THE BIOLOGY OF THE TUBERCLE BACILLUS

II. THE ASPARAGIN AND GLYCEROL METABOLISM OF THE TUBERCLE BACILLUS

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Received for publication, August 14, 1931

In order to understand the activities of a disease-producing organism in the host, it is desirable to determine, as nearly as possible, the metabolism of that organism. If the food of the organism were known and the method of utilization were determined, such knowledge might suggest a method of controlling the food supply in some way so as to limit the growth of the organism within the body. It is well known, for instance, that the glycerol content of a medium has a marked influence upon the growth of the tubercle bacillus, and that without nitrogenous compounds the organism will not grow. Doubtless these substances exert a similar influence within the body. How are they utilized? What products are formed? What effect have these products upon the surrounding medium? The answers to these questions may serve, by analogy, to explain some of the reactions occurring in the body of a tuberculous individual.

One method of approach to this problem is to analyze the medium on which the organisms are grown. This is done in order to show what substances are metabolized, and what changes take place. Considerable work has been done on this subject in the past, but most of the analyses have been made on media of unknown composition, such as glycerol broth, and these analyses have been chiefly of the terminal products of metabolism. In the present work, the medium was of known composition, and it was analyzed weekly in order to show the progressive utilization of the asparagin and glycerol. Is this utilization accomplished by both virulent and avirulent organisms in the same manner?
Which atom of nitrogen in the asparagin is most easily metabolized? Is the glycerol completely or partially used? What effect does the metabolism of these substances have upon the pH of the medium? To answer these questions was the object of this research.

REVIEW OF THE LITERATURE

Theobald Smith (1910) first observed that virulent and avirulent tubercle bacilli differed in their reactions in glycerol broth; the virulent organism produced a terminal acidity, while the avirulent organism produced a terminal alkalinity. He also noted that the addition of glycerol increased the growth of the virulent organism, thus indicating its utilization. In the case of Mycobacterium tuberculosis-bovis, however, the alkalinity of the glycerol broth offered inconclusive evidence as to the utilization of glycerol.

Kendall, Day and Walker (1914) showed that saprophytic tubercle bacilli tend to produce alkalinity when grown in glucose, mannitol or plain broth. This alkalinity, they concluded, was due to variations in the ammonia produced during metabolism. In glycerol broth, a progressive acidity was produced, which, however, might be preceded by an alkalinity. The reactions were not fixed, but varied greatly with the composition of the medium. Long and Major (1921) obtained similar results; a progressive alkalinity occurred when the organisms grew in amino acids, acid amines, and ammonium salts. The addition of glycerol, however, produced an acid reaction which was sufficiently great to mask the alkalinity. Frouin and Guillaume (1923) obtained similar results in regard to the utilization of various carbohydrates. Fosca (1924) also found a progressive acidity produced by various types of tubercle bacilli.

Weinzirl and Knapton (1927) found that virulent organisms produced progressive acidity on Long's synthetic medium while the avirulent organisms first produced marked acidity, then a regression to alkalinity. Weinzirl and Ott (unpublished data) showed that when virulent bacilli were grown on Long's synthetic medium, the elimination of asparagin did not stop the growth of
the bacilli, but that no growth occurred when the glycerol was omitted. With avirulent bacilli the elimination of asparagin caused a progressive alkalinity without a previous acidity. From these results they attributed the acidity to the action on the glycerol, and the final alkalinity to the action on the asparagin.

Long and Finner (1927) showed that the growth and amount of lipin present in the bacilli is related to the amount of glycerol present in the medium, thus indicating a marked utilization of glycerol in the metabolism of the organisms.

In the work of Renfrew, Bass and Johnson (1928, 1929) with human and avian tubercle bacilli grown on Long's synthetic medium it was shown that the period of growth was characterized by constant changes in the ammonia content and pH, which became comparatively constant when growth ceased. A correlation existed between the growth of the bacilli and the pH curves. The pH curve of the human strain showed progressive and permanent acidity, while that of the avian strain became acid and then alkaline.

