THE CHEMICAL STUDY OF BACTERIA.

XXIX. A PROXIMATE ANALYSIS OF A DEFATTED RESIDUE OF AVIAN TUBERCLE BACILLI.

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INTRODUCTION.

For some time a chemical study of tubercle bacilli (Strain H-37) has been in progress in this laboratory. A recent extensive study of the lipoids has been made by Anderson (1). Aside from that investigation, attention has been centered chiefly on the nitrogen constituents of the cell—more particularly proteins and nucleic acid. The nitrogen content of the defatted cell is 10 to 11 per cent, indicating a large proportion of protein, for which the distribution of amino acids has been determined (2, 3). The aqueous or saline extracts of the cell residue yield only 1 to 2.5 per cent of protein, but in addition, contain nitrogen in dialyzable, non-protein combination (4). This water-soluble protein has the characteristic biological activity associated with tuberculin preparations (4, 5). A second and larger yield of protein may be obtained by extraction with 0.5 per cent sodium hydroxide solution. Van Slyke analyses indicate a higher content of basic amino acids for this protein than for the water-soluble protein (6). However, since the protein from the alkaline extract has very little tuberculin potency in skin tests, it has received relatively little attention.

The findings with regard to the constitution of tuberculinic acid place it among the animal nucleic acids. The purines adenine and guanine were identified by Levene (7) and by Long (8). Johnson and Brown isolated the pyrimidines thymine and cytosine (9), and later Johnson and Coghill identified methyleytosine (10). As further evidence for the classification of tuberculinic acid, the

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presence of a hexose sugar was demonstrated by the isolation of levulinic acid (11).

In a recent paper Johnson and Renfrew (12) have reported a carbohydrate obtained from the aqueous extract of the defatted Strain H-37 residue. In the present paper we note, in relation to the analysis of the avian residue, the isolation of a carbohydrate fraction from the alkaline extract of the bacillus, Strain H-37; this preparation has a higher pentose content (as determined by a furfural distillation) than the carbohydrate from the aqueous extract. Laidlaw and Dudley (13) have previously described a carbohydrate which gave specific precipitin reactions; their product was obtained from a human strain of tubercle bacilli.

During the course of the above investigations, a general scheme of procedure for the analysis of bacteria was developed by Johnson (14) and later elaborated by Johnson and Anderson (15). This procedure has been followed in a large measure in the study of the avian strain of tubercle bacilli.

EXPERIMENTAL.

The avian tubercle bacilli (Strain 531) were grown on Long's synthetic medium (16) by the H. K. Mulford Company at Glenolden, Pa. A chemical study of weekly samples from this growth (Lot II) has already been reported (17). More recently Anderson (18) has described the extraction of lipoid from these organisms by digestion in a 50 per cent alcohol-ether mixture and in chloroform. The defatted residue (Fraction G of the chart outlined by Johnson (14)), was finally dried by passing an air current over thin layers of the cellular material.

200 gm. units of the air-dried residue were used in the following study. Since this material lost 17 per cent in weight when dried over dehydrite at 60° in vacuo, percentage yields are calculated on the basis of 166 gm., dry weight.

Preparation of Avian Water-Soluble Protein.—166 gm. of defatted, desiccated, and finely pulverized bacilli were allowed to macerate for 6 hours in a ball mill with 600 cc. of water and a little toluene. About 380 cc. of a densely milky liquid were recovered during centrifugalization of the macerated paste, and the moist residue was returned to the ball mill for a second extraction. The combined extracts were clarified by filtration through car-
fully washed paper pulp and through a Berkefeld candle. A third extract was clarified and analyzed separately to determine the completeness with which water-soluble substances had been removed.

The Berkefeld filtrate (Fraction R (14)) had a pH of 6.5, corresponding closely to the reaction of similar extracts of the human organism. The solution contained 0.70 gm. of nitrogen or 4.5 per cent of the total nitrogen of the defatted cells. Maximum flocculation of protein was obtained in the presence of 2 to 4 per cent acetic acid. The water-soluble protein, thus prepared, did not reduce Benedict's solution either before or after hydrolysis. The yield was about 0.48 gm. (0.38, 0.58, 0.56, 0.42 gm.) with a nitrogen content of 14.46 per cent.

