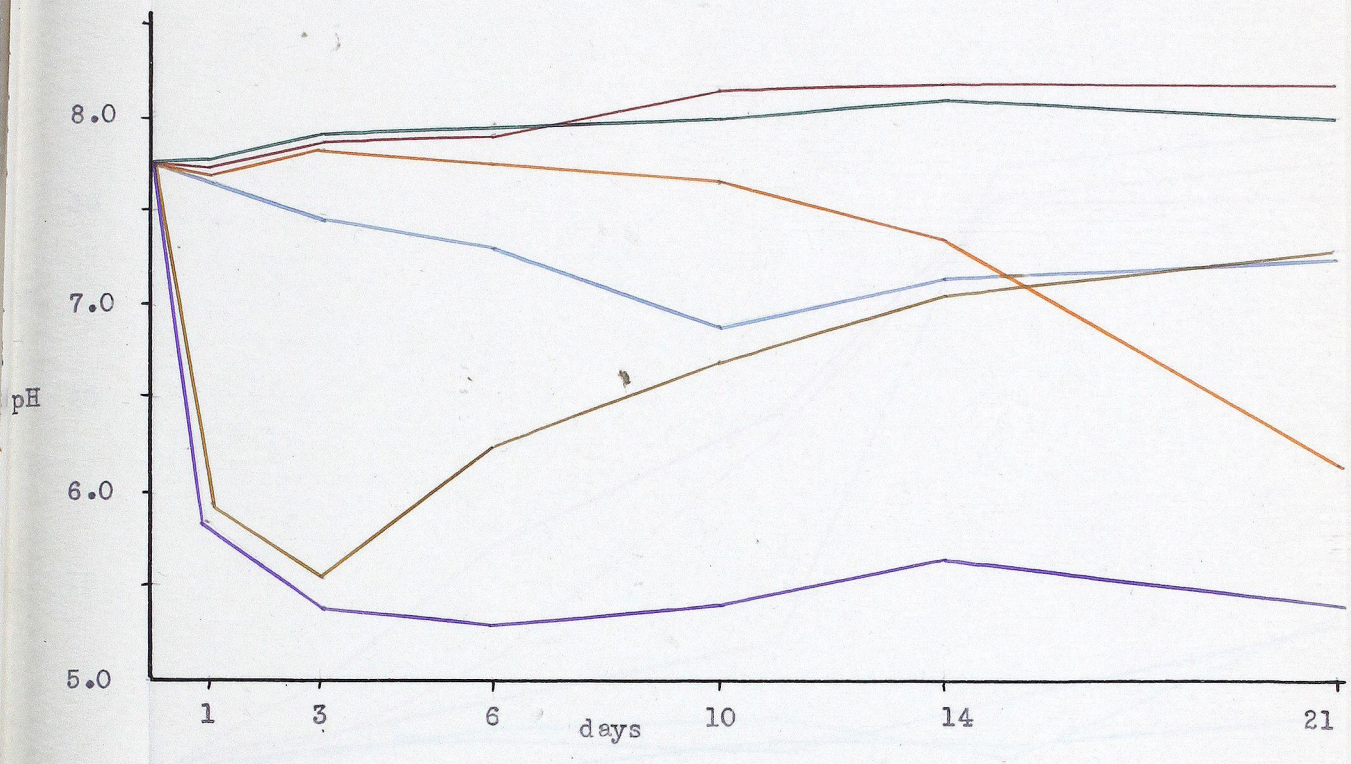


Figure 39

M/5 Buffer Concentration

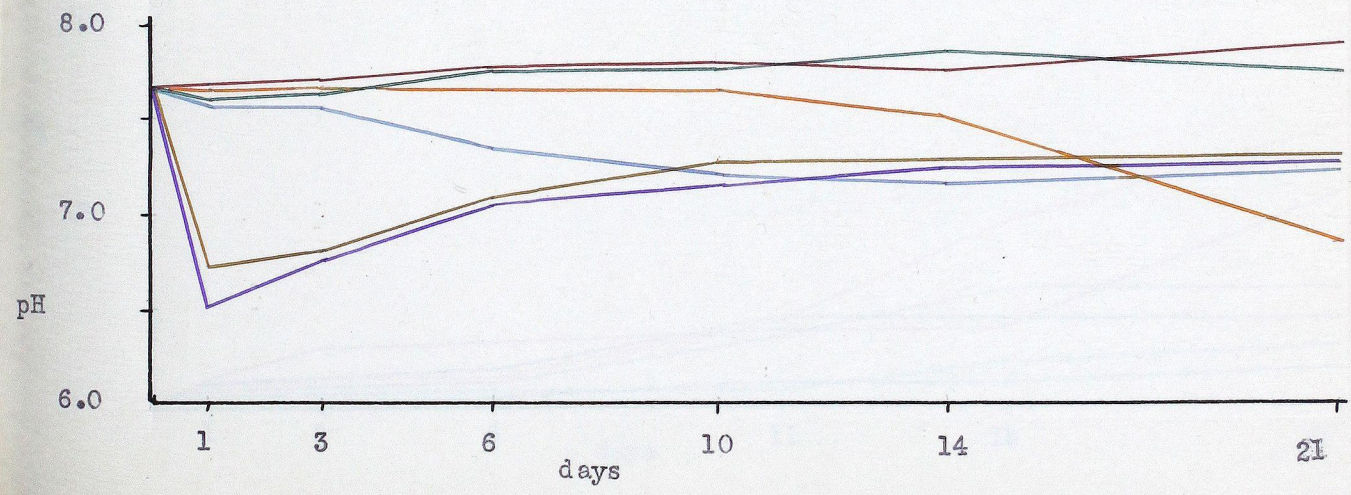


Figure 40

Pseudomonas putrafaciens Arginine

M/20 Buffer Concentration

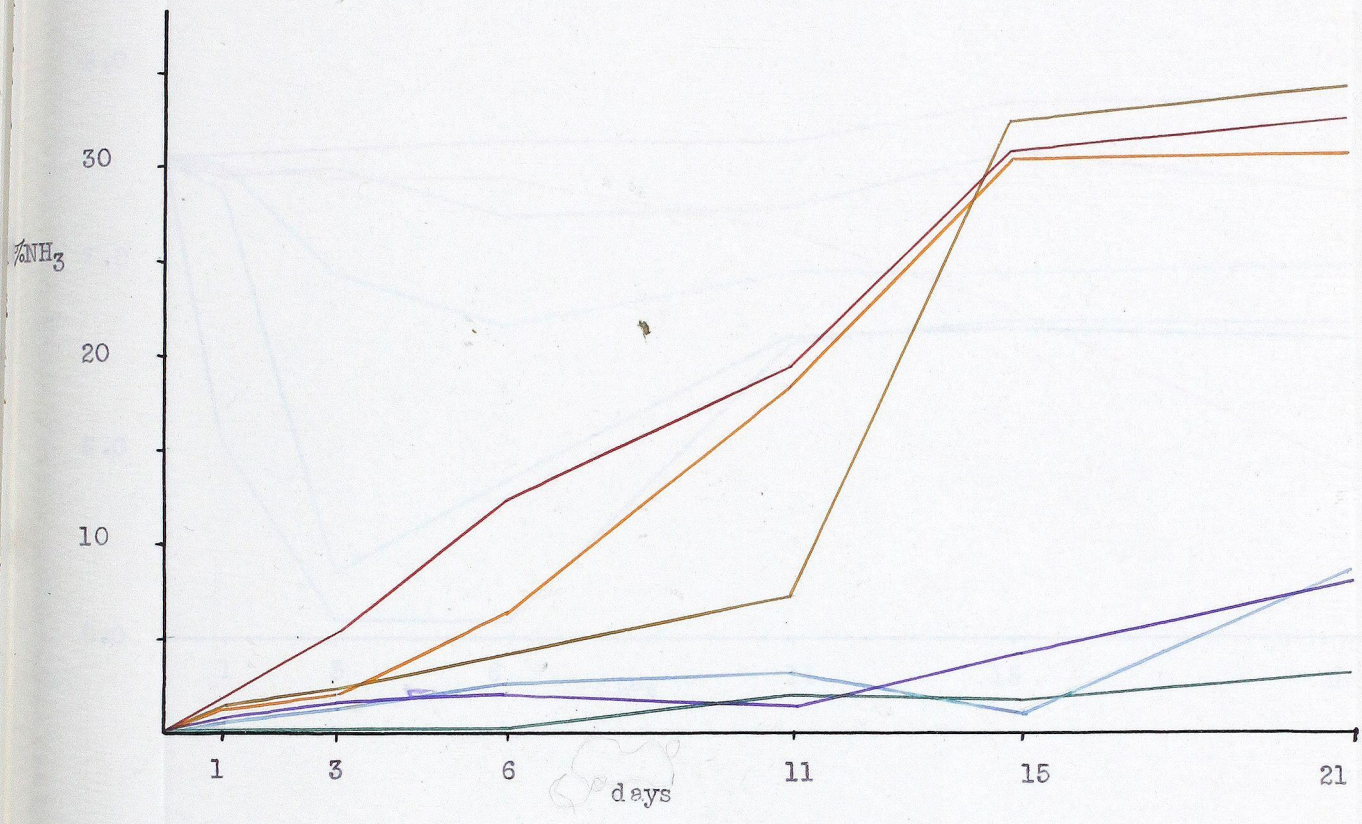


Figure 41

M/5 Buffer Concentration

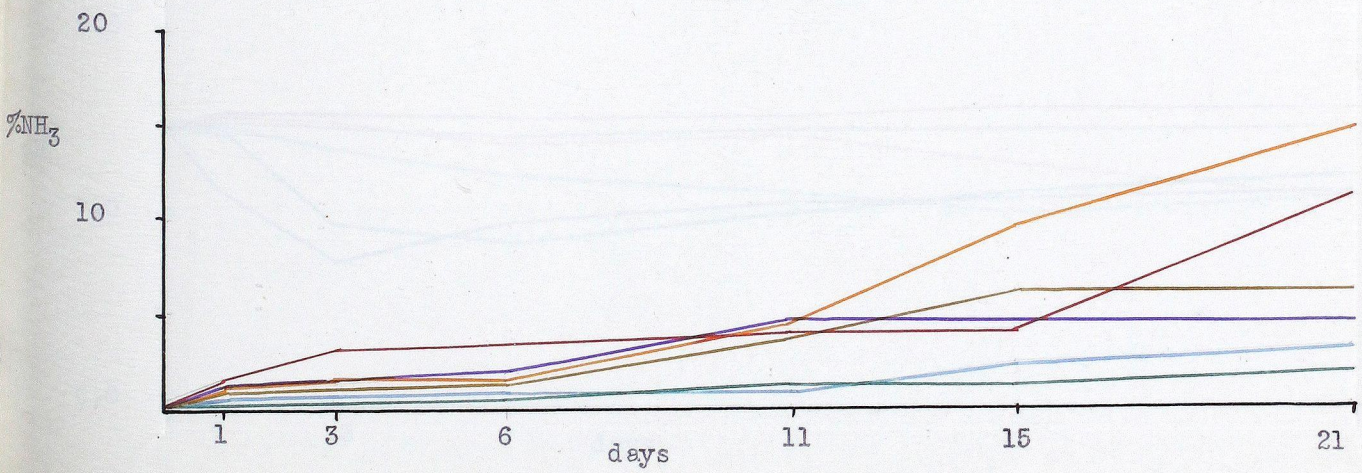


Figure 42

Pseudomonas putrafaciens — Arginine

M/20 Buffer Concentration

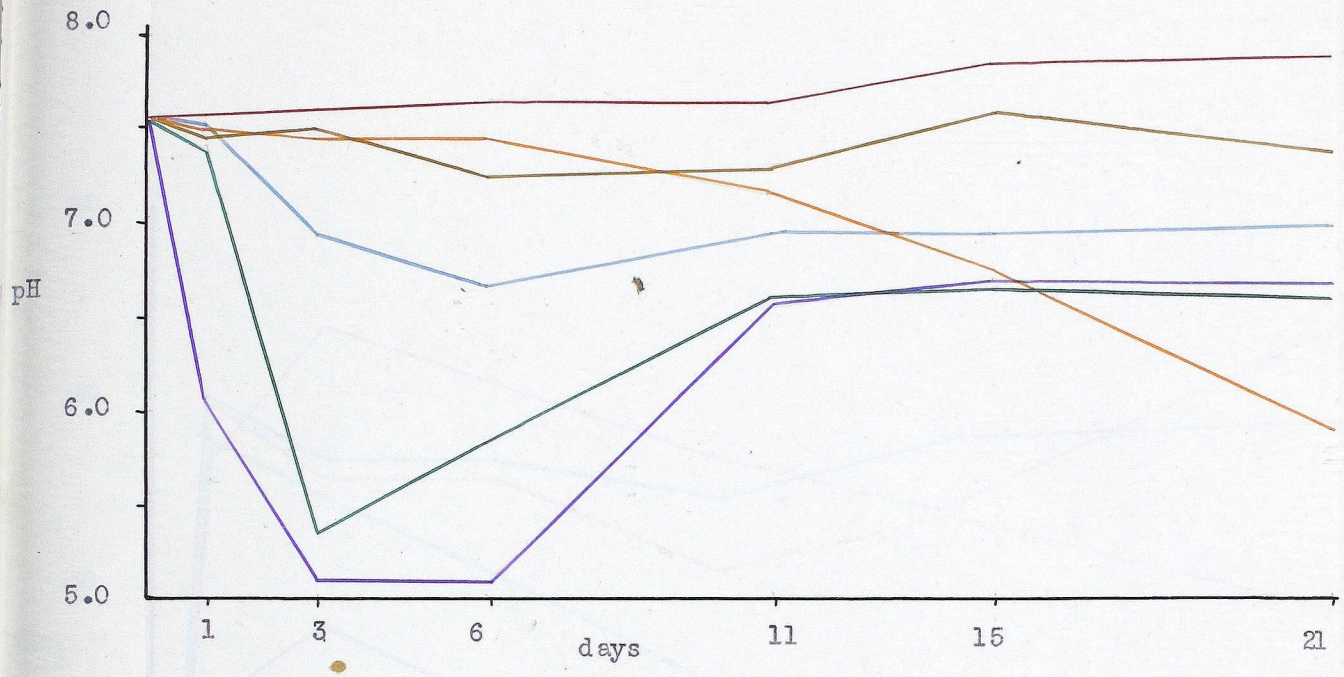


Figure 43

M/5 Buffer Concentration

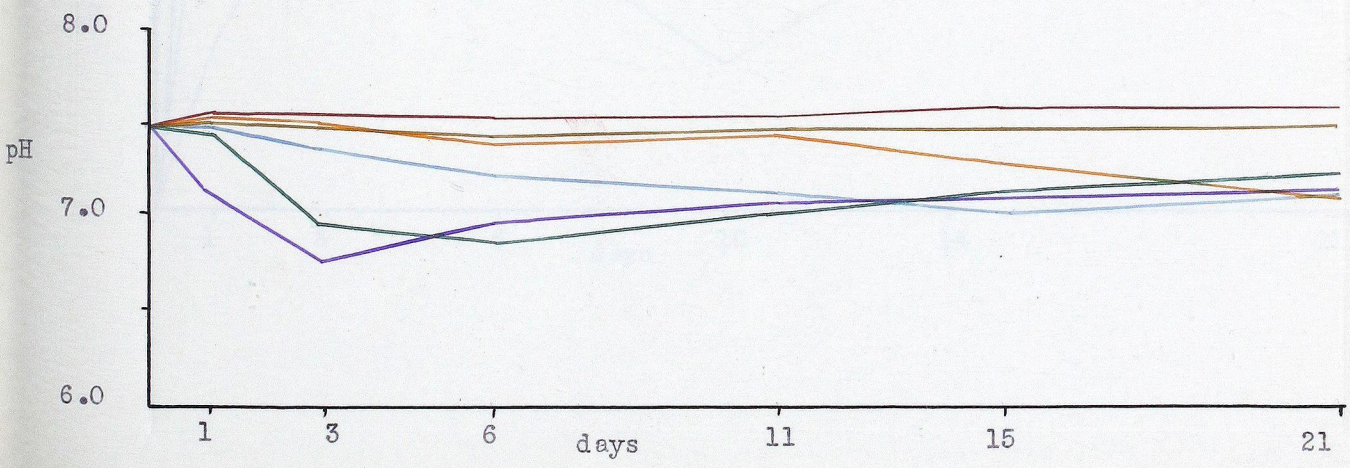


Figure 44

Proteus ichthyosmius Aspartic Acid

M/20 Buffer Concentration

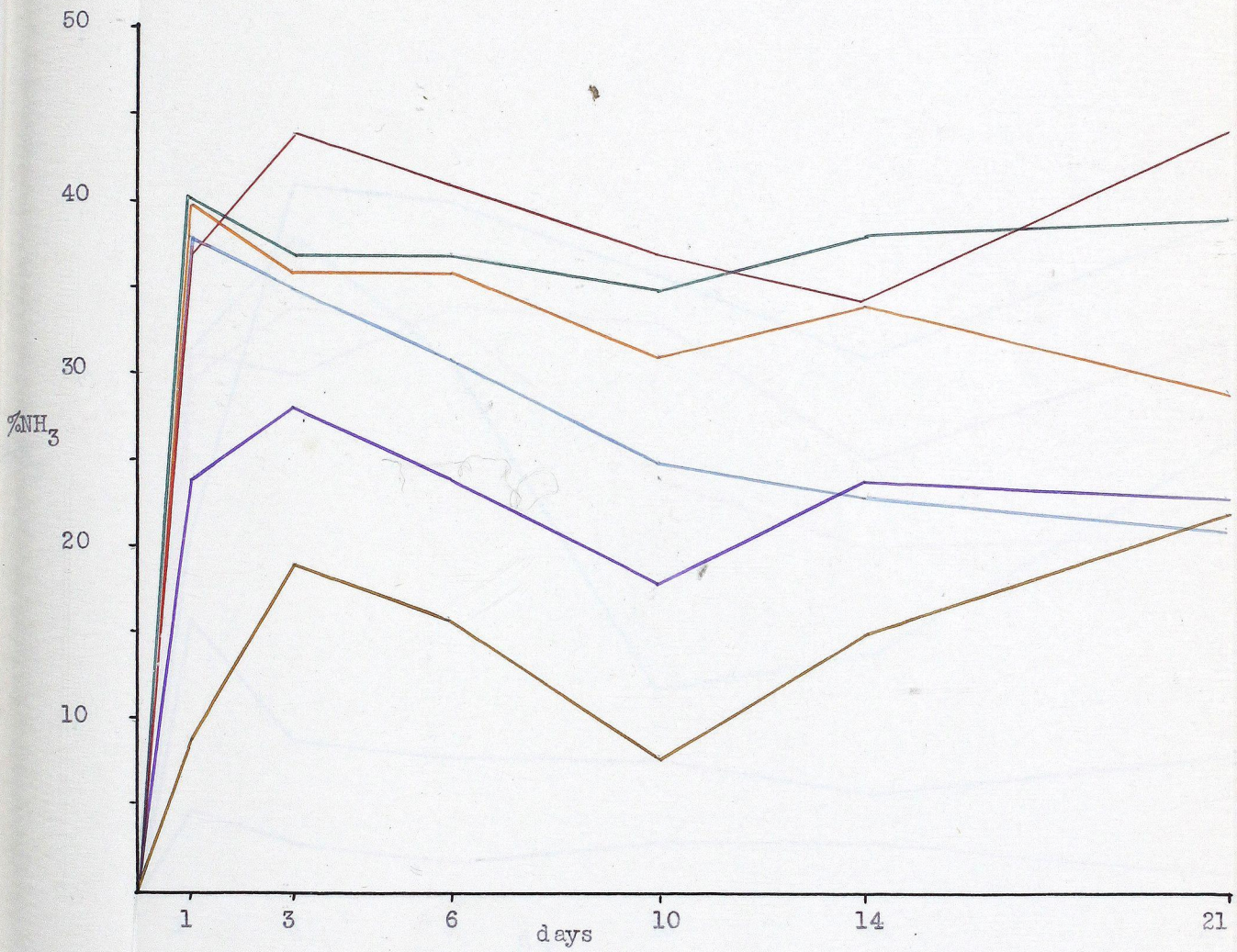


