Studies on Protein Synthesis by the Genus Azotobacter

by

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INTRODUCTION

The subject of the biological fixation of nitrogen has received the attention of many investigators, and numerous articles have been published which are concerned with the fixation of atmospheric nitrogen by non-symbiotic as well as by symbiotic organisms. Since the majority of these investigations have been of a physiological rather than of a biochemical nature, the present investigation was commenced in order to determine, to some extent, the products of metabolism of *Azotobacter*, to compare the biochemical activities of the various species which comprise the genus, and to attempt to explain the mechanism of protein synthesis by these organisms.

The genus *Azotobacter*, as described by Bergey (4), consists of the following six species: *A. chroococcus* Beijerinck, *A. vinelandii* Lipman, *A. beijerinckii* Lipman, *A. Woodstownii* Lipman, *A. vitreus* Lohnis and Westerman. There is some question, however, whether the two latter species are true *Azotobacter*. The author has shown elsewhere (26) that they are not vigorous nitrogen fixing organisms. Lohnis and Smith (52) studied the cultural characteristics and life histories of many strains of *Azotobacter* and their investigations "have shown that only two species of *Azotobacter* are characterized thus far: *Azotobacter chroococcum* and *A. agile* Beij. (syn. *A. Vinelandii* J. G. Lipman). *A. Beijerinckii* J. G. Lipman is a variety of *A. chroococcum* and *A. vitreum* Lohnis is probably a variety of *A. agile*. *A. Smyrni* C. B.

Lipman et Burgess can not be accepted as a species; according to all marks ascribed to it by its authors it is a large sporulating growth type of A. chroococcum. A. Hilgardii C. B. Lipman and A. Woodstownii J. G. Lipman are both incompletely described and should not be retained."

Aso and Yoshida (1), by means of serum reactions (complement fixation) have been able to distinguish various types of *Azotobacter*. Their results show that *Azotobacter* can be classified into three types: A. chroococcum, A. vinelandii, and A. vitreus. A. chroococcum and A. beijerinckii are of the same type, and apparently the latter is a strain of the former.

The partial composition of *Azotobacter* cells has been determined by various investigators. Stoklasa (60) reports the nitrogen content of A. chroococcum as varying from 9.8 to 14.1 per cent; Gerlach and Vogel (20) found the variation to be from 10.0 to 12.0 per cent; Omeliansky and Sieber (53) report a value of 2.07 per cent. Hunter (37) found the nitrogen content to vary widely, depending upon the type of medium used. He found the nitrogen content of cells grown on solid media is less than that of cells grown on liquid media. Waksman (64) believes that this difference is due to the fact that a large part of the membranous material and the slime surrounding the cells consists of carbohydrates, free from proteins. In the case of liquid media this material is filtered out, which tends to increase greatly the amount of protoplasm in the residual material.

The nitrogen fixed by *Azotobacter* does not remain in a soluble form, but is converted into complex nitrogen com-

pounds. This was recognized by Berthelot (5) and later studied by Lipman (49) who found *Azotobacter* to contain 10.45 per cent total nitrogen. The basic nitrogen was found to be 2.76 per cent, non-basic 6.39 per cent, and ammonia nitrogen 0.98. These values would correspond to 26.40 per cent, 61.00 per cent and 9.37 per cent of the total nitrogen, respectively. Lipman considered the non-basic fraction to consist of amino acids or simple peptides, and that alanin was one of the early substances synthesized. Further studies by Halversen (29) showed that in two days, 89.6 per cent of the nitrogen fixed by *A. chroococcum* was in the form of protein, and in four days, the protein nitrogen was 96.2 per cent of the total. The most complete protein analysis was made by Omeliansky and Sieber (53) shortly after the introduction of the Van Slyke method of protein analysis. Their results are discussed elsewhere in this paper.

Azotobacter produces a slimy carbohydrate substance which consists largely of polysaccharides. Beijerinck and Van Delden (3) considered this to be similar to pectin, while Hoffman (35) reported the presence of pentosans. More recent work by Hamilton (30) has shown that in the case of *A. chroococcum*, the carbohydrate material is a gum, which is soluble in water. This gum gives a positive test with Molisch's reagent, and contains only a trace of nitrogen. It does not reduce Fehling's solution (even after boiling with acid). Since this gum was levo-rotatory, Hamilton con-

cluded that it should be classed as a true gum, and not as a dextrin. He states: "The results..... place the gum in 'Class I' of Haas and Hill, and under this classification it would be termed an arabin". The author does not fully agree with Hamilton's classification of this gum, but feels that it should be considered as being a polysaccharide of Class II of Haas and Hill (33). This group is made of "Gums which comprise (a) natural gums and pentosanes; (b) mucilages and pectic bodies", while Class I includes "Starches and dextrins, including glycogen, inulin, mannane and galactane ($C_6H_{10}O_5$)_n". Haas and Hill classify the gums into the following sub-groups:

- "A. Gums, such as arabin (gum arabic), which are soluble in water.
- B. Gums which are partially soluble in water.
- C. Mucilages and pectic bodies which swell with water to form jelly."

It appears, therefore, that the gum of A. chroococcum should be placed should be placed in Class II of Haas and Hill, and be listed as a water soluble gum.

In a preceding paragraph, reference was made to wide variations in the nitrogen content of *Azotobacter* as reported by various investigators. Similar variations are found in the results for moisture, ash and carbon determinations. These varying results do not imply experimental errors on the part of the respective investigators, but merely show the great variation which may result from the use of different strains of the same organism under different cultural conditions. Within recent years, many studies have been made concerning bacterial variation. In general, these studies have been limited to pathogenic organisms, and the dissociative aspects of soil bacteriology have received but

little attention. The studies of Lohnis and Smith (52) on life cycles of *Azotobacter*, and Israilevsky and Starygin (38) probably represent the principal works along these lines. The latter authors pointed out that the symbiotic nitrogen fixing organisms may exhibit dissociation phenomena, and that under certain conditions, smooth and rough colonies may be formed. Smooth colonies are the usual form of the majority of bacteria, while the rough colonies as variants, sometimes exhibit modified physiological, morphological and biochemical activities. These variants may be produced in many ways, such as by rapid transfer of cultures, the use of different media, changes in the pH of the culture media, incubation temperature, et cetera. While the relations of bacterial variation to the physiological activities of *Azotobacter* does not lie within the scope of this paper, the subject is mentioned as a possible explanation of the varying results which have been reported in the literature. In general, Ashby's nitrogen-free mannitol agar has been used by most investigators. Some have used other carbohydrates, such as starch, dextrin and dextrose. It is quite probable that changes in carbohydrates, as well as the agar content of the media, the length of the incubation period, the temperature of incubation and the reaction of the media may produce profound changes in the growth of *Azotobacter*. The effects of these factors upon the physiological activity of *Azotobacter* will be the subject of a subsequent report.

The present investigation had for its purpose, a more extensive chemical analysis of *Azotobacter*, as well as a comparison of the composition of the various species of the genus, and an attempt to explain the mechanism of protein synthesis from the results obtained.

EXPERIMENTAL METHODS AND RESULTS

Preliminary tests were made to determine the best method of securing *Azotobacter* cells for analysis. Hoffman and Hammer (36) grew *A. chroococcum* on Ashby's agar and scraped the growth from the surface with a glass slide. The cells were dried, pulverized and analyzed. Hunter (37) used the same method for removing growth from solid media, and employed a supercentrifuge to separate the cells from liquid media. On account of limited incubation space the plate method was chosen for this investigation, and it incidentally afforded a comparison with the results of Omeliansky and Sieber (53).

The preliminary tests showed that the cells contained from 91 to 95 per cent of moisture, therefore dehydration was necessary. Several procedures have been used by various investigators; some have air-dried the cells, others have dehydrated in vacuo, and others have dried them at the temperature of boiling water. It is apparent that some of these methods may cause a chemical alteration of the material. Omeliansky and Sieber (53) found that desiccation in vacuo was not satisfactory due to the fact that a gummy residue was formed which was difficult to pulverize. Complete dehydration was seldom obtained, and butyric fermentation of the residue usually resulted. The author experienced these same difficulties, so dehydration by means of

a solvent was tried. Wilkerson and Gortner (73) used anhydrous acetone to dehydrate pig embryos in a study of the chemistry of embryological development. This dehydrating agent was employed in this study and proved to be satisfactory. It does not denature proteins and does not dissolve fats to any extent. It has the disadvantage, particularly when hot, of dissolving phospho-proteins, and phospholipids, but it is probable that these substances do not occur in large amounts in the cells of *Azotobacter*.

