THE NITROGEN NUTRITION AND METABOLISM OF EFFECTIVE AND INEFFECTIVE STRAINS OF *RHIZOBIUM meliloti*

by

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GENERAL INTRODUCTION

Man is dependent, either directly or indirectly, upon plant protein for the maintenance of his life processes. The nitrogen necessary for the synthesis of this protein is derived mainly from the soil and must be constantly replenished. In addition to losses of soil nitrogen via plant uptake the processes of erosion, leaching, sewage loss and biological denitrification annually remove tremendous quantities of combined nitrogen from the terrestrial cycle. Fertilization has been employed for many years for replenishment purposes, but is expensive, especially the synthetic nitrogenous fertilizers which depend upon high temperature and pressure for their production. By far the greatest potential source of this element is the atmosphere and an economical and efficient means of converting this free molecular nitrogen into forms which plants can assimilate is through biological nitrogen fixation. Ever since the classical experiments of Hellriegel and Wilfarth it has been realized that a major factor in biological nitrogen fixation is the symbiotic relationship between bacteria of the genus Rhizobium and certain leguminous plants. These bacteria dwell within root nodules and, in combination with the host plant, are capable of fixing large quantities of free nitrogen. Not all strains of Rhizobium are capable, however, of fixing nitrogen. Strains exist which vary from a condition of effectiveness, through ineffectiveness, to parasitism. These two latter strains are a matter of some concern as they are potentially able to cause crop failures in fields which are low in

inorganic nitrogen.

In spite of intensive investigation, beginning in 1888 when Beijerinck first isolated the nodule organism, there is still much to be learned about these microorganisms from the standpoint of mechanism of nitrogen fixation, biochemical basis of ineffectiveness and parasitism, and metabolism in general. Since the key intermediate of fixation is believed to be ammonia and the first product easily detected is glutamic acid the intracellular mechanisms involving ammonia and amino acids are of particular importance.

The present investigation was commenced under the auspices of the Department of Bacteriology, Ontario Agricultural College and the Legume Research Committee in Ontario. Its purpose was to make a somewhat general survey of the alfalfa-sweet clover organism (Rhizobium meliloti) from the standpoint of organic and inorganic nitrogen metabolism and nutrition. From a practical viewpoint it was hoped that information would be gained which would:

(a) help in the development of a rapid biochemical test for effectiveness, ineffectiveness, and parasitism among the legume bacteria,

(b) aid in the formulation of a superior synthetic medium for the cultivation and study of these microorganisms, and

(c) contribute to an understanding of the enzymic capabilities of rhizobia so that further studies on the mechanism of nitrogen fixation, interconversion of cellular nitrogen or transfer of

fixed nitrogen to the host plant could be carried out more intelligently.

This study is complicated, as are all investigations on living cells or crude cellular preparations, by the tremendous interplay of intracellular enzymes. In addition, little is known of the rates of reactions which occur in the living cell. Any particular rate is necessarily dynamic in that it is accelerated or retarded depending upon the nature of the physiochemical environment, and upon the operation of interconnected processes. All that can be said after such a study is that the organism is potentially capable of carrying out such and such a reaction, but even that represents a glitter of light in the darkness of cellular metabolism.

PART I

NITROGEN NUTRITION AND METABOLISM

INTRODUCTION AND LITERATURE REVIEW

The literature dealing with the legume nodule bacteria is filled with innumerable reports on the nutrition of these unusual microorganisms. Many of these reports are contradictory and the resulting confusion probably stems from the varied experimental conditions which the different investigators used. In some cases the media used as a base contained soil or plant extracts whose exact composition are unknown, even today. Most of the older workers employed unwashed cells as inocula and the presynthesized materials adhering to these cells were neglected in the interpretation of the experimental findings. Nevertheless, many of the reports are valuable in that they do indicate whether or not the rhizobial strains employed could utilize the various test substances, but the ability of such substances to initiate the growth of washed cells in a chemically defined inorganic basal medium could not be stated with any degree of certainty. Stated in another fashion the compounds under test could be classified as stimulatory or non-stimulatory only, and not as essential or non-essential. Another important feature that must not be disregarded in reading the available literature is the influence of physiochemical factors on the nutrition of the bacteria. Such factors as pH, Eh, temperature etc. are extremely important and it is to be lamented that much of this information has been omitted from earlier experimental reports. Species or strain differences are, of course, additional major

factors and investigations which at first appear contradictory are often a result of such inherent differences among microorganisms.

The question as to whether or not rhizobia are capable of initiating growth in chemically pure inorganic synthetic media is extremely important, but in all probability can never be answered in either the affirmative or the negative for all strains of the organism. Each strain would be expected to behave in its own peculiar fashion in this respect. Wilson and Wilson (101) found that continuous transfer of Rh. meliloti and Rh. trifolii was possible in a medium containing purified salts plus alcohol extracted glucose, sucrose or synthetic glycerol. They concluded, however, that there were at least three principal groups of organisms: a) those that grew very poorly in the absence of biotin and reached a population of about one tenth the maximum b) those that attained practically maximum growth in the absence of biotin and c) those strains that were unable to grow unless biotin was supplied. The nitrogen source in their medium was ammonium chloride. West and Wilson (98) stated that "small inocula, washed free of metabolic products of the culture from which they were taken, produce little or no growth in the absence of the Rhizobium factor". Although the "factor" in this case was a heat labile substance produced by actively growing cultures of the organism and thought to contain thiamin and riboflavin the fact is emphasized in a more recent report (48) that the "factor" may actually be any one of a number of amino acids which supply essential amino groups to the organism.

For the purposes of clarity this review has been divided into several sections, all related to the nitrogen nutrition of the nodule bacteria. The first section deals with the utilization of inorganic nitrogen while the second has reference to organic nitrogenous compounds. The final section briefly discusses the effect of pH and temperature on rhizobia.

I. Inorganic

As far back as 1890 Prazmowski (73) recommended the use of a nitrogen-poor medium for the growth of rhizobia. This recommendation was supported by such men as Zinsser (107) and Dawson (23). Harrison and Barlow (39) and Greig-Smith (36), among others, actually advocated the use of media devoid of any added combined nitrogen source. Of course traces of nitrogenous compounds in the mineral salts used in the preparation of such media would have ensured the presence of at least some combined nitrogen, otherwise the organisms, which cannot fix free nitrogen in the absence of the host plant, would have suffered from nitrogen starvation. Fred, Baldwin, and McCoy (26) reported growth stimulation of nodule organisms by the addition of nitrate to a medium such as Ashby's. Beijerinck (8), Müller and Stapp (63), Leonard (55), and Pohlman (72) all grew rhizobia in nitrate media. However, all these workers used unwashed inocula and in some cases (55), soil and plant-extract basal media as well. Müller and Stapp (63) found that ammonium salts could be utilized by unwashed cells of these bacteria, but tried

unsuccessfully to grow them in media containing nitrite. Pohlman's work (72) emphasized species differences when he reported that Rh. japonicum utilized ammonium sulphate in a mannitol mineral-salts medium to better advantage than Rh. meliloti, possibly because the soybean organism is capable of neutralizing the sulphuric acid liberated, thus prolonging the period of favorable growth.

Allison and Hoover (3), using a yeast-extract mineral-salts medium, published a report stating that potassium nitrate and ammonium chloride were utilized, but later (42) reported that the growth of certain species in a synthetic medium composed of sugar, inorganic salts and nitrate was negligible if pure ingredients were used. Although Thorne and Walker (90) advocated the addition of cysteine to the above medium to overcome the high oxidation-reduction potential, Nilsson, Bjälfve, and Burstrom (66) observed that no growth took place in this medium even when reducing substances were added or when ammonium chloride was substituted for the nitrate. Allyn and Baldwin (5) investigated the effect of the redox potential on unwashed cells of nodule bacteria and found that they grew in a solid mannitol nitrate medium, but not in mannitol nitrate broth unless cysteine was added. Thorne and Walker (89) in further studies maintained that in their work several Rhizobium species were unable to maintain growth in a chemically pure mineral-salts sucrose medium with potassium nitrate as the nitrogen source. Nevertheless, these strains could be cultivated indefinitely if the potassium

nitrate was replaced by NH_4Cl or asparagine. Jordan (48) using well washed rhizobial cells stated that the strains of alfalfa-sweet clover bacteria used in his studies would not utilize ammonium chloride as the sole source of nitrogen in a chemically defined medium containing purified sucrose and mineral salts. Richardson and Jordan (77) extended these results to include ammonium sulphate, calcium nitrate, potassium nitrate, potassium nitrite, sodium nitrate and sodium nitrite. Ishizawa (43) also reported that certain cowpea rhizobia were unable to utilize ammonium salts or nitrate.

A number of workers have studied the reduction of nitrate by rhizobia. Prucha (75), Hills (40), and Wilson (99) found no nitrate reduction for the field pea, alfalfa, and soybean organism respectively. Ishizawa (43) reported that a few strains of bean and cowpea rhizobia were unable to reduce nitrate in nitrate yeast-water mannitol medium. Nevertheless, more recent workers such as Wilson and Chin (102) are of the opinion that all strains of rhizobia may reduce nitrates to nitrites when a certain concentration of nitrate is provided. Wilson (100) claimed that the nitrite will react in acidic media with a number of compounds to liberate gaseous nitrogen. Pohlman (72) found that nitrites tended to accumulate in cultures of Rh. meliloti and Rh. japonicum and there was some indication of a slight nitrite utilization by both organisms. Ishizawa (43) showed that distinct differences are present amongst different strains of rhizobia in their ability to utilize ammonium salts, nitrate and nitrite. Although the alfalfa organism was unable to utilize nitrite certain species

were capable of doing so.

There is little experimental evidence concerning the ability of rhizobia to form ammonia from nitrates although Zipfel (108) noted that the organisms which nodulate pea, red clover, horse bean and garden bean all reduce nitrate to nitrite, but not to ammonia. However, these results may be due to the rapid utilization by the organisms of any ammonia formed.

Several investigators have studied the stimulatory powers of added inorganic nitrogen sources upon the uptake of oxygen by cells suspended in carbohydrate media. Walker, Anderson, and Brown (97) in a series of microrespirometer studies reported that unwashed cells of Rh. leguminosarum in a glucose mineral-salts medium were not stimulated in their oxygen uptake by ammonium chloride or sodium nitrate over a period of thirty hours. Neal and Walker (64) investigated the oxygen consumption by twice-washed alfalfa rhizobia growing for twenty-four hours in Warburg vessels containing either sodium nitrate or ammonium chloride mineral-salts basal medium plus any one of several different carbon sources. The uptake of gaseous oxygen was greater in the ammonium chloride medium than in the nitrate base.

Burris and Wilson (15) employed "resting cell" techniques in a series of manometric experiments designed to determine the manner of utilization of hemoglobin-like compounds by rhizobia. Their results

showed that certain inorganic nitrogen salts (as well as many amino acids) increased the respiration of washed suspensions of four strains of rhizobia furnished with glucose. With certain strains $Ca(NO_3)_2$, $(NH_4)_2HPO_4$, $(NH_4)_2SO_4$, $NaNO_2$ and NH_4NO_3 were, however, decidedly inhibitory towards oxygen uptake.

As a resume it may be said that certain species or strains of nodule bacteria are capable of initiating growth in a chemically defined medium containing ammonium salts or nitrate as the sole source of nitrogen. To the majority of strains, however, these inorganic nitrogen sources are purely stimulatory, not only towards cell proliferation, but also towards cellular respiration as typified by "resting cell" experiments. In most cases the nitrate is reduced to nitrite and thence to ammonia, which, however, is probably rapidly metabolized and may not be detectable in culture media. Although nitrite may be stimulatory to some species in most cases it is not utilized to any detectable extent.

2. Organic

During a study of the effects of organic nitrogen compounds on rhizobial growth, Müller and Stapp (63) found that asparagine, sodium asparaginate, and uric acid would serve as nitrogen sources for unwashed cells while glycine and hippuric acid were unsuitable. Alanine, leucine, and urea gave only weak growth. Pohlman (72) reported that unwashed cells of Rh. meliloti and Rh. japonicum utilized glycine, dl-alanine,

dl-amino-n-butyric acid, dl-valine, d-glutamic acid, l-cystine, l-tyrosine, dl-phenylalanine, urea, asparagine and peptone, usually with an attack upon the amino group and with the liberation of free ammonia in the case of glycine and dl-alanine. In certain cultures a slow oxidation of tyrosine to dihydroxyphenylalanine and finally to melanin took place. West and Wilson (98) mentioned that β-alanine was essential for the growth of Rh. trifolii, but Nielsen and Johansen (65) disagreed since they found that this particular amino acid was neither essential nor stimulatory to their strains of Rh. meliloti, Rh. trifolii and Rh. lupini. Allison and Hoover (3) used both urea and asparagine in their basal medium as the sole source of nitrogen and discovered that urea was toxic to the clover organism but not to the alfalfa Rhizobium. This inhibition was believed to be due to an excessive rise in pH.

Richardson and Jordan (77) in a recent study found that washed rhizobia were unable to utilize acetamide, amylamine, butylamine, butyramide, formamide, guanidine hydrochloride, hexylamine, oxamide, propionamide, propylamine, succinimide, thiourea, urea, valeramide, hydrazine or hydroxylamine as the sole source of nitrogen in a chemically defined medium. Glutamine was utilized with the production of considerable quantities of free ammonia. Histamine was also utilized, but to a very small extent and no ammonia was found, although this could conceivably be due to a rapid anabolism of the small amount of ammonia formed.

Walker, Anderson and Brown (97) reported that alanine and urea

did not increase the oxygen uptake by unwashed cells of Rh. trifolii, Rh. phaseoli, and Rh. leguminosarum in a glucose mineral-salts medium whereas Rh. meliloti was stimulated by these compounds. This latter stimulation was of the same order as the stimulation of all four strains by yeast extract, which gave increased amounts of oxygen consumption in proportion to the amount added. In this regard Allen and Allen (2) suggest several plausible explanations for the general utilization of yeast extract by rhizobia such as a) the availability of various essential protein degradation products, b) the presence of certain substrates capable of stimulating respiration, c) the stimulatory effect of various vitamins and accessory growth factors, d) the presence of certain trace elements, and e) the poising of the Eh at an optimum value.

Burris and Wilson (14) reported that impure, but not pure fractions of biotin preparations stimulated respiration. None of a number of vitamins and accessory growth factors could replace this impure fraction. Of a number of amino acids examined, aspartic acid, asparagine, histidine, and arginine were found to be very stimulatory. Casein hydrolysate completely replaced the stimulatory activity of the impure biotin fraction and histidine and arginine closely approached it. This action of the nitrogenous compounds apparently did not arise via a stimulation of cell division. These same workers (15) in a further study found that certain amino acids increased the respiration of washed suspensions of four strains of rhizobia furnished with glucose. This stimulation was immediate and was not due

to cellular proliferation. The total increase in oxygen uptake was dependent upon the concentration of the nitrogen compound supplied and young cells were stimulated somewhat more than older cells. Cysteine was inhibitory to three of the four strains in respect to the uptake of gaseous oxygen.

Jordan (48), in a study involving a large number of alfalfa-sweet clover rhizobia and mutants derived from them by X-ray irradiation, stated that washed cells required amino acids for growth in a synthetic, purified mineral-salts sucrose medium at pH 7.0. This requirement could be satisfied by any one of a large number of amino acids, which varied slightly from culture to culture. These amino acids were not concentrated intracellularly as in the case of certain, if not all, Gram positive bacteria and could not be replaced by inorganic nitrogenous compounds either in the presence or absence of any one of a number of vitamins, purines or pyrimidines. Therefore, the stimulation of these organisms by yeast extract was thought to be principally due to the high concentration of amino acids contained in this material, although the beneficial influence of other materials could not be entirely neglected.

These reports may seem contradictory in many cases but, as stated before, may not actually be so since strain differences and environmental conditions are all capable of modifying results. Certainly it is apparent that most amino acids are capable of initiating growth of washed cells in a synthetic medium devoid of any other nitrogen source and that in addi-

tion they can, for the most part, stimulate the cellular respiration of "resting cells" suspended in a carbohydrate substate.

There are amazingly few reports dealing with the mechanisms of amino acid utilization by the nodule bacteria. As mentioned previously Pohlman (72) believed that the mode of attack was directed against the amino group and, in the case of two amino acids, with the formation of free ammonia. Wilson (103) in his "resting cell" procedure found that ammonia was liberated by endogenous respiratory activities, due probably to deamination of cellular amino acids. Virtanen et al. (95) decided that the mode of attack against aspartic and glutamic acids was one of decarboxylation, with the resulting formation, in the former case, of beta-alanine. Billen and Lichstein (11) further studied the aspartic acid decarboxylase of Rh. trifolii and estimated the pH optimum at 5.7. Jordan (49) emphasized the importance of transamination reactions in Rhizobium metabolism when he demonstrated the formation of glutamic acid from alpha-ketoglutaric acid when aspartic acid, alanine, leucine, and valine acted as amino donors.

In relation to the activity of rhizobia towards synthetic peptides, Berger, Johnson, and Peterson (9) compared the relative peptidase contents of a number of micro-organisms. An extract from ten grams of wet Rh. trifolii cells possessed activity against dl-alanylglycine, dl-leucylglycine, diglycine, dl-alanyldiglycine, dl-leucyldiglycine and triglycine. The resulting rates were of a low order compared to many of

the other organisms tested. Burris and Wilson (15) showed that glycylglycine was capable of stimulating the uptake of gaseous oxygen by four Rhizobium strains supplied with glucose.

There have been no published reports to the author's knowledge which deal primarily with the utilization of D-amino acids or pure D-amino acid peptides by members of the genus Rhizobium.

3. pH and Temperature

Fred, Baldwin and McCoy (26) have summarized the topic concerning the effect of hydrogen ion concentration on the growth and longevity of rhizobia in liquid media and in the soil. Opinions vary but, in general, the various species are affected differently by acidity and possess about the same tolerance to alkalinity. Rh. meliloti is the most acid sensitive and is favored by slight alkalinity or neutrality. Rh. japonicum and Rh. lupini are the most acid tolerant species. Using the Warburg technique Thorne and Walker (89) studied the growth and respiration of representative strains of Rh. meliloti and Rh. japonicum as a function of medium pH. The growth of the former in a glucose yeast-extract medium was found to decrease from a maximum at pH 7.0 to zero between pH 4.6 and 4.9 on the acid side of neutrality and pH 9.6 on the alkaline side. The growth of Rh. japonicum in arabinose yeast-extract medium decreased from a maximum at pH 6.7 - 6.9 to zero at pH 4.2 and 9.5. The initial respiration rate for the two species reached

a maximum between pH 7.2 and 8.0 and decreased to a very small value at the critical pH. Dehydrogenase activity of the nodule bacteria occurs between pH 4.0 and 10.0 with an optimum in the alkaline range of pH 8.0 to 8.2 (86). Ishizawa (44) decided that a neutral or alkaline pH (7-7.5) was better for alfalfa, soybean and cowpea bacteria, which were all acid sensitive having a critical pH of 5.5 - 6.0.

Allison and Minor (4) summed up the status of the knowledge on the temperature requirements of the nodule bacteria in the statement, "The literature is surprisingly deficient in data showing quantitatively the effect of temperature on the growth rates of rhizobia". These workers used a basal mineral medium containing sucrose, yeast water and asparagine and cell numbers were counted with a Levy-Hausser chamber. Using nine Rhizobium cultures, representing nine species they showed that the optimum growth rate for most of these organisms lay between 29 and 31°C although Rh. meliloti grew best at 35°C. No growth occurred within 24-48 hrs. with most species held at 37°C, but a temperature of 41°C was required to prevent the growth of Rh. meliloti. The growth rate at 15°C was only one quarter to one half as great as at 30°C. Ishizawa (45) confirmed Allison and Minor's work with the conclusion that the optimum temperature for Rh. meliloti was about 34°C.

It must be realized that a great deal of work must still be done on Rhizobium physiology, not only in the pursuit of new lines of investigation, but also for the purpose of a reappraisal of former work in the light of

newer discoveries and techniques. It is only through an exact understanding of the biochemical activities of these microorganisms that one can hope to discern differences between parasitic, ineffective and effective rhizobia and to gain further insight into the actual mechanism of the process of symbiotic fixation.

A. The utilization of L-, D-, and DL-amino acids and the effects of pH and temperature.

In almost every text-book of biochemistry amino-acids are spoken of as the "building blocks" for protein synthesis. Although this statement has not yet been absolutely proved, it is, nevertheless, almost impossible to overemphasize the value of these compounds to the living cell. Amino acids are usually designated by the prefix L- or D-, denoting a particular optical isomer, since all of these acids except glycine have an asymmetric alpha-carbon atom. The configuration of the naturally prevalent amino acids is similar to that of L-lactic acid and hence these compounds are said to possess the L- configuration. By far the majority of problems in bacterial physiology have dealt with L-amino acids, but there is sufficient information available to make a study of the D-isomers extremely fascinating.

(a) Strain description

The strains of Rhizobium meliloti used in this and subsequent investigations were designated R_{21}, R_{20}, and R_{20-27A}. Their effective-

ness in nitrogen fixation and their colonial descriptions are given in TABLE I.

TABLE I

TABLE INDICATING THE SOURCE, EFFECTIVENESS, AND COLONIAL DESCRIPTIONS OF EACH OF THE RHIZOBIUM TEST STRAINS

Strain	Source	Effectiveness on alfalfa	Colonial description
R_{21}	Ottawa	effective	convex, translucent with whitish center, entire margin, extremely mucoid and glistening
R_{20}	Ottawa	parasitic to ineffective	convex, translucent with whitish center, entire margin, slightly mucoid and glistening
R_{20-27A}	X-ray mutant of R_{20} (47)	effective	pulvinate, translucent with whitish center, entire margin, mucoid appearing but "rough" or pebbled on the surface. Interior of colony appears marbled with light and dense areas

On modified Lochhead's medium strain R_{21} (the most copious gum producer) and R_{20-27A} exhibited a more profuse and rapid growth than R_{20}. Both R_{21} and R_{20} were found to have the same fermentative ability

on arabinose, galactose, glucose, glycerol, lactose, mannose, and xylose, from which they produced acid. They were able to utilize, but produced no acid from, dextrin, dulcitol, inulin, levulose, maltose, mannitol, sucrose, fumarate, succinate, or pyruvate when these compounds were used as the sole carbon source. They produced ammonia in milk and brought about complete digestion of the casein. All three strains were unable to grow in a liver infusion broth, but utilized a wide variety of amino acids as the sole source of nitrogen in a chemically defined medium (48).

(b) Media

The formulae for a modified Lochhead's medium, Modified Medium "79" and a modified Wilson and Wilson's medium are given in the appendix. The latter medium was composed of specially purified ingredients and constituted the nitrogen-free base employed in much of the present work. The chemicals used in the formulation of these media were of "Analar" (C. P. analyzed) grade purchased from British Drug Houses, Ltd.

(c) Purification of bacterial strains

The organisms were grown at 30°C. for 48hr. on slants of Lochhead's medium and portions of this growth emulsified in Modified Medium "79". These suspensions were each passed through a Handuroy "Germ Separator" (38) and the aerosol thus formed allowed to fall on open plates

of Lochhead's medium, which were immediately covered and incubated at 30°C. until colonies developed. Representative colonies were picked, emulsified in liquid medium and again passed through the aerosol device. Single cell isolations were made with a de Fonbrune pneumatic micromanipulator, using the growth which developed on these second plates. The most successful technique was as follows: a sterile cover slip, having a thin layer of solidified clear agar on one side was placed, agar side down, over a sterile moist chamber containing water-soaked filter paper. The cover slip was sealed to the chamber with vaseline and a circle drawn on the upper surface with a wax pencil. On the agar directbeneath this circle a minute amount of log phase bacterial growth was placed by means of a slender platinum needle and spread slightly so that some cells would be well separated from the parent mass. The moist chamber was then placed on the mechanical stage of a binocular microscope, from which the top condenser lens was removed, and the bacterial cells brought into focus. Right-angled micropipettes with a 4.5 micron lumen opening were manufactured in a de Fonbrune microforge, sterilized in individual glass tubes, partially filled with sterile Modified Medium "79", and attached to a syringe by means of a flexible rubber tube. The tips of these instruments were moved immediately below individual bacterial cells which were then drawn in by capillarity or by the application of very slight suction. The cells and the contained liquid were expelled onto predetermined spots on the surface of a plate of solidified

Lochhead's medium, which was subsequently incubated at 30°C. until colonies developed. Usually, ten isolations were made from each bacterial strain, with a percentage of success which varied between 70-80%.

(d) Effectivity tests

The bacterial cultures were tested on Ontario Variegated alfalfa using the sterile glass assemblies of Leonard (56) and the techniques previously described by Jordan and Garrard (50). The treatments (uninoculated and inoculated with R_{21}, R_{20} and R_{20-27A}) were replicated and the various assemblies placed on the greenhouse bench in a 4 x 4 Latin Square. Supplementary lighting was provided to ensure a 14 hr. photoperiod. The plants were harvested after a growing period of five weeks and the aerial portions excised and dried at 115°C. to constant weight. The nitrogen contents of representative portions of dried material were determined using a micro-Kjeldahl procedure which will be described in a later section of the thesis. The results were statistically analyzed for significance using the analysis of variance (see appendix). Only the R_{21} and R_{20-27A} inoculated plants were significantly better than the uninoculated controls and hence only these two bacterial strains can be regarded as effective.

(e) Determination of maximum culture growth using the Photonephelometer.

The term "nephelos" refers to the ability of a suspended phase to scatter light by virtue of optical discontinuity and does not refer to a trans-

mission phenomenon. The light scattered by particles (Tyndall light) is proportional to the number and size of these small bodies and, in the case of bacterial cells, to the total amount of protoplasm. It is thus an excellent indicator of growth, which in its true sense is defined as an increase in protoplasm and not merely an increase in cell numbers. The determination of culture nephelos was made with a Coleman Model 7 Photonephelometer which was calibrated with a commercially prepared standard each time it was used. The "null" method was employed, which has an estimated instrument error or 0.2%. The cuvettes used were originally optically matched, but minute scratches occurred with constant handling. To correct for such optical imperfections ten cuvettes were chosen at random and filled with a bacterial suspension of low, medium, or high nephelos. Readings were taken on each cuvette with each solution and the standard deviations calculated (see appendix). These values were $\pm$2.57, $\pm$ 2.98 and $\pm$ 2.52 for the low, middle and high ranges respectively and indicated that deviations in nephelos from the true value are not related to the nephelos magnitude. Since significance at the 5% point requires a difference exceeding $\pm$ 1.96 standard deviations, an arbitrary difference of four standard deviations between any two nephelos readings has been required for significance. This amounts to about 11 nephelos units (4 x the ave. S.D. of 2.69 = 10.76) and any readings differing by less than this amount are to be regarded as non-significant.

The photonephelometric method depends upon the assumption that there

is a linear relationship between concentration and instrument response. The validity of this assumption for viable cells was shown by actual test. Dilutions of a broth culture of R_{21} were made and the nephelos determined. These dilutions were then plated out in triplicate on Lochhead's medium using a conventional bacteriological technique. The plates were incubated at 30°C. for three days and the colony counts were plotted against the nephelos readings. FIGURE 1 illustrates the linear response obtained.

When bacterial growth curves were plotted in terms of nephelos it was found that the values reached a maximum and then decreased. One to two days after this initial decrease the nephelos readings rose. The decrease was attributed to a cessation of growth followed by the settling out of the bacterial cells while the second increase appeared to coincide with the onset of autolysis. For comparative purposes the primary maxima were considered.

