

# CERTAIN PHASES OF NITROGENOUS METABOLISM IN BACTERIAL CULTURES<sup>1</sup>

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The classification of bacteria has been studied from different viewpoints since the researches of Pasteur. With the exception of the three great groups of bacteria, the form of the cell is not a very satisfactory means of differentiation. There is left, then, one method of study that is applicable and that method is the study of the metabolism of the bacteria. The determination of the ability of the bacteria to ferment various carbohydrates has been studied extensively. The study of the nitrogenous metabolism has, however, been confined for the greater part to the identification of such compounds as indol or the determination of the ability of the bacteria to grow in certain nitrogenous media. The paucity of such data is the result of the complication of the subject with an unusual number of factors and, furthermore, of the fact that precise chemical methods have been wanting to give quantitative results.

## LITERATURE

Hirschler (1886) was probably the first to observe that the presence of carbohydrate inhibited the production of such nitrogenous decomposition products as indol, phenol and the cresols. Smith (1897) and Peckham (1897) independently came to the conclusion that the production of acid in the medium containing carbohydrate inhibited the formation of indol. This early work

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was confirmed later by Glenn (1911) who found that the addition to the medium of more than 0.5 per cent lactic acid inhibited the production of indol. Fischer (1915) concluded, as a result of colorimetric determinations of indol, that neither hydrogen ion content nor total acidity affected its production and that the reason for its absence in sugar media appeared to be the inactivation of the proteolytic enzymes by glucose. Logie (1919-1920) tested for indol colorimetrically with Ehrlich's reagent and found that it decreased in the presence of sugar and increased in its absence. His conclusion that the presence of sugar increased the utilization of indol appears well founded.

Gelatin liquefaction has also been used as an index of the type of nitrogenous metabolism. Glenn (1911) concluded from a series of experiments that the failure of bacteria to liquefy gelatin was due to the acidity developed in media containing carbohydrates. Kendall and Walker (1915) found that a bacteria-free filtrate of a plain broth culture of *P. vulgaris* would liquefy gelatin in the presence of glucose as readily as in its absence. They concluded that the proteolytic enzyme was not produced in cultures in glucose media and that the glucose was fermented first, thus "sparing" the protein of the medium.

The effect of various concentrations of a carbohydrate on the amount and rate of formation of metabolic products has been demonstrated by several workers in this field. Clark and Lubs (1915) showed that an increase in the concentration of glucose, to the limit of the experiment, which was 8 per cent, increased the total amount of gas produced. Bronfenbrenner and Schlesinger (1918) concluded from the results of an extensive series of experiments that the many discrepancies in the results of fermentation studies, which are so apparent in the literature of this subject, have been due largely to the fact that the media have not usually been compared with sufficient attention to the relative proportions of peptone and buffer. At the present time, Kendall and his co-workers and other investigators claim that bacteria prefer carbohydrates and utilize these substances more readily than nitrogenous materials, thus "sparing" the protein of the medium. Kendall and his co-workers have shown that in the

presence of carbohydrate the rate of production of ammonia is usually decreased although the reverse is the case in a few instances.

It is evident from the results quoted above that the presence of carbohydrate in the medium affects the type of the nitrogenous metabolism of the bacteria, so that certain products may not be produced or, if produced, the rate of production may be materially changed. Some investigators have interpreted these changes in the nitrogenous metabolism as indicative of the degree of proteolysis. Kendall and Walker (1915) interpreted ammonia production as an index of proteolysis and concluded that the decreased production of ammonia is proof of the "protein-sparing" action of carbohydrates. Berman and Rettger (1918) used the biuret test of Vernon (1903-1904) as an indicator of proteolysis.

Since amino acids are results of protein hydrolysis, I have assumed that the production of amino nitrogen should give a better index than either ammonia production or the biuret reaction—in other words, that in an actively growing bacterial culture the amino acids are formed as a result of proteolysis. However, I realized that the amount of amino nitrogen of a medium is the difference between the amount produced and the amount utilized by the bacteria and the index is arbitrary to that extent. A similar criticism may be made concerning ammonia production and the biuret reaction as an index of proteolysis. However, the amino nitrogen has the advantage that its production is the result of the direct action of the proteolytic enzymes upon the protein of the medium.

At this point it may be profitable to consider briefly the previous work on amino acid production by bacteria. Before the publication of improved methods, Taylor (1902) made determinations of the amino acids by the laborious method of chemical isolation. He reported that *Bact. coli* did not produce amino acids from casein and concluded that the organism was able to split the protein to albumoses only. With *P. vulgaris*, however, he demonstrated the formation of lysin and histidin. Rosenthal and Patai (1914) increased the virulence of streptococci, staphylococci and *Bact. coli* by means of animal passage, and found that

the more virulent strains gave higher amino nitrogen values when judged by the formol titration method of Sørensen. After the completion of the experimental part of my study Kendall and his co-workers (1922) reported that the amino nitrogen in cultures varied with the organism and also with the presence or absence of sugar. In many tests the amino nitrogen showed a decrease in plain media and an increase in glucose media; although other tests with the same organism showed the reverse. Sears (1916) found that certain bacteria, with the exception of a few strongly proteolytic organisms such as *Ps. pyocyanea* and *B. subtilis*, gave fluctuating values of amino acids from which he concluded that these substances were continuously formed and broken down. Jones (1916) working with *P. vulgaris* in gelatin cultures confirmed the observation of earlier investigators that the gelatin is not liquefied in media containing sugar and came to the conclusion that the proteolytic enzyme was not produced in media containing a fermentable carbohydrate. Waksman (1917) working with *Aspergillus niger* and later (1920) with the Actinomycetes showed that these organisms did not produce as much amino acid nitrogen in glucose media as in plain media. With *Bact. coli* the amino nitrogen was slightly lower in glucose broth than in plain broth. There was an increase in the amino nitrogen in plain broth with *Bact. coli* of 5 mgm. per 100 cc. of the medium. With *Aspergillus niger* there was a decrease in the total amino nitrogen. Robinson and Tartar (1917) working with *B. subtilis*, *B. mycoides* and *Proteus vulgaris* found that the mono-amino and di-amino acids were decreased in blood, egg and peptone media. Wolf and Harris (1916-1917) showed that the total amino acid nitrogen content was decreased by *C. sporogenes* in a medium made by the tryptic digestion of casein. This was confirmed later by Harris (1919-1920) who showed also that the presence of a fermentable carbohydrate increased the total amino nitrogen. Wolf (1918-1919a) obtained the same results with *P. vulgaris*, that is, the presence of glucose increased the amount of amino nitrogen. Wolf and Harris (1918-1919) working with *C. histolyticus* and Wolf working with *Vibrio septique* (1918-1919b) and *C. sporogenes* (1918-1919c) showed that these organisms

increased the total amount of amino nitrogen in cultures. Foster (1921) reported that a decrease in amino nitrogen was associated with an increase in ammonia in cultures of *Streptococcus hemolyticus*. Raistrick and Clark (1921), working with a synthetic medium of tryptophane and tyrosin as the only nitrogen sources, found that the amino nitrogen decreased both in the presence and absence of glycerol. They found that ammonia was not produced in the medium containing glycerol. Their conclusion was that glycerol did not have a "protein-sparing" action.

It is clear from the above brief review of the literature that the studies of the production of amino nitrogen in bacterial cultures have not been very extensive. Furthermore, the results of the various investigators differ very greatly and in many cases the extent of the change in the total amount of the amino nitrogen is surprisingly insignificant.

#### EXPERIMENTAL

In view of the influence of the medium and other variable factors upon the nitrogenous metabolism of bacteria, the prime importance of having a standard medium, simple in composition and readily reproduced, is at once self evident. The first phase of my work, therefore, consisted in obtaining a suitable medium having the necessary properties and in which the bacteria would grow. After a number of attempts I selected the following: Two per cent Difco peptone made up with mono-potassium and di-sodium phosphates, so that the final hydrogen ion concentration was approximately pH 7.5 and the total phosphorus equivalent to  $M/20$ . Glucose, 1 per cent, was added whenever carbohydrate was desired. This was the medium used throughout my work and its method of preparation will be given later in detail. While it was realized that peptone media are not as simple as desirable it was not possible to obtain growth of *C. botulinum* in synthetic media with the amino acids which were available.

The bacteria which were studied were limited to *Bacterium coli*, *Pseudomonas pyocyanea*, *Bacillus subtilis*, *Clostridium botulinum* (type A and type B) and *Clostridium sporogenes*. *Bact.*

*coli* was selected because it represents a good type which exhibits active fermentative powers. *Ps. pyocyanea* was taken as a type which exhibits weak fermentative power and produces large quantities of ammonia. *B. subtilis*, *C. botulinum* and *C. sporogenes* were selected from the family Bacillaceae which actively decomposes protein. In this group *B. subtilis* was taken as the representative of the aerobes and *C. botulinum* and *C. sporogenes* as representatives of the anaerobes. *C. sporogenes* was selected to compare with the toxin producing organism, *C. botulinum*.