The following procedure was finally adopted: twenty-four hours prior to the inoculation of plates, the stock cultures were transferred to slants of Ashby's mannite agar. These cultures had been grown for approximately two years on Ashby's agar. At the end of the stated time, a small amount of growth was transferred to a slide, stained with methylene blue, and examined microscopically in order to check the purity of the culture. In no case were contaminating organisms observed. Sterile tap-water blanks were then inoculated with each of the cultures used. Several hours prior to this, large plates of Ashby's agar had been poured and allowed to solidify. The medium had the following composition (17).

Mannitol	10.0 gm.
Dipotassium phosphate(K_2HPO_4)	0.5 gm.
Magnesium sulphate($MgSO_4 \cdot 7H_2O$)	0.2 gm.
Sodium Chloride($NaCl$)	0.2 gm.
Manganese sulphate($MnSO_4 \cdot 4H_2O$)	trace
Ferric chloride($FeCl_3 \cdot 6H_2O$)	trace
Agar	15.0 gm.
Distilled water	1000.0 cc.

When the agar had solidified, 1 cc. of the water suspension was added to each plate, which was then rotated so as to give an even distribution of the suspension upon the surface of the media. All glassware and media were carefully sterilized, and every precaution was taken to prevent contamination. All chemicals which were used in the preparation of the media during the course of the experiment had been previously tested for absence of nitrogen. In each series of plates, one culture dish was inoculated with 1 cc. of sterile water, to serve as a check. In no case did any colonies develop on the controls.

It was necessary to use glass culture dishes, 200 x 20 mm., because a mass culture apparatus was not available. After inoculation, the plates were incubated for four days at 30°C. The growth was then scraped from the surface with a glass slide and transferred to a jar containing anhydrous acetone. The material was allowed to stand several days at room temperature, and the acetone changed at frequent intervals. When a sufficient quantity of the material had been secured, the acetone was removed by filtration through hardened filter paper, and the material was then air-dried, a fan being used to hasten the removal of the acetone. The residue was then ground in an agate mortar, extracted and dried as before, and then finally ground in a ball mill. By this method, it was possible to secure approximately 25 grams of material from each species. This material was then

analyzed according to the methods given in following pages.

The collection of this material required approximately seven months. Each plate yielded only from one to two grams of bacterial material, which contained approximately 95 per cent moisture. Consequently, one hundred plates gave only about five grams of the dehydrated material. This method seems preferable to that of Omeliansky and Sieber (53) since possible chemical alteration of the material is greatly reduced. They grew A. chroococcum on 2 per cent dextrin agar for 6 days at 30°C., the growth was scraped off with a glass slide and an equal amount of distilled water was added. The suspension was filtered through glass wool to remove particles of agar. The tarry mass was transferred to a crystallizing dish and placed on the bottom of a 37°C. incubator until dry. In the method used in the present investigation, no difficulty was experienced in removing the growth from the surface. Every precaution was taken to prevent the removal of agar, sacrificing yield to minimize possibility of contamination.

Three of the stock cultures used were obtained from culture collections in the United States and Europe. The culture of A. chroococcum was number 50-C, which is described in another publication (26), and which had been isolated from a red sandy soil from Oak Creek Canyon, about 75 miles northeast of Prescott, Arizona. These cultures have been carefully examined over a period of a few years, and corres-

pond well to the descriptions given by Bergey (4) and by Waksman (64)*. As stated above, the cultures were examined before each series of plates were inoculated. No attempts were made to correlate morphological character with physiological activity. Plans are now being made, however, for future investigations along these lines.

The incubation period was set at four days for two reasons; first, Halversen (29) has shown that at that time 96 per cent of the total nitrogen is in the form of protein, and it is also recognized that older cultures may autolyze, liberating ammonia; and second, the culture of A. chroococcum was an active pigment former, but this pigment did not develop until later than the fourth day. Rippel and Ludwig (58) have claimed that the black pigment formed by A. chroococcum is due to the conversion of tyrosine to melanin. Consequently, the incubation period was set so as to harvest this strain before pigment formation had commenced.

Only a single strain of each species of Azotobacter was employed in this investigation. The author realizes the desirability of using more than a single culture of each species, but due to the time required to accumulate sufficient material and to make the involved analyses, this did not appear feasible. However, the material collected from these

*In order to further establish the identity of the stock cultures, they were examined by Mr. N. R. Smith, Senior Bacteriologist, Bureau of Chemistry and Soils, U. S. Department of Agriculture, who found them to be typical.

cultures which were used is representative, since all were treated in exactly the same manner during the course of the investigation; it may be emphasized that the material collected from each species came from approximately one thousand different plates and was collected over a relatively long period of time.

The material secured in the manner just described was then analyzed. A gross chemical analysis was made according to the methods of the Association of Official Agricultural Chemists (2). The results of this analysis are given in Table I.

TABLE I

Gross Chemical Composition of Azotobacter

	<u>A. Agilis</u>	<u>A. Vinelandii</u>	<u>A. Beijerinckii</u>	<u>A. Chroococcum</u>
Moisture	8.53%	9.06%	5.68%	5.46%
	8.57	9.05	5.53	5.36
Average	8.55	9.06	5.61	5.41
Ash	7.60	7.10	4.03	3.97
	7.50	7.02	4.07	4.03
Average	7.55	7.06	4.05	4.00
Protein (Nx6.25)	61.19	55.94	25.00	23.69
	61.19	56.56	24.37	25.31
Average	61.19	56.25	24.68	25.00
Fat	0.61	1.92	0.94	3.25
	0.63	1.99	0.93	3.25
Average	0.62	1.96	0.94	3.25
Carbohydrates	22.09	25.67	64.72	62.34
(by difference)				
Total	100.00	100.00	100.00	100.00

This table shows that A. agilis and A. vinelandii contain larger amounts of protein and smaller amounts of carbohydrates than A. beijerinckii and A. chroococcum, and that the two latter species are characterized by larger quantities of carbohydrates and smaller percentages of protein.

In order to secure further information concerning the composition of the various species, the nitrogen distribution was determined by Cavett's micro-modification (14) of Van Slyke's method (63). The carbohydrate distribution (proximate analysis) was also made, using the method of Waksmann and Stevens (66).

In the Van Slyke method of protein analysis, a sample is hydrolyzed with hydrochloric acid, and the excess acid is then removed by distilling under diminished pressure. Ammonia is determined by adding a slight excess of calcium hydroxide, and subsequent distillation and titration. The nitrogen thus obtained is reported as ammonia or amide nitrogen, since the larger part comes from acid amide linkages ($-\text{CONH}_2$). Gortner and Holm (23) have shown, however, that especially on prolonged hydrolysis, there may be a considerable amount of deaminization of amino groups, which would naturally give high results. The black residue which is formed during hydrolysis is then separated by filtration or by centrifuging, and is analyzed for nitrogen which is reported as humin or melanin nitrogen. The studies of Gort-

ner and his associates (21) have shown that humin is a condensation product of tryptophane with an aldehyde, and that under properly controlled conditions, the humin nitrogen may serve as a quantitative index of the tryptophane content of a protein. In the ordinary protein hydrolysate, however, the humin nitrogen is at best qualitative as regards tryptophane. The filtrate from the humin separation is then treated with phosphotungstic acid which precipitates the diamino acids (arginine, lysine, histidine, and cystine), or the so-called "bases". These are determined individually as follows: one molecule of arginine is quantitatively converted by alkaline hydrolysis into one molecule each of ornithine and urea. Hydrolysis, followed by an ammonia determination, gives the arginine content. Formerly, cystine was estimated from an organic sulphur determination but in the method of Cavett (14) it is determined directly by the colorimetric method of Folin and Marenzi (16). In the original Van Slyke method (63) lysine and histidine were calculated from the various nitrogen determinations which had been made upon the bases. In the method of Cavett, histidine is determined directly by the method of Koessler and Hanke (31,44) and lysine is calculated by difference. The filtrate from the bases is analyzed for total and amino nitrogen. The amino nitrogen is derived chiefly from the monoamino-monocarboxylic acids and the monoamino-dicarboxylic acids; the non-amino (total nitrogen of the filtrate minus the amino

nitrogen) comes from proline, hydroxy-proline and partly from tryptophane. Tyrosine and tryptophane were determined on another sample by the colorimetric method of Folin and Ciocalteu (15).