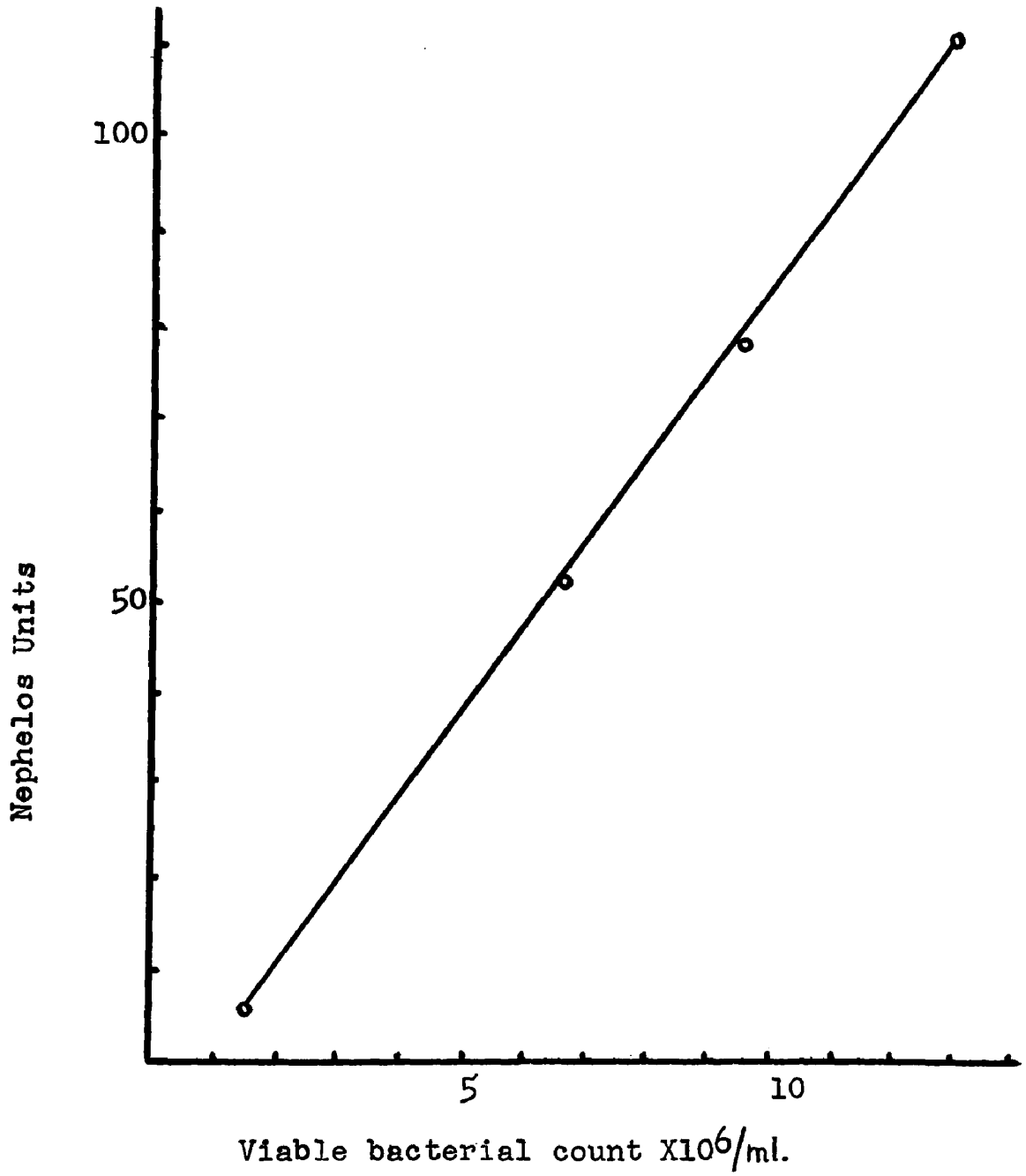


Fig. 1 Correlation between viable bacterial counts and nephelos readings.

Methods:

A number of 6-304B Photonephelometer cuvettes were cleaned by warming in 33% nitric acid and thoroughly rinsed 10 times in ordinary distilled water and twice in double glass-distilled water. They were then plugged with cheesecloth-covered cotton and sterilized by autoclaving. Modified Wilson and Wilson's medium was prepared, adjusted to a pH of 6.0, 7.0, or 7.5 and sterilized by Seitz filtration, using a special device in which the sterilized fluid was allowed to collect in a graduated dispensing column. The first 20ml. of medium passing through the filter pad were discarded and 9.5 ml. aliquots of the remaining medium were aseptically added to the sterile cuvettes. A pH value greater than 7.5 was not used since turbidity resulted in the medium.

Stock amino acid solutions were prepared with double glass-distilled water, the concentrations being such that the addition of 0.5 ml. to 9.5 ml. of basal medium would yield a solution containing the equivalent of 0.0001 moles of amino acid per liter. This is the optimum molarity of several amino acids in regard to utilization by Rhizobium species (76). The pH of the acidic and basic amino acids were carefully adjusted to 7.0 with a glass-electrode potentiometer before being brought to their final volume. After sterilization in an autoclave at 15 lb. pressure for 6 min., 0.5 ml. aliquots of these solutions were added to their respective cuvettes.

The amino acids used consisted of the neutral aliphatic amino acids

glycine, leucine, and valine, the neutral sulphur containing acid methionine, the acidic amino acid glutamic and the basic amino acid histidine. They were all obtained from Nutritional Biochemicals Corp. (Cleveland, Ohio, U.S.A.) and met the following specifications:

nitrogen	100% $\pm$ 1% of theory
moisture	less than 0.30%
ash	less than 0.10%
chloride	less than 0.03%
ammonia	less than 0.03%
heavy metals	less than 0.003%
iron	less than 0.005%
specific rotation	100% $\pm$ 2% of theory

The stock solutions were analyzed by paper partition chromatography using water-saturated phenol (104), and were found to be pure, each giving rise to only one ninhydrin positive spot of appropriate Rf (12).

Thirty milliliters of a 24 hr. 30°C. culture of the test organism in Modified Medium "79" were centrifuged and the sedimented cells washed three times in 0.85% saline prepared from purified NaCl (101) and double glass-distilled water. The resulting cell mass was brought upto a final volume of 30 ml. with the same fluid and the nephelos aseptically adjusted to 55.0 (ca. 0.1 mgm. bacterial nitrogen /ml.) using saline as a blank. Aliquots of 0.2 ml. served as the inocula for the amino acid cuvettes. The total initial volume of each cuvette was 10.2 ml. and

was experimentally determined to take into consideration the amount of evaporation during the incubation period. With a vessel of water in the incubator to serve as a humidifier 0.2 ml. of culture evaporated in five to six days. This corresponded to the approximate maximum in the growth of the test organisms and at this point the concentration of the amino acid was more exactly 0.0001 M.

In each experimental series one strain of organism was tested for the ability to utilize, as the sole source of nitrogen, glycine and the L-, D-, and DL- forms of all the other amino acids at the three different pH's. Inoculated and uninoculated control cuvettes, containing no amino acids, were included in each run, the latter serving as a blank for the nephelos readings which were taken daily in the Photonephelometer. Incubation was carried out at 30°C. until the readings began to decrease in magnitude. After incubation the pH of each culture was taken with a potentiometer and Gram stains were made as an index of purity.

The "optimum" temperature for the growth of all three test organisms in a yeast-extract mineral-salts medium was determined by inoculating three series of cuvettes, each containing 10 ml. of Modified Medium "79", with washed cells of R_{21}, R_{20}, and $R_{20\text{-}27A}$. The series were incubated at 35°C., 30°C. and 27°C. One cuvette in each series served as an uninoculated control and daily nephelos readings were made on each culture.

The action of a temperature lower than 30°C. on the spectrum of

amino acids utilized by rhizobia was studied with a triplicate series of cuvettes containing 9.5 ml. of basal medium, 0.5 ml. of a stock solution of glycine or the L- or D- isomers of the other amino acids, and 0.2 ml. of washed cell inoculum (nephelos 55.0). The pH of the basal medium in each cuvette was the optimum for the contained amino acid, as determined in the previous experiment on pH. These values are given in TABLE 2. The incubation temperature was 27°C. and nephelos readings were taken each day until the maxima were reached.

TABLE 2

TABLE INDICATING THE INITIAL pH VALUES OF THE AMINO ACID MEDIA IN THE "TEMPERATURE EFFECT" CUVETTES

Cuvette	Amino Acid	Organism		
		R_{21}	$R_{20\text{-}27A}$	R_{20}
1	glycine	7.5	7.5	7.0
2	L-leucine	7.5	7.5	7.0
3	D-leucine	7.5	7.5	7.0
4	L-valine	7.5	7.5	7.0
5	D-valine	7.5	7.0	7.0
6	L-methionine	7.5	7.5	7.0
7	D-methionine	7.0	7.0	7.0
8	L-glutamic acid	7.5	7.5	7.0
9	D-glutamic acid	7.5	7.0	7.0

Table 2 (cont'd)

Cuvette	Amino Acid	Organism		
		R_{21}	R_{20-27A}	R_{20}
10	L-histidine	7.5	7.5	7.5
11	D-histidine	6.0	7.5	6.0
12	inoculated control	7.0	7.0	7.0
13	uninoculated control	7.0	7.0	7.0

A duplicate series of chemically clean test-tubes were filled with 9.5 ml. of sucrose-free modified Wilson and Wilson's base. One series contained 0.5 ml. of L-histidine stock solution and the other contained 0.5 ml. of L-glutamic acid stock solution. The tubes in each lot were inoculated with washed cells of R_{20}, R_{21}, R_{20-27A} and a number of other strains of rhizobia which are indicated in TABLE 3. The production by X-ray irradiation of the mutants listed has been previously described (47). Incubation was at 27°C for eight days and the presence or absence of visual growth recorded. This experiment indicated the ability of the bacterial strains to utilize these two amino acids as sole sources of both carbon and nitrogen.

TABLE 3

RHIZOBIUM STRAINS, OTHER THAN R_{21}, R_{20-27A}, AND R_{20}, TESTED FOR THEIR ABILITY TO UTILIZE L-HISTIDINE OR L-GLUTAMIC ACID AS SOLE SOURCES OF BOTH CARBON AND NITROGEN

Strain	Source	Effectiveness in nitrogen fixation*
R_{23}	O.A.C. stock culture	effective
R_{20-28}	X-ray mutant of R_{20}	effective
R_{20-31}	X-ray mutant of R_{20}	effective
R_{20-16}	X-ray mutant of R_{20}	ineffective
R_{20-17}	X-ray mutant of R_{20}	ineffective
R_{20-19}	X-ray mutant of R_{20}	ineffective
R_{20-20}	X-ray mutant of R_{20}	ineffective

* tested on Grimm alfalfa. Those strains designated as "ineffective" may also have parasitic tendencies (50).

Results:**

FIGURES 2, 3, and 4 graphically represent the maximum growth attained by the three Rhizobium strains in the amino acid media at the three different pH's. The final pH values were generally found to be several

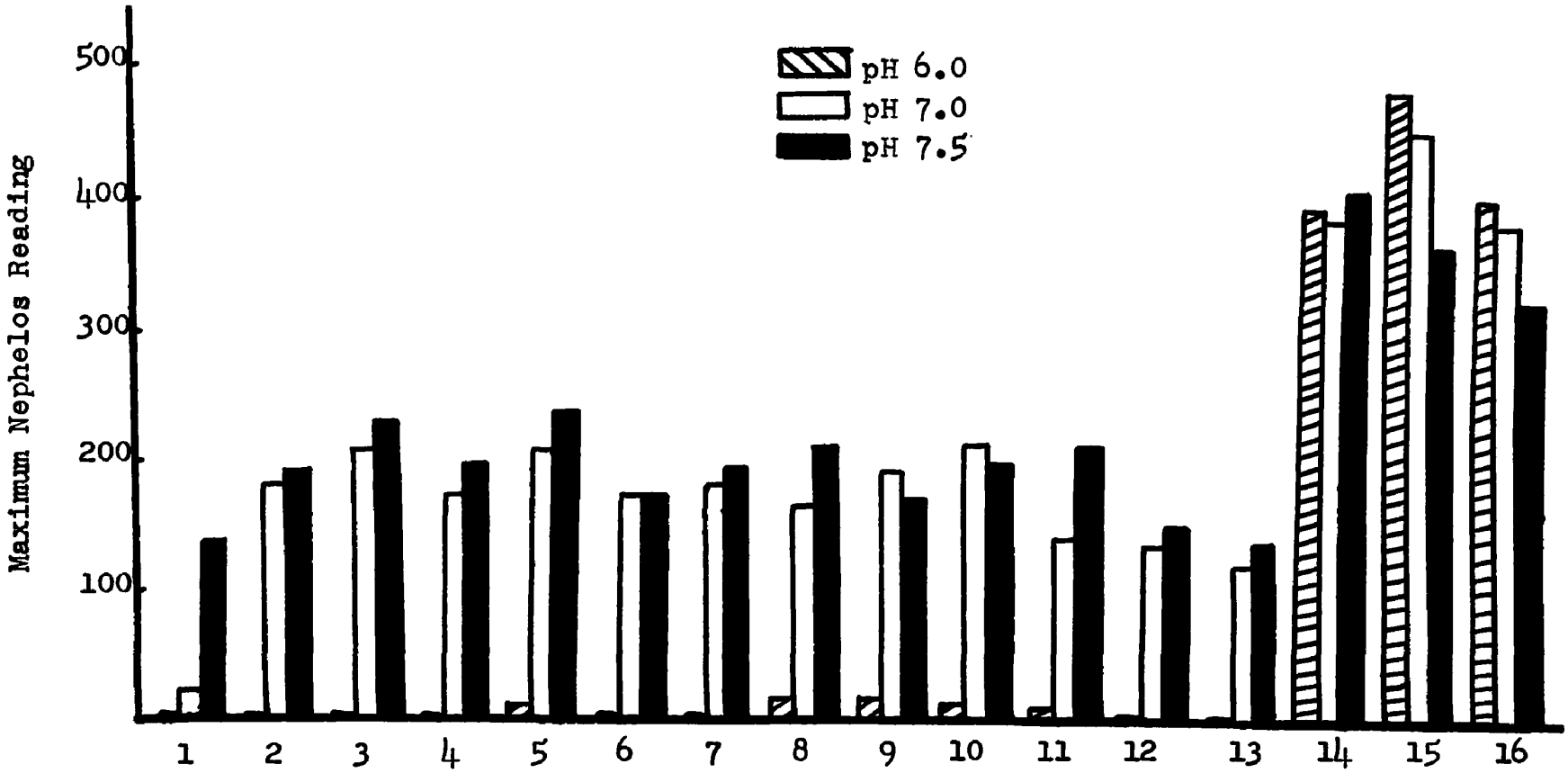


Fig.2 Vertical bar chart showing the influence of medium pH on the growth of R_{21} at 30°C on (1) glycine (2) L-leucine (3) D-leucine (4) DL-leucine (5) L-valine (6) D-valine (7) DL-valine (8) L-methionine (9) D-methionine (10) DL-methionine (11) L-glutamic acid (12) D-glutamic acid (13) DL-glutamic acid (14) L-histidine (15) D-histidine (16) DL-histidine

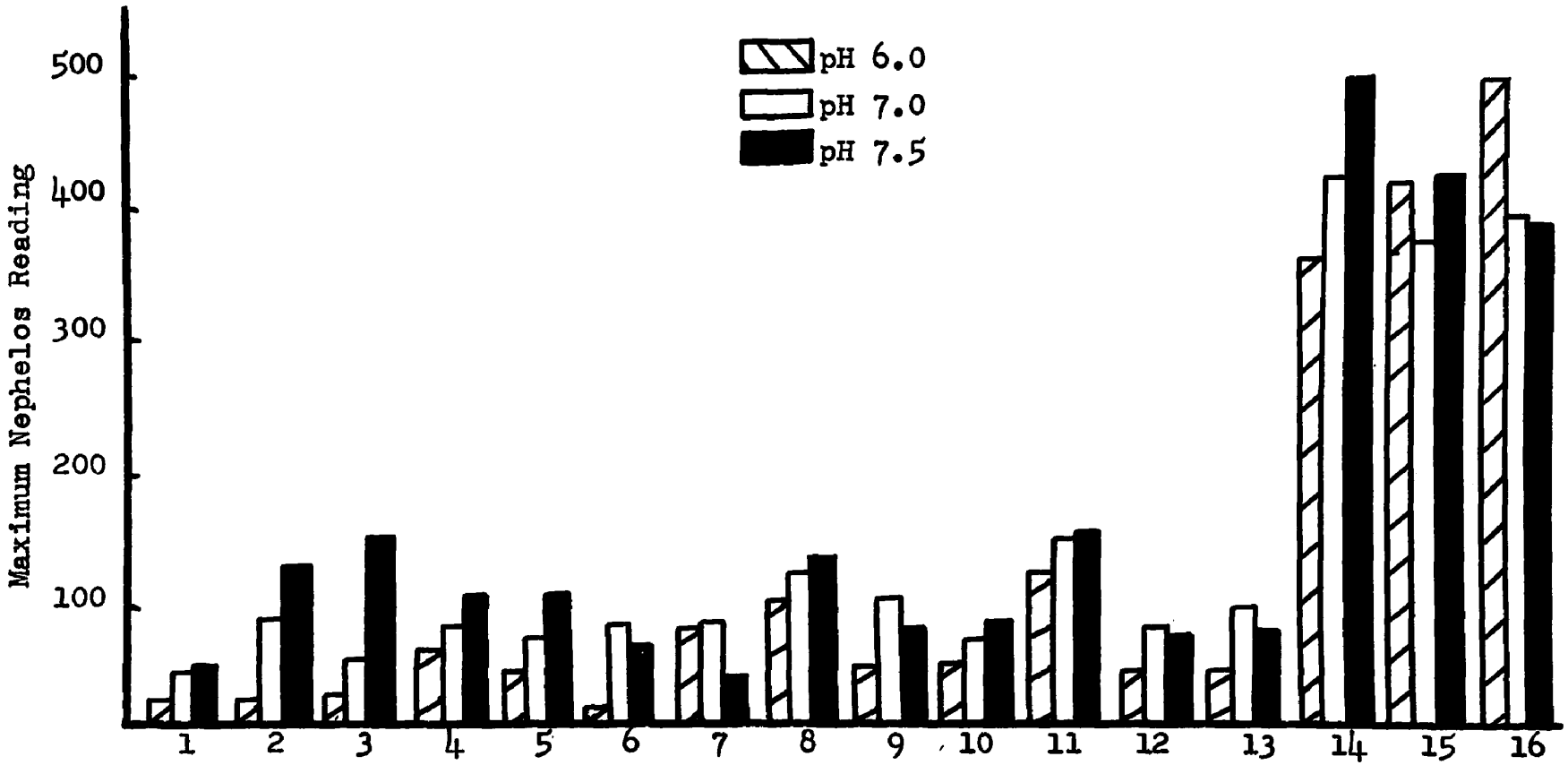


Fig. 3 Vertical bar chart showing the influence of medium pH on the growth of R20-27A at 30°C on (1) glycine (2) L-leucine (3) D-leucine (4) DL-leucine (5) L-valine (6) D-valine (7) DL-valine (8) L-methionine (9) D-methionine (10) DL-methionine (11) L-glutamic acid (12) D-glutamic acid (13) DL-glutamic acid (14) L-histidine (15) D-histidine (16) DL-histidine

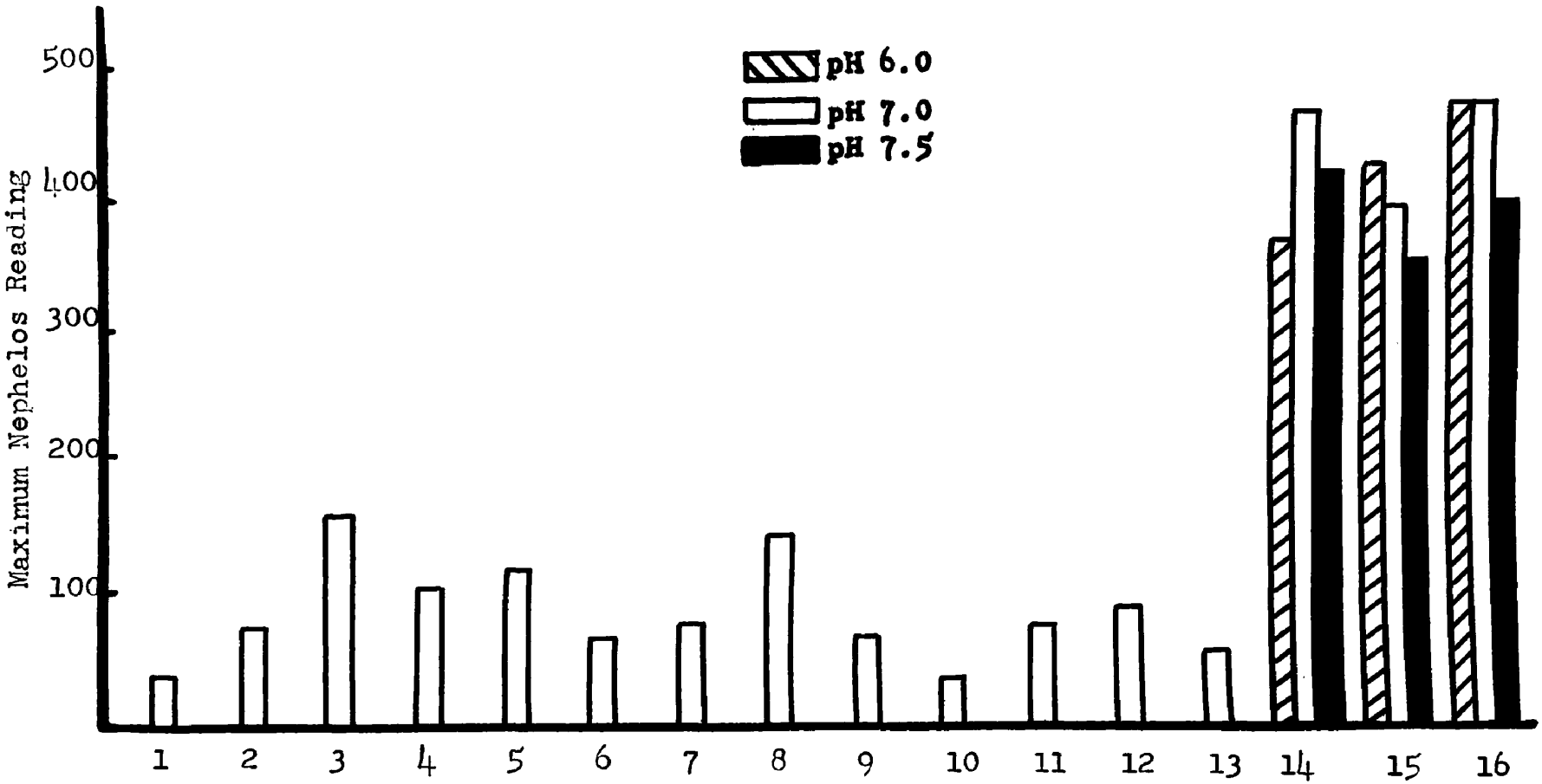


Fig. 4 Vertical bar chart showing the influence of medium pH on the growth of R20 at 30°C on (1) glycine (2) L-leucine (3) D-leucine (4) DL-leucine (5) L-valine (6) D-valine (7) DL-valine (8) L-methionine (9)D-methionine (10) DL-methionine (11) L-glutamic acid (12) D-glutamic acid (13) DL-glutamic acid (14) L-histidine (15) D-histidine (16) DL-histidine

tenths of a unit lower than the original (see appendix).

All the strains of the nodule bacteria were readily capable of using D-amino acids as the sole nitrogen source. In several cases the organisms made better use of these "unnatural" isomers than they did of the corresponding "natural" ones.

With R_{21} a pH of 7.5 was optimum for utilization of all the amino acids with the exception of D- and DL- histidine (pH 6.0 best) and D-methionine (pH 7.0 best). All three forms of histidine supported exceptional growth at all pH's, including 6.0 at which pH growth did not occur with the majority of the acids except to a very limited extent with L-valine, L-glutamic acid and L, D- and DL- methionine. The different isomers of any one amino acid were found to vary in their ability to be utilized, depending on the pH. The growth observed in the racemic mixtures indicated that both inhibition and stimulation could occur. These last effects, together with those found with R_{20} and R_{20-27A}, are summarized in TABLE 4.

For R_{20-27A} a pH of 7.5 was found to be optimum for the utilization of 10 of the 16 different forms of amino acids tested, while a pH of 7.0 was best for 5 of the remainder.

** Numerical data pertaining to most of the charts and graphs presented in this thesis are given in the appendix.

Maximum growth in DL-histidine was observed at pH 6.0.

TABLE 4

INHIBITION AND STIMULATION FOUND IN THE RACEMIC MIXTURES OF VARIOUS AMINO ACIDS WHEN TESTED AGAINST THREE STRAINS OF RHIZOBIUM

Strain	pH	Inhibition	Stimulation
R_{21}	7.0	histidine (77.0%)*	
		glutamic (86.5%)	
	7.5	histidine (84.9%)	
		glutamic (74.3%)	
R_{20-27A}	6.0	methionine (64.5%)	leucine (285.4%)
		glutamic (55.9%)	valine (272.5%) histidine (127.7%)
	7.0	methionine (58.6%)	
		glutamic (85.6%)	
	7.5	methionine (53.3%)	
		glutamic (67.5%)	
		leucine (72.4%)	
		valine (46.5%)	
R_{20}	6.0	--	histidine (121.5%)
	7.0	--	histidine (111.2%)

*numbers in brackets refer to the percent of the theoretical growth. This

theoretical growth is equal to one-half the growth on the L-isomer + one-half the growth on the D-isomer at the same pH, assuming no inhibition. Only values less than 90% or more than 110% of the theoretical are listed.

Histidine in all its forms behaves in a fashion similar to that observed with R_{21} in that it promotes excellent growth at all pH's. R_{20-27A} is decidedly more acid tolerant than R_{21}, growth occurring at 6.0 with every amino acid. This organism, however, does not make as good use of the amino acids (at their optimum pH) as does R_{21}, but due to inevitable variations in inoculum size no critical comparisons were attempted.

R_{20} is definitely fastidious in its pH requirements. With 13 out of the 16 amino acids against which it was tested it grew only at pH 7.0, although with histidine it grew well at all three pH's.

The approximate optimum temperatures for the growth of R_{21}, R_{20-27A} and R_{20} in the yeast-extract mineral-salts medium containing no added carbohydrate were 35°C., 30-35° C, and 27°C. respectively. The optimum for R_{20} is somewhat lower than that given in various literature reports for Rhizobium meliloti. The effects of a 27°C. incubation temperature on amino acid utilization are shown in FIGURE 5. Generally, with the exception of L-glutamic acid and R_{20-27A}, the best growth was attained at 30°C. rather than 27°C., even with R_{20}. No significant difference was noticed, however, in the utilization of glycine and L-methionine by R_{20-27A} and glycine and L-glutamic acid by R_{20}. A more careful consideration of the data indicates that with certain amino

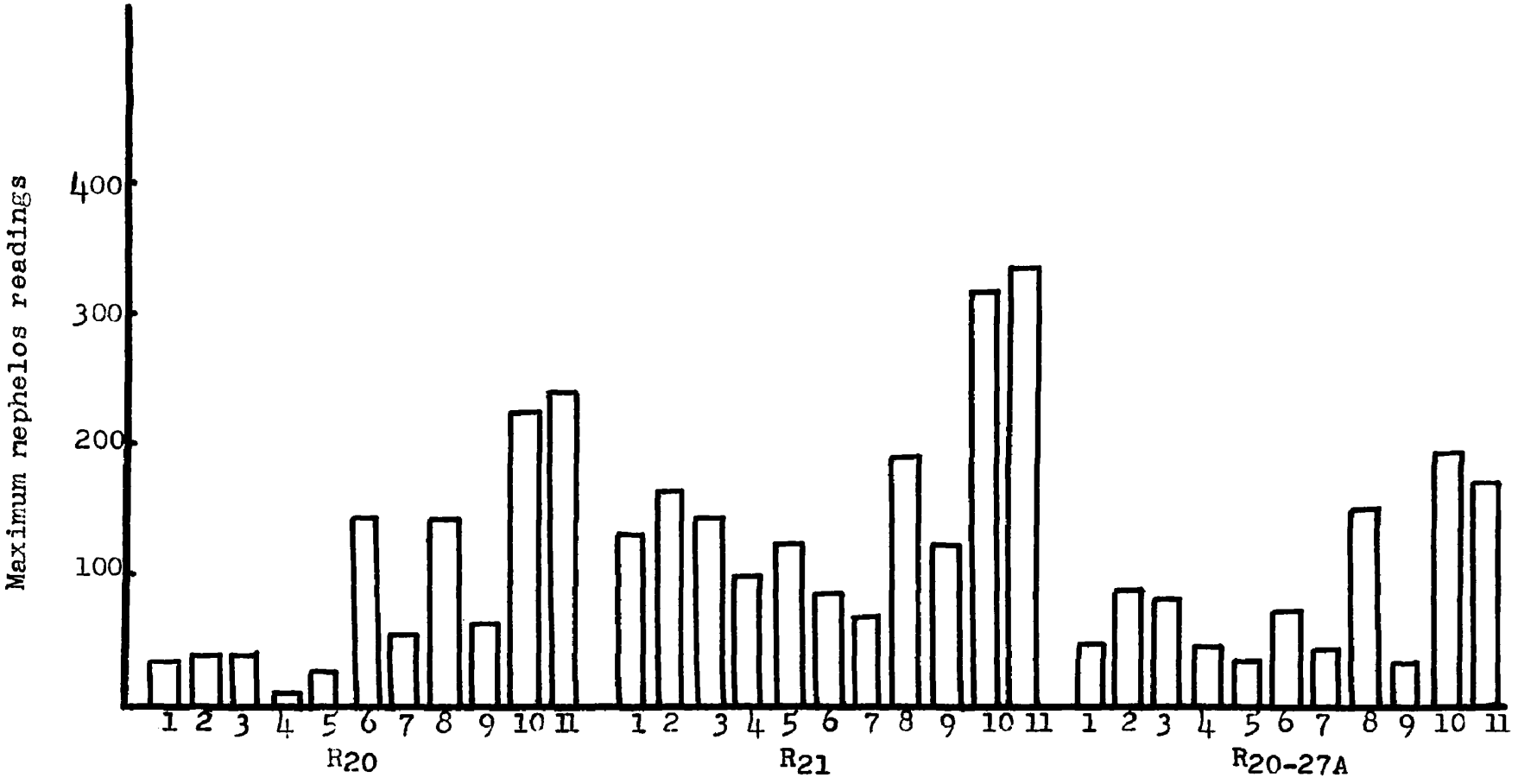


Fig. 5 Vertical bar chart showing the influence of temperature (27oC) on the utilization of amino acids at optimum pH by three Rhizobium strains: (1) glycine (2) L-leucine (3) D-leucine (4) L-valine (5) D-valine (6) L-methionine (7) D-methionine (8) L-glutamic acid (9) D-glutamic acid (10) L-histidine (11) D-histidine

acids there is an "isomeric effect" which is temperature dependent (see TABLE 5). That is, one isomer is more effective at 27°C. while the other is more effective at 30°C.