#### METHODS

##### *Amino nitrogen determination*

The nitrogenous metabolism of the bacteria was followed in this study by means of the amino nitrogen and the ammonia nitrogen. At the same time determinations of the glucose content, the hydrogen ion concentration, the amount of phosphorus and the number of bacteria were made.

There were two published methods for the determination of amino acids at the time this study was commenced. The formol titration method of Sørensen (1907-1908) was tried out but was found to be unsatisfactory on account of the color of the culture medium and the presence of phosphates and carbon dioxide. The medium which I used was buffered with phosphates to approximately  $M/20$  in terms of phosphorus. Van Slyke's method (1911) using the micro apparatus (1913-1914) was then tried. Control analyses could not be obtained on a sterile 1 per cent peptone solution when this method was used. That the failure to obtain consistent control analyses was not due to the apparatus or reagents was shown by the fact that consistent blanks were obtained by myself and were checked by another worker in the laboratory. The variation in per cent in one particular series of tests was 18.4. Other series gave similar results and occasional tests showed errors even higher than the one indicated.

Dr. Folin very kindly permitted me to use his newly devised method for the determination of amino nitrogen a little over a

year before its publication (1922) and gave me some of the reagent necessary for the determinations. In view of the fact that I have used Folin's method for the determination of amino nitrogen throughout my work a brief discussion of the special applications of the method in bacterial culture media seems pertinent.

In the beginning a grave source of error was detected in the lot of glycocoll which was sold as chemically pure. It was found that the glycocoll solution contained more ammonia nitrogen than amino nitrogen. The water used was ammonia-free and was not the source of the error. It is very important, therefore, to test all lots of glycocoll for ammonia before use as a standard. Dr. Folin very kindly gave me some pure glycocoll with which all standards were prepared that are reported in this paper.

Some time later I made up the reagent of the Folin method for amino nitrogen and experienced difficulty because the standards showed a precipitate after the addition of the thiosulfate and acetic acid solutions. It was thought that there might be some undesirable products left after purification of the reagent and, in order to check this, some of the reagent which Dr. Folin had prepared was obtained from him to compare with the reagent which I had prepared.

It was found with the reagent which I had prepared that there was a certain amount of precipitate formation depending upon the amount of the acetic acid and thiosulfate solutions added. In view of the possibility that this precipitate formation may occur with other workers, it is suggested that the reagent be tested in the nephelometer against a dark field before determinations of amino nitrogen are carried out. In the results which follow, all tests were carried out with 2 cc. of the solution of the amino nitrogen reagent and 2 cc. of the thiosulfate solution. Under these conditions the amount of light which came through the nephelometer was barely perceptible when compared to the other side which had been darkened by holding the hand over the plunger.

*Folin method for determination of amino nitrogen.* The first problem with this method of the determination of amino nitrogen was to study its constancy with peptone solutions. In order to

do this a flask of 1 per cent Difco peptone was made and sterilized. Samples were removed from day to day and a determination of the amino nitrogen was made. The largest percentage error was 3.5.

Later a 2 per cent Difco peptone solution was used and the results are shown in table 1.

Taking the lowest reading as the correct one, the largest percentage error is 3.0. As shown in table 1, the fact that the results of four out of the five samples were the same suggests that the low determination was faulty. From these findings it is concluded that the method gives consistent results in determining amino nitrogen in peptone solutions.

TABLE 1

*Amino nitrogen determinations of a 2 per cent peptone solution. Determinations made on different days*

DAY	AMOUNT OF PEPTONE SOLUTION	READING IN MM. STANDARD AT 20 MM.	AMOUNT IN MGM. OF $\text{NH}_2\text{N}$
	cc.		
1	0.5	19.0	0.157
1	0.5	19.7	0.152
1	0.5	19.0	0.157
1	0.5	19.0	0.157
2	0.5	19.0	0.157

The next problem was to find whether the addition of a known amount of amino acid nitrogen as the standard glycocoll could be detected. In order to ascertain this a sample of 2 per cent Difco peptone was used as follows: 1 cc. of a 2 to 4 dilution of the peptone gave 0.102 mgm.  $\text{NH}_2\text{-N}$ . 1 cc. of a 2 to 4 dilution of the peptone plus 0.05 mgm. of amino nitrogen gave 0.150 mgm.  $\text{NH}_2\text{-N}$ . The difference is well within experimental error and shows that the true reading may be obtained after an addition of a standard glycocoll solution. This is illustrative of several similar tests. The same test was applied to culture media after the growth of bacteria and it was found that the presence of metabolic products of the bacteria did not interfere with the test. From these results I concluded that the Folin method for the determination of amino nitrogen was applicable in peptone solutions.



*Removal of ammonia.* The reagent used in the Folin method for the determination of amino nitrogen reacts with ammonia which, if present, must be removed. It was suggested that the simplest and easiest method for the removal of the ammonia would be with permutit (Folin and Bell, 1917). It was found however, that the permutit removed variable quantities of amino nitrogen and for that reason was inapplicable in this work. The removal of ammonia by the aeration method of Folin (1902-1903) was tested and found to be accurate in the presence of peptone under the conditions of the experiments. Consistent determinations of the amino nitrogen were obtained by using 50 cc. in aeration cylinders when compared with 1 cc. in test tubes. It was further determined that the addition of sodium carbonate and potassium oxalate to the peptone solution in the aeration cylinders did not affect the amino nitrogen.

*Ammonia determination.* The ammonia content of all solutions was determined by the aeration process of Folin (1902-1903). Tests were carried out to find the speed of the air current and the length of time necessary for complete removal. Much difficulty was experienced with foaming. It was found after trying kerosene and other substances that secondary octyl alcohol (E. K. Co.) was best for this purpose. Tests were made and it was shown that this alcohol did not affect either the readings of the amino nitrogen or the ammonia titration. The procedure finally adopted was the addition of 0.8 to 1 cc. of the alcohol and aeration for two and one half hours.

*Phosphorus determination.* The buffer content of the medium was judged solely by its phosphorus content. The total phosphorus was determined by titration with uranium acetate.

*Hydrogen ion concentration.* The determination of the hydrogen ion concentration was made by means of the apparatus described by Bovie (1915). In all solutions the reading was not made until the shifting of the needle had stopped. The only change in the apparatus as originally described was that a galvanometer was used instead of the electrometer.

*Glucose determination.* The amount of glucose in the medium was determined by the titration method of Folin and Peck (1919),

except when the total amount of sugar was 250 mgm. per 100 cc. or lower, in which case the colorimetric method of Folin and Wu (1920) was used.

*Number of bacteria.* The aerobic bacteria were plated in plain agar and the counts were made after an incubation period of forty-eight hours at 37°C. An estimate of the approximate number of the anaerobic bacteria was obtained by the method of Breed (1911).

#### *General procedure*

The strains of bacteria used were obtained from the sources indicated below and were purified from time to time throughout the experiment. In the case of the aerobes the bacteria were purified by plating on plain agar. The anaerobic bacteria were purified by dilution in glucose agar shake cultures. The cultures were diluted so that only one colony grew in the tube, this procedure being repeated several times with each culture. Tests were made with *C. botulinum* for toxin production. The cultures were obtained from the following sources:

*C. botulinum* (type A) was isolated by myself from the olives causing the food poisoning outbreak in New York City in 1920.

*C. botulinum* (type B) was obtained from Mr. P. F. Orr.

*C. sporogenes* as obtained from Miss Ruth B. Edmondson of the Bureau of Chemistry, U. S. Department of Agriculture, who had received it from Dr. Savage of England.

*Bact. coli*, *B. subtilis* and *Ps. pyocyanea* were obtained from Dr. Ernst of the Department of Bacteriology, Harvard Medical School.

The medium used was a 2 per cent solution of Difco peptone. The method for making the medium was the same in all cases and was as follows: Thirty-two grams of the peptone were dissolved in about 900 cc. of cold distilled water, 16 cc. of M/2 monopotassium phosphate and 144 cc. of M/2 di-sodium phosphate were added and the whole diluted to 1500 cc. The medium was then placed in a flask and heated for fifteen minutes at 15 pounds pressure. The solution was filtered, the loss due to evaporation and absorption by the filter paper was made up and

then it was divided into two lots of 750 cc. each. One lot was diluted with 50 cc. of distilled water and placed in a large bottle holding approximately 900 cc. The second lot was placed in a bottle without further dilution. The bottles were plugged with cotton and a square of paper was tied over the cotton and neck of the bottle. The bottles were then sterilized at 15 pounds pressure for thirty minutes. Eight grams of glucose were dissolved in distilled water, diluted to 50 cc. and sterilized at 15 pounds pressure for fifteen minutes.

The anaerobic bacteria were grown in a partial vacuum obtained by a water pump. Each time the bottles were opened for the purpose of obtaining samples, the vacuum was reestablished and the bottles sealed. Samples were removed on the first, second, fourth and tenth days of growth. The anaerobic bacteria were tested for contamination each time by removing about 1 cc. and plating aerobically. If aerobic growth were shown the bottle was discarded.

