POLYSACCHARIDE COMPLEXES ISOLATED FROM *MYCOBACTERIUM TUBERCULOSIS* (HUMAN STRAIN)

by

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INTRODUCTION

Despite the application of a wide variety of methods for separating naturally occurring macromolecules, the most effective vaccines generally are those made from suspensions or solutions of whole bacterial cells. In the search for potential immunogens and antigens from various cells, much attention has been given in the past to the isolation of relatively pure chemical entities[9, 10, 11]. The general biological activity of complex substances such as lipopolysaccharides, mucopolysaccharides and mucoproteins has not received particular attention. It was suggested to us some years ago by Professor W. T. ASTBURY F.R.S. that interesting results might be obtained by study of the effect of strong urea solutions on bacterial cells and we have found that this reagent does indeed have quite a remarkable solubilising action on the components of many bacterial cells. We have paid particular attention to the action of urea and other basic organic compounds on *M. tuberculosis* in attempts to obtain essentially protein-free mucolipoids from heat-killed cells. It was considered that the serological and immunological properties of such fractions would be of some interest.

Accordingly, heat-killed cells (human strain) have now been extracted with saturated solutions of a number of reagents such as urea, β-hydroxypropionamidine, dimethylethanolamine, diethylaminopentan-2-ol, piperidine and ethylene glycol[7]. In view of the large amount of lipoidal material contained by the organism, the above solvents represented a series of substances of graded lipophylic character. The action of a saturated aqueous solution of urea on moist cells at 37° C is quite striking for it causes considerable swelling and results in a gelatinous mass at the end of about 30 hours. On dilution of the mass with water, followed by high speed centrifugation, a heavy deposit of bacterial debris is flung down whilst the solution contains considerable amounts of extracted material including lipoids, nucleoproteins and polysaccharides. After a wide series of preliminary studies it was found that a fraction of the material thrown out from the centrifuged liquid by addition of several volumes of alcohol, possessed interesting serological properties and provided a useful starting point for further investigations.

In a typical extraction of cells with urea, the same cells were twice extracted, the

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liquid being removed by high speed centrifugation after each extraction. The urea was removed by dialysis of the pooled extracts and the complex molecules isolated either by freeze-drying or by precipitation with ethanol. The resulting fractions were tested by the collodion particle agglutination (C.P.A.) test[1,3], against sera from tuberculous patients. The patients selected had all more or less extensive disease and at the time were improving under treatment. From the previous experience of one of us (E.N.) with specific serological reactions in tuberculosis, these were likely to possess certain antibodies to a relatively high titre.

In this test the material under investigation is coated on to a colloidal suspension of collodion particles in a buffer solution. Utilizing the test, RIORDAN[8] found that some fractions from *M. tuberculosis* agglutinated in the presence of serum from tuberculous animals and there was evidence that the polysaccharide moiety of the active fractions was primarily involved in the agglutination.

More recently MIDDLEBROOK AND DUBOS[6] developed a haemagglutination reaction which could well be diagnostic for active tuberculosis. A complex polysaccharide fraction prepared by them and coated on to washed sheep erythrocytes was used as an antigen.

EXPERIMENTAL

Mycobacterium tuberculosis cells

The organisms employed in these investigations were *M. tuberculosis* (humain strain) grown at the Veterinary Laboratories of H.M. Ministry of Agriculture and Fisheries (Weybridge), killed by steam-heating. The cells were freed from excess moisture by filtration and air drying.

Collodion Particle Agglutination test

B.D.H. collodion (500 ml) was stirred into water (2 l); the mass which separated was washed thoroughly with water and dried to constant weight at 40° C. A solution (5%) was then prepared of the material in acetone and to this was added 25% aqueous acetone (in three times distilled water) until a faint opalescence appeared. More distilled water (1,200 ml) was added to the solution which was then aerated for 5 hours until all the acetone had been removed. The resulting suspension was filtered through glass wool and centrifuged at 3000 r.p.m. for 5 minutes. When the supernatant was almost clear, it was decanted and the precipitated particles washed thrice with "three times distilled" water, and finally resuspended in 400 ml of distilled water.

A 0.1% solution in water of the bacterial complex under investigation was prepared where possible. Complexes insoluble in water were ground with half their weight of gum acacia and the mixture diluted with distilled water (1 mg/ml).

The suspension of collodion particles was mixed with twice its volume of solution (or suspension) of the test material and the resulting liquid kept at 0° C for 12 hours. A volume of distilled water equal to that of the solution under test was added to the above suspension and one tenth of the final volume of a buffer solution at p_H 6. The latter had the effect of stabilising the suspension for a period of several weeks at 0° C.

The original fraction *A* coated on to collodion particles was tested against 10 sera from patients undergoing treatment for pulmonary tuberculosis at the Harefield Hospital. In addition homologous rabbit antisera from three rabbits immunised by biweekly intravenous injections of 10 mg of fraction *A* were used as positive controls. Negative controls with normal rabbit sera and sera from tuberculin negative nurses were also frequently run alongside the tests. Altogether some 125 fractions were tested against sera derived from 46 patients. Each batch of approximately six fractions was tested against 4–6 sera of which at least two were used in previous tests and were known to contain antibodies against *M. tuberculosis* and fraction *A*.

