

THE CHEMICAL CONSTITUTION OF THE TUBERCLE BACILLUS.

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SHORTLY after Koch's discovery of the tubercle bacillus Ehrlich⁽¹⁾ made the important observation that this micro-organism can be distinguished from others by certain tinctorial characteristics, of which the most remarkable is its resistance, when once stained, to the decolorising action of mineral acids. This acid-resisting property or "acid-fastness" has since Ehrlich's discovery been used for the microscopic diagnosis of the bacillus. In his original communication Ehrlich recommended the addition of anilin water to the stain (fuchsin), the decolorising action being subsequently carried out by a 33% solution of nitric acid. With slight modifications, viz. the substitution of anilin water by carbolic acid and of the nitric acid by 25% sulphuric acid, the method is universally known at the present day as that of Ziehl-Neelsen^(2 and 3).

That this acid-fastness of the tubercle bacillus and its congeners is due to some peculiarity in its chemical constitution can scarcely be doubted, and attempts have not been wanting to isolate the substance.

In general it has been stated that the essential substance, in virtue of which the bacillus is acid-fast, is *fat*, and various researches have in reality shown that in the bodies of the bacilli there are considerable quantities of fat and fatty acid, and that the amounts of these constitute a peculiarity of the bacillus.

Hammerschlag⁽⁴⁾ for instance found 27% of substance soluble in alcohol and ether.

De Schweinitz and Dorset⁽⁵⁾ found the amount of fat even higher, viz. 37%. On saponifying the fat and finally decomposing with sulphuric

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acid they demonstrated the existence of palmetic, arachidic and lauric acids. Klebs⁽⁶⁾ found in the pure ethereal extract a solid reddish fat melting at 42° C. and constituting 20·5 % by weight of the bacillary mass. In addition he found a colourless fat insoluble in ether, but soluble in benzene, and having a melting-point over 50° C. This fat was present to the extent of 1·4 %. By Klebs the acid-fastness is referred to these fats.

Ruppel⁽⁷⁾⁽⁸⁾ described three kinds of fatty substance which can be successively extracted by cold alcohol, hot alcohol, and by ether. Cold alcohol extracted about 8 % of the total weight of the body substance of the bacillus. On evaporation a quantity of free fatty acid was found and a fat which was readily saponified. By hot alcohol a peculiar colourless mass was obtained which began to liquefy at 65° C. but it did not become perfectly clear when heated even to 200° C. This substance was very difficult to saponify but appeared to be composed of fatty acid, esters, and higher alcohols. Further extraction of the bacilli by ether yielded a substance melting at 65°—70° C. The total amount of substances extracted by alcohol and by ether varied from a minimum of 8—10 % to a maximum of 26 % of the total weight of the bacilli.

Aronson⁽⁹⁾ extracted the dried bacilli with an alcohol-ether mixture containing 1 % hydrochloric acid. After evaporating off the alcohol-ether a brown mass was obtained amounting to 20—25 % of the original T. B. An analysis of this substance showed it to contain 17 % of free fatty acid, the remainder consisting of a body which Aronson supposed to be of the nature of a *wax*. On prolonged boiling with alcoholic potash the wax yielded an insoluble residue which dissolved in acetic anhydride to form an acetate, showing that it contained a hydroxyl group and was probably an alcohol.

De Giaxa⁽¹⁰⁾ obtained 35·2—40·4 % by weight of substances soluble in alcohol and ether. Levene⁽¹¹⁾ obtained 31·56 % of fatty substances.

Recently Kresling⁽¹²⁾ has investigated more closely the nature of the fatty substances in the tubercle bacillus. He found that the best extractive was chloroform, the substance extracted by this reagent possessing the following properties :

Melting point	46° C.
Acid value	23·08.
Reichert Meissl value	2·007.
Hehner's value	74·236.
Saponification value	60·70.
Ether value	36·62.
Hüb's iodine value	9·92.

According to Kresling the so-called "fat" of the tubercle bacillus is a mixture of neutral fat, free fatty acid, esters, and higher alcohols (lecithin and cholesterin) and a number of extractives soluble in ether, alcohol, chloroform, and benzene. A quantitative estimation (calculated from the above values) of the fat substance soluble in chloroform showed,

Free fatty acid	14.38 %.
Neutral fats and fatty acid esters	77.25 "
Alcohols obtained from fatty acid esters	39.10 "
Lecithin	0.16 "
Substances directly soluble in water	0.73 "
Substances (soluble in water) formed during the complete saponification of the fat	25.764 %.