Merrill (1930, 1931) in his work with the genus Mycobacterium cultured on broth enriched with various carbohydrates, showed that the increase in alkalinity is associated with an increase in the ammonia content of the medium, which is approximately equivalent to the increase in titratable alkalinity, and which varies directly with the pH. He concludes that if the carbohydrate molecule is attacked at all it is oxidized completely to carbon dioxide and water without the accumulation of demonstrable amounts of any intermediate products in the medium. In the repetition of this work he replaced the broth medium with a glucose synthetic medium, and found that all the carbon could be accounted for as carbon dioxide plus the carbon in the organisms and that occurring in the medium as sugar. The acidity of the medium was accounted for on a basis of the removal of ammonia from an inorganic compound, ammonium sulphate. No attempt was made to follow the course of the utilization of the nitrogen and carbon during the growth of the organisms, and conclusions were based upon the results of determinations made at the end of forty-two days.
METHODS

Medium employed. It was desirable to use a medium of the simplest composition upon which an abundant growth of the tubercle bacillus could be obtained, and in which the sources of nitrogen and carbon were limited. Many different media were tried, but the following was found to give a satisfactory growth of the tubercle bacilli: asparagin 5 grams; dipotassium phosphate (anhydrous) 1.50 grams; magnesium sulphate 1 gram; ferric chloride 0.08 gram; glycerol 50 cc.; and distilled water 1000 cc.

The ingredients were dissolved in the water in the order named, the solution titrated to a pH of 7.2, dispensed in 500 cc. flasks with 250 cc. in each, and sterilized in the autoclave at 12 pounds pressure for fifteen minutes. A small amount of precipitate of magnesium and iron phosphates formed, but was disregarded since it did not interfere with the growth.

Cultivation of organisms. The flasks of medium were inoculated with two strains of tubercle bacilli: Mycobacterium tuberculosis, human strain "H 37," received from the Trudeau Foundation; and Mycobacterium tuberculosis, human strain "H 520," an avirulent strain received from the University of British Columbia. The former grows slowly and is pathogenic; the latter grows abundantly on plain agar within twenty-four hours and will not cause tuberculosis in guinea pigs when administered intraperitoneally in 10 mgm. doses. The cultures were grown on the surface of the synthetic medium at an incubation temperature of 37°C. Small beakers were inverted over the mouths of the cotton stoppered flasks to prevent contamination. Cultures were removed for analysis on the third, seventh, tenth, and fourteenth days, and weekly thereafter for eight weeks. Uninoculated flasks of medium were subjected to the same conditions and were removed for analysis weekly in order to determine the changes due to evaporation of the medium. The organisms were removed by decantation through a filter paper, and the following determinations made upon the filtrate:

Hydrogen-ion concentration. The hydrogen-ion concentration was determined colorimetrically, using brom-thymol-blue and brom-cresol-purple as indicators. The pH was checked through-
out by the potentiometer method using a quinhydrone electrode and a saturated calomel cell.

_Titratable reaction._ To 25 cc. portions of the medium was added 0.5 cc. of a 0.1 per cent solution of phenol red, and the culture titrated with N/40 sulphuric acid or sodium hydroxide to the same pH as the uninoculated control. This was done because an accurate endpoint could not be obtained by titrating to a color change. The results thus represent the increase in the titratable acidity or alkalinity of the culture relative to the control. This method gave quite constant results in duplicate determinations.

_Ammonia._ The ammonia was determined by the aeration method of Folin, the air being washed by passing through sodium hydroxide and sulphuric acid before use. The determinations were made upon 25 cc. portions of the cultures, to which from 0.5 to 1 gram of NaOH and 5 grams of NaCl were added. The ammonia was collected in N/40 sulphuric acid, and the amount of ammonia determined by back-titration using methyl red as an indicator. By determinations made upon known ammoniacal solutions, this method was found to be accurate within 0.3 cc. of N/40 reagent, or 0.12 mgm. of ammonia. Since the amount of ammonia in these cultures was found to be small, some of the results given are within the limits of experimental error. The standard solutions were checked every three weeks in order to insure their accuracy.