Figure 45

Proteus ichthyosmius Aspartic Acid

M/5 Buffer Concentration

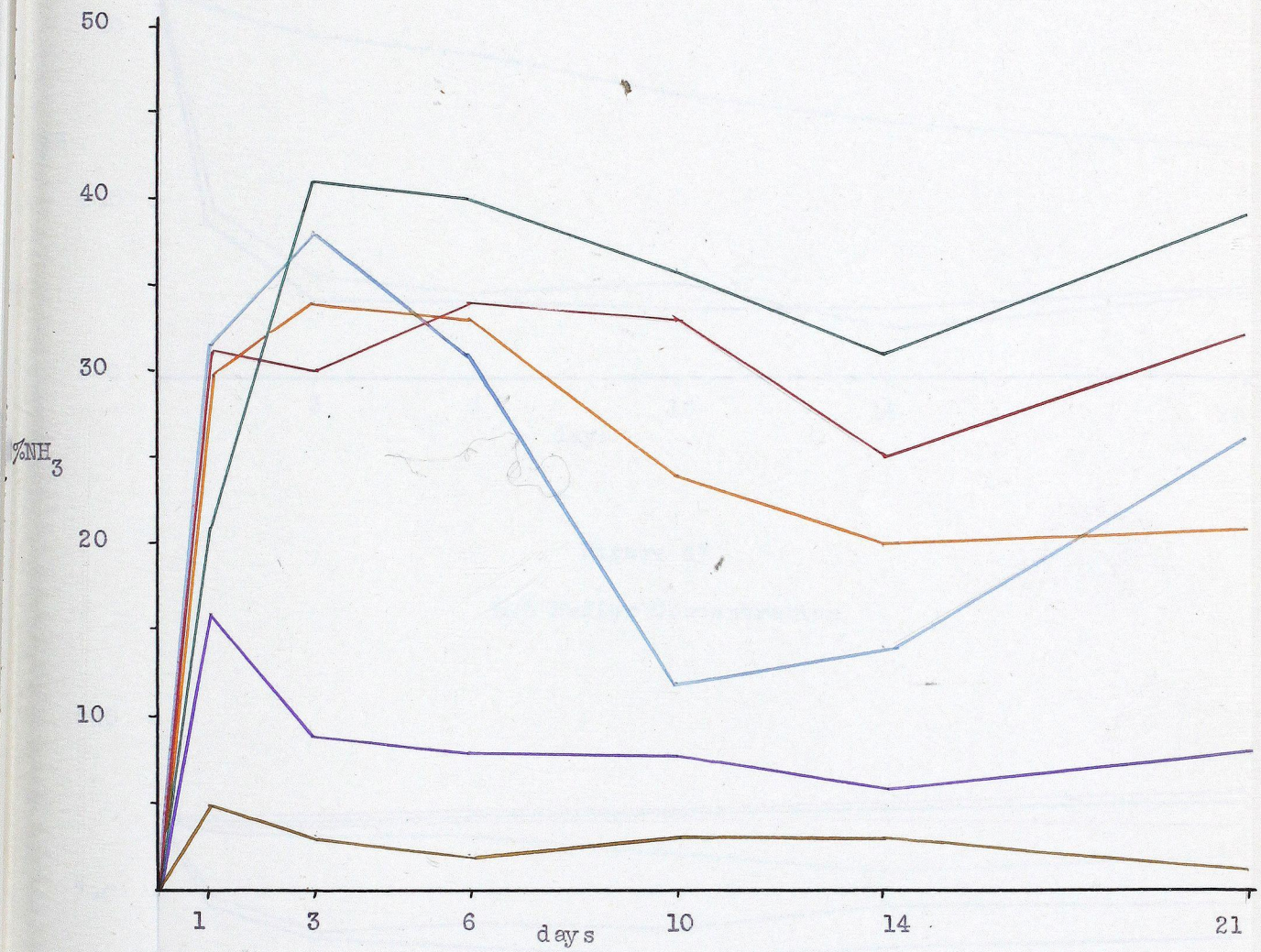


Figure 46

Proteus ichthyosmius — Aspartic Acid

M/20 Buffer Concentration

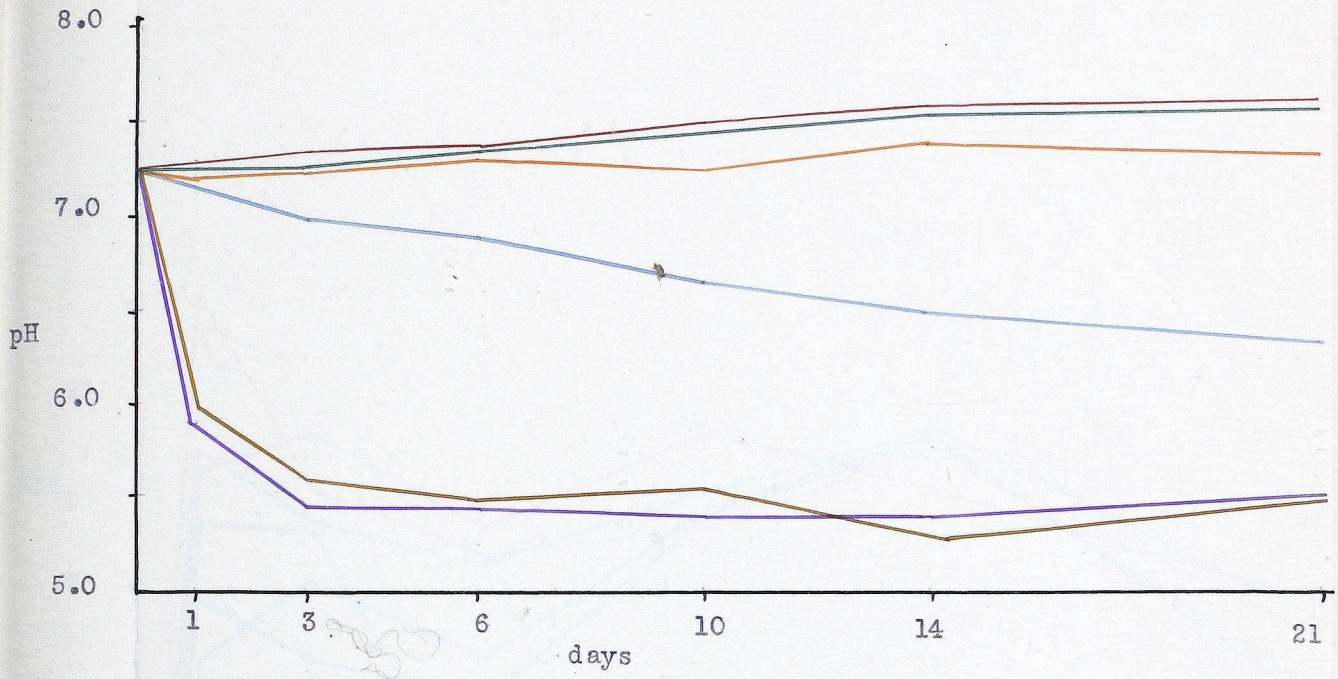


Figure 47

M/5 Buffer Concentration

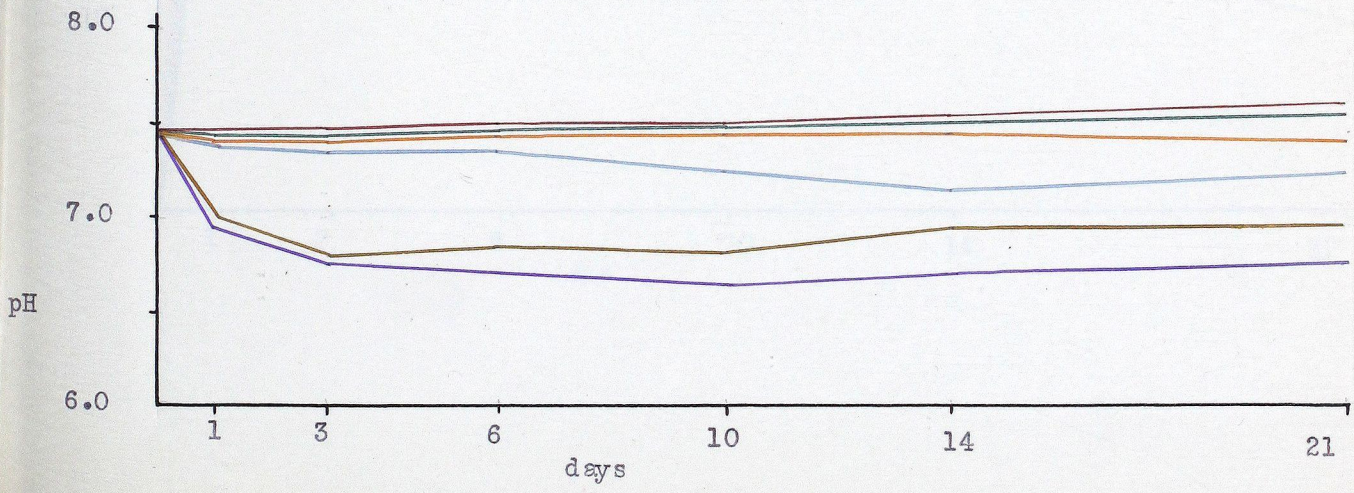


Figure 48

Pseudomonas putrefaciens Aspartic Acid

M/20 Buffer Concentration

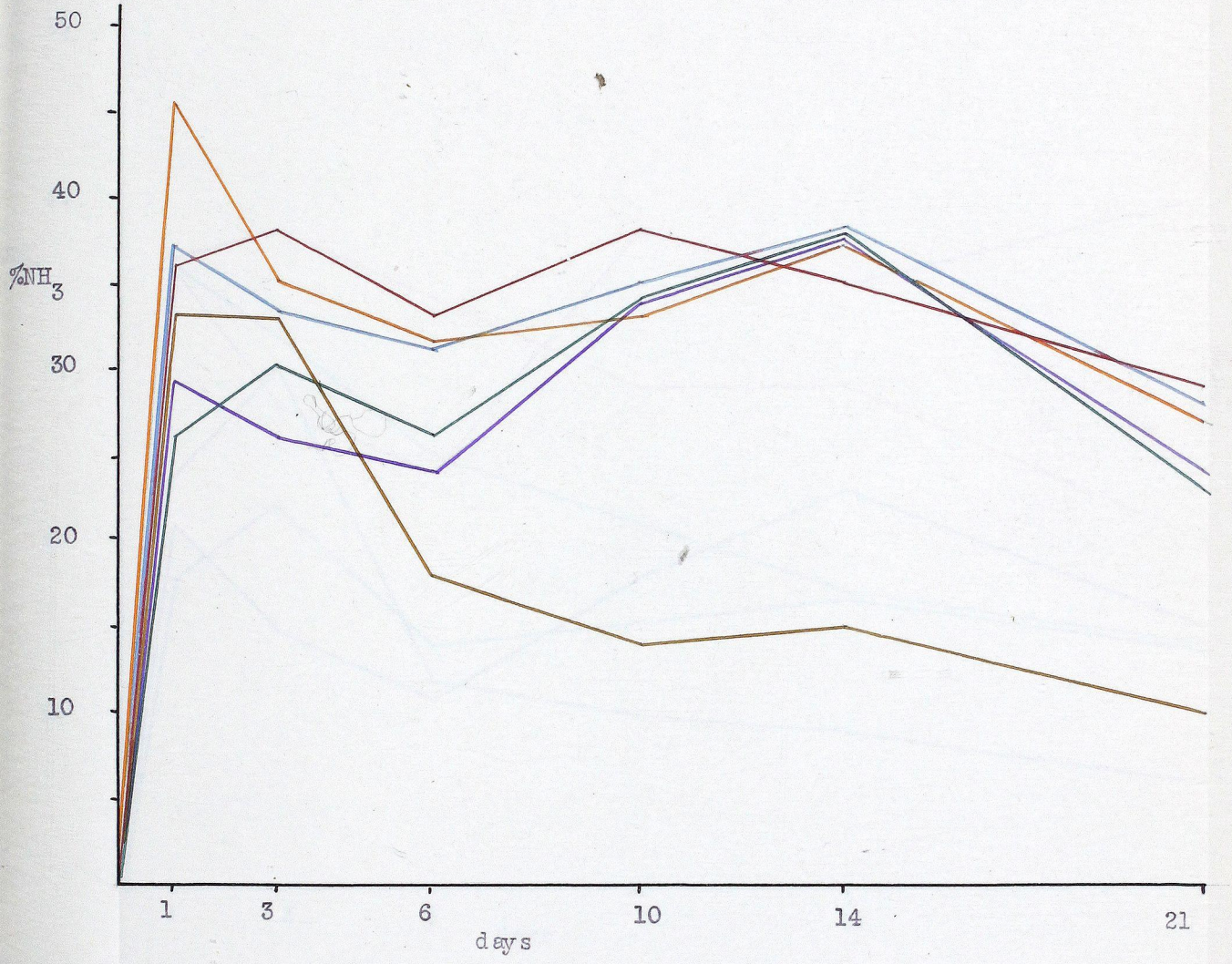


Figure 49

Pseudomonas putrefaciens — Aspartic Acid

M/5 Buffer Concentration

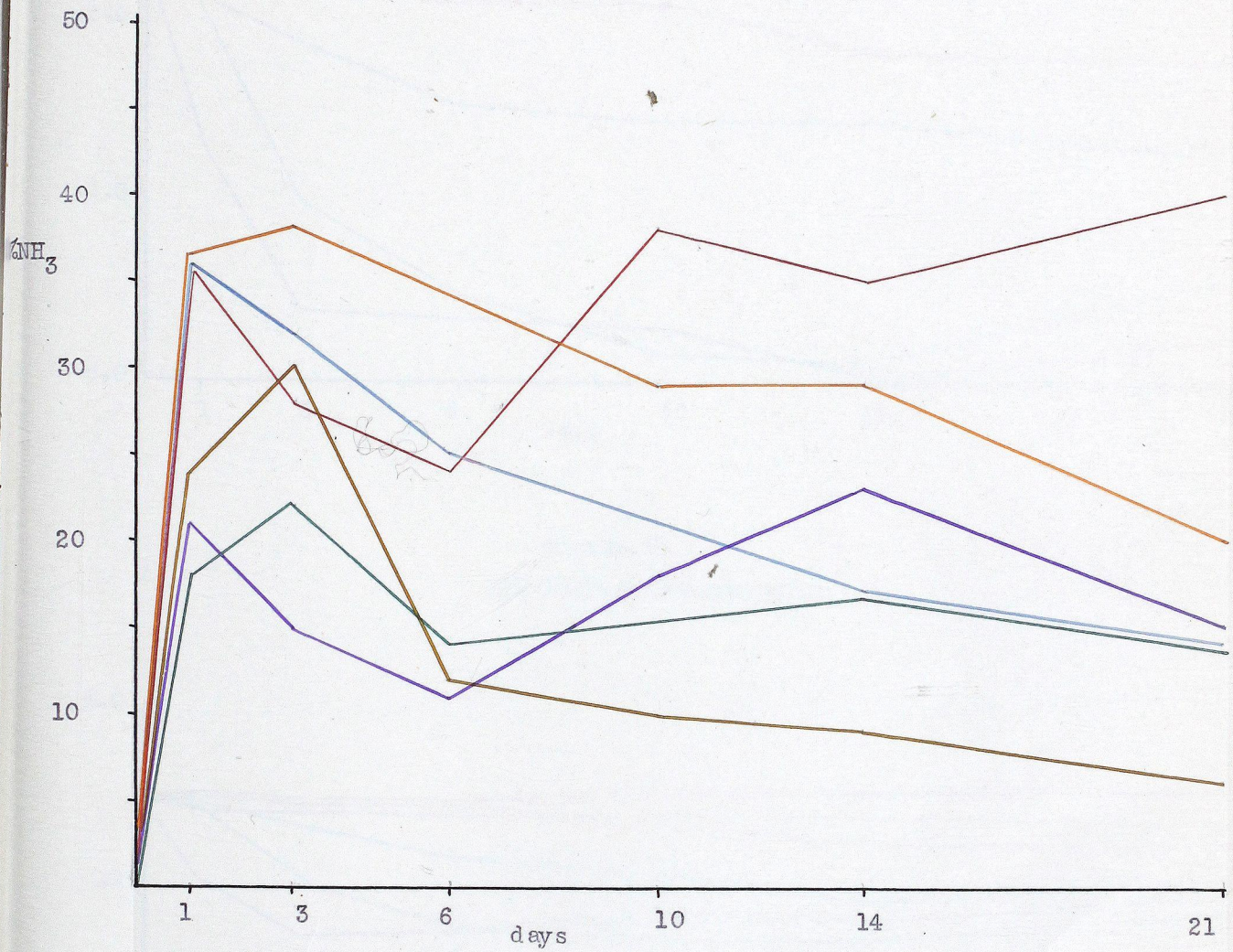


Figure 50

Pseudomonas putrefaciens — Aspartic Acid

M/20 Buffer Concentration

