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Effect of Amino Acids on the Nature of the Nitrogenase System in Aerobacker Aerogens

Duane C. Yoch

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EFFECT OF AMINO ACIDS ON THE ADAPTIVE NATURE OF THE NITROGENASE SYSTEM IN AEROBACTER AEROGENES

BY
DUANE C. YOCH

A thesis submitted in partial fulfillment of the requirement for the degree Master of Science, Department of Bacteriology, South Dakota State University
1965

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EFFECT OF AMINO ACIDS ON THE ADAPTIVE NATURE OF THE
NITROGENASE SYSTEM IN AEROBACTER AEROGENES

This thesis is approved as a creditable and independent investigation by a candidate for the degree, Master of Science, and is acceptable as meeting the thesis requirements for this degree, but without implying that the conclusions reached by the candidate are necessarily the conclusions of the major department.

Thesis Adviser

Date

Head, Bacteriology Dept.

Date
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INTRODUCTION

In the past 30 years many aspects of biological nitrogen fixation have been studied intensively by many different workers. These studies have included: the agents of fixation, the physiology of fixation, inhibitors of fixation, cell free nitrogen fixation and symbiont relationships during fixation. A very general classification of the study presented here would place it with the physiology of fixation.

Ten years ago workers in the field postulated the nitrogenase system was adaptive in nature. This was the result of several workers finding it necessary to add a small amount of fixed nitrogen to their cultures before they would fix atmospheric nitrogen. Thus far the only work that has been done on the inductive phenomenon of the nitrogenase system has been by other workers in this laboratory. Results from recent study in this laboratory of the effects of nitrogen supplements on nitrogen fixation suggest that amino acid supplements and the free amino acid pool play a significant role in the formation of the nitrogen fixing enzyme(s). This study was carried out to investigate further the effect of exogenous and intracellular amino acids on the synthesis of the nitrogenase system.
Nitrogen fixation was first observed in the coli-aerogenes group of bacteria by Skinner (1928). He observed fixation in two and possibly three organisms of the 23 he had isolated from sewage. He called these isolates *Bacterium aerogenes* [Aerobacter aerogenes].

Our knowledge of nitrogen fixation by *Clostridium* and *Azotobacter* was at this time, well established by the findings of Winogradsky (1898), and Bertholet (1894), Béijerinck and van Delden (1902) and others. Brestol and Page (1923) had reported fixation by algae and Duggar (1916) reported it in fungi. Although there are prior references to nitrogen fixation on what were probably aerobacter strains, Skinner has received most of the credit for his observations of fixation in this genus. Using the sensitive isotope method of Burris *et al.* (1943), Newton (1952) surveyed several strains of this organism under a variety of well-controlled conditions and obtained slight but definite fixation. Ehat and Palacios (1949) noted an increase in the level of nitrogen in soil when the soil was inoculated with *A. aerogenes*. Hamilton and Wilson (1955) finally confirmed the equivocal findings of fixation in *Aerobacter aerogenes* and added several new strains to the now expanding list. By culturing the organisms anaerobically in a well buffered medium they could show the organisms fixed sufficient nitrogen to be measured by a semi-micro kjaldahl method (Wilson and Knight, 1952). Their attempts to grow the organism...
on Burk's nitrogen-free medium were unsuccessful; it had always been necessary to add a small amount of fixed nitrogen to the medium. The quantity added was 30 μg/ml as yeast water nitrogen. Hamilton and Wilson (1955) postulated that the necessity of adding minute quantities of fixed nitrogen to initiate fixation was that the organism's nitrogenase system was adaptive.

The next major observation of nitrogen fixation by *Aerobacter aerogenes* was by Jensen (1956) in Denmark. He obtained fixation by 2 of 3 strains he had isolated from water. The composition of the medium he used is essentially that of Hamilton and Wilson (1955) except that he found it necessary to add yeast extract to his medium. He could not get fixation without the yeast extract and so postulated that it must have been a growth factor that was necessary for the fixation of molecular nitrogen. Pengra and Wilson (1958) made an extensive study of the physiology of fixation by *Aerobacter aerogenes* using one of the strains Hamilton (1955) had previously worked on. They used a modification of the medium of Monod and Wellman (1947) with much success. It differed from Burk's nitrogen-free medium, which to this time had always been used for fixation experiments of the aerogenes group, in that it contained a soluble buffer to accommodate the high acid production of these organisms. They were also able to demonstrate fixation without the 30 μg/ml of ammonical nitrogen Hamilton and Wilson (1955) had found necessary. When a small quantity of ammonium acetate-nitrogen was added to a culture that
had an N₂ atmosphere from the start, a second lag was noted. This was a typical example of diphasic growth which Rickenberg and Lester (1955), Vogel (1961) and others have associated with the adaptation of an enzyme. An elaboration of this experiment using the N₁⁵ isotope showed that N₂ gas was not taken up until after the ammonia was exhausted from the medium. Following the exhaustion of ammonia a second lag occurred. It was not until the end of this lag that growth began on molecular nitrogen. They suggested that the second lag period corresponded to the adaptive lag during which the enzyme was induced.

Goerz and Pengra (1961) while working with a species of Achromobacter observed a similar adaptive lag. The data of Pengra and Wilson (1958) indicate that there was no production of the nitrogenase enzyme during growth on ammonia. This was assumed because the culture failed to grow immediately on nitrogen gas after the depletion of ammonia from the culture. In light of the work of Vogel (1957) these results might be interpreted to mean that as long as ammonia is present in the culture it will repress the formation of the nitrogen fixing enzyme. The other possibility is that during the adaptive lag the nitrogenase system is being formed from the nitrogenous reserve built up on ammonical nitrogen.

