# XXVIII. THE SPECIFIC CARBOHYDRATE OF THE TUBERCLE BACILLUS.

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The first experiments on the hydrolysis of the specific carbohydrate from tubercle bacilli [Laidlaw and Dudley, 1925] indicated that only a part of the total hydrolytic products could be accounted for as reducing sugars. Later work by Masucci, McAlpine and Glenn [1930] and Renfrew [1930] has shown that amongst the products acidic substances are formed. The present communication deals with the attempted identification of these acids.

Prolonged acidic hydrolysis led to the exclusive formation of non-acidic, alcohol-soluble substances, but hydrolysis with 2·5 % sulphuric acid for a short time yielded mannose, *d*-arabinose, galactose, and an acidic residue, which, when isolated as the calcium salt, amounted to 30 % of the weight of the carbohydrate. In different experiments the calcium content of this salt varied from 4 to 6 % and attempts to purify it showed it to be heterogeneous; *e.g.* fractional precipitation of an aqueous solution with alcohol yielded a series of salts with calcium contents ranging from 10 to 2·5 %. A similar product was formed by hydrolysis of the carbohydrate with aqueous oxalic acid and longer treatment with *N* sulphuric acid led first to the production of a smaller amount of acid of approximately the same average molecular weight and finally to complete loss of acidic character. In all these repeated hydrolyses of the acidic material mannose is produced almost exclusively of any other reducing sugar.

The carbohydrate used in these experiments was free from nitrogen and sulphur and thus the possibility that the acidic constituents consisted partly of amino-acids or acid sulphuric esters was excluded. The small content of organically combined phosphorus accounts for only a small fraction of the total acid produced.

Although it has not been found possible to identify the acidic constituents, the results so far obtained make it possible to submit a tentative suggestion as to the general type of the structure of the material. It appears to contain a comparatively stable acidic nucleus, consisting of a polymerised glucoside-like compound of mannose and sugar acids, which is less firmly combined with arabinose, mannose and galactose molecules. A part of the acids may consist of substances of the sugar phosphoryl ester type.

It is difficult to obtain evidence as to whether the specific substance consists of a series of carbohydrates of the same type with specific properties, or

whether it contains a small amount of a highly active specific substance mixed with inactive substances of a closely similar type. The first view is considered the more probable since, by fractional precipitation and adsorption, products with very different optical rotations were obtained, which, however, displayed essentially equal specific activities.

## EXPERIMENTAL.

### *Growth of the bacillus.*

Cultures of the human strain 100 of the tubercle bacillus were made at 37° on a modified Long's medium devised by Capt. Douglas. It had the following composition: tap-water, 1000 cc.; glycerol, 50 cc.; sodium citrate, 6 g.; potassium dihydrogen phosphate, 2 g.; ammonium chloride, 1 g.; hydrated magnesium sulphate, 0·5 g.; ferrous ammonium citrate, 0·05 g.; glycine, 4 g.; guanidine hydrochloride, 5 cc. of a 2·2 % solution.

### *Isolation of the carbohydrate.*

The original method of Laidlaw and Dudley was modified in order to shorten the process. Moist tubercle bacilli were suspended in sufficient 4 % sodium hydroxide solution (containing 4 % of sodium acetate) to form a very thin paste and heated on the water-bath until most of the material had passed into solution (4–5 hours). After cooling the liquid was rendered acid with acetic acid and the precipitate removed. An excess of copper sulphate was added and the impure salt of tuberculinic acid filtered off. After removal of excess of copper by means of hydrogen sulphide, the liquid was reduced to a small volume at 40° and dialysed in collodion sacs until free from dialysable substances. The liquid was then evaporated to a thin syrup and poured into ten volumes of absolute alcohol. The crude carbohydrate was purified and separated from glycogen as previously described [Laidlaw and Dudley, 1925]. Later experiments showed that the lengthy process of dialysis could be avoided by precipitation of the carbohydrate with basic lead acetate in the presence of ammonia.

After removal of the precipitate produced by acetic acid, the filtrate was treated with an excess of normal lead acetate and the precipitate discarded. Addition of a slight excess of basic lead acetate, after basifying the filtrate with ammonia, produced a heavy precipitate. This was suspended in water and treated with acetic acid until no more material passed into solution. The undissolved material was discarded and the dissolved substances reprecipitated with ammonia. This process was repeated until the material dissolved completely in dilute acetic acid. The lead salts were then decomposed with hydrogen sulphide and the carbohydrate was isolated as before.