On account of the many chemical and bacteriological manipulations which had to be crowded into a short time, it became necessary to systematize the work and the general plan of procedure was as follows: Fifty cubic centimeters of the culture were measured with a graduated pipette and placed in a cylinder for the ammonia aeration. Three grams of potassium oxalate and two grams of sodium carbonate were added to drive the ammonia over. From 0.8 to 1 cc. of the secondary alcohol was added to prevent foaming. The ammonia was blown over by a fairly strong air current into  $N/10$  HCl, using 0.5 cc. of methyl red as the indicator. The aeration was continued for two and one half hours. During the time the culture was being aerated, the hydrogen ion concentration and glucose content were determined. The phosphorus content was titrated if the determination were a control determination, made that is, just after inoculation. It was not titrated in the subsequent determinations. If growth had taken place the aerobic cultures were plated on plain agar; the anaerobic cultures were counted by the Breed technique. After the ammonia aeration the acids were titrated with  $N/10$  NaOH and the calculation of the ammonia nitrogen content was

made in milligrams per 100 cc. of the culture. The culture which was left in the aeration cylinder was diluted to 100 cc. in the cylinder and dilutions made from that for the amino nitrogen content. Two separate dilutions were made from which 1 cc. was placed in a test tube graduated to 25 cc. The dilutions were made in dry test tubes and all measuring of the dilution water and the culture was done with a standardized Ostwald pipette. The remainder of the procedure for the detremination of the amino nitrogen was the same as described by Folin (1922). The following day the colors were read against a standard. The two dilutions should not vary more than 2 per cent from the mean. If care is taken in preparing the dilutions the variations will not be more than 1 per cent. The readings as given later are the mean of the two dilutions.

#### METABOLISM OF BACTERIA

##### *Bacterium coli*

The results of the analyses of the medium during the growth of this bacterium are recorded in table 2. The experiment is divided into two series which were done at different times and are tabulated separately as series 1 and series 2. The results are shown graphically in graphs 1 to 3 inclusive.

The amino nitrogen curves obtained from the medium containing glucose show higher values than from the medium without glucose. The effect of the presence of fermentable carbohydrate is apparent at once in the large increase of the amino nitrogen and the rapid change of the hydrogen ion concentration. The amino nitrogen curves of the carbohydrate free medium show fluctuating values with a final reading slightly higher than the control.

The curves of the ammonia nitrogen do not show the rapid increases that the amino nitrogen curves show. They do indicate some quantitative differences between the carbohydrate and the non-carbohydrate media, the latter being slightly higher. In general *Bact. coli* shows a strong fermentative power but does not produce large quantities of ammonia. These results agree with those of Sears (1916) and Kendall and Bly (1922) but do not

TABLE 2  
*Bacterium coli*

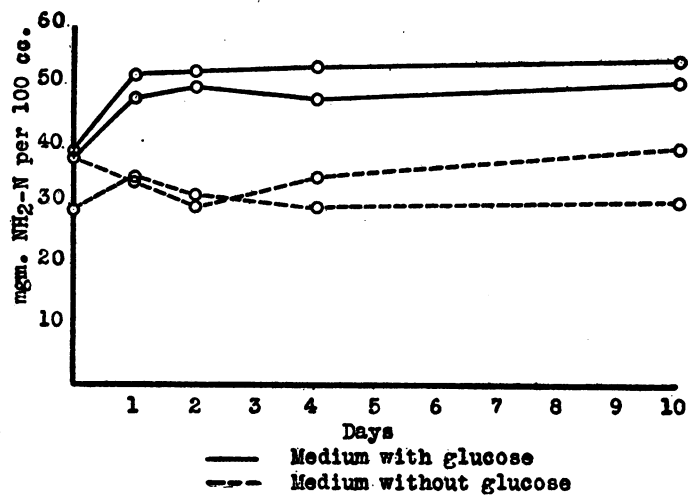
	DAY OF GROWTH											
	0		1		2		4		10			
	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B		
Series 1*												
Ammonia nitrogen†.....	1.0	1.0	3.3	4.4	2.8	4.1	5.8	8.3	3.2	11.3		
Amino nitrogen†.....	38.0	29.2	48.4	35.1	50.0	32.1	48.4	30.0	51.7	31.3		
Glucose†.....	1041.0	0	378.0	0	348.0	0	342.0	0	140.0	0		
pH.....	7.7	7.7	6.4	7.4	5.6	7.4	5.3	7.6	4.7	7.7		
Organisms per cubic centimeter.....			360,000,000	176,000,000		112,000,000	690,000	160,000,000	100,000	40,000,000		
Series 2†												
Ammonia nitrogen†.....	1.2	1.3	3.0	5.5	5.9	8.7	3.5	8.1	3.4	11.9		
Amino nitrogen†.....	39.7	38.7	52.1	34.7	52.8	30.0	53.4	35.0	54.8	40.9		
Glucose†.....	1000.0	0	357.0	0	280.0	0	280.0	0	280.0	0		
pH.....	7.5	7.7	5.1	7.2	5.1	7.3	5.2	7.4	5.1	7.7		
Organisms per cubic centimeter.....			1,090,000,000	320,000,000	30,000,000	320,000,000	4	320,000,000	0	146,000,000		

\* The media in this series contained 162 mgm. P per 100 cc.

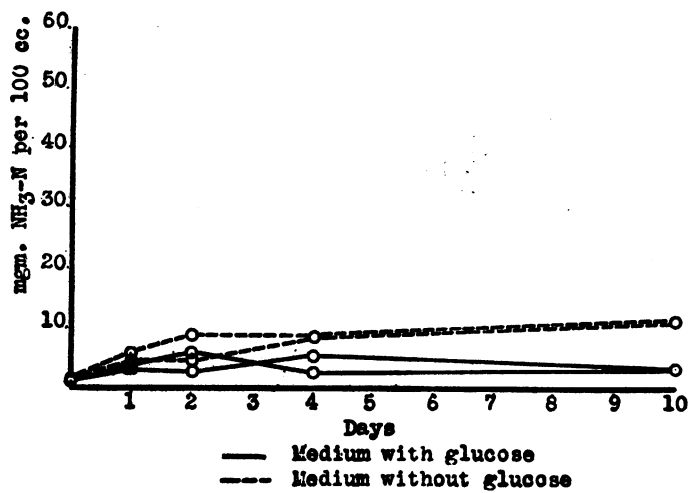
† Milligrams per 100 cc. of the medium.

‡ The media in this series contained 139 mgm. P per 100 cc. (Bottle A) This column indicates the medium with glucose. (Bottle B) This column indicates the medium without glucose.

Graph No. I  
Bact. coli  
Amino nitrogen

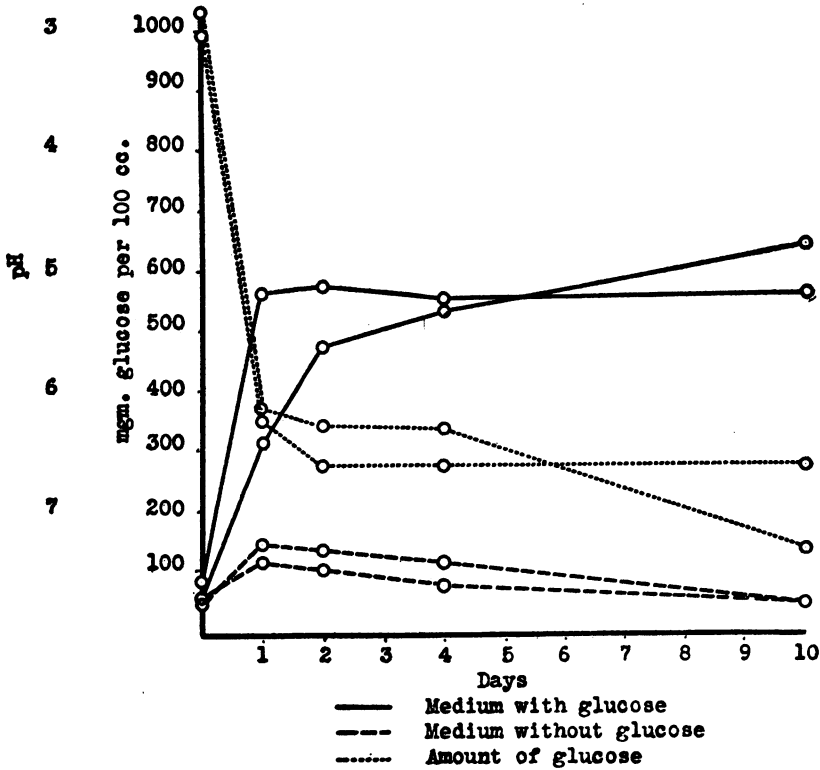


Graph No. II  
Bact. coli  
Ammonia nitrogen



agree with those of Waksman (1920) who found very little change, or those of Berman and Rettger (1918) who found no change in the amino nitrogen in media with and without glucose.

Graph No. III  
 Bact. coli  
 Hydrogen ion concentration  
 and amount of glucose



*Pseudomonas pyocyanea*

The results of the analyses with *Ps. pyocyanea* are shown in table 3. The experiment is divided into two series which were done at different times and are tabulated as series 1 and 2. The graphs of the analyses are shown in graphs 4 to 6 inclusive.