While the Van Slyke method has been criticized because it gives no information concerning the amino acids of the filtrate, and while certain errors are recognized, yet it is valuable in protein studies, due to its rapidity as compared to other methods of protein analysis and also because smaller samples are required (0.5 gm. by the method of Cavett). This method gives good results with pure proteins, but in the case of substances containing carbohydrates, inorganic salts, and other substances, certain difficulties are encountered. These were recognized by Van Slyke (63) and are discussed by Wilkerson and Gortner (73). For example, Gortner and Blish (22) have shown that the presence of carbohydrates will affect the amount of tryptophane converted to humin, and Hauge (34) has pointed out that the presence of fats will increase the acid soluble humin and decrease the amino nitrogen in the basic and non-basic fractions. If any amines are formed during hydrolysis, they will appear as ammonia. The author recognized these difficulties before the determinations were commenced, but a separation of the protein and non-protein fractions did not seem feasible, and as Wilkerson and Gortner (73) have stated: "Inasmuch as all samples were treated under exactly the

same rigid procedures, we feel that we are justified in concluding that the results we have obtained are comparable."

The nitrogen distribution of *Azotobacter*, as determined by the methods discussed in the preceding paragraphs, is given in Table II.

TABLE II

The Distribution of Nitrogen in the Acid Hydrolysate of *Azotobacter*
(Results expressed as percentage of total nitrogen, not corrected
for the solubility of bases.)

	<u>A. Agilis</u>		<u>A. Vinelandii</u>		<u>A. Beijerinckii</u>		<u>A. Chroococcum</u>	
Amide N	13.22		20.11		18.36		17.90	
	13.62	13.42%	20.61	20.36%	18.14	18.25%	17.90	17.90%
Humin N	13.26		15.88		13.42		9.25	
	13.10	13.18	15.77	15.82	13.92	13.67	10.00	9.62
Basic N								
Arginine N	19.59		16.66		20.26		16.25	
	18.79	19.19	16.66	16.66	20.26	20.26	16.25	16.25
Histidine N	0.16		0.16		0.17		0.12	
	0.16	0.16	0.16	0.16	0.17	0.17	0.11	0.11
Cystine N	0.34		0.29		1.02		0.50	
	0.29	0.31	0.29	0.29	1.04	1.03	0.50	0.50
Lysine N		7.24		5.11		4.51		3.14
Filtrate N								
Amino N		44.33		39.25		40.08		50.34
Non amino N		<u>1.44</u>		<u>2.25</u>		<u>1.95</u>		<u>2.16</u>
Recovery		99.27		99.90		99.92		100.02
Tyrosine N	1.00		1.22		0.78		0.62	
Tryptophane	0.41		0.19		0.15		0.11	

A proximate analysis was made in order to secure further information regarding the composition of *Azotobacter*. The method of Waksman and Stevens (66) was used. It was first employed by its originators in a study of the composition of peat (65), and later extended by them to all plant materials (66). More recently, it has been used by Porges (55) in a study of *Aspergillus niger*. The method is briefly given in the following paragraph.

A sample of the moisture free material (5.0 gms.) was extracted for 16 hours in a Bailey-Walker extraction apparatus with anhydrous ether. After drying, the extracted material was weighed, and the loss reported as ether-soluble organic matter. The residue was transferred to a beaker and extracted for one-half hour on a steam bath with 95 per cent alcohol. The alcoholic extract was filtered through a hardened filter paper which had been previously dried and weighed. The residue was washed well with alcohol and the filtrate made to a volume of 250 cc. An aliquot (50 cc.) was transferred to a platinum dish, dried and weighed. The residue was then ignited and weighed to determine the ash content. The total organic content minus the ash gives the alcohol-soluble organic matter. Total nitrogen and sugar determinations were also made upon separate aliquots. The residue from the alcohol extraction was returned to the original beaker and was extracted with distilled water for 24 hours at room temperature. The aqueous extract was fil-

tered through the same filter paper, the residue washed with distilled water, and the filtrate made to volume (250 cc.). Total organic matter, total nitrogen, sugars and ash were determined on separate aliquots as outlined above. The residue from the cold water extraction is transferred again to the original beaker and is extracted with hot water for one hour. The hot water extract was filtered, washed, and analyzed for total organic matter, ash, nitrogen and sugars. The residue was then dried and weighed. After drying, the residue was hydrolyzed for five hours in flowing steam with 2 per cent HCl. The acid solution was filtered through the original filter paper, washed free from acid with hot water, and dried to determine its weight. Total nitrogen and reducing sugars were determined on the filtrate. The hemi-cellulose content was calculated by multiplying the amount of reducing sugar by the factor 0.9. The residue, after it had been dried and weighed, was transferred to a mortar and was ground. Duplicate 1 gm. samples were placed in beakers and 10 cc. of 80 per cent H_2SO_4 were added to each. At the end of two hours, 150 cc. of distilled water were added to each beaker, which were then placed in an autoclave and hydrolyzed at 15 pounds pressure for one hour. The acid solution was filtered through filter papers which had been previously dried and weighed. The residue was washed with water until free from acid, dried and weighed. One sample was placed in a platinum crucible and ignited to

determine the ash content, and total nitrogen was determined upon the other. The weight of the residue from the HCl hydrolysis minus the weight of ash and the weight of protein ($N \times 6.25$) gave the amount of lignin in the sample. Since 1 gm. portions of the residue from the hemicellulose determination were used, the amount of lignin in the sample must be multiplied by the weight of the HCl residue in order to obtain the total amount of lignin in the original sample. Ash was determined on a separate sample, and the nitrogen was reported as crude protein, which was calculated from the total nitrogen determined by the Kjeldahl method. The quantities of protein determined in the various fractions were subtracted from the total protein since they were included as organic matter.

Porges (55) summarizes the method of Waksman and Stevens thus: "The fractions differentiated by this method of procedure contained organic matter soluble in the solvents used and may be divided as follows:

1. In the ether extract are found the fats and oils, and also a part of the resins and waxes.

2. In the alcohol extract are found the waxes, resins, pigments, alkaloids, and some of the sugars.

3. In the cold-water extract are found the various sugars, amino acids, some proteins, various organic acids, and alcohols.

4. In the hot-water extract are found the starches, pectins, tannins, proteins, amino acids and other organic acids.

5. In the dilute-acid extract are found the carbohydrates insoluble in water but soluble in dilute acid. These are the hemicelluloses, including pentosans and hexosans, which account for the greater part of this extract, and starches. In addition there are present amino acids and proteins.

6. In the 80 per cent H_2SO_4 extract are found those carbohydrates not soluble in dilute acids but soluble in concentrated acids and which give glucose as a product of hy-

drolysis. These include the celluloses. Amino acids and proteins are also found in this extract.

7. The residue from the H_2SO_4 extract contains lignin-like products and proteins not acted upon by the various treatments. As mentioned previously, the amount of lignin-like material is obtained by subtracting the weight of the protein and ash from the weight of the residue.

8. The ash or inorganic fraction contains the various minerals which are available for the organism.

9. The total protein is calculated from the nitrogen determined by the Kjeldahl method. The nitrogen found in the various fractions should equal the total nitrogen."

In the analysis of complex organic materials, it is practically impossible to secure one hundred per cent recovery. The difficulties involved in this method were recognized by Waksman and Stevens (65) and have also been discussed elsewhere by the author (27). The results of the proximate analysis, which was made by the method given above, appear in Table III.