Preparations were also made from the medium upon which tubercle bacilli had been grown in the same way. The carbohydrate thus obtained possessed

properties similar to those previously recorded, but although no glycogen could be detected as a contaminant, the optical rotatory power was very variable.

| Origin of preparation | $[\alpha]_{5461}$ in water |
|---|---|
| Autoclaved bacilli ... ... ... | +32·1° |
| Autolysed bacilli (kept at 40° for several months) ... ... ... | +50·3 |
| Untreated bacilli ... ... ... | +83·1 |
| Old tuberculin (from Long's medium) | +37·3 |
| Long's medium (12 weeks' culture) | +89·1 |

These samples gave about 4 % of ash upon ignition, but this content could be reduced to below 1 % by repeated precipitation with alcohol from solutions containing hydrochloric acid. The rotatory powers of the samples were not altered by the addition of 2 % of hydrochloric acid. Most samples contained no nitrogen detectable by the Kjeldahl method, but contained about 0·8 % of phosphorus. Samples whose ash-values had been reduced below 1 % were strongly acidic in reaction. The precipitating power against immune serum varied from 1 in 5 millions to 1 in 3 millions. Glycogen was isolated only from the untreated bacilli. The yield of carbohydrate from a 12 weeks-old culture on the modified Long's medium (17,000 cc.) was 8–12 g. from the bacilli and 5–10 g. from the medium. In older cultures more appeared in the medium.

*Fractionation of carbohydrate.*

Fractional precipitation of a solution of the carbohydrate (0·9 g., $[\alpha]_{5461}$ + 50·3°) in water (4 cc.) with alcohol (12 cc.) yielded a precipitated fraction (0·33 g., $[\alpha]_{5461}$ + 59·8°) and an unprecipitated fraction (0·5 g., $[\alpha]_{5461}$ + 47·3°). Other fractionations gave similar results, as did fractional precipitation by alcohol of a solution of the carbohydrate in formamide. Precipitation of a solution of the carbohydrate (0·2 g., $[\alpha]_{5461}$ + 83·1°, from untreated bacilli) in water (2 cc.), with insufficient saturated basic lead acetate solution to give complete precipitation (1 cc.) and 2*N* ammonium hydroxide (0·5 cc.), followed by recovery of the carbohydrate from the precipitate and the filtrate by decomposition with hydrogen sulphide, yielded fractions with + 91·4° (0·126 g.) and + 71·0° (0·038 g.) respectively.

It was observed that an aqueous solution of the carbohydrate containing lithium, barium, strontium or calcium chloride and just insufficient alcohol to cause precipitation gave a precipitate upon addition of ammonia. This solid contained a large amount of the metal and possessed an alkaline reaction. A solution of carbohydrate (1·0 g., ash content 0·91 %, $[\alpha]_{5461}$ + 89·1°) in water (9 cc.) and ethyl alcohol (8 cc.) containing saturated calcium chloride solution (0·8 cc.), was treated with concentrated aqueous ammonia (1 cc.) and the precipitated solid removed and washed with 75 % alcohol. The washings and filtrate were poured into a large excess of absolute alcohol. The precipitate

(0·22 g.) gave 8·2 % ash, had $[\alpha]_{5461}$ + 82·4° and contained 0·83 % P, whilst the substance (0·7 g.) recovered from the mother-liquors gave 2·3 % ash, had $[\alpha]_{5461}$ + 111·8° and contained 0·45 % P.

*Acetylation of the carbohydrate.*

The carbohydrate (0·11 g., $[\alpha]_{5461}$ + 52·8°) was warmed with a mixture of pyridine (1 cc.) and acetic anhydride (1 cc.) at 40°. The solid slowly dissolved and after some hours a flocculent precipitate separated. After 24 hours the whole mixture was poured on to ice and the precipitated solid removed (0·108 g.). The product was almost insoluble in water, ether, light petroleum and carbon disulphide but dissolved in alcohol, acetone, chloroform or benzene. Addition of small amounts of water to the alcoholic solution produced an amorphous precipitate. The acetylated product had $[\alpha]_{5461}$ + 42·9° in ethyl alcohol. The carbohydrate was recovered from the acetyl derivative (0·1 g.) by warming at 100° with *N* sodium hydroxide (5 cc.) for 2 hours. The recovered material had $[\alpha]_{5461}$ + 95·4° (prepared from a sample of carbohydrate $[\alpha]_{5461}$ + 91·4°) and possessed essentially the same specific activity.