_Amino-nitrogen._ The amino nitrogen was determined by the Van Slyke method (1912, 1913, 1915). The protein was removed from the culture medium by mixing equal volumes of medium and 20 per cent trichloracetic acid. After standing an hour the protein precipitate was removed by centrifugation. Of this mixture, 2 cc., representing 1 cc. of medium, were analyzed with the micro apparatus. This method for determining amino nitrogen is accurate to 1 per cent with quantities as small as 0.5 mgm. of amino nitrogen.

_Total nitrogen._ The total nitrogen was determined by Gunning's modification of the macro-Kjeldahl method.

_Protein nitrogen._ The protein nitrogen was determined in-
directly by the determination of the nitrogen remaining in the medium after the precipitation of the protein with trichloracetic acid as described under the method for amino nitrogen. The method used was the same as that for total nitrogen. The difference between the total nitrogen determinations and the determinations following the precipitation of the protein indicated the amount of protein nitrogen. This method allowed for some error due to the fact that some of the protein was probably converted into soluble acid albuminates and was not removed from the solution.

Rest Nitrogen. This value was calculated by subtracting the combined amino nitrogen and protein nitrogen from the total nitrogen. It represents chiefly amino nitrogen from the asparagin, with ammonia, proteoses, basic amines, or any other nitrogenous substances which may be produced by the organisms during their growth.

Glycerol. The glycerol was determined by method I, in the Methods of Analysis of the Association of Official Agricultural Chemists (1925) for the official determination of glycerol in dry wines. In this method 100 cc. of the medium were used for analysis, the glycerol being determined by evaporation and subsequent extraction with boiling alcohol and an alcohol-ether mixture. The glycerol was then determined gravimetrically. This method allows for a considerable error due to the fact that some glycerol is volatile at 90° to 100°C., although milk of lime was added to decrease this volatility. The results, however, are sufficiently accurate to show the course of the glycerol utilization.

The results obtained by all of the above methods were checked by duplicate determinations and the two figures averaged for use in establishing the curves.

RESULTS

In an attempt to follow the reaction curves produced by the tubercle bacilli in a simplified medium of known composition, and to show the relationship between the reaction curves and the metabolism of the asparagin and glycerol by the organisms, quantitative methods were relied upon. The metabolism of the asparagin was followed by four nitrogen determinations: (1)
nitrogen in the form of free or combined ammonia; (2) amino nitrogen; (3) protein nitrogen, and (4) total nitrogen. By comparison of these results, the amount of asparagin utilized can be determined, and to a certain extent its manner of utilization. It was assumed that the nitrogen which was not in the medium had been incorporated into the bodies of the organisms. It is possible, but not very probable, that a small amount of nitrogen may have escaped into the air.

The glycerol metabolism was followed by direct quantitative determination of the amount of glycerol in the medium, the decrease in glycerol content indicating its utilization by the organism. Whether or not such utilization was complete was difficult to prove, but it was thought that if there were partial oxidation of the glycerol to organic acids, as some investigators (Long, Campbell and Smith (1922)) have supposed, lactic and acetic acids would, presumably, be formed to some extent. In order to determine whether or not these acids were formed, the ferric chloride test for lactic acid were tried using ether extraction, and the U. S. P. tests for acetic acid and acetates. Satisfactory positive tests were not obtained for either of these acids, which would seem to indicate that if partial oxidation of the glycerol to these organic acids took place, it was only to a very slight extent.

The titratable reaction was determined to see whether or not there was a direct relationship between the pH curves and the titratable acidity or alkalinity, and whether or not the titratable alkalinity could be accounted for by the ammonia production.

The data obtained for Mycobacterium tuberculosis, human strain H 37, are presented in table 1.

Table 1 indicates the following: (1) the pH shows a progressive rise of acidity from pH 7.2 to 5.2 which has been paralleled throughout by the titratable reaction; (2) The ammonia formed has been slight, but the maximum amount seems to occur at the time of maximum growth; (3) the amino nitrogen, total nitrogen, and rest nitrogen show, in general, a decrease; (4) the protein does not appear in appreciable quantity until the fifth week; (5) the glycerol has been practically all utilized at the end of the ten weeks' incubation.