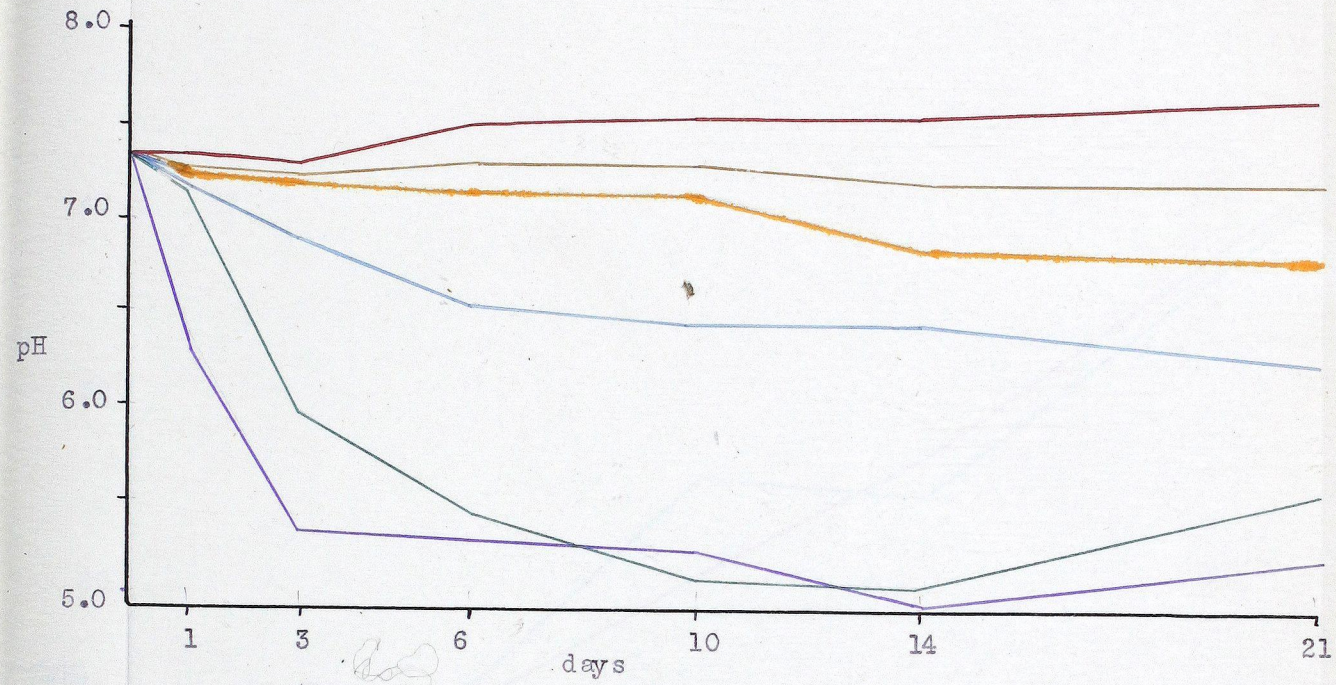


Figure 51

M/5 Buffer Concentration

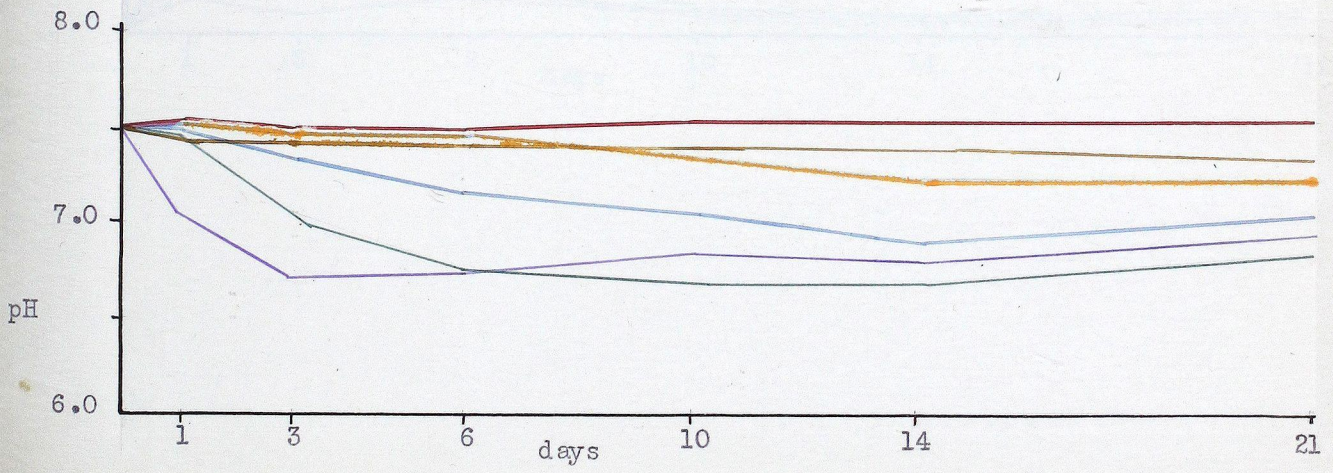


Figure 52

Proteus ichthyosmius

Glutamic Acid

M/20 Buffer Concentration

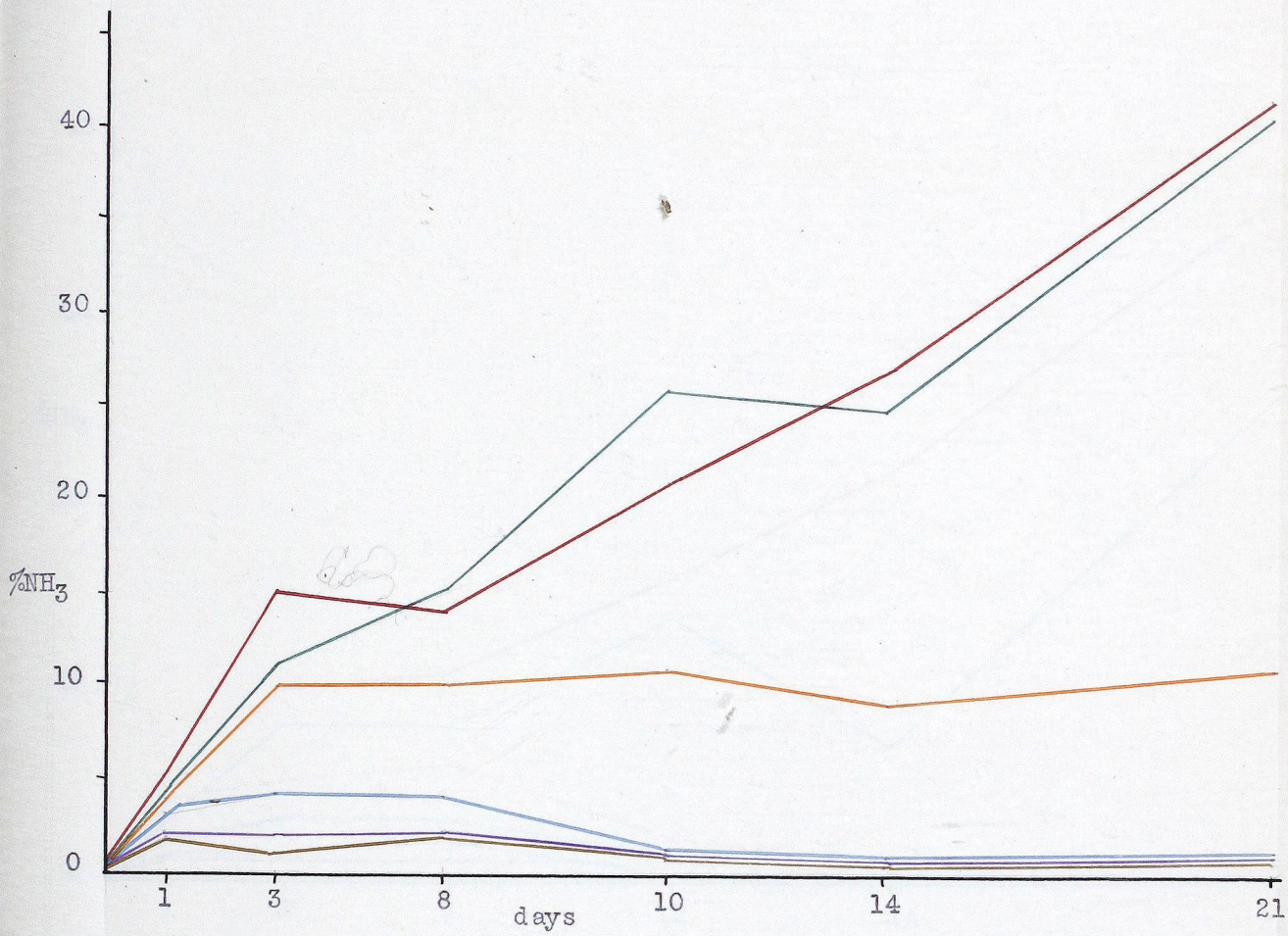


Figure 53

Proteus ichthyosmius — Glutamic Acid

M/5 Buffer Concentration

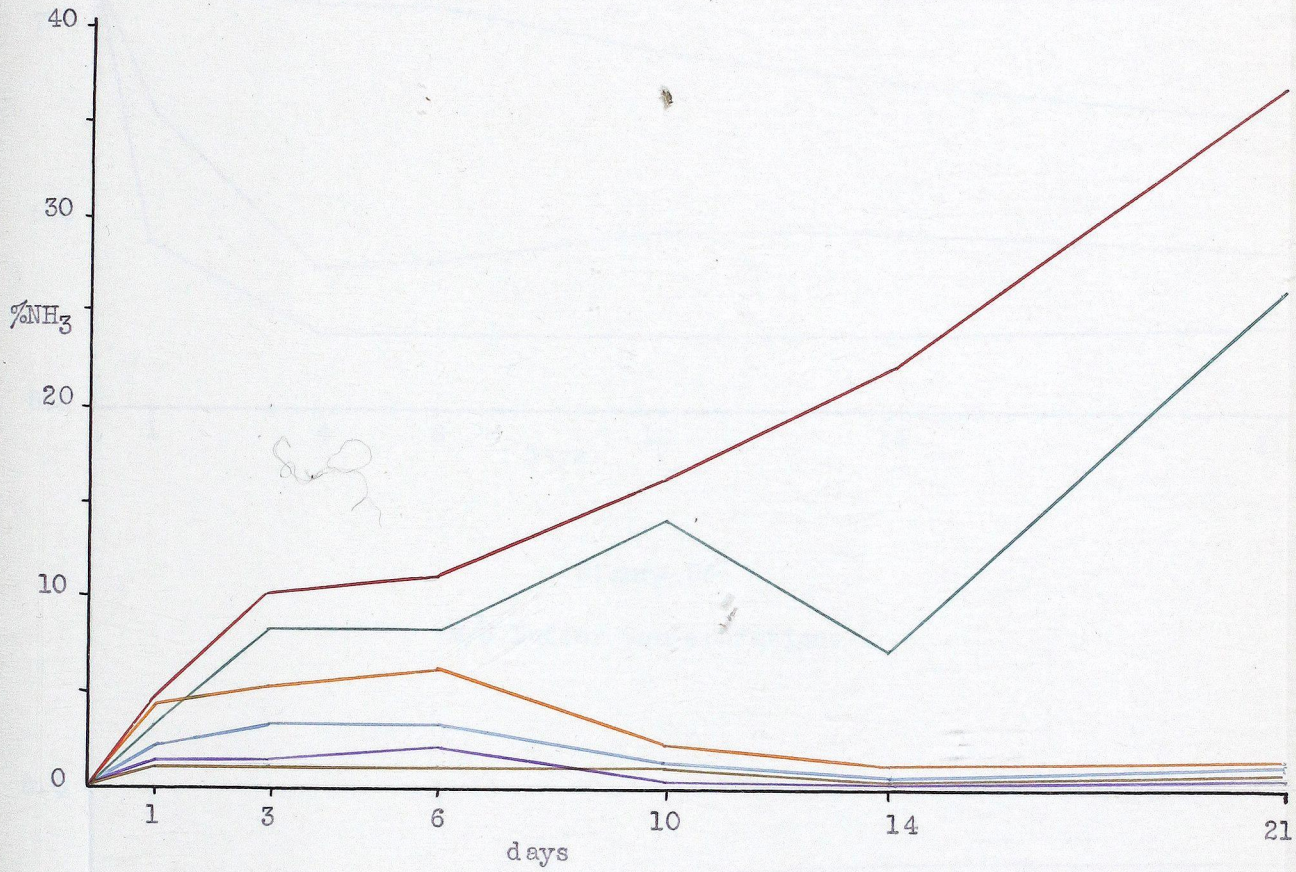


Figure 54

Proteus ichthyosmius — Glutamic Acid

M/20 Buffer Concentration.

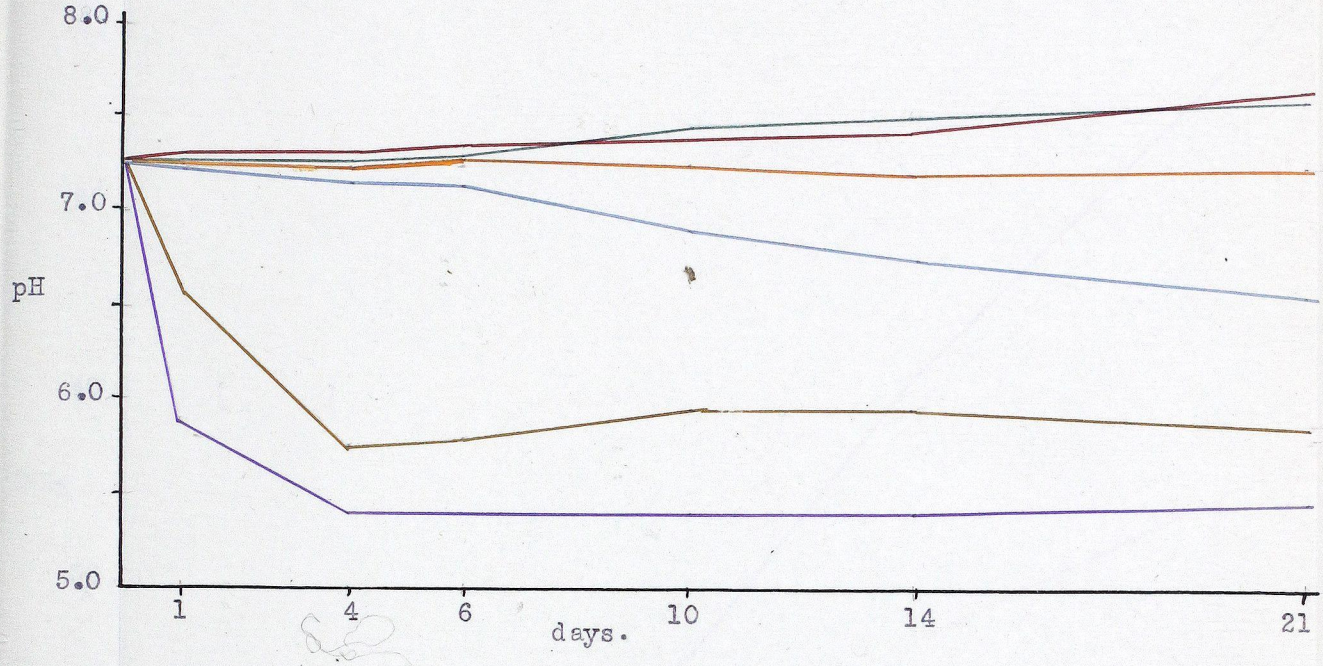


Figure 55

M/5 Buffer Concentration.

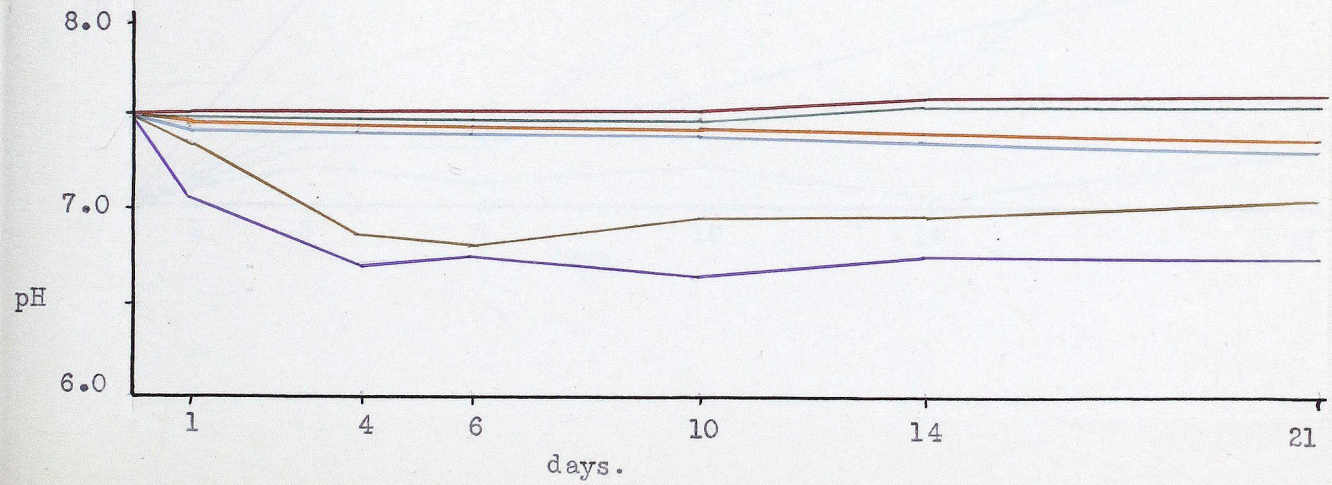


Figure 56

Pseudomonas putrefaciens Glutamic Acid

M/20 Buffer Concentration.

