THE CHEMICAL STUDY OF BACTERIA.

XII. THE ALBUMIN-GLOBULIN FRACTION OF THE TUBERCLE BACILLUS.*

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INTRODUCTION.

This paper, and one to follow, are preliminary reports dealing with the preparation, methods of purification, and proximate analysis of certain specific proteins present in the tubercle bacillus. Due to the great complexity of an aqueous extract of any complete organism, such as a bacterium, it is very difficult to separate a given product from such solutions in the state of purity which is to be desired. This is particularly true of the protein constituents of cells which, in addition to being separated from one another, must also be freed from adsorbed impurities. Many experimental difficulties are encountered in the development of a technique which can be recommended for general application in chemical work of this character, and until much larger quantities of bacteria are available, the further purification of these protein fractions must be postponed. In the light, however, of important results which have been reported by other workers in the biological field, and whose researches are dependent on the progress and success of our work, it has become particularly advisable to publish certain findings which have been made to date.

In a previous publication (1) has been charted the procedure which has so far been developed for the separation of the various constituents of the bacterial cell. The present work has been

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carried out in accordance with that plan except for several minor operations which are now discussed in greater detail. All of the bacilli used were grown by the H. K. Mulford Company of Philadelphia. The medium used was made entirely from chemicals of established purity. This eliminates the possibility that any product obtained from the cells was not synthesized by the bacteria. The bacteria used were a human strain, H37. The culture media had the composition shown in Table I.

After 6 to 8 weeks growth under sterile conditions, the bacteria were filtered off on Buchner funnels, washed free from chlorides with distilled water, and dried in a vacuum oven at a temperature which was never greater than 50°C. They were then ground in a mortar or porcelain ball mill, and the resulting nearly white powder dried to constant weight in a vacuum desiccator over sulfuric acid.

After separation from the culture media, it is necessary that the bacteria be thoroughly washed with water and filtered as dry as possible before being placed in the vacuum drying oven, otherwise a clean white powder will not be obtained after grinding.

EXPERIMENTAL.

Before the bacteria are treated to remove their water-soluble constituents it is necessary to extract them with an organic solvent to remove fat. Although the ordinary bacteria contain only about 2 to 5 per cent of fat, the bacteria of the acid-fast group, to which the tubercle bacillus belongs, are characterized by their high lipid content, running from 15 to 50 per cent of the weight of

<table>
<thead>
<tr>
<th>TABLE I.</th>
<th>Composition of Medium.</th>
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<tbody>
<tr>
<td>Glycerol</td>
<td>50.0</td>
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<tr>
<td>Asparagine</td>
<td>5.0</td>
</tr>
<tr>
<td>Sodium citrate</td>
<td>6.0</td>
</tr>
<tr>
<td>KH₂PO₄</td>
<td>2.0</td>
</tr>
<tr>
<td>NH₄Cl</td>
<td>1.0</td>
</tr>
<tr>
<td>MgSO₄·7H₂O</td>
<td>0.5</td>
</tr>
<tr>
<td>Ferrous ammonium citrate</td>
<td>0.05</td>
</tr>
<tr>
<td>Water</td>
<td>1000</td>
</tr>
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</table>
the desiccated cells. This fat encases the bacteria and cements them together and must be removed in order to get at the interior of the cell.

To avoid any changes which heat might induce in the protein constituents, this defatting process was done at room temperature. Experience with different procedures showed that decantation with cold ether was the best method. Four such treatments, allowing the ether to stand on the bacteria for 2 or 3 days each time, give practically a complete extraction, the fourth extract being nearly fat-free. This treatment does not remove all the fat and waxes of the bacteria, but merely that portion which constitutes the protective coating of the cells and is easily soluble in ether. Unless this is first removed it is not possible to accomplish a quick extraction of the protein fraction with cold water. With the bacteria used in this research, the yield of fat varied from 18 to 20 per cent of the weight of the dried cells.

The subsequent treatment to obtain the water-soluble proteins will be discussed under two sections. In the first treatment distilled water was used immediately after the defatting of the bacteria, and in the second, the initial solvent used was a 5 per cent aqueous solution of sodium chloride.

A. Water as Initial Solvent.

100 gm. of dry bacteria were defatted as described above, 20.3 per cent of fat being obtained. Three extractions with cold distilled water were then made, the bacteria being allowed to macerate for 36 hours each time. Toluene was used as a preservative. These extracts, made at room temperature, were then combined to give a total volume of 2380 cc. This solution was centrifugalized as well as possible in a cup centrifuge rotating at a speed of 1500 revolutions per minute, and the protein precipitated by addition of acetic acid.

The protein thus obtained was separated by means of the centrifuge and redissolved in the least possible amount of 1 per cent sodium hydroxide solution. A small quantity of insoluble residue was removed by centrifugalizing and the protein reprecipitated with acetic acid and again submitted to this same process of purification. After the third precipitation with acetic acid, the protein was washed once with 2 per cent acetic acid, twice with 95
per cent alcohol, twice with ether, and then dried in a vacuum desiccator over sulfuric acid. The yield was 6.0 gm. of a nearly white powder.