Table 2 shows the quantitative data obtained during the growth
TABLE 1
Giving data obtained for Mycobacterium tuberculosis, human type 37

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<th>AGE (days)</th>
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<th>Amine N</th>
<th>Total N</th>
<th>Protein N</th>
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TABLE 2
Giving data obtained for Mycobacterium tuberculosis, human type 520

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<th>Total N</th>
<th>Protein N</th>
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of *Mycobacterium tuberculosis*, human type 520 (saprophytic), upon the synthetic medium. In general, the results show that the metabolism of the saprophytic H 520 closely parallels that
of the virulent H 37. The chief differences are: (1) the changes occur more rapidly with the saprophytic organism than with the virulent organism; (2) the pH of the saprophytic organism becomes acid and then alkaline while that of the virulent organism remains acid.

Table 3 shows the quantitative data obtained from the analysis of the medium in uninoculated control flasks exposed to the same incubational conditions as those of the inoculated flasks. These determinations serve merely as checks upon those of tables 1 and 2.

**TABLE 3**

*Giving data obtained for the control flasks of uninoculated medium*

| days | pH | TITRATABLE REACTION | NH₃ | AMINO N | TOTAL N | PROTEIN N | RIBEN N | GLYCEROL |
|------|----|---------------------|-----|---------|---------|-----------|--------|
|      |    | cc. N/40 per 100 cc. | mgn. per 100 cc. | mgn. per 100 cc. | mgn. per 100 cc. | mgn. per 100 cc. | grams per 100 cc. |
| 0    | 7.2|                     |      |         |         |           |         |
| 3    | 7.2|                     |      |         |         |           |         |
| 7    | 7.2|                     |      |         |         |           |         |
| 10   | 7.2|                     |      |         |         |           |         |
| 14   | 7.2|                     |      |         |         |           |         |
| 21   | 7.1|                     |      |         |         |           |         |
| 28   | 7.1|                     |      |         |         |           |         |
| 35   | 7.1|                     |      |         |         |           |         |
| 42   | 7.1|                     |      |         |         |           |         |
| 49   | 7.0|                     |      |         |         |           |         |
| 56   | 7.0|                     |      |         |         |           |         |
| 63   | 7.0|                     |      |         |         |           |         |
| 70   | 7.0|                     |      |         |         |           |         |

A graphic representation of the changes produced by the organisms is shown in figures 1 to 8.

**DISCUSSION**

In an interpretation of the results based upon analytic determinations of substances in a culture medium, it is necessary to remember the error contingent upon the effect of the organisms' excretions and lysis. Since the organism maintains a balance between the food ingested and the waste products excreted, the medium contains not only the unused food substances placed in
**HYDROGEN POTENTIAL**

![Graph showing hydrogen potential over time for samples H.37, H.520, and control.](image)

**TITRATABLE REACTION**

![Graph showing titratable reaction over time for samples H.37 and H.520.](image)

Fig. 1

Fig. 2
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Fig. 3

Fig. 4
TOTAL NITROGEN

PROTEIN-NITROGEN

Fig. 5

Fig. 6
REST-NITROGEN

![Graph showing the nitrogen levels over days for H.37 and H.520.]

GLYCERIN

![Graph showing the glycerin levels over days for H.37, H.520, and control.]

Fig. 7

Fig. 8
it originally, but also the excretory and lytic products of the organism. If the organism metabolised its food completely to carbon dioxide and water, such excretion products would have little effect upon these determinations. But apparently it does not do this, as evidenced by the fact that protein and other nitrogenous compounds which must have come from the organism, since there were none in the original medium, appear after three or four weeks' growth. This protein may be due in part to lysis of the organism, and in part to its solution. It seems probable that the organism might eliminate not only protein but also other complex nitrogenous compounds, such as proteoses, peptones and amines. On this account, in a cautious interpretation of results based upon these determinations, no rigid lines can be drawn to show just how much of the materials have been utilized and in what manner such utilization has taken place.

