CXXXVIII. THE WATER-SOLUBLE PROTEINS OF THE TUBERCLE BACILLUS.

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The toxic action on the infected host of substances derived from the tubercle bacillus is associated in most cases with proteins or products of their degradation. Particularly is this true of tuberculin preparations, whether derived from the bacilli or from media on which the bacilli have been grown. Examination of these toxic substances clearly demands a preliminary investigation into the native proteins of the tubercle bacillus, since one of these proteins is almost certainly responsible for the sensitisation of the host towards the allergic-like activity of tuberculins of low antigenic power.

The isolation of proteins from the tubercle bacillus without the use of drastic reagents is rendered difficult by the resistant nature of the waxy envelope of the bacillus; this protects the living protein from reagents such as dilute sulphuric acid and is not easily ruptured by grinding. Solvents which would dissolve the wax would almost certainly cause partial denaturation of the protein under ordinary conditions. Coghill [1926] dried tubercle bacilli below 50°, ground the dried bacilli in a ball-mill, defatted at room temperature with ether, and extracted the protein with water, saline and alkalis. The extracted material was subsequently coagulated with acetic acid. No claim was made that this preparation resembled closely the material of the original bacillus. Later Johnson and his co-workers [1927; 1928] concluded that defatting with solvents other than ether, heating at 100°, or autoclaving considerably reduced the yield of soluble proteins. Methods calculated to cause less alteration in the bacillary protein were employed by Heidelberger and Menzel [1932] who isolated the proteins of *B. phlei* and later [1933] of the tubercle bacillus by drying the frozen bacilli, defatting with ether and acetone in the cold and grinding in a ball-mill.

The process used in the present work was essentially that devised by Hardy and Gardiner [1910] for the preparation of fat-free undenatured proteins. An ice-cold suspension of the living bacilli, grown on synthetic medium, was treated with a large excess of absolute alcohol at − 10°, which was replaced by mixtures of alcohol and ether containing increasing proportions of ether until a suspension of the organisms in anhydrous ether was obtained; the whole of this treatment being carried out at − 3°. The suspension was then filtered into Soxhlet thimbles and exhaustively extracted with ether. The bacilli were dried in a vacuum, ground in a ball-mill and extracted with water. Microscopical examination of the bacillary dust[1] after grinding showed it to be completely non-acid-fast.

[1] Since the bacillary dust, although containing no living organisms, produces severe reactions if breathed in minute quantities, special precautions are necessary, and were adopted, in handling the material.

Saturation of the aqueous extract with ammonium sulphate produced a thick curd of protein which was then separated into globulin and albumin fractions by precipitation of an aqueous solution by half and complete saturation with ammonium sulphate. The two fractions were reprecipitated twice under similar conditions and then formed water-soluble, heat-coagulable products. The main properties are given in the following table:

	Reaction	Albumin	Globulin
Colour reactions	Biuret	Strong	Strong
	Millon	Yellow only	Yellow only
	Glyoxylic acid	Trace	Strong
	Molisch	Strong	Trace
	Sakaguchi	Deep red	Deep red
	Xanthoproteic	Positive	Positive
	Lead acetate and sodium hydroxide	Negative	Negative
	$[\alpha]_{5461}$	$+19{\cdot}8°$	$-67{\cdot}1°$
	P %	2·5	0·25
	Total N %	11·1	14·2
	Nitrogen partition expressed as % of total N	(By the method of Thimann [1926])	
	Amide	2·6	4·33 %
	Humin	3·06	Trace
	Basic	29·6	24·5
	Non-basic	61·7	75·5
	Reducing carbohydrate after hydrolysis expressed as glucose	11·7	0·3 %

The outstanding evidence of difference between the two fractions is shown in the glyoxylic acid and Molisch reactions and also in the nitrogen, phosphorus and carbohydrate contents. The globulin fraction, which gives a strong glyoxylic acid reaction, is apparently almost completely separated from the protein of low nitrogen content, which is associated or combined with nearly all the carbohydrate of the original mixture. The difference in the glyoxylic acid reaction has not been followed up since the amounts of material available were not enough for the isolation of tryptophan. It is satisfactory, however, to note that the occurrence of tryptophan in the globulin and not in the albumin fraction indicates that the method of fractionation has achieved a definite chemical separation, and not merely a separation into fractions of closely similar properties arising from the same parent protein.

Whilst these protein fractions show the characteristic properties of albumins and globulins in their behaviour towards half-saturated ammonium sulphate, saturated sodium chloride and saturated sodium and magnesium sulphate solutions, the globulin fraction is not precipitated from aqueous solution on prolonged dialysis and is easily soluble in distilled water. The water-soluble proteins isolated by Coghill [1926] also remained in solution on prolonged dialysis, and this fact led him to conclude that probably no globulin was present in the tubercle bacillus. Although it is true that neither fraction is an orthodox representative of its class, it has been thought convenient to adopt a nomenclature which indicates best the general properties of the fractions.