Mortenson (1961) working on nitrogen fixation by cell free extracts of Clostridium pasteurianum found that preparations from cells grown on ammonia did not yield nitrogen-fixing extracts when subjected to
the same procedures that gave active extracts from nitrogen-grown cultures. He therefore concluded that fixation activity appears to be an inducible (or repressible) character as did previous workers with A. aerogenes. Patil (1963) carried out further experiments on the adaptive nature of the nitrogenase system. He noted that the cell washings and a cell dialysate shortened the initial lag of the nitrogen-fixing culture. It was first thought that a growth factor of some sort was contributing to the shortening of the lag. However, upon analysis of the cell dialysate he found that it consisted mainly of a number of amino acids. In another series of experiments he added 10 micrograms of amino acids as vitamin-free casein hydrolysate-nitrogen to a culture and to a parallel culture an equal amount of ammonical nitrogen. Both sets of cultures were under N₂ gas from the time of inoculation. The results were very interesting in that the culture containing the ammonical nitrogen again showed the diphasic growth indicating that the ammonia had been assimilated before any growth occurred on N₂ gas. However, the culture containing the casein hydrolysate-nitrogen gave smooth and continuous growth. The culture had made a smooth transition from growth on casein hydrolysate-nitrogen to molecular nitrogen. Patil concluded from these data that the casein hydrolysate exerts a stimulatory action on induced formation of the nitrogenase system. Thus, casein hydrolysate seems to supply the essential or stimulatory amino acids for the formation of nitrogenase.

Halvorson and Spiegelman (1953) working on the formation of maltozymase in yeast found that after prolonged or repeated starvation
yeast cells could not form maltozymase unless amino acids were supplied exogenously. Millen and Lichstein (1951) have reported that a combination of six amino acids; namely, methionine, cystine, valine, glutamic acid, tyrosine and lysine were as effective as hydrolysed casein in inducing formic hydrogenlyase formation in *Escherichia coli* during growth on a mineral salts glucose medium. From the data of these workers it would seem that enzyme induction is dependent on an amino acid or a mixture of amino acids to act as precursors for the formation of the enzymes.

Taylor (1947) using the method of Gale (1947) determined the free amino acid levels of a considerable number of bacteria. She found no significant amounts of amino acids in gram-negative bacteria. Both yeasts and gram-positive bacteria were found to contain an appreciable amino acid pool. Although Taylor (1947) was not able to detect a free amino acid pool in gram-negative bacteria, Mandelstam (1955); Britton et al. (1955); Markovitz and Klein (1955) and Zaitseva et al. (1963) were all able to demonstrate an amino acid pool in gram-negative bacteria.

These studies, an extension of the work of Patil (1963), were designed to study the effect of amino acids on the diauxic lag. The amino acid pool has also been investigated to determine its role in the adaptive formation of the enzyme. The fact that cell-free nitrogen fixation has not yet been achieved with *A. aerogenes* limits the investigation to some extent. The nature of the nitrogenase system makes it difficult to apply the methodology used in studying
other adaptive enzyme systems. One of the reasons for difficulty is that the inducer, molecular nitrogen, is necessarily the substrate. This fact rules out a whole series of possible experiments. In these studies enzyme activity could not be measured directly. Cell growth is, at present, the method available for measuring the activity of the induced enzyme. The difficulties inherent in analyzing intact cell systems, which by necessity might concern problems of permeability, availability of substrates and cofactors, have been previously stressed (Cohn, 1957 and Pardee, 1959). These studies cannot, therefore, prove or disprove the existence of an adaptive enzyme, but can only show the effects of various amino acid supplements on the diphasic lag which is indicative of an adaptive enzyme. As most of the inducible enzymes so far discovered are catabolic in nature (Pardee, 1959), the fact that nitrogenase is adaptive is of significant interest because it is involved in synthetic reactions.
MATERIALS AND METHODS

Organism Employed

*Aerobacter aerogenes* strain M5al, an isolate from a butylene glycol fermentation, was used in these studies. The organism was isolated by Dr. McCoy of the Department of Bacteriology, University of Wisconsin. Although this strain was characterized by Hamilton and Wilson (1955), they called the organism M5aL. It is believed this was a typographical error. Pengra and Wilson (1958) in their physiology study of this organism also called it M5aL. Patil (1963) and Yoch and Pengra (1964) after realizing the previous error both called this strain of *A. aerogenes*, M5al. It will be referred to as strain M5al in this study.

The genus name of this organism, as are all other aerobacter-like nitrogen fixers, is in contention. Edwards and Ewing (1962) present very interesting biochemical and antigenic comparisons of the genera *Aerobacter* and *Klebsiella*. Comparing the biochemical characteristics of our strain, M5al, it would appear that this is of the *Klebsiella* group. E. W. Ewing, Communicable Disease Center, Atlanta, Georgia classified this strain as a species of *Klebsiella* because of a quellung reaction with the sera of types 8, 11, 21, 26 and 69. Centifanto and Silver (1964) have isolated a leaf-nodule endophyte of *Psychotria bacteriophila* that is very similar biochemically to our M5al. Three different laboratories have independently classified the isolate within the *Klebsiella* taxon. Pengra
(1964) also raised the question of the taxonomic position of some 60 water and soil nitrogen fixers that he had isolated. Biochemical tests on them and antigenic tests on a selected group showed them to be in Edwards and Ewing's (1962) klebsiella group. However, he feels that "as long as the two genera are described as they are, these organisms should be in the genus Aerobacter."