The pH curves, shown in figure 1, agree closely with those given by other investigators. A progressive acidity was noted in the cultures of the virulent H 37, and acidity which returned to alkalinity in the cultures of the virulent H 520. The alkalinity shown at the third week by the pH curve of H 37 is also shown by the titratable reaction and an increase in the ammonia content of the medium. No cause could be determined for this alkalinity. Both Theobald Smith (1910) and Merrill (1930) report that in some cultures of virulent tubercle bacilli they have found an initial alkalinity occurring before the progressive acidity. The pH curve for the avirulent bacilli (H 520) progressed to an acidity of 5.6 and then returned to an alkalinity of 7.2, the same as that of the initial uninoculated medium. The pH of the uninoculated control progressed from 7.2 to 7.0 during the duration of the experiment. This change was possibly due to absorption of carbon dioxide from the air, and to evaporation of the medium with a resultant change in its buffering power.

The titratable reaction of the culture medium, expressed in cubic centimeters of N/40 acid or alkali paralleled the pH closely, as is shown by figure 2. The alkalinity occurring in the last few week's growth of the avirulent organisms (H 520), as expressed by the titratable alkalinity, may be due to the fact that the me-
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dium was titrated to the same reaction as the control. Since the latter became acid from 7.2 to 7.0 in the last seven weeks of incubation, the addition of acid was required to make the reaction of the culture and control identical, as the pH of the culture returned to the initial pH.

The ammonia determinations, figure 3, showed a progressive increase in the amount of ammonia present in the medium up to the time of maximum growth of the organisms as indicated by their bulk. The increase was marked in H 520 up to the third week, following which there was a gradual recession of ammonia. With H37, the increase was more gradual and reached a maximum about the sixth or seventh week, following which there was a marked recession. This decrease in the ammonia content may have been due to the loss of ammonia from the medium into the air, and also to the utilization of the ammonia by the organisms. Since asparagin was the only source of nitrogen, and thus of ammonia in the medium, the ammonia must have come from the breaking down of the asparagin by the organisms, probably by enzymic action in the medium or by absorption of the whole molecule and resultant excretion of the ammonia. As will be shown below, the asparagin was almost completely broken down during the progress of the experiment. Since more nitrogen would be essential to the further growth of the organisms, it seems likely that the ammonia and other nitrogenous excretory and lytic products may have served as partial sources of nitrogen for further growth. The cause of the two extreme variations in the ammonia content of the H 37 medium can only be determined by further experiments. The ammonia curve for H 520 was at its highest point at approximately the same time that the pH was the most acid, and decreased as the pH became alkaline, indicating that the alkalinity was not due to ammonia formation.

The amino nitrogen determinations, figure 4, made upon the medium after the precipitation of the protein with trichloracetic acid, show practically complete utilization of the amino nitrogen by both organisms. The virulent H 37 organisms metabolized the nitrogen only slightly during the first six weeks, but from the sixth to the eighth week there was marked utilization as shown by
the decrease in the amino nitrogen content, until only a small amount was left in the medium. The avirulent H 520 metabolized the amino nitrogen slightly during the first and second weeks, but a marked decrease in the amino nitrogen content occurred in the third, fourth, and fifth weeks when no nitrogen could be detected by this method. At the ninth and tenth weeks a slight amount of amino nitrogen was noted. This was probably due to the lysis of some of the organisms and to the partial hydrolysis of their proteins. The control flasks showed an increase of approximately 1 mgm. of amino nitrogen per 100 cc. of the medium during the period of incubation. This increase was presumably due to evaporation of the medium.

The curves shown in figure 5 indicate the progressive decrease in the total nitrogen content of the medium. This decrease is approximately the same with both organisms, although it occurs much more rapidly with H 520 than with H 37. The lowest point is reached at the fourth week in the H 520 cultures, and not until the eighth week in the H 37 cultures. Up to these points the greatest amount of nitrogen was probably in the form of asparagin, but from this time on it seems likely that the largest amount of nitrogen was in the form of protein nitrogen and other nitrogenous excretory and lytic products of the organisms.

The protein nitrogen (fig. 6), appeared between the third and fifth weeks in the culture medium of the H 37 organisms, and about the second week in that of the H 520 organisms. In both, the protein nitrogen increased rapidly and then decreased, the decrease being more marked in the case of H 37. This may possibly be explained by the fact that the H 37 medium became quite acid, which would tend to favor proteolysis, while the H 520 medium returned to approximately neutrality, which would probably retard the proteolytic process.