By treating a solution of the fat in benzene with sodium alcoholate Kresling succeeded after three days in producing saponification, and on removal of the soap and evaporation of the filtrate he obtained 380 grm. of an alcohol the melting-point of which was 43.5—44° C.

The results which we have to record are part of a general research on the chemical constitution of the tubercle bacillus. We have thought it of importance however to deal with the subject in parts, especially as the results which we have obtained in connection with the acid-fast substance complete the observations of Kresling.

Before proceeding to detail these results we have to express our sincerest thanks to Professor Bang and Dr Striboldt of Copenhagen and Professor O. Malm of Christiania for having placed at our disposal large quantities of tubercle bacilli necessary for the examination. We also desire to thank Dr J. Lewkowitsch for most valuable advice and assistance, especially in the determination of the purity of the substances which we have been able to extract.

Altogether we have experimented on several kilogrammes of bacilli—the deposit obtained by filtering autoclaved cultures for the preparation of tuberculin (T.O.)

The research has been mainly chemical, but all the products were tested for acid-fast properties by the usual methods.

From the experiments of Aronson and others it was known that the acid-fast substance is more or less extractable by substances which dissolve fats. After a long series of tentative experiments we adopted the following methods:

A weighed quantity of tubercle bacilli dried to constant weight was extracted in a 2-litre flask connected with a vertical condenser, the extracting substances being,

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(1) Methylated spirit followed by Aronson's mixture (alcohol-ether + 1% HCl).

(2) Methylated spirit followed by benzene, chloroform, or petroleum ether.

(3) Aronson's mixture alone.

In each case the extraction was allowed to proceed over a water-bath for at least several hours; in some cases for several days. As a result it was found that although methylated spirit was allowed to act for days it was unable to completely deprive the bacillus of its acid-fastness. When the extract was filtered through a hot funnel a white precipitate deposited on cooling. This white precipitate was acid-fast, the acid-fast substance being therefore partly soluble in spirit.

1. On applying Aronson's mixture to the bacilli which had thus been treated with spirit a further extract was obtained which on filtration deposited a white acid-fast precipitate. This process had to be repeated six or ten times before the bacillus was completely deprived of its acid-fast properties.

2. In a second series of experiments the white acid-fast substance was obtained by extracting with benzene, chloroform, or petroleum ether as a substitute for Aronson's mixture.

3. By using Aronson's mixture from the commencement large quantities of the acid-fast precipitate were obtained.

In this way filtrates and acid-fast precipitates were collected.

Filtrates. The clear filtrates which reacted strongly acid to phenolphthalein were evaporated slowly to dryness and treated with sodium carbonate solution in the boiling water-bath and again evaporated. The resulting dry mass was then shaken up in a separating funnel with a mixture of ether and water until the ether extracted no more. In this way we obtained an ethereal extract I, and a watery extract II, the latter containing the fats in the form of soap.

I. *Ethereal Extract.* On evaporating to dryness this yielded a residue with a peculiar odour, and which presented the following reactions:

(1) It was *not* acid-fast.

(2) It was blackened by osmic acid.

(3) It was readily stained by Sudan III, Fettponceau, and Indophenol, in 70% alcoholic solutions.

(4) Acrolein reaction positive.

(5) It did not contain phosphorus.

(6) No cholesterin crystals were found microscopically, although Salkowski's reaction was positive.

The iodine value obtained by a modified Hübl's method¹ showed in two samples 40·8% and 38·7% respectively. From this result the percentage of olein was calculated by Hübl's formula² to be 47·3% and 44·9%. 3·2 grms. of the fat were saponified by alcoholic potash and the resulting soap decomposed by H₂SO₄. The mixture of fatty acids obtained was semisolid at ordinary temperatures, and the solids were then separated from the fluid fatty acids by the following method:

The mass was melted in a beaker and dissolved in 70% alcohol. It was then filtered hot and allowed to cool. The solid acids separating out as a crystalline mass, were removed by filtration, and the precipitate thoroughly washed with 70% alcohol. In this way a filtrate and a precipitate were obtained:

a. The filtrate containing the liquid fatty acids was evaporated to dryness at a low temperature, and the residue was found to be instantly blackened by osmic acid and was probably oleic acid.