Although there is some confusion as to what genus this organism belongs and there has been confusion as to the strain, in this study it will be called Aerobacter aerogenes strain 45al. Previous studies with this organism have shown it to be free from gum formation (Pengra and Wilson, 1955). Patil (1963) found that the organism clumped heavily. This was overcome by using sodium chloride in the growth medium as described in the next section on medium. The organism readily utilizes combined nitrogen in the form of casein hydrolysate or ammonical nitrogen.

The organism was maintained on nutrient agar slants. Transfers were made monthly. Because of the highly specific medium used, the same culture was often used in several experiments without fear of contamination.

Medium Used for Growth Studies

The medium originally suggested by Monod and Wollman (1947) for Escherichia coli has been modified by Pengra and Wilson (1958) and used in their studies on the physiology of fixation by A. aerogenes. The medium was further modified (Patil, 1963) by
incorporating a physiological concentration of sodium chloride and reducing the concentration of monobasic and dibasic phosphate salts to half. The addition of the relatively large amount of sodium chloride prevented clumping of the cells. This was necessary to facilitate turbidity determinations of the growing culture.

As Pengra and Wilson (1959) could show no requirement for calcium while the organism was fixing molecular nitrogen or assimilating combined nitrogen, calcium was eliminated from the medium with no apparent effects. The additional sodium chloride did not affect growth of the organism on either gaseous or combined nitrogen. The final composition of the medium was:

**Solution I**

\[ \text{Na}_2\text{HPO}_4 \quad 6.25 \text{ g} \]

\[ \text{KH}_2\text{PO}_4 \quad 0.75 \text{ g} \]

Distilled water \quad 500 \text{ ml}

**Solution II**

\[ \text{MgSO}_4 \cdot 7\text{H}_2\text{O} \quad 0.2 \text{ g} \]

\[ \text{NaCl} \quad 8.5 \text{ g} \]

Fe-Mo soln. (Wilson and Knight, 1952) \quad 1.0 \text{ ml}

Sucrose or Mannitol \quad 15-20 \text{ g}

Distilled water \quad 500 \text{ ml}

The two solutions were autoclaved separately and mixed just before use. The pH of the medium after mixing was 7.5.
The buffered saline used in these studies contained Na₂HPO₄, 6.25 g.; KH₂PO₄, 0.75 g.; and NaCl, 8.5 g. dissolved in a liter of distilled water. After autoclaving the pH was 7.5.

**Nitrogen Supplements Added to Medium**

**Amino acids**: Stock solutions of the individual amino acids were made up to contain 150 µg N/ml. Another amino acid solution was used, but this contained 0.5 µg/ml of each amino acid found in yeast extract. These solutions were sterilized by autoclaving and stored under refrigeration.

**Ammonium acetate solution**: The stock solution was prepared in distilled water and sterilized by filtration through a "Millipore" filter.

**Casein hydrolysate**: A stock solution of casein hydrolysate was made up to contain 1000 µg N/ml. This was prepared by dissolving the required amount of vitamin-free Casamino acids (Bacto) in distilled water. The solution was sterilized by autoclaving.

**Yeast extract**: A stock solution of yeast extract was prepared by dissolving the required amount of Bacto yeast extract in distilled water. The solution was sterilized by autoclaving.

**Procedures used in Growth Studies**

The procedures used for the growth studies were essentially those of Pengra and Wilson (1958). Turbidity readings for part of the growth studies were made from 250-ml Erlenmeyer flasks equipped
with colorimeter tube side arms. The side arms were fused to the flask at a 20 degree angle below the horizontal. The culture was merely tipped into the side arm of the flask and the turbidity was read. This technique allowed turbidity readings to be taken without disturbing the gas phase. The flasks were closed with rubber stoppers fitted with a glass tube vent on which was placed a short length of rubber tubing closed with a screw type pinch clamp. The flasks were evacuated three times and subsequently flushed with high purity nitrogen. After final evacuation 0.9 of an atmosphere of nitrogen was placed in each flask. A screw clamp on the rubber tube contained the nitrogen. The negative pressure was necessary because of the fermentation gases formed. The flasks were shaken on a "Brunswick" rotary shaker and were incubated at 31 degrees centigrade. When mass cultures were needed in the latter part of these studies a 2 liter Erlenmeyer flask containing 1.5 liters of medium was used. The flasks were closed with three-holed rubber stoppers carrying a gas inlet tube, to the end of which was attached a sintered glass gas dispersion tube, an outlet tube, and a sampling tube. High purity tank nitrogen was slowly bubbled through the culture. The organism was transferred from a slant culture to a 50 milliliter portion of growth medium in a 250 milliliter flask. The culture was treated as described above. To initiate growth it was necessary, in most cases, to add up to 10 micrograms of nitrogen as either ammonium acetate or an amino acid source, such as yeast extract. The culture was harvested in its exponential growth phase by centrifugation and used to inoculate either the 50 milliliters
of medium in the "side-arm" flask or the 1.5 liters of medium in the 2 liter flask. Turbidities were measured with a Klett-Summerson photoelectric colorimeter using a 660 millimicron filter.