This protein gives negative tests for sugar with both Fehling's and Benedict's solutions, but a very strong Molisch test. It also gives all the ordinary protein reactions. The results of analyses and nitrogen distribution are recorded in Table II. The sulfur content of the basic fraction of this protein was so low that it could not be satisfactorily determined. The calculations for nitrogen distribution were made assuming the absence of cystine, although there is possibly a trace present.

A second experiment was run with a few variations in procedure. In this case 340 gm. of bacteria were taken. Extraction of the cells with ether removed 18.4 per cent of fat. The bacteria residue was extracted with distilled water at room temperature for 2 days and then centrifugalized as well as possible in a cup centrifuge at a maximum speed of 1500 r.p.m. During this time chloroform

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No. 502 according to our laboratory record. This fraction, corresponding to section (S) of our published scheme of analysis (1), has been reported to us as being a very strong tuberculin by Dr. Esmond R. Long of the Sprague Institute, University of Chicago (unpublished).
was used as a preservative. This brings it to the stage where in
the above experiment the protein was precipitated with acetic
acid. In this experiment, however, the water extract was twice
run through a Sharples supercentrifuge at a speed of 45,000 R.P.M.
In this manner we succeeded in obtaining a clear and light brown
fluorescent liquid. Acetic acid was added to this to maximum
precipitation, and without further purification, the protein which
separated was washed with dilute acetic acid, 95 per cent alcohol,
and ether, and finally dried in a vacuum desiccator over sulfuric
acid. The desiccated protein weighed 3.03 gm. and contained
15.5 per cent of nitrogen. It gave the usual protein reactions, in-
cluding the Molisch test, but it responded to no other tests for
sugar either before or after hydrolysis.

The bacteria residue from the first aqueous extraction was given
a second and third extraction with water in just the same manner

| TABLE III. |
|---------------------|-------|--------|--------|-------|
| Partition of Extractable Protein | | | | |
| Weight of protein, gm. .......... | 3.03  | 0.35   | None.  | 3.38  |
| Nitrogen in protein, per cent. .... | 15.5  | 11.1   |        |       |

as the first. The results of these extractions are summarized in
Table III

B. 5 Per Cent Sodium Chloride as Initial Solvent.

100 gm. of bacteria were defatted by maceration and decanta-
tion with ether, yielding 18.4 gm. of fat. They were then treated
with 600 cc. of a 5 per cent sodium chloride solution and shaken
occasionally over a period of 2 weeks, chloroform being used to
maintain a sterile condition. This caused them to swell to ten
times their original volume. The supernatant extract was then
carefully decanted and the residue washed four times by decanta-
tion with 200 cc. portions of 5 per cent sodium chloride solution.

The brine extract and washings were combined and then cen-
trifugingized in the cup centrifuge at a speed of 1500 R.P.M. The
solution, which was now slightly fluorescent and of a yellowish
brown color, was twice run through the Sharples supercentrifuge
at a speed of 35,000 R.P.M. It came out sparkling clear and showed an acidity corresponding to pH 6.0. This solution was then dialyzed free from chlorides in a parchment sac in the presence of toluene. This caused the separation of a minute amount of a colorless flocculent precipitate, which, however, gave a negative biuret test, showing its non-protein character. Judging from this result we conclude that the tubercle bacillus must contain a very small amount of a globulin, if any.

After dialysis, the aqueous solution was saturated with ammonium sulfate and the precipitated protein filtered off, dissolved in fresh water, and again dialyzed under aseptic conditions until Nessler's solution gave only a slight test for ammonia and the test for sulfate radical was entirely negative. Two volumes of 95 per cent alcohol were then added and the resulting protein precipitate was washed twice each with 95 per cent alcohol and ether and finally dried in a vacuum desiccator over sulfuric acid. As it dried black, it was redissolved in water, precipitated with alcohol again, and dried by washing twice with 95 per cent alcohol, once with absolute alcohol, twice with absolute ether, and once with dry toluene. In applying these washings use was made of the centrifuge as efficient filtering was impossible.

This protein, after being dried in a vacuum desiccator, was obtained as a light gray powder which contained 11.3 per cent nitrogen. It gave all the usual protein tests, including a strong Molisch reaction, but did not contain enough potential reducing substances to give even a trace of reduction with Benedict's solution after hydrolysis.