TABLE III

The Proximate Analysis of Azotobacter
(moisture free basis)

	<u>A. Agilis</u>		<u>A. Vinelandii</u>		<u>A. Beijerinckii</u>		<u>A. Chroococcum</u>	
Ether-soluble	0.66		2.10		0.99		3.43	
Organic matter	0.68	0.67%	2.18	2.14%	0.98	0.99%	3.43	3.43%
Alcohol-soluble	1.68		1.28		1.47		1.68	
Organic matter	1.68	1.68	1.24	1.26	1.40	1.44	1.66	1.67
Cold-water soluble	8.60		9.48		14.42		10.63	
Organic matter	8.59	8.60	9.60	9.54	14.40	14.41	10.67	10.65
Hot-water soluble	15.80		19.05		18.88		14.68	
Organic matter	15.86	15.83	19.01	19.03	18.90	18.89	14.60	14.64
Hemicelluloses	4.60		4.62		0.13		0.13	
	4.60	4.60	4.62	4.62	0.13	0.13	0.13	0.13
Celluloses	0.12		0.12		0.13		0.13	
	0.12	0.12	0.12	0.12	0.13	0.13	0.13	0.13
Lignin-like Material		3.65		3.72		33.18		33.01
Crude Protein		50.98		47.22		15.40		17.98
Ash	8.26		7.75		4.26		4.22	
	8.20	<u>8.23</u>	7.65	<u>7.70</u>	4.32	<u>4.29</u>	4.26	<u>4.24</u>
Recovery		94.36		95.35		88.86		85.88

DISCUSSION

An examination of Table I shows a very close chemical relationship between A. agilis and A. vinelandii, and between A. beijerinckii and A. Chroococcum. The first two are characterized by much larger percentages of ash and protein, and a relatively lower percentage of carbohydrates.

In Table III a similar relationship is found. A. agilis and A. vinelandii contain smaller amounts of water soluble organic matter and lignins, and a larger amount of hemicelluloses. In Table II, there are not enough consistent variations to warrant a generalization. The results however were expressed in terms of percentage of total nitrogen. Table I shows, however, that the material did not consist entirely of protein, but contained ash, carbohydrates and fat. The results of the Van Slyke analysis were then expressed in terms of total material. These results are given in Table IV.

TABLE IV

The Distribution of Nitrogen in Azotobacter
(Results expressed as percentage of original material, air-dry basis.)

	A. Agilis	A. Vine- landii	A. Beijer- inckii	A. Chroo- cocum
Amide N	1.340%	1.832%	0.720%	0.716%
Humin N	1.312	1.425	0.540	0.385
Basic N	(2.70)	(2.00)	(1.025)	(0.800)
Arginine N	0.95	0.75	0.40	0.325
Histidine N	0.078	0.073	0.003	0.002
Cystine N	0.034	0.026	0.037	0.020
Lysine N	1.638	1.151	0.585	0.453
Filtrate N				
Total N	4.55	3.76	1.67	2.10
Amino N	<u>4.41</u>	<u>3.53</u>	<u>1.55</u>	<u>2.01</u>
Non-amino N	0.04	0.23	0.12	0.09
Recovery	9.90	9.00	3.95	4.00
Total N (Kjel- dahl method)	9.95	9.00	3.95	4.00

An examination of this table shows the same similarity between A. agilis and A. vinelandii and between A. beijerinckii and A. chroococcum as shown by Table I. While the results given do not agree absolutely, they are of the same order of magnitude. The similarities agree, in general with those found in Table I and IV, but are not reflected in Table II because of the differences in total nitrogen content. When converting the results into percentage of total nitrogen, the actual variations are so greatly magnified that these relationships are not apparent.

The relationships mentioned above are of interest in the light of the results of Lohnis and Smith (52) and Aso and Yoshida (1), which were discussed in the introduction of this paper. Their findings indicate that the genus *Azotobacter* may be classified into three chief types: A. chroococcum, A. vinelandii, and A. vitreus. It seems that A. beijerinckii may be either a strain of, or closely related to A. chroococcum, and that a similar relationship exists between A. vinelandii and A. agilis. It is questionable whether A. woodstownii and A. vitreus should be considered as members of the genus. The results given in Tables I and III support, in general, the results obtained by the investigators mentioned above.

Table I also shows that A. agilis and A. vinelandii have a higher nitrogen content than do the other two species. The author has shown elsewhere (26) that over a wide range

of temperatures, A. agilis and A. vinelandii were more active nitrogen fixing organisms than A. beijerinckii or A. chroococcum. The ratio of total nitrogen fixed is not proportional to the nitrogen content of the particular species, however, which would indicate that although A. chroococcum and A. beijerinckii contained a smaller percentage of nitrogen, they must have a higher growth rate. Many soil bacteriologists have considered A. chroococcum to be the most active species of the genus, but this is probably because few studies have been made in which the physiological activity of the various species have been compared, and also because A. chroococcum is more widely distributed than the other species.

An examination of Table II shows that the amide nitrogen varies from 13.42 to 20.36 per cent of the total nitrogen, and the humin nitrogen varies from 9.60 to 15.82 of the total. Larmour (48) has compiled the results of the analyses of many different proteins, and when the results of this investigation are compared to the results given in his compilation, these values for humin are much higher than any values which he cites. It has already been mentioned that the carbohydrate fraction may lead to high values for humin, by the formation of an aldehyde by the action of concentrated HCl on the carbohydrates and a subsequent condensation of tryptophane with this aldehyde to form humin. It is possible in this case, that the carbohydrate fraction

may be partially responsible for the high values for humin. When compared to the results given by Larmour (48), the values for amide nitrogen are also high, although he does cite a few cases where the amide nitrogen may be 25 per cent of the total nitrogen. This is particularly true in the case of gliadin. If any deaminization of amine acids occurred during hydrolysis, this would lead to high results, or the presence of free ammonia would have the same effect. It is quite probable that the nitrogen fixing organisms in general may be characterized by relatively large percentages of humin and ammonia nitrogen. Paresi and Masetti-Zannini (54), in a study of the proteins of the nodules of Vicia Faba, report the following results: ammonia nitrogen, 18.80 per cent; humin nitrogen, 12.53 per cent; basic nitrogen 16.91 per cent; and non-basic nitrogen 50.87 per cent (recovery, 99.11 per cent). In this case, the relatively low values for basic nitrogen and similarly high values for non-basic nitrogen are of interest because they indicate that the principal amino acids are found in the non-basic fraction, and are therefore principally monoamino-mono carboxylic acids.

In the case of *Azotobacter*, the principal amino acid of the basic fraction is arginine, but a considerable amount of lysine is also found. A small amount of cystine and a smaller quantity of histidine were found in this fraction. Arginine and lysine, however, are the principal amino acids

of the bases.

As shown in Table II, the average tyrosine content of the four species examined is approximately one per cent of the total nitrogen, and the tryptophane content is much less. It is interesting to note that A. chroococcum contained the smallest percentage of tyrosine. Rippel and Ludwig (58) have claimed that the black pigment formed by this species was due to the conversion of tyrosine to melanin. The stock culture of A. chroococcum used in this experiment formed an intense black pigment and had been selected especially for that reason. The incubation period was set at four days in order that the organisms might be harvested before pigmentation had commenced. Ranganathan and Norris (57) were not able to demonstrate the presence of tyrosinase in a culture of A. chroococcum. If the pigment formed by this species is due to the conversion of tyrosine to melanin, it is difficult to explain why the other species do not form the pigment as readily under identical conditions, since they all contain as much or more tyrosine than A. chroococcum. This is particularly true in the case of A. beijerinckii which appears to be closely related to A. chroococcum.

In each species, the non-basic fraction contains the major portion of the total nitrogen. In the Van Slyke analysis, only the six amino acids mentioned above, were determined. Tyrosine and tryptophane were the only amino acids

of the basic fraction which were determined. The non-amino nitrogen of the filtrate from the bases may be due, in part at least, to proline and hydroxyproline. Therefore, approximately 38 to 40 per cent of the total nitrogen is unaccounted for, and probably occurs as other amino acids: glycine, alanine, valine, phenyl alanine, etc. Unfortunately, satisfactory tests are not available at present for identifying these amino acids.

A nitrogen distribution of A. chroococcum was made by Omeliansky and Sieber (53), but to the author's knowledge, such investigations have not been extended to the other species. For purposes of comparison, their results as well as those obtained in the present investigation are given in Table IV.