Assay For Free Amino Acid Pool

The amino acid pool was obtained from the cells of a 600 milliliter portion of a 1.5 liter culture. The culture had a continuous flow of nitrogen bubbling through it as described in the section on growth. The method used for extracting the pool was, in general, that of Gale (1947). The dry weight of the cell paste was calculated from a standard curve. The cell paste was then suspended in 10 milliliters of water and heated at 100 degrees centigrade for 20 minutes to liberate the intracellular amino acids. The suspension was centrifuged and the clear supernatant extract poured off.

The preparation of the sample for analysis was according to the procedure recommended for blood plasma samples that are to be analysed for amino acids on the Beckman Spinco Model 120 amino acid analyzer. The sample was deproteinated with 15 milliliters of a saturated picric acid solution. The precipitate was centrifuged for 10 minutes at 35,000 x g and the clear supernatant liquid decanted. To remove the picric acid from the sample it was passed through an ion exchange column. The resin used was Fisher's Rexyn CO 3 in the chloride form. Sufficient resin was used to give a bed 5 centimeters high. The bed was washed with 15 milliliters of 1N HCl, followed by water until the effluent was neutral. After the sample had been
passed through the prepared resin bed, the bed was washed with three milliliter portions of 0.02N HCl. The effluent and washings were then evaporated under reduced pressure to the desired concentration. In some cases material remained suspended in solution. It was removed by adding a few milligrams of Celite (analytical filter aid) and filtering the suspension by gravity through a paper previously washed with 1N HCl and water.

In order to determine the total ninhydrin-positive material that makes up the free amino acid pool a modification of the method of Moore and Stein (1948) was used. The 0.2M citrate buffer used in this procedure was prepared from 21.008 grams of citric acid (reagent grade) and 200 milliliters of N NaOH diluted to 500 milliliters. The pH of the buffer when diluted with an equal volume of water was 5.0 ± 0.1. The ninhydrin solution was prepared by dissolving 0.80 grams of reagent SnCl₂·H₂O in 500 milliliters of the citrate buffer. This solution was added to 20 grams of ninhydrin dissolved in 500 milliliters of methyl cellosolve. The reagent was transferred to a reservoir bottle and a stream of nitrogen was run through the solution for about 30 minutes. When stored under N₂, the solution can be kept for at least a month without deterioration. A diluent solution consisting of equal volumes of water and n-propanol was also required. A standard solution of aspartic acid was used to prepare the standard curve. In the original method of Moore and Stein (1948), acidic fractions were carefully neutralized with NaOH using a preliminary sample to estimate a methyl red end point. In these studies an
alternative method of evaporating aliquots to dryness was used. The small amount of acidity from the non-volatile hydrochlorides is well within the buffer capacity of the ninhydrin reagent. A 0.2 milliliter aliquot of each sample was transferred to a matched colorimeter tube and placed in a rack in a vacuum desiccator. The desiccator was partially immersed in a hot water bath and evacuated continuously with an aspirator. When the sample was evaporated to dryness, 1.5 milliliters of the ninhydrin solution was added to each tube. Aluminium caps were placed on the tubes after they were shaken to dissolve and mix the sample. The samples were then heated for 20 minutes in a boiling water bath. To each tube 5 milliliters of the water-propanol diluent was then added and 15 minutes was allowed for the color to develop fully. Readings were taken on a "Spectronic 20" (Bausch and Lomb Optical Company) photoelectric colorimeter using 565 millimicron wave length. Using the above sample preparation and colorimetric methods the samples were designated as total ninhydrin positive material.

The sample preparation method for blood plasma as described above was also used for samples on which an amino acid analysis was done. There was one exception to the procedure in that all samples were evacuated to dryness and then redissolved in 2 milliliters of pH 2.2 citrate buffer. The analyses were done on a Beckman Spinco Model 120, amino acid analyzer.
Paper Chromatography of Amino Acids

Solutions of amino acids to be used as standards were made up in distilled water to contain 150 micrograms of nitrogen per milliliter. Unknown samples were concentrated to approximately the same nitrogen level. The standards and the unknown samples were then spotted on Whatman No. 1 filter paper. The spots were put on a baseline 1 inch from the bottom of the paper. The mobile phase of the solvent system consisted of phenol and water, 115:10 in a battery jar. The stationary phase consisted of 20 milliliters of 0.3\% NH$_4$OH (1.0 ml. of 58\% NH$_4$OH/100 ml. of water). This part of the solvent system was put in a small beaker and placed in the bottom of the battery jar. The chromatogram was placed in the battery jar so that the bottom of the paper was in contact with the mobile phase of the solvent. Development was allowed for 5-6 hours. After the chromatogram was dried over night it was rinsed in ether to remove the phenol and again allowed to dry $\frac{1}{2}$ hour. The chromatogram was then sprayed with the ninhydrin solution (0.2\% in ethyl alcohol) and dried in a oven at 98 degrees centigrade.
RESULTS AND DISCUSSION

While conducting a survey for the presence of aerobacter-like nitrogen fixers in South Dakota soils and waters it was noted that after isolating a number of organisms from enrichment cultures they would not fix molecular nitrogen in a purely inorganic medium. In recalling Jensen’s (1956) observations concerning the need for yeast extract, added 20 micrograms per milliliter of yeast extract to the nitrogen-free medium. Fixation was then observed in 80% of the isolates.