Isolation of Carbohydrate Fraction from Protein Filtrate.—In the study of the tubercle bacillus (Strain H-37) a carbohydrate (12) was separated from the acetic acid filtrate after the removal of protein. A similar, but much smaller, amount of material, precipitable with basic lead acetate, has been found in the corresponding aqueous extract of the avian organism.

The acetic acid solution (Fraction T) was first concentrated to a volume of 150 to 200 cc. in vacuo at 40°. A precipitate, obtained at this point after the addition of 2 volumes of 95 per cent alcohol, was removed and is recorded in this paper as avian Fraction Aq-3. The alcoholic supernatant liquid was concentrated to 75 cc. and treated with a 20 per cent lead acetate solution till no further precipitation occurred. The final concentrate at a volume of 30 cc. was precipitated with basic lead acetate (Goulard's solution (19)) and excess ammonium hydroxide. 1.9 to 2.3 gm. or a 1.4 per cent yield of carbohydrate was recovered from the basic lead acetate precipitate after removal of the lead as sulfide and the dehydration of the concentrated syrup with absolute alcohol.

The slight reducing properties of the avian carbohydrate were greatly increased after acid hydrolysis. 0.0362 gm. was heated with 5 per cent sulfuric acid for 8 hours on a boiling water bath. The reducing value of this hydrolysate as determined by the Shaffer-Hartmann micro method was equivalent to 0.0177 gm. of glucose—or 48.8 per cent of the weight of carbohydrate taken. A sample of the carbohydrate from Strain H-37 bacilli was hydrolyzed at the same time and gave 52 per cent of reducing sugars
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calculated as glucose. That hydrolysis is rather difficult is shown by the values for solutions treated 5 hours with 0.5 N hydrochloric acid at 80-100°. The glucose equivalent for the avian sugar was 37 per cent and for the human 39 per cent.

The avian carbohydrate contains small amounts of both nitrogen and phosphorus and gives 8.2 per cent pentose as calculated from the furfural phloroglucinol obtained in a furfural distillation.

Avian Fraction Aq-3.—The avian Fraction Aq-3 was a white powder, only partially soluble in water. A clear solution, which was easily obtained by the addition of a little 0.1 N hydrochloric acid, clouded with a rather gelatinous precipitate when the reaction was made neutral or alkaline. A solution of Fraction Aq-3 gave a fairly heavy precipitate with molybdate reagent but did not reduce Benedict's solution. Tests for glycogen and protein were negative. The hydrolyzed solution of Fraction Aq-3 was an actively reducing solution and gave a heavy precipitate when tested with molybdate reagent. A Weidel test was negative.

Preparation of Avian Alkali-Soluble Protein.—The insoluble residue from the aqueous extraction was not dried, but was again returned to the ball mill with 500 cc. of dilute alkali. After two extractions with 0.5 per cent sodium hydroxide, the pasty residue was macerated with 400 cc. of water. The two alkaline solutions were combined, clarified, and filtered through a Berkefeld candle. The nitrogen content of the Berkefeld filtrate was 1.26 gm. of nitrogen, or 8.1 per cent of the total nitrogen in the original defatted bacilli. This solution had a pH of about 9.2. Heavy flocculation of protein followed the addition of acetic acid to a concentration of 0.3 to 0.5 per cent.

The alkali-soluble protein weighed about 2.9 gm. and on analysis gave 15.86 per cent of nitrogen. This preparation gives a Molisch reaction but does not reduce Benedict's solution either before or after hydrolysis. The final 400 cc. of water washings of the residue contained 0.8 gm. of protein.

Table I indicates comparative yields of protein and carbohydrate from avian and from human bacilli, which have been defatted in accordance with the method described by Anderson (18).