*Adsorption experiments.*

Attempts to adsorb the carbohydrate on benzoic acid, α-naphthol and diphenylmethane-2 : 4′-dicarboxylic acid were unsuccessful. Adsorption took place readily, however, on Lloyd's reagent. A solution of the carbohydrate (0·313 g., $[\alpha]_{5461}$ + 84°) in 0·06 *N* hydrochloric acid (30 cc.) was shaken with Lloyd's reagent (4·2 g.) for 2 minutes and filtered. The filtrate was freed from aluminium and dialysed, giving finally 0·14 g. carbohydrate, $[\alpha]_{5461}$ + 69·4°. The adsorbate was eluted with *N*/20 sodium hydroxide (30 cc.), the eluate freed from aluminium and dialysed. From the eluate 0·11 g. carbohydrate was obtained with $[\alpha]_{5461}$ + 94·1°. Both products had very similar precipitating powers towards immune serum.

*Hydrolysis experiments.*

Preliminary experiments showed that no change in rotatory power or production of reducing sugars was brought about by the action of saliva, malt diastase or taka-diastase. The stomach juices of the snail, which have been used by Karrer to hydrolyse various cellulose derivatives, were equally without action on the specific carbohydrate. 0·1 *N* hydrochloric acid at 40° has almost no action on the carbohydrate. At 100°, however, 1 % sulphuric acid produced the rapid formation of reducing sugars, the reduction value as measured by the Hagedorn-Jensen method reaching a maximum in 2 hours. A very slight fall in reducing power occurred after this period, amounting to a 6 % loss of maximum reducing power after 5¼ hours. In order to isolate the hydrolytic products the carbohydrate (2 g.) was heated at 100° for 3 hours in 2·5 % sulphuric acid (100 cc.). The observed optical rotation of the solution fell rapidly from

$\alpha$ + 1·89° to + 0·01° in 2 hours. After 3·5 hours a slight negative rotation was observed (− 0·02°). After quantitative removal of the sulphuric acid with baryta the liquid was digested with an excess of calcium carbonate at 100° until it became permanently neutral to litmus. A separation of the acidic components was effected by evaporation of the neutral filtrate to a small volume followed by precipitation with ten volumes of methyl alcohol. The insoluble material was washed repeatedly to remove adhering sugars.

The alcohol-soluble portion was freed from alcohol, dissolved in water (15 cc.) and treated according to the method of Chargaff and Anderson [1930]. Addition of phenylhydrazine (1·5 g.) dissolved in alcohol (3 cc.) to the liquid resulted in the formation of mannosephenylhydrazone (0·14 g.). After crystallisation from 60 % ethyl alcohol it formed small rhombs and had M.P. 194° (not depressed by admixture of authentic mannosephenylhydrazone). The filtrate from this hydrazone was shaken with freshly distilled benzaldehyde (2 g.), kept for 12 hours, filtered and extracted three times with ether. After evaporation (to 7 cc.) the liquid was treated with $\alpha$-benzylphenylhydrazine (1·2 g.) in alcohol (2 cc.) and sufficient alcohol to give a homogeneous solution. After keeping for 12 hours the mass of fine needles (0·67 g.) was removed and crystallised from 60 % alcohol. *d*-Arabinosebenzylphenylhydrazone was thus obtained with M.P. 175–176°. The filtrate was then boiled with benzaldehyde (1·2 g.) for 2 hours. After dilution with water (50 cc.) the liquid was filtered, extracted four times with ether, and evaporated to 3 cc. Addition of freshly distilled $\alpha$-phenylmethylhydrazine (1·5 g.) and sufficient alcohol to give a clear solution produced a small amount of a substance crystallising in elongated rectangular plates (0·04 g.). After crystallisation from aqueous alcohol this substance had M.P. 157° and did not depress the melting-point of pure galactose-$\alpha$-phenylmethylhydrazone.

The alcohol-insoluble portion (0·6 g.), consisting of calcium salts, gave 3·1 % of ash on ignition, possessed a weak reducing power towards Fehling's solution and yielded a strongly acidic substance after precipitation from dilute hydrochloric acid with alcohol. Another sample of the calcium salt prepared in a similar way gave a reduction value equivalent to 4·9 % glucose by the Hagedorn-Jensen method. All samples of the salt prepared gave a negative naphthoresorcinol reaction for glycuronic acid.

Samples of calcium salt prepared from the carbohydrate in the same way possessed calcium contents varying from 4 to 6 %. Fractional precipitation of the salt (1·5 g.) from aqueous solution (6 cc.) by alcohol yielded a fraction slightly soluble in water (0·1 g.) and three very soluble fractions (0·25, 0·50, and 0·41 g.) which possessed calcium contents 10·03, 5·45, and 2·54 %, respectively. The last three fractions were non-reducing to Fehling's solution. The last two fractions were combined and heated with *N* sulphuric acid at 100° with the object of obtaining a simpler hydrolytic product. Two fractions of calcium salt were isolated (0·044 g., Ca 8·55 %; 0·007 g., Ca 7·28 %), and from the alcohol-soluble hydrolytic products mannosephenylhydrazone (0·15 g.)

was obtained by the action of phenylhydrazine. Only a very small amount of an osazone could be prepared from the mother-liquors of the mannosephenylhydrazone.