TABLE 5

THE EFFECT OF TEMPERATURE ON THE UTILIZATION OF THE L- AND D- ISOMERS OF CERTAIN AMINO ACIDS BY RHIZOBIA

Organism	Amino acid	Temperature	Activity
R_{21}	leucine	27°C.	L*
		30°C.	D
	valine	27°C.	D
		30°C.	L
R_{20}	leucine	27°C.	N. S. D.
		30°C.	D
	valine	27°C.	D
		30°C.	L
	glutamic acid	27°C.	L
		30°C.	D
R_{20-27A}	leucine	27°C.	N. S. D.
		30°C.	D
	valine	27°C.	N. S. D.
		30°C.	L

*L= L-isomer yields best growth D=D-isomer yields best growth
N. S. D. =no significant difference between isomers

L-histidine was found to be capable of serving as the sole source of nitrogen and carbon for all the strains tested while L-glutamic was active for all the strains except R_{20-19} and R_{20-20}.

Repetition of the experiments in this section gave essentially the same overall results as those listed.

Discussion:

Rydon (80) in a review article, lists a number of microorganisms which have been reported as utilizing D-amino acids. Negative results with certain bacteria cannot always be attributed to the lack of a capable enzyme within the cell since certain workers (10) have indicated that the failure of resting suspensions to metabolize D-amino acids is often due to an inability of these compounds to penetrate the cell barrier. This feature may also explain the relatively slow rate of D-isomer utilization in some instances. In consideration of the data the Rhizobium strains tested in the present investigation cannot be regarded as unusual with respect to their ability to use the "unnatural" isomers of amino acids.

In contrast to internal mammalian cells, which function under a condition of homeostasis, bacterial cells are continually subjected to the effects of their external environment. Although some microorganisms may control their intracellular pH to some extent by deamination of amino acids by high pH and decarboxylation at low pH (30), it is difficult to believe that no pH shift at all occurs. The pH optima of enzymes are scattered around the physiological range, and small shifts in pH will increase the activity of some enzymes, decrease that of others and leave that of the rest unchanged. Small changes in pH will, therefore, markedly

influence metabolic reactions dependent on the functioning of series of enzymes. Fisher (25) states another possible effect of pH on cellular function in these words, "it is to be expected that it will modify the degree of ionization of constituents of the boundary layer of the cell, which will cause changes in the pattern and the properties of this layer as a whole, a possible consequence being alterations of the permeability of the layer, which, by modifying exchange of metabolites between the cell and the environment could modify the pattern of metabolism". The results of medium pH on amino acid utilization by rhizobia could, therefore, be explained by consideration of the effects of the pH on either the enzymes or the cell membranes.

It is generally concluded that bacterial cells are unable to maintain themselves at temperatures above or below that of their environment. The heat produced during metabolism, for example, is lost by convection and radiation and is not used for temperature maintenance. The "optimum" temperature for a bacterial cell is only a reflection of the effect of this factor on enzymatic activity. If a temperature of 30°C. produces more growth with one particular amino acid than a temperature of 27°C it may be concluded that the enzyme(s) concerned acts at an increased rate at the higher temperature, that a secondary "inhibiting" reaction is suppressed at the higher temperature or that the reaction normally occurring at the lower temperature is suppressed and replaced by a more efficient pathway of utilization. If it is assumed that at least two different enzymes

are implicated in the metabolism of the L- and D-isomers of any one of several amino acids then the effects noted in TABLE 5 may be explained by one or more of the aforementioned mechanisms. As a general statement, the L-isomer of valine is preferentially utilized at 30°C. and the D-form at 27°C., while the converse is true for L- and D- leucine. Since it would be expected that a number of different routes of amino acid metabolism would exist in intact Rhizobium cells it would be hazardous to make any specific statement with regard to a particular acid.

The reports of a number of previous workers, as mentioned in the literature review, have been confirmed in that Rhizobium meliloti is acid sensitive, stimulated by a neutral or slightly alkaline pH and has a temperature optimum which lies approximately within the range 27°-35°C.

The ability of histidine to serve as a nitrogen source for the rhizobia is outstanding and possibly results from the fact that this amino acid is somewhat complex and might therefore be comparatively difficult to synthesize by the organisms. It would then act as a limiting factor in growth unless supplied preformed in the medium. This implies that all the other essential amino acids can be readily formed from histidine, but that the converse is not true. This concept is reasonable since histidine is known to be convertible through a series of unknown intermediates into formate and glutamic acid. Transamination of the latter could then "funnel" the histidine amino nitrogen into other amino acids. Little is known of the synthesis of histidine except that the pyrimidine-like carbon

of the imidazole ring is derived from formate.

The inhibition of any enzyme by a substrate analogue and the action of an enzyme on a substrate are closely related, as both involve the attachment of the substrate/inhibitor to certain reactive groups on the enzyme molecule. An inhibitor of this type is simply a potential substrate which, although it can attach itself to the enzyme, is sufficiently dissimilar to the normal substrate as to be not acted upon by the enzyme after the attachment has occurred. With compounds as similar as the L- and D-isomers of amino acids it is not surprising that reports of D-amino acid inhibition of bacterial growth are relatively plentiful. Such inhibition usually stems from the fact that the molecules of the D-amino acid are not utilized and "clog" up reactive sites on the L-amino acid enzyme. In the rhizobia examined, however, the D-isomer is also utilized for growth purposes and in some cases better than the corresponding L-form. Therefore, in order to account for the deviations in growth as compared with the theoretical - see TABLE 4 - a somewhat hypothetical explanation must be postulated. Assuming that the enzymes first attacking the amino acids in a racemic mixture are stereospecific and in non-limiting and approximately equal amounts and assigning an arbitrary growth value of 50 to an L-amino acid molecule and a value of 25 to a D-amino acid molecule a hypothetical racemic mixture of two L- and two D- molecules would invoke an overall growth response of 150. If a mutual inhibition occurs between these molecules, that is, if one molecule of the D-isomer

is affixed to an L-amino acid enzyme site and is not utilized, and vice versa, then the growth response would be 75 units (= 1/2 = 50% of the theoretical, where all the molecules are utilized). Deviations in the relative amounts of the two enzymes, such as might occur with pH (or temperature fluctuations, would necessarily cause proportionate deviations in the percentage of the theoretical that would be observed. If the L-amino acid enzyme molecules were numerically reduced less D-amino acid molecules would be affixed to them and it might appear that more of the latter should be utilized as a result of D-amino acid enzyme activity. At the same time, however, more L-amino acid molecules would be available for inhibition of the D-enzyme. The net result would vary statistically depending on the proportion of competing molecules. This concept may explain the various degrees of inhibition found in the racemic mixtures listed in TABLE 4.

The stimulatory effects brought about by the racemic mixtures of certain amino acids at the lower pH's is inexplicable at present.

B. The Utilization of Synthetic Di- and Tri- peptides

In 1949 Dunn and McClure (24) found that partial hydrolysates of protein were more effective sources of essential amino acids for Lactobacillus casei than complete hydrolysates. Peters and Snell (70) observed similar results with Lactobacillus delbrueckii and attributed the findings to the presence of unidentified peptides. There are now a num-

ber of reports in the literature dealing with the growth stimulating effects of peptides as compared to the effects of their constituent amino acids. It was felt that the beneficial results of amino acid peptides might also be manifested in rhizobia and the following series of experiments were devised to ascertain the presence and extent of such activity.

Methods:

The peptides tested were obtained from Nutritional Biochemicals Corp., (U.S.A.) and consisted of glycylglycine, glycylglycylglycine, L- and D- leucylglycine, L- and D- leucylglycylglycine, and glycyl-L-tryptophane. Stock solutions were prepared with double glass-distilled water so that the addition of 0.5 ml. to 9.5 ml. of a fluid would result in a final peptide concentration of 0.0001M. These solutions were sterilized at 15 lb. pressure for 8 min. and 0.03 ml. aliquots spotted on a Whatman #1 filter paper sheet and chromatographed in water-saturated phenol at 27°C., according to the method of Woiwod (104). After drying, the sheet was sprayed with 0.1% alcoholic ninhydrin solution. It was found that strong heating of the area on the filter paper to which the peptide solution was being transferred during the "spotting" procedure, resulted in a splitting of the peptide. Therefore, heating was eliminated in this operation.

At the same time that an organism was tested for the effect of pH on its amino acid utilization it was also examined for growth in peptide media. Thus, the growth values obtained on the various amino acids can be accur-

ately compared with the peptide growth values. For each of the three test organisms (R_{21}, R_{20-27A}, and R_{20}) 9.5 ml. of pH 7.0 modified Wilson and Wilson's medium and 0.5 ml. of sterile stock peptide solution were added to each of seven sterile, chemically clean cuvettes. One cuvette, without any peptide added, served as an inoculated control, and another, the uninoculated control, was used as a blank for the nephelos readings. Washed cell inoculum was prepared, as previously described, and a 0.2 ml. aliquot was used to inoculate the various cuvettes. The tubes were then incubated at 30°C. and daily nephelos readings taken with the Photonephelometer. Final pH values were determined with a Beckman glass-electrode potentiometer.

A cell-free extract of R_{21} was prepared from a three liter, 24 hr. aeration culture (Modified Medium "79") by centrifugation in a Scharples centrifuge, washing the sedimented cells in 0.85% saline and grinding for 12 min. with aluminum oxide using a Potter homogenizer and an ice-bath. The cellular extract was centrifuged at 20,000 x g in a Servall vacuum-type centrifuge in order to sediment the cellular debris and the translucent straw-colored supernatant diluted with saline to a final volume of 5 ml. (0.412 mgm. bacterial nitrogen/ml.). Aliquots of 0.5 ml. were added to peptide solutions containing 5 mgm. of peptide per 0.5 ml. double glass-distilled water. The peptides used were L-, and D- leucylglycine, L- and D- leucylglycylglycine, and glycyl-L-tryptophane. In addition L- and D- leucylglycine solutions were prepared containing a

final concentration of sodium cyanide amounting to 0.01M. After incubation for one hour at 30°C., 0.04 ml. quantities of each solution were spotted on filter paper sheets and chromatographed in water-saturated phenol. Controls included cell-free extract alone, known amino acids and peptides, test solutions sampled at zero time and test solutions containing known amino acids (in order to demonstrate uniform movement of these acids in the test mixture). Spot tests for ammonia were carried out using Nessler's reagent.

Results:

Chromatographic analysis demonstrated the purity of the peptides used and ensured that the sterilization method did not result in any splitting of the peptide bonds. The Rf values found for the various amino acids and peptides are given in TABLE 6.

TABLE 6

TABLE SHOWING THE Rf VALUES OF AMINO ACIDS AND PEPTIDES OBTAINED DURING PAPER PARTION CHROMATOGRAPHY IN WATER-SATURATED PHENOL AT 27°C.

Compounds	Rf value
glycine	0.41
leucine	0.82
tryptophane	0.73

Compounds	Rf value
glycylglycine	0.42
glycylglycylglycine	0.48
glycyl-L-tryptophane	0.71
L- and D- leucylglycine	0.76
L- and D- leucylglycylglycine	0.79

The utilization of the di- and tri- peptides as sole sources of nitrogen by R_{21}, R_{20-27A}, and R_{20} is pictorially represented in FIGURE 6. Except for an overall reduction in the magnitude of growth (and in the utilization of L-leucylglycylglycine as compared to L-leucylglycine) the response pattern of R_{20-27A} is very similar to that of R_{21}. The R_{20} pattern, however, is noticeably different. With this organism there was a remarkable growth response brought about by glycyl-L-tryptophane, better growth on glycylglycylglycine as compared with L-leucylglycine and a greater growth attained on D-leucylglycylglycine than on L-leucylglycylglycine. In fact, this particular organism made better use of those peptides which contained D-amino acids than the other two strains of rhizobia. With all three strains, however, L-leucylglycine promoted better growth than D-leucylglycine. The final pH values ranged from 6.2 to 6.8.

The chromatographic results indicated that R_{21} at least partially hydrolyzed all the peptides against which it was tested. In all cases glycine was found as the free acid.

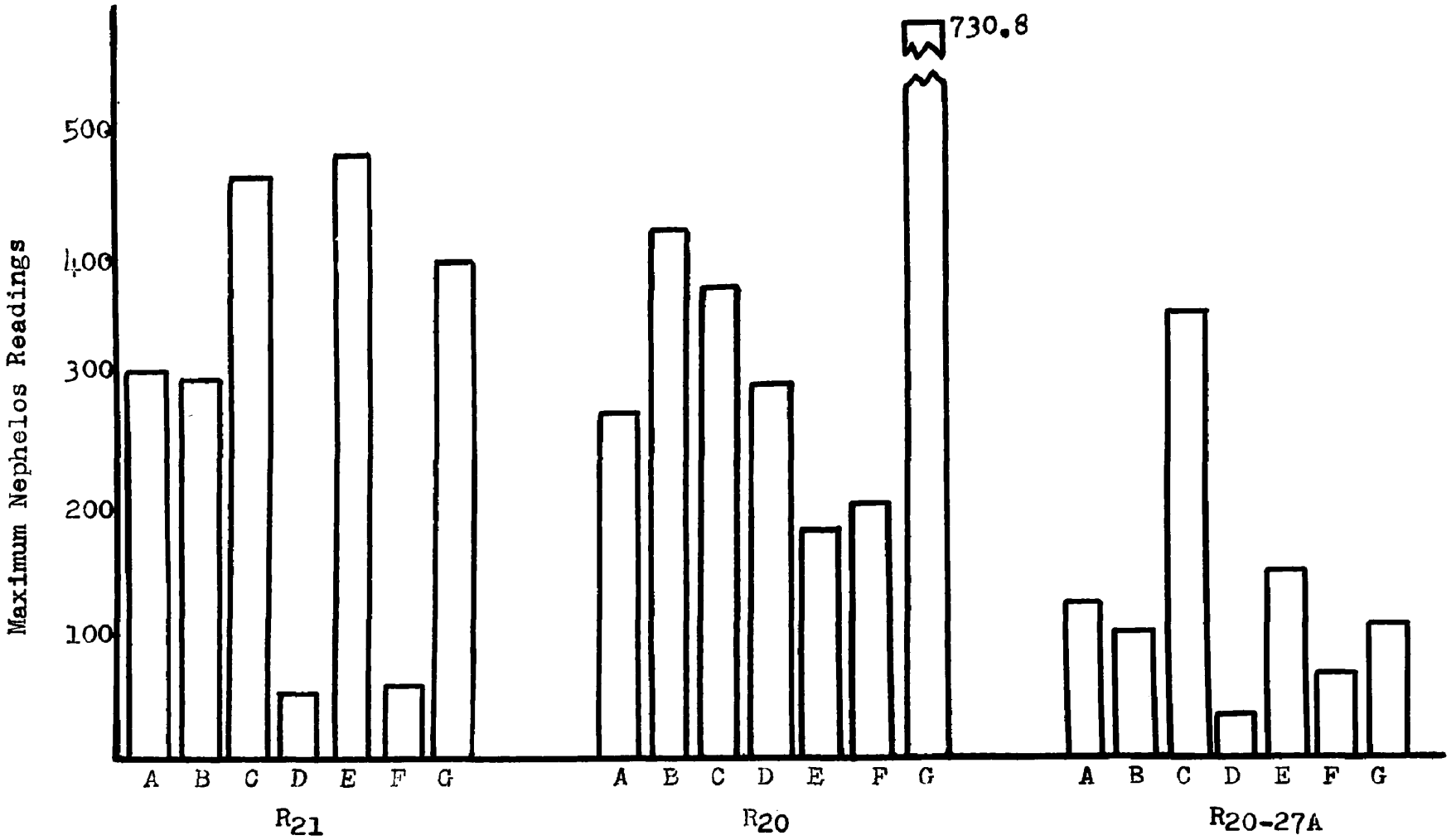


Fig. 6 Vertical bar chart showing the maximum growth attained by three Rhizobium strains in N-free basal medium containing synthetic peptides. (A) glycylglycine (B) glycylglycylglycine (C) L-leucylglycine (D) D-leucylglycine (E) L-leucylglycyl-glycine (F) D-leucylglycylglycine (G) glycyl-L-tryptophane.

Since the Rf value of leucine was similar to that of the leucine peptides no critical attempt was made to identify this amino acid, although several different solvent systems were tried in an effort to obtain better resolution. Elution of the glycine spots in 10 ml. of 50% ethanol: water and comparison of the resulting solutions in a Coleman Spectrophotometer at 570 millimicrons (12) revealed that the amount of glycine produced from D-leucylglycylglycine was only about one-half that produced from the other peptides, which produced similar amounts of glycine. Ammonia was detected in every test solution except in those containing cyanide, and implied that a cyanide sensitive deamination of the liberated amino acids occurred.

TABLE 7 indicates the stimulative and inhibitory activity resident in certain of the peptides, as far as rhizobial growth is concerned.

Discussion:

Although hydrolysis of the peptides by the cell-free bacterial extracts was demonstrated the experimental results are not consistent with the concept of complete hydrolysis. The di- and tri- peptides of glycine, and glycine plus L- leucine, with two exceptions, stimulated the growth of the three Rhizobium strains above the values that would be expected if complete hydrolysis were assumed. The two exceptions occurred with R_{20-27A} and the peptides glycylglycylglycine and L-leucylglycine, both of which yielded "observed" growth values which were not significantly

TABLE 7

STIMULATION AND INHIBITION OF RHIZOBIUM GROWTH BY SYNTHETIC PEPTIDES

	R_{21}			R_{20-27A}			R_{20}		
	Obs.	Calc.	Result	Obs.	Calc.	Result	Obs.	Calc.	Result
glycine	23.0			38.2			34.0		
glycylglycine	308.0	46.0	S(262.0)	120.8	76.4	S(44.4)	277.5	68.0	S(209.5)
glycylglycylglycine	302.0	69.0	S(233.0)	107.4	114.6	N.S.D.	426.5	102.0	S(324.5)
L-leucine	170.2			80.9			74.5		
L-leucylglycine	466.0	193.2	S(272.8)	363.5	119.1	S(244.4)	379.0	108.5	S(270.5)
L-leucylglycylglycine	483.0	216.2	S(266.8)	153.8	157.3	N.S.D.	187.2	142.5	S(44.7)
D-leucine	195.0			48.6			156.5		
D-leucylglycine	54.0	218.0	I(164.0)	37.2	86.8	I(49.6)	302.0	190.5	S(111.5)
D-leucylglycylglycine	60.0	241.0	I(181.0)	70.2	125.0	I(54.8)	208.0	224.5	I(15.8)

Obs. = Observed Values = The maximum nephelos readings attained in an N-free synthetic medium containing these peptides or amino acids.

Calc. = Calculated Values = Values obtained by assuming complete hydrolysis of the peptide. For example, the calculated value for glycylglycine would be twice the observed value for glycine.

S = Stimulation, I = Inhibition. The magnitude of such activity is indicated by the nephelos values in the brackets.

N.S.D. = No significant difference

different from values calculated on the basis of complete peptide degradation. This organism, therefore, apparently has the enzymic capacity to hydrolyze these two peptides completely. With glycylglycylglycine the enzyme (or enzymes) concerned may attack both ends of the tripetide chain simultaneously, thereby leaving no stimulative dipeptide fractions.

It cannot be said with certainty that the utilization of amino acids for protein synthesis by bacteria involves prior incorporation into small peptides, but considerable evidence for this concept has been obtained from observations on bacteria such as Lactobacillus casei which requires, for optimal growth, the presence of preformed peptides (29). Other organisms, as well as rhizobia, use peptides for growth more efficiently than they do the amino acids which compose them. This approach to the study of protein synthesis is at present being explored by Fruton and Simmonds (28). If it is assumed that the stimulative peptides can be directly utilized for protein synthesis by rhizobia the amount of observed stimulation is probably representative of a balance between the rate of incorporation of the peptides into cellular proteins and the relative peptidase activity of these microorganisms. If the peptidase activity of a particular strain is greater than the incorporation rate then the amount of stimulation by a peptide would be much lower than if the converse were true. This probably explains the different degrees of stimulation observed with the bacterial strains used in the present work.

An inhibitory effect was noted when R_{21} and R_{20-27A} were grown in the presence of the D-leucine peptides. This may partly be the result of an inability to carry out transpeptidation reactions involving D-amino acids. It is also necessary, however, to postulate that an inhibitory action occurred between glycine and D-leucine. If incorporation did not occur by reason of the contained D-amino acids it would have been expected that hydrolysis would have proceeded to completion, the amino acid products being removed from the site by various types of secondary reactions, such as deamination. With such hydrolysis the "observed" growth of the organism should have been equal to the "calculated" growth with no evidence of inhibition. The mere fact that inhibition was present makes it appear that one of the liberated amino acids blocked the ulilization of the other as else that a mutual inhibition occurred. Confirmation of such inhibition was obtained by comparing the growth of washed R_{21} cells in basal medium containing glycine, D-leucine, or glycine + D-leucine. A definite reduction in growth was found in the solution containing both amino acids when its maximum nephelos was compared with the theoretical. This action is probably similar to that observed by Kihara and Snell (51), who showed that high levels of glycine inhibited the utilization of D-alanine in Lactobacillus casei, but did not affect L-alanine.

Other reasons for the stimulative results of certain peptides, as suggested by other workers, may or may not apply to rhizobia. These reasons include:

(a) the protection afforded against the destruction of the individual amino acids by other enzymatic forces (51),

(b) the protection afforded against amino acid inhibitors, as shown by several groups of investigators (41), (74),

and (c) the ability of the organism to concentrate the peptide, but not the amino acid (71).

One of the more interesting effects noticed was the stimulation of Rhizobium R_{20} by D-leucylglycine. One might postulate incorporation of this peptide into the protein of the microorganism, but the possiblity of other unknown mechanisms occurring must first be investigated. At present the effect is inexplicable on the basis of former work.

In the absence of further data little can be said about the nature of the intracellular peptide-splitting enzymes present in rhizobia. These bacteria probably contain a glycine dipeptidase, which is a rather specific enzyme according to Smith (81). In view of the fact that D-leucylglycine is attacked as readily as L-leucylglycine by these strains of nodule bacteria the responsible enzyme (or enzymes) must be radically different from most of the classical peptidases, such as leucine aminopeptidase, which exhibits a stereochemical specificty for L-amino acid peptides. It may be that the L-leucine peptides are broken down in the rhizobia by leucine aminopeptidase and that a separate enzyme is responsible for the attack upon the D-forms. The peptidase activity of rhizobia appears somewhat similar to that of Leuconostoc mesenteroides which Smith (8),

in his review article, states as having two dipeptidases and at least two tripeptidases. In addition, this organism also exerts a rapid activity on the D-forms of a variety of peptides, a phenomenon which it now shares with certain strains of Rhizobium.

The enzyme responsible for the breakdown of glycyl-L-trytophane is unknown and represents an interesting possibility for further research.

C. The Utilization of Ammonium, Nitrate and Nitrite Salts in the Presence of Amino and Carboxylic Acids.

Although R_{21}, R_{20-27A}, and R_{20} were not capable of using ammonium, nitrate or nitrite salts "per se" as sole source of nitrogen (48) there was a possibility that utilization might occur in the presence of certain other compounds, particularly amino acids. These experiments were devised to test this hypothesis.

Methods:

Sterile, chemically clean optical cuvettes were filled with 9.0 ml. aliquots of sterile pH 7.0 modified Wilson and Wilson's medium together with 0.5 ml. of sterile ammonium chloride, sodium nitrate, or sodium nitrite stock solutions so that the final concentrations were equivalent to 0.01, 0.001 and 0.0001 moles of salt/liter. These stock solutions were prepared from C.P. grade compounds (British Drug Houses Ltd. - "Analar" grade) and were sterilized by sintered glass filtration. To each cuvette

was added 0.5 ml. of stock L-leucine solution (final concentration = 0.0001M) and 0.2 ml. of washed R_{20}, R_{21}, or R_{20-27A} cells. Inoculated and uninoculated controls were prepared which contained leucine basal medium and 0.5 ml. of sterile distilled water in place of the salt solution. Incubation was carried out at 30°C. and daily nephelos readings recorded for each cuvette. Ammonia and nitrite tests (60) were made after several days and again at the period of maximum growth.

To a series of nine sterile cuvettes 9.0 ml. of sterile pH 7.0 modified Wilson and Wilson's medium were added. To cuvettes #1 to #4 0.5 ml. of stock ammonium chloride solution was added (to give a final concentration of 0.01M) together with 0.5 ml. of 0.5M alpha-ketoglutaric acid (Nutritional Biochemicals Ltd.) or sodium fumarate (British Drug Houses Ltd.). Cuvette #5 contained ammonium chloride basal medium and 0.5 ml. of sterile distilled water, while the remaining tubes held basal medium plus the carboxylic acids and 0.5 ml. of sterile water. After inoculation with 0.2 ml. of washed R_{21} cells these cuvettes, together with inoculated and uninoculated basal medium controls were incubated at 30°C. and nephelos readings made at 24 hr. intervals.

Attempts were made to replace the carboxylic acids by gaseous carbon dioxide. Basal medium containing 0.01M ammonium chloride was inoculated with washed R_{21} cells and placed in a large dessicator together with suitable controls. Measured amounts of air were withdrawn

from the chamber by means of a syringe fitted with a two-way valve and replaced with an equivalent volume of gaseous carbon dioxide (Mathison Co. U.S.A.). The dessicator was placed in a 30°C. incubator for seven days, at which time the tubes were removed and examined. Carbon dioxide concentrations of 5-10% were employed.

Washed cells of Rhizobium R_{21} were added to cuvettes containing 9.0 ml. of modified pH 7.5 basal medium, 0.5 ml. of stock L-histidine solution (final concentration = 0.0001M) and 0.5 ml. of either water or an ammonium chloride solution. Using ammonium chloride solutions of different concentrations final concentrations ranging from 0.to 0.02M were obtained. After incubation at 30°C. the maximum nephelos readings were plotted against the molarity of ammonium chloride to yield a growth response curve. (FIGURE 7)

Results:

FIGURE 8 illustrates the maximum growth in nephelos units found during the utilization of ammonium, nitrate, and nitrite salts by nodule bacteria growing in the presence of amino acid nitrogen. No free ammonia was located in the nitrate or nitrite tubes and no nitrite was found in the nitrate tubes. There was definite evidence that the inorganic nitrogen salts (including nitrite, which some workers regard as being non-utilizable by alfalfa rhizobia) were utilized when amino acids were present to initiate growth. The optimum molarity of ammonium chloride in

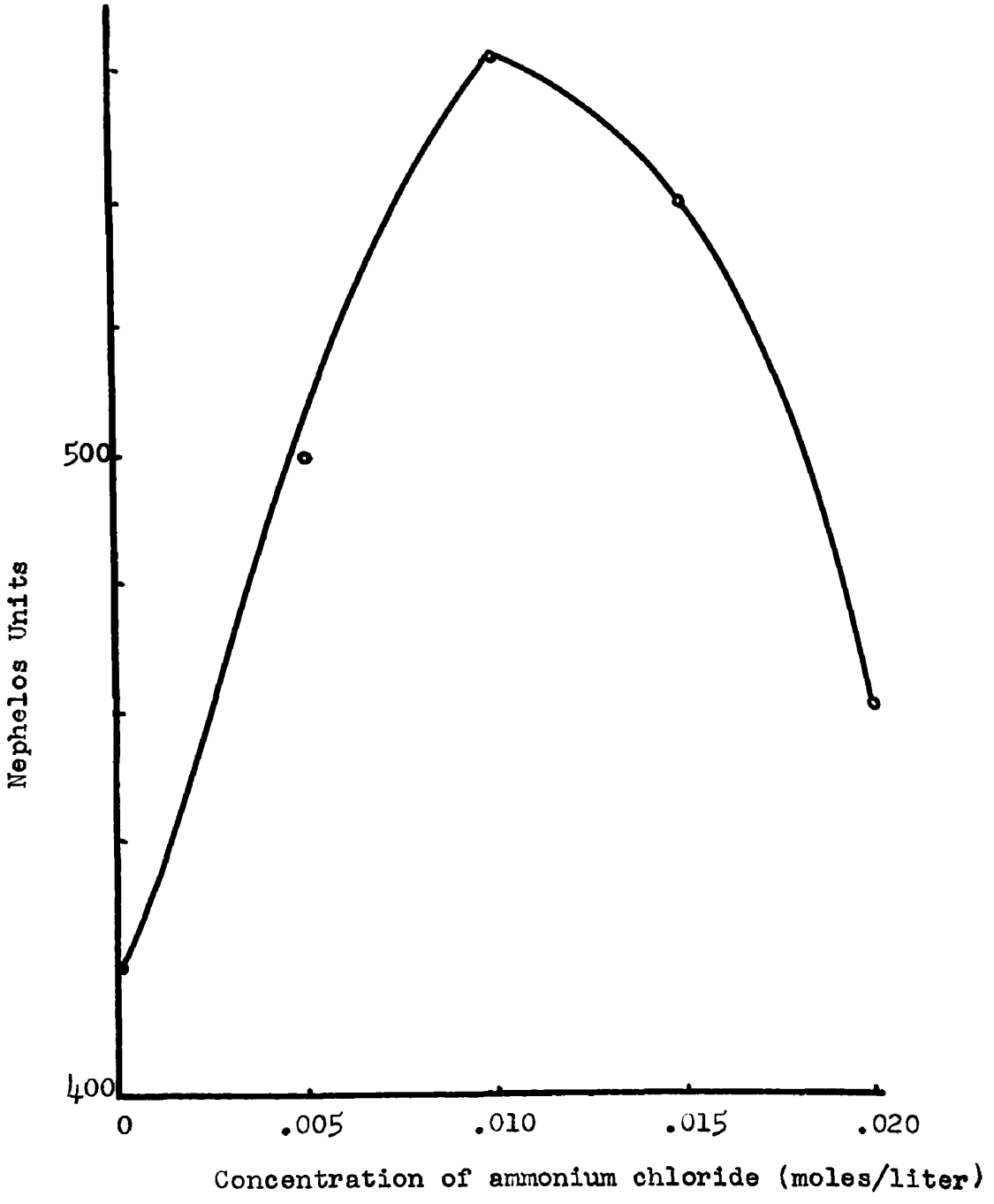


Fig. 7 Growth response curve of Rhizobium R21 in histidine basal medium containing various concentrations of ammonium chloride.