TABLE V
The Nitrogen Distribution of A. Chroococcum

		Omeliansky & Sieber		Author's Results
Ammonia		9.90%		17.90%
Humic N		3.70		9.60
Basic N				
Arginine N	10.39		16.25	
Lysine N	14.50		3.14	
Histidine N	1.63		0.11	
Cystine N	none	26.24	0.50	20.00
Filtrate N				
Amino	55.22		50.34	
Non-amino	4.77	<u>55.22</u>	2.11	<u>52.55</u>
Recovery		99.94		100.05

The results given in the preceding table show only approximate agreement. The values reported by the author for humin and ammonia nitrogen are much greater than those of Omeliansky and Sieber. They also report smaller amounts of arginine, hence the value of lysine was greater, since it was calculated by difference. They also found larger amounts of histidine by calculation than the author found by a direct colorimetric determination. They report no cystine, which was probably based upon an organic sulphur determination whereas the author found it present by colorimetric methods. The determination of cystine in proteins, however, offers considerable difficulty, and the methods existing even today are open to question. Gortner and Sandstrom (24) using mixtures of known amino acids, were able to recover only 62 to 73 per cent of the cystine which was added. The results of protein analyses made by various investigators vary widely, as an examination of the analyses compiled by Larmour (48) will show. This lack of agreement may be due to several factors: the Van Slyke method has been modified considerably since it was employed by Omeliansky and Sieber; they used dextrin as a source of carbohydrate, while the author used mannitol; their media contained 2 per cent agar, while the author's contained 1 1/2 per cent; their incubation period was six days as compared to four days in this present investigation. The same temperature was employed. It may be that these factors are responsible, as well as

marked differences in the strains of *Azotobacter* which were used. The length of the incubation period may also play an important part. Jones (40) has found that *A. chroococcum* forms capsules about the fifth or sixth day, and this may also be responsible for the different results obtained. The results do agree, however, in that arginine and lysine are the principal amino acids of the basic fraction, and that the larger part of the amino acids are found in the non-basic portion.

The results given in Table III show that *A. vinelandii* and *A. chroococcum* contain slightly larger quantities of fats and oils (ether soluble material) than the two other species, but there was little variation in the amounts of alcohol soluble organic matter (waxes, resins, etc.). *A. beijerinckii* and *A. chroococcum* contain larger amounts of water soluble organic matter than *A. agilis* and *A. vinelandii*. This material consisted chiefly of protein and a material which gave a positive test for carbohydrates with Molisch's reagent, yet it neither reduced Fehling's solution nor gave a positive test for starch. This material may be the gum which Hamilton (30) described for *A. chroococcum*. At least, the carbohydrate material in the water soluble fractions could be placed in the same classification of Haas and Hill (33) as the material which Hamilton described. Further tests were not made on this fraction. The hemicellulose content of *A. agilis* and *A. vinelandii*

was greater than that of A. beijerinckii and A. chroococcum, but the lignin content of the latter two species was much greater. On hydrolysis with dilute hydrochloric acid, there is evidence that certain reducing hexosans were formed, as shown by a positive test with Fehling's solution.

THE MECHANISM OF PROTEIN SYNTHESIS BY AZOTOBACTER

Many theories have been advanced to explain the metabolism of Azotobacter and the mechanism of fixation of atmospheric nitrogen by these organisms. Excellent summaries of these theories are given by Greaves (25) and by Waksman (64). The bulk of experimental evidence, particularly the investigations of Winogradsky (75,76) and Kostychev (45, 46,47), now points to ammonia as the first product of fixation. It has been demonstrated that large amounts of ammonia are found in the early stages of fixation. It has been claimed that this direct combination of nitrogen and hydrogen may be considered as a "biological Haber-Bosch process" (46) and that it is brought about by an intracellular enzyme which has been tentatively named "azotase" (10). This reaction was usually considered to be endothermic, but Burk (11) has shown from thermodynamic calculations that little energy is required for the biological fixation of nitrogen. Bortels (8) has suggested that molybdenum may act as a catalyst, while Blom (6) has advanced a theory of fixation in which iron acts as a catalyst to activate gaseous nitrogen and water to form hydroxylamine as one of the intermediate products.

If the fixation of nitrogen requires but small amounts of energy, the carbohydrates which are necessary for the development of Azotobacter must be used principally for growth.

Therefore, the decomposition of carbohydrates supplies the energy for growth through respiration. The general subject of respiration has undergone many changes within recent years, and several theories have been advanced to explain its mechanism. Two of the more important of these are the theories of Warburg (67) and of Wieland (66,69,70,71,72). Although these have been considered as opposing theories, they have been combined into a compromise theory by Kluyver and his associates (41,42,43). Wieland pointed out that oxidations could take place in the absence of free oxygen, and postulated that such oxygen came from water and that the mobilization of hydrogen was the vital part of oxidations. The intramolecular combination of oxygen with a compound, therefore, was due to the formation of a hydrate, which was followed by dehydrogenation. These transfers of hydrogen were assisted by the presence of specific enzymes and substances capable of acting as hydrogen acceptors or donors. Accordingly, the oxidation of hydroquinone to quinone, or the dehydrogenation of acetaldehyde to acetic acid may be shown by the following reactions:

or

According to this theory, biochemical oxidations may be considered as transfers of hydrogen from the oxidized substance or from the hydrate, in the presence of some substance which may act as a hydrogen acceptor or donor. Warburg considered oxidation as a surface reaction, in which some element, such as iron, by changes of valence might serve as an activator for oxygen. The compromise theory of Kluyver is essentially a combination of these: the idea of Wieland being retained, and Warburg's required catalyst being added.

There are many substances which may act as hydrogen acceptors or donors: methylene blue is a common example, and in the animal body, glutathione plays an important part in the oxidation-reduction system. Callow and Robinson (13) have reported the presence of glutathione in many species of bacteria, and while their results have been questioned, there exists in bacteria some substance capable of giving a positive test with sodium nitroprusside. This test is specific for the sulphydryl ($-SH$) group, although it is now recognized that several other substances may give a positive test with sodium nitroprusside reagent.

In order to determine whether *Azotobacter* contained any substance capable of acting as a hydrogen acceptor or donor, the material which had been used in the other determinations was tested for glutathione, using the technique of Callow and Robinson (13). Each species gave a positive

reaction. Although this test alone is not sufficient evidence to conclude that glutathione is present in *Azotobacter*, it does not seem unlikely, and probably a part of the cystine which was found in the Van Slyke analysis might result from the oxidation of cysteine, which is a part of the glutathione molecule (a tripeptide of glutaminic acid, glycine and cysteine).

Wieland (72) suggested that in the case of the nitrogen fixing organisms, the reduced hydrogen acceptors did not depend upon oxygen for dehydrogenation, but upon nitrogen, thereby forming ammonia directly. The mechanism of this reaction might be explained by the general equation which Kluyver (41) has applied to respiration and photosynthesis:

(A may be O, S, or nothing)

In the case of nitrogen fixation, the equation would be:

This is essentially the same mechanism which Blom (6) has suggested. He assumes that organic iron compounds are the catalysts:

Loeb (50) has suggested that the ammonia formed by direct fixation was assimilated by the organism and synthesized into protein, with formamide as an intermediate product. Loew and Aso (51) advanced the theory that gaseous nitrogen and water combined to form ammonium nitrite, which, after reduction to ammonia, reacted with the intermediate products of carbohydrate metabolism to give amino acids, which condensed to form proteins.

Recent studies have shown that carbohydrate breakdown, as accomplished by various micro-organisms probably proceeds according to a mechanism common to all. Space will not permit a discussion of this mechanism, but these theories are fully discussed by Harden (32), Kluyver (41,42,43), and Buchanan and Fulmer (9). Stephenson (59) has summarized carbohydrate breakdown into the following steps:

1. The disruption of the hexose molecule into two having the formula $C_3H_6O_3$, which tend to pass by loss of water into $C_3H_4O_2$, probably representing methyl glyoxal or some activated form of it.