The supernatant solutions after acetic acid precipitation of the alkali-soluble protein were concentrated to small volume and fractionally precipitated with alcohol. After acid hydrolysis
these fractions, designated Fractions O3 and O4 have marked reducing properties.

**Bacterial Residue Insoluble in 0.5 Per Cent NaOH (Residue P).**—
The weight of the bacterial residue recovered from the alkaline extraction was about 100 to 110 gm. This residue contains 10.05 per cent of nitrogen, which may be regarded as largely protein nitrogen in view of the satisfactory results for amino acid determinations (2) and Van Slyke (3) analyses when the insoluble bacterial residue of Strain H-37 is treated as whole protein. The avian residue gives positive biuret and Millon tests and after hydrolysis reduces Benedict's solution. An attempt was made to isolate an osazone from the hydrolysate of the avian residue, Residue P, after the removal of amino acids with mercuric acetate. Only a very small amount of a yellow osazone with the apparent

| TABLE I. |
| Comparative Yields of Bacterial Products. |

| 100 gm. unit. | H₂O-soluble protein. | Carbohydrate from aqueous solution. | Alkali-soluble protein. |
|---------------|---------------------|-----------------------------------|------------------------|
| Avian (Strain 531)                      | 0.35                | 1.4                               | 2.9                    |
| Human (Strain H-37)                     | 0.50                | 3.9                               | 9.6                    |

properties of phenylglucosazone was obtained. This osazone, which was soluble in alcohol but insoluble in acetone, melted at 175–180°; a mixture of phenylglucosazone with the unknown product melted at 189–192°.

**Liberation of Additional Quantities of Lipoid after Treatment of Defatted Residue G or Residue P.**—It has been previously noted by Long (20) and others that even after exhaustive extraction with fat solvents, the bacterial residue still contains lipoid materials which may be brought into soluble form by acid hydrolysis. 10 gm. portions of the defatted avian Residue G (14) were hydrolyzed for 2 hours with 20 per cent hydrochloric acid. The residue (about 2 gm.) from the filtered solution was thoroughly washed, dried, and extracted with chloroform. Evaporation of the chloroform leaves a waxy substance which is soluble in chloroform and in ether but insoluble in acetone or alcohol. For the avian bacillus
this lipoid fraction represented 15 per cent of the weight of the residue hydrolyzed and for the human about 8 per cent. A Liebermann-Burchard test gave no evidence of cholesterol in the avian wax.

Only 11 per cent of lipoid was obtained after hydrolysis of Residue P (14), which had been freed from substances soluble in 0.5 per cent sodium hydroxide. This lower percentage yield as compared with the original bacterial Residue G would suggest a loss, as yet unaccounted for, in the chloroform-soluble fraction during alkaline extraction. There is still very little lipoid in directly soluble form in Residue P, since several successive extractions removed only 2.5 per cent of a chloroform-soluble, semitransparent, waxy material.

A lipoid determination was also carried out under conditions more closely resembling those described by Long (20). 10 gm. of the defatted avian Residue G were heated 2 hours on a steam bath with 70 cc. of N hydrochloric acid. The yield of 9.4 per cent of lipoid was probably low because of losses in the repeated filtration needed to clarify the chloroform solution. The wax thus obtained was of a more brittle consistency than that of the corresponding product from treatment with 20 per cent acid. Even after the chloroform solution of the lipoid had been filtered through a Berkefeld candle, the waxy product from treatment with N hydrochloric acid gave slightly positive tests for both nitrogen and phosphorus. A Liebermann-Burchard test for cholesterol was negative, and the chloroform layer in the Salkowski test reddened only after 6 to 8 hours.

DISCUSSION.