TABLE 3  
*Ps. pyocyanea*

	DAY OF GROWTH									
	0		1		2		4		10	
	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B
Series 1*										
Ammonia nitrogen†..	11.5	1.2	8.4	13.8	14.2	16.2	16.4	24.2	19.4	40.0
Amino nitrogen†..	38.8	36.7	46.0	46.0	45.0	45.0	59.4	47.4	64.2	44.7
Glucose†....	1059.0	0	1041.0	0	1000.0	0	581.0	0	51.0	0
pH.....	7.50	7.65	7.85	8.0	8.0	8.0	7.30	8.2	7.40	8.40
Organisms per cubic centimeter			530,000,000	138,000,000	670,000,000	270,000,000	780,000,000	210,000,000	260,000,000	150,000,000
Series 2†										
Ammonia nitrogen†..	1.1	1.1	12.4	13.6	13.6	Lost	14.1	28.8	18.6	59.8
Amino nitrogen†..	31.8	31.9	47.9	40.4	47.9	40.0	55.1	48.2	64.6	51.3
Glucose†....	1020.0	0	1008.0	0	984.0	0	827.0	0	393.0	0
pH.....	7.65	7.70	7.75	7.75	8.00	8.15	7.70	7.95	7.65	8.20
Organisms per cubic centimeter			420,000,000	430,000,000	160,000,000	300,000,000	160,000,000	200,000,000	232,000,000	59,000,000

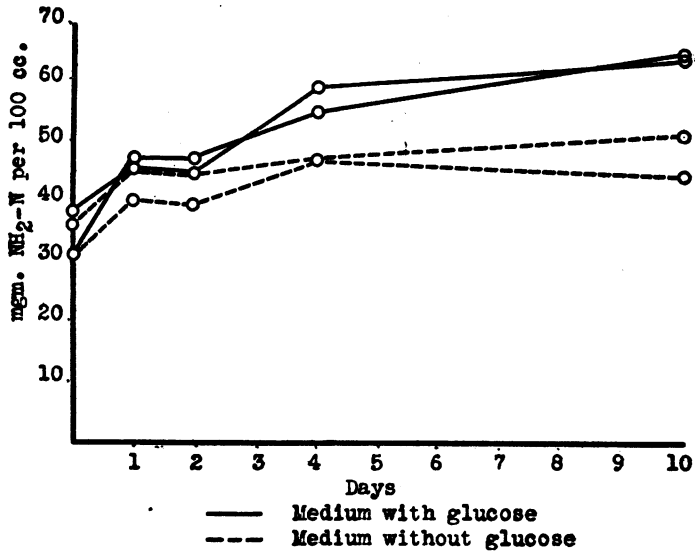
\* The media in this series contained 162 mgm. P per 100 cc.

† Milligrams per 100 cc. of the medium.

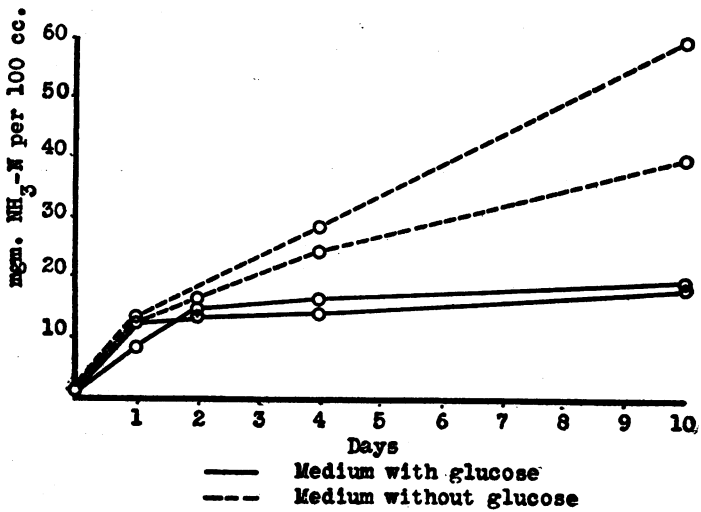
‡ The media in this series contained 162 mgm. P per 100 cc. (Bottle A) This column indicates the medium with glucose. (Bottle B) This column indicates the medium without glucose.



Graph No. IV  
*Ps. pyocyanea*  
 Amino nitrogen

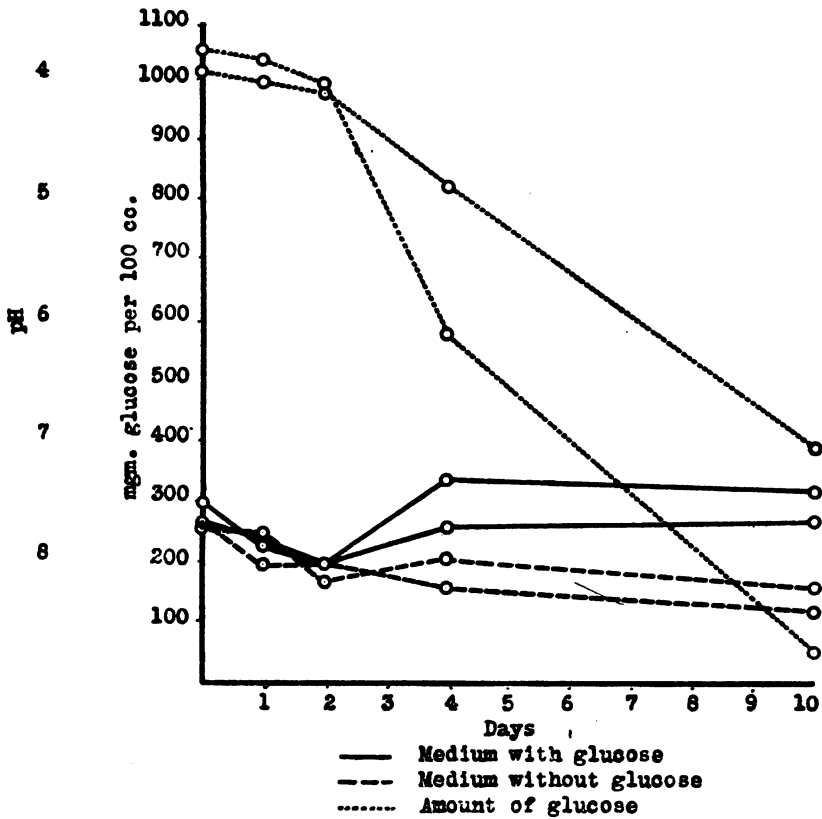


Graph No. V  
*Ps. pyocyanea*  
 Ammonia nitrogen



*Pseudomonas pyocyanea* does not give rapid increases in the amino nitrogen in the first twenty-four hours of growth. There is a slight decrease in the amino nitrogen on the second day. At the present time there are no data available to explain this

Graph No. VI  
*Ps. pyocyanea*  
 Hydrogen ion concentration  
 and amount of glucose



drop. With *Ps. pyocyanea* as with *Bact. coli* the medium containing the glucose shows a more rapid increase and a higher level in the amino nitrogen. The ammonia nitrogen shows a greater difference between the carbohydrate and the non-carbohydrate media than do the ammonia curves of *Bact. coli*.

Attention is especially drawn to the fact that the hydrogen ion concentration curves in the presence of glucose do not show a greater acidity than pH 7.0, despite the fact that the amount of glucose shows a rapid diminution. It is important to consider this fact in testing the ability of bacteria to ferment carbohydrates when acid production is used as the criterion. For example, an indicator in a growing culture of *Ps. pyocyanea* in the presence of glucose would not show acid production, and the inference might therefore be drawn that the glucose had not been destroyed, whereas, I have shown the contrary to be the case. When the curves of the hydrogen ion concentration and glucose content of *Ps. pyocyanea* and *Bact. coli* are compared the difference in the metabolism of the two bacteria is apparent at once.

*Bacillus subtilis*

The results of the analyses of the media with *B. subtilis* are shown in table 4. The experiment was divided into two series which were done at different times and are tabulated as series 1 and 2. The graphs of the analyses are shown in graphs 7 to 9 inclusive.

The variation in the two series in the amino nitrogen is somewhat greater than in the case of some of the other bacteria which were studied. This may be due to differences in the inoculation of the media, to the rate of growth or other factors. The presence of glucose does not appear to increase the amino nitrogen as markedly as with other bacteria tested. It is to be noted that this organism does not produce a great change in the hydrogen ion concentration in the presence of glucose. This was also noted in the case of *Ps. pyocyanea*. These two bacteria are able to destroy large amounts of glucose without producing an acid reaction. The practical importance of this observation has already been noted.

*Clostridium botulinum (type A)*

The results of the studies of this bacterium are shown in table 5 and in graphs 10 to 12 inclusive.