This same level of yeast extract was also added to medium which was inoculated with the known nitrogen fixer, \textit{A. aerogenes} M5al. This organism, as Fengra and Wilson (1958) had shown, does not require a fixed source of nitrogen to initiate fixation. As is shown in Figure I, the addition of the yeast extract reduced the initial lag phase of the culture by 2 to 3 hours.

At this point the composition of yeast extract was examined. This information was available in a special report of the General Society for Microbiology (Sykes, 1956). Here it was found that 45 percent of the weight of yeast extract consists of amino acids and that nearly all the known amino acids are present. Also found in small amounts are ammonia nitrogen, 0.08 percent and purine base nitrogen, 0.62 percent. Yeast extract also contains numerous growth factors, trace elements and naturally occurring vitamins of the \textit{B}-complex. Trace elements were eliminated as the possible stimulating factor when other workers in this laboratory ashed yeast.
Figure I. Effect of yeast extract on nitrogen fixation

Yeast extract at 20 µg of N/ml, I; Control with no fixed nitrogen added, II.
extract and added the ash back to a culture of strain M5al and found no stimulatory activity.

Extensive work was also done on the possibility that a vitamin of the B-complex might be the stimulatory factor. Emphasis was placed on biotin as the possible stimulator. Carnahan and Castle (1958) found that biotin was necessary to attain maximum growth of nitrogen fixing clostridia. Biotin was also shown to be a growth stimulant for the root nodule bacteria (West and Wilson, 1940). The methods of Lichstein (1949) and Lichstein and Christman (1948) were followed to no avail in trying to activate biotin into serving as the stimulator for nitrogen fixation. The stimulatory activity of a ferrichrome compound was also checked with strain M5al. This compound was kindly supplied to us by J. B. Neilands, University of California. This ferrichrome compound actually increased the initial lag phase, the lag increasing with increased ferrichrome concentration. With the trace elements and vitamins giving no results they were eliminated. This left the amino acids as the possible stimulators or activators of fixation.

Figure II shows the result of adding yeast extract, vitamin-free casein hydrolysate and a solution containing 0.5 micrograms per milliliter of all amino acids found in yeast extract to three different portions of the test medium. As can be seen from the graph all three sources of amino acids served equally well in eliminating the initial lag phase of A. aerogenes. The three amino acid sources also served equally well in initiating growth in the soil and water.
Figure II. Effects of added amino acid supplements on nitrogen fixation

Casamino acids, I; 1.0 µg of each amino acid found in yeast extract, II; yeast extract, III; control with no amino nitrogen, IV.
isolates. It is possible that an amino acid(s) is serving as a growth factor for the isolates or that a source of fixed nitrogen is necessary, although equal levels of ammonium acetate-nitrogen would not initiate growth. Once growth was attained with the isolates the amino acids were not necessary to initiate growth in subsequent passages of the cultures to new nitrogen-free media. As A. aerogenes is a soil organism, soil extract prepared according to Pramer and Schmidt (1964) was used to find out if it would supply the necessary amino acids to eliminate the initial lag of this organism. No stimulation was obtained with soil extract.

With these preliminary data and the results of previous workers in this laboratory, our conclusions were similar to those of Patil (1963), in that, the amino acids are serving as precursors for the adaptive formation of the nitrogenase system. It was our aim from this point on to obtain more conclusive evidence as to whether or not the nitrogen fixing system was inducible and to determine the role of free amino acids in this system.

If the nitrogen fixing system is adaptive as supposed, it would then be logical to assume that molecular nitrogen is the inducer. In order to shed more light on these assumptions an experiment was designed on the basic premise that if an enzyme is constitutive it will be present even when the necessary substrate or inducer is absent from the growth medium. If the enzyme is inductive it will be absent if the inducer is absent. In the nitrogenase system the substrate and
inducer are one and the same, that is, molecular nitrogen.

Mortenson (1961) has given evidence for an inductive nitrogenase system in *Clostridium pasteurianum*. He found that the cell free extracts of cells grown on ammonia did not have the ability to fix nitrogen, whereas extracts of *N₂*-grown cells could fix nitrogen.

Figure III shows the growth curves of cultures in which the inoculum for part of the experiment had been grown on casein hydrolysate-nitrogen (curves I and III) and the other part on nitrogen gas (curves II and IV). The cells were washed with buffered saline and inoculated into medium which contained 10 micrograms of casein hydrolysate-nitrogen per milliliter. The cultures all had a helium cover. The cultures with the casein-grown and *N₂*-grown inocula were allowed to attain maximum growth on the limiting concentrations of casein hydrolysate-nitrogen. Maximum growth was reached in both cultures at about 3½ hours. The flasks were then immediately evacuated and refilled with molecular nitrogen. A helium atmosphere was allowed to remain on designated cultures to act as controls (curves III and IV).