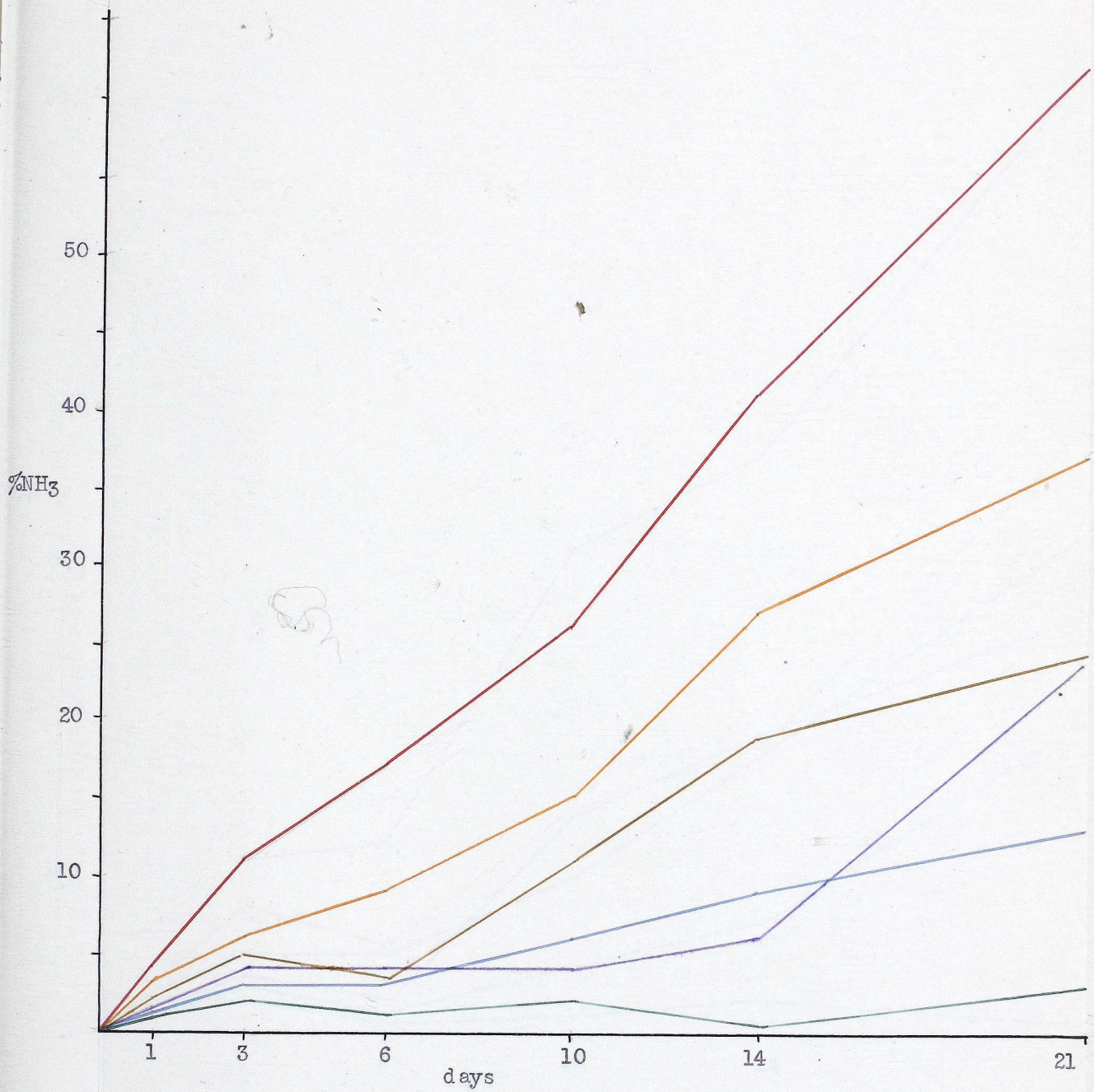


Figure 57

Pseudomonas putrefaciens

Glutamic Acid

M/5 Buffer Concentration

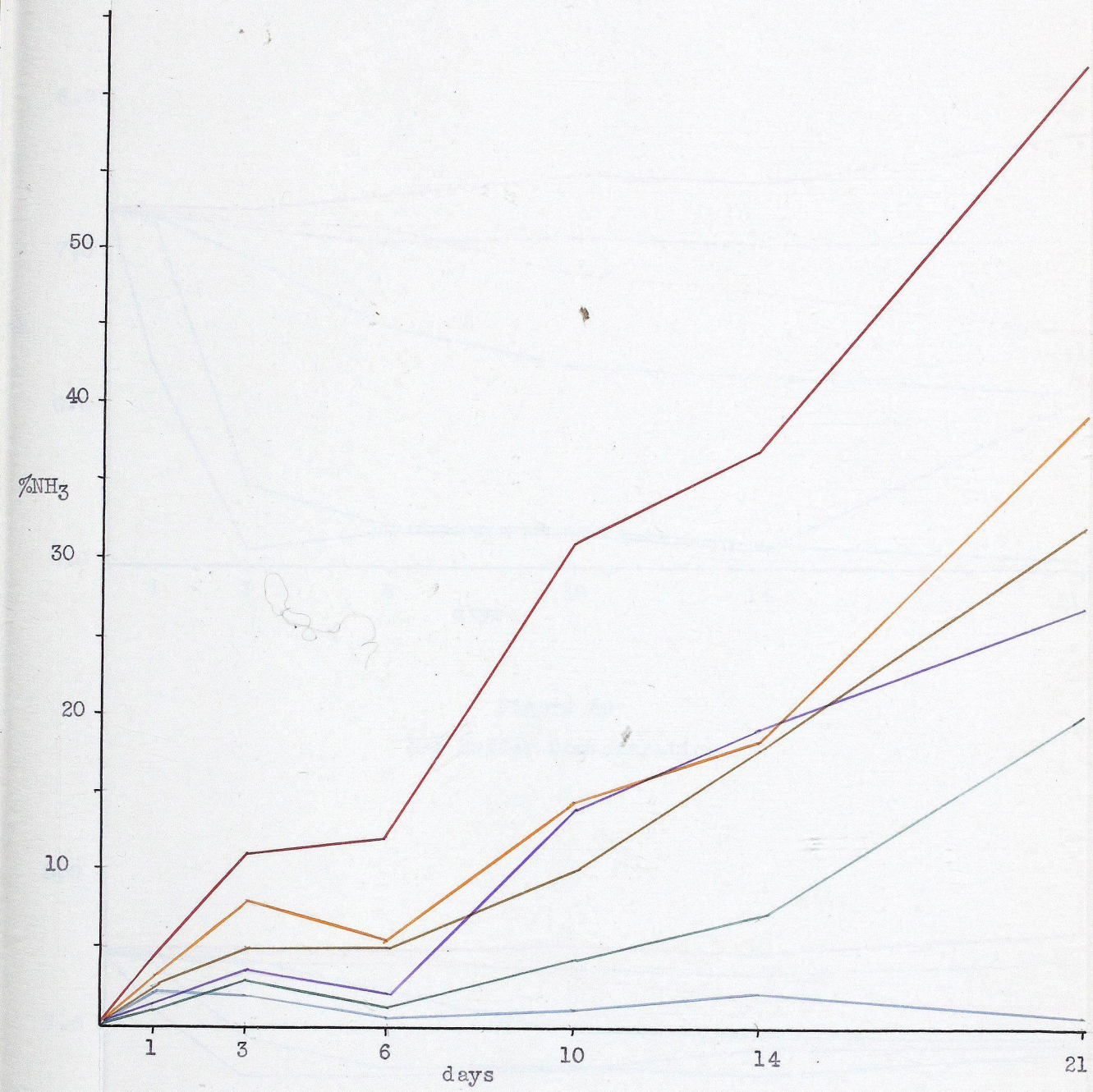


Figure 58

Pseudomonas putrafaciens — Glutamic Acid

M/20 Buffer Concentration

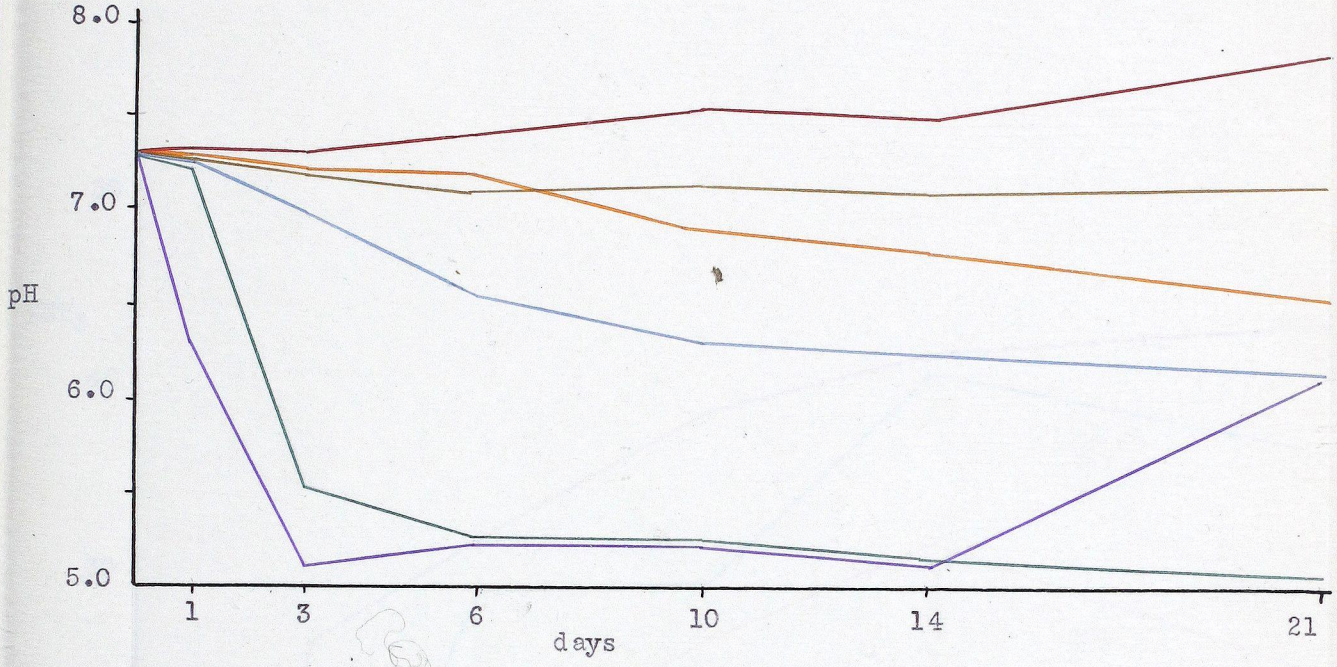


Figure 59

M/5 Buffer Concentration

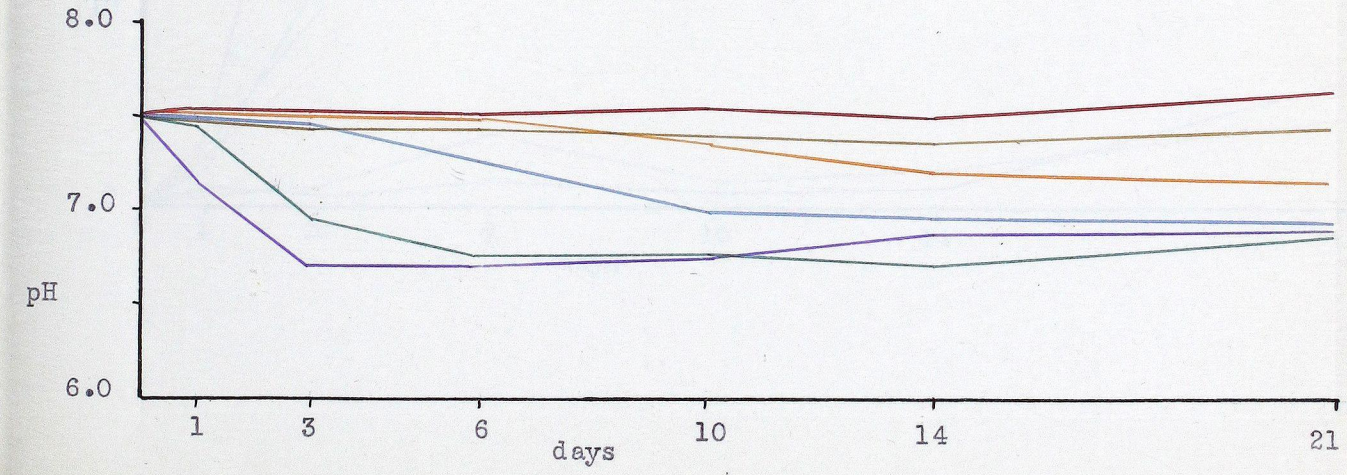


Figure 60

Proteus ichthyosmius — Histidine

M/20 Buffer Concentration.