The large difference between the Molisch reactions of the two fractions suggested at first that the polysaccharide material had been salted out or adsorbed on to the albumin fraction. Later experiments showed, however, that the carbohydrate is associated in some more definite manner with the albumin, since repeated precipitation of this fraction from solution with trichloroacetic acid—a procedure which would be expected to break down a loose complex of the protein and the polysaccharide—yielded a denatured protein still giving a

strong Molisch reaction. Only on boiling with trichloroacetic acid is a product with a weak carbohydrate reaction obtained, and this treatment liberates at the same time some of the organically bound phosphorus.

Experimental.

Preparation of the proteins of the tubercle bacillus.

A 10-weeks old culture of tubercle bacilli, grown on the synthetic medium (15 l.) previously described [Gough, 1932], was filtered through fine copper gauze and, after draining, the bacilli were transferred to a Büchner funnel fitted with a double layer of butter muslin. After pressing out and washing twice with water, the bacilli were suspended in sufficient water to form a thin paste, chilled until ice crystals began to form and poured into 2 l. of neutral absolute alcohol at − 10°. After standing overnight the alcohol was decanted and replaced by a further 2 l. of cold alcohol. This operation was repeated at 24 hour intervals with 2 l. amounts of alcohol containing increasing amounts of dry ether (25, 50, 75 %). The bacilli were finally washed twice with similar amounts of dry ether. The entire treatment with solvents up to this stage was conducted at − 3°. It was found convenient to conduct the washing in a large filter-flask fitted with a wide-bore siphon tube and a cotton plug in the side-tube. The supernatant wash-liquors were removed by forcing air through the side-tube with a rubber bulb blower, so that the liquid passed out through the siphon tube and the subsequent formation of easily air-borne powders round the neck of the flask was avoided. The bacilli were shaken with ether, siphoned over into Soxhlet extraction thimbles, and extracted with ether for about 60 hours. Each extraction apparatus was fitted with a calcium chloride tube and the flasks contained pieces of sodium to ensure the absence of water. After the ether had been removed from the thimbles in a vacuum, the contents were put into a large porcelain ball-mill. Owing to the fact that the dead bacilli at this stage easily form a dust cloud, which may give rise to a tuberculin reaction in the experimenter, the thimbles were emptied into the mouth of the ball-mill under a duster and a gas mask was worn. After grinding for 48 hours the dust was found to be completely non-acid-fast. The contents of the mill were then extracted successively with 2 l. water, 1 l. 5 % sodium chloride solution, and with 1 l. 0·5 % sodium hydrogen carbonate solution. The extracts, which formed almost permanent suspensions of bacillary particles and porcelain dust, were centrifuged, filtered through a thin layer of kieselguhr and saturated with neutral ammonium sulphate. Almost all the protein appeared in the first extract; the other extracts were neglected. After standing for some hours the curd of protein was removed, dissolved in water (200 cc.) and treated with an equal volume of saturated ammonium sulphate solution. The precipitated material was removed by filtration, dissolved in water (50 cc.) and reprecipitated with an equal volume of saturated ammonium sulphate solution. Finally the "globulin" fraction was dissolved in water (100 cc.) and dialysed in a collodion sac until free from ammonium sulphate. Even after long dialysis against distilled water no precipitate formed inside.

The mother-liquor from the first precipitation of the "globulin" fraction was completely saturated with ammonium sulphate and the precipitated material removed and dissolved in water (50 cc.). The liquid was again brought to half-saturation, filtered from a slight precipitate, and finally fully saturated. This "albumin" fraction was then dissolved in water and dialysed until free from ammonium sulphate. Both preparations were isolated from their dialysed solutions by freezing in shallow dishes followed by evaporation in the frozen

state in a desiccator, over sulphuric acid and sodium hydroxide, evacuated to a pressure of 0·2 mm. on an oil-pump.

From about 46 g. of dry extracted bacilli 1·7 g. albumin and 2·4 g. globulin were obtained. The preparation was repeated with 28 g. of dry extracted bacilli. In order to obtain a more complete separation of the fractions, the "albumin" fraction was reprecipitated three times from filtered solutions in ammonium sulphate of 60 % saturation by adding solid ammonium sulphate. The "globulin" fraction was precipitated three times at half-saturation.

Both protein fractions are easily soluble in water and are precipitated from these solutions by the addition of acid or by an equal volume of alcohol. The globulin fraction is also precipitated by saturation of its aqueous solution by sodium chloride, sodium or magnesium sulphate, whilst the albumin fraction is not precipitated under these conditions.

The optical rotatory power of the protein fractions.

A solution of 0·0958 g. of the albumin fraction in 10 cc. of water had α_{5461} + 0·19°, a value which remained constant for 2 days. Upon the addition of 0·1 cc. 50 % sodium hydroxide solution the observed rotation fell immediately to − 0·25° and after keeping 6 days at 37° slowly rose to the constant value of − 0·10°. The globulin fraction (0·1103 g. in 10 cc. of water under similar conditions) showed a constant value of α − 0·74° which changed slowly to − 0·54° on addition of sodium hydroxide after 24 hours.