TABLE 4  
*Bacillus subtilis*

	DAY OF GROWTH											
	0		1		2		4		10			
	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B		
	Series 1*											
Ammonia nitrogen†.....	1.5	1.5	3.5	2.3	9.6	2.8	40.4	8.6	37.7	86.5		
Amino nitrogen†.....	34.7	31.6	37.2	37.0	42.8	41.1	52.0	48.4	68.5	57.5		
Glucose†.....	1020	0	986	0	833	0	753	0	367	0		
pH.....	7.55	7.55	7.45	7.60	7.25	7.60	7.05	7.80	7.50	8.75		
Organisms per cubic centimeter.....			5,000,000	2,000,000	4,000,000	700,000	27,000,000	750,000,000	Lost	Lost		
	Series 2†											
Ammonia nitrogen†.....	1.8	1.5	2.4	3.6	4.4	15.4	14.0	43.6	84.0	100		
Amino nitrogen†.....	29.7	29.5	34.7	33.8	39.7	40.9	37.5	37.5	51.6	32.7		
Glucose†.....	1086	0	1000	0	880	0	568	0	28	0		
pH.....	7.55	7.55	7.35	7.25	7.00	7.65	7.00	8.00	8.20	8.85		
Organisms per cubic centimeter.....			8,600,000	1,600,000	Lost	Lost	18,000,000	3,000,000	100,000,000	34,000,000		

\* The media in this series contained 150 mgm. P per 100 cc.

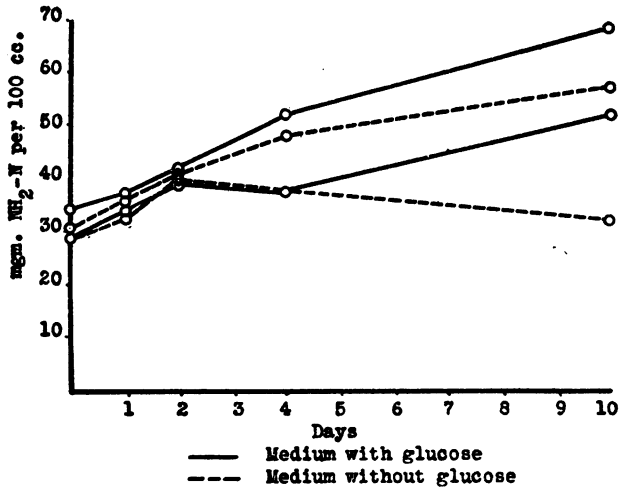
† Milligrams per 100 cc. of the medium.

‡ The media in this series contained 144 mgm. P per 100 cc.

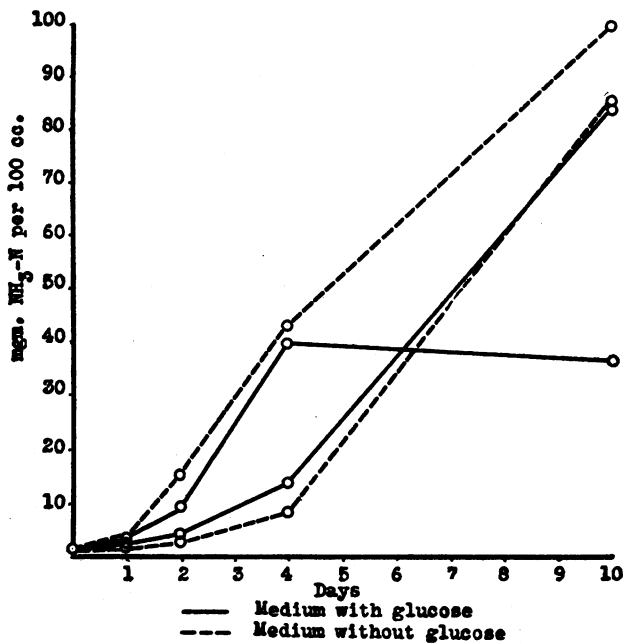
(Bottle A) This column indicates the medium with glucose.

(Bottle B) This column indicates the medium without glucose.

Graph No. VII  
*B. subtilis*  
 Amino nitrogen

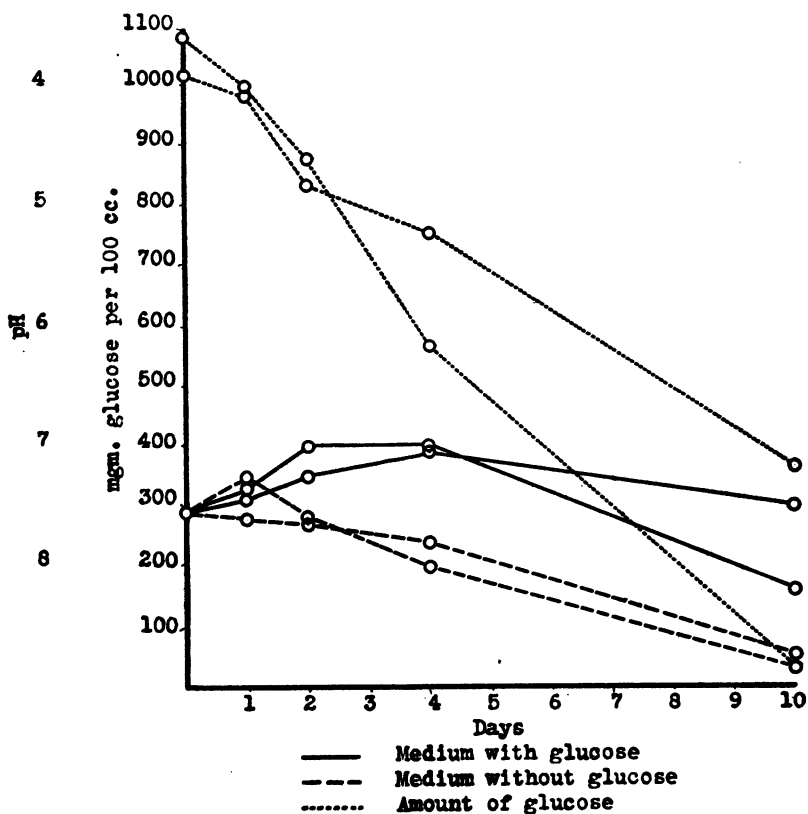


Graph No. VIII  
*B. subtilis*  
 Ammonia nitrogen



The amino nitrogen curves in the medium containing glucose do not agree very closely in total amounts of the nitrogen. However, it is to be noted that the type of curve is the same in both

Graph No. IX  
*B. subtilis*  
 Hydrogen ion concentration  
 and amount of glucose



series. The medium without carbohydrate shows a decrease on the tenth day. The sharp drop in the amino nitrogen on the second and fourth days corresponds to the times when the bacteria show a definite decrease in numbers. While spores could not be demonstrated in stained smear preparations, a large number of shadow forms were noted.

The types of the ammonia nitrogen curves in the two series are very similar. The effect of the presence of the glucose is shown by these curves in that the production of ammonia is decreased. The organism is able to use glucose very rapidly as shown in graph 12.

TABLE 5  
*C. botulinum (type A)*

	DAY OF GROWTH									
	0		1		2		4		10	
	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B
Series 1*										
Ammonia nitrogen†	1.4	1.3	19.2	27.4	20.6	32.1	22.5	35.6	41.8	46.4
Amino nitrogen†	38.1	36.0	54.0	37.7	46.5	41.3	48.1	35.2	56.5	30.0
Glucose†	1054.0	0	496.0	0	367.0	0	178.0	0	33.0	0
pH	7.75	7.75	6.80	7.60	6.80	7.60	6.80	7.75	6.50	7.70
Series 2‡										
Ammonia nitrogen†	2.4	1.7	18.8	25.8	18.5	29.2	20.1	49.4	22.2	58.2
Amino nitrogen†	39.2	37.5	79.7	32.8	76.5	36.5	71.7	44.2	84.9	40.6
Glucose†	1082.0	0	757.0	0	453.0	0	208.0	0	192.0	0
pH	7.50	7.50	6.30	7.65	5.65	7.70	5.55	7.70	5.40	7.70

\* The media in this series contained 150 mgm. P per 100 cc.

† Milligrams per 100 cc. of the medium.

‡ The medium marked (A) in this series contained 144 mgm. P per 100 cc.; that marked (B) contained 153 mgm. P per 100 cc.

(Bottle A) This column indicates the medium with glucose.

(Bottle B) This column indicates the medium without glucose.

*Clostridium botulinum (type B)*

The results of the analyses are shown in table 6 and the graphs of the analyses are shown in graphs 13 to 15 inclusive.

The amino nitrogen and the ammonia nitrogen curves are very similar to those of *C. botulinum (type A)*. The amino nitrogen shows a drop on the fourth day and then a gradual increase to the tenth day. The amino nitrogen curves always

show an increase in the total amount in the medium containing glucose. Kendall, Day and Walker (1922) working with six strains found an increase in three strains of *C. botulinum* and a decrease in three strains. Their results are puzzling.