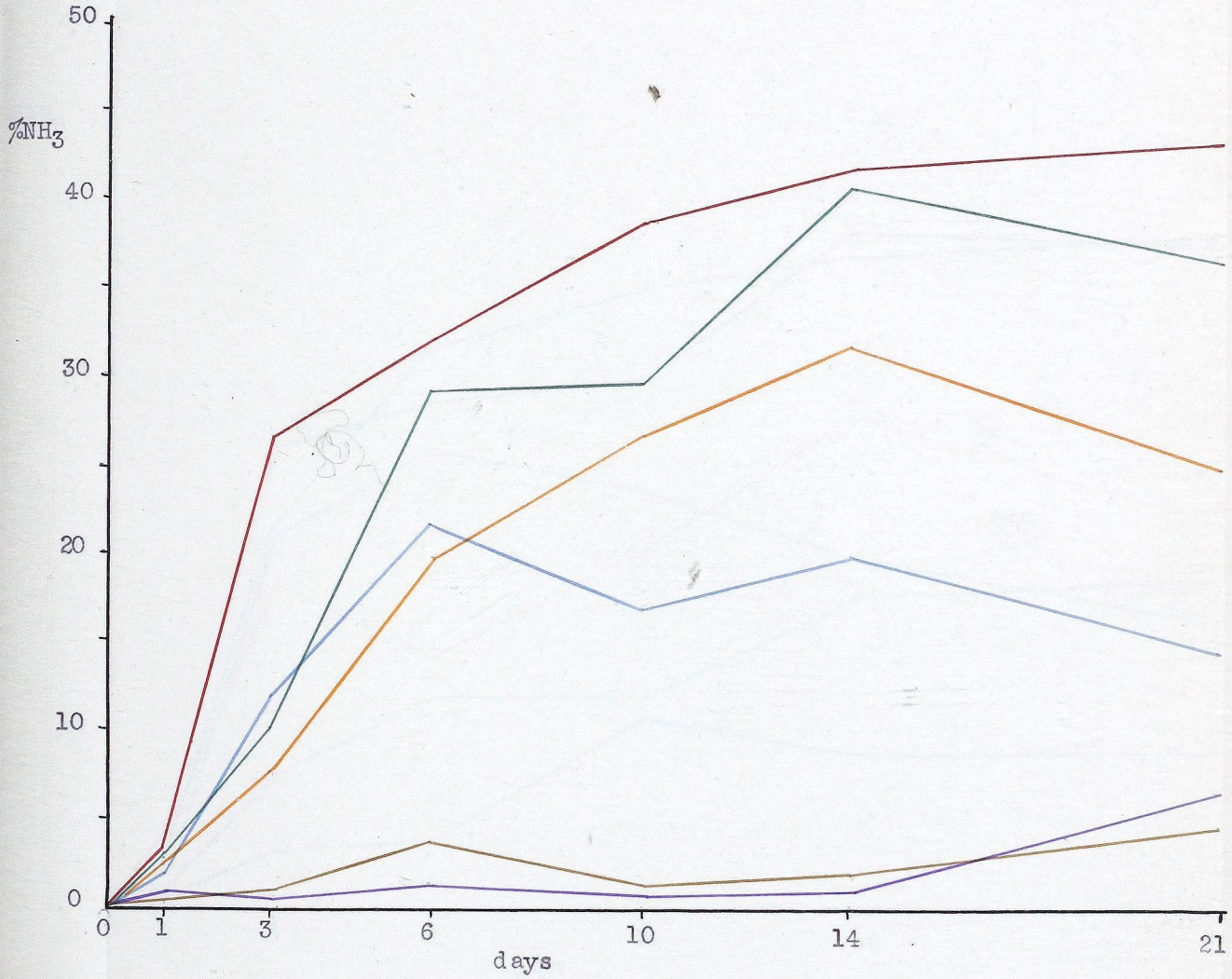


Figure 61

Proteus ichthyosmius — Histidine

M/5 Concentration

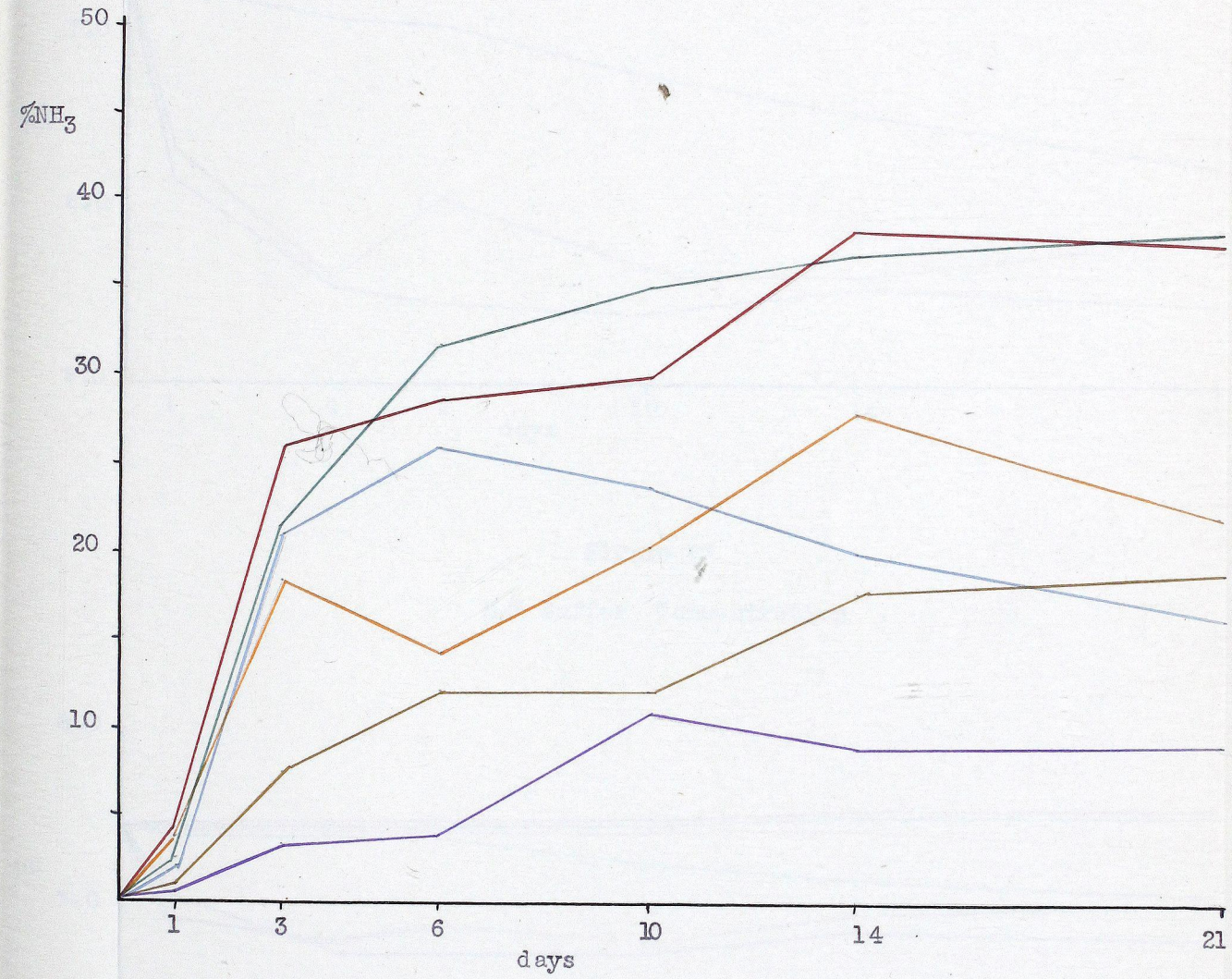


Figure 62

Proteus ichthyosmius — Histidine

M/20 Buffer Concentration

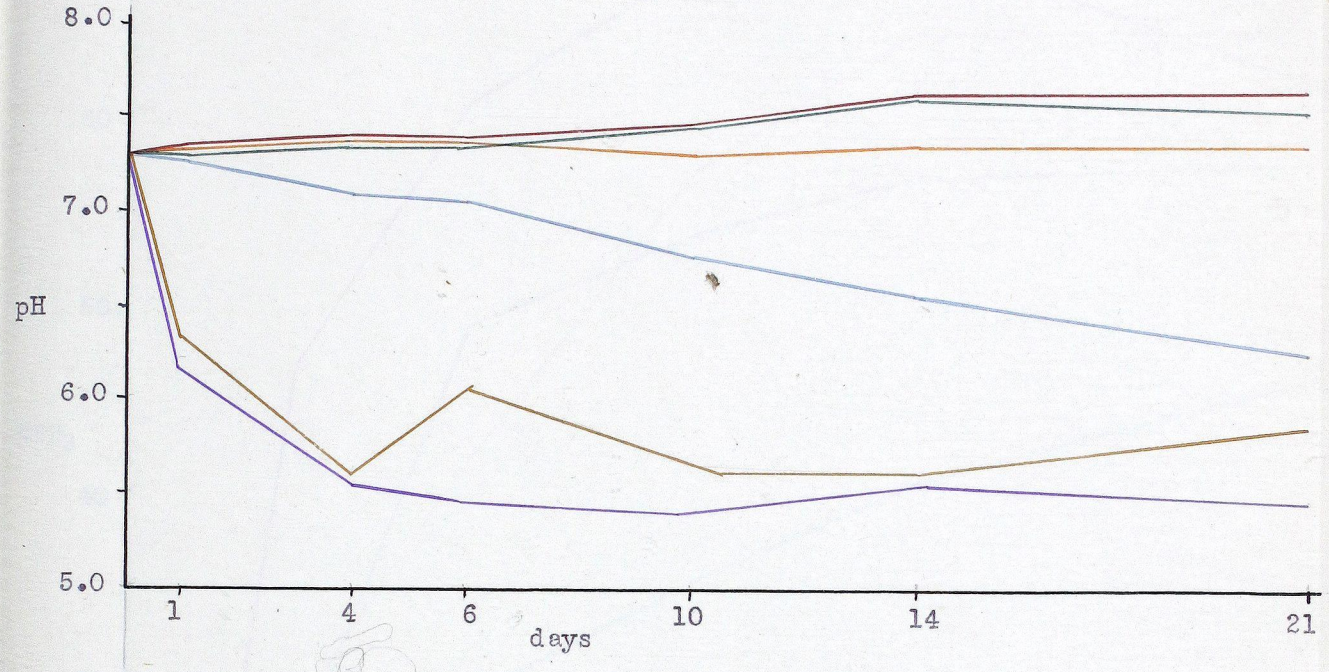


Figure 63

M/5 Buffer Concentration

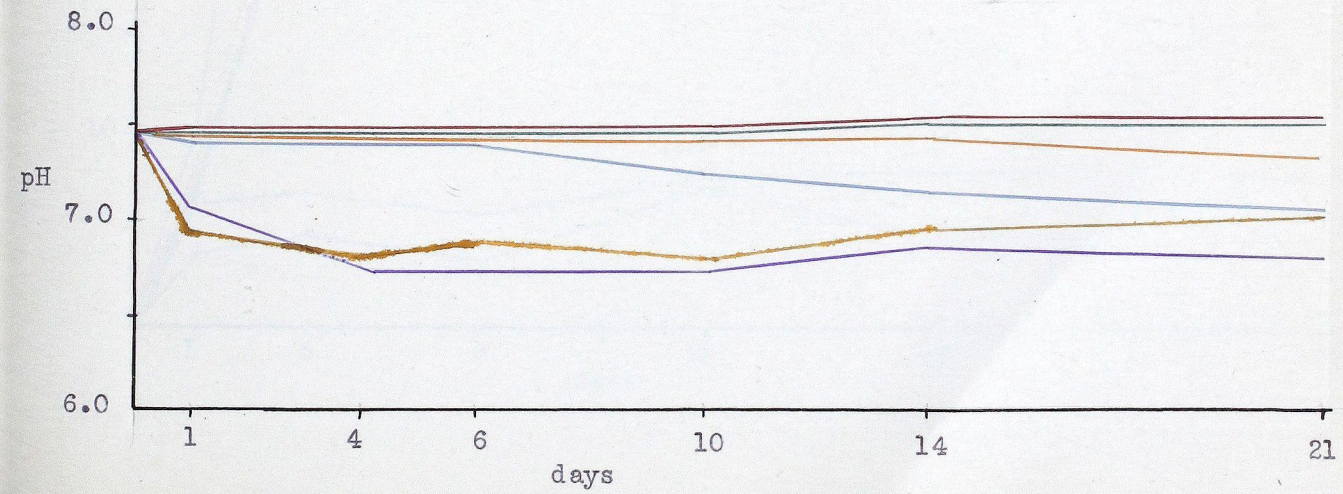


Figure 64

Pseudomonas putrefaciens Histidine

M/20 Buffer Concentration

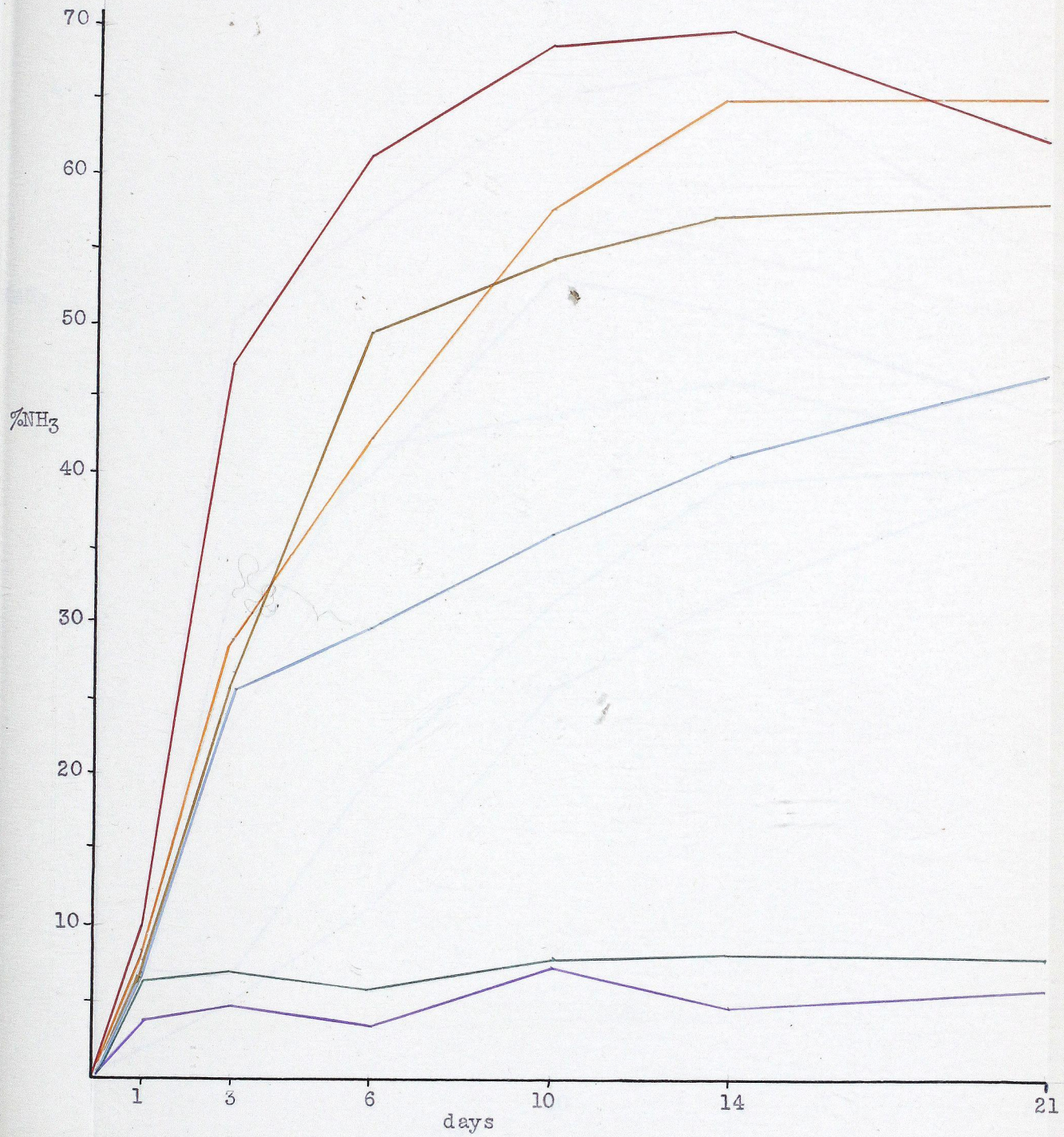


Figure 65

Pseudomonas putrefaciens — Histidine

M/5 Buffer Concentration

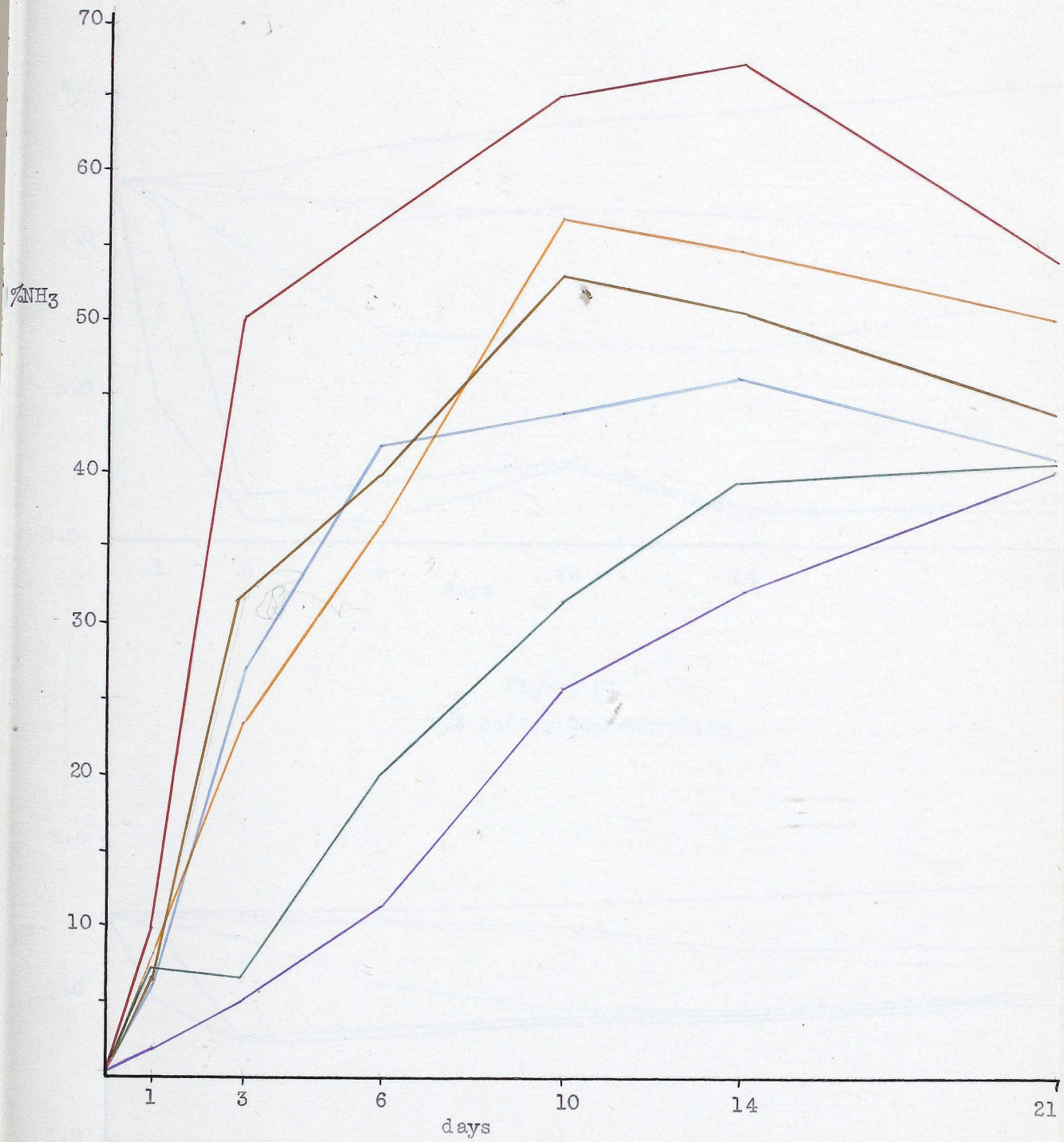


Figure 66

Pseudomonas putrefaciens — Histidine

M/20 Buffer Concentration

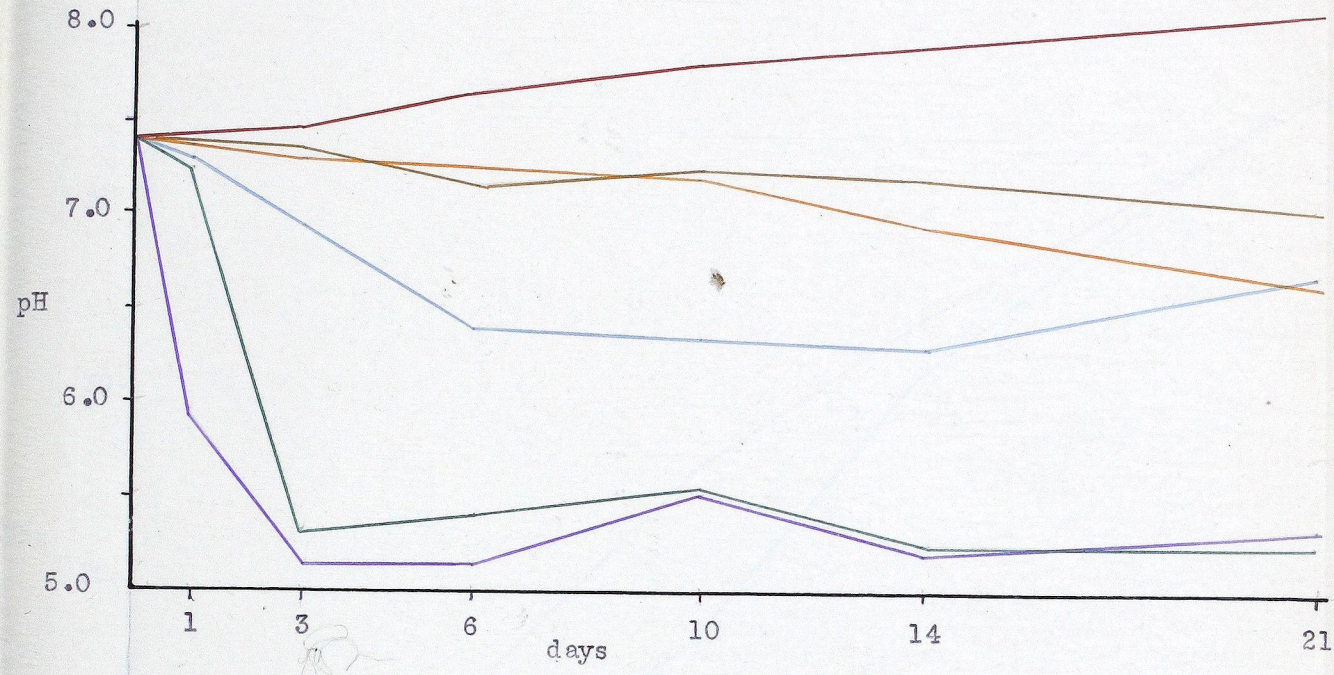


Figure 67

M/5 Buffer Concentration

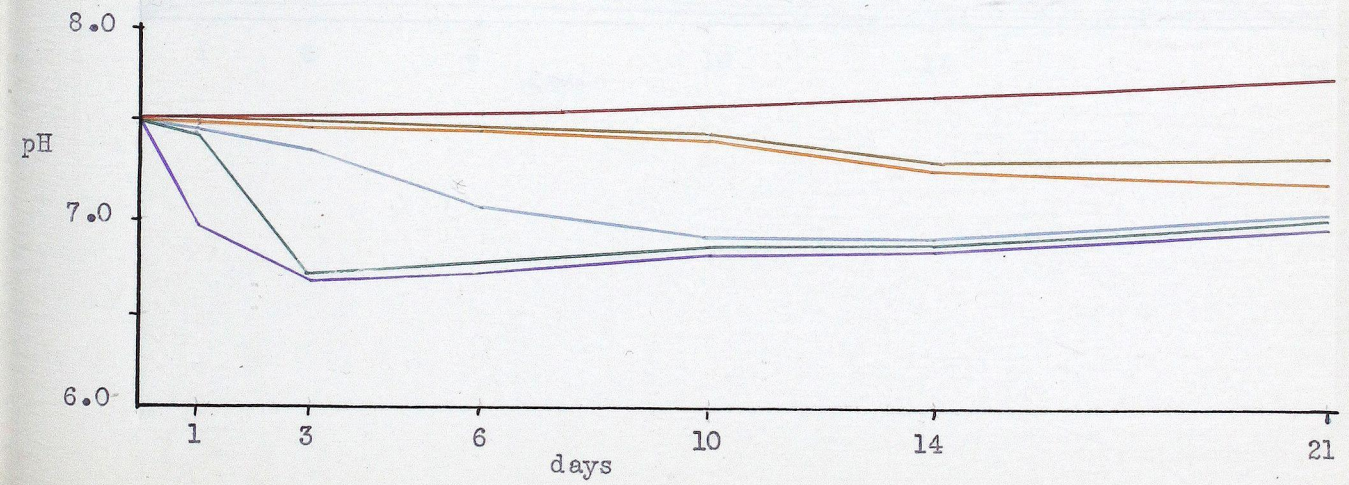


Figure 68

Proteus ichthyosmius

Proline

M/20 Buffer Concentration

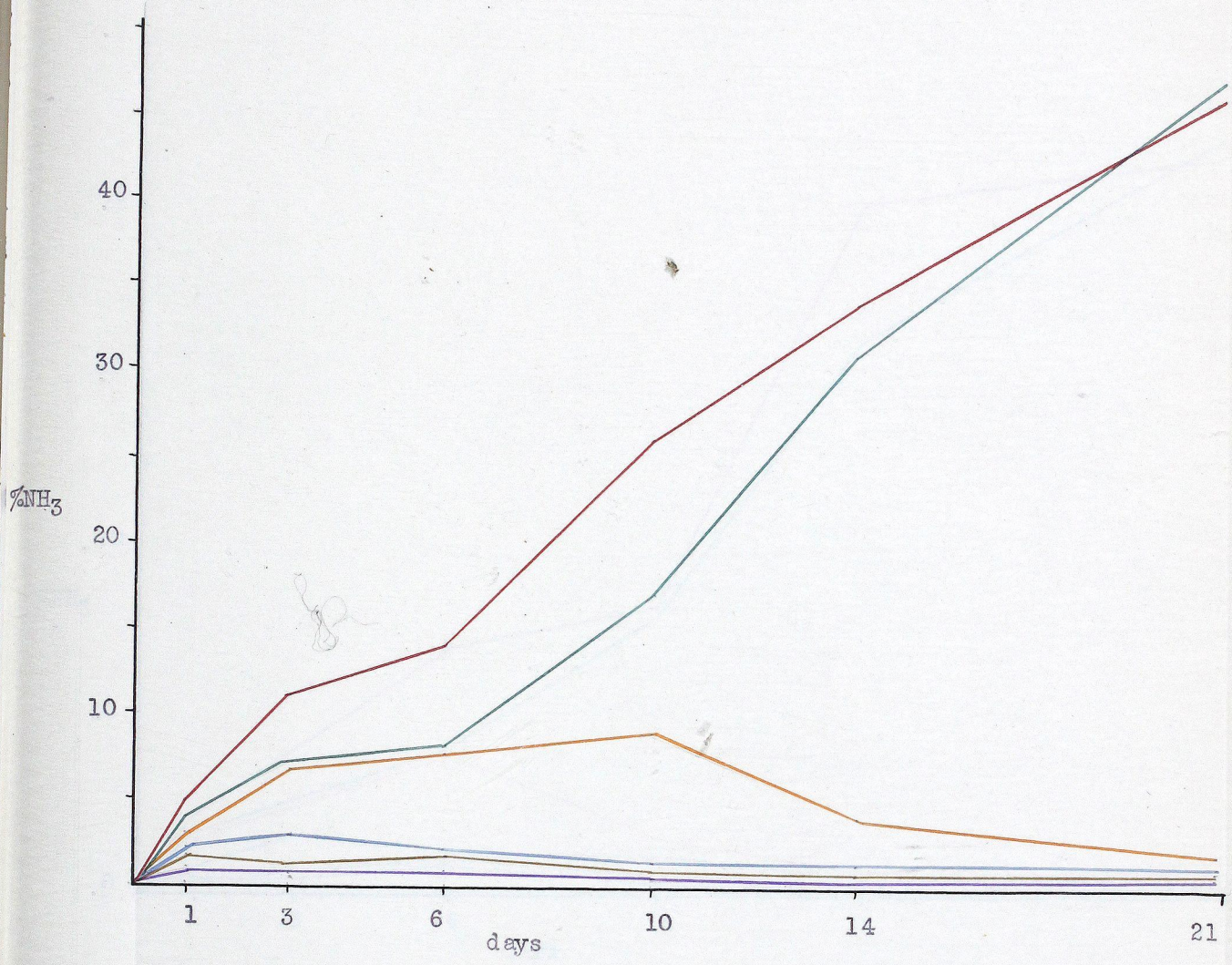


Figure 69

Proteus ichthyosmius — Proline

M/5 Buffer Concentration

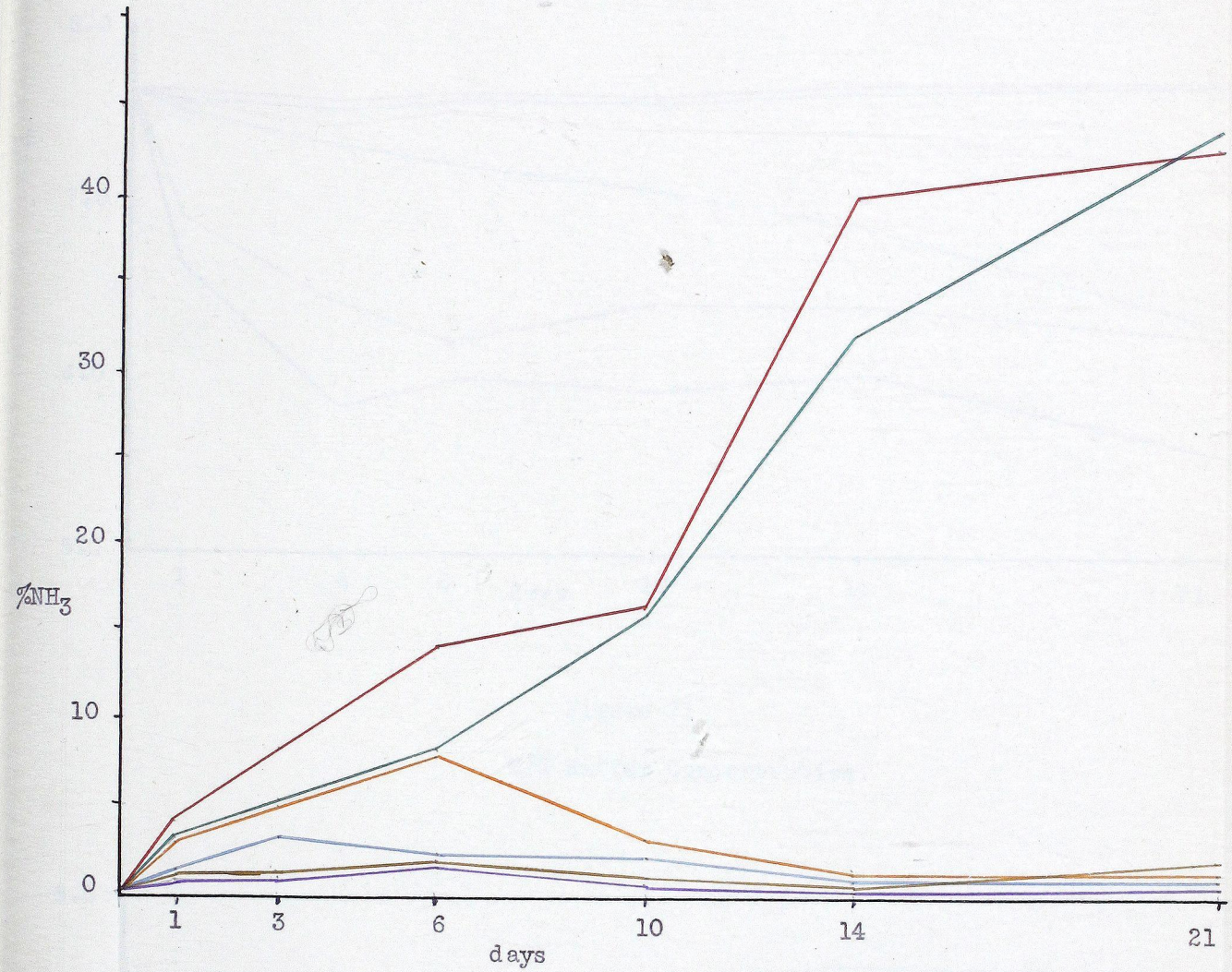


Figure 70

Proteus ichthyosmius — Proline

M/20 Buffer Concentration.