β. The precipitate was separated by fractional precipitation by means of an alcoholic solution of lead acetate, the exact technique being that described in Salkowski's *Practicum*.

The melting-points were determined in a capillary tube tied to the bulb of a thermometer, the latter being placed in a test-tube which was immersed in a flask containing conc. sulphuric acid.

The fractions gave the following melting-points:

- (1) 57° C.
- (2) 54·5° C.
- (3) not determined.
- (4) „ „

From these results a comparison with standard melting-points shows that the acids are probably isocetinic (55° C.) and myristinic (53·8° C.).

These solid acids were not blackened by osmic acid and were not in the slightest degree acid-fast.

II. *Watery Extract.* The watery solution (from the original filtrate which had been shaken up with a mixture of ether and water) was decomposed by sulphuric acid in the heat, when a precipitate settled out. No fatty acid however collected on the surface. In order to

¹ Benedikt und Ulzer, *Analyse der Fette*, Berlin, 1897, p. 150.

² Benedikt, *l.c.* p. 173.

obtain the fatty acid the decomposed fluid was shaken with ether in a separator till no more dissolved out. The ether was then evaporated to dryness when fatty acid remained. When collected in quantity this fatty acid had a melting-point of 41° C. It was not acid-fast. As the product was doubtfully pure we are unable to say what it was, but its melting-point corresponded to lauric acid.

Besides these acids the filtrate contained lipochromes, a solution of which in chloroform gave a very distinct band at β , a little to the right of Fraunhofer's line *E*. An aromatic body was also found, but was not investigated further.

The acid-fast white precipitates. The precipitates obtained by methylated spirit as an extractor were different from those obtained by extraction with Aronson's mixture, ether, etc., although they both agreed in being acid-fast.

Precipitate from methylated spirit extract. This became syrupy at 117° C. and on heating further became black and gave off a pungent vapour. Even when twice purified by solution in spirit and filtration this precipitate still showed the same peculiarities. On heating the purified precipitate with Aronson's mixture under a condenser and filtering, a white precipitate settled out of the filtrate on cooling. This precipitate had a melting-point of 47° C., this also being the melting-point of the substance obtained by extraction of T.B. with Aronson's mixture, benzene, petroleum ether, or chloroform. The whole purified methylated spirit precipitate was, however, not soluble in these fluids, as a darkly coloured substance still remained. This substance, strange to say, was still acid-fast.

With the investigation of this insoluble substance we have not proceeded, and we are quite in the dark as to its relation with the other acid-fast white precipitates obtained by chloroform, etc., although we believe its acid-fastness to be due to adherent wax.

As stated above the precipitate obtained by Aronson's mixture, chloroform, etc., melted at 47° C. and had the following properties:

It was intensely acid-fast. On staining with hot anilin-water, fuchsin, or carbol fuchsin for 5 minutes, it still retained its stain unaltered after immersion in 25% sulphuric acid for *eight* days. Even fuming nitric acid required several minutes to remove the stain. Counter-staining with alcoholic solution of methylene blue did not displace the fuchsin.

There can be no doubt that this substance is the chief ingredient in the bacillus which gives the latter its peculiar staining properties,

and its isolation in a state of purity was therefore a matter of considerable interest.

Saponification was attempted by dissolving it in warm petroleum ether, or in warm benzene, and adding absolute alcohol to the resulting solution until the white precipitate which formed on the first addition had redissolved and an opalescent solution had been obtained. Pieces of metallic sodium were then dropped into the solution and kept in motion by means of a glass rod until they had dissolved, only one or two pieces being added at a time. The sodium was added until no more effervescence ensued, after which the mixture was heated under a condenser for 3—4 hours.

The solution was then allowed to stand in a corked Erlenmeyer flask overnight, when a more or less copious precipitate of soap had fallen down. This was filtered by suction and the precipitate thoroughly washed with petroleum ether, and then removed to a flat dish and allowed to stand exposed to the air to remove the petroleum ether.

The filtrate poured into a shallow basin was allowed to slowly evaporate in air. In this way a white powder was obtained consisting of the alcohol mixed with sodium ethylate and sodium hydrate. This powder was partially soluble in boiling water, in which it formed an opalescent solution. To separate the alcohol the powder was placed in a large separating funnel and mixed with about 500 c.c. of methylated ether and about 100 c.c. of water. On shaking, a milky solution was obtained which reacted strongly alkaline to litmus. A 10% solution of sulphuric acid was added till the reaction became distinctly acid, when the ether at once became clear. After standing a few moments the ether was run off and allowed to evaporate in the air, when a white flaky powder was obtained. This however still contained 7% of ash, so that the above process had to be repeated till an ash-free product was obtained.