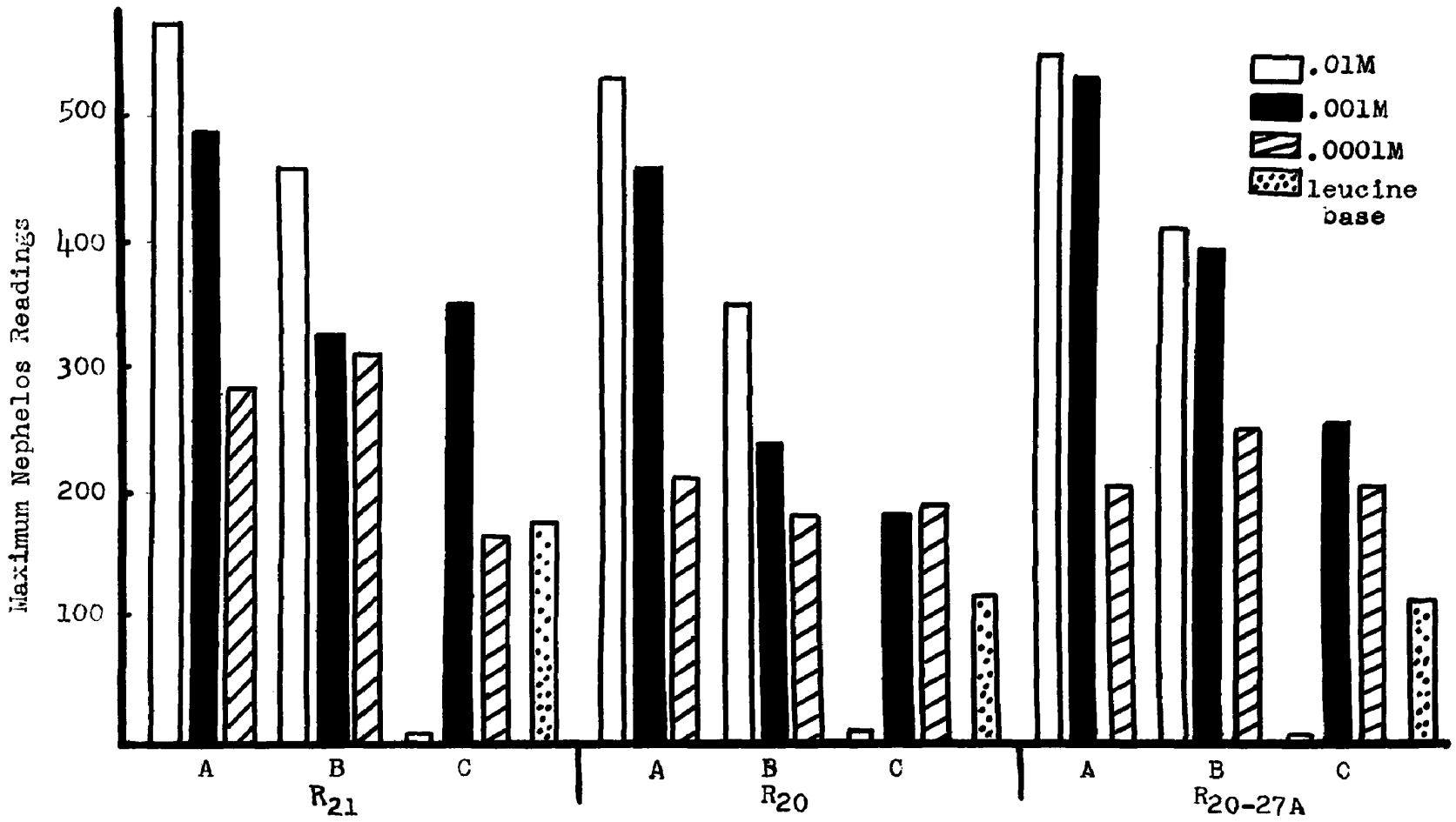


Fig. 8 Vertical bar chart showing the maximum growth attained by three Rhizobium strains in leucine basal medium containing three different concentrations of (A) ammonium chloride (B) sodium nitrate and (C) sodium nitrite.

histidine medium was 0.01 (see FIGURE 7). Table 8 gives values obtained with the carboxylic acids and indicates that the amino acids can be replaced by certain organic acids plus inorganic nitrogenous salts. No growth was evident in any of the ammonium chloride tubes incubated under increased carbon dioxide tensions.

TABLE 8

MAXIMUM NEPHELOS READINGS ATTAINED BY R_{21} IN SYNTHETIC BASAL MEDIA CONTAINING AMMONIUM CHLORIDE AND CERTAIN CARBOXYLIC ACIDS

Carboxylic Acid	*plus* NH_4Cl	minus NH_4Cl
alpha-ketoglutarate	204.0	24.0*
pyruvate	158.0	15.0*
fumarate	32.0	16.0*
none	0.0	0.0

*not significantly different from each other

Discussion:

Although ammonium ions are not utilized by the test Rhizobium strains as the sole source of nitrogen in sucrose basal medium they are readily utilized, as are nitrate and nitrite ions, when either amino or certain carboxylic acids are present. These results may be interpreted

by assuming that in "resting" rhizobia intracellular carbonaceous acids important in the synthesis of amino acids from ammonia do not accumulate in quantities compatible with growth. When carboxylic or amino acids are supplied in the external medium sufficient quantities of these essential "ammonia-fixing" acids become available for synthetic purposes. These results are not by any means novel since Virtanen and Rautanen (96) have reported that Das observed growth of Aerobacter aerogenes on ammonium nitrogen in the presence of fumaric, pyruvic, alpha-ketoglutaric and oxalacetic acids. One of the most prominent acids in amino acid synthesis is alpha-ketoglutaric acid from which amino acids can be formed by a reversal of L-glutamic acid dehydrogenase activity (94). This reaction, together with that of aspartase, forms one of the two known pathways of amino acid synthesis via ammonia utilization (67). This may explain the high activity of alpha-ketoglutaric acid in the present experiment. The other carboxylic acids, including those produced from any amino acid present, may not be in themselves reactive, but are capable of forming alpha-ketoglutarate within the cell, perhaps by a cyclic system of reactions such as the Kreb's cycle. The comparatively small amounts of growth observed in the presence of the three organic acids, but in the absence of any added ammonium salt probably indicates that trace amounts of inorganic nitrogen present in the medium constituents were utilized. Ammonium chloride was found to be a better source of nitrogen in the organic acid media than either nitrate or nitrite, which

probably emphasizes its prime importance. The two latter compounds are no doubt converted to ammonia prior to utilization, since both are readily reducible in the sequence: nitrate ⟶ nitrite ⟶ ammonia (94).

The optimum concentration of ammonium chloride and nitrate was approximately 0.01M, while at this concentration nitrite was markedly inhibitory. It is interesting to note that in Meyerhof's work, as reported by Stephenson (82), the rate of nitrite oxidation by Nitrobacter was greatest at a nitrite concentration of 0.1% and fell off rapidly as the concentration increased. Stephenson believes that this could be explained by the consideration that nitrite acts as a cell poison, possibly by reacting with the free amino groups of cellular proteins. The maximum growth on nitrite in the present work was at a final sodium nitrite concentration of 0.001M and the inhibitory activity of greater amounts than this may be a result of the above mentioned toxic effects.

Clifton(19) mentions that the assimilation of ammonia by bacteria and yeast may be dependent upon energy yielding processes. If the amino and carboxylic acids are more efficient energy producers for this purpose than the sucrose present in the basal medium their influence on ammonia assimilation may be partially explained in this manner as well.

Some heterotrophic bacteria are known to require the presence of added carbon dioxide for optimum growth and the substitution of several dicarboxylic acids for this gas can satisfy the requirement (1). The utilization of carboxylic acids by rhizobia is apparently not due to a CO_2

requirement, since this gas could not replace the acids in ammonium ion utilization.

Although no evidence was found for the conversion of nitrate to nitrite, or of nitrite to ammonia these reactions may actually exist, the end products being either very transitory in nature or rapidly utilized.

The utilization of inorganic nitrogen sources does not appear promising as an indication of effectiveness or ineffectiveness in the nodule bacteria, however the data have resulted in the formulation of a synthetic medium for further growth studies on washed cells of Rhizobium meliloti. It is composed of sucrose, 2.50 gm.; ammonium chloride, 0.53 gm.; dipotassium hydrogen phosphate, 0.50 gm.; sodium chloride, 0.10 gm.; magnesium sulphate, 0.20 gm.; calcium sulphate, 0.20 gm.; ferric chloride, 0.10 mgm.; histidine, 7.50 mgm.; glass-distilled water, 1000 ml. The pH is adjusted to 7.5 and sterilization accomplished by Seitz filtration. The purification of the sucrose and mineral salts is carried out using the methods of Wilson and Wilson (101).

D. The general metabolism of certain amino acids

The Rhizobium strains herein investigated are apparently dependent upon the presence of preformed amino acids (or ammonium ions plus certain organic acids) for growth in a chemically defined medium - at least so far as small inocula are concerned. The actual enzymatic

mechanisms by which these organisms metabolize amino acids is somewhat obscure and further information is needed. For this reason the present section of the work was commenced.

Any living cell possesses an amazing array of enzymic potentialities, some of which are exceedingly complex and, in many cases, not as yet visualized by the cellular physiologist. The processes which occur are even more complicated in that they may be easily modified by changes in the physiochemical environment. In addition, a number of metabolic pathways may exist for any one compound and the importance of any particular route may be easily under - or overrated. In the words of Cohen and McGilvery (21): "It is plain that no detailed picture of nitrogen metabolism in terms of specific enzymatic reactions can be drawn. In the tissue slice, tissue brei, and crude tissue extract, the breakdown of a particular amino acid may invoke transamination, transimination, decarboxylation, and possibly other yet unknown non-oxidative reactions yielding different nitrogen compounds more susceptible to attack, so that a direct interpretation of the results obtained with such systems is usually difficult and subject to many doubts". In spite of this aura of pessimism it was felt that the labyrinth of intracellular reactions could at least be partially resolved by a number of biochemical procedures. Utilizing some of these procedures a general survey of the amino acid metabolism of the effective Rhizobium strain R_{21} was made. It was hoped, thereby, to gain an understanding of the "normal" general

metabolic paths inherent in rhizobia so that an approach to subsequent studies of "abnormal" metabolism, as manifested in the ineffective or parasitic strains, could be made with more assurance. No attempt was made to elucidate the transformations specific for individual amino acids.

In order to eliminate needless repetition several of the experimental methods used repeatedly in this section will be given below:

(a) Preparation of cell suspensions and cell-free extracts.

Three liters of Modified Medium "79" were inoculated with a 10 ml. logarithmic phase culture of R_{21} and incubated under continuous aeration from a sintered glass sparger for 48 hr. After this period the cells were checked for purity by the Gram stain, sedimented in a Sharples centrifuge, washed twice with 0.85% sterile saline and brought to the desired volume with buffer of appropriate pH. This preparation constituted a "washed cell suspension". For the preparation of cell-free extracts the washed cells were sedimented, mixed with finely powdered aluminum oxide and ground for 12 min. in a Potter homogenizer, using an ice bath to reduce frictional heat. The resulting mixture was centrifuged in a Servall vacuum centrifuge for 15 min. at 18,000 x g in order to sediment the cellular debris. The resulting translucent supernatant composed the "cell-free extract".

(b) Determination of "total cell nitrogen"

Aliquots of the cellular preparations were heat-digested with the mixture of Campbell and Hanna (17), which employs a copper-selenium catalyst and both phosphoric and sulphuric acids. After digestion the mixtures were transferred quantitatively to a Markham micro-Kjeldahl apparatus and after the addition of 48% sodium hydroxide the ammonia was steam distilled into 4% boric acid containing a mixed indicator (22). The ammonium borate thus formed was titrated with standard acid. A reagent blank was run with each duplicate determination and the amount of standard acid used substracted from the titration values obtained for the test mixtures. Using a standard solution of ammonium sulphate the recovery by this method was calculated at 99.6%.

(c) Paper-partition chromatography

The technique used was essentially that of Woiwod (104). Aliquots of the mixtures to be analyzed were spotted on Whatman #1 filter paper sheets with gentle warming and the sheets placed in an equilibrated chromatography tank for several hours. The solvent was then added and descending paper chromatography carried out for 24-28 hr. at 27°C. The sheets were dried in a warm oven, sprayed with a 0.1% alcoholic solution of ninhydrin and heated. Water-saturated phenol was one of the better solvent systems found for the detection of amino acids in various test mixtures. Butanol-acetic tended to produce a "tailing" effect.

(d) Manometric techniques

A Warburg constant-volume microrespirometer was used in all cases. After preparing the flasks and manometers they were placed on the water bath and shaken continuously at 96 oscillations/min. A 10 min. period was allowed for temperature equilibration, after which time the manometers were set at 150 mm., the stopcocks closed, and readings taken every five minutes until the change in each flask was approximately the same. The contents of flasks and sidearms were then mixed and readings made periodically until the end of the run.

The organic chemicals used were all obtained from Nutritional Biochemicals Ltd. (Ohio, U.S.A.).

1. Decarboxylation

Certain amino acids (arginine, lysine, histidine, ornithine, tyrosine, glutamic acid, beta-hydroxyglutamic acid, and aspartic acid) are decarboxylated by some bacteria to form carbon dioxide and the corresponding amine, according to the reaction:

$$R.CH(NH_2)COOH \longrightarrow R.CH_2NH_2 + CO_2$$

The coenzyme in most cases is pyridoxal phosphate. Gale (32) found that the enzymes concerned were specific for one amino acid and that the amino acid substrates must be of the L-configuration and possess more

than two unsubstituted polar groups. These enzymes, with the possible exception of glutamic acid decarboxylase, are strictly adaptive, act only at an acidic pH and are formed to a maximum extent at about the time active cell division ceases.

Although decarboxylation occurs there is some doubt as to the actual metabolic significance of the reaction. It is apparently not a route for amino acid synthesis because the equilibrium lies far to the right, but it may serve to liberate carbon dioxide for the synthesis of organic acids via a Wood-Werkman type reaction. The amines produced are probably further metabolized with the liberation of ammonia.

An aspartic decarboxylase has been studied in some Rhizobium species (11), but comparatively little work has been done on the decarboxylation of histidine and glutamic acid. In a series of unpublished experiments the author found that carbon dioxide is aerobically liberated from these particular amino acids by the activity of cell-free extracts of the nodule bacteria. These results were not in themselves too significant, however, since carbon dioxide evolution was also observed from other amino acids such as valine and methionine. This fact, coupled with the finding that both the L- and D-isomers were attacked cast some doubt as to whether or not a glutamic or histidine decarboxylase existed. It was more likely that the gas was derived from the keto acids produced from these amino acids by prior enzymatic activity, but further work was required for substantiation.

Methods:

Cell-free extracts and washed R_{21} cells were prepared as previously described and diluted with M/20 pH 6.0 phosphate buffer. Duplicate Warburg flasks, with sidearm gas vents, contained 0.2 ml. of water in the center wells, 0.5 ml. of cell preparation in the sidearms and 0.8 ml. of M/20 pH 6.0 buffer plus 0.5 ml. 0.125M neutralized L- or D-glutamic acid (or histidine) in the main compartments. The average micro-Kjeldahl nitrogen content of the cell-free extract was 0.54 mgm./ml. The vessels were seated on the manometers and joined in series with short pieces of rubber tubing running from the sidearm vent of one flask to the open stopcock arm of the manometer belonging to the adjacent flask. Prepurified nitrogen gas (Matheson Co. U.S.A.) was then slowly passed through all the flasks so that the contained air was flushed out. After the passage of several liters of gas the pressure within the flasks was raised slightly by partially compressing the nitrogen outlet tube running from the end flask of the series. The manometer stopcocks and sidearm vents were immediately closed and the manometers placed on the water bath. As the flasks warmed up the stopcocks were opened momentarily to release gas. The bath temperature was 30°C. and the flasks were continuously agitated.

A two-manometer technique for the simultaneous determination of oxygen uptake and carbon dioxide evolution was carried out with washed cells and L-glutamic acid, without using any nitrogen flush. The buffer pH was

7.0 and the carbon dioxide flask-constants were calculated using the alpha prime correction factor for retention of carbon dioxide in the buffer (93). The contents of the flasks were the same as previously described except that the oxygen flasks contained 0.2 ml. of 10% KOH in the center wells while the carbon dioxide flasks contained 0.2 ml. of water.

The aerobic evolution of carbon dioxide from Rhizobium cells growing in the presence of glycine was studied by means of radioactive tracer techniques. Two hundred milliliters of modified Wilson and Wilson's medium containing 15 mgm. of non-labelled glycine and 5 mgm. of glycine - 1 - C^{14} (specific activity = 0.025 microcuries/mgm. = 925 d.p.s./mgm.) was adjusted to pH 7.5, sterilized by Seitz filtration, and placed in a specially designed flask having a test-tube sealed into the bottom. R_{21} cells were washed and added to this medium as the inoculum. The apparatus employed is shown in FIGURE 9. Air was bubbled through at a rate of approximately 64 bubbles/min. and daily nephelos readings were made with the Photonephelometer by cutting off the aeration and using the attached test-tube as a cuvette. The barium carbonate that accumulated in the trapping vessels was filtered off every 24 hr., carefully dried and 95.0 mgm. accurately weighed out. This amount was evenly spread over the bottom of a special, shallow aluminum pan whose area of 4.7 cm. was the same as that of the end window of the Geiger-Müller counter used. This pan was inserted in a plastic sample holder which held it immediately below the thin-mica end window of the counting tube. A lead castle pro-

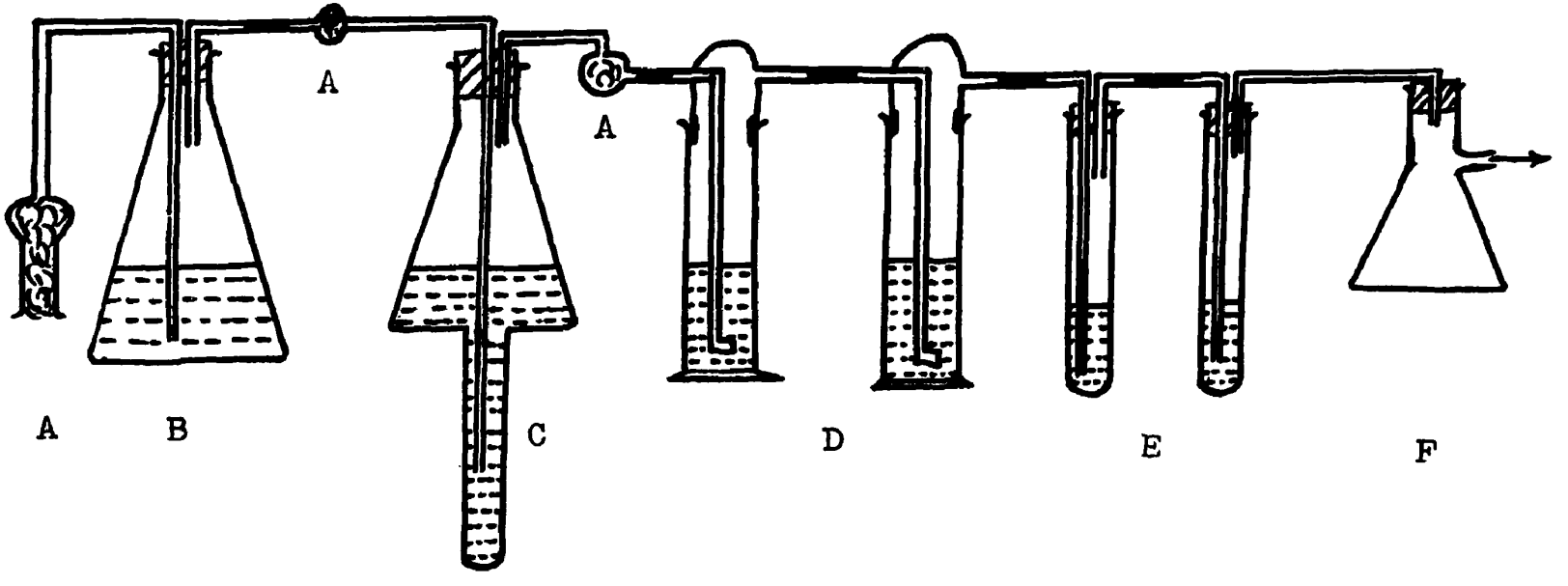


Fig. 9 Diagram of apparatus used in the recovery of $C^{14}O_2$ produced from glycine-1-C^{14} by R_{21}. (A) cotton filters (B) sterile acidified H_2O (C) culture in aeration flask (D) gas washing units + barium hydroxide (E) test tubes + barium hydroxide (F) water trap.

vided adequate shielding. The counting tube was operated at one kilovolt, which represented the mid-point of the plateau on a counting rate-voltage curve. The decade scaler (Electronic Associates Ltd. EA-SC3T) was operated for ten minutes and the counts averaged as "counts per minute (c.p.m.)". No resolving time corrections were made since the counts were not that numerous.

After the first day small aliquots of the culture were removed daily, centrifuged and 0.1 ml. of the supernatants spotted on a Whatman #1 filter paper sheet and chromatographed in water-saturated phenol. After development the colored ninhydrin-positive areas were cut out, extracted for 30 min. by shredding in 3 ml. of 50% (v/v) aqueous ethyl alcohol and the optical density of the extracts determined in a Coleman Junior Spectrophotometer at 570 millimicrons (12).

Upon the completion of the experiment the cells were centrifugalized from the medium, ground with aluminum oxide, centrifuged at 20,000 x g, and an aliquot of the resulting clear extract dried and examined for radioactivity. The protein present in the dried aliquot and remaining fluid extract was precipitated with heat and trichloroacetic acid and after high speed centrifugation and thorough washing of the solid residue the residual radioactivity of the "protein" and evaporated "non-protein" fractions were determined. Values were corrected to a dry weight basis.

The activity of washed intact R_{21} cells on two of the keto acids of

importance in intermediary metabolism was investigated using a double-manometer technique similar to that employed for L-glutamic acid. Both aerobic and anaerobic (nitrogen flush) conditions were used with 0.5M alpha-ketoglutaric acid and 0.5M sodium pyruvate as substrates. Under anaerobic conditions the buffer pH was 6.0 (35), while under aerobic conditions the buffer was of pH 7.0. The optimum pH for the aerobic decarboxylation of these keto acids is 8.0 (84), but a pH of 7.0 was chosen since the alpha prime correction factor for carbon dioxide retention in the buffer is subject to considerable error at pH values above 7.0. The nitrogen content of the cell suspension amounted to 4.61 mgm. N/ml. After the reaction was completed the contents of the aerobic Warburg vessels were checked for the presence of aldehydes by the Schiff reagent, with the realization that a negative result would be of little value since any aldehyde formed might be immediately reduced or dismutated.

Results and Discussion:

The cellular preparations of Rhizobium R_{21} did not form any detectable amounts of carbon dioxide anaerobically from histidine, glutamic acid, sodium pyruvate or alpha-ketoglutaric acid. It may be concluded, therefore, that amino acid decarboxylases for histidine and glutamic acid were absent under the experimental conditions designated. Under aerobic conditions, however, carbon dioxide was rapidly evolved from at least three of the four compounds. TABLE 9 lists experimentally determined values for pyruvate, alpha-ketoglutaric acid and L-glutamic acid.

TABLE 9

$Qco_2(N)$ VALUES, $Qo_2(N)$ VALUES AND $\frac{CO_2}{O_2}$ RATIOS FOR THE OXIDATIVE PRODUCTION OF CARBON DIOXIDE FROM SEVERAL SUBSTRATES BY R_{21}

Substrate	$Qco_2(N)$*	$Qo_2(N)$**	$\frac{CO_2}{O_2}$
sodium pyruvate	196.4	190.8	1.16
alpha-ketoglutaric acid	161.7	150.8	1.13
L-glutamic acid	173.6	170.3	0.98
endogenous	60.4	79.3	0.77

* microliters of CO_2 evolved per hour per mgm. cell nitrogen - endogenous not subtracted.

** microliters of O_2 taken up per hour per mgm. cell nitrogen - endogenous not subtracted.

The values given are averages obtained from duplicate flasks.

The endogenous $\frac{CO_2}{O_2}$ ratio probably represents a respiratory quotient indicative of protein oxidation, a conclusion further strengthened by the finding of endogenously produced ammonia (see "Oxidation and Deamination" section). The ratio for L-glutamic acid was approximately one and was similar to that found for both keto acids. This ratio is usually representative of carbohydrate oxidation (7).

The removal of the amino groups from most of the glutamic acid molecules by transamination would yield keto acids which could be metabolized to give such a value.

FIGURE 10 graphically illustrates the relationships between the disappearance of glycine, the production of $C^{14}O_2$ and the growth curve of the test organism. The release of radioactive carbon dioxide is directly correlated with the disappearance of glycine and the growth of R_{21}. The carbon dioxide produced by R_{21} in glycine medium is, therefore, partially derived from the carboxyl group of this amino acid. This fact, together with the other results, indicates that keto acids are probably produced from at least some amino acids by processes such as deamination and transamination and that these keto acids can be oxidatively decarboxylated. It is extremely unlikely that glycine decarboxylation can occur since no such decarboxylase is known. In addition glycine has only two polar groups and the pH of the medium used was not conducive to potential decarboxylase activity.

