THE CHEMISTRY OF THE LIPIDS OF TUBERCLE BACILLI

XXVI. SEPARATION OF THE LIPOID FRACTIONS FROM THE LEPROSY BACILLUS*

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(Received for publication, November 3, 1931)

INTRODUCTION

The causative agent of leprosy was first recognized by Hansen (1) in Norway who isolated the leprosy bacillus in 1873. The organism was studied by Neisser (2) and he determined that it was acid-fast. The bacillus was successfully cultivated by Clegg (3) in the Philippines in 1909 and shortly afterwards by Duval (4) in Louisiana. Duval (5) succeeded in producing typical leprous lesions in the monkey by the injection of massive doses of the artificially cultivated bacillus.

A review of the chemistry of the leprosy bacillus is given by Long (6) but it is evident from a perusal of the literature that only fragmentary information exists concerning the chemical composition of this organism. This lack of knowledge is surprising because leprosy has been a dreaded disease in all parts of the world from the earliest times to the present.

The staining properties of the fatty substances obtained from the leprosy bacillus by extraction with various solvents together with the acid fastness of the extracted bacilli were studied by microchemical methods by Unna, Jr. (7), while the first macro-

* An abstract of this paper was read before the Division of Medicinal Chemistry at the meeting of the American Chemical Society at Buffalo, 1931.

The present report is a part of a cooperative investigation on tuberculosis and it has been supported partly by funds provided by the Research Committee of the National Tuberculosis Association.

† Holder of the Leonard Wood Memorial Fellowship at Yale University for the year 1930-31.
chemical investigation of the lipoids was reported by Gurd and Denis (8) who used the organism that had been isolated by Duval.

These investigators found that the dry bacillus yielded about 34 per cent of fat together with a small quantity of a waxy solid containing phosphorus and which was regarded as lecithin. It was further found that the fat contained unsaturated fatty acids and a pigment which was classified as a lipochrome. After the fat had been saponified, a small amount of unsaponifiable matter was obtained which gave color reactions similar to those of cholesterol.

Long and Campbell (9) in a comparative study of the lipids of acid-fast bacteria found that the leprosy bacillus gave 9.7 per cent of total lipid. The lipid had a saponification number of 188 and contained 27.2 per cent of unsaponifiable matter. Other investigations dealing with the metabolism of Bacillus lepræ have been published by Long (10) and by Reed and Rice (11). According to Kendall, Day, and Walker (12) various acid-fast bacteria including Bacillus lepræ produce soluble lipase.

Since leprosy and tuberculosis are closely related diseases, it was decided to include the leprosy bacillus in the program of the cooperative chemical and biological investigation on tuberculosis which is being conducted under the auspices of the Medical Research Committee of the National Tuberculosis Association. Such a comparative study might reveal significant differences in chemical composition and in biological reactions of the lipoid fractions obtained from two closely related organisms which differ in virulence or pathogenicity.

In the extraction and separation of the lipoid fractions from Bacillus lepræ we have followed the methods which have been developed in this laboratory in connection with investigations on the tubercle bacilli (13). We were naturally anxious to secure the lipoid material in a condition as similar as possible to that in which it existed in the living cells. We therefore employed the mildest methods of extraction, using cold alcohol and ether followed by chloroform, and the extracts were concentrated at a temperature not exceeding 40°.

Through the cooperation of the Medical Research Committee of the National Tuberculosis Association and the Mulford Biological Laboratories, Sharp and Dohme, we were provided with a very large quantity of living Bacillus lepræ. The bacilli were
extracted with large quantities of alcohol and ether and then with chloroform.

The alcohol-ether extract was separated into phosphatide, acetone-soluble fat, and a small amount of wax. From the aqueous solution which remained after the ether and alcohol had been evaporated we isolated a considerable amount of polysaccharide. The chloroform extract yielded a large amount of wax on the evaporation of the solvent. All of these compounds were similar in properties to corresponding fractions obtained from tubercle bacilli, but they were much more highly pigmented.

Biological experiments are under way to determine the reactions produced by the lipoids of the Bacillus leprae and the results will be reported separately.