The graph shows a lag of nearly 3 hours in the growth of the culture with the casein-grown inoculum (curve I). This lag indicates that the nitrogenase system was not present, at least in functional quantities, to use the molecular nitrogen as a substrate. In this case it must have been serving as an inducer for the formation of the nitrogenase system. At about 5½ hours the enzyme system was fully adapted and growth proceeded on nitrogen gas.
The culture whose inoculum was grown on molecular nitrogen (curve II) shows that the rate of growth decreased slightly for a short time and then resumed growth at an increased exponential rate a few minutes after the culture was flushed with nitrogen gas. This decrease in the growth rate probably resulted from the "diluting out" of the nitrogenase system (Wainwright and Pollock 1949; Rickenberg et al., 1953). This would occur as the \( \text{N}_2 \)-grown inoculum was growing on the 10 micrograms per milliliter of casein hydrolysate-nitrogen that had been added to the medium. This decrease in growth rate would then be due to the time it took for the induced synthesis of a functional quantity of nitrogenase. As for the culture with the "casein-grown" inoculum, one would expect an infinite "diluting out" of the nitrogen fixing system and therefore a much longer lag phase. This lag would represent the time needed to synthesize a functional quantity of the enzyme.

If the permease system for nitrogen gas is considered to be part of the nitrogenase system, it could be a permeability problem that is causing the huge difference in the lag when molecular nitrogen is added to the cultures. If it is permeability, the nitrogen gas could not immediately get into the cell to act as either inducer or substrate. If this is the case, the \( \text{N}_2 \) permease is considered to be part of the nitrogenase system, the explanation would be as stated in the preceding paragraph.

Halvorson (1960) in his review on induced protein synthesis suggests that enzyme synthesis proceeds at a maximal rate within a
few minutes after the addition of the inducer. This work is substantiated by the work of Vogel (1961); Duerksen and Halverson (1959) and Jacob and Monod (1961). The lag in the growth curve of the casein-grown culture in Figure III seems to indicate that the nitrogenase system is not complete and operative for about 3 hours after contact with the inducer, molecular nitrogen. This observation is not necessarily inconsistent with Halverson's (1960) above statement. As mentioned earlier, we are measuring induction indirectly by cell turbidity. Until there was a functional quantity of the nitrogen fixing enzyme present for cell growth, our indirect method could not measure its presence. This could very well be the reason for the long lag in the culture with the casein-grown inoculum.

As was stated earlier, one of our major aims in this study was to learn more about the role of free amino acids in the inductive synthesis of the nitrogenase system. Figure II suggests the importance of amino acids in eliminating the prefixation lag of strain M5al. The need of adding low concentrations of yeast extract to the medium of soil and water isolates to initiate growth further exemplifies the importance of amino acids in nitrogen fixation.

Patil (1963) has shown still another way in which amino acids in the form of casein hydrolysate, stimulate nitrogen fixation. He used limited supplements of both casein hydrolysate-nitrogen and ammonium acetate to demonstrate the stimulatory effect of the amino acids (as can be seen in Figure IV), in the presence of ammonical nitrogen, two exponential growth phases, one on ammonical nitrogen
Figure III. Effect of adding N$_2$ gas to cultures that have attained maximum growth on a limited supply of fixed nitrogen. The inocula used were from N$_2$-grown and casein hydrolysate-grown cultures.

Casein hydrolysate-grown inoculum, I; N$_2$-grown inoculum, II; Casein hydrolysate and N$_2$-grown inocula that remained under helium, III and IV respectively.
and the second on molecular nitrogen, were separated by an adaptive lag (flat portion of curve III). In the presence of casein hydrolysate-nitrogen (curve I) no prefixation lag was seen. The growth curve was continuous and uninterrupted by any secondary lag. This means that while the culture was growing on the amino acids of casein hydrolysate the nitrogenase system was being induced. It was assumed that one or more of the amino acids present in casein hydrolysate was necessary for the formation of nitrogenase. This type of response is not unlike the results of Pinsky and Stokes (1952) who reported that formic hydrogenlyase activity in *Escherichia coli* was maximum when hydrolyzed casein was used as an exogenous nitrogen supply, whereas ammonia in contrast had no stimulatory effect on the formation of the enzyme.

In order to study the effect of a single amino acid on nitrogen fixation, L-aspartic acid and L-glutamic acid at 10 micrograms of nitrogen per milliliter were added to separate flasks of medium. In Figure IV one can compare the effectiveness of the individual amino acids (curves IV and V) to casein hydrolysate (curve I) as to their ability to overcome the adaptive lag caused by ammonia (curve III) and give smooth and continuous growth. As Figure IV shows, aspartic acid (curve IV) is as effective as casein hydrolysate (curve I) in giving a smooth transition between growth on a fixed nitrogen source and molecular nitrogen. Glutamic acid (curve V) was effective in eliminating the diauxic lag, but growth was slow and sluggish for the first 4 hours. This initial sluggish growth might be from an
insufficient rate of uptake of glutamic acid to provide for exponential growth.

Thirteen other amino acids were tested for their ability to replace casein hydrolysate and give a smooth, uninterrupted growth curve. Rather than show all the graphs for these amino acids they will be compared to curves I-VI of Figure IV. Other amino acids that gave growth curves very similar to that of aspartic acid (curve IV) were: serine, threonine, leucine, and histidine. Amino acids with a growth curve very similar to that of glutamic acid (curve V) were: tyrosine, phenylalanine, alanine, lysine, valine and methionine.

Two amino acids, isoleucine and glycine have growth curves similar to the control that had no fixed nitrogen supplement added, only an \( N_2 \) atmosphere (curve VI). Nitrogen fixation was inhibited for 12 hours in the cultures that had proline added to the medium as an amino acid supplement.