Not all of the radioactive carbon escaped into the atmosphere as carbon dioxide. Part of it was incorporated into cellular material, particularly protein (see TABLE 10). The mechanism of incorporation is unknown but may have involved a number of processes such as direct assimilation of glycine and/or assimilation of part of the evolved carbon dioxide by reason of a Wood - Werkman type reaction which could result in the primary synthesis of a keto acid. This latter mechanism could account for the

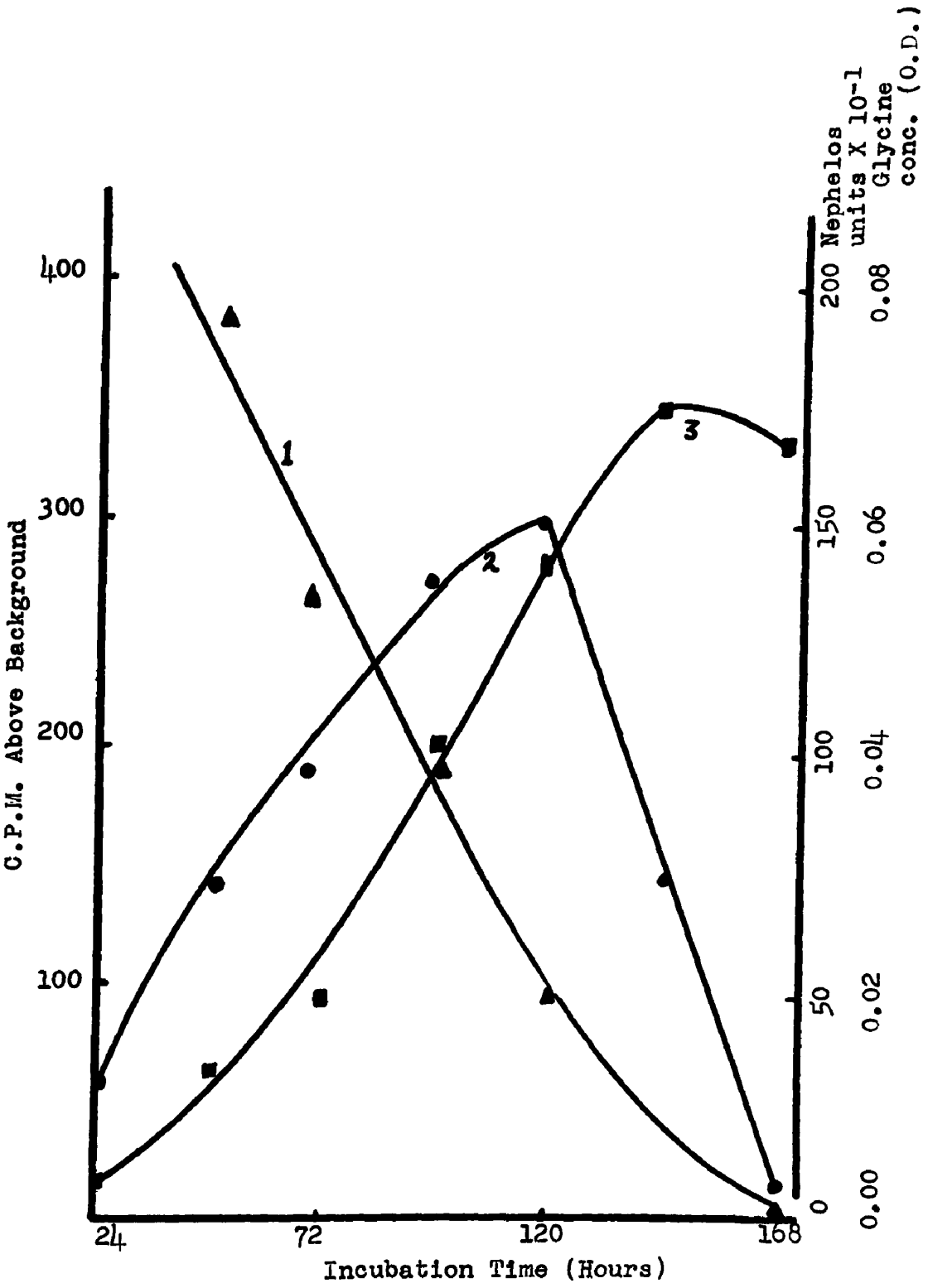


Fig. 10 Graphs illustrating the production of radioactive carbon dioxide from glycine-1-C^{14} by R21. (1) the disappearance of added glycine (2) the output of $C^{14}O_2$ (3) the growth curve of R21

radioactivity found in the "non-protein" cell fraction. It is unlikely that this radioactivity is derived from unassimilated intracellular glycine since amino acids are apparently not accumulated intracellularly to any detectable extent by this organism (48).

TABLE 10

RADIOACTIVITY PRESENT IN THE "PROTEIN" AND "NON-PROTEIN" FRACTIONS OF RHIZOBIUM R_{21} AFTER GROWTH IN GLYCINE-1-C^{14} MEDIUM

	"Protein" fraction	"Non-protein" fraction
Total count (c.p.m./mgm. dry wt)	89.3	59.7
Background count (c.p.m.)	15.5	15.5
Count above background (c.p.m./mgm. dry wt.)	73.8	44.2
% of original count*	62.5	37.5

* total original count (minus background and before fractionation = 119.0 c.p.m./mgm. dry wt.

The Schiff test was negative and no statement can be made as to the intermediate formation of aldehydes during decarboxylation of the keto acids, although it would be assumed that aldehyde would only be produced by anaerobic decarboxylation.

2. Oxidation and deamination

In addition to the possibility of having its carboxyl group removed via decarboxylation an amino acid may lose its amino group by the process of oxidation. Although the same organism may carry out both processes at one time or another they do not occur simultaneously. Deamination occurs at an alkaline pH when the amino acid carboxyl group is ionized ($R.CH(NH_2).COO^-$), while decarboxylation takes place at an acidic pH where the amino group is ionized ($R.CH(NH_3^+).COOH$). The general amino acid oxidases (deaminases) are of two major types - those which attack L-amino acids and those which act on the D-isomers. These enzymes exhibit differences depending upon their source, but they are essentially stereospecific. Glycine, glutamic acid, serine, threonine, cysteine and cystine are not attacked. A specific glycine oxidase exists, however, and L-glutamic acid may be deaminated via a stereospecific L-glutamic acid dehydrogenase. L-aspartic acid may have its amino group removed as a result of aspartase activity, a reaction often referred to as desaturation deamination since one of the end products is an unsaturated acid (fumaric). This latter reaction can only be demonstrated by inhibiting succinic dehydrogenase by cyclohexanol and fumarase activity by toluene. Serine, threonine, and cysteine may undergo dehydration deamination to yield pyruvic acid and ammonia, the reaction occurring in several steps.

Whereas the D- and L- amino acid oxidases and glycine oxidase appear to be flavoprotein enzymes capable of using molecular oxygen as their acceptor (a slight anaerobic activity is known to occur) L-glutamic dehydrogenase requires D. P. N. or T. P. N. and can be termed an anaerobic dehydrogenase. Regardless of which of these enzymes brings about deamination the process itself is always oxidative in nature and occurs in two steps (7).

1. $R.CH(NH_2).COOH \longrightarrow R.C = NH.COOH + 2H$
2. $R.C = NH.COOH + H_2O \longrightarrow R.C = O.COOH + NH_3$

It is probable that the imino acid intermediate is also produced in the deamination of the hydroxy acids, serine and threonine. The hydrogen peroxide formed when the hydrogen of reaction #1 is transferred to oxygen brings about a decomposition of the keto acid formed in the second reaction. The overall reaction, therefore, is:

$R.CH(NH_2).COOH + O_2 \longrightarrow R.COOH + CO_2 + NH_3$

Since the deamination of amino acids plays a very important metabolic role its presence was investigated in Rhizobium R_{21}.

Methods:

Washed R_{21} cells were diluted to 14 ml. with pH 8.0 M/40 phosphate buffer and aerated aseptically for 2 hr. Two 1.2 ml. lots were recentrifuged, the supernatant discarded and the cells resuspended with

1.2 ml. of pH 6.0 and pH 7.0 buffer. These suspensions contained 1.74 mgm. bacterial nitrogen/ml. Duplicate Warburg vessels were prepared containing 0.2 ml. 10% KOH in the center wells, 0.5 ml. of washed cells in the side arms and 0.8 ml. pH 8.0 M/40 phosphate buffer plus 0.5 ml. 0.125M L-amino acid solution in the main compartments. The glutamic acid and histidine solutions were previously neutralized to pH 7.0. With glycine duplicate flasks were prepared using buffers of pH 6.0, 7.0 and 8.0 and containing washed cells resuspended in buffers of corresponding pH. Endogenous respiration flasks contained double-distilled water in place of the amino acid solution. The bath temperature was 30°C. After two hours the contents of the flasks were centrifuged and the supernatants quantitatively analyzed for ammonia by Nesslerization, as carried out by Peech (69). The method was as follows:
1 ml. of the test solution was pipetted into a 125 ml. Erlenmeyer flask to which was added 45 ml. double glass-distilled water, 1 ml. 10% sodium tartrate and 2.5 ml. of Nessler's reagent, with shaking after each addition. After 25 min. the transmittance was measured in a Coleman Junior Spectrophotometer using a 6-304B cuvette and a wavelength of 450 millimicrons. The percent transmittance was referred to a previously prepared concentration - transmittance (calibration) graph and the ammonia concentration determined as ammonium. From this concentration the endogenous ammonia value was subtracted and the resulting figure was used to calculate the millimoles of ammonia found /flask/2 hr./

mgm. cell nitrogen.

$Qo_2(N)$ values were calculated from the slopes of the linear portions of the oxygen uptake curves (0-30 min.) The endogenous respiration was subtracted from the total oxygen uptake in these calculations, although Burris and Wilson (15) observed that glycine stimulated the endogenous respiration of certain rhizobia.

The endogenous oxygen uptake of the test organism was quite high and was not appreciably reduced by doubling the aeration period after washing. Later work indicated that it could be reduced by storing the washed cells for three days at 5°C. in a small amount of physiological saline.

The above manometric procedure was duplicated using D-amino acids and a washed and aerated cell suspension containing 0.686 mgm. of bacterial nitrogen per ml. In addition, the action of certain inhibitors on glycine oxidation was studied. These compounds (sodium benzoate, sodium azide and sodium cyanide) were added so as to give an initial concentration in the flasks of 0.01 moles/liter.

An attempt was made to isolate the keto acid (glyoxylic) produced by the oxidative deamination of glycine. The procedure used was a combination of the methods of Janke and Tayenthal (46) and Cavallini and Frontali (18) and was as follows: 20 ml. of a glycine solution containing 5 mgm. of amino acid/ml., 20 ml. of a heavy suspension of washed R_{21} cells suspended in pH 8.0 M/40 phosphate buffer (or 5 ml. of ground

cells, including cell-wall fragments) and 5 ml. of a 5% alcoholic suspension of 2, 4-dinitrophenylhydrazine were added together and incubated overnight at 30°C. In several instances catalase, prepared from beef liver by the procedure of Sumner and Somers (85) was used in place of the dinitrophenylhydrazine. (In this case the reagent of Friedemann and Haugen (27) was added after the incubation period and before ether extraction.) The suspensions were deproteinized with a 10% solution of trichloracetic acid, centrifuged, and the supernatants extracted several times with ethyl ether. The ether solutions were then brought to dryness in a vacuum dessicator over "Drierite" and KOH, one milliliter of 1N ammonium hydroxide and an equal amount of chloroform added, and the mixtures shaken gently. After centrifugation the supernatant ammonia solutions were spotted on a Whatman #1 filter paper sheet and chromatographed in a solution of n-butanol, ethanol, and water (40/10/50 by volume.) Controls included the dinitrophenylhydrazones of known keto acids and a processed reaction mixture which contained no added glycine.

The methylene blue reduction technique was employed for the detection of an L-glutamic acid dehydrogenase in R_{21}. Thunberg tubes of conventional design contained 3 ml. of pH 8.0, M/40 phosphate buffer, 3 ml. of M/50 neutralized L-glutamic acid, 1 ml. of 1:10,000 aqueous methylene blue and, in the cap, 1 ml. of cell suspension. The cells were prepared by centrifuging a 100 ml. Modified Medium "79" culture of R_{21}, which had been incubated 72 hr. at 27°C. The cells were then washed twice in 0.85%

saline, resuspended in 0.2 ml. of saline and stored for three days at 5°C. Just prior to use they were diluted with 1 ml. of pH 8.0 buffer and standardized in the spectrophotometer to 18% T at 420 millimicrons, using the same buffer as a diluent. Endogenous tubes contained water in place of the substrate and control tubes contained cells killed by boiling and the addition of chlorobutanol. The tubes were evacuated for 3 min. with a vacuum pump, equilibrated in a 42°C. water bath for 10 min. and the cells added from the caps. The rate of dye reduction was followed in a spectrophotometer at a wavelength of 540 millimicrons (86).

Results and Discussion:

Rhizobium R_{21} was found to consume oxygen during amino acid metabolism. The uptake curves are shown in FIGURES 11 and 12. The gradual increase in rate after the initial 30 min. period, which was also noticed by Burris and Wilson (15), is attributed to cellular growth and is not to be confused with the rapid increase in rate found immediately upon the addition of substrate. The zero time point on these curves represents the point of substrate addition. TABLE 11 indicates the $Qo_2(N)$ values obtained with the various amino acids, together with the millimoles of ammonia (as NH_4^+) recovered.

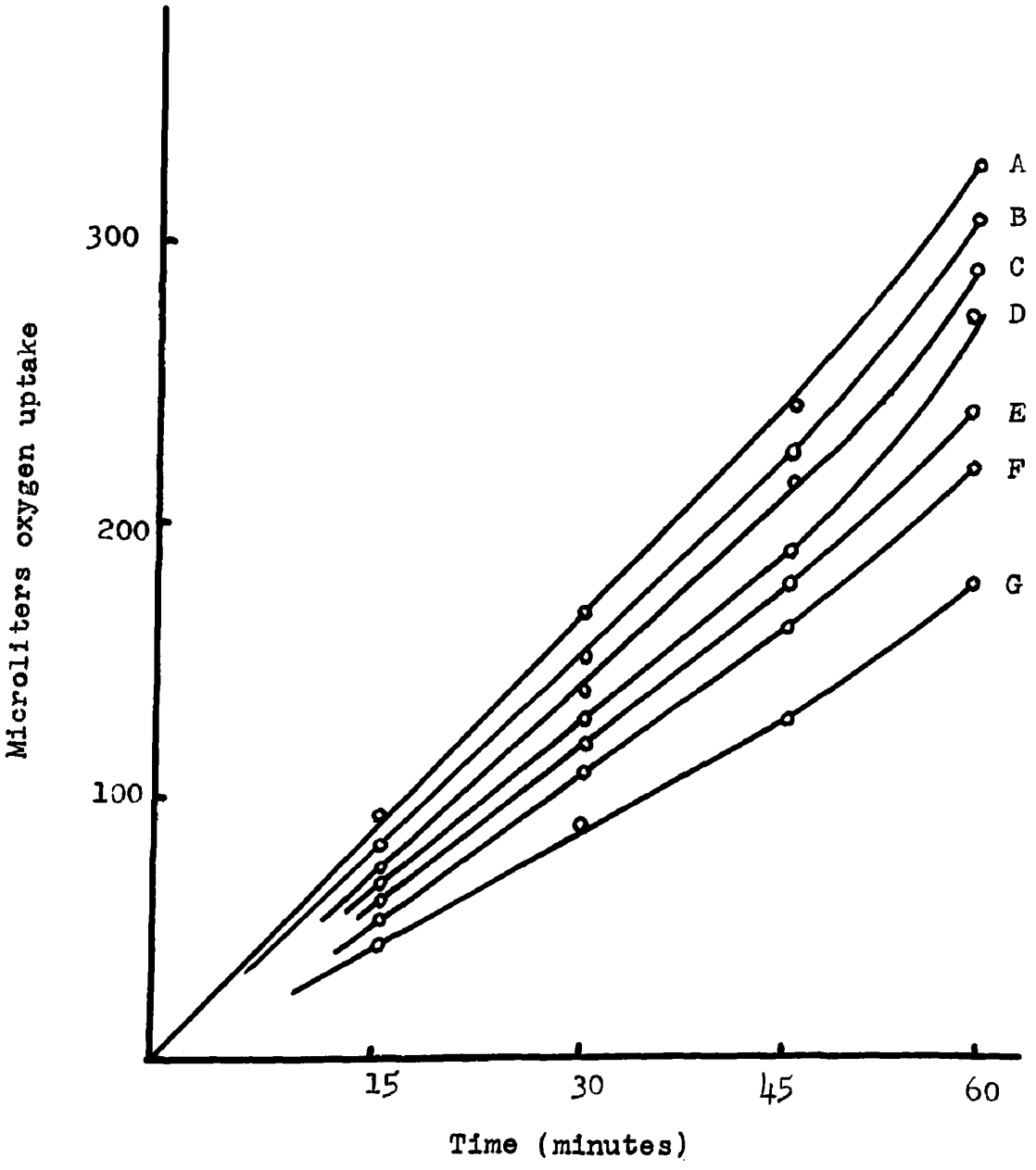


Fig. 11 Oxygen uptake of Rhizobium R_{21} on glycine and L-amino acid substrates. (A) L-glutamic acid (B) glycine (C) L-histidine (D) L-valine (E) L-methionine (F) L-leucine (G) endogenous

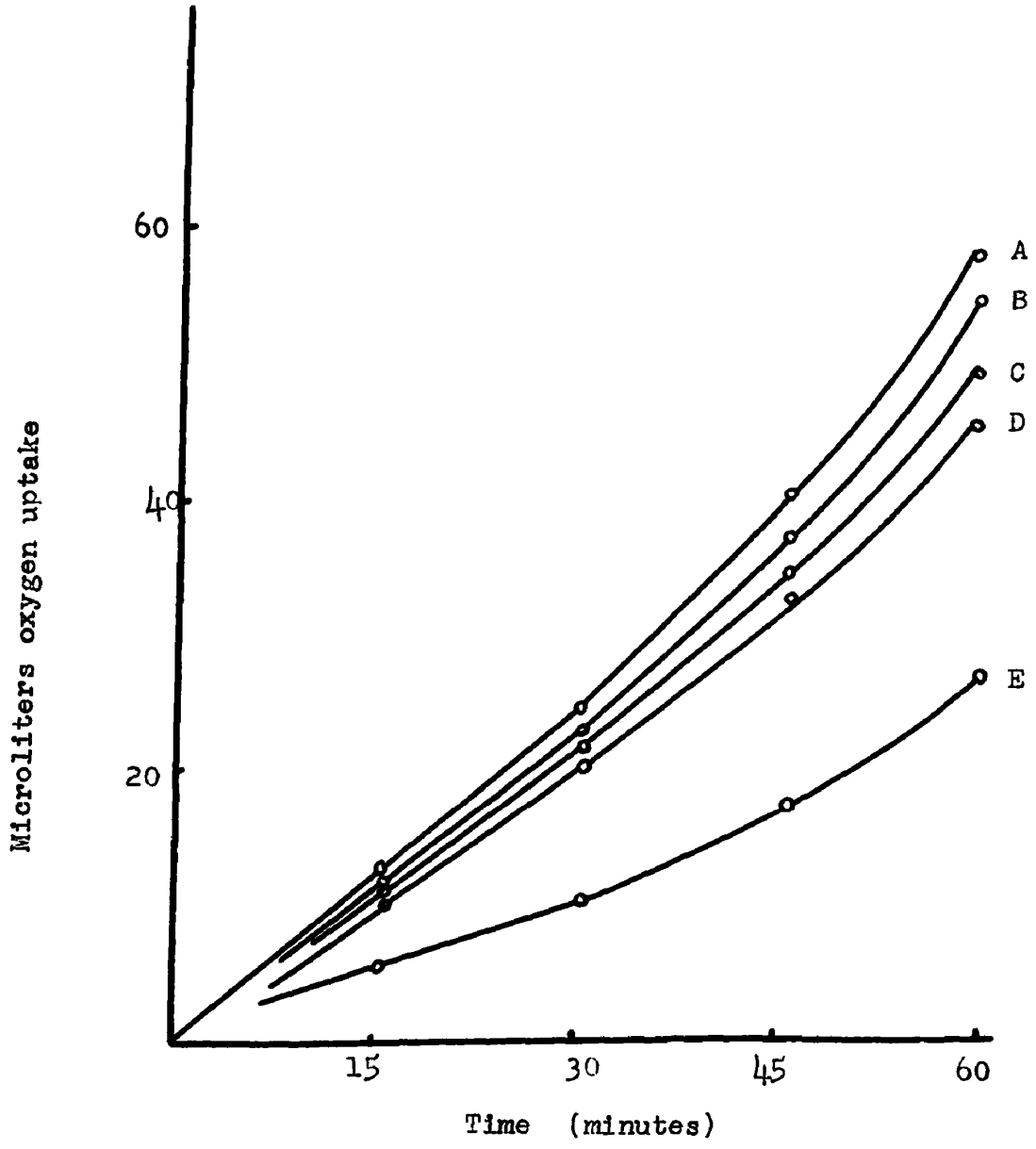


Fig. 12 Oxygen uptake of Rhizobium R_{21} on D-amino acid substrates. (A) D-glutamic acid and D-leucine (B) D-methionine (C) D-histidine (D) D-valine (E) endogenous

TABLE 11

$Qo_2(N)$ VALUES AND MILLIMOLES OF AMMONIA RECOVERED DURING THE ACTIVITY OF RHIZOBIUM R_{21} ON L- AND D- AMINO ACIDS

Amino acid *	Average number of mM ammonia recovered/flask/ 2 hr./mgm. cell nitrogen **	$Qo_2(N)$ **
L-glutamic acid	1.4	168.1
glycine	4.8	128.1
L-histidine	3.0	113.1
L-valine	2.1	81.9
L-methionine	1.8	68.3
L-leucine	1.4	33.6
D-leucine	10.5	78.2
D-glutamic acid	3.8	77.0
D-methionine	15.2	72.9
D-histidine	14.3	70.8
D-valine	8.4	68.8

* 35 mM/flask

** endogenous subtracted

It must be emphasized that these sets of values are only of a relative nature. The small amounts of ammonia recovered with the L-amino acids may imply that transamination occurred and that the oxygen uptake resulted from the metabolism of the keto acids formed. The larger amounts of ammonia found with the D-amino acids may infer a slower rate of transamination and a correspondingly higher rate of oxidative deamination. These rates would be expected to vary, depending on the amino acid substrate. The high oxygen consumption and low ammonia recovery from L- and D-glutamic acid probably reflects the high activity of this amino acid in transamination (see section on "Transamination"). The ammonia produced endogenously (average of 0.7 mM/flask/2hr./mgm. cell nitrogen) may have resulted from deamination of intracellular amino acids, as suggested by Wilson (15). The seemingly low overall ammonia values could be a result of partial assimilation of the evolved gas, or its escape into the atmosphere due to the basic nature of the buffer used.

In the absence of any concrete knowledge of the rates of metabolic reactions within living Rhizobium cells any definite statements concerning the amounts of ammonia found or the quantities of oxygen consumed would be presumptuous.

FIGURE 13 shows the effect of pH on the oxygen uptake in the presence of glycine. A pH of 8.0 was distinctly superior to more acidic pH's and this is to be expected if the process involved is one of deamination,

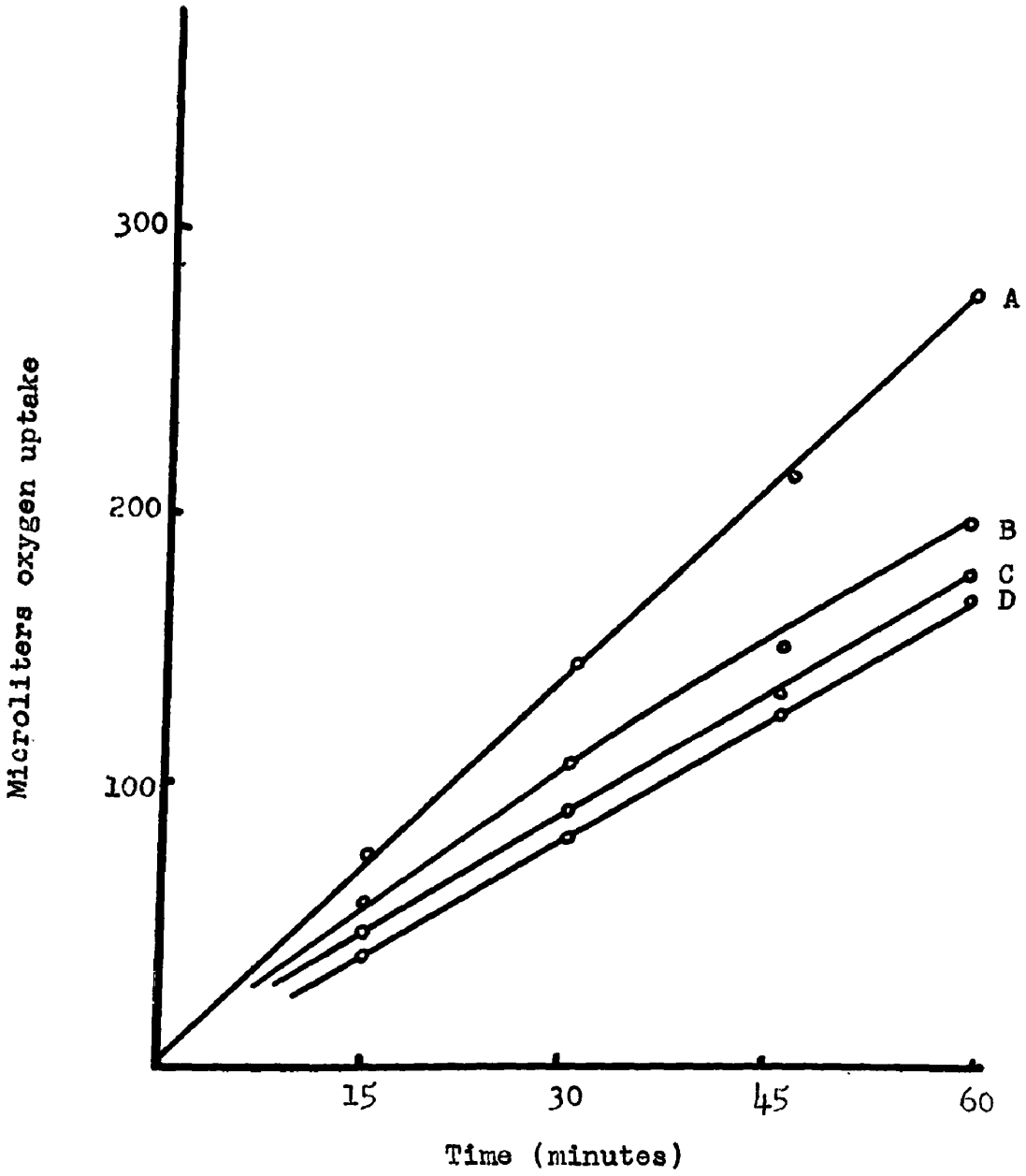


Fig. 13 The effect of pH on the oxygen uptake of Rhizobium R_{21} on glycine substrate. (A) pH 8.0 (B) pH 7.0 (C) pH 6.0 (D) endogenous

since the pH optimum for this particular reaction lies in the range 8 - 9 (31).

The effects of added inhibitors are indicated in FIGURE 14. Cyanide is usually considered to be an inhibitor of metalloprotein enzymes such as cytochrome oxidase, although it is known to inhibit transamination (20) and certain other reactions as well. Of all the enzymes deaminating amino acids only the L-amino acid oxidase of Proteus vulgaris and a similar enzyme found by Krebs in liver and kidney breis are said to be inhibited by cyanide (54). Nevertheless, glycine oxidation was completely inhibited by this agent, although there is a possiblity that the inhibition is not primary, but that it is directed against a secondary reaction such as that involved in the transportation of glycine across the cell wall (34, 68, 79), if this occurs by methods other than simple diffusion. The cyanide caused an 85% reduction in ammonia formation from glycine, compared with 63.2% and 11.3% reduction by azide and benzoate respectively.

Azide appeared to inhibit the oxygen uptake on glycine completely and to partially decrease the endogenous respiration. A supplementary experiment was carried out to study the effect of azide on the endogenous respiration alone and it definitely indicated that the oxygen uptake in the absence of substrate is only slightly affected by this inhibitor. The endogenous respiration of R_{21} is, therefore, somewhat similar to that of Aerobacter aerogenes, which Tissières (91) reports to be azide-resistant.