2. Oxidation of methyl glyoxal into pyruvic acid.

3. Decarboxylation of pyruvic acid to acetaldehyde and carbon dioxide.

4. Acetaldehyde may be reduced to alcohol, or by a Cannizzaro reaction, one half the molecules are oxidized to acetic acid and half are reduced to alcohol. (See Figure I, F to S).

The last step in the above outline shows the formation of alcohol and acetic acid from glucose (Figure I, P and O). This same scheme may be used to explain the formation of other organic acids. Naturally the end products of carbohydrate decomposition vary with the particular carbohydrate being decomposed, and with the particular organism bringing about the decomposition. In metabolism, human as well as bacterial, there is always a certain amount of synthesis accompanying carbohydrate decomposition, so it is not surprising to find that complex substances may be synthesized from the intermediate products of metabolism. These relationships in human metabolism are discussed and graphically represented by Bodansky (7).

The formation of several organic acids is given by the following equations (41,59):

Acetaldehyde, however could be converted into both acetic acid and alcohol as follows (Figure I, L and M):

The formation of other acids might be similarly explained, using the same mechanism.

Lactic acid (Figure I, I)

BUTYRIC ACID (Fig. I, S)

PROPIONIC ACID (41, 62) (Fig. I, S)

In a similar manner, other organic acids might be formed. In this connection, it is interesting to note that of the 22 known amino acids, 8 are derivatives of propionic acid (alanine, serine, cystine, cysteine, phenyl alanine, tyrosine, histidine and tryptophane); 4 are derived from valeric or isovaleric acid (valine, α -amino valeric acid, isoleucine and arginine); 3 from caproic or isocaproic acid (leucine, caprine and lysine); 2 from 2-pyrrolidine carboxylic acid (proline and hydroxyproline); and one each from acetic (glycine), succinic (aspartic acid) and glutaric acids (glutaminic acid).

Figure I gives a diagrammatic representation of a possible system of metabolism of *Azotobacter*. This chart is based largely upon a somewhat similar one given by Bodansky (7) to show the intermediate carbohydrate metabolism in the human body, as well as from the discussions of respiration and fermentation as given by Kostychev (45), Kluyver (41) and others (9,56,59). In preparing this figure, the author assumes that nitrogen is converted directly into ammonia (Figure I, T), which in turn reacts with the acids produced during carbohydrate breakdown (Figure I, W), and that the active hydrogen produced during the processes is utilized for the fixation of nitrogen (Figure I, X). While this figure may be open to criticism, it is offered as a possible scheme of metabolism. There seems, however, to be sufficient experimental evidence to warrant certain of these assump-

In regard to the formation of hexose-phosphates, this step is recognized in alcoholic fermentation and in human metabolism; *Azotobacter* requires small amounts of phosphorus for growth, and for this reason has been used in a method to determine phosphate deficiencies in soils. The amino acids which were determined show that certainly caproic, valeric and propionic acids were produced (Table II, Figure I, S,T,U). Stoklasa (60) has shown that *Azotobacter* may produce ethyl alcohol, glycocoll, acetic, butyric, lactic and formic acids, as well as carbon dioxide and hydrogen (Figure I, M,L,G,S,I,P,V). These observations have been confirmed by the work of Ranganathan and Norris (57), who found that carbon dioxide, ethyl alcohol, aldehydes, and formic, acetic, lactic and tartaric acids (Figure I, P,M,K,L,I) were produced in the fermentation of dextrose by *Azotobacter*.

Mannite is used extensively in nitrogen fixation studies because it usually gives higher yields of nitrogen fixed. This may be due to the fact that, because of structure, mannitol yields hydrogen more readily than the keto or aldo sugars. Mannitol ($C_6H_{14}O_6$) by loss of two hydrogen atoms would be converted into fructose, which by rearrangement could be converted into glucose (Figure I,A), which would then be decomposed in the manner discussed in preceding paragraphs.

The formation of ring and cyclic compounds (Figure I, R) might be explained to some extent by the studies of Wind-

as and Knoop (74) who have found that methyl imidazol, which is structurally similar to histidine, could be formed by the reaction of glucose and ammonia, presumably taking place through the formation of pyruvic aldehyde and formaldehyde (Figure I,J).

A relationship may exist between gum formation (Figure I,Q) by *Azotobacter* and protein synthesis. Hamilton has shown that the amount of gum produced by *A. chroococcum* was not greatly affected by the various simple carbohydrates, although the more complex carbohydrates (polysaccharides) lead to increased gum production. In peptone solutions, however, no gum is formed. This may be due to the fact that because of composition, or a favorable carbon: nitrogen ratio, the organisms are able to utilize peptone directly. Hamilton (30) has shown that the gum of *A. chroococcum* does not contain nitrogen, so its production may be associated with protein synthesis, probably in the production of active hydrogen for fixation. The decomposition of carbohydrates liberates energy for these organisms. If, however, a small amount of nitrogen is fixed, due to the utilization of nitrogen compounds, small quantities of activated hydrogen are required, which means low respiration, and a small amount of available energy. Gum formation, therefore may be considered as a sort of "luxury synthesis" by *Azotobacter*. This may account for the difference in the hemicellulose content reported in Table III. *A. agilis*

and A. vinelandii fixed larger amounts of nitrogen, hence more energy was available for the synthesis of hemicelluloses. In the case of A. beijerinckii and A. chroococcum, smaller amounts of nitrogen were fixed and consequently less hemicelluloses were formed. Although this, as well as previous investigations (26) have shown that these two latter species are not as vigorous nitrogen fixers as the two former species, yet, in a given incubation period, they will fix approximately three fourths as much nitrogen as A. agilis and A. vinelandii. The total nitrogen content of A. beijerinckii and A. chroococcum is less than that of the two other species, so the former may have a higher growth rate, and therefore have higher energy requirements. The energy in excess of that required for growth is probably used for the synthesis of gums.

Kostychev (45) states: "The following sequence has been established for Azotobacter agilis: the molecular nitrogen is reduced by the bacteria to ammonia which is transferred to amino acids and from these finally into proteins. Under properly selected conditions, ammonia and amino acids can be detected in liquid substrates of Azotobacter. Thus the fixation of nitrogen is a reduction process. The reducing power of Azotobacter is surprising: the conditions of N-fixation are comparable to those in the technical operations of Haber-Bosch. According to my own experiments, Azotobacter also reduces nitrates to NH_3 at an astonishing rate and HNO_2 is formed as an intermediate product." The same author points out that nitrogen fixation is a synthesis of ammonia, and therefore is an exothermic reaction, which has also been pointed out by Burk (11). Accordingly, the process of fixation is more or less independent of the amount of sugar consumed. It is recognized that sugars are util-

ized more efficiently when present in small amounts and that two or three-fold additions of sugar will not lead to doubled or trebled fixation.

The nitrogen fixing organisms have a high carbohydrate requirement, and Waksman (64) has shown graphically, from the results of Krainsky and Bonazzi that there is a close relationship between carbon dioxide produced and nitrogen fixed. Gainey (19) has found a close correlation between nitrogen fixed and the energy of various fatty acids which may be used by *Azotobacter*. The author is inclined to agree with Kostychev that carbon dioxide is the product of ordinary metabolism (carbohydrate breakdown) and the active hydrogen for nitrogen fixation is formed by the processes of fermentation. It seems, however, that it should come largely from fermentation since nitrogen fixation does not depend directly upon the energy produced by respiration.

If the process of nitrogen fixation is primarily a synthesis of ammonia, the depressing effect of combined nitrogen on fixation may be due to the inability of *Azotobacter* to utilize these substances in the synthesis of ammonia. Nitrates, in very small amounts, are readily used, but in larger quantities, they have a depressing effect on the fixation of atmospheric nitrogen. This may be due to a "draining" of active hydrogen for the reduction of the excess nitrates to ammonia and the subsequent transformation into proteins. The fixation of nitrogen may be considered as a

reversible reaction and according to the law of mass action, the accumulation of an excess of ammonia would tend to reverse the process, which would result in a decreased rate of fixation. Also, large amounts of ammonia might be directly toxic to *Azotobacter*. The author has shown elsewhere (28) that at higher pH, phosphorus is not available for *Azotobacter*, and that in an alkaline solution these organisms first reduce the pH to about 7.6 before there is any phosphate utilization.