The data presented in Figure V offers a partial explanation as to why aspartic acid served so well in replacing casein hydrolysate and isoleucine did not serve at all. Figure V shows the uptake of aspartic acid from the medium to be very rapid during the time the diauxic lag was occurring in the culture with the ammonia supplement. This indicates that \textit{A. aerogenes} K51 has an aspartic acid permease which facilitates the rapid entry of aspartic acid into the cell. It also indicates that since the growth curve of the culture with the aspartic acid supplement (curve I) does not
Figure IV. Effect of limiting concentrations of amino acids in replacing casein hydrolysate as a means of overcoming the diauxic lag caused by ammonia.

Casein hydrolysate, I; ammonium acetate control under He, II; ammonium acetate, III; aspartic acid, IV; glutamic acid, V, each at 10 µg of N/ml of medium. Control with no fixed nitrogen. All under N₂ atmosphere.
have a diauxic lag, the exogenous aspartic acid that has been taken up by the organism must have been used in transamination to make the amino acids needed in forming the nitrogenase system. It might be assumed that serine, threonine, leucine and histidine, the other amino acids that gave growth curves similar to aspartic acid also have readily available permease and transaminase systems which allow them to act as did aspartic acid. There was no disappearance of isoleucine from the medium, as Figure V indicates. As expected the growth curve (III) parallels the control which contained no fixed nitrogen (curve IV). Apparently *A. aerogenes* M5al does not have an isoleucine permease system. This same explanation might also be applied to glycine which gave a growth curve similar to isoleucine. It is also possible that glycine is entering the cell but is not serving in transamination to form other amino acids. An experiment showing the uptake of glycine by the cells would have to be done before this question could be settled. As for the inhibition of fixation by proline, more work is necessary before an explanation can be offered.

The explanation given for the rather slow initial growth on glutamic acid will have to suffice for the other six amino acids that gave growth curves similar to glutamic acid when compared to casein hydrolysate. Either the amino acids were not entering the cell as fast as needed or the amino acids were not serving as substrates for transamination reactions at the necessary rate to support rapid
Figure V. Nitrogen fixation in the presence of limiting amounts of fixed nitrogen and amino acid uptake during fixation

Aspartic acid, I; ammonium acetate, II; and isoleucine, III, each at 10 µg of N/ml of medium.
Curve IV is the control with no fixed nitrogen.
growth. More work is needed to determine whether the permease systems for these amino acids are deficient or the transaminases system is deficient.

Since limiting concentrations of many amino acids gave smooth and continuous growth as opposed to the diauxic lag with a similar amount of ammonia, we postulated that it might be possible to eliminate the diauxic lag by adding organic acids to the medium along with the ammonia. These organic acids might then serve as carbon skeletons for possible amination which would result in amino acids. The amination reactions could be detected by following growth. If the diauxic lag did not occur when the limited concentration of ammonia and the organic acids were added to the medium, it would have to be assumed that amination reactions had taken place. The resultant amino acids would then have had to enter into transamination to form the other amino acids necessary for the synthesis of the nitrogenase system. The organic acids used in these studies were alpha-keto glutaric, pyruvic, fumaric and malonic. These acids were used at varying concentrations from 75 to 500 micrograms per milliliter. In the cases where NADH was a required cofactor it was used at a concentrations of 0.0001 M. Although amination reactions do occur (Woelf, 1929; De van, 1938; Greenberg, 1961) with these organic acids in microorganisms, we could not detect them in A. aerogenes M5AL Our criteria were to be elimination of the diauxic lag.

Up to this point our study had dealt mainly with the effect of amino acid supplements on nitrogen fixation. The effects were
displayed in initiating fixation in aerobacter-like isolates, shortening the prefixation lag and eliminating the diauxic lag which occurs when similar concentrations of ammonia are added to the medium. It was assumed that by adding the amino acids to the medium a build-up of the amino acid pool was occurring in the organism. These amino acids in the pool were then immediately available to be synthesized into protein, including the nitrogenase system.

The diauxic lag that occurs when low concentrations of ammonia are added to the medium afforded ideal conditions to examine the composition and the changes in the amino acid pool during the diauxic lag. Halvorson and Spiegelman (1952) were the first workers to examine the effect of variations of internal free amino acids on the capacity of yeast cells to synthesize enzymes. These data as well as those obtained with bacteria (Pinsky and Stokes, 1952; Ushiba and Magasanik, 1952) lead to the conclusions that the primary pathway of induced enzyme formation involves synthesis of new enzymes from free amino acids.

Figure VI shows the growth curve of *A. aerogenes*, the inoculum for which was grown on substrate levels of ammonium acetate under helium. The small amount of fixed nitrogen in the medium under a nitrogen atmosphere caused the diauxic lag to occur in the growth of the organism. The arrows indicate the times at which cell samples A and B were taken to be analyzed for the content and size of their free amino acid pool. The pools were extracted using the method of Gale (1947). Amino acid analysis on the Beckman Spinco Model 120 shows
Figure VI. Nitrogen fixation in the presence of a limiting concentrations of ammonia

Changes in the amino acid pool during the diauxic lag measured at times A and B.
that ninhydrin-positive materials other than amino acids and ammonia are also extracted by this technique (Gale, 1947). The cells from sample A have a pool considerably larger than those of sample B as ninhydrin-positive material. In Table I the changes in the free amino acid pool during the diauxic lag are expressed. All the measurable components of the pool showed a decrease in concentration between the onset (sample A) and the termination (sample B) of the diauxic lag. Aspartic acid decreased from 238 micrograms (sample A) to a mere trace (sample B). At the present time we can offer no explanation for this. The disappearance of aspartic acid during the lag could not be shown by paper chromatography. This was because of the large number of other ninhydrin-positive components in small concentrations. The glutamic acid in the pool was reduced from 212 to 163 micrograms, a depletion of 23 percent between samples A and B. The depletion of leucine and ammonia from the cells between the onset and the termination of the diauxic lag was very small. Although the amount of ammonical nitrogen in the pool was comparable to that of aspartic and glutamic acid it apparently was not converted to amino nitrogen by the process of amination.