The rest nitrogen (fig. 7), consisted chiefly of the imino nitrogen of the asparagin in the early part of the experiment, and of the nitrogenous excretory and lytic products of the organisms in the latter part. In the H 37 medium the decrease in the rest nitrogen (chiefly imino nitrogen) was approximately the same as the decrease in the amino nitrogen for the first two weeks. Dur-
ing the third and fourth weeks, however, the decrease was very marked until at the end of the fifth week it reached the lowest point, while the amino nitrogen had scarcely decreased at all. At this same time the protein nitrogen was approaching a maximum, and it would appear likely that the imino nitrogen may be entirely metabolized and that the remaining nitrogen figured here was due to other nitrogenous compounds. This same relationship existed in the avirulent H 520 medium, although the changes occurred more quickly than in the H 37 medium. These results would seem to indicate that the organisms utilized the imino nitrogen more readily than the amino nitrogen.

In a consideration of the above results with respect to the pH reactions produced in the medium, several possibilities presented themselves. The organisms could produce an acidity (1) by removing an alkaline substance from the medium, (2) by the partial utilization of a neutral compound leaving an acid one, (3) by the excretion of an acidic compound, or (4) by some combination of these; the processes reversed would cause an alkalinity. The pH changes may have been due to the metabolism of the inorganic salts, glycerol, and asparagin in this medium.

No determinations were made of the inorganic constituents of the medium, hence the effect of their metabolism upon the medium cannot be stated.

The glycerol metabolism may account in part for the acidity produced in the medium. Long (1922) believes that this is the case, but Merrill (1930) believes that the glycerol as well as other carbohydrates are completely metabolised to carbon dioxide and water. Our results seem to favor the latter view, since the glycerol as such was completely removed from the medium and no tests were obtained for lactic and acetic acids. However, it is possible that other acids or acidic compounds were produced from the glycerol.

The acidity may be due to the removal of the basic nitrogenous radicals from the neutral asparagin by enzymic or hydrolytic processes. It seems likely that this is the case and that the carbon of the asparagin is not readily utilized, since the virulent organism will not grow with asparagin as the only source of
carbon. If the acidity were produced in this manner, leaving an acidic organic compound in the medium, the return of the pH to neutrality in the medium of the avirulent organism might be explained by the subsequent utilization of this compound as a source of carbon. Thus it seems likely that in our medium the pH curve may have been to a large extent dependent upon the metabolism of the asparagin.

CONCLUSIONS

1. In this work two strains of tubercle bacilli were employed, namely, a virulent H 37 and avirulent H 520. These seemed to be alike in their metabolism on the synthetic medium employed except that the avirulent H 520 bacilli grew more rapidly and produced a terminal alkalinity, while the virulent H 37 grew more slowly and produced a terminal acidity.

2. The titratable reaction and the pH curves closely paralleled each other throughout the course of the experiment. Apparently there was no relationship between the titratable alkalinity and the ammonia.

3. The ammonia content of the medium in the case of both organisms was small, but showed a maximum content at the time of greatest growth, followed by a recession. This would indicate that the alkalinity produced in the H 520 medium was not due to ammonia.

4. The nitrogen determinations showed that practically all of the nitrogen of the asparagin was utilized by both organisms. Apparently the organisms utilized the imino nitrogen of the asparagin more readily than the amino nitrogen.

5. The glycerol was practically completely utilized by both organisms, the decrease in the amount in the medium occurring much more rapidly with the H 520 than with the H 37. It appeared likely that the utilization was complete and that the glycerol was not partially oxidized to organic acids.

6. The acidity produced by the organisms in the medium seemed to a considerable extent to be due to the metabolism of the asparagin. The terminal alkalinity produced by the avirulent organism may be explained by the assumption that there oc-
curred further utilization of acidic compounds produced from the asparagin.

REFERENCES

Methods of Analysis of the Association of Official Agricultural Chemists, 1925. Washington, D. C.