In general, combined nitrogen depresses the fixation of atmospheric nitrogen by *Azotobacter*, as Burk and Lineweaver (12), Zoond (77) and Fuller and Rettger (18) have demonstrated. Thompson (61) found that, with the exception of *A. vinelandii* which utilized small amounts of various amino acids, several other species of *Azotobacter* were not able to use the same compounds. Fuller and Rettger (18) showed that various organic nitrogenous compounds practically inhibited fixation, but *Azotobacter* was able to utilize peptone, aspartic acid, glycocoll, and small amounts of tryptophane, glutamic acid and tyrosine. It seems probable, however, that the proteins of *Azotobacter* are synthesized principally by the organisms from gaseous nitrogen and carbohydrates, and not by direct assimilation of amino acids as is the case in animal nutrition.

Since arginine and lysine were found to make up a large percentage of the protein fraction of *Azotobacter*,

and since these contain longer carbon chains (lysine having 6 carbon atoms in a straight chain, and arginine having 5 in the chain and 1 in the side group), it might be argued that these are synthesized directly from a hexose sugar and ammonia. This does not seem likely, however, unless the hydrogen necessary for ammonia synthesis is considered as coming from the decomposition of another fraction of the carbohydrate. It seems more logical to believe that these amino acids of longer carbon chains are built up by the condensation of smaller units. Otherwise, it is difficult to explain why, in the case of lysine, amino groups are found on the alpha and epsilon carbon atoms. Furthermore, it is recognized in human metabolism that most oxidations occur on the beta carbon atom.

SUMMARY

Four species of *Azotobacter* were grown on Ashby's mannite agar and the growth was analyzed.

A. vinelandii and *A. agilis* are quite similar in their gross chemical composition; a close similarity also exists between *A. chroococcum* and *A. beijerinckii*. This is especially true of their nitrogen fixing abilities.

A Van Slyke nitrogen distribution did not reveal any consistently wide differences in percentages of compounds present in the four species, although it does indicate a similarity between *A. agilis* and *A. vinelandii*, and *A. beijerinckii* and *A. chroococcum*.

Of the amino acids, arginine and lysine were found in amounts, with smaller amounts of tyrosine, cystine, tryptophane and histidine. Approximately 40% of the total nitrogen is in the form of ether amino acids (glycine, alanin, etc.).

Qualitative tests showed the presence of a substance giving a positive reaction with sodium nitroprusside. This substance may be glutathione.

Semi-quantitative determinations indicated that the protein groups present were chiefly globulins, glutelins and albumins.

Ammonia is probably formed directly from dissolved nitrogen gas and active hydrogen which is produced during the

decomposition of carbohydrates. Organic acids, whose formation from carbohydrates is discussed, react with the ammonia to form amino acids, which are then converted into proteins.

A diagrammatic representation of the metabolism of Azotobacter is given.

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REFERENCES

1. Aso, K., and Yoshida, R. 1928. Application of the serum reaction in the classification of *Azotobacter*. Proc. and Papers of the International Congress of Soil Science. Vol. 3, Comm. 3 & 4, p. 150-151.
2. Association of Official Agricultural Chemists. 1931. Methods of Analysis. Washington, D. C.
3. Beijerinck, M. W. and Van Delden, A. 1902. Ueber die assimilation des freien stickstoffs durch bakterien. Centbl. Bakt. (II) Bd. 9:3-43.
4. Bergey, D. H. 1930. Manual of Determinative Bacteriology. The Williams and Wilkins Co., Baltimore.
5. Berthelot, M. 1892-3. Recherches nouvelles sur les micro-organismes fixateurs de l'azote. Compt. Rend., 115: 569-574; 842-849.
6. Blom, J. 1931. Ein versuch, die chemischen vorgange bei der assimilation des molekularen stickstoffs durch mikroorganismen zu erklaren. Centbl. Bakt. (II) 84: 60-86.
7. Bodansky, M. 1927. Introduction to Physiological Chemistry. John Wiley and Sons, New York.
8. Bortels, H. 1930. Quoted by Waksman from Arch. Mikrob., 1:333-342.
9. Buchanan, R. E. and Fulmer, E. I. 1928, 1930. Physiology and Biochemistry of Bacteria. Vol. I & III. The Wil-

- liams and Wilkens Co., Baltimore.
10. Bureau of Chemistry and Soils. 1931. U.S. Dept. of Agriculture, Report of the Chief.
 11. Burk, D. 1927. The free energy of nitrogen fixation by living forms. *Jour. Gen. Physiol.*, 10:559-573.
 12. Burk, D. and Lineweaver, H. 1930. The influence of fixed nitrogen on *Azotobacter*. *Jour. Bact.*, 19:389-414.
 13. Callow, A.B. and Robinson, M.E. 1925. The nitroprusside reaction of bacteria. *Biochem. J.* 19:19-24.
 14. Cavett, J.W. 1932. A modification of the Van Slyke nitrogen distribution method. *Jour. Biol. Chem.* 95: 335-343.
 15. Folin, O. and Ciocalteu, V. 1927. On tyrosine and tryptophane determinations in proteins. *Jour. Biol. Chem.*, 73:627-650.
 16. Folin, O. and Marenzi, A.D. 1929. An improved colorimetric method for the determination of cystine in proteins. *Jour. Biol. Chem.*, 83:103-108.
 17. Fred, E.B. and Waksman, S.A. 1928. *Laboratory Manual of General Microbiology*. McGraw-Hill Book Co., New York.
 18. Fuller, J.E., and Rettger, L.J. 1931. The influence of combined nitrogen on growth and nitrogen fixation by *Azotobacter*. *Soil Sci.*, 31:219-234.
 19. Gainey, P.L. 1928. Sources of energy for *Azotobacter* with special reference to fatty acids. *Ann. Mo. Bot. Garden*, 15:113-168.

20. Gerlach, M. and Vogel, I. 1903. Weitere versuche mit stickstoffbindenden bakterien. III. Centbl. Bakt. (II), 10:636-643.
21. Gortner, R.A. 1929. Outlines of Biochemistry. John Wiley and Sons, New York.
22. Gortner, R.A. and Blish, M.J. 1915. On the origin of the humin formed by the acid hydrolysis of proteins. Jour. Am. Chem. Soc. 37:1630-1636.
23. Gortner, R.A. and Holm, G.E. 1917. The effect of prolonged acid hydrolysis upon the nitrogen of fibrin with especial reference to the ammonia fraction. Jour. Am. Chem. Soc. 39:2736-2745.
24. Gortner, R.A. and Sandstrom, W.M. 1925. Proline and tryptophane as factors influencing the accuracy of Van Slyke's method for the determination of nitrogen distribution in proteins. Jour. Am. Chem. Soc. 47: 1663-1671.
25. Greaves, J.E. 1918. Azofication. Soil Sci., 6: 163-217.
26. Greene, R.A. 1932. The effect of temperature upon nitrogen fixation by Azotobacter. Soil Sci., 33; 153-161.
27. Greene, R.A. 1932. Composition of the fiber and waste of Agave lechuguilla. Bot. Gaz., 93: 484-491.
28. Greene, R.A. 1933. Some factors limiting the applicability of biological methods for determining the availability of plant food elements in calcareous soils.
Accepted for publication, Soil Sci.

29. Halversen, W.V. 1927. The nitrogen metabolism of nitrogen-fixing bacteria. Iowa State Coll. Jour. of Sci., 1: 395-411.
30. Hamilton, W.B. 1931. Gum production by *Azotobacter chroococcum* of Beijerinck and its composition. Jour. Bact., 22: 249-254.
31. Hanke, M.T. and Koessler, K.K. 1920. Studies on proteinogenic amines. VII. The quantitative colorimetric estimation of histidine in proteins and protein containing materials. Jour. Biol. Chem., 43: 527-542.
32. Harden, A. 1923. Alcoholic Fermentation. 3rd Ed., Longmans, Green & Co., New York.
33. Hass, P. and Hill, T.G. 1923. An Introduction to the Chemistry of Plant Products. Vol. I. Longmans, Green & Co., London.
34. Hauge, S.M. 1931. The Van Slyke method for protein analysis as affected by fats. Jour. Am. Chem. Soc., 53: 3049-3052.
35. Hoffman, C. 1913. Quoted by Waksman from Centbl. Bakt. (II) 36: 474-476.
36. Hoffman, C. and Hammer, B.W. 1910. Some factors concerned in the fixation of nitrogen by *Azotobacter*. Wis. Agri. Exp. Sta. Res. Bul. 12: 155-172.
37. Hunter, O.W. 1923. Protein synthesis by *Azotobacter*. Jour. Agri. Res. 24: 263-274.
38. Israillsky, W.P. and Starygin, L. 1930. Die Dissoziation

bei einigen Bakterienarten. Centbl. Bakt. (II) 81:
1-11.