EXPERIMENTAL

The Mycobacterium leprae used in this work is known as the Hygienic Laboratory Strain No. 370 (Apa case) and it was isolated from a case of human leprosy in Honolulu about 1909. The organism has been carried in the Mulford Biological Laboratories at Glenolden, Pennsylvania, since February 4, 1926, and is there known as Strain 1629. The bacillus grows well on a synthetic medium and is highly chromogenic.

For the present work we were provided with 3000 cultures of Bacillus leprae and this is the largest quantity of acid-fast bacteria that we have ever worked up at one time. The cultures were grown for 6 weeks in 1 liter Pyrex bottles, each bottle containing 200 cc. of the Long synthetic medium (14). The living bacilli were collected on large Buchner funnels, washed with water, and immediately placed in a mixture of equal parts of alcohol and ether contained in 5 gallon Pyrex bottles.

During the filtration streams of carbon dioxide were passed over the funnels; the solvents used for extraction had been saturated with carbon dioxide. In all the subsequent operations that will be described below, air was always displaced by carbon dioxide. All solvents were freshly distilled and saturated with carbon dioxide before they were used. The alcohol had been distilled over potassium hydroxide.

It will be mentioned here and not referred to again that all of the bacterial extracts were filtered through Chamberland candles.
under carbon dioxide pressure before the lipoids were isolated. The filtrates thus obtained were brilliantly clear and we believe that all cell debris had been removed.

**Extraction with Alcohol-Ether**

The bacteria were equally distributed between seven 5 gallon bottles. The containers were securely stoppered, thoroughly shaken, and transported to the Sterling Chemistry Laboratory. The bottles were shaken occasionally for about 1 week; the bacterial cells were then allowed to settle and the clear, deep reddish colored supernatant extract was siphoned off with carbon dioxide pressure. The bacterial residues were distributed evenly between four 5 gallon Pyrex bottles and again extracted two times in a similar manner, 5 liters of ether and 1 liter of alcohol being used for each bottle. The bacteria were finally filtered on Buchner funnels and washed with ether. The alcohol-ether extracts and washings were combined and worked up, as will be described later.

**Extraction with Chloroform**

Two extractions with chloroform were carried out in the following manner. The bacterial residues were returned to the four large bottles, 4 liters of chloroform being added to each bottle, and the mixtures were shaken occasionally for about 1 week. Bacterial suspensions in chloroform do not settle and it is necessary therefore to recover the extract by filtration on Buchner funnels. After filtering, the treatment with chloroform was repeated and the mixtures were again filtered. The bacterial residues were finally shaken with a mixture of 4 liters of ether and 1 liter of alcohol for the purpose of removing the chloroform which adhered to the cells. After filtering and washing with the alcohol-ether mixture, the bacterial cells were dried in vacuo at 40° and reserved for the isolation of water-soluble constituents such as proteins, carbohydrates, nucleic acid, etc. The dried bacterial residue formed a chocolate-colored crumbly mass weighing 3389.8 gm.

**Chloroform-Soluble Wax**

The last alcohol-ether extract mentioned above was concentrated to dryness, when a waxy residue was obtained which weighed 23.3 gm., and this was combined with the main lot of the
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chloroform-soluble material. The chloroformic solutions were evaporated to dryness under reduced pressure, leaving a deep reddish waxy residue. The material constitutes the crude wax; the total weight was 444.8 gm. It was analyzed for phosphorus and nitrogen but only traces of these elements were found, thus indicating that the wax fraction was practically free from phosphatide. The material is reserved for future investigations.

Examination of the Alcohol-Ether Extract

The extract was perfectly clear and of deep reddish color. The ether was removed at a temperature of 35–40° by a current of carbon dioxide and the alcohol was distilled off under reduced pressure at the temperature mentioned above, carbon dioxide being admitted through the capillary tube. The dark red lipoid material which remained in the aqueous suspension was extracted with ether. The aqueous solution was reserved for the isolation of a polysaccharide, as will be described later.

Separation of the Acetone-Soluble Fat

The ethereal extract was dried with sodium sulfate, filtered, washed, and was then concentrated by a current of carbon dioxide to a volume of 2 liters. An equal volume of acetone was added, when a dark red oily mass was precipitated. The clear supernatant liquid was decanted and concentrated until most of the ether had been removed. More acetone was added and the solution was cooled in ice water and the precipitate which separated was filtered off and washed with cold acetone. The acetone solution was concentrated under reduced pressure, yielding a dark red oily fat which did not solidify entirely on cooling. A further small quantity of a similar fraction was recovered in purifying the phosphatide and it was combined with the main lot, giving a total of 289.5 gm. of fat soluble in cold acetone.