On examination the ash-free powder was found to be intensely acid and alcohol-fast. It was examined by Dr J. Lewkowitsch, who found it to give the following values :

Iodine value	9.39%
Saponification value	49.40 "
Melting-point	44.4 "
Increase of weight on acetylating	1.2 "
Saponification value of acetylated product	69.0 "

From these results it was evident that complete saponification of the wax had not been effected. At the same time the increase of weight on

acetylating showed that a large amount of a body containing a hydroxyl group (an alcohol) was present.

Saponification of the wax by metallic sodium was therefore unsuitable in obtaining the pure alcohol, and further attempts were made with alcoholic potash, the method we have ultimately adopted being the following :

Dried tubercle bacilli were extracted with boiling methylated spirit until the bulk of the fatty substances had been removed. The remainder, which was still acid-fast, was then boiled under a condenser with alcoholic potash for several hours, cooled and allowed to stand, when the alcohol was decanted off. The residue was then repeatedly shaken with petroleum ether and the extract was allowed to evaporate, when a white residue was obtained. This was again saponified by alcoholic potash. At this stage of the process we have found that certain precautions must be taken in order to obtain a pure preparation of the alcohol. The white mass was shaken up with boiling absolute alcohol and the resulting solution filtered through a hot funnel.

From the filtrate on cooling a white precipitate separates out, to disappear again on warming. Great care must be taken that the alcoholic solution becomes entirely clear on warming, as in the residue from the petroleum ether there is frequently a certain amount of a gummy like substance insoluble in warm absolute alcohol.

Pure caustic potash dissolved in as little water as possible was then added to the alcoholic solution in the amount of 3%, and the flask placed on a boiling water-bath and connected with a reflux condenser. It was allowed to boil for 4—5 hours, then cooled and the contents removed to a large separating funnel and thoroughly shaken with petroleum ether. Extraction with petroleum ether was continued until on evaporation of the ether no residue was obtained.

The ethereal extracts were then repeatedly shaken up with distilled water in the separator until the washings were no longer alkaline to litmus. To attain this end it is necessary to shake and wash for several days.

The washed petroleum ether extract was then evaporated and the alcohol obtained as a white flaky powder.

The saponification value showed that this powder was the *pure* alcohol of the tubercle bacillus, and it was further found that it is the alcohol which gives the tubercle bacillus its acid- and alcohol-fastness ; when small particles of the alcohol spread on a slide were stained with carbol fuchsin the stain was found to remain unaltered after prolonged

immersion in equal parts of 50% HNO_3 in methylated spirit and then in spirit. The fatty acids separated from the wax were not acid- or alcohol-fast, the fuchsin being instantly discharged, even with weak acid.

We have now obtained about 1 gramme of pure alcohol, and hope to be able to determine its elementary composition and molecular weight shortly.

Summary of Results.

1. Dried tubercle bacilli extracted with hot solutions of spirit, alcohol, alcohol-ether, Aronson's mixture, yield large percentages of fatty substances.

2. On filtering the boiling extracts a white acid-fast precipitate deposits on cooling.

3. The filtrates on evaporation can be saponified by soda, and on subsequent agitation with ether and water two extracts can be obtained—an ethereal and a watery extract.

4. The ethereal extract contains fat which is *not* acid-fast.

5. By saponifying the dried ethereal extract with alcoholic potash and decomposing the resulting soaps with sulphuric acid a mixture of fatty acids is obtained containing probably oleic, isocetic and myristic acids. None of these are acid-fast.

6. The watery extract (soap) on decomposition yielded a fatty acid with a melting point corresponding to lauric acid.

7. The filtrates also yield lipochromes to which the cultures of tubercle bacillus owe their colour.

8. The white acid-fast precipitate obtained by the original extraction can be saponified, but with great difficulty.

9. By prolonged boiling with alcoholic potash the acid-fast precipitate is decomposed and results in the deposition of an acid-fast snow-white flaky powder, and a non-acid-fast filtrate of fatty acids.

10. The chemical examination of the white flaky powder shows it to be an alcohol.

11. Acid- and alcohol-fastness of the tubercle bacillus is due to the presence of an alcohol.

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