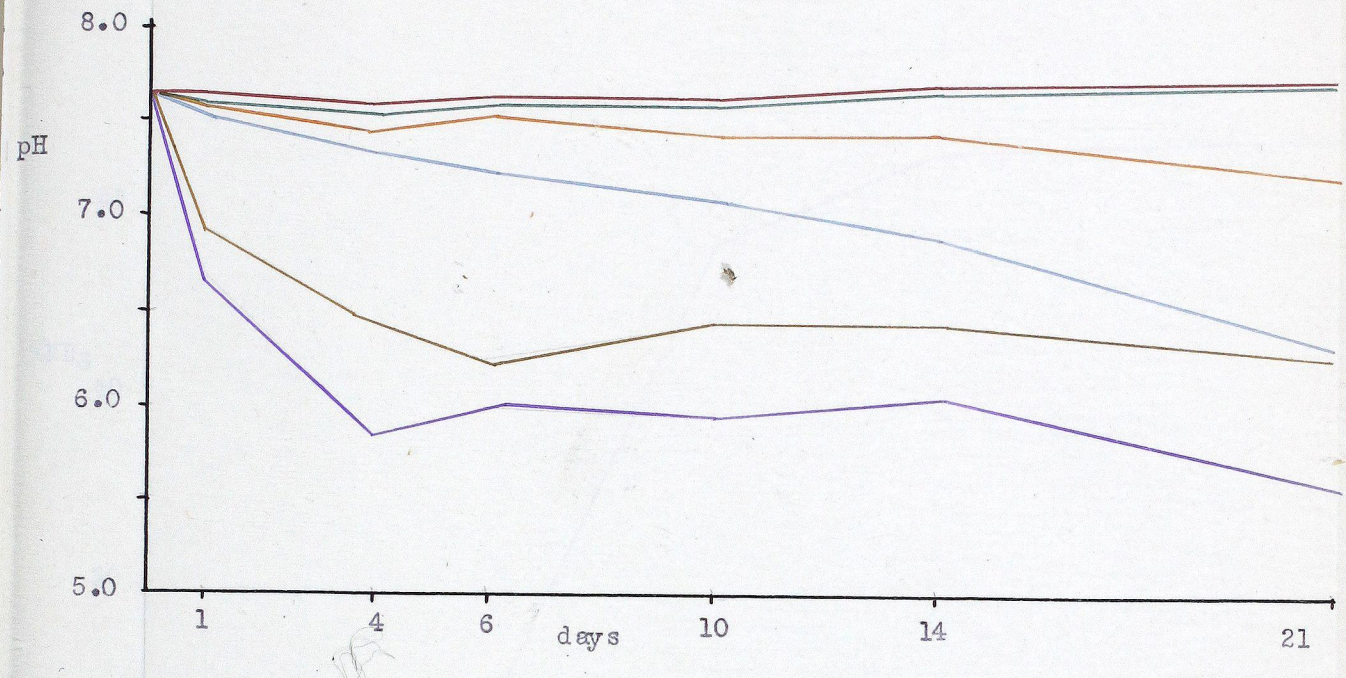


Figure 71

M/5 Buffer Concentration.

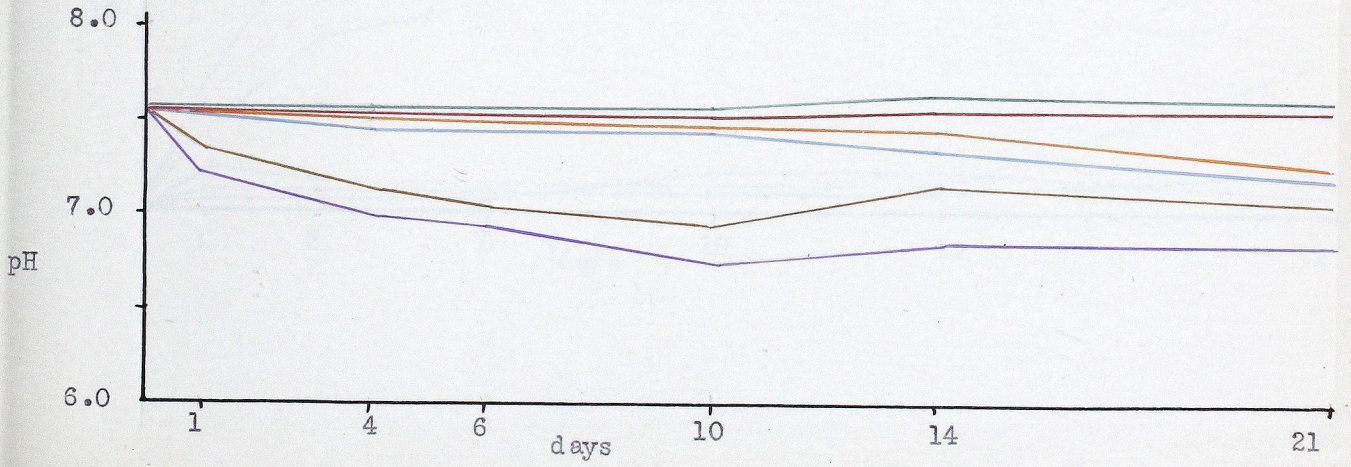


Figure 72

Pseudomonas putrefaciens

Proline

M/20 Buffer Concentration

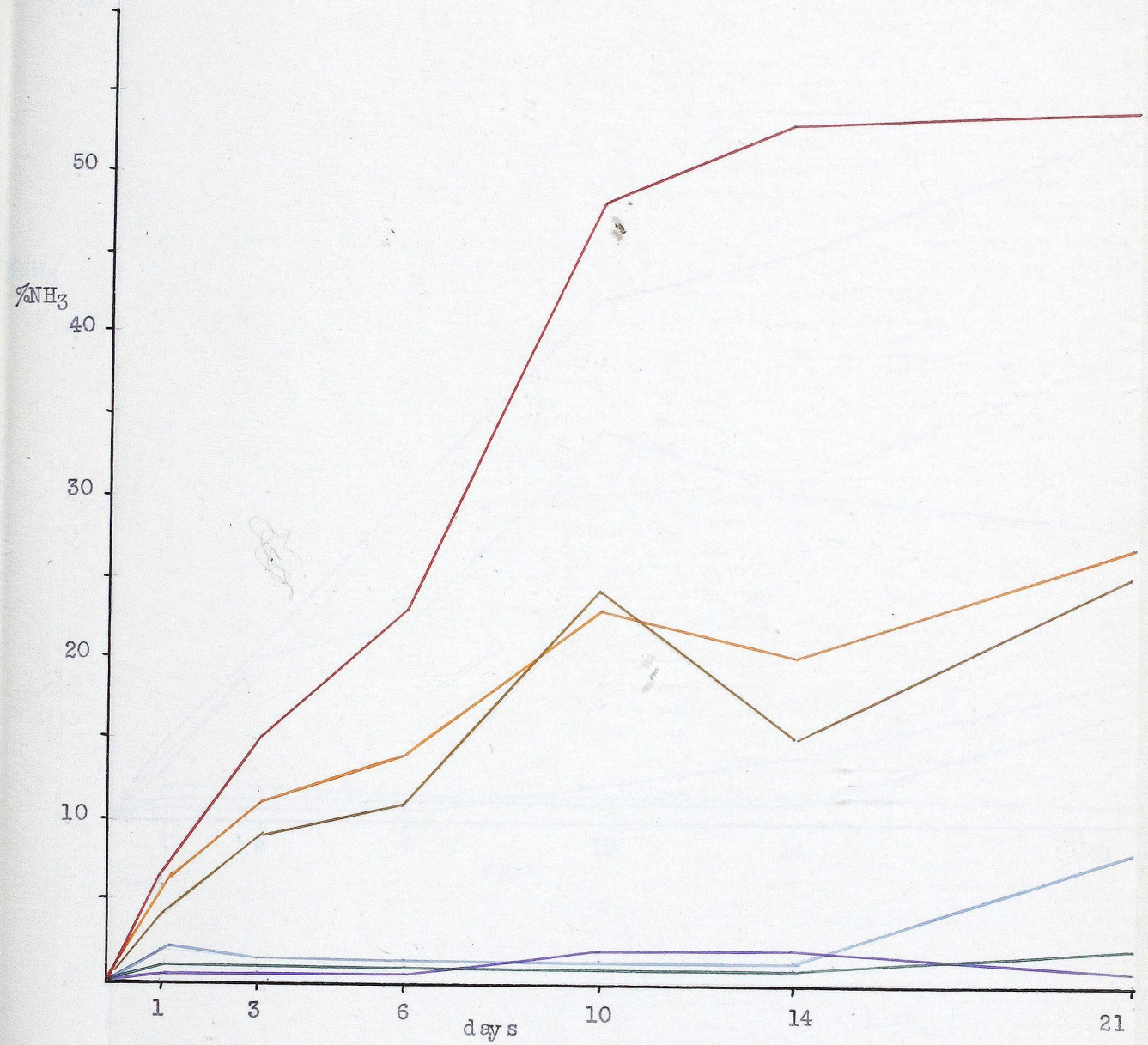


Figure 73

Pseudomonas putrefaciens Proline

M/5 Buffer Concentration

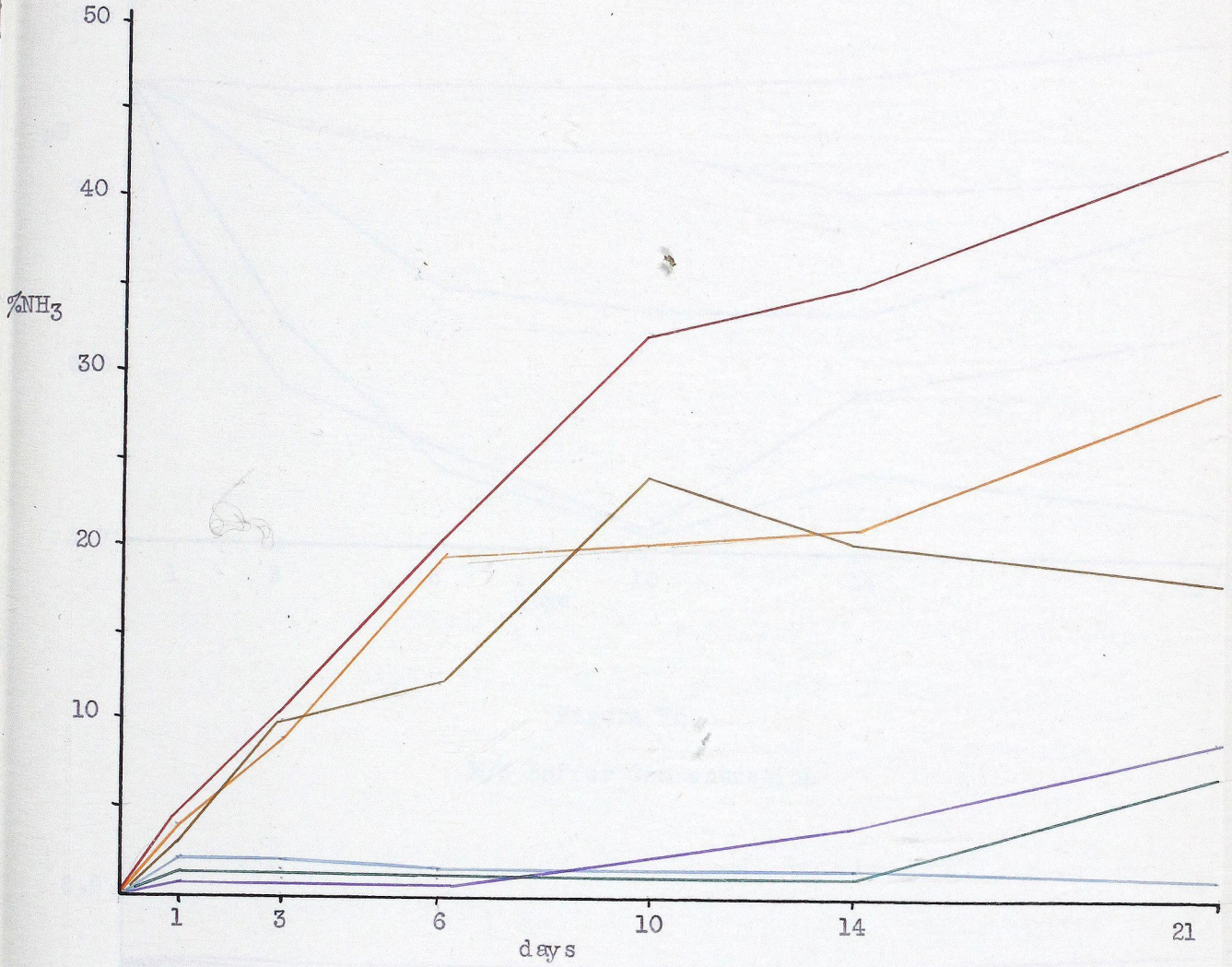


Figure 74

Pseudomonas putrefaciens Proline

M/20 Buffer Concentration

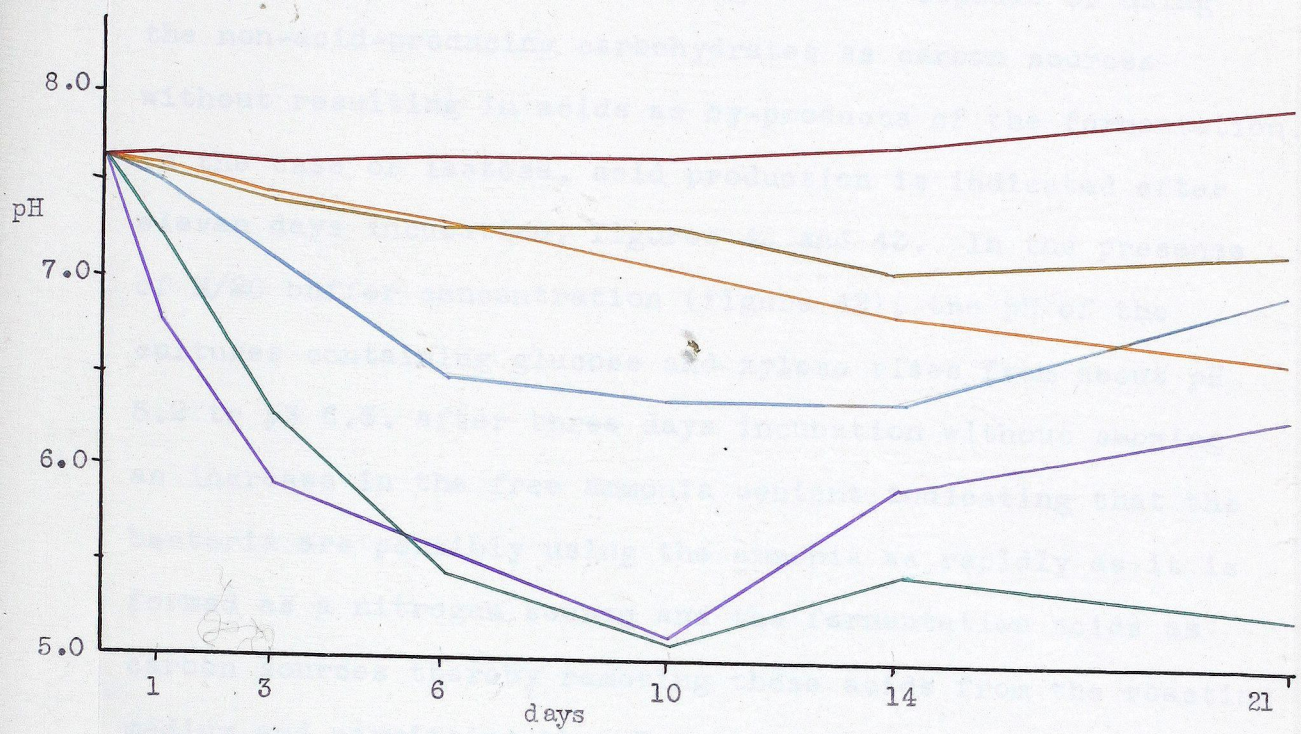
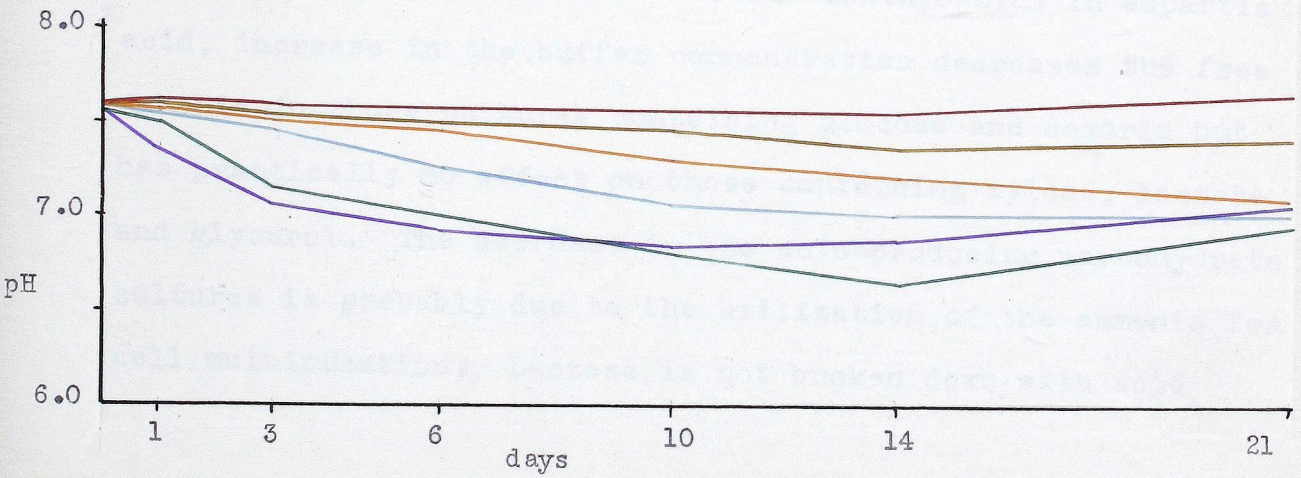


Figure 75

M/5 Buffer Concentration



control, figures 40 and 41. There appears to be no adequate explanation of this finding. The cultures of this species containing arginine show good growth in the presence of both the acid-producing and the non-acid-producing carbohydrates. This suggests that this microorganism is capable of using the non-acid-producing carbohydrates as carbon sources without resulting in acids as by-products of the fermentation. In the case of lactose, acid production is indicated after eleven days incubation, figures 42 and 43. In the presence of M/20 buffer concentration (figure 42), the pH of the cultures containing glucose and xylose rises from about pH 5.2 to pH 6.6. after three days incubation without showing an increase in the free ammonia content indicating that the bacteria are possibly using the ammonia as rapidly as it is formed as a nitrogen source and the fermentation acids as carbon sources thereby removing these acids from the reacting medium and permitting the pH to rise. An alternative explanation is that the bacteria have some mechanism for the production of alkaline compounds other than ammonia to neutralize part of the acidity.

In the cultures of Proteus ichthyosmius in aspartic acid, increase in the buffer concentration decreases the free ammonia in those cultures containing glucose and dextrin but has practically no effect on those containing xylose, lactose and glycerol. The decrease in the acid-producing carbohydrate cultures is probably due to the utilization of the ammonia for cell multiplication. Lactose is not broken down with acid

production by Proteus ichthyosmius in the presence of aspartic acid (vide supra arginine) while glycerol is seen to exhibit slow acid production.