TABLE 6  
*C. botulinum* (type B)

	DAY OF GROWTH									
	0		1		2		4		10	
	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B
Series 1*										
Ammonia nitrogen†	2.1	2.1	18.7	31.6	22.8	55.6	28.0	61.4	28.0	71.6
Amino nitrogen†	38.7	39.0	58.8	43.1	72.0	43.5	64.9	46.5	64.8	45.6
Glucose†	1091.0	0	959.0	0	806.0	0	500.0	0	250.0	0
pH	7.50	7.55	7.00	7.50	6.85	7.50	6.45	7.50	5.80	7.50
Series 2‡										
Ammonia nitrogen†	1.8	1.8	14.2	25.0	20.2	61.2	22.5	70.5	31.1	70.1
Amino nitrogen†	34.7	34.7	48.4	41.1	61.1	45.4	54.6	39.6	70.0	58.8
Glucose†	1041.0	0	892.0	0	463.0	0	239.0	0	42.0	0
pH	7.45	7.55	7.35	7.65	6.65	7.60	6.25	7.60	5.90	7.70

\* The media in this series contained 144 mgm. P per 100 cc.

† Milligrams per 100 cc. of the medium.

‡ The media in this series contained 162 mgm. P per 100 cc.

(Bottle A) This column indicates the medium with glucose.

(Bottle B) This column indicates the medium without glucose.

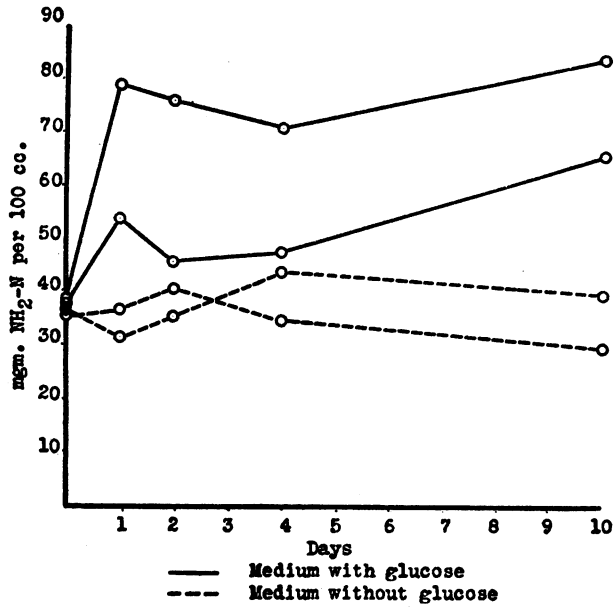
### *Clostridium sporogenes*

The results of the analyses are shown in table 7 and in graphs 16 to 18 inclusive.

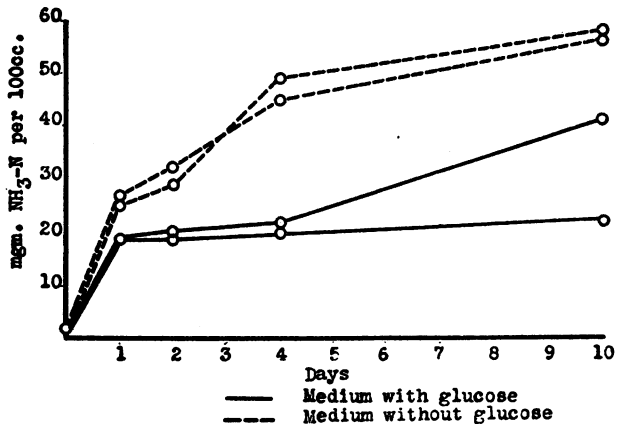
The amino nitrogen curves of this organism are very similar to those of *C. botulinum*. The presence of glucose increases the amino nitrogen and there is also a drop in the total amount on the fourth day. Wolf and Harris (1916-1917) obtained marked decreases in the amino nitrogen in a peptone solution made by the tryptic digestion of casein. In the same medium, however, an



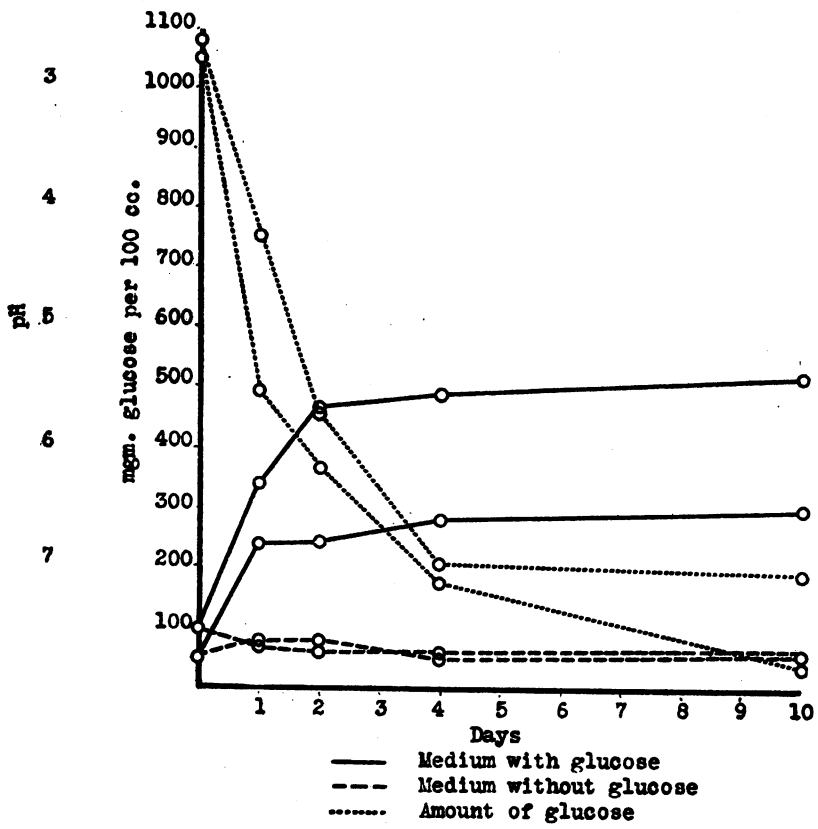
Graph No. X  
*C. botulinum* (type A)  
 Amino nitrogen



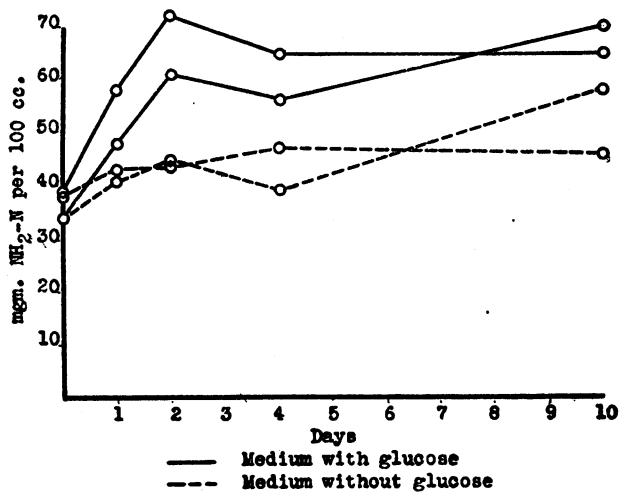
Graph No. XI  
*C. botulinum* (type A)  
 Ammonia nitrogen



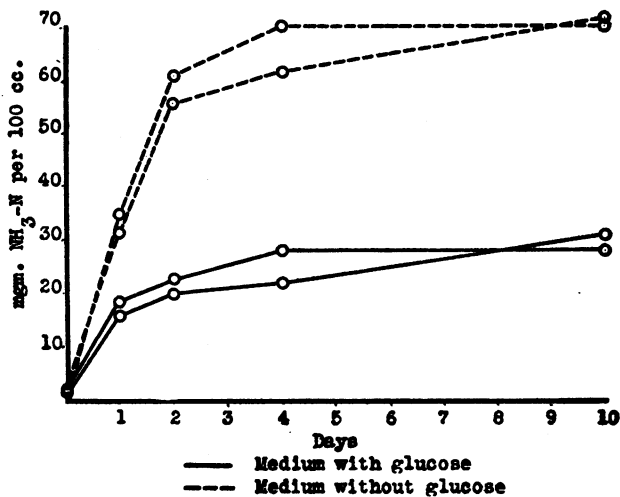
Graph No. XII  
*C. botulinum* (type A)  
 Hydrogen ion concentration  
 and amount of glucose