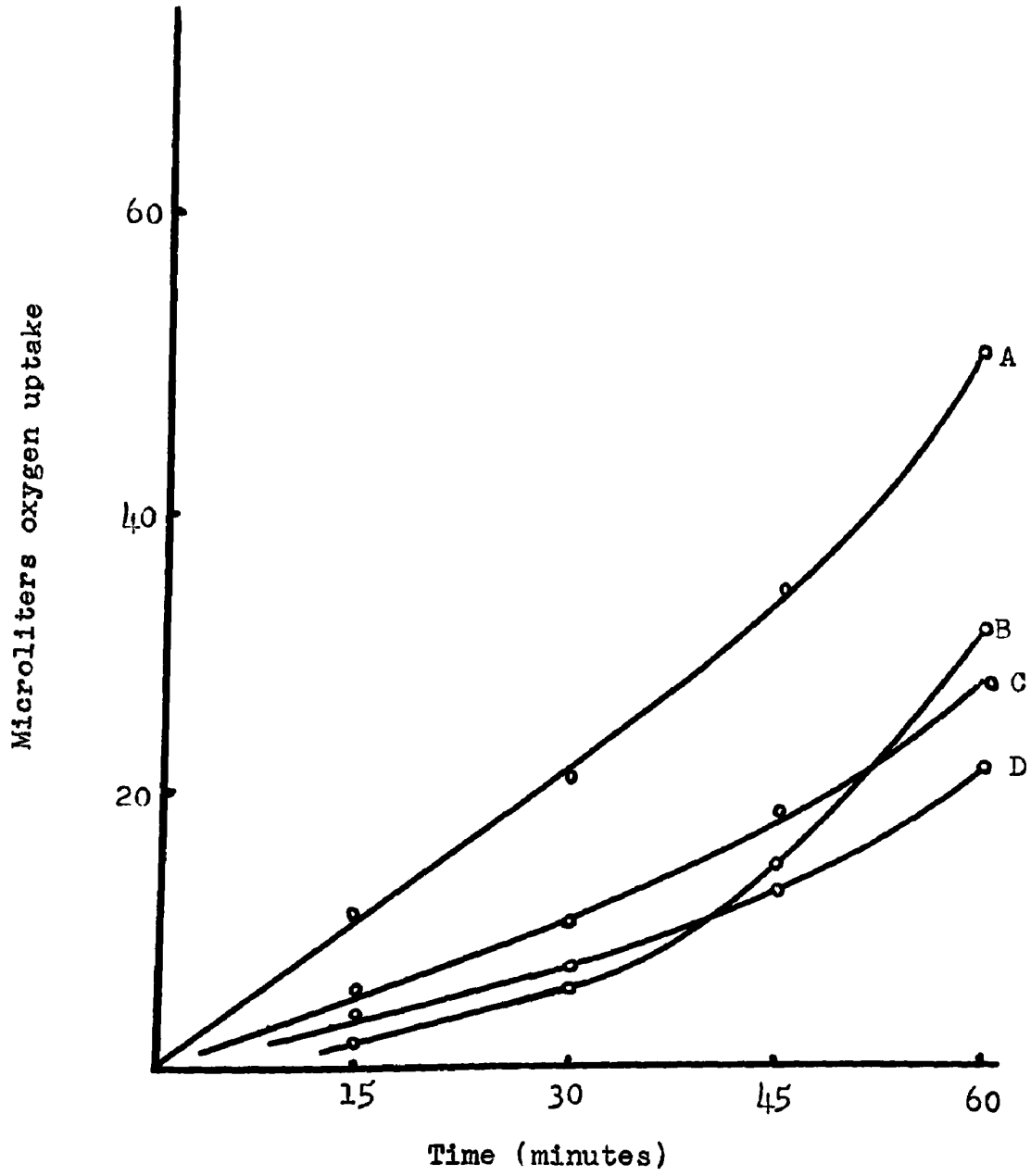


Fig. 14 The effect of inhibitors on the oxygen uptake of Rhizobium R_{21} on glycine substrate (A) glycine (B) glycine + .01M benzoate (C) endogenous (D) glycine + .01M azide. Complete inhibition observed with .01M cyanide

With benzoate, an inhibition was apparent during the initial 30 min. period, but after this time there was a stimulation of the oxygen uptake. This phenomenon may be attributed to an adaptive mechanism; the benzoate acting as an inhibitor until such time as adaptive enzymes are produced which can oxidize it. On the other hand it may be the result of slow entrance of this compound into the cell.

Glyoxylic acid, resulting from the oxidative deamination of glycine, was identified as the 2, 4-dinitrophenylhydrazone, but only in the mixtures which originally contained dinitrophenylhydrazine. No acid was isolated from those mixtures to which catalase had been added and the implication is that this acid is metabolized by a mechanism other than mere hydrogen peroxide decarboxylation.

The anaerobic reduction of methylene blue in the presence of intact R_{21} cells and L-glutamic acid is shown in FIGURE 15. The initial lag may be due to the time required for the acid to permeate the cell barrier. An L-glutamic acid dehydrogenase is therefore present and potentially enables this organism to "fix" ammonia by a reversal of its activity.

All available evidence indicates the presence of an L- and D-amino acid oxidase, a glycine oxidase and an L-glutamic acid dehydrogenase in Rhizobium R_{21}. The deamination of D-amino acids would provide a ready means for the utilization of these isomers since the resulting ammonia could be used to aminate certain organic acids with the resulting produc-

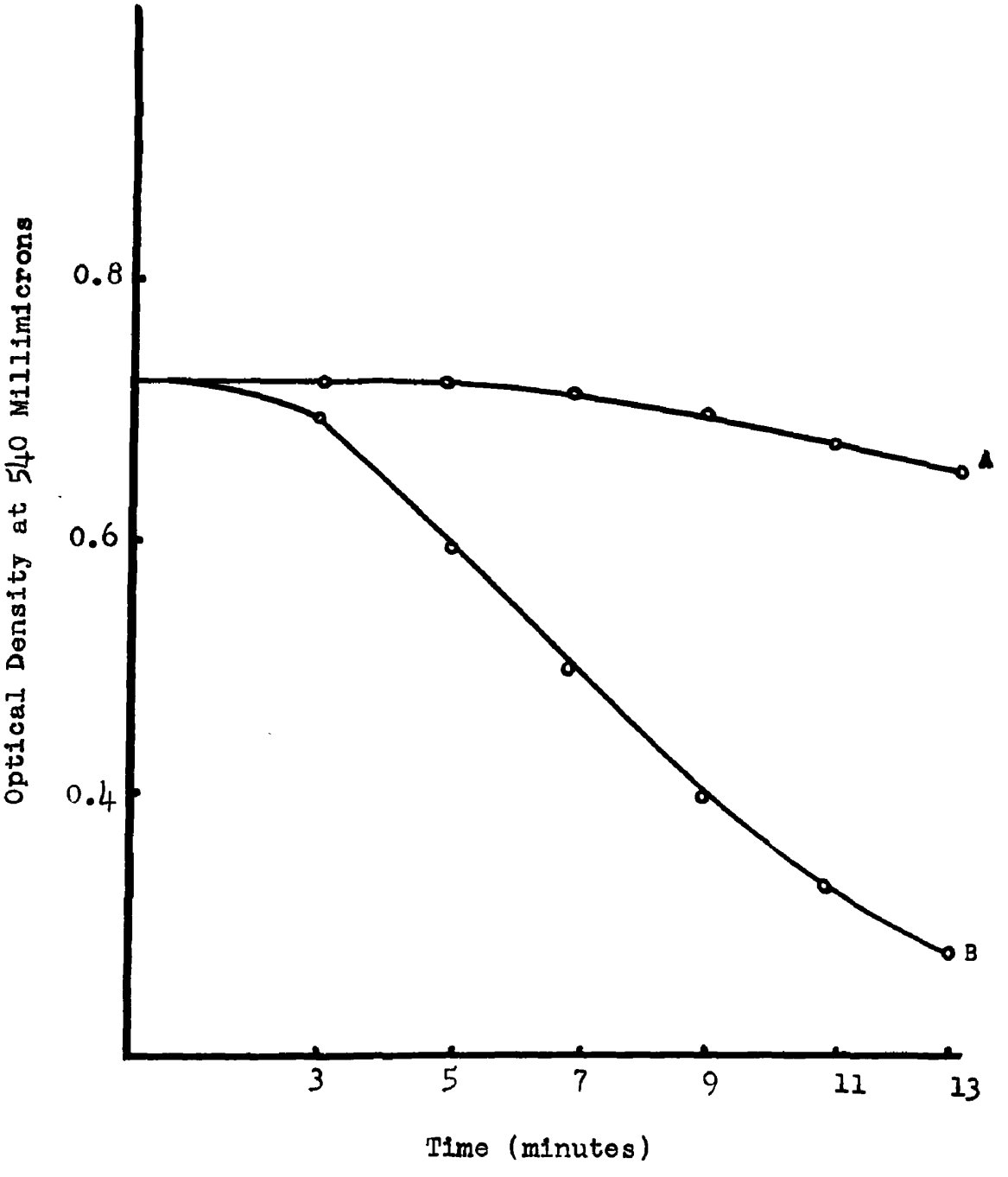


Fig.15 The anaerobic reduction of methylene blue by R_{21} in the absence (A) and presence (B) of L-glutamic acid.

tion of L-amino acids which could be metabolized in the normal way. Lyman and Kuiken (59) report that lactobacilli can utilize D-amino acids if the medium contains pyridoxamine, the suggested mechanism being deamination of the D-isomers to the corresponding keto acids which are then reaminated and utilized as such. A somewhat similar mechanism may operate in rhizobia.

3. Transamination

Transamination, which involves the transfer of the amino group of an alpha-amino acid to the alpha position of an alpha-keto acid was first described by Braunstein and Kritzman (13). Since that time a number of such reactions have been found in various tissues and bacteria, pyridoxal phosphate being the coenzyme involved. In all except a relatively few cases glutamic acid is one of the reactants. Studies on the utilization of L- and DL-amino acids by parasitic and effective strains of Rhizobium suggested that some of these compounds might be linked by transamination mechanisms (48), and this supposition was supported by the author's finding of a number of such reactions in cell-free extracts of these microorganisms (49). The fact that rhizobia are also capable of utilizing D-amino acids suggested that transamination might be involved in these cases as well, although most of the previous reports have indicated that in these reactions the original and synthesized amino acids are of the L-configuration.

Methods:

Cell-free extracts of Rhizobium R_{21} were prepared in the usual manner and diluted with pH 8.0 M/20 phosphate buffer to a final volume of 9 ml. (1.23 mgm. bacterial nitrogen/ml.). Acetone-dried cells were obtained by mixing 10 volumes of ice-cold acetone with washed cells and rapidly evaporating off the solvent in a vacuum dessicator over "Drierite" and glycerol. After dilution to 9 ml. with pH 8.0 buffer a micro-Kjeldahl nitrogen determination revealed an average of 5.27 mgm. N/ml. Reaction mixtures were then prepared having the composition given in TABLE 12.

TABLE 12

COMPOSITION OF TRANSAMINATION REACTION-MIXTURES

	Mixtures				
	1	2	3	4	5
0.3 ml. M/20 pH 8.0 phosphate buffer	+	+	+	+	+
0.1 ml. 0.5M alpha-keto acid	P	KG	-	KG	KG
0.2 ml. 0.125M D-amino acid	DA	DA	DA	DV	DA
0.1 ml. (1.0 mgm.) adenosine triphosphate**	+	+	+	+	+
0.1 ml. (3 micrograms) pyridoxal HCl	+	+	+	+	+
0.8 ml. cell preparation	+	+	+	+	+*

P = sodium pryuvate

KG = neutralized alpha-ketoglutaric acid

DA = neutralized D-aspartic acid

DV = D-valine

* boiled

** disodium salt - 95% pure

These mixtures were placed in Thunberg tubes with the cellular preparations in the caps. The control mixture, number five, was heated for 5 min. in a boiling water bath and its cell material similarly heat-treated. After evacuation with a vacuum pump for 3 min. the tubes were sealed, the enzyme preparations added and anaerobic incubation carried out for 1 hr. at 30°C. The reaction was then immediately stopped by placing the unsealed tubes in a boiling water bath for five minutes and, after centrifugation, 0.03 ml. aliquots of the various supernatants were spotted on Whatman #1 filter paper and chromatographed in water-saturated phenol. Controls included known amino acids, cellular preparations alone, reaction mixtures sampled at zero time and the complete mixture plus either L-glutamic acid or L-alanine. The latter was included to demonstrate uniform movement of the glutamic acid and alanine spots. In order to detect any aspartase activity or possible anaerobic amination of alpha-ketoglutarate the entire procedure was repeated with reaction mixtures containing ammonium chloride and either sodium fumarate (0.5M) or neutralized alpha-ketoglutaric acid (0.5M).

Mixtures containing D-aspartic acid were incubated for 3 hr. and analyzed manometrically using a double-manometer technique and an L-glutamic acid decarboxylase prepared from Clostridium perfringens (welchii) (33). (The exact technique will be given in the following section on "Racemase Activity"). Warburg vessels contained 1 ml. of test mixture, 0.2 ml. of 10% KOH or water in the center wells, and 0.8 ml. of decarboxylase preparation in the side arms.

Since all of the L-amino acids used in this thesis, with the exception of glycine and L-histidine, had been shown to act as amino donors in the synthesis of glutamic acid by the test organism (49), it was felt that these two amino acids should also be tested for activity. Consequently 0.125M glycine and L-histidine were incubated in reaction mixtures containing alpha-ketoglutaric acid and after the reaction period these mixtures were heat-treated, centrifuged and analyzed chromatographically for glutamic acid.

Results and Discussion:

The cell-free extract was found to have synthesized glutamic acid in those mixtures which contained alpha-ketoglutaric acid and either D-aspartic acid or D-valine. No alanine was detected in the D-aspartic acid - pyruvate mixture and no new amino acids were noticed in the zero time controls. In addition, no ninhydrin positive spots representing synthesized amino acids were found with control mixtures

three and five or with the cell preparations alone.

When acetone-dried cells were used minute amounts of glutamic acid and alanine were found in all the mixtures including the heat-treated control and were not enzymatically produced during the incubation period. Subsequent experimentation indicated that these amino acids did not arise as a result of the heat treatment, but that they may have been absorbed onto the cell walls prior to drying and liberated upon the addition of buffer. In addition, trace amounts of an unknown amino acid with an Rf similar to serine was found in all the mixtures except the control and appeared to have been enzymatically formed. Large amounts of glutamic acid were found in those mixtures containing alpha-ketoglutaric acid and either D-aspartic acid or D-valine, while considerable amounts of alanine were observed in the D-aspartic acid-pyruvate mixture. The amount of alanine formed in the latter case was considerably in excess of that which could have been produced by a transamination reaction involving pyruvate and the trace amount of contaminating glutamic acid which was present. Since D-aspartic-alanine transamination activity was only found to be associated with acetone-dried cells it was possible that the method used in preparing the cell-free extracts had brought about inactivation of the enzyme (s) concerned. However, the amount of such activity might have been small enough so that it could only be detected in very large amounts of cellular material and in the present case much more material was added as

acetone-dried cells than as cell-free extract. Both cell preparations synthesized glutamic acid from glycine and L-histidine.

The manometric procedure revealed that at least a small part of the glutamic acid synthesized from D-aspartic acid was of the L-configuration. During the 3 hr. incubation period the amount of L-glutamic acid produced was calculated at 0.14 mgm.

Although it is tempting to assume that a true D-aspartic L-glutamic acid transamination had taken place (thus postulating a new enzyme) certain other possibilities must be examined. Oxidative deamination, which could possibly result in the synthesis of L-glutamic acid via amination of alpha-ketoglutaric acid by the liberated ammonia, can be eliminated as a factor in the present work. Nevertheless, it is possible that the synthesized glutamic acid was originally of the D-configuration and was subsequently racemized. There is also a possibility of the occurrence of a series of reactions somewhat similar to those postulated by Thorne (88). For example, the following sequence could have taken place:

(a) D-aspartic acid + pyruvate $\rightleftharpoons$ D-alanine + oxalacetic acid

(b) D-alanine $\xrightleftharpoons{\text{racemase}}$ D-alanine + L-alanine

(c) L-alanine + alpha-ketoglutaric acid $\rightleftharpoons$ L-glutamic acid + pyruvic acid.

Reaction (c) is known to occur in R_{21} (49) and the process would, there-

fore, depend upon the presence of D-aspartic—D-alanine transaminase and an alanine racemase. Alanine has been found to be synthesized from D-aspartic acid and pyruvate in the acetone-dried cells, but the presence of an alanine racemase is debatable without further work. Thorne (89) in a more recent report stated that sonic extracts of Bacillus subtilis contained transaminases which resulted in the synthesis of D-glutamic acid from either D-aspartic acid or D-alanine, and less actively from D-methionine and D-serine. Such reactions, in the presence of certain racemases would provide a mechanism for the conversion of D-amino acids into L-amino acids prior to further metabolism.

4. Racemase activity

The name "racemase" has been proposed for an enzyme catalyzing the interconversion of D- and L-amino acids. Two such enzymes have been reported. Alanine racemase is present in many microorganisms and has been partially purified by Wood and Gunsalus (105) from Streptococcus faecalis extracts. It apparently has a requirement for pyridoxal phosphate as a coenzyme. Ayengar and Roberts (6) have demonstrated a glutamic acid racemase with an optimum pH of 8.0 in acetone powders of Lactobacillus arabinosus. The mechanism by which these enzymes operate is as yet unknown.

In order to elucidate as fully as possible the mechanism of D-amino acid utilization by rhizobia it was considered necessary to investigate the

occurrence of a racemase within these microorganisms. The results observed in the previous section on D-amino acid transamination might, for example, be explicable on the basis of racemization.

Methods:

Preliminary work on an "aspartic racemase" involved aerobic incubation of intact R_{21} cells or cell-free extracts in pH 8.0 buffer containing either L- or D-aspartic acid. After inhibition of the activity by heat the mixtures were centrifuged and the supernatants analyzed manometrically for L- and D-aspartic acid using freshly prepared Proteus vulgaris L-amino acid oxidase (83) and hog kidney D-amino acid oxidase. Although the data appeared to indicate the absence of any aspartic racemization there was the inherent possiblity that oxidative deamination would interfere in the interpretation of the results. Consequently the procedure was modified to eliminate deamination.

Cell-free Rhizobium R_{21} extracts (0.50 - 1.30 mgm. bacterial nitrogen/ml.) and acetone-dried cells (1.57 - 1.85 mgm. N/ml.) were prepared. (In some cases, during the growth of the cells the particular amino acid under test was added several hours prior to harvesting, but this procedure was later discarded.) The reaction mixtures used had the following composition:

2.0 ml. M/20 pH 8.0 phosphate buffer

0.8 ml. 0.125M neutralized L-aspartic acid or L-alanine

0.4 ml. (12 micrograms) pyridoxal hydrochloride

0.4 ml. (4 mgm.) adenosine triphosphate (disodium salt)

L-aspartic acid and L-alanine control solutions and their enzyme preparations were heat-treated and the various mixtures placed in Thunberg tubes with 1.5 ml. of cellular material and processed in the same fashion as in the transamination experiments. After incubation at 30°C. for 1 hr. the reaction was stopped by immersing the unsealed tubes in boiling water for 5 min. The various mixtures were then centrifuged and 0.03 ml. aliquots of the supernatants analyzed by paper-partition chromatography in water-saturated phenol. Tests for ammonia were carried out with Nessler's reagent and proved negative.

The mixtures were examined manometrically for the presence of D-amino acids. Duplicate Warburg flasks contained 0.2 ml. 10% KOH in the center wells, 1 ml. of test mixture in the main chambers and 0.8 ml. of D-amino acid oxidase in the side arms. The bath temperature was 37°C. In several experiments the oxidase was prepared from guinea pig kidney cortex by the method of Krebs (52) but in later work a commercially available hog kidney D-amino acid oxidase was employed. (Nutritional Biochemicals Corp., Ohio. 1 gm. = 150,000 units). A solution of this was prepared which contained 1500 units/0.8ml.

Test mixtures were prepared containing D-glutamic acid in place of L-aspartic acid or L-alanine. Manometric analyses were made on

these mixtures, after reaction, using a standard double-manometer technique for carbon dioxide (93), and an L-glutamic acid decarboxylase preparation obtained from Clostridium perfringens (33). The preparation of the enzyme suspension was as follows: a flask containing 200 ml. of Cooked Meat Medium (Difco) was inoculated with a log phase culture of Cl. perfringens* and incubated at 37°C. for 24 hr. This culture was filtered through a Buchner funnel, centrifuged, and the cells washed in 0.85% saline and diluted with saline to a final volume of 6.2 ml. (1.83 mgm. cellular N/ml.). This suspension was immediately used to obviate any deterioration. The duplicate Warburg flasks contained 1.0 ml. of test mixture in the main chamber, 0.2 ml. 10% KOH or water in the center wells and 0.8 ml. of decarboxylase preparation in the side arms. The bath temperature was 37°C. In addition to a boiled control mixture this method required an endogenous respiration flask containing pH 8.0 buffer, KOH, and Cl. perfringens suspension.

Results and Discussion:

No significant difference in oxygen uptake was found between the reaction mixture originally containing L-aspartic acid and its respec-

* This culture had been carefully checked for activity on both L- and D-glutamic acid and was found to act only on the L-isomer.

tive control, when examined by D-amino acid oxidase. The L-glutamic acid decarboxylase yielded no significant amounts of carbon dioxide in excess of the control when added to flasks containing D-glutamic acid test mixture. It may, therefore, be concluded that no aspartic or glutamic acid racemization occurred under the experimental conditions used.

The oxygen uptake curve, plotted from date obtained from the action of D-amino acid oxidase on an L-alanine mixture is given in FIGURE 16 and is indicative of the formation of a D-amino acid by Rhizobium R_{21}. Paper-partition chromatography failed to reveal the presence of any amino acid not present in the control and, therefore, the D-isomer is concluded to be that of alanine. In this particular case the acetone-dried cells (1.57 mgm. N/ml.) produced 0.35 mgm. D-alanine from 1.7 mgm. L-alanine (in 1 ml. aliquot of test mixture) in 1 hr. at 30°C. - a 20.5% conversion. No oxygen uptake was found upon manometric analysis of the heated L-alanine control mixture.

The synthesis of small quantities of L-glutamic acid from D-aspartic acid, as observed with R_{21}, could be explained by a series of reactions involving D-alanine racemase, as previously suggested. If D-amino acids other than D-aspartic could be converted to D-alanine then racemization of this amino acid would provide a pathway for the utilization of D-isomers. Nevertheless, since nothing is known of the relative rates of D-amino acid conversion via D-amino acid transamina-

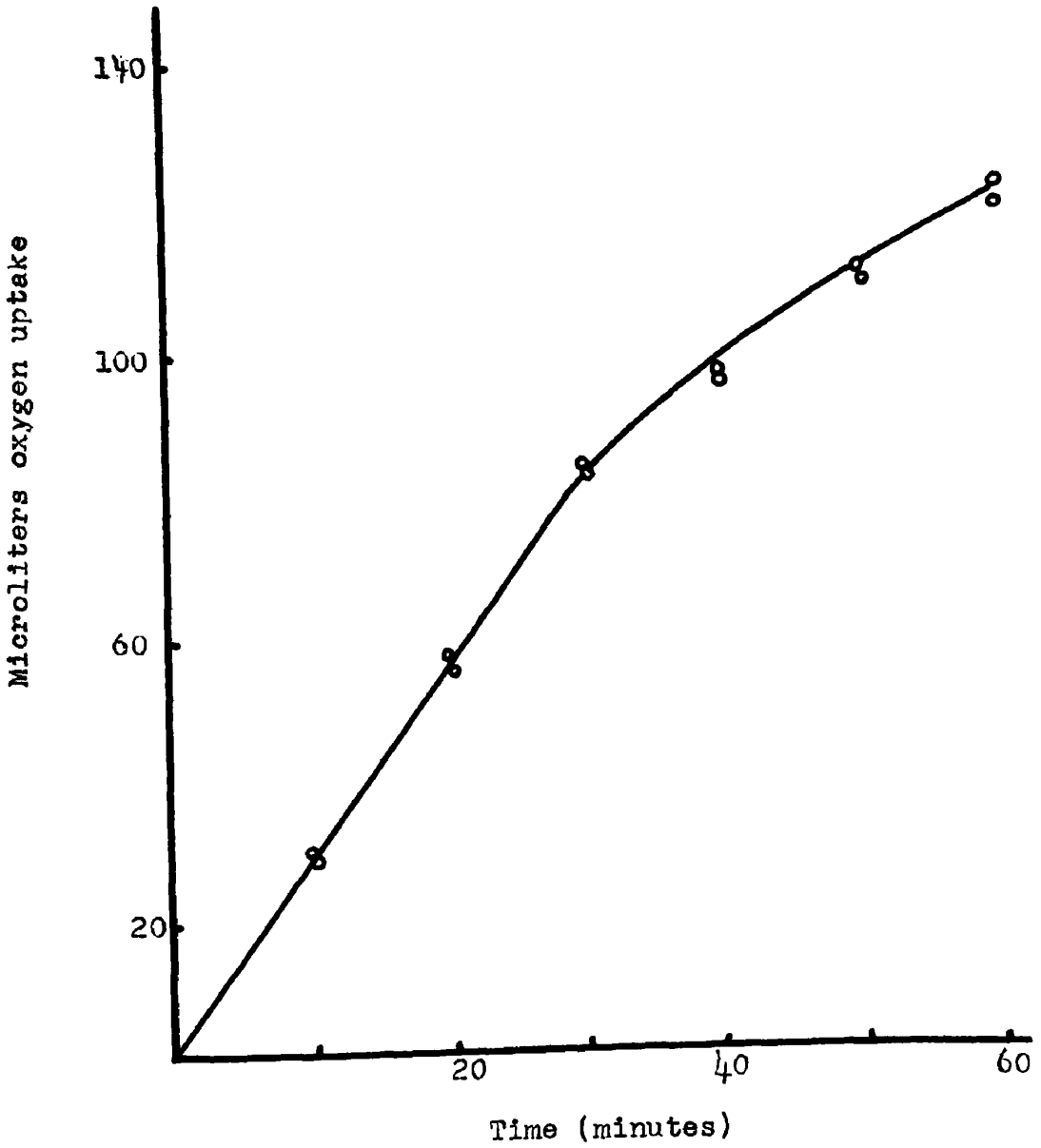


Fig. 16 Oxygen uptake curve of D-amino acid oxidase in a racemase test mixture originally containing L-alanine and acetone-dried Rhizobium R_{21} cells.

tion and racemization care must be taken not to assign to these processes any major position in D-amino acid metabolism without considerable reservation. They may only be of minor importance, with the major route being deamination and subsequent amination of one or more keto acids with the resultant production of L-amino acids.

Although the results presented in section D are not in themselves absolutely conclusive they do suggest a possible integrated scheme of non-specific reactions for the utilization and conversion of L- and D-amino acids by Rhizobium R_{21}. FIGURE 17 illustrates the possible reactions of such a scheme, with the emphasis being upon the production of L-glutamic acid. The importance of this particular amino acid is continually being brought to the foreground in recent scientific literature as it provides a common meeting place for nitrogen sources prior to their incorporation into protein or their transformation into amino acids by transamination.

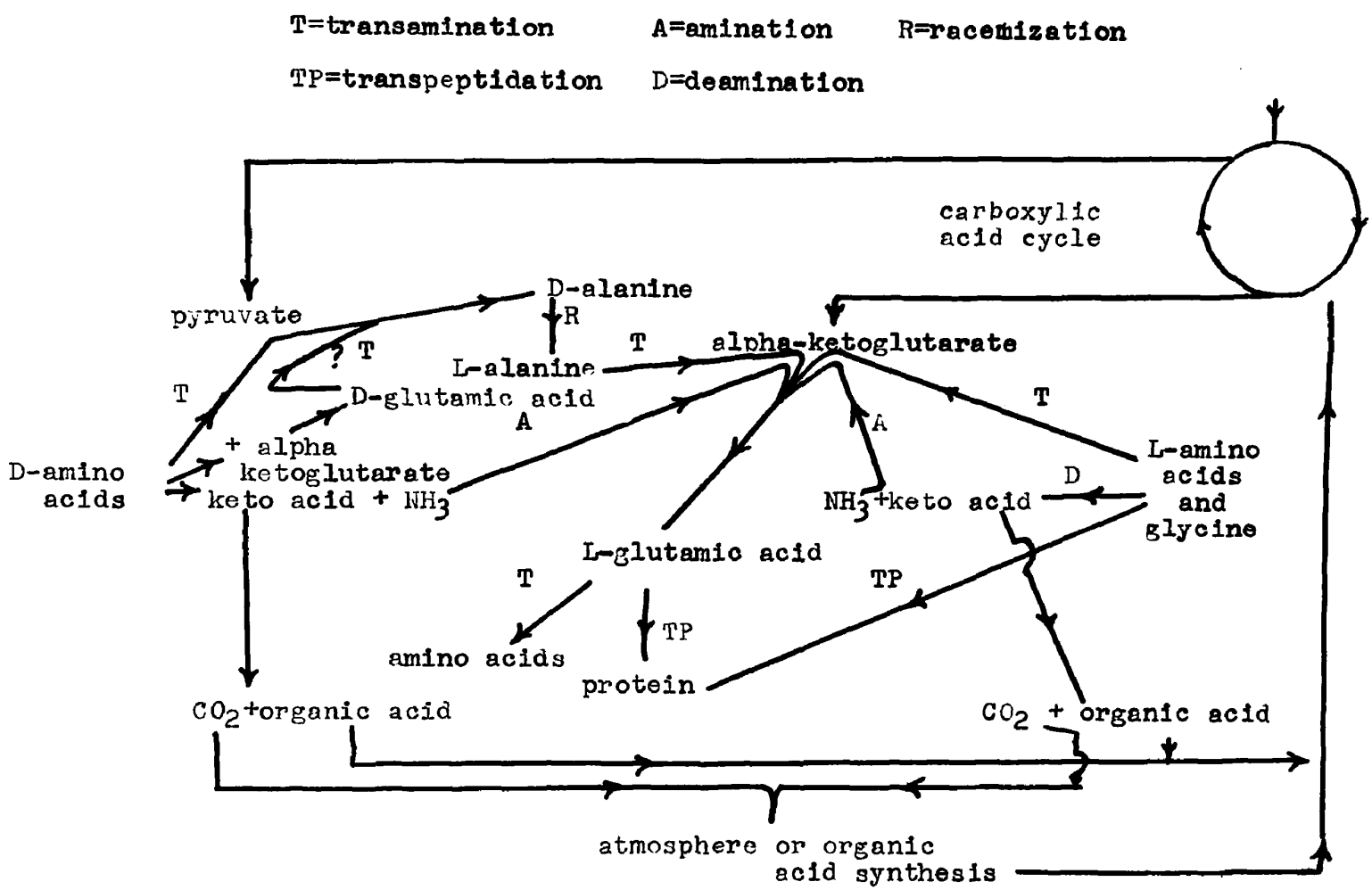


Fig.17 Possible non-specific metabolism of amino acids in Rhizobium R21

PART II

GROWTH STIMULATION IN AMINO ACID MEDIA

Introduction and Literature Review

As in the case of investigations on the nitrogen nutrition, the literature dealing with the growth factor requirements of rhizobia must be carefully interpreted (in the present study the term "growth factors" will imply vitamins and nucleic acid fractions. In some cases amino acids may be classified as growth factors, but they will be omitted from this discussion). The same restrictions apply to these reports as to the nitrogen data in that, in many instances, unwashed cells and complex media were used. The synthesis, essentiality, or non-essentiality of biotin, thiamine, riboflavin, pyridoxine, nicotinic acid, p-aminobenzoic acid, beta-alanine, and pantothenic acid in regard to the nodule bacteria have been adequately reviewed in tabular form by Allen and Allen (2). Comparatively few facts can be uncovered concerning the alfalfa-sweet clover organism (Rh. meliloti). Some strains of this organism require biotin while others synthesize it (101). Thiamine (65), pantothenic acid (62), and beta-alanine (65) are regarded as non-essential, as are pyrimidine, thiazole, beta-oxypropionic acid and pimelic acid (67). Burton and Lochhead (16) tested seventy strains of rhizobia representing six species and found that Rh. meliloti was sharply distinguished from the other species by its ability to produce significantly higher quantities of vitamin B_{12}. Lilly and Leonian (57) stressed the importance of temperature and aeration upon the stimula-

tory activity of seven vitamins on the growth of Rh. trifolii. An increase in temperature from 25°C to 30°C greatly increased the need of this organism for vitamins.