Figures 48 to 51 inclusive graphing the results of the action of Pseudomonas putrefaciens on aspartic acid reveal two outstanding points. The first is that increase in the buffer concentration increases the range in ammonia content of the cultures in the presence of the two groups of carbohydrates, acid-producing and non-acid-producing. The low concentration of buffer, M/20, is insufficient to maintain the pH of the cultures containing xylose and glucose with the result that the acidity is too great to permit bacterial activity and the ammonia is not utilized for cell reproduction. The increased buffer content maintains the pH level and the amount of ammonia decreases in those cultures with fermentable carbohydrates. The second observation is that the quantity of ammonia in the cultures with dextrin in the presence of both buffer concentrations decreases without any change in pH. This suggests, again, that Pseudomonas putrefaciens is capable of utilizing dextrin as a carbon source without forming acids.

Ammonia formation from glutamic acid by Proteus ichthyosmius takes place slowly over the three weeks of incubation. Consequently, as the results showing only small quantities of free ammonia in the cultures containing the acid-producing carbohydrates indicate, the bacteria are probably using the ammonia as rapidly as it is formed as a

nitrogen source for cell multiplication. In the cultures containing acid-forming carbohydrates, increase in the buffer concentration slightly decreases the ammonia content probably because the more favourable hydrogen-ion concentration of the higher buffer permits more active bacterial reproduction.

The rate of ammonia formation from glutamic acid by Pseudomonas putrefaciens for the first ten days of incubation is about the same as that for Proteus ichthyosmius, but increases after the ten day interval to such an extent that ammonia formation is more rapid than its utilization as a nitrogen source by the bacteria and as a result free ammonia accumulates in the cultures. The other point of note from figures 56 and 58 is the sudden increase in activity in the culture containing glucose in the presence of the low buffer concentration after fourteen days incubation as shown by the increase in ammonia content and in pH.

In studies on the activity of Proteus ichthyosmius on histidine, the only effect of the increase buffer concentration in addition to maintaining the pH of the cultures is to increase the quantity of ammonia in the cultures containing dextrin and glucose. This increase is, in all probability, the result of controlling the acidity thereby preventing the decrease in bacterial activity that takes place when the pH drops too low.

Similar results were obtained for Pseudomonas putrefaciens in histidine except that xylose and not dextrin is the carbohydrate which with glucose shows increased

ammonia content in their respective cultures when the buffer concentration is increased fourfold. It is to be recalled that acid production from xylose and non-acid production from dextrin distinguish Pseudomonas putrefaciens from Proteus ichthyosmius.

The cultures of Proteus ichthyosmius containing proline show practically no response in the form of increased or decreased ammonia formation to the increase in buffer concentration. The pH of the M/20 buffer cultures containing the other four amino acids, suggesting that the bacterial activity does not decrease in the case of proline to the extent that it did with the other amino acids and that cell multiplication takes place equally well in the presence of both buffer concentrations.

A similar picture was obtained for the action of Pseudomonas putrefaciens on proline except that the increase in buffer concentration caused a marked decrease in the rate of ammonia accumulation after six days incubation.

The Breakdown of Arginine by Proteus ichthyosmius and Pseudomonas putrefaciens.

The decomposition of arginine with the conversion of seventy to eighty percent of its total nitrogen into ammonia by Proteus ichthyosmius and about thirty percent by Pseudomonas putrefaciens, suggested that the pathway of the breakdown by investigated. In order to carry out this suggestion, the above two species of bacteria were inoculated individually into buffer cultures of arginine, ornithine

TABLE 31.

Ammonia Formation from Arginine and its Decomposition Products.

Proteus ichthysomius

Compound	cc. N/100 H_2SO_4	% T. N. into NH_3
Arginine	12.85	64.25
	13.95	69.75
Ornithine	7.0	70.0
	7.0	70.0
Delta-Amino Valeric Acid	0.5	10.0
	0.55	11.0
Urea	1.0	10.0
	1.0	10.0

Pseudomonas Putrefaciens

Arginine	9.0	45.0
	9.4	47.0
Ornithine	7.05	70.5
	6.9	69.0
Delta-Amino Valeric Acid	0.3	6.0
	0.35	7.0
Urea	0.65	6.5
	0.65	6.5

(prepared as outlined by Hunter (37)), delta-amino n-valeric acid and urea respectively in duplicate and incubated at 30° C. for twenty-one days. The result of this experiment (XIV) are recorded in table 31.

It was found, in the case of Proteus ichthyosmius, that the ammonia formed from arginine was greater than the total of that formed from ornithine and urea, and that that formed from ornithine was seven times greater than that from delta-amino valeric acid. On the other hand, the ammonia formed by Pseudomonas putrefaciens from arginine was only slightly more than that from ornithine and urea while that formed from ornithine was more than seven times greater than that from delta-amino valeric acid. The results also show clearly that both species of bacteria employed have equal ability to deaminate both the alpha and the delta animo groups of the ornithine used in this experiment, while neither species can form significant quantities of ammonia from delta-amino valeric acid and urea. A discussion of the significance of these results in relation to the probable course of breakdown of arginine will be given later in the thesis.

Ammonia Formation from Non-Amino Acid Nitrogenous Compounds.

During the course of this study, the question arose as to whether amino or imino groups of nitrogenous compounds other than amino acids could be converted into amminia by the surface taint producing bacteria. In order to obtain some data on this aspect of the problem, an experiment (XV)

TABLE 32.

Ammonia Formation from Amino-Group Containing Compounds other than Amino Acids.

Compound	Prot. ichthyosmius		Pseudo. putrefaciens	
	5 days	22 days	5 days	22 days
Asparagin	6.80	4.95	6.80	5.45
glutamine (control 2.3 - not subtracted)	4.0	4.35	4.1	5.9
adenine	4.05	4.70	1.15	3.55
guanine	3.90	8.20	4.5	2.80
uracil	.35	.8	.25	.75
uric acid	.65	1.25	.55	.85
nicotinic acid	.4	.95	2.6	3.6
betaine	.45	.65	.45	.75
urea	.3	.8	.25	.6

Results expressed as cubic centimeters of N/100 sulphuric acid equivalent to the ammonia formed in the culture from one cubic centimeter of M/20 solution of the nitrogenous compound. Application of the formula at the beginning of the Experimental section will convert results to per cent ammonia formed of the total nitrogen.
(see discussion)

employing the action of Proteus ichthyosmius and Pseudomonas putrefaciens on the following compounds was carried out: adenine, asparagin, betaine, glutamine, guanine, nicotinic acid, uracil, urea and uric acid. The experiment was set up as outlined at the beginning of this section of the thesis with the above nitrogenous compounds replacing the amino acids. Controls containing the nitrogenous compounds without inoculation with one of the bacterial species were employed to determine the breakdown, if any, of the compounds by the alkaline aeration of the Van Slyke procedure. The cultures were set up in duplicate, one set being removed after incubation at 30° C. for five and twenty-two days respectively. The results are given in table 32.

The first point of interest from these results is that glutamine is the only one of the compounds tested in the control series that shows a significant liberation of ammonia during the alkaline aeration. The quantity liberated is equivalent to about fifty percent of one nitrogen. The nitrogen group affected is probably the amide group which decomposes readily under strongly alkaline conditions. Because of this decomposition, it is difficult to determine if the decomposition of the amide group in the glutamine cultures containing the bacteria is caused by the bacteria prior to aeration or takes place chemically during the actual ammonia determination. The amide group of asparagin did not decompose significantly during the alkaline aeration. This striking

difference between the amides of aspartic acid and glutamic acid respectively will be discussed more fully later.

The quantity of ammonia formed from asparagin by Proteus ichthosmius and Pseudomonas putrefaciens is equivalent to between fifty and seventy percent of the total nitrogen. This finding shows clearly that these species are capable of splitting off both the amino and the amide groups of l-asparagin. The results obtained for glutamine, on the other hand, do not necessarily indicate that both groups are attacked by these bacterial species. The difficulties inherent in the method for the determination of ammonia from glutamine by the Van Slyke procedure to which reference has previously been made, renders impossible a clear-cut interpretation of the figures obtained for the action of these micro organisms on this compound. No definite conclusion can be reached as to their action on the amide group of glutamine. There is, however, some ammonia formed in excess of the control but whether it comes from the amino or the amide group cannot be determined.

The object of studying the breakdown of adenine, guanine, uric acid and uracil was to determine of Proteus ichthyosmius and Pseudomonas putrefaciens had the ability to open the purine and pyrimidine rings with subsequent deamination and formation of ammonia. Uracil (2, 6, dioxy pyrimidine) contains two imino groups. Uric acid (2, 6, 8, trioxy purine) has four nitrogen groups, two in the iminazol ring and two in the pyrimidine ring. Adenine (6 amino purine) has a free amino group in addition to the four

nitrogens in the purine ring, while guanine (2 amino, 6 oxy purine) has, in addition to a free amino group, an oxy group. Whereas the amino group in adenine is in position 6, that in quanine is in position 2. The findings of the experiments employing these four compounds show clearly that the pyrimidine ring of uracil and the purine ring of uric acid are both opened to a slight extent with the formation of small quantities of ammonia and that the free amino group of both adenine and guanine is deaminated.

When the results for the twenty-two day period of incubation are considered, it is seen that in the case of Proteus ichthyosmius rupture of one of the rings in guanine with the resultant formation of ammonia has also occurred. It would appear most likely that the formation of ammonia has resulted from cleavage of the iminazol nucleus and has arisen from either or both of the imino groups present in this ring.

The results obtained when nicotinic acid was employed are of interest. The findings show clearly that the pyridine ring is opened by each of the organisms studied, Pseudomonas putrefaciens possessing the more marked ability to elaborate ammonia from this ring structure. The opening of five distince nitrogen containing ring structures with subsequent deamination resulting in the formation of ammonia has thus been demonstrated for these species of micro-organisms. These bacterial species have previously been shown to attack the iminazol, pyrollidine, pyrimidine and purine rings.

The two other compounds studied, betaine (from

glycine) and urea gave small quantitied of ammonia under the influence of the two bacterial species employed. These results, however, were not sufficiently significant to cause speculation. The failure to hydrolyse urea to any extent by two organisms that have been shown to decompose arginine readily is discussed elsewhere.

PART I. DISCUSSION

The experiments reported upon herein constitute the first study of the conditions governing deamination by the two species of bacteria - Proteus ichthyosmius and Pseudomonas putrefaciens - that has been reported. Similar studies employing different techniques and more rapidly acting bacteria such as Bacterium coli have been recorded. The technique followed in previous studies, i.e. that using the Warburg apparatus, however, could not be followed in this study because the two species of surface taint bacteria employed are relatively slow to attack the amino acids, sometimes requiring a number of days whereas Bacterium coli has the ability to deaminate in the matter of a few hours or less. The method of ammonia determination also differed in this study from most of the previous studies. Since the colorimetric determination of ammonia employing Nessler's reagent is inhibited by the presence of certain nitrogeneous compounds, outstanding among which are histidine and tryptophan (50), this procedure, commonly used by other workers, could not be employed directly in this study. Distillation of the ammonia from the culture followed by Nesslerization would have overcome this difficulty but such a procedure is too slow to permit completing the large number of determinations required in the experiments described above. As an alternate, the Va- The alternative procedure that was chosen was the Van Slyke aeration procedure. This procedure, although possibly not yielding as accurate results as may be obtained employing Nessler's reagent, was found to aerate ninety-six to ninety-

eight percent of the ammonia added in a test series and had the (required) advantage of permitting the carrying out of the large number of determinations required in this study.

The decomposition of arginine by Proteus ichthyosmius has been shown to yield seventy-five to eighty percent of its nitrogen as ammonia. This means that at least three, and in all probability four, of the nitrogens of arginine are converted partially or completely into ammonia by this species. If the enzyme arginase is produced by Proteus ichthyosmius, the products of hydrolysis by this enzyme would be ornithine and urea. The results of experiment XIV have shown that Proteus ichthyosmius converts seventy percent of the nitrogen of ornithine into ammonia but only ten percent of that of urea. These findings suggest that the elaboration of bacterial urease for the splitting of urea may depend upon the prior elaboration of arginase. The failure to split urea to a significant extent shows that this species is unable to form urease in the presence of urea alone. The results reported upon herein support the findings described by Hill in work on the splitting of the arginine molecule and may lend credence to the theory put forth by him with respect to the activity of arginine dihydrolase.

The rate and quantity of ammonia formed from arginine by Pseudomonas putrefaciens is not so great as that formed by Proteus ichthyosmius. Is would appear that the alaboration of the enzymes necessary for the breakdown of arginine is much slower and not so complete in the case of Pseudomonas putrefaciens as compared with Proteus

ichthyosmius. The total quantity of ammonia formed by Pseudomonas putrefaciens from arginine is, however, in excess of that formed by the same species from ornithine and urea, suggesting again that there is an associative action present in the elaboration of the different enzymes required for the complete degradation of arginine.

The breakdown of ornithine has been found to be similar for both bacterial species - seventy percent of its nitrogen being converted into ammonia. This finding shows clearly that these bacterial species have the ability to attack both the alpha and the delta amino groups of ornithine to a considerable extent. When, however, the results showing almost insignificant quantities of ammonia formed from the deamination of delta amino valeric acid (the compound formed by the deamination of the alpha amino group of ornithine) are considered, it can readily be seen that the elaboration of the deaminase for attacking the delta amino group is dependent upon the prior elaboration of the alpha amino deaminase - a sequence of events similar to that found for the elaboration of ammonia from arginine.

Aspartic and glumatic acids are both dicarboxylic mono-amino acids differing in structure only by the presence of an additional CH_2 group in the case of glutamic acid. A comparison of the rates of ammonia formation from these two amino acids, however, reveals striking differences. The dl-aspartic acid employed was deaminated to the extent of fifty percent of its nitrogen within twenty-four hours by each of the two bacterial species. A longer period of

incubation did not result in further formation of ammonia. Previous studies (50) have shown that the l-isomer of this amino acid is deaminated very rapidly suggesting that one hundred percent of the l-compound in the dl-mixture is attacked while the d-compound is not attacked. When d-glutamic acid is considered, it is found that deamination takes place slowly over the complete period of incubation - forty percent of the total nitrogen being converted into ammonia after twenty-one days incubation. One reason for this slower rate of deamination when compared with that found in the case of aspartic acid may be that the d-isomer is not readily attacked. It would be necessary to employ the l- or the dl-isomer in order to make a direct comparison of the rates of deamination of these two amino acids.

The conversion by Pseudomonas putrefaciens of about seventy percent of the nitrogen of histidine into ammonia shows clearly that this bacterial species has the ability to open the iminazol ring and to deaminate the compound thus formed. Cleavage of the ring with the liberation of one molecule of ammonia followed by oxidation, oxidative decarboxylation and deamination respectively may result in the formation of glutamic acid (see figure 2). In the experiments reported upon herein, l-histidine was employed. If the degradation of histidine takes place as suggested, l-glutamic acid should be formed. Providing the first two ammonia molecules in the degradation are released to the extent of approximately one hundred percent, about ten percent of the third molecule present in the suggested glutamic acid are converted into

ammonia showing a slower rate of breakdown than takes place when d-glutamic acid is employed by itself.

The cleavage of the iminazol ring by Proteus ichthyosmius is not so rapid nor so complete as that found for Pseudomonas putrefaciens - about forty percent of the total nitrogen being converted into ammonia. The ring is definitely opened, however, by Proteus ichthyosmius since a maximum of thirty-three percent ammonia would be formed by deamination of the side chain amino group alone.

The decomposition of proline with the liberation of between forty and fifty percent of its nitrogen as ammonia takes place equally well under the influence of the two bacterial species employed. Proline is not a true amino acid but contains its nitrogen as an imino group in the pyrollidine ring structure. Opening of this ring is a prerequisite of ammonia formation from this compound at all times. The pyrollidine ring may be broken on either side of the imino group. Hydrolytic cleavage on the side next to the carboxyl group yields alpha hydroxy delta amino valeric acid. If the deamination of this compound is similar to that of delta amino valeric acid, it is possible that this method of opening the ring does not take place. Oxidative opening of the ring on the side of the imino group away from the carboxyl group would give glutamic acid. Weil-Malherbe and Krebs have concluded that proline is oxidized by kidney tissue to glutamic acid which in turn may be further oxidized to alpha keto glutaric acid with the liberation of one molecule of ammonia. Further evidence in support of cleavage of the

pyrollidine ring in this manner may be obtained when the figures from the different experiments for glutamic acid and proline are compared. For example, figures 52 and 53 and figures 68 and 69 depicting the ammonia formation by Proteus ichthyosmius from glutamic acid and proline respectively show clearly that the rate of formation and the final quantities produced are highly comparable.

The results obtained in the experiments outlined above add further evidence to the hypothesis shown in figure 2 that the six amino acids studied are interrelated. The alpha keto and alpha hydroxy acids of delta amino valeric acid and alpha keto glutaric acid are probably the links joining arginine, ornithine and proline to histidine, aspartic acid and glutamic acid. The quantities of ammonia formed during the decomposition of these six amino acids by the two bacterial species are sufficient to suggest that their breakdown proceeds through the number of oxidations and deaminations outlined. Arginine, under the influence of Proteus ichthyosmius shows the liberation of four nitrogens in the form of ammonia indicating the formation of alpha keto glutaric acid or one of its decomposition products. The action of Pseudomonas putrefaciens on arginine does not liberate so much ammonia as Proteus ichthyosmius but the decrease is probably due to the failure to hydrolyse urea since it has been shown that this species is capable of liberating seventy percent of the nitrogen of ornithing in the form of ammonia.

The breakdown of proline by both bacterial species

also points to the formation of alpha keto glutaric acid or one of its products. While the possibility of the course of breakdown from alpha keto delta amino valeric acid proceeding by reductive deamination to the simpler compounds of valeric acid must be considered, the evidence obtained in the expiraments of the effect of oxygen supply on ammonia formation, particularly in the case of proline, strongly suggests that oxidative deamination rather than reductive deamination takes place.

The experimental evidence obtained for the decomposition of histidine by Pseudomonas putrefaciens shows clearly that the three nitrogens are liberated in the form of ammonia adding further support to the hypothesis that this amino acid is degraded to alpha keto glutaric acid or lower. The findings in the experiments employing Proteus ichthyosmius on histidine suggest that only two nitrogens are converted into ammonia or that a smaller percentage of the histidine is attacked with the formation of ammonia equivalent to the three nitrogens. The evidence is stronger for the second alternative when it is recalled that increase in age of the growth culture of this bacterial species markedly decreases the quantity of ammonia formed from histidine. The younger growth cultures produced sufficient ammonia to indicate that deamination of the three nitrogen groups occurs.

The direct oxidative deamination of glutamic acid gives alpha keto glutaric acid or one of its related compounds. Aspartic acid when subjected to aimilar oxidative deamination yields oxalacetic acid which is also formed from alpha keto

glutaric acid by oxidative decarboxylation. The ammonia formed from these two amino acids by the bacterial species studied indicates that they are deaminated completely in the case of the l-isomer of dl-aspartic acid and partially in the case of d-glutamic acid.

The experimental evidence obtained for the influence of carbohydrate on ammonia formation and its subsequent utilization as a nitrogen source for cell multiplication is considerable. In examining this evidence it must be remembered that the figures represent the free ammonia content of the cultures which is an expression of the resultant of amino acid degradation and subsequent cell synthesis. Thus it can readily be understood that a decrease in ammonia content does not necessarily mean that less ammonia is being formed but rather, in the case of those cultures showing considerable bacterial growth, that the ammonia has been used for cell synthesis.

The findings of experiment IX in which the effect of the presence of glucose in the growth medium on subsequent ammonia formation is studied, show clearly that the organisms grown of the glucose-containing agar are not so active as those grown on the glucose-free agar. This decrease in activity may be due to the general decrease in activity of this organism - Proteus ichthyosmius - as exhibited by the marked retardation in the rate of growth on the glucose-containing agar as compared to the glucose-free agar. Epps and Gale (23) in an investigation to find an explanation for similar findings obtained employing Escherichia coli, observed

that the degree of inhibition of deamination by the presence of glucose bore no relation to the effect produced by the addition of fermentation acids to the growth medium in place of the glucose. They also showed that neutralization of the fermentation acids of glucose during growth does not alter yhe degree of inhibition of subsequent deamination. Further investigation similar to that outlined by Epps and Gale is necessary before an adequate explanation of these findings can be advanced.