<table>
<thead>
<tr>
<th>TABLE IV. Summary of 5 Per Cent Aqueous Sodium Chloride Extracts.</th>
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<tr>
<td>Experiment I.</td>
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<tr>
<td>--------------</td>
</tr>
<tr>
<td>Bacteria</td>
</tr>
<tr>
<td>Fat removed</td>
</tr>
<tr>
<td>N in defatted bacteria</td>
</tr>
<tr>
<td>&quot; &quot; NaCl extract</td>
</tr>
<tr>
<td>Globulin</td>
</tr>
<tr>
<td>Water-soluble protein</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>gm.</th>
<th>per cent of total N</th>
<th>gm.</th>
<th>per cent of total N</th>
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<tbody>
<tr>
<td>8.65</td>
<td></td>
<td>8.47</td>
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<tr>
<td>18.4</td>
<td></td>
<td>20.6</td>
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<tr>
<td>10.6</td>
<td></td>
<td>10.7</td>
<td></td>
</tr>
<tr>
<td>35.5</td>
<td></td>
<td>36.0</td>
<td></td>
</tr>
<tr>
<td>11.3</td>
<td></td>
<td>9.35</td>
<td></td>
</tr>
<tr>
<td>0.65</td>
<td></td>
<td>0.74</td>
<td></td>
</tr>
</tbody>
</table>
A second experiment was run in exactly the same manner, to serve as a check on the first. In this case no precipitate was obtained upon dialysis of the original extract, which corroborates the non-protein character of the precipitate obtained at this point in the first experiment. The results of these two experiments are conveniently summarized in Table IV.

**DISCUSSION.**

A careful analysis of the analytical data recorded in Table IV reveals some very important facts which will have an immediate influence on the future course of our researches on tubercle bacilli. As mentioned in the first part of this paper, it must be borne in mind that the water extract of bacteria is a very complicated mixture. Table IV shows that extraction with cold 5 per cent sodium chloride solution removes 36 per cent of all the nitrogen of the bacteria. In Experiment I, after dialysis only 0.655 gm. of nitrogen remained within the parchment sac, showing that at least 2.42 gm., or 28 per cent of all the nitrogen in the tubercle bacilli, is in the form of non-protein, dialyzable compounds. In what organic combinations it exists is a problem which must be decided by future research. To date the only definite nitrogen-containing constituent that has been separated from it is a small amount of free tyrosine, and from this result it might be expected that other amino acids are floating free within the bacterial cell. Whether this aqueous extract contains sugar in other combinations than are found in nucleic acids remains to be determined.

Another interesting point to be emphasized is in connection with the persistent Molisch test which is encountered in all the preparations. This test is so delicate that it will serve to detect the smallest traces of a carbohydrate grouping, so it is not surprising that proteins prepared from a bacterial extract should give the reaction. A fresh extract of the tubercle bacillus will not reduce Fehling’s or Benedict’s solutions until after acid hydrolysis, when it gives a very strong reduction. This is due to the presence of glycogen and nucleic acid existing in the free condition (3), which breaks down on hydrolysis, and also to the polysaccharide combination which Mueller (4) and Laidlaw and Dudley (5) have succeeded in isolating, and which is the specific substance in the precipitin reaction. Thus in the water extract of this bacteria
we are dealing with proteins, nucleic acid, glycogen, a polysaccharide, free amino acids, inorganic salts, and probably several other organic compounds as yet unknown.

An interesting difference is noted in the nitrogen content of the protein fractions prepared by acetic acid precipitation of a water extract. In the first case, where the extract was centrifugalized at a speed of only 1500 R.P.M. the protein had a nitrogen content of 12.3 per cent. In the next experiment, the Sharples supercentrifuge was used, and by means of this high speed a jelly-like substance is removed having a nitrogen content varying from 5 to 9 per cent of this element in different experiments. As a result of removing this substance, the yield of the protein fraction is reduced from 6 to 1 per cent, but the nitrogen content jumps from 12.3 to 15.5 per cent. The protein with low nitrogen percentage and, we assume, of very high impurity, will act satisfactorily as an antigen in complement fixation reactions, while the product with the high nitrogen, which indicates a higher state of purity of the protein, is inactive when used biologically in the same manner (6). What this protein impurity is remains to be established by further work.

Although the literature on the proteins of the tubercle bacillus is quite extensive, only a brief mention will be made of it here. The emphasis in this work was placed on obtaining from the cell a protein in its original form if possible. To accomplish this end the bacteria were never heated, nor treated with mineral acids at all, or acetic acid above 2 per cent strength. No alkali was used in their preparation, except in one instance. The only reagents which have been used in any manner, except the one case of dilute alkali, were dilute acetic acid and the inert solvents ether, alcohol, chloroform, and toluene. On the contrary, the literature deals with proteins which have been obtained by autoclaving, acid and alkali extractions, and other means which would lead to deep seated changes in the protein molecule. The products obtained hitherto have lost the structural characteristics of the proteins existing in the cell.

Due to the lack of materials, no quantitative analyses were made for sulfur or phosphorus, although qualitative tests show these elements to be present in very slight amounts.

In regard to the amino acid analyses, the high percentage of the
hexone bases is the most striking characteristic. The arginine, in particular, is much higher than is generally found in proteins with the exception of the protamines and so called histones.

**SUMMARY.**

1. A water-soluble protein having the properties of an albumin has been isolated in an unaltered condition from the tubercle bacillus.

2. Evidence is presented which would indicate that the globulin content of the tubercle bacillus is very small if any.

3. This water-soluble protein shows an unusually high content of basic amino acids.

4. The protein preparations described have been reported as having a pronounced biological activity, and further investigation dealing with their specific properties is now in progress.

**BIBLIOGRAPHY.**

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