Since hydrolysis with sulphuric acid caused considerable darkening, a milder hydrolytic agent was sought and it was found that hydrolysis of the carbohydrate (4·4 g.) with oxalic acid (5 g.) in water (50 cc.) at 100° for 12 hours gave rise to a much larger yield of calcium salt (1·3 g., Ca 6·22 %). Further hydrolysis of this salt with *N* sulphuric acid at 100° resulted in some darkening and loss of much total acidic content. The acid from this hydrolysis, isolated as the barium salt (0·13 g., Ba 19·6 %), possessed almost the same equivalent as the original material. Reducing sugars were produced as in the previous experiment. The barium salt which was extremely soluble in water gave an intense yellow colour with ferric chloride solution and also gave the reaction of Denigès [1909] for glycollic acid. Attempts to isolate calcium glycollate by conversion into the calcium salt and seeding with calcium glycollate were not successful. At this stage it was thought possible that the salt might contain some aldehyde-acid unit of the glycuronic acid type which decomposed when the glucoside linking was broken. An attempt therefore was made to effect hydrolysis and oxidation at the same time with the production of a dicarboxylic acid. Oxidation of a sample of the calcium salt (0·12 g., Ca 6·22 %) with *N* hydrobromic acid (2·5 cc.), containing 0·5 cc. of bromine in 50 cc., in a sealed tube at 102° for 22 hours, resulted in complete loss of acidic value.

Fractionation of the hydrolytic acids was also made by means of lead salts. A sample of carbohydrate (4·4 g.), isolated from tubercle medium, was hydrolysed with oxalic acid as before and the acid products were isolated as the calcium salts (0·68 g., Ca 3·0 %). The calcium ion was quantitatively removed with oxalic acid from a solution of the total salts in water (20 cc.) and the resulting acid divided into two fractions by the addition of alcohol. The precipitated material (0·08 g.) was heated for 10 hours with oxalic acid (1 g.) and water, when small amounts of phosphate ion and reducing sugars were formed. The recovered material, which was free from organically combined phosphorus, gave no precipitate with basic lead acetate in neutral solution; on the addition of ammonia a heavy precipitate was produced. The material unprecipitated by aqueous alcohol in the first separation of the acidic constituents was isolated by evaporation, followed by precipitation from a large volume of alcohol. Evaporation of the alcohol liquors yielded a minute amount of an acidic syrup which gave a strong reaction for glycollic acid but which could not be converted into a crystalline calcium salt. The alcohol-insoluble material (0·4 g.), which was slightly acid to Congo red and non-reducing towards Fehling's solution, was treated with lead acetate in water (8 cc.) and a small amount of mainly inorganic material, containing 0·2 % P, removed. After neutralisation with ammonia, lead acetate was added to the filtrate, the precipitate removed, the mother-liquor made alkaline with ammonia and an excess of basic lead

acetate added. Decomposition of the precipitate from neutral solution with hydrogen sulphide gave a small amount of acidic substance, containing less than 0·2 % of phosphorus, which gave a glycollic acid reaction. The precipitate formed in alkaline solution was similarly decomposed and yielded an acidic gum (0·15 g.) insoluble in alcohol and acid to Congo red (P, 0·78 %; 5·132 mg. required 0·42 cc., 0·0135 *N* sodium hydroxide for neutralisation; equivalent, 905). Attempts to form a crystalline brucine salt were not successful. A barium salt of the acid was prepared by digestion of the acid with an excess of barium carbonate and separated into two fractions by partial precipitation from a small volume of water with ethyl alcohol. The less soluble fraction contained 1·66 % and the more soluble 0·42 % of phosphorus.

## Summary.

1. Methods of isolation of the specific carbohydrate from tubercle bacilli and from the medium on which they have been grown are described.

2. Acetylation of the carbohydrate with acetic anhydride and pyridine yields a water-insoluble derivative from which the carbohydrate may be regenerated by alkaline hydrolysis without detectable loss of specific precipitating activity.

3. Hydrolysis of the carbohydrate with acids yields mannose, *d*-arabinose, galactose and a mixture of acids which partially lose their acidic nature and give mannose on prolonged hydrolysis. Evidence of the presence of an acid, possibly glycollic, was obtained, but no glycuronic acid could be detected.

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