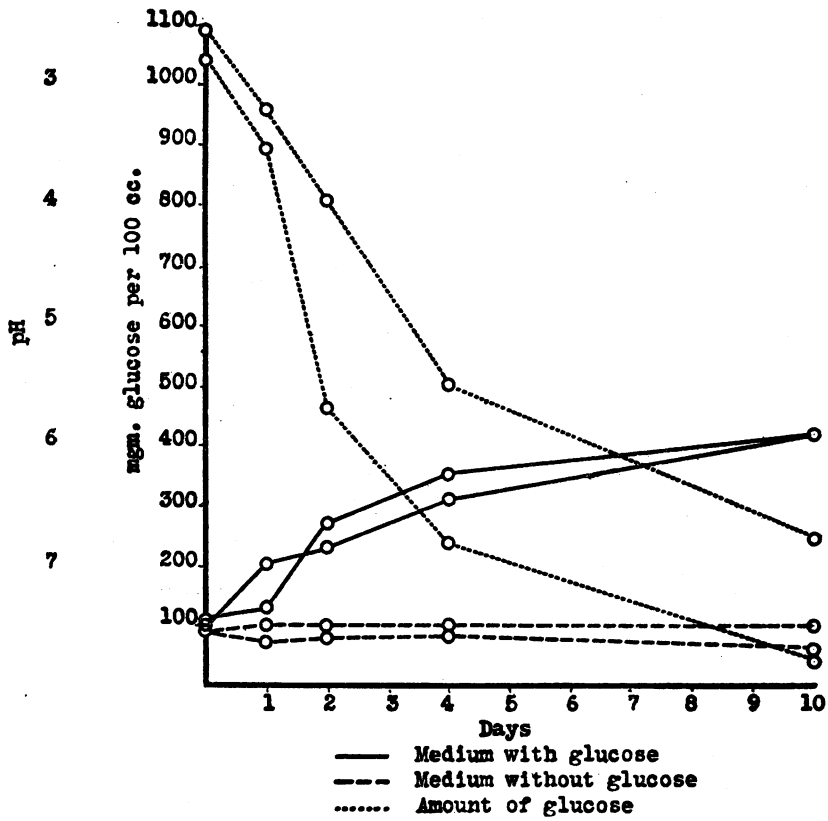
Graph No. XIII  
*C. botulinum* (type B)  
 Amino nitrogen



Graph No. XIV  
*C. botulinum* (type B)  
 Ammonia nitrogen



Graph No. XV  
*C. botulinum* (type B)  
 Hydrogen ion concentration  
 and amount of glucose



increase was obtained when glucose was present (Harris (1919-1920)). In a minced meat medium Wolf (1918-1919 c) obtained large increases in the amino nitrogen in the absence of carbohydrate.

TABLE 7  
*C. sporogenes*

	DAY OF GROWTH									
	0		1		2		4		10	
	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B	Bottle A	Bottle B
Series 1*										
Ammonia nitrogen†	1.1	1.2	21.2	39.2	19.4	46.2	19.4	44.4	19.6	50.2
Amino nitrogen†	36.7	34.7	60.4	35.0	63.9	39.0	58.5	40.7	71.6	48.4
Glucose†	1096.0	0	735.0	0	505.0	0	384.0	0	312.0	0
pH	7.50	7.50	6.50	7.55	6.30	7.75	6.15	7.50	6.05	7.55
Series 2‡										
Ammonia nitrogen†	1.2	1.8	25.0	41.2	20.1	49.0	20.6	55.7	19.8	63.2
Amino nitrogen†	37.1	36.7	59.0	42.8	69.3	39.5	64.7	35.2	72.4	44.0
Glucose†	1100.0	0	771.0	0	563.0	0	416.0	0	357.0	0
pH	7.30	7.55	6.45	7.45	6.25	7.65	6.15	7.50	6.05	7.35

\* The media in this series contained 153 mgm. P per 100 cc.

† Milligrams per 100 cc. of the medium.

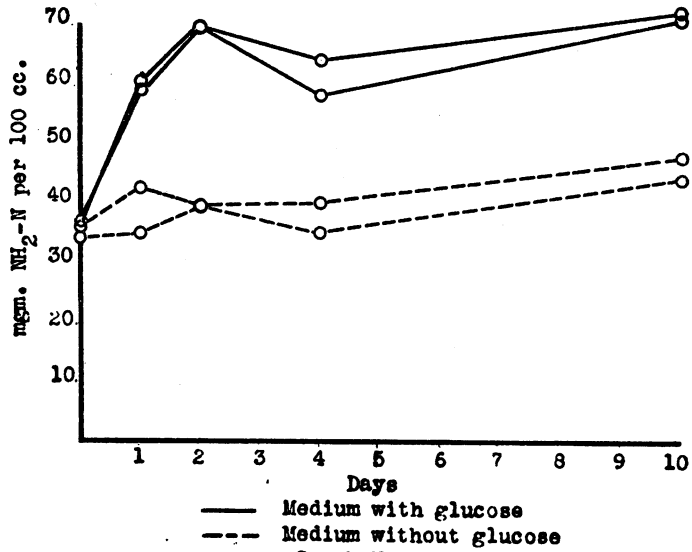
‡ The medium in this series marked (A) contained 153 mgm. P per 100 cc.; that marked (B) contained 153 mgm. P per 100 cc.

(Bottle A) This column indicates the medium with glucose.

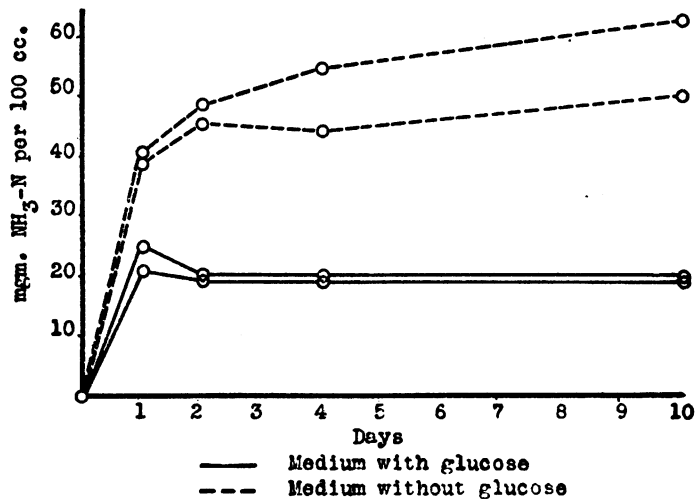
(Bottle B) This column indicates the medium without glucose.

The ammonia nitrogen in the carbohydrate medium is much lower than in the medium not containing a carbohydrate. Similar results have been recently reported by Kendall, Day and Walker (1922).

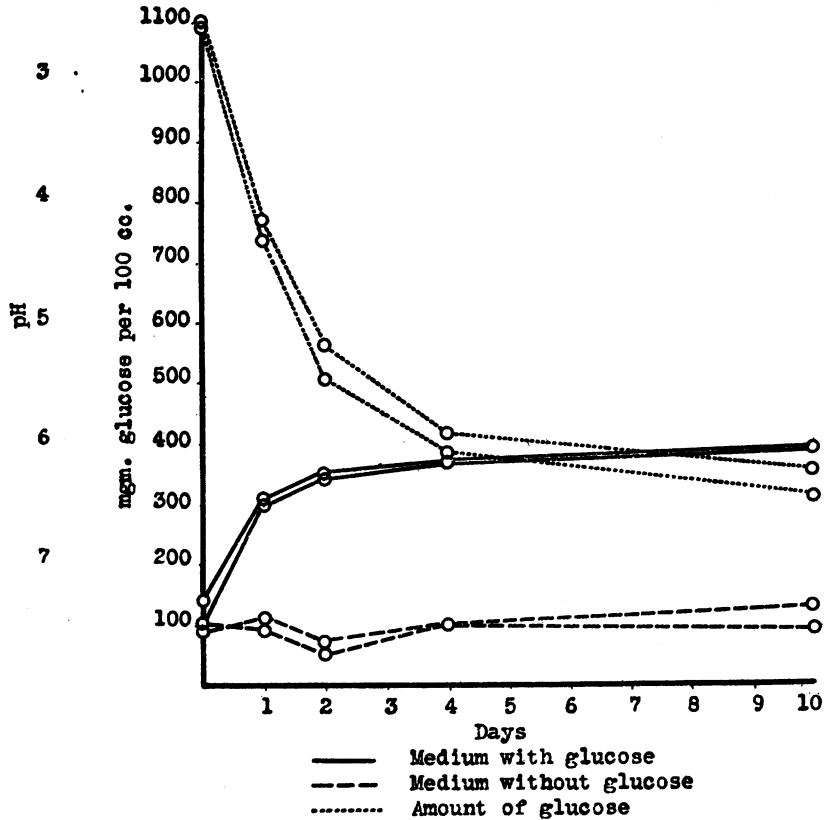
Graph No. XVI  
*C. sporogenes*  
 Amino nitrogen



Graph No. XVII  
*C. sporogenes*  
 Ammonia nitrogen



Graph No. XVIII  
*C. sporogenes*  
 Hydrogen ion concentration  
 and amount of glucose



SUMMARY AND DISCUSSION

In order to study the nitrogenous metabolism of bacteria, I gave special attention to the determination of the changes in the amino nitrogen and ammonia nitrogen in growing cultures of representative bacteria. Analyses were made of the medium just after it was inoculated and again on the first, second, fourth and tenth days of growth. The usefulness of work of this type depends entirely upon the trustworthiness of the methods used.

A large part of this study, therefore, resolved itself into an investigation of the methods applicable to the conditions of the experiment.