Certain differences in the proportionate amounts of the fractions of the avian bacillus as compared with the human strain are brought out in the above chemical study. Under the same experimental conditions distinctly less carbohydrate, precipitable with basic lead acetate, was obtained from the avian aqueous extract: 1.4 per cent from the avian organism and 3.9 per cent from Strain H-37, or a 1:3 ratio. Since this product is rather easily extracted and isolated, the difference in yields seems marked and unquestionable; however, this difference is less striking than


**TABLE II.**

*Nitrogen Distribution in Defatted Avian Tubercle Bacilli.*

|                    | Avian Strain 531. |                    | Human Strain H-37 |
|--------------------|-------------------|--------------------|-------------------|
|                    | Experiment 1.      | Experiment 2.       |                   |
| Defatted bacteria. | 100 gm. 9.33%     | 100 gm. 9.33%      | 100 gm. 10.72%    |
| Aqueous extract.  |                   |                    |                   |
| Protein           | 0.288 gm. 14.66%  | 0.293 gm. 13.78%   |                   |
| Non-protein N*    | 4.44%             | 4.1%               |                   |
| Total N content of extract | 4.45% | 4.6% | 1.21% | 11.3% |
| Alkaline extract. |                   |                    |                   |
| Protein           | 2.2 gm. 15.86%    | 3.1 gm. 16.24%     |                   |
| Non-protein N*    | 4.5%              | 5.3%               |                   |
| Total N content of extract | 8.1% | 8.4% | 2.04% | 10.0% |
| Insoluble residue†| 74 gm. 10.05%     | 73.2 gm. 12.38%    |                   |
| Total N accounted for | 92.6% | 86.2% | 70.7% |

* Non-protein nitrogen calculated by difference.
† Residue nitrogen was not accurately determined in consequence of unavoidable losses in recovery of residue.
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the comparative 1:6 values determined for copper-reducing substances in the study of the culture medium of these two organisms.

The differences in nitrogen distribution for the avian and human strains may be seen in Table II. The data for the human strain are average results from extractions of unit lots of Strain H-37 bacilli defatted by Anderson (1). A consideration of Table II shows a definitely lower percentage of water-soluble non-protein nitrogen for the avian bacillus, and a smaller quantity of alkali-soluble protein as compared with Strain H-37. It is interesting to note that the amount of alkali-soluble protein extracted from the avian bacillus closely parallels the results obtained by Coghill and Bird (21) for a non-pathogenic organism, the timothy bacillus. The amounts of water-soluble protein, extracted from exhaustively defatted residues of both avian and human strains, are about the same; such very low yields, however, make quantitative comparisons unsatisfactory. A more significant comparison might be obtained if the yield of water-soluble protein were known for an avian residue treated only with ether, inasmuch as protein yields seem to be higher under these conditions (12).

It seems very possible that the lipoid which is liberated in available form only after acid treatment of the bacilli may have biological properties of marked interest. Long (20) has studied this fraction in relation to the acid-fast properties of the bacterial cell. However, we are not familiar with any investigation of this material under conditions comparable to the studies of Sabin and Doan with the phosphatides from tubercle bacilli.

The proximate chemical analysis of the aqueous and alkaline extracts of avian and human bacilli does not provide any single outstanding factor which, ipso facto, would serve to distinguish between the two types. There are marked similarities in the general fractions separated. Nevertheless, there are differences in the relative amounts of various fractions from the two types and a more precise chemical study may show differences in the chemical components of corresponding fractions. Whether the substances isolated will have biological specificity remains to be determined and the character of further chemical work must in a large measure depend upon the biological findings.
SUMMARY.

1. The defatted avian residue has been subjected to extraction with water, sodium chloride solution, and a solution of 0.5 per cent sodium hydroxide. The nitrogen distribution under these conditions has been determined.

2. The protein comparable to Protein 304 (12) of the human strain, the carbohydrate material from Fraction T (14) comparable to Carbohydrate 81 (12) of the human strain, the protein extracted by 0.5 per cent sodium hydroxide, and certain lipoid fractions have been isolated from the avian organism for testing.

3. The present yields of the carbohydrate fraction and the yields of the alkali-soluble protein are low in comparison with similar fractions from Strain H-37.

4. Further quantities of lipoid of a waxy character have been obtained from human and avian strains after acid treatment of bacterial residues which had previously been thoroughly extracted with fat solvents.

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