Purification of the Phosphatide

The crude acetone-insoluble material which was separated during the isolation of the acetone-soluble fat was precipitated repeatedly from ethereal solution by acetone. The crude phosphatide, which was finally obtained by pouring the ethereal solution into cold methyl alcohol, was a bright red amorphous powder. In
working up the mother liquors from these precipitations the ether was first removed by distillation in a current of carbon dioxide. The resulting acetone solution was cooled in cracked ice, when a considerable quantity of wax-like material was precipitated as a nearly white amorphous powder and was removed by filtration. The final acetone mother liquor yielded on concentration a small amount of fat which, as mentioned above, was combined with the acetone-soluble fat.

The crude phosphatide which weighed 100.5 gm. could not be freed from the adhering pigment by precipitation with acetone. The product was still bright red after it had been precipitated twenty times from ether with acetone. The use of methyl alcohol in place of acetone was more effective in removing the coloring matter, and by precipitating the phosphatide five times from chloroform with methyl alcohol it was obtained as a straw-colored amorphous powder. In order to obtain the phosphatide in powder form it was necessary to pour the chloroformic solution into cold methyl alcohol; when the process was reversed a sticky salve-like mass was obtained.

The purified phosphatide melted with decomposition at 231°. On analysis it gave 7.36 per cent of ash, 1.75 per cent of phosphorus, and a barely measurable trace of nitrogen.

**Separation of Crude Polysaccharide**

The aqueous alcoholic solution which remained after the lipoids had been extracted was concentrated under reduced pressure to a volume of about 400 cc. An aqueous solution of 20 per cent lead acetate was added until no further precipitation occurred, and the precipitate after it had been filtered and washed with water was discarded. The filtrate and washings were combined, concentrated under reduced pressure, and precipitated by adding an excess of basic lead acetate and ammonia. The heavy white precipitate was filtered off after the mixture had stood overnight and was washed with water. It was then suspended in water, decomposed with hydrogen sulfide, and the lead sulfide was filtered off and washed with water. The clear filtrate was concentrated in vacuo to a syrup and the latter was dried in a vacuum desiccator. The thick syrup was dehydrated by grinding in a mortar under absolute alcohol until a nearly white amorphous powder was ob-
tained. After this substance had been dried in a vacuum desiccator, it weighed 41.2 gm. The substance gave no reduction when boiled with Fehling's solution but after it had been boiled for some time with dilute sulfuric acid it gave a heavy reduction with Fehling's solution. It is evidently a polysaccharide similar to corresponding fractions obtained from the other acid-fast bacteria.

A summary of the various products isolated from the leprosy bacillus is given in Table I and for comparison we also include similar fractions isolated from the human tubercle bacillus.

The most notable difference in the lipoids of the two organisms is the lower phosphatide and wax content of the Bacillus lepra. It must be remembered, of course, that the values given in Table I do not represent the total lipoids of the bacillus but only those portions that can be extracted by alcohol-ether and by chloroform at room temperature.

The bacterial residue which had been defatted as described above still contained a large amount of lipoid material. Some 3 or 4 per cent of lipoids could be removed by prolonged grinding of the defatted bacteria in a ball mill with alcohol and ether, but when some of the defatted bacteria was refluxed for 4 hours with a mixture of equal parts of alcohol and ether containing 1 per cent of hydrochloric acid, we obtained 30 per cent of soluble material of which more than one-half or 17 per cent of the bacteria represented ether-soluble lipoids and 13 per cent was soluble in water. It is...
evident therefore that the leprosy bacillus contained over 30 per cent of total lipoids.

It is a pleasure to acknowledge our indebtedness to the Medical Research Committee of the National Tuberculosis Association for financial assistance, and to the Mulford Biological Laboratories, Sharp and Dohme, who provided the Bacillus lepræ. We are also indebted to the Leonard Wood Memorial Fund for providing a fellowship.

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