Jordan (48) reported that under the experimental conditions he employed none of fifteen vitamins, purines, and pyrimidines was able to initiate the growth of washed cells of the alfalfa-sweet clover organism in a chemically defined ammonium chloride medium and hence these cells were able to synthesize these compounds when a utilizable nitrogen source was present. It was in an attempt to extend this particular investigation to amino acid utilization that the present section of the work was commenced. Although the growth factors previously examined were apparently not utilized for the initiation of cellular proliferation the fact remains that they may be concerned in growth stimulation. Since absolute differences in growth stimulation may result in detectable differences among parasitic, effective, and ineffective rhizobia this portion of the research is further emphasized. Also, the knowledge of those compounds which are capable of bringing about large increases in growth while present in minute quantities would be invaluable in the formulation of new media for the propagation of large numbers of effective, viable bacteria for the production of high grade legume inoculants.

Methods:

A number of stock vitamin solutions were prepared (TABLE 13) using double glass-distilled water. The various concentrations were those designated by Roepke, Libby and Small (78) with the exception of pyridoxal hydrochloride, ascorbic acid, and vitamin B_{12}.

TABLE 13

TABLE SHOWING THE VITAMINS TESTED, THEIR SOURCE AND STOCK SOLUTION CONCENTRATION

Vitamin	Source	Concentration (mgm./25 ml. water)
ascorbic acid	British Drug House	1.0
B_{12}	Merck and Co.	0.0005
biotin	Nutritional Biochemicals	0.005
calcium pantothenate	British Drug House	1.0
folic acid	Nutritional Biochemicals	0.0005
inositol	British Drug House	10.0
nicotinamide	Nutritional Biochemicals	2.0
para-aminobenzoic acid	Nutritional Biochemicals	0.2
pyridoxal HCI	Nutritional Biochemicals	1.0
pyridoxine HCI	Nutritional Biochemicals	1.0
riboflavin	Eastman-Kodak Co.	1.0
thiamin	Eastman-Kodak Co.	0.8

Ten milliliters of sterile modified Wilson and Wilson's basal medium, 0.2 ml. of a sterile cysteine solution (10 mgm. cysteine/25 ml. water), 0.5 ml. of vitamin solution, and 0.2 ml. of washed cells of R_{21}, R_{20-27A}, and R_{20} were added aseptically to a series of sterilized optical

cuvettes. These tubes, together with inoculated and uninoculated controls, were incubated at 27°C. and daily readings taken with a Photonephelometer. A special uninoculated control containing cysteine basal medium, 0.2 ml. of water and riboflavin solution was prepared to serve as a blank for the inoculated riboflavin tube which had a slight yellowish coloration. This experiment was repeated using 0.0001M histidine basal medium.

Three series of cuvettes containing 9.5 ml. of modified Wilson and Wilson's medium, 0.5 ml. of leucine solution (final concentration = 0.0005M) and 0.5 ml. of a purine or pyrimidine solution (final concentration 0.0001M) were inoculated with 0.2 ml. of washed cells of R_{21}, $R_{20\text{-}27A}$ and R_{20}. The test compounds were all obtained from Nutritional Biochemicals Corp. (U.S.A.) and consisted of adenine sulphate, guanine HCl, xanthine, thymine, uracil, and cytosine. The basal medium pH in the series inoculated with R_{21} and $R_{20\text{-}27A}$ was 7.5, while for the R_{20} inoculated series it was 7.0. Incubation was at 30°C. and nephelos readings were taken at definite intervals.

In a brief study of inhibition by structural analogues of purines and pyrimidines two additional series of cuvettes containing 9.0 ml. of pH 7.5 modified Wilson and Wilson's medium, and 0.5 ml. of stock leucine solution (final concentration = 0.0001M) were set up as shown in TABLE 14. The inhibitor, purine and pyrimidine solutions contained 10 mgm. of each compound/ 10 ml. double glass-distilled water. Therefore, the

0.5 ml. aliquots added to each cuvette contained 500 micrograms of ingredient (106). The tubes were inoculated with 0.2 ml. of washed R_{21} cells and incubated, with suitable controls at 30°C.

TABLE 14

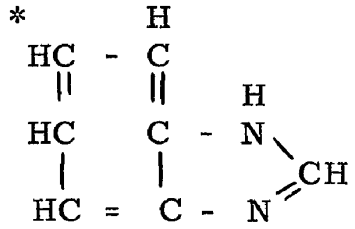
SUPPLEMENTS ADDED TO LEUCINE BASAL MEDIUM FOR DETERMINATION OF STRUCTURAL ANALOGUE INHIBITION OF R_{21}

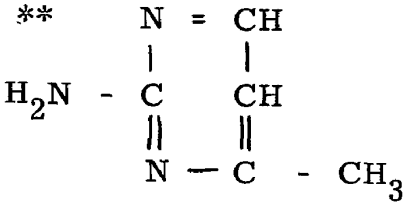
Series A

Cuvette #	Benzimidazole* Solution	Adenine Solution	Water
1	0 ml.	0 ml.	1.0 ml.
2	0 ml.	0.5 ml.	0.5 ml.
3	0.5 ml.	0 ml.	0.5 ml.
4	0.5 ml.	0.5 ml.	0 ml.

Series B

Cuvette #	2-amino-4-methyl pyrimidine**solution	Thymine Solution	Water
1	0 ml.	0 ml.	1.0 ml.
2	0 ml.	0.5 ml.	0.5 ml.
3	0.5 ml.	0 ml.	0.5 ml.
4	0.5 ml.	0.5 ml.	0 ml.





Results and Discussion:

Of the 12 vitamins tested only nicotinamide yielded maximum growth responses above that of the inoculated cysteine or histidine basal madia alone. The growth curves on cysteine medium are shown in FIGURE 18. Those obtained on the histidine media are almost identical except in the overall magnitude of growth. The types of curves found with R_{21} and R_{20-27A} probably indicate that the nicotinamide merely served as an additional nitrogen source for these organisms. With Rhizobium R_{20} the curve was more representative of an accessory growth factor response, the main effect, in addition to providing more growth, being to reduce the long initial lag period. The growth lag, as calculated according to Monod (61), was decreased from 43.0 nephelos units to 22.5. The cause of this lengthy lag period in R_{20} is unknown, but may be the result of an adaptive response. The nicotinamide may merely serve as a more readily available nitrogen source for R_{20} than the amino acid present and therefore is utilized first. When the amino acid begins to be utilized the general effect would be a reduction in the lag.

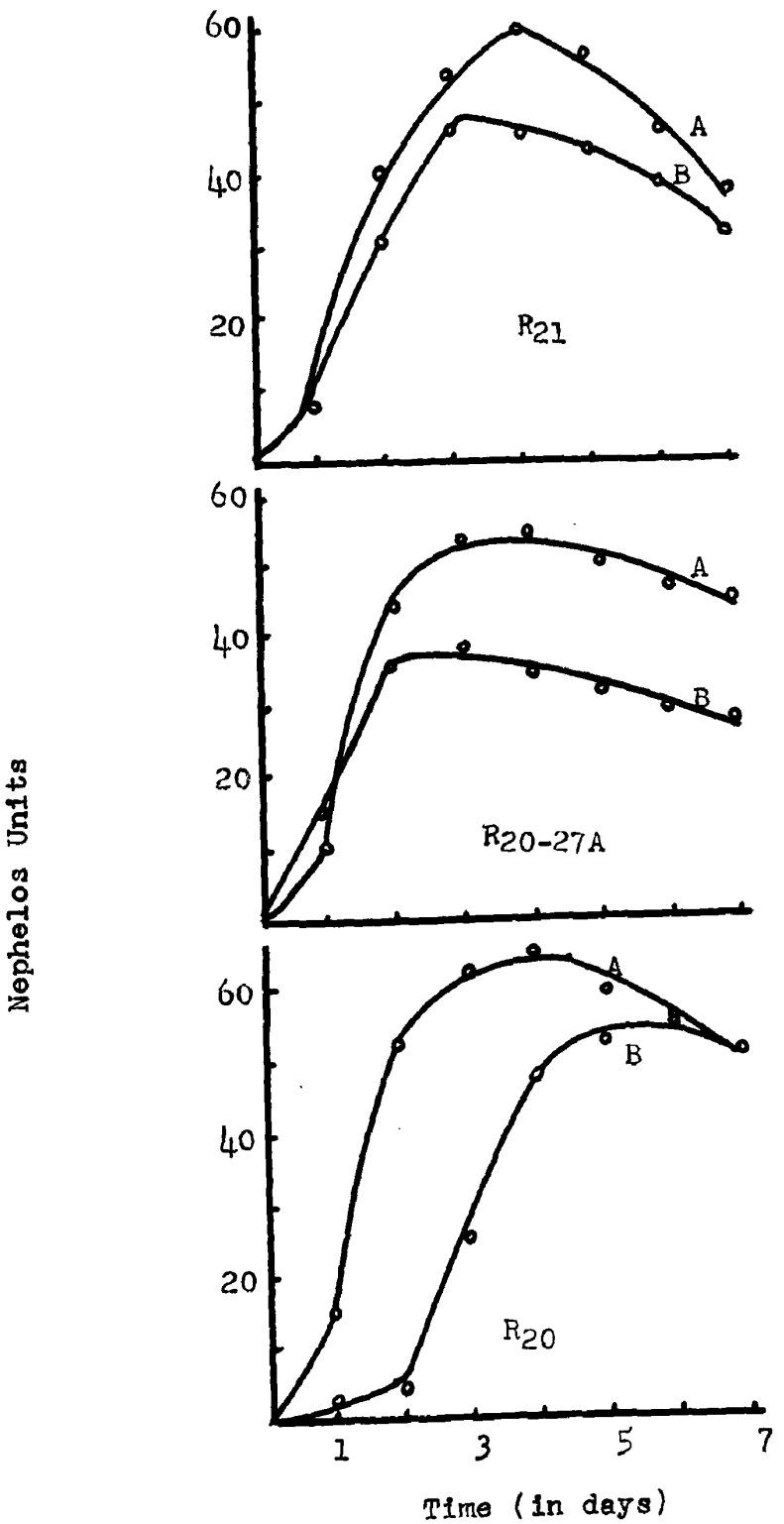


Fig.18 Growth curves of three Rhizobium strains in cysteine basal medium in the presence (A) and absence (B) of added nicotinamide.

FIGURE 19 represents the maximum growth observed on the purine- and pyrimidine-containing media. R_{20} was inhibited by these compounds for some unknown reason, but the other two strains readily utilized them. The growth curves were very similar to those given in FIGURE 14 for R_{21} and are indicative of utilization as additional nitrogen sources. The author has previously shown that these compounds cannot be used as sole sources of nitrogen (48), however, from the present work it appears that utilization can occur when amino acids are present in the medium. One of the degradation products of both purines and pyrimidines is ammonia (92) and this compound could be utilized by rhizobia in the same manner as that described in the section dealing with growth in the presence of ammonium chloride and either keto- or amino acids. Transaminases exists in some organisms which catalyze glutamate formation from alpha-ketoglutarate when pyridoxamine, adenine, guanine or cystosine act as amino donors (37) and these enzymes may function in rhizobia once a supply of acceptor keto acids (or substances giving rise to such acids) are added.

The action of the structural analogues is shown in TABLE 15. The results of series B are complicated owing to the fact that the "inhibitor" used was actually utilized by the test organism. A calculated inhibition was observed in both series of cuvettes, but in series B the added thymine appeared to interfere with the utilization of the 2-amino-4-methyl pyrimidine rather than vice versa. The action of benzimidazole

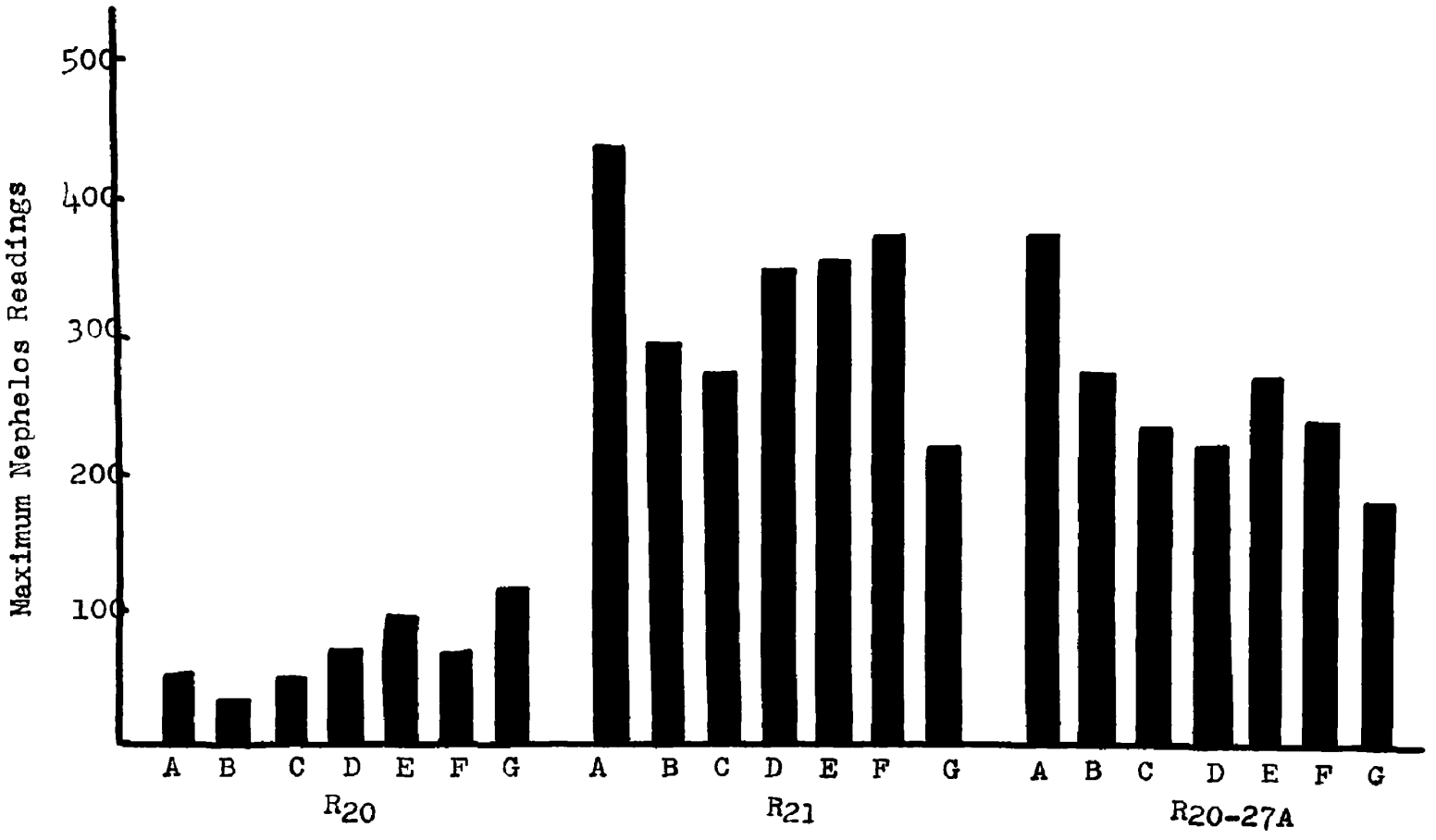


Fig. 19 Vertical bar chart showing the effect of certain purines and pyrimidines on the growth of three Rhizobium strains in leucine basal medium.(A) adenine sulphate (B) guanine HCl (C) thymine (D) uracil (E) cytosine (F) xanthine (G) leucine base only.

may have resulted from an interference with purine utilization or/and to an inhibition of unrelated enzymes (53). It was hoped that these metabolic analogues would serve as specific inhibitors of those enzymes concerned in the immediate utilization of adenine and thymine and would hence be invaluable as an aid in elucidating the types of reactions that occur. It is evident, however, that until the inhibitory activity of benzimidazole in leucine base is explained the use of this compound as a specific inhibitor of "purine-attacking enzymes" is contraindicated. The utilization of 2-amino-4-methyl pyrimidine by the test organism indicates that attempts to use this compound as a "blocking agent" would be useless.

TABLE 15

ACTION OF "INHIBITORS" ON THE UTILIZATION OF ADENINE AND AND THYMINE IN LEUCINE BASAL MEDIUM

Series	Cuvette	Supplement	Maximum nephelos
A	1	none	54.0
	2	benzimidazole	38.0
	3	adenine	304.0
	4	both	178.0

Calculated inhibition in cuvette #4 $= 100 - \left(\frac{178.0}{38.0+(304.0-54.0)} \times 100\right) = 61.8\%$

Table 15 (cont'd)

Series	Cuvette	Supplement	Maximum nephelos
B	1	none	59.0
	2	2-amino-4-methyl pyrimidine	340.4
	3	thymine	279.0
	4	both	302.4

Calculated inhibition in cuvette #4 $= 100 - \left(\frac{302.4}{340.4 + (279.0-59.0)} \times 100\right) = 46.0\%$

In conclusion it may be said that, with the exception of nicotinamide and R_{20}, none of the compounds tested show promise as growth stimulants. The increases brought about by the purines and pyrimidines can be more economically attained by the use of inorganic ammonium salts and little, if any, benefit would be obtained by their inclusion in a synthetic medium. It is debatable whether or not the action of R_{20} on added nicotinamide is related to ineffective or parasitic tendencies on the part of this nodule organism, but it does provide a lead for further research.

SUMMARY

One effective and two ineffective strains of Rhizobium meliloti were examined in regard to their ability to utilize organic and inorganic sources of nitrogen. Previous work had shown that washed cells of these organisms would grow in a purified synthetic medium containing amino acids, but that these acids could not be replaced by other organic or inorganic nitrogenous compounds.

In the present investigation all three strains were found to utilize both the L- and D-isomers of glycine, leucine, valine, methionine, glutamic acid, and histidine when these compounds served as the sole sources of nitrogen in a purified sucrose-mineral salts-medium. The maximum growth on any one isomer was a function of pH and temperature, but generally a pH of about 7.5 and a temperature above 27°C. were best. Inhibitory effects were found in certain racemic mixtures and explained by mutual stereochemical interference of the enzymes attacking the L- and D-isomers. Histidine was remarkable in that both its forms yielded the greatest amount of growth at all pH values (6.0, 7.0, 7.5) with all three organisms, including the ineffective strain which did not grow at pH 6.0 on any other amino acid. This ability of histidine is probably a result of its relative complexity which makes it difficult to synthesize. The three strains were found to be acid sensitive, inhibition of growth occurring at pH 6.0.

In addition to amino acids the organisms were found to be capable of using a variety of di- and tri-peptides of glycine and L- and D-leucine as sole nitrogen sources. Partial hydrolysis occurred, but stimulatory effects were also observed and explained on the basis of direct incorporation of the peptides into proteins.

These rhizobia utilized ammonium, nitrate, and nitrite salts providing that amino or certain carboxylic acids were also present. The latter could not be replaced by excess CO_2. The suggested mechanism is the accumulation of organic acids which can be aminated by ammonia to form amino acids. Compounds such as purines and pyrimidines, which can be degraded to ammonia substituted for the inorganic nitrogenous salts with two of the bacterial strains.

No glutamic or histidine decarboxylase activity was located, but a manometric examination of one of the effective strains demonstrated an uptake of gaseous oxygen on both L- and D-amino acids. Ammonia was formed oxidatively, but the amounts varied with individual acids, being particularly low with glutamic acid which is a good amino group donator. The keto analogue of glycine, formed as a result of oxidation, was isolated. The respiratory quotient on glutamic acid was approximately one, the same as that observed with several keto acids, and is indicative of carbohydrate metabolism such as might occur through degradation of keto acids. The endogenous quotient was suggestive of protein metabolism.

Studies on glycine oxidation revealed that a pH of 8.0 was better than lower pH values in regard to oxygen uptake and that cyanide and azide inhibited the reaction in intact cells.

Transaminase experiments demonstrated that cell-free extracts of at least one of the Rhizobium strains were capable of synthesizing glutamic acid from alpha-ketoglutaric acid when glycine, L-histidine, D-aspartic acid, or D-valine acted as amino group donators. A small amount of L-glutamic acid was located in the case of D-aspartic acid, and could have been formed as a consequence of a series of reactions similar to those postulated by Thorne. In addition, acetone-dried cells synthesized alanine from pyruvate and D-aspartic acid. Alanine racemase activity was located, but racemization of added aspartic or glutamic acids did not occur under the conditions used.

An L-glutamic acid dehydrogenase was found using the Thunberg technique and provides a potential mechanism for "ammonia fixation" by a reversal of its activity.

None of 12 vitamins were found to be stimulatory to the growth of the three strains in cysteine or histidine media, with the exception of nicotinamide. This compound appeared to serve merely as an additional source of nitrogen, although it resulted in a reduction of a long initial lag period with the ineffective strain.

The ineffective strain varied from the effective strains in its extreme acid sensitivity, its better utilization of D-amino acid peptides

particularly D-leucylglycine, its lower optimum temperature, its lag phase reduction by nicotinamide, and its inability to utilize purines and pyrimidines. It is unknown whether or not any of these features are correlated with ineffectiveness in nitrogen fixation. Such differences may only be due to the inherent differences normally found among different strains of this organism and more work is required along this line of endeavour.

A synthetic mineral salts - histidine-sucrose medium has been formulated for growth studies on washed Rhizobium meliloti cells.

The experimental results suggest that in rhizobia most amino acids are primarily deaminated or transaminated and that the keto-acids produced are oxidized or either directly or indirectly aminated to reform amino acids. Racemization, coupled with transamination appears to play a role in the utilization of D-amino acids, but deamination and subsequent amination of one or more keto-acids is probably of greater significance.

REFERENCES

1. Ajl, S.J. and Werkman, C.H. Replacement of CO_2 in heterotrophic metabolism. Arch. Biochem. 19:483-492.1948.

2. Allen, Ethyl K. and Allen, O.N. Biochemical and symbiotic properties of rhizobia. Bact. Rev. 14:273-330.1945.

3. Allison, F.E. and Hoover, S.R. An accessory factor for legume nodule bacteria. J. Bact. 27:561-581.1934.

4. Allison, F.E. and Minor, F.W. The effects of temperature on the growth rates of rhizobia. J. Bact. 39:365-371.1940.

5. Allyn, W.P. and Baldwin, I.L. The effect of the oxidation-reduction character of the medium on the growth of an aerobic form of bacteria. J. Bact. 20:417-438.1930.

6. Ayengar, P. and Roberts, E. Utilization of D-glutamic acid by Lactobacillus arabinosus: glutamic racemase. J. Biol. Chem. 197:453-460.1952.

7. Baldwin, E. Dynamic aspects of biochemistry. 2nd. ed. Cambridge Univ. Press, England. 1952.

8. Beijerinck, M.W. Die Bacterien der Papilionaceenknollchen. Bot. Ztg. 46:726-735.1888.

9. Berger, J., Johnson, M.J., and Peterson, W.H. The proteolytic enzymes of bacteria. II. The peptidases of some common bacteria. J. Bact. 36:521-545.1938.

10. Bernheim, F., Bernheim, Mary, and Webster, M. Dorothy. Oxidation of certain amino acids by "resting" Bacillus proteus. J. Biol. Chem. 110:165-172.1935.

11. Billen, D. and Lichstein, H.C. Aspartic acid decarboxylation by Rhizobium trifolii. J. Bact. 57:267-268.1949.

12. Block, R.J. Paper chromatography. Academic Press, Inc., New York.1952.

13. Braunstein, A.E. and Kritzman, M.G. Über den Ab-und Aufbau von Aminosäuren durch Umaminierung. Enzymologia, 2:129-146.1937.

14. Burris, R.H. and Wilson, P.W. Respiratory enzyme systems in symbiotic nitrogen fixation. Cold Spring Harbour Symposia Quant. Biol. 7:349-361.1939.

15. Burris, R.H. and Wilson, P.W. Effects of haemoglobin and other nitrogenous compounds on the respiration of the rhizobia. Biochem. J. 51:90-96.1952.

16. Burton, Marg. O. and Lochhead, A.G. Production of vitamin B_{12} by Rhizobium species. Can. J. Bot. 30:521-524.1952.

17. Campbell, W.R. and Hanna, M.I. The determination of nitrogen by modified Kjeldahl methods. J. Biol. Chem. 119:1-7.1937.

18. Cavallini, D. and Frontali, N. Quantitative determination of keto acids by paper partition chromatography. Biochem. et Biophys. Acta, 13:439-445.1954.

19. Clifton, C.E. in Bacterial physiology, ed. by C.H. Werkman and P.W. Wilson. Academic Press, Inc., New York. 1951.

20. Cohen, P.P. in Symposium on respiratory enzymes. Univ. of Wisconsin Press. Madison. 1942.

21. Cohen, P.P. and McGilvery, R.W. in Respiratory enzymes, ed. by A. Lardy. Burgess Pub. Co., Minn. 1949.

22. Cole, J.O. and Parks, C.R. Semimicro-Kjeldahl procedure for control laboratories. Ind. and Eng. Chem. Analyt. Ed. 18: 61-62.1946.

23. Dawson, M. Further observations on the nature and functions of the nodules of leguminous plants. Roy. Soc. (London) Phil. Trans., Ser. B, 193:51-67.1900.

24. Dunn, M.S. and McClure, L.E. The response of Lactobacillus casei to partial hydrolysates of protein. J. Biol. Chem. 184:223-233. 1949.

25. Fisher, R.B. Protein metabolism. Methuen and Co., Ltd. London. 1954.

26. Fred, E.B., Baldwin, I.L., and McCoy, Elizabeth. Root nodule bacteria and leguminous plants. Univ. Wisc. Studies in Science. 5. 1932.

27. Freidemann, F.E. and Haugen, G. Pyruvic acid. II. The determination of keto acids in blood and urine. J. Biol. Chem. 147:415-441. 1943.

28. Fruton, J.S. in Scientific American Reader. Simon and Schuster, New York. 1953.

29. Fruton, J.S. and Simmonds. Sofia. General biochemistry. J. Wiley and Sons, Inc., New York. 1953.

30. Gale, E.F. and Epps, H.M.R. The effect of the pH of the medium during growth on the enzymic activities of bacteria (E. coli and M. lysodeikticus) and the biological significance of the changes produced. Biochem. J. 36:600-618. 1942.

31. Gale, E.F. Factors influencing the enzyme activities of bacteria. Bact. Rev. 7:139-173. 1943.

32. Gale, E.F. The bacterial amino acid decarboxylases. Adv. in Enzymology, 6:1-32. 1946.

33. Gale, E.F. The use of enzyme preparations from bacteria for the estimation of amino acids. Biochem. J. 41:vii-ix. 1947.

34. Gale, E.F. The nitrogen metabolism of gram-positive bacteria. Bull. Johns Hopkins Hosp. 83:119-175. 1948.

35. Green, D.E., Herbert, D., and Subrahmanyan, V. Carboxylase. J. Biol. Chem. 138:327-339. 1941.

36. Greig-Smith, R. The determination of rhizobia in the soil. Centbl. Bakt. 2 Abt. 34:227-229. 1912.

37. Gunsalus, Carolyn, F., and Tonzetich. J. Transaminases for pyridoxamine and purines. Nature, 170:162. 1952.

38. Handuroy, P. Le "separateur de germes". Bull. Assoc. Diplomes Microbiol. Fac. Pharm. Nancy, 39:5-9. 1950.

39. Harrison, F.G. and Barlow, B. The nodule organism of the Leguminosae, its isolation, cultivation, identification, and commercial application. Centbl. Bakt. 2 Abt. 19:264-272. 1907.