39. Johnson, T.B. 1926. The chemical study of bacteria. XI.
the development of a systematic analytical method for
the comparative study of bacterial cells. Am. Rev.
of Tub., 14: 164-171.
40. Jones, D.H. 1913. A morphological and cultural study
of some Azotobacter. Centbl. Bakt. (II) 38: 14-25.
41. Kluyver, A.J. 1931. The chemical activities of micro-
organisms. University of London Press, London.
42. Kluyver, A.J. and Donker, H.J.L. 1925. The unity in the
chemistry of the fermentative sugar dissimilative
processes of microbes. Proc. Kon. Akad. V. Wet.
Amsterdam, 28: 297.
43. Kluyver, A.J. and Donker, H.J.L. 1925. The catalytic
transfers of hydrogen as the basis of the chemistry
of dissimilation processes. Proc. Kon. Akad. V. Wet.
Amsterdam, 28: 605.
44. Koessler, K.K. and Hanke, M.T. 1919. Studies on protein-
ogenous amines. II. A microchemical colorimetric meth-
od for estimating imidazole derivatives. Jour. Biol.
Chem., 59: 497-519.
45. Kostychev, S. 1931. Chemical Plant Physiology. P.
Blakiston's Sons Co., Philadelphia.
46. Kostychev, S. and Ryskaltschuk, A. 1925. Les produits de
la fixation de l'azote atmospherique par l'*Azotobacter*

- agile. Compt. Rend. 180: 2070-2072.
47. Kostychev, S., Ryskaltshuk, A. and Schwezowa, O. 1926.
Biochemische untersuchung uber "Azotobacter agile".
Z. Physiol. Chem. 154: 1-17.
48. Larmour, R.K. 1928. Correlations between the total nitrogen of the bases and arginine nitrogen and between the total nitrogen of the bases and lysine nitrogen, of various proteins. Trans. Royal Soc. Canada. 3d Series, 22: 349-363.
49. Lipman, J.G. 1904. Soil bacteriological studies. N.J. Agri. Exp. Sta. 25th Ann. Rpt. : 237-289.
50. Loeb, W. 1913. Uber das verhalten des formamid unter den wirkung der stillen entladung. Ber. Deut. Chem. Gesell., 46: 684-697.
51. Loew, O. and Aso, K. 1906. On the change of the availability of nitrogen in soils. II. Bul. Col. Agri., Tokio Imp. Univ., 7: 567-574.
52. Lohnis, F. and Smith, N.R. 1923. Studies upon the life cycles of the bacteria. Jour. Agri. Res., 23: 401-432.
53. Omeliansky, W.L. and Sieber, N.O. 1913. Zur frage nach der chemischen zusammensetzung der bakterienkorper des Azotobacter chroococcum. Z. Physiol. Chem., 88: 445-459.
54. Parisi, E. and Masetti-Zannini, C. 1926. Le sostanze proteiche dei tubercoli delle leguminose. Staz. Sper.

- Agri. Ital., 59: 207-228.
55. Porges, N. 1932. Chemical composition of *Aspergillus niger* as modified by zinc sulphate. Bot. Gaz., 94: 197-205.
56. Rahn, O. 1932. Physiology of Bacteria. P. Blakiston's Sons Co., Philadelphia.
57. Ranganathan, S. and Norris, R.V. 1927. Nitrogen fixation by *Azotobacter chroococcum*. Jour. Ind. Inst. Sci., 10A., part 8: 79-96.
58. Rippel, A. and Ludwig, O. 1925. The darkening of *Azotobacter chroococcum* Beij. by melanin formation. Centbl. Bakt. (II), 84: 161-166 (Abstd. in Chem. Absts. 19: 2684).
59. Stephenson, M. 1930. Bacterial Metabolism. Longmans, Green & Co., London.
60. Stoklasa, J. 1908. Beitrag zur kenntness der chemischen vorgange bei der assimilation des elementaren stickstoffs durch *Azotobacter* und *radiobacter*. Centbl. Bakt. (II), 21: 620-632.
61. Thompson, L.G. 1932. Nitrogen changes produced in certain nitrogeneous compounds by *Azotobacter* and the nitrogen fixed in the presence of these compounds. Jour. Agri. Res., 45: 149-161.
62. Van Niel, C.B. 1928. The propionic acid bacteria. N.V. Vitgeverszaak J.W. Boissevain & Co., Haarlem, Holland.
63. Van Slyke, D.D. 1911. The analysis of proteins by deter-

- mination of the chemical groups characteristic of the different amino acids. Jour. Biol. Chem. 10: 15-55.
64. Waksman, S.A. 1932. Principles of Soil Microbiology. Williams and Wilkins Co., Baltimore.
65. Waksman, S.A. and Stevens, K.R. 1928. Contribution to the chemical composition of Peat: I Chemical nature of organic complexes in peat and methods of analysis. Soil Sci. 26: 113-137.
66. Waksman, S.A. and Stevens, K.R. 1930. A system of proximate chemical analysis of plant materials. Ind. and Eng. Chem., Analyt. Ed., 2: 167.
67. Warburg, O. 1924. Über eisen, sauerstoffübertragenden bestandteil des atnungsferments. Biochem. Z., 152: 479.
68. Wieland, H. 1912. Über hydrierung und dehydrierung. Ber. Deut. Chem. Gesell., 45: 494-493.
69. Wieland, H. 1912. Zur verbrennung des kohlenoxyds. Ibid: 679-685.
70. Wieland, H. 1912. Studien über den mechanismus der oxydationsvorgänge. Ibid: 2606-2615.
71. Wieland, H. 1913. Über den mechanismus der oxydationsvorgänge. Ber. Deut. Chem. Gesell., 46: 3327-3342.
72. Wieland, H. 1922. Über den verlauf der oxydationsvorgänge. Ber. deut. Chem. Gesell., 55: 3639-3643.
73. Wilkerson, V.A. and Cortner, R.A. 1932. The chemistry of embryonic growth. III. A biochemical study of the em-

- bryonic growth of the pig with special reference to the nitrogenous compounds. *Am. Jour. Physiol.*, 102: 153-166.
74. Windas, A. and Knoop, F. 1905. Ueber führung von Trauhenzucker in methylimedazol. *Ber. deut. Chem. Gesell.* 38: 1166-1170.
75. Winogradsky, S. 1893. Sur l'assimilation del'azote gazeux de l'atmosphere par les microbes. *Compt. Rend.*, 116: 353.
76. Winogradsky, S. 1930. Sur la synthese de l'ammoniaque par les Azotobacters du sol. *Compt. Rend.*, 190: 661-664.
77. Zoond, A. 1926. The relation of combined nitrogen to physiological activity of Azotobacter. *British. Jour. Exp. Biol.*, 4: 105-113.

physiological growth of the pig with special reference to
the nitrogenous compounds. Am. Jour. Physiol., 1931;
123-125.

76. Kinsch, A. and Knapp, V. 1930. Ueber Fehrbildung von Trau-
bennachre in nachfolgender. Ber. deut. Chem. Gesell.
113: 1130-1131.

77. Kinsch, A. 1932. Zur Kenntnis der Ammoniak-
bildung bei der Nitrifizierung. Comp. Rend.,
193: 123.

78. Kinsch, A. 1933. Zur Kenntnis der Ammoniak-
bildung bei der Nitrifizierung. Comp. Rend., 193;
123.

79. Kinsch, A. 1934. The relation of combined nitrogen to
physiological activity of Anabaena. Jour.
Exp. Biol., 4: 123-125.

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