In experiments of the effect of the presence of acid-producing and non-acid-producing carbohydrates in the buffer medium upon ammonia formation, the findings have been clearly shown to be dependent upon both the carbohydrate and the amino acid employed. The fermentation of the carbohydrate with acid production and the breakdown of the amino acid with ammonia formation appear to be independent processes. The rate at which these two processes take place, however, has been shown to materially affect the final outcome in so far as the quantity of free ammonia is concerned. When the results for the deamination rates of aspartic and glutamic acids are considered, this finding is seen to be clearly demonstrated. While in the absence of carbohydrate, the final quantities of ammonia formed from these amino acids are approximately fifty and forty percent respectively, in the presence of an acid-producing carbohydrate, considerable free ammonia results from aspartic acid and practically no ammonia formation is indicated in the case of glutamic acid. The considerable quantities of free ammonia formed from

aspartic acid in the presence of fermentable carbohydrate is largely dependent upon the fact that the rate of ammonia formation from this amino acid proceeds as rapidly as does the fermentation of the carbohydrate. In the case of glutamic acid, the final result of practically no ammonia present in a culture containing an acid-producing carbohydrate may depend upon the speed of acid production outdistancing the formation of ammonia, resulting in the lowering of the pH of an inadequately buffered culture to a level inhibitive to further bacterial activity. On the other hand, with an adequate buffer supply, the available carbohydrate may be used for purposes of cell multiplication utilizing the ammonia produced by deamination as rapidly as it is formed as a source of nitrogen. Whatever the explanation in the case of glutamic acid, practically no free ammonia is to be found in the cultures containing acid-forming carbohydrates.

The findings observed in these experiments add further evidence to the hypothesis of Raistrick and Clark that carbohydrate, far from having a protein-sparing effect, actually enables the bacteria to utilize more protein or protein products than they would in the absence of carbohydrate. The idea of ammonia utilization as a nitrogen source is a factor that is not encountered in studies employing Bacterium coli or other rapid deaminating species of bacteria. The slowness of the process in the case of the surface taint producing bacteria and the consequent influence of the possible utilization of ammonia makes more difficult the interpretation of data obtained for the slower deaminat-

ing species.

One of the important problems that arises from these observations is the question as to the relative suitability of the methods of studying deamination - the simple method using such bacterial species as Bacterium coli and the Warburg apparatus or the more complicated procedure employing the slower acting bacteria such as those used in this study. The answer to this question is dependent upon the object of the study - whether the investigation is primarily concerned with the breakdown of the amino acid or with the activity of the particular bacterial species. In this investigation, the more important factor was the study of the action of two species of bacteria known to be among those responsible for surface taint in butter on compounds that might be the precursors of the substance or substances formed in the elaboration of the sweaty-feet odour of typical surface taint.

Previous investigations of the effect of the presence of carbohydrate on deamination have been principally concerned with the one carbohydrate - glucose. Nisimura (51) used a number of carbohydrates, but limited the amino acid studied to tyrosine. The experiments reported upon herein were carried out employing eleven distinct carbohydrates, subsequently reduced to five key carbohydrates in the experiments demonstrating the influence of variation in buffer concentration. The findings show clearly that the nature of the carbohydrate has a marked effect upon the rate and the final ammonia formation. In the absence of adequate buffer in the medium, the pH of the cultures containing the rapid

acid-producing carbohydrates is readily lowered to a level unfavorable to further bacterial activity. Increasing the buffer capacity of the culture overcame this difficulty and the ammonia formation showed itself to be positive or negative depending upon the relative rapidity of fermentation and deamination. The rate of acid production from the various carbohydrates has been shown to vary considerably - glucose and sucrose appearing to be the most rapidly fermented, while glycerol is one of the slowest acid-formers. As previously noted, the acid production from lactose after a fourteen day period of incubation particularly in the presence of arginine is an example of a carbohydrate that provides available carbon sources after the nitrogen source has been formed in large quantities and permits bacteria multiplication to proceed in the latter days of the three week period of incubation.

Thus as can readily be seen from the findings of these experiments considerable data has been added to our knowledge of amino acid breakdown. The study of the effect of the presence of various carbohydrates has increased our information concerning this little explored aspect of amino acid decomposition. The provision of an available carbohydrate adds the complication of bacterial growth to the already involved study of the enzymes responsible for amino acid catabolism and subsequent ammonia formation.

PART II. Studies on the Isolation and Identification of Odouriferous Compounds.

HISTORICAL

The problem of the nature of the compound or compounds formed during the development of surface taint in butter has confronted investigators since studies of the defect were begun a number of years ago. Campbell(9) found that when indole, a derivative of tryptophan was added to milk, an odour strongly resembling that of surface taint was emitted. As already mentioned, however, Neilson (50) has shown that indole is not found in the sera of surface taint butters and therefore cannot be the cause of the odour of the defect.

In the same study (50), a number of organic compounds related to the amino acids were added to milk and the odours emitted recorded. It was found that betaine, delta amino valeric acid (a derivative of both arginine and ornithine) and beta amino butyric acid gave odours reminiscent of surface taint. The following combinations of chemicals also emitted odours closely resembling the characteristic "sweaty-feet" odour; betaine and delta amino valeric acid; betaine and beta amino butyric acid; betaine and isovaleric acid; betaine and para hydroxy phenylacetic acid; and betaine, is-valeric acid and para hydroxy phenylacetic acid. The odour emitted from betaine itself was enhanced when the other compounds were also added to the milk. These findings suggest that the bacterial synthesis of betaine may be concerned in the development of surface taint in butter.

Wolochow, Thornton and Hood (71) of Alberta, in studies on odour production by Pseudomonas putrefaciens, one of the causative agents of surface taint in butter, have found that the "sweaty-feet" odour that is absent in skim milk cultures at pH 7.6 becomes apparent when the milk cultures of the organism were aerated in sulphuric acid solution but were not present when aerated in sodium hydroxide solution. This evidence suggests that the compound responsible for the odour is a volatile acid which is present as a salt in alkaline solutions and is freed as an acid at lower pH's.

In a later study, Dunkley, Hunter, Thornton and Hood (12) obtained evidence indicating that there is a definite connection between isovaleric acid and the "sweaty-feet" odour. Under certain unknown conditions, the "sweaty-feet" odour seems to arise possibly chemically from isovaleric acid but is distinctly different in odour from this acid. Wolochow, etc. have expressed the opinion that Pseudomonas putrefaciens may produce in milk a substance which is in the reduced state and, if odourous, is present in insufficient concentration to be detected by the sense of smell. Exposure to air oxidizes this substance to a detectably odourous state. More strongly oxidizing levels will reversibly change the compound to a non-odourous state. Further support for this opinion comes from the findings obtained from commercial and experimentally produced surface taint butter in the laboratories of the Department of Dairying at the University of British Columbia. The odour of surface taint may be absent from a sample of defective butter

when the bottle containing it is first opened, but if the bottle be closed and re-examined about ten minutes later, the odour is often present. Continued exposure has been found to decrease the concentration of the odour until it is no longer detectable.

Fosdick and Rapp (27), in the course of an investigation on the degredation of glucose by Staphlococcus albus under aerobic conditions produced a reaction mixture of a particularly foul odour which was not characteristic of any known product of fermentation.

Subsequent extraction and purification left a solution containing two organic compounds, one acidic in nature and the other neutral. Neither compound could be isolated in pure form without decomposition but identification tests indicated that the acidic compound was alpha-keto-gama-hydroxy valeric acid while the neutral one was the corresponding aldehyde. These compounds are closely related to the amino acids arginine, ornithine, histidine, proline and glutamic acid, and may possibly be related to the characteristic surface taint odour.

EXPERIMENTAL.

In order to determine if the surface taint producing bacteria are capable of forming odouriferous compounds from sodium pyruvate similar to those obtained by Fosdick and Rapp the following experiment (XVI) was undertaken. Three 50 cc. flasks were set up in duplicate and contained in addition to the 8 cc. of phosphate buffer (M/15 at pH 6.8)

(1) 1.0 cc. of M/20 sodium pyruvate plus 1.0 cc. of ten percent washed cell suspension.

(2) 0.1 cc. of M/20 sodium pyruvate plus 0.9 cc. of water plus 1.0 cc. of ten percent washed cell suspension.

(3) 1.0 cc. of M/20 sodium pyruvate plus 1.0 cc. of one percent washed cell suspension

respectively.

One of the duplicates was inoculated with a cell suspension of Proteus ichthyosmius and the other with that of Pseudomonas putrefaciens. A control containing 9 cc. of buffer plus 1 cc. of ten percent cell suspension was set up for each of the two bacterial species. The flasks were incubated at 30° C. and the odours formed observed at daily intervals. The flasks were observed to emit odours suggestive of surface taint. The odours from the flasks containing sodium pyruvate were more pronounced than the controls containing bacterial cells only, although these also gave off putrid odours.

With these results in mind, an experiment was set up to find out if the compounds causing the odours produced by the two bacterial species from sodium pyruvate could be isolated and identified. Two 250 cc. flasks containing 80 cc. M/15 phosphate buffer (pH 6.8), 10 cc. M/20 sodium pyruvate and 10 cc. of a ten percent washed cell suspension of P. putrafaciens were prepared and incubated at 23° C. for 48 hours. The flasks were removed from the incubator and one of the flasks treated as follows:

First the contents of the flask were centrifuged to remove the bacterial cells and the supernatant poured off

into a liquid-ether-extraction flask where it was extracted for 16 hrs. The ether extract (1) was taken up in water and gave a putrid odour. The residual liquid from the extraction was taken down to pH 4.0 with 20% phosphoric acid, filtered and the filtrate extracted with ether for another 16 hours. This extract (2) also gave a putrid odour when taken up in water.

The second flask was taken down to pH 4.0 directly with phosphoric acid, filtered and the filtrate extracted with ether for 16 hours. This extract (3) when mixed with water also gave a putrid odour. An attempt was made to obtain a 2,4, dinitro phenylhydrazone precipitate from these extracts but the quantities of material were too small to give anything more than a slight precipitate.

In the procedure of Dunkley, etc. the odouriferous compounds were separated from the remainder of the milk culture by steam distillation of the acidified culture. This procedure would further hydrolyze, to a considerable degree, the products of the bacterial protein decomposition and it would not be possible to determine which portion of the distillate was from bacterial hydrolysis and which was from chemical hydrolysis. The hydroxy and keto acids of the lower fatty acids are usually ether soluble and therefore should be removed by ether extraction from a culture containing them. In order to obtain data on this hypothesis a flask containing 100 cc. of skim milk and 10 cc. of a ten percent washed cell suspension of Pseudomonas putrafaciens was prepared and incubated at 23° C. for twenty days. The protein

of the milk was then largely converted to soluble decomposition products and emitted a foul, putrid odour. The contents of the flask were centrifuged and the pH of the supernatant taken. It was pH 5.9. The supernatant which was a bright yellow colour suggesting the presence of a flavine, was then extracted in a liquid ether extractor for 12 hours. The extract thus formed (1) was soluble in water and gave off a pungent fruity odour. The residual fluid from this extraction was taken down to pH 4.0 with phosphoric acid and re-extracted with ether for a further 12 hours. This extract (2) was only sparingly soluble in cold water and emitted a mixture of odours resembling those from well ripened Oka cheese. The extract (2) was treated further as follows:

It was tested for solubility in a number of solvents and found to be slightly soluble in cold ether and hot water, soluble in hot ether, and very soluble in 95% alcohol. The extract was finally dissolved in alcohol and filtered. The filtrate was evaporated to dryness giving a yellowish white residue to which was added a small quantity of hot water. The residue clumped together in the water and oiled off into solution when the water was raised to boiling point. The solution was evaporated to dryness giving a yellow-brown oily residue which when dissolved in hot ether crystallized out from the ether as white needles with a melting point of 175-177° C. The quantity of crystals was insufficient to permit further identification.

With the object of obtaining information on the ether solubility of the decomposition products of certain

amino acids whose chemical structure suggested that odouriferous compounds might result from their breakdown a further experiment (XVII) was undertaken. The amino acids employed were arginine, histidine and proline and the bacteria used were Proteus ichthyosmius and Pseudomas putrefaciens. Six flasks containing 160 cc. of M/15 phosphate buffer (pH 7.4), 20 cc. of M/20 Amino acid and 20 cc. of one percent washed cell suspension were prepared and incubated at 30° C. Each flask was treated individually as follows:

Flask (1) containing Proteus ichthyosmius in arginine was removed from the incubator after 7 days and the pH taken electrometrically. It was pH 8.0. The contents of the flask were taken down to pH 4.0. with phosphoric acid and extracted with ether for four hours. The small amount of extract obtained failed to give off a putrid odour and did not dissolve readily in water.

Flask (2) containing Proteus ichthyosmius in histidine was treated the same as flask (1.). The pH following incubation was pH 7.15. The extract did not emit a putrid odour. In an attempt to isolate the ether insoluble decomposition products of histidine, the residual fluid following ether extraction was filtered, raised to pH 10.0 with sodium hydroxide and evaporated to dryness in vacuo. The residue was taken up in 95% alcohol and refluxed for about one half hour. The alcohol mixture was then filtered and the filtrate evaporated to dryness. The residue was taken up in a small quantity of distilled water and a few cc. of 19% phosphotungstic acid in 5% sulphuric acid were added. The

heavy white precipitate formed was filtered off, washed and dried carefully.

Flask (3) containing Pseudomonas putrefaciens in histidine was removed from the incubator after 14 days and treated after the same manner as flask (2). The pH after incubation was pH 7.55. A similar phosphotungstic precipitate was obtained. As a control check the phosphotungstic precipitate of histidine was prepared and found to resemble closely the precipitates obtained from flask (2) and (3). The compounds precipitated were basic in nature otherwise they would not have combined with phosphotungstic acid.

Flask (4) containing Pseudomonas putrefaciens in the presence of proline was taken from the incubator after 14 days' incubation and treated the same as flask (1). The pH following incubation was pH 7.45. No putrid odours were observed in the ether extract.

Flask (5) containing Pseudomonas putrefaciens and arginine was permitted to incubate for 20 days, following which it was treated the same as flask (1). After incubation the pH was 7.75. The extract obtained emitted no putrid odours.

Flask (6) containing Proteus ichthyosmius in proline had a pH of 7.45 after 20 days' incubation and when treated in a similar manner to flask (1) gave an extract free from putrid odours.

DISCUSSION

The findings obtained from the experiments described in the above section serve to discourage certain lines

of thought from the investigation into the cause of surface taint rather than to give positive evidence in any one direction. The inability to isolate odouriferous compounds from the decomposition products of arginine, histidine and proline - three amino acids whose hydrolytic products suggest odour formation - indicates that possibly these acids are not directly responsible for the elaboration of the sweaty-feet odour of typical surface taint. It must be remembered, however, that the techniques employed in this investigation may not have provided the correct conditions of potential for the emittance of the odours. Also the odours from the compounds may have been masked by other cactors or substances, or the compounds themselves may not have been ether soluble. This branch of the investigation into the cause of surface taint cannot be dispensed with as yet because of insufficient evidence to prove or disprove the hypothesis that the acidic decomposition products of certain amino acids may be responsible for the sweaty-feet odour. The much sought after odour has been shown to be present at different times during the course of the experimental section of Part I in cultures containing the amino acids used above - arginine, histidine and proline.

The elaboration of the typical odour of surface taint appears to be dependent upon a number of physical, chemical and biological factors. The first of these which may be physical or chemical is that the odour is not formed in butter made from raw cream nor in raw milk inoculated with surface taint producing bacteria, but is formed when the milk

or cream employed is pasteurized. It would appear that the elaboration of surface taint is dependent upon a subtle change induced by the heat on the protein complex of the milk or cream. A second factor is that the compound or compounds are not always present in the odouriferous state, but require a certain oxidation-reduction potential before they emit the odour. Evidence for this point comes, as previously mentioned, from the appearance and disappearance of the surface taint odour in butter exposed to the air. The outstanding biological factor is that all species of putrefactive bacteria do not cause surface taint in butter. The number of species of bacteria capable of elaborating the defect has been shown to be very limited - Pseudomonas putrefaciens being the major causative agent and Proteus ichthyosmius also producing the defect but not so regularly.

The source of the compound responsible for surface taint may not necessarily be one of the amino acids studied nor an amino acid at all. The precursor of the odouriferous substance may be a larger molecule such as a simple peptide. Evidence for this idea comes from the ability to isolate odour-forming compounds from milk combined with the inability to show the presence of such compounds among the decomposition products of certain amino acids. The difficulties encountered regarding the stability of the odour or the substance responsible for the odour further complicate the problem of determining the cause of surface taint. Considerable investigation is still required before more definite conclusions regarding the source of the sweaty-feet odour can be reached.

SUMMARY AND CONCLUSIONS

The problem under investigation has been outlined and the following two approaches to its solution have been made:

1. An investigation of the conditions affecting ammonia formation from arginine, aspartic acid, glutamic acid, histidine and proline by two species of surface taint producing bacteria - Proteus ichthyosmius and Pseudomonas putrefaciens.
2. A study of the production, isolation and identification of compounds evolving odours identical with or intimately related to the characteristic surface taint odour.

The literature concerning the conditions governing deamination of amino acids has been reveiwed in detail.

The general methods and procedures employed have been outlined.

It has been shown that the general optimum range in pH for the formation of ammonia from amino acids is from pH 6.0 to pH 8.5 and that the individual amino acids vary within this range.

Increase in the age of the growth culture has been found to decrease the subsequent ammonia formation from histidine and proline but to have no effect on the amount elaborated from arginine, aspartic acid and glutamic acid.

The conditions of oxygen supply during growth and subsequent deamination did not materially affect the

ammonia formation from arginine, aspartic acid, glutamic acid and histidine but did affect that from proline. Anaerobic conditions of growth and deamination gave only one-half the quantity of ammonia formed under aerobic conditions.

Graphs are presented comparing the ammonia formation from the five amino acids by Proteus ichthyosmius and Pseudomonas putrefaciens at intervals over a period of one year.

Proteus ichthyosmius has been shown to produce acid and gas from glycerol, mannitol, glucose, galactose, sucrose, maltose, dextrin and salicin and to have no immediate action but slowly to become alkaline in xylose, lactose, dulcitol and the control lacking a carbohydrate. Pseudomonas putrefaciens, on the other hand, produces acid and gas from xylose, mannitol, glucose, sucrose, maltose and salicin, slowly produces acid with no gas from glycerol and has no immediate action but slowly becomes alkaline in lactose, dextrin, dulcitol and the control without a carbohydrate. The differentiating carbohydrates for these microorganisms are xylose and dextrin - Proteus ichthyosmius ferments dextrin but not xylose while Pseudomonas putrefaciens ferments xylose but not dextrin.

The presence of glucose in tryptic casein digest growth agar decreases the general rate of growth and subsequent activity of the bacterial cells of Proteus ichthyosmius.

The effect of the presence of carbohydrates in the

buffer medium has been shown to be dependent largely upon the rate of fermentation and subsequent acid production that takes place. The carbohydrates themselves do not materially affect the ammonia formation from the various amino acids but the fermentation acids lower the pH of the cultures to level unfavourable to further bacterial activity.

It was found that the addition of adequate buffer to the cultures controlled the hydrogen-ion concentration thereby permitting bacterial activity to continue. This activity included not only further deamination but also the utilization of the available carbon sources from the fermented carbohydrates along with the ammonia as a nitrogen source for cell multiplication.

A study has been made of ammonia formation from a number of non-amino acid nitrogenous compounds. It has been shown that in addition to the iminazol and pyrollidine rings, the bacterial species employed herein have the ability to open the pyrimidine, purine and pyridine rings with subsequent formation of ammonia.

A detailed discussion of the breakdown of the six amino acids - arginine, ornithine, aspartic acid, glutamic acid, histidine and proline - by the two species of bacteria - Proteus ichthyosmius and Pseudomonas putrefaciens - has been given.

A comparison of the approach made to the problem of deamination in this investigation with that made by other workers and the factors involved is outlined.

With the object of isolating and identifying the

compound or compounds responsible for the sweaty-feet odour of surface taint, a series of experiments was undertaken, (Part II).

While the findings of this part of the investigation did not yield positive results, they have provided data on the nature of the decomposition products of the amino acids employed and have suggested further studies that may be undertaken.

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