40. Hills, T.L. Influence of nitrates on nitrogen assimilating bacteria. J. Agr. Res. (U.S.A.) 12:183-230. 1918.

41. Hirsch, M.L. and Cohen, G.N. Amino acid utilization in bacterial growth. I. Peptide utilization by a leucine-requiring mutant of Escherichia coli. Biochem. J. 53:25-30. 1953.

42. Hoover, S.R. and Allison, F.E. A growth and respiratory factor for certain rhizobia. Trans. 3rd. Internat'l. Congress Soil Sc. 1:158-160. 1935.

43. Ishizawa, S. Studies on the root-nodule bacteria of leguminous plants. I. Characters in artifical media. (Part 3) Physiological characters (d) nitrogen source. J. Sci. Soil and Manure. (Japan) 24:29-35. 1953.

44. Ishizawa, S. Studies on the root-nodule bacteria of Leguminous plants. I. Characters in artifical media. (Part 4) The effect of pH on the growth of rhizobia. J. Sci. Soil and Manure (Japan) 24:169-172. 1953.

45. Ishizawa, S. Studies on the root-nodule bacteria of Leguminous plants. I. Characters in artifical media. (Part 5) Effects of temperature on the growth of rhizobia. J. Sci. Soil and Manure. (Japan) 24:227-230. 1953.

46. Janke, A. and Tayenthal, Wilhelmine. Probleme des Stickstoff-kreislaufes. III. Mitteilung. Über den Abbau des Glykokolls durch Bakterien. Biochem. Z. 289:76-86. 1937.

47. Jordan, D.C. Studies on the legume root nodule bacteria. II. The production and behavior of colonial mutants produced by X-ray irradiation. Can. J. Bot. 30:125-130. 1952.

48. Jordan, D.C. Studies on the legume root nodule bacteria. III. Growth factor requirements for effective, ineffective, and parasitic strains. Can. J. Bot. 30:693-700. 1952.

49. Jordan, D.C. Transamination in cell-free extracts of effective and parasitic rhizobia. J. Bact. 65:220-221. 1953.

50. Jordan, D.C. and Garrard, E.H. Studies on the legume root-nodule bacteria. I. Detection of effective and ineffective strains. Can. J. Bot. 29:360-372. 1951.

51. Kihara, H. and Snell, E.E. Peptides and bacterial growth. II. L-alanine peptides and growth of Lactobacillus casei. J. Biol. Chem. 197: 791-800. 1952.

52. Krebs, H.A. Metabolism of amino acids. III. Deamination of amino acids. Biochem. J. 29:1620-1644. 1935.

53. Kushner, D.J. and Pascoe, Enid. The action of benzimidazole on enzymatic adaptation. Can. J. Biochem. and Phys. 33:1-9. 1955.

54. Lardy, H.A. Respiratory enzymes. Burgess Publishing Co., Minn. 1949.

55. Leonard, L.T. Nitrite production by some strains of cowpea and soybean organisms. J. Bact. 17:19. 1929.

56. Leonard, L.T. A simple assembly for use in the testing of cultures of rhizobia. J. Bact. 45:523-525. 1943.

57. Lilly, V.G. and Leonian, L.H. The interrelationships of iron and certain accessory factors in the growth of Rhizobium trifolii, strain 205. J. Bact. 50:383-395. 1945.

58. Lochhead, A.G. personal communication via Mr. F. Newbould.

59. Lyman, C.M. and Kuiken, K.A. The effect of vitamin B_6 on the utilization of D-amino acids by lactic acid bacteria. Federation Proc. 7:170. 1948.

60. Manual of methods for pure culture study of bacteria, ed. by Committee on Bact. Tech. Soc. Am. Bact. Biotech Publishing Co., Geneva, New York. 1946.

61. Monod, J. The growth of bacterial cultures. Ann. Rev. Microbiol. 371-394. 1949.

62. McBurney, C.H., Bollen, W.B., and Williams, R.J. Pantothenic acid and the nodule bacteria-legume symbiosis. Proc. Nat'l Acad. Sci. U.S. 21:301-304. 1935.

63. Müller, A. and Stapp, C. Beitrage zur Biologie der Leguminosenknöllchenbakterien mit besonderer Berücksichtigung ihrer Artverschiedenheit. Arb. Biol. Reichsanst. Land u. Forstw. 14:455-554. 1925.

64. Neal, O.R. and Walker, R.H. Physiological studies on Rhizobium. IV. Utilization of carbonaceous materials. J. Bact. 30:173-187. 1935.

65. Nielsen, N. and Johansen, G. Untersuchungen uber biologische Stickstoffbindung. V. Untersuchungen uber die Wirkung verschiedener Wuchsstaffe auf 4 Stämme von B. radicicola. Compt. rend. Trav. Lab. Carlsberg. Sér, Physiol. 23: 173-193. 1941.

66. Nilsson, R., Bjälfve, G., and Burstrom, D. Über Zuwachsfaktoren bei Bact. radiciola. Lantbruks - Hogskol. Ann. 5:291-322. 1938.

67. Oginsky, Evelyn, L. and Umbriet, W.W. An introduction to bacterial physiology. W.H. Freeman and Co., San Francisco. 1954.

68. Osterhout, W.J.V. The mechanism of accumulation in living cells. J. Gen. Physiol. 35:579-594. 1952.

69. Peech, M. Determination of exchangeable cations and exchange capacity of soils. Rapid micromethods utilizing centrifuge and spectrophotometer. Soil Sc. 59:25-38. 1945.

70. Peters, V.J. and Snell, E.E. Peptides and bacterial growth. VI. The nutritional requirements of Lactobacillus delbrueckii. J. Bact. 67:69-70. 1954.

71. Peters. V.J., Prescott, J.M., and Snell, E.E. Peptides and bacterial growth, IV. Histidine peptides as growth factors for Lactobacillus delbrueckii 9649. J. Biol. Chem. 202:521-532. 1953.

72. Pohlman, G.G. Changes produced in nitrogenous compounds by Rh. meliloti and Rh. japonicum. Soil Sc. 31:385-406. 1931.

73. Prazmowski, A. Die Wurzelknöllchen der Erbse. Landw. Vers. Sta. 37:161-238. 1890.

74. Prescott, J.M., Peters, V.J., and Snell, E.E. Peptides and bacterial growth. V. Serine peptides and growth of Lactobacillus delbrueckii 9649. J. Biol. Chem. 202:533-540. 1953.

75. Prucha, M.J. Physiological studies of Bacillus radicicola of Canada field peas. N.Y. (Cornell) Agr. College Mem. 5:1-83. 1915.

76. Richardson, D.A. M.S.A. thesis. University of Toronto. 1954.

77. Richardson, D.A. and Jordan, D.C. unpublished data.

78. Roepke, R.R., Libby, R.L., and Small, Margaret, H. Mutation or variation in Escherichia coli with respect to growth requirements. J. Bact. 48:401-412. 1944.

79. Rosenberg, T. and Wilbrandt, W. Enzymatic processes in cell membrane penetration. Intern. Rev. of Cytol. 1:65-92. 1952.

80. Rydon, H.N. D-amino acids in microbiological chemistry. Biochem. Soc. Symposia, 1:40-60. 1948.

81. Smith, E.L. in The enzymes, ed. by J.B. Sumner and K. Myrbäck. Academic Press, Inc., New York. 1951.

82. Stephenson, Marjory. Bacterial metabolism. 3rd. ed. Longmans, Green and Co., London. 1949.

83. Stumpf, P.K. and Green, D.E. L-amino acid oxidase of Proteus vulgaris. J. Biol. Chem. 153:387-399. 1944.

84. Stumpf, P.K., Zarudnaya, K., and Green, D.E. Pyruvic and alpha-ketoglutaric oxidase of animal tissue. J. Biol. Chem. 167:817-825. 1947.

85. Sumner, J.B. and Somers, G.F. Chemistry and methods of enzymes. 2nd. ed. Academic Press, Inc., New York. 1947.

86. Tam, R.K. and Wilson, P.W. Respiratory enzyme systems in symbiotic nitrogen fixation. III. The dehydrogenase systems of Rhizobium trifolii and Rhizobium leguminosarum. J. Bact. 41:529-546. 1941.

87. Thorne, C.B. in Symposium on amino acid metabolism. McCollum-Pratt Institute. The Johns Hopkins University, Baltimore. 1954.

88. Thorne, C.B. Transamination of D-amino acids. Bact. Proceed. p. 104. 1954.

89. Thorne, D.W. and Walker, R.H. Physiological studies on Rhizobium. III. Respiration and growth as influenced by the reaction of the medium. J. Bact. 30:33-43. 1935.

90. Thorne, D.W. and Walker, R.H. Physiological studies on Rhizobium. VI Accessory growth factors. Soil Sc. 42:231-240. 1936.

91. Tissières, A. Effect of azide on the respiratory system of Aerobacter aerogenes. Nature, 166:1115.1950.

92. Umbreit, W.W. Metabolic maps. Burgess Publishing Co., Minn. 1952.

93. Umbreit, W.W., Burris, R.H., and Stauffer, J.F. Manometric techniques and tissue metabolism. Burgess Publishing Co., Minn. 1949.

94. Vitanen, A.I. in Symposium on microbial metabolism. VI th. Internat'l Congress Microbiol. Rome. 1953.

95. Virtanen, A.I. and Laine, T. The decarboxylation of d-lysine and l-aspartic acid. Enzymologia, 3:266-270.1936.

96. Vitanen, A.I. and Rautanen, N. in The enzymes, ed. by J.B. Sumner and K. Myrbäck. Academic Press, Inc., New York. 1952.

97. Walker, R.H., Anderson, D.A., and Brown, P.E. Physiological studies on Rhizobium. II. The effect of nitrogen source on oxygen consumption of Rh. meliloti, Rh. trifolii, and Rh. phaseoli. Soil Sc. 38:207-217.1934.

98. West, P.M. and Wilson, P.W. Growth factor requirements of the root nodule bacteria. J. Bact. 37:161-185.1939.

99. Wilson, J.B. Physiological studies of the Bacillus radicicola of the soybean and of the factors influencing nodule production. N.Y. (Cornell) Agr. Expt. Sta. Bull. 386:369-413.1917.

100. Wilson, J.B. The legume bacteria liberate gaseous nitrogen from nitrate. Soil. Sci. Soc. of Amer. Proc. 12:215-216.1947.

101. Wilson, J.B. and Wilson, P.W. Biotin as a growth factor for rhizobia. J. Bact. 43:329-341.1942.

102. Wilson, J.K. and Chin, C. Symbiotic studies with isolates from nodules of Astragalus. Soil Sc. 63:119-127.1947.

103. Wilson, P.W. Respiratory enzyme systems in symbiotic nitrogen fixation. I. The "resting cell" technique as a method for study of bacterial metabolism. J. Bact. 35:601-623.1938.

104. Woiwod, A.J. A technique for examing large numbers of bacterial culture filtrates by partition chromatography. J. Gen. Microbiol. 3:312-318.1949.

105. Wood, W.A. and Gunsalus, I.C. D-alanine formation: a racemase in Streptococcus faecalis. J. Biol. Chem. 190:403-416.1951.

106. Woolley, D.W. Some biological effects produced by benzimidazole and their reversal by purines. J. Biol. Chem. 152:225-232. 1944.

107. Zinsser, O. Über das Verhalten von Bakterien insbesondere von Knöllchenbakterien in lebenden pflanzlichen Geweben. Jahrb. Wiss. Bot. 30:423-452.1897.

108. Zipfel, H. Beitrage zur Morphologie und Biologie der Knöllchenbakterien des Leguminosem. Centbl. Bakt. 2 Abt. 32: 97-137.1911.

APPENDIX

PART I

Media

(1) Wilson and Wilson's Medium modified by the omission of ammonium chloride.

Ref: 101

K_2HPO_4	0.5 gm.	extracted with absolute ethyl alchol for 24 hr.
NaCl	0.1 gm.	
sucrose	2.5 gm.	
$MgSO_4.7H_2O$	0.2 gm.	ignited in oven for 2 hr.
Ca SO_4	0.2 gm.	
$FeCl_3$	.001 gm.	- sublimed

Special water to 1000 ml. - distilled, treated with $KMnO_4$ and $AgNO_3$ and redistilled in an all-glass apparatus.

pH adjusted with C. P. KOH solution.

(2) Modified Medium "79"

Ref: 103

Remodified by the omission of $CaCO_3$

K_2HPO_4	0.5 gm.
$MgSO_4.7H_2O$	0.2 gm.
NaCl	0.1 gm.
Yeast Extract (Difco)	5.0 gm.
Distilled water to 1000 ml.	

pH should be 7.0

(3) Modified Lochead's Medium

Ref: 58

(a)	agar	15.0 gm.
	tap water	600 ml.
(b)	KH_2PO_4	0.4 gm.
	$MgSO_4.7H_2O$	0.1 gm.
	NaCl	0.1 gm.
	$CaSO_4$	0.05 gm.
	tap water	200 ml.
(c)	sucrose	10.0 gm.
	yeast extract (Difco)	1.0 gm.
	tap water	200 ml.

The three solutions are made up separately, dissolved in the steamer and mixed together.

TABLE 1

STANDARD DEVIATIONS OF NEPHELOS READINGS

Nephelos readings		
Series A (low)	Series B (medium)	Series C (high)
36.5	63.0	107.5
40.2	66.7	103.4
40.2	69.0	101.0
40.0	62.5	106.9
40.0	63.5	108.5
44.0	70.0	108.5
41.5	67.2	107.5
41.5	69.0	104.2
45.0	64.0	100.0
45.0	69.0	106.0
413.9	663.9	1053.5
Mean = 41.4	Mean = 66.4	Mean = 105.3
Zdx^2 = 63.8	Zdx^2 = 66.3	Zdx^2 = 88.8
S.D. = 2.52	S.D. = 2.57	S.D. = 2.98

TABLE 2

VIABLE BACTERIAL COUNT VS. NEPHELOS READINGS - RESPONSE CURVE

Nephelos	Viable count X 10^6
4.0	1.52
50.3	6.85
78.4	9.62
112.2	12.73

TABLE 3

DRY WEIGHT AND NITROGEN CONTENT OF ONTARIO VARIEGATED ALFALFA INOCULATED WITH THREE RHIZOBIUM STRAINS.

FEB. - MARCH. 1955

Bacterial strain	Position in latin square Row	Column	Dry wt./ plant (gm.)	Nitrogen content (mgm./gm. dry wt.)
R_{21}	1	4	.0100	30.35
	2	1	.0070	33.16
	3	3	.0110	25.36
	4	2	.0111	29.44
$R_{20\text{-}27A}$	1	1	.0102	27.27
	2	2	.0125	25.34
	3	4	.0107	23.95
	4	3	.0135	24.28
R_{20}	1	3	.0059	13.07
	2	4	.0059	21.99
	3	2	.0043	23.36
	4	1	.0061	19.25
Control (not inoc.)	1	2	.0065	18.60
	2	3	.0059	18.40
	3	1	.0059	18.39
	4	1	.0059	19.33

TABLE 4

ANALYSIS OF VARIANCE FOR MGM. N/GM. DRY WT. OF PLANT TISSUE - LATIN SQUARE - ALFALFA INOCULATED WITH R_{21}, R_{20-27A}, AND R_{20}

Sum of squares for total = 8999.46

$$\text{correction} = \frac{(Zx)^2}{N^2} = 8580.32$$

8999.46 - 8580.32 = 419.14

Sum of squares for treatments = 8903.63

8903.63 - 8580.32 = 323.31

Sum of squares for rows = 8594.60

8594.60 - 8580.32 = 14.28

Sum of squares for columns = 8625.51

8625.51 - 8580.32 = 45.19

Sum of squares for random error = 36.36

Difference between	Degrees of Freedom	Sum of Squares	Variance	F for significance	
				5%	1%
Treatments	3	323.31	107.77	6.87	9.78
Rows	3	14.28	4.76		
Columns	3	45.19	15.06		
Error	6	36.36	12.12		
Total	15	419.14			

ANALYSIS OF VARIANCE (CONT.)

Significance between treatments

Error (random variance) for each treatments = 12.12

Error (random variance) for each mean = $\frac{12.12}{4}$ = 3.3

Random variance between means = 3.3 x 2 = 6.6

Standard deviation of difference between means due to random error = $\sqrt{6.6}$ = 2.6

t = 2.45 necessary mean difference = 2.45 x 2.6 = 6.37

Table of means of treatments in order of magnitude

Treatment	Means
R_{21}	29.57
$R_{20\text{-}27A}$	25.20
R_{20}	19.17
Control	18.68

R_{21} and $R_{20\text{-}27A}$ are significantly better than the control, but R_{20} is not.

TABLE 5-

INFLUENCE OF pH ON AMINO ACID UTILIZATION

	Maximum Nephelos Values								
	R_{21}			R_{20}			R_{20-27A}		
pH	6.0	7.0	7.5	6.0	7.0	7.5	6.0	7.0	7.5
glycine	0.0	23.0	141.0	0.0	34.0	0.0	17.0	38.2	38.0
L-leucine	0.0	170.2	181.0	0.0	74.5	0.0	17.0	80.4	115.0
D-leucine	0.0	195.0	214.5	0.0	156.5	0.0	24.0	48.6	136.3
DL-leucine	0.0	168.7	188.0	0.0	99.0	0.0	58.5	76.6	91.0
L-valine	11.5	194.0	224.5	0.0	115.0	0.0	37.7	67.0	95.5
D-valine	0.0	160.0	193.5	0.0	67.5	0.0	11.5	79.9	56.0
DL-valine	0.0	168.0	184.5	0.0	76.0	0.0	67.5	70.0	35.2
L-methionine	16.2	156.0	200.0	0.0	145.5	0,0	92.5	110.2	120.5
D-methionine	12.0	186.0	160.0	0.0	64.0	0.0	44.0	92.1	68.8
DL-methionine	11.5	202.0	189.2	0.0	34.3	0.0	44.0	59.3	77.5
L-glutamic acid	9.5	140.0	207.5	0.0	76.5	0.0	106.0	136.5	140.5
D-glutamic acid	0.0	130.0	148.5	0.0	93.7	0.0	35.5	71.2	65.5
DL-glutamic acid	0.0	116.8	132.2	0.0	56.0	0.0	39.5	88.9	69.5
L-histidine	378.5	371.6	390.0	355.0	449.5	409.5	334.5	395.0	467.5
D-histidine	462.0	438.5	345.0	410.0	380.0	340.2	390.0	382.0	399.2
DL-histidine	381.5	369.0	312.0	460.5	461.0	385.0	462.5	366.2	357.5

TABLE 6

FINAL pH PRODUCED IN AMINO ACID MEDIA BY THREE RHIZOBIUM STRAINS

	R_{21}			R_{20}			R_{20-27A}		
pH	6.0	7.0	7.5	6.0	7.0	7.5	6.0	7.0	7.5
glycine	6.30	6.90	7.10		6.95		-	-	-
L-leucine	6.15	6.80	6.95		6.86		6.00	7.00	7.19
D-leucine	5.98	6.70	6.90		6.72		6.10	7.01	7.17
DL-leucine	5.90	6.75	6.92		6.79		6.00	6.99	7.27
L-valine	5.60	6.85	7.00		6.75		6.47	7.05	7.36
D-valine	6.00	6.85	7.20		6.85		6.18	7.06	7.32
DL-valine	6.00	6.85	7.00		6.80		6.12	7.06	7.40
L-methionine	6.05	6.45	6.75		6.77		6.09	6.93	7.30
D-methionine	6.00	6.52	6.99		6.80		6.09	7.02	7.22
DL-methionine	6.02	6.59	6.95		6.80		6.09	7.00	7.28
L-glutamic acid	6.10	6.80	7.00		6.85		6.30	7.10	7.40
D-glutamic acid	6.00	6.90	7.12		6.80		6.32	7.10	7.40
DL-glutamic acid	6.00	6.95	7.12		6.82		6.25	7.10	7.43
L-histidine	5.50	6.35	6.60	6.02	6.45	6.82	5.57	6.58	6.76
D-histidine	5.40	6.25	6.50	5.55	6.50	6.80	5.57	6.58	6.83
DL-histidine	5.38	6.35	6.50	5.45	6.42	6.75	5.57	6.58	6.80

TABLE 7

INFLUENCE OF 27°C. INCUBATION TEMPERATURE ON THE UTILIZATION OF L- AND D- AMINO ACIDS BY THREE *RHIZOBIUM* STRAINS

Amino acid	Maximum growth in nephelos units		
	R_{21}	R_{20}	R_{20-27A}
glycine	134.0	36.0	49.0
L-leucine	165.2	40.0	84.0
D-leucine	146.0	40.0	80.0
L-valine	100.0	8.0	44.0
D-valine	125.0	25.0	36.0
L-methionine	86.0	144.0	71.0
D-methionine	69.0	54.0	46.0
L-glutamic acid	191.4	141.0	151.0
D-glutamic acid	122.0	66.0	33.0
L-histidine	321.6	226.0	195.0
D-histidine	337.0	241.0	173.0

TABLE 8

OPTIMUM TEMPERATURE FOR GROWTH IN MODIFIED MEDIUM "79"

Temperature	Organism	Maximum nephelos values
27°C.	R_{20}	73.4
	R_{21}	139.0
	R_{20-27A}	86.0
30°C.	R_{20}	71.0
	R_{21}	438.0
	R_{20-27A}	110.0
35°C.	R_{20}	55.0
	R_{21}	494.0
	R_{20-27A}	116.0

TABLE 9

MAXIMUM GROWTH (AS NEPHELOS UNITS) ATTAINED BY THREE RHIZOBIUM STRAINS ON A LEUCINE MEDIUM CONTAINING INORGANIC NITROGEN COMPOUNDS

Compound	NH_4Cl				$NaNO_3$				$NaNO_2$			
Molarity	0	0.01	0.001	0.0001	0	0.01	0.001	0.0001	0	0.01	0.001	0.0001
R_{21}	174.0	570.0	487.4	283.0	174.0	455.0	322.0	310.0	169.0	5.0	350.0	260.0
R_{20}	116.0	532.0	460.0	209.4	116.0	350.0	236.8	180.0	108.0	8.0	180.0	192.6
R_{20-27A}	114.0	552.0	537.0	203.0	116.0	414.0	398.0	252.0	120.0	4.0	258.0	210.0

TABLE 10

GROWTH RESPONSE OF R_{21} ON HISTIDINE MEDIUM CONTAINING VARIOUS CONCENTRATIONS OF NH_4Cl.

Molarity of NH_4Cl	Maximum growth (nephelos units)
0	400.2
0.005	500.4
0.010	563.0
0.015	540.3
0.020	463.1

TABLE 11

RESPIRATION OF R_{21} ON GLUTAMIC ACID, PYRUVIC ACID AND ALPHA-KETOGLUTARIC ACID

Time (min.)	Glutamic acid			pyruvic acid		
	O^*_2	CO_2**	$\frac{CO_2}{O_2}$	O_2*	CO_2**	$\frac{CO_2}{O_2}$
5	42.9	50.3	1.20	28.6	31.0	1.08
10	87.4	83.6	0.96	73.1	83.0	1.13
15	133.6	121.5	0.91	119.2	137.5	1.15
20	176.5	158.9	0.90	167.0	203.0	1.21
25	209.9	193.0	0.92	206.7	252.6	1.22
		ave. = 0.98			ave. = 1.16	

TABLE 11 (CONT.)

Time (min.)	alpha-ketoglutaric acid			endogenous		
	O_2*	CO_2**	$\frac{CO_2}{O_2}$	O_2*	CO_2**	$\frac{CO_2}{O_2}$
5	29.0	38.9	1.34	34.1	24.7	0.79
10	65.8	75.0	1.12	62.0	45.0	0.73
15	105.6	113.2	1.07	91.4	69.7	0.76
20	145.3	155.7	1.07	122.4	94.5	0.77
25	179.0	192.0	1.07	141.0	114.7	0.81
		ave. =	1.13		ave. =	0.77

* microliters O_2 taken up

** microliters CO_2 evolved

O_2 and CO_2 values are averages of duplicate flasks.

TABLE 12

DATA REGARDING THE GROWTH OF R_{21} ON GLYCINE-1-C^{14}

Incubation time (days)	Background (c.p.m.)	Sample count (c.p.m.)	c.p.m. above background	Nephelos*	O.D.**
1	19.1	71.0	51.9	60.8	-
2	14.3	158.9	144.6	297.5	.076
3	14.4	204.0	189.6	487.0	.052
4	16.6	282.5	265.9	999.0	.039
5	18.2	310.7	292.5	1415.0	.018
6	17.2	159.6	142.4	1730.0	.012
7	17.2	29.9	12.7	1690.0	.000

* corrected for imperfections in the reading tube

** O.D. = optical density of solutions obtained by extracting chromatogram areas.

TABLE 13

OXIDATION OF L- AND D-AMINO ACIDS BY RHIZOBIUM R_{21}

Amino acid	Oxygen uptake in microliters*			
	Time (min.)			
	15	30	45	60
L-valine	62.0	123.2	187.5	278.5
L-histidine	70.5	136.8	209.0	291.7
L-methionine	55.8	117.3	172.3	239.0
L-leucine	50.0	102.2	159.7	220.4
L-glutamic acid	84.2	160.7	230.4	326.1
glycine pH 8.0	72.0	143.3	212.3	285.0
glycine pH 7.0	56.3	110.3	150.8	202.1
glycine pH 6.0	45.0	93.0	134.2	182.9
endogenous	40.0	87.0	127.0	179.4
D-valine	10.0	21.6	33.9	46.2
D-histidine	7.2	14.0	22.4	32.2
D-methionine	12.7	22.3	38.2	57.2
D-leucine	12.5	23.2	41.8	60.4
D-glutamic acid	12.7	23.0	41.8	59.0
endogenous	5.7	9.9	18.5	28.0
glycine	13.5	19.5	34.5	52.5
glycine + azide	4.7	6.3	12.6	22.1

TABLE 13 (CONT.)

Amino acid	Oxygen uptake in microliters*			
	Time (min.)			
	15	30	45	60
glycine + benzoate	1.4	5.8	14.5	31.9
glycine + NaCN	0.0	0.0	0.0	0.0

* averages of duplicate flasks

TABLE 14

ANAEROBIC REDUCTION OF METHYLENE BLUE BY R_{21} IN THE PRESENCE OF L-GLUTAMIC ACID

Time (min.)	Optical density*	
	L-glutamic acid	endogenous
0	0.690	0.710
3	0.690	0.710
5	0.590	0.720
7	0.485	0.710
9	0.390	0.690
11	0.325	0.670
13	0.285	0.650
14	0.275	0.640
15	0.275	0.630

* average of triplicate determinations

TABLE 15

ACTION OF L-GLUTAMIC ACID DECARBOXYLASE ON TRANSAMINATION TEST MIXTURES ORGINALLY CONTAINING ALPHA-KETOGLUTARIC ACID AND D-ASPARTIC ACID

	Microliters CO_2 evolution				
	Time (minutes)				
	10	20	30	40	50
Standard**	36.0	70.1	85.0	93.0	101.5
Test mixture	10.0	21.0	30.1	37.6	46.0

* averages of duplicate flasks - endogenous subtracted

** containing 0.5 mgm. L-glutamic acid/flask

TABLE 16

ANALYSIS OF L-ALANINE REACTION MIXTURE BY D-AMINO ACID OXIDASE -(ALANINE RACEMASE)

Flask contents	Oxygen uptake in microliters*						
	Time (min.)						
	10	20	30	40	50	60	70
L-alanine mixture	29.3	56.2	84.0	95.8	110.9	122.2	132.9
control	0.0	0.0	0.0	1.0	0.0	0.0	1.0

* averages of duplicate flasks.

APPENDIX

PART II

TABLE 17

DATA PERTAINING TO THE GROWTH OF THREE RHIZOBIUM STRAINS IN CYSTEINE MEDIUM WITH OR WITHOUT ADDED NICOTINAMIDE

Time (days)	Nephelos readings					
	R_{21}		R_{20-27A}		R_{20}	
	A	B	A	B	A	B
1	8.7	7.9	16.0	10.0	3.6	14.0
2	30.4	41.0	37.0	44.0	4.0	54.3
3	47.2	54.7	39.5	54.3	25.2	63.5
4	47.1	61.2	35.7	55.2	49.0	65.5
5	42.1	58.7	33.0	51.0	53.1	58.1
6	40.2	47.0	30.0	47.0	53.9	54.8
7	32.0	38.0	29.5	47.0	51.4	51.3

A = without added nicotinamide B = with added nicotinamide

TABLE 18

UTILIZATION OF PURINES AND PYRIMIDINES (0.0001M) BY THREE *RHIZOBIUM* STRAINS GROWING IN LEUCINE BASAL MEDIUM

Compound	Maximum growth in nephelos units		
	R_{20}	R_{21}	R_{20-27A}
adenine sulphate	53.0	438.0	377.0
guanine HCl	36.0	296.6	275.0
xanthine	71.0	375.2	242.0
thymine	51.0	276.6	239.0
uracil	74.0	354.0	224.0
cytosine	96.0	358.0	271.0
leucine only	113.0	222.0	182.0