So far as amino nitrogen is concerned, I have shown that the method of Folin (1922) gives consistent results in the presence of peptone. Thus, a sterile peptone solution gives consistent results when tested from day to day. Further, the addition of a standard glycocoll solution gives the correct increase in the amino nitrogen in sterile peptone solutions and in cultures of bacteria after growth has taken place. While it is realized that not all of the amino nitrogen may react with the reagent used in this method, it must be remembered that other methods fail to give correct readings with some of the amino acids. This is shown clearly with the simplest amino acid, glycocoll, in the Van Slyke method. It is desirable to have a method which will give the total amino nitrogen with accuracy. However, any method used in the study of bacterial metabolism should give, first of all, consistent values for the total amino nitrogen of a sterile peptone solution. Since the Folin method does give consistent values for the amino nitrogen in peptone solutions, I have adopted it as the best method available for the purpose of this investigation.

The results of my study clearly show that (1) the total amount of amino nitrogen may be increased to but a slight extent during bacterial growth in media not containing glucose, while (2) a very marked increase in amino nitrogen may take place in media containing glucose.

With the greater number of strains of bacteria used in this study there was a sharp drop in amino nitrogen on the second or fourth day of growth. The decrease takes place in the case of the anaerobic bacteria about the time they show definite autolysis and a sharp decrease in the numbers of bacteria. This decrease in amino nitrogen is not constant in the case of the aerobic bacteria. Moreover, it could not be correlated with the number of living cells.

I found the production of amino nitrogen always greater in the presence of glucose. These results do not agree with some of the results of other investigators. Berman and Rettger (1918)



found that the concentration of amino nitrogen remained unchanged in cultures of *Bact. coli* in plain as well as glucose media. They stated that the presence of carbohydrate did not affect the nitrogen metabolism. My results do not warrant such a conclusion. Kendall and his co-workers found in their extensive studies of bacterial metabolism that the amino nitrogen is changed very little during the growth of various bacteria. Kendall, Day and Walker (1922) found that three strains of *C. botulinum* produced less amino nitrogen in carbohydrate media than in non-carbohydrate media. However, the reverse was true with three other strains of *C. botulinum* which they studied. Unless a difference in the methods would account for these results, the discrepancies are difficult to reconcile. Sears (1916) working with *Ps. pyocyanea* grown aerobically showed that more amino nitrogen was produced in media containing carbohydrate than in media free of carbohydrate. On the contrary, when the organism was grown anaerobically less amino nitrogen was produced in media containing carbohydrate than in media free of carbohydrate. In view of his results it is difficult to understand Sears' conclusion that the amino nitrogen production of *Ps. pyocyanea* when grown aerobically does not differ materially from the amino nitrogen production of the same organism when grown anaerobically.

My results lead me to conclude that the amino nitrogen produced in non-carbohydrate cultures of bacteria is utilized for the growth and the energy requirements of the cells almost as rapidly as formed, and that the production of amino nitrogen in carbohydrate media is much greater than its utilization. There may be several reasons for the increased amino nitrogen production in media containing carbohydrate. Theoretically, the amino acids used for the energy requirements of the bacteria may not be necessary to the same extent if a fermentable carbohydrate be present, and therefore, the amino nitrogen would show as an increase in the medium. The presence of carbohydrate may increase the availability of nitrogenous products other than amino nitrogen which would also augment the total amino nitrogen. Again, we must consider that the increase in the hydrogen ion

concentration may influence the type of the nitrogenous metabolism. Further, there may be an increased enzyme activity. We lack sufficient data to prove whether one or all of the above factors may be concerned in the accumulation of amino nitrogen in media containing a fermentable carbohydrate.

Since the utilization of amino nitrogen may keep pace with its production under certain conditions, it is evident that the amino nitrogen cannot be used as an index of proteolysis under such conditions. However, amino acids are used by the bacteria, and therefore even if the total amino nitrogen remains unchanged, it is safe to assume that there must have been a certain amount of proteolysis to replace the amino nitrogen utilized. The direct proof can not be obtained from the determinations of total amino nitrogen. If the amino nitrogen were not used in the metabolism of bacteria, an increase would always be expected which could be taken as a quantitative index of proteolysis. From the above considerations it is concluded that amino nitrogen may be taken only as an approximate index of proteolysis in bacterial cultures which show a material increase in total amino nitrogen. This is shown clearly in media containing a carbohydrate. In media not containing a carbohydrate the amount of amino nitrogen cannot be taken as an index of proteolysis except in the cases where the amino nitrogen is increased. In any case, it is clear that the amino nitrogen in bacterial cultures can not be taken as a quantitative index of proteolysis.

The total amount of ammonia produced depends upon many factors, especially the composition of the medium (presence or absence of carbohydrate) and upon the species of organism used. *Bact. coli* produces very little ammonia when compared to *B. subtilis*, *Ps. pyocyanea* and the anaerobes, *C. botulinum* and *C. sporogenes*. In all of my experiments in this study the presence of a fermentable carbohydrate has resulted in a lessened production of ammonia when compared to a non-carbohydrate medium. The cause of this decreased rate of production of ammonia has not been definitely proven. It has been claimed by Kendall and Walker (1915) that this decreased production of ammonia is due to a decreased rate of proteolysis. Gordon (1917) has

shown that *Bact. coli* is able to use certain ammonium salts, for example, ammonium chloride, as the only nitrogen source when a carbohydrate is present. Therefore, the fact that ammonia is decreased in carbohydrate media does not necessarily indicate a smaller amount of proteolysis; it may indicate a greater utilization of the ammonia. Further, the increase in the hydrogen ion concentration may influence the type of metabolism and thus affect ammonia production.

If ammonia production is taken as an index of proteolysis, as proposed by Kendall and Walker (1915), their conclusion that there is less proteolysis in media containing a carbohydrate would be justified. However, if the production of amino nitrogen is taken as an index of proteolysis, the fact that there is a large increase in the amino nitrogen in carbohydrate media would indicate that there is greater proteolysis. Since the number of bacteria in a medium containing carbohydrate is many times greater than in a medium not containing carbohydrate, it is evident that the total nitrogenous metabolism in the presence of sugar must of necessity be greater than in the absence of carbohydrate. Ammonia if it be taken as an index would indicate a decreased total nitrogenous metabolism, therefore, under these circumstances, it seems justifiable to conclude that ammonia production in bacterial cultures is not a reliable index of proteolysis. The change in the rate of ammonia production in media containing carbohydrate may indicate the extent of either deamination or utilization of ammonia or the balance of the two processes. While amino nitrogen may not be an absolute index of proteolysis it does indicate, contrary to ammonia, an increase in the rate of nitrogenous metabolism in bacterial cultures in the presence of carbohydrate.

Since the cultures show a large increase in the total amount of amino nitrogen in media containing glucose, the suggestions that the proteolytic enzyme was inactivated by glucose as proposed by Fischer (1915) or that glucose prevented the formation of the proteolytic enzymes as proposed by Kendall and Walker (1915) and Jones (1916) do not seem plausible.

The organisms studied divide themselves into two groups with reference to hydrogen ion concentration. One group, including *Bact. coli*, *C. sporogenes* and *C. botulinum*, shows a large reduction of glucose with a markedly increased production of acid as determined by the hydrogen ion concentration. The other group, which includes *B. subtilis* and *Ps. pyocyanea*, destroys approximately the same amount of glucose without the production of acid, as determined by the hydrogen ion concentration. It is important to note that the medium in which both these groups of bacteria were grown had approximately the same composition with reference to the initial amount of glucose and buffer content.

It is therefore apparent that some organisms have the power to destroy glucose without markedly increasing the hydrogen ion concentration; thus indicating a different type of carbohydrate metabolism. If the hydrogen ion concentration is not increased the inference commonly drawn is that the glucose is not destroyed. My data show that the contrary may take place and the inference is therefore not justified. The conclusion is clear, then, that the hydrogen ion concentration of cultures of different bacteria in media containing approximately the same amount of buffer does not necessarily prove whether the carbohydrate has or has not been destroyed.

#### CONCLUSIONS

The presence of glucose in peptone media increases the rate of production of amino nitrogen in growing cultures of *Bact. coli*, *Ps. pyocyanea*, *B. subtilis*, *C. botulinum* and *C. sporogenes*.

The amino nitrogen found in bacterial cultures may be taken as an approximate index of proteolysis under certain conditions.

The Folin method for the determination of amino nitrogen is applicable in the studies of bacterial metabolism in peptone media.

The ammonia found in bacterial cultures is not a reliable index of bacterial proteolysis.

The presence of a fermentable carbohydrate in bacterial cultures affects the nitrogenous metabolism as judged by the total amino nitrogen and the total ammonia nitrogen.

The rate of production of amino nitrogen or ammonia nitrogen indicates different types of metabolism of bacteria.

Some bacteria destroy glucose without a marked increase in the hydrogen ion concentration. Therefore the hydrogen ion concentration may not be an index of the destruction of glucose in bacterial cultures.

I wish to acknowledge my indebtedness to Dr. M. J. Rosenau whose generous assistance has made it possible to carry out this investigation. I desire to express my appreciation to Dr. O. Folin for permission to use before publication his method for the determination of amino acid nitrogen and for his many helpful suggestions. I am also greatly indebted to Dr. J. Bronfenbrenner and Dr. C. H. Fiske for their aid throughout this study. Thanks are due to Dr. Harry Weiss for aid in preparing the charts.

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