Immunological properties of the proteins.

The tuberculin activity of the fractions was low compared with those of recently prepared tuberculin concentrates. A 1 % solution of either fraction had an activity approximately equal to that of the standard.

Both proteins give precipitates with anti-tubercle serum (Diaplyte serum) at a dilution of 1 in 62,500. After treatment with 0·5 % sodium hydroxide for 10 hours the albumin retained almost all its precipitating activity whilst that of the globulin was completely destroyed. This result would be expected, if it is assumed that the serum contains both protein and carbohydrate antibodies, and that the treatment with sodium hydroxide destroys the precipitating power of protein but not of carbohydrate.

Carbohydrate determinations.

A modified copper solution similar to that used by Seibert and Munday [1931] was prepared by dissolving anhydrous sodium carbonate (25 g.), sodium hydrogen carbonate (20 g.), Rochelle salt (25 g.), copper sulphate (hydrated, 7·5 g.), and 0·01 N potassium iodate solution (100 cc.) in water and diluting to 1000 cc. The oxidising power of this solution towards small amounts of glucose was determined by heating 5 cc. with 5 cc. of known glucose solutions in a boiling water-bath for 10 mins., rapidly cooling, adding 1 cc. of 2·5 % potassium iodide and 2·5 cc. of 2N sulphuric acid and titrating with 0·005N sodium thiosulphate solution. With amounts of glucose up to 0·7 mg. the amount of copper reduced was proportional to the weight of glucose taken.

For the determination of carbohydrate in protein, 5 cc. of a solution (containing about 10 mg. protein) were heated with an equal volume of 0·5 N sulphuric acid in a boiling water-bath for 4½ hours, cooled, 5 cc. 0·5 N sodium carbonate and 5 cc. saturated mercuric chloride solution added, and the total liquid diluted to 30 cc. The diluted liquid was filtered through a paper covered

with a thin layer of kieselguhr, saturated with hydrogen sulphide, filtered, and the dissolved hydrogen sulphide removed by passing air through the solution for ¾ hour. In order to be sure that this process did not lead to the loss of reducing sugar or, alternatively, to the appearance of other reducing substances in the final solution, test determinations were made in which a sample of horse serum-albumin, which had an almost negative Molisch reaction, and a mixture of this albumin and glucose were used. A negligible "glucose" value for the albumin alone and accurate results for the albumin-glucose mixture (3 and 4·1 mg. found; 3 and 4 mg. glucose added) were obtained.

Relation of the alcohol-soluble proteins of the Salmonella *to the proteins of tubercle.*

The occurrence of proteins soluble in 95 and 75 % aqueous alcohol acidified with hydrochloric acid which have been isolated from *Salmonella* bacilli by White [1932; 1933] raised the question of the possibility of the existence of similar proteins in the tubercle bacillus. A sample of tubercle bacilli (about 7 g.), treated according to Hardy but not exhaustively extracted with ether, was boiled with absolute alcohol containing 5 % of its volume of *N* hydrochloric acid for 1½ hours, and the bacilli were removed and boiled under similar conditions with 75 % alcohol containing 5 % *N* HCl. The first extract gave no precipitate upon neutralisation and, on evaporation, yielded only a small amount of lipoid substances. The 75 % alcohol extract, however, yielded on neutralisation a large precipitate. After twice repeated precipitation from acid alcohol, the dried product (0·3 g.) was insoluble in water, neutral 75 % alcohol and in very dilute acids, but dissolved slowly in water on the addition of a trace of alkali. It gave strong biuret, Sakaguchi and glyoxylic acid reactions, but no Molisch reaction.

Examination of the proteins already isolated from tubercle bacilli showed that, whilst both fractions were insoluble in neutral 75 % alcohol, the globulin alone was easily soluble in warm acidified 75 % alcohol or in alcohol made alkaline with a trace of sodium hydroxide. When precipitated from the acid alcohol by neutralisation the globulin was recovered in an insoluble denatured form which appeared identical with the "T" protein isolated by direct extraction of the bacilli.

It therefore seems that a certain similarity exists between the protein constitution of the tubercle bacillus and that of the *Salmonella* group in so far as the globulin fractions are concerned. No cross reactions occur between the tubercle globulin and a number of anti-"T" protein sera prepared from various strains of *Salmonella* by Mr P. Bruce White. There is also a slight similarity between the albumin and globulin from tubercle and the "residual protein" and "alcohol-soluble protein" respectively isolated from *B. typhosus*, *B. coli communis* and *Staphylococcus aureus* by Goadby [1928; 1932], although in these cases the last-named proteins were soluble in neutral 70 % alcohol.

Summary.

1. A method for the isolation of the water-soluble proteins of the tubercle bacillus in an undenatured condition is described.

2. Fractionation of the mixed proteins with half- and fully-saturated ammonium sulphate solutions yields two proteins, possessing many of the properties of albumins and globulins, which show different chemical and immunological characteristics.

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