Table I shows a depletion of 1374 to 885 micrograms or 34 percent in ninhydrin-positive material. This is somewhat comparable to the 42.5 percent decrease in the amino acid pool reported by Halvorson and Spiegelman (1952). It is assumed that the 34 percent depletion of the pool between samples A and B was from the utilization
of the amino acids and other ninhydrin-positive material in the synthesis of the nitrogenase system.

Table I. Changes in the free amino acid pool during the diauxic lag of Aerobacter aerogenes M5al

<table>
<thead>
<tr>
<th></th>
<th>ug/100 mg dry cells</th>
<th>ug N/100 mg dry cells</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>sample A*</td>
<td>sample B**</td>
</tr>
<tr>
<td></td>
<td></td>
<td>A*</td>
</tr>
<tr>
<td>Ninhydrin-positive material</td>
<td>1347</td>
<td>885</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>238</td>
<td>trace</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>212</td>
<td>163</td>
</tr>
<tr>
<td>Leucine</td>
<td>21</td>
<td>20</td>
</tr>
<tr>
<td>Ammonia</td>
<td>26</td>
<td>25</td>
</tr>
</tbody>
</table>

* Sample A taken at onset of lag.
**Sample B taken at termination of lag.

Chromatograms from the amino acid analyses showed that many other amino acids were present in the pool in trace quantities plus several unidentifiable peaks. A number of investigators (Halvorson and Spiegelman, 1952; Blair and Rouser, 1952) have shown that under a variety of conditions the components of the free amino acid pool behave in a quantitatively similar manner. It is, therefore, possible that the amino acids that were present only in trace quantities (0.5-5.0 micrograms) were also depleted slightly in sample B.

We feel that the observed depletion in the amino acid pool during the diauxic lag is another bit of evidence in support of an adaptive nitrogenase system. It is not known if the pool, in this case, is furnishing all or only a part of the necessary amino acids for enzyme synthesis. It is possible that, as happens during nitrogen
starvation, those proteins which tend to degrade easily will do so and contribute their amino acids to the pool (Halverson and Spiegelman, 1953). It will be necessary to examine the protein turnover of adapting cells before this point can be clarified.
SUMMARY AND CONCLUSIONS

Several workers in the field of nitrogen fixation in the past 10 years have reported the necessity of adding a source of fixed nitrogen to the medium before nitrogen fixation would occur in Aerobacter aerogenes. These observations eventually lead to the postulation that the nitrogenase system is adaptive. Other workers in our laboratory have shown that the addition of limited supplements of casein hydrolysate has a stimulatory effect under various conditions of nitrogen fixation by Aerobacter aerogenes M5al. The addition of amino acid supplements has been shown in this study to initiate fixation in aerobacter-like isolates, shorten the prefixation lag phase and eliminate the diauxic lag of a culture grown on a limited ammonia supply under nitrogen gas. It is assumed that the amino acids that are added to the medium caused an increase in the amino acid pool of the organism. These amino acids were then available for the rapid synthesis of the nitrogenase system.

Data have been presented to show that cells from casein-grown cultures did not begin to fix nitrogen immediately when the inducer, molecular nitrogen, was added to the culture flask. A lag of several hours occurred before fixation began. Fixation began in cells from an N₂-grown inoculum almost immediately after the inducer was added. It would seem logical to assume that upon addition of the inducer the culture with N₂-grown inoculum had a fully functional nitrogenase system which allowed fixation to begin immediately. The lag
in the culture with the casein-grown inoculum must then have been
due to the time required to induce the formation of the nitrogenase
system. It was noted that when amino acids were added to the medium
individually they would replace casein hydrolysate as a stimulator
of fixation to various degrees. Aspartic acid was among several
amino acids that was most effective in replacing casein hydrolysate.
Proline actually inhibited fixation for 12 hours. The other amino
acids all served some where between these two extremities. Data
indicate that in some cases the ability of an amino acid to replace
casein hydrolysate was dependent upon the permeability of the amino
acid. It was assumed that if an amino acid was taken-up by the cells
and yet did not replace casein hydrolysate the transaminase mechanism
of the cell was not functional for this amino acid.

Organic acids were added to the medium in various concen-
trations with limited amounts of ammonia. This was done in order to
determine if an amination reaction would occur to form required amino
acids. It was assumed that if the required amino acid was formed,
the diauxic lag resulting from the addition of limited concentrations
of ammonia to the medium would be overcome and growth would be smooth
and continuous. None of the organic acids that were tried served.

It seemed very likely possible that the amino acid supplements
were causing a build-up of the amino acid pool. These amino acids
were then immediately available to be used in synthesis of the enzyme.
The changes in the amino acid pool were measured during the diauxic
lag. A 34 percent decrease in ninhydrin-positive material was noted during the diauxic lag. Without being too presumptuous, it might be stated that the decrease in the pool was related to the adaptive formation of the nitrogenase system.