Serum dilutions of 1/8 and 1/64 and a saline control were used throughout in 0.5 ml. The antigens were adjusted to an opacity corresponding to tube No. 5 of the MCFARLAND scale, and 0.5 ml added to each serum dilution and saline control. The tubes were shaken, kept in the refrigerator (4° C) overnight and read after four minutes centrifuging at 1200 r.p.m. by gently shaking the tubes. Agglutination in the serum dilutions was compared with the ease of resuspension of the particles in the saline control. As a rule no attempt was made to titrate to an endpoint beyond the 1/64 serum dilution. A fraction was regarded as being serologically active, *i.e.* as a good antigen, when agglu-

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tinated by the majority of sera in a dilution of 1/64, moderately good in a titre of 1/32, and weak when agglutinated by 1/8 or 1/16 dilution only.

The purified carbohydrate complex fractions were tested by the precipitin reaction against patients' sera. In preliminary tests serum dilutions of 1/8 to 1/32 were tested against antigen dilutions of 1/1000 to 1/5,000,000. Serum dilutions of 1/16 was found to give the most satisfactory readings, therefore, all further tests were done with 1/8 and 1/16 serum dilutions only.

The described serological control of the various fractionation procedures was chosen for technical reasons. The amount of material available was often very small and the large number of fractions made the preparation of homologous antisera impracticable. Many or most of the fractions were probably mainly of hapten character and would not induce antibody formation. By using sera from tuberculous patients a good measure of the serological activity of the various reasonably well defined fractions from *M. tuberculosis* could be obtained.

Extraction Procedure

A typical extraction was carried out as follows: Moist cells (414 g) *i.e.* 83 g dry weight, were stirred with an equal weight of solid urea. The mixture was maintained with occasional stirring at 37° C for 12 hours and more urea (30 g) was added. During this time, the cells were converted to a viscid liquid mass. After being kept for a further 88 hours at 37° C, the liquid was diluted with water (100 ml) and centrifuged at high speed repeatedly. The supernatant liquor was acidified (p_H 6) with dilute acetic acid and again centrifuged. The remaining cells in the solution could be removed by means of a Seitz filter or by addition of a little alcohol until a faint precipitate formed which was separated.

The solution was finally stirred with ethanol (3 volumes) and the precipitated complex collected, and dried either from the frozen state or by washing with ethanol and ether (2.1 g).

Further fractionation could be performed on the moist complex extracting it by shaking with sodium acetate solution (100 ml of 2% each portion) four times. An insoluble active fraction (*A*) was thus obtained (C.P.A. test ++). Great care was taken by microscopic examination, redissolution in urea followed by high speed centrifugation etc., to make sure that fraction *A* contained no cell cytoskeletons or cell debris.

The mass of extracted cells were available for re-extraction by the same method.

Extracting agents

Using the procedure set out above heat killed cells were extracted with various reagents using the following proportions.

TABLE I

Reagent	Mass of moist cells (g)	Amount of reagent	Yield of "active" fractions (g)	Total yield (g)
Urea	414	444 g	1.8	5.5
β-hydroxpropionamidine	118	26 g	0.33	0.33
Dimethylethanolamine	111	50 ml	—	1.61
Diethylaminopentan-2-ol	100	50 ml	0.1	1.14
Piperidine	101	50 ml	—	0.51
Water at 100° C	265	3 l	0.9	1.3
Water at 37° C	75	50 ml	0.1	6.8
Ammonium hydroxide	120	30 ml of 0.88	0.65	2.0
NaCl at p_H 10	100	15 g	0.4	1.0
Guanidine	50	15 g	—	0.8

Properties of the active complex

1. *Lipoid-content of urea-extract (fraction A).* It was found that no difference in C.P.A. activity could be detected between specimens of fraction *A* which had been "freezedried" or those which had been dried rapidly with a simple washing with absolute ethanol and ether.

Prolonged extraction of fraction *A* (0.4 g) which had acid-fast staining properties, with ether in a Soxhlet apparatus for 20 hours removed some lipoid material (0.068 g = 27%). The solid remaining had no C.P.A. activity and had lost its acid fastness. An attempt was made to isolate active material from moist cells (900 g) which had been "defatted" in a Soxhlet apparatus with acetone

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(1 l) and with ether (1 l) for 20 and 48 hours respectively, using urea (50 g) dissolved in the minimum amount of water. None of the resulting fractions were active in the C.P.A. test.

2. *Solubility of the active complex.* (i) Moist complex, extracted by urea and precipitated with ethanol, was shaken with buffer solutions between pH 2 and pH 11, each for 1 hour. The insoluble material was separated centrifugally and dried with ethanol and ether and the soluble fractions were precipitated from the supernatant liquors with ethanol (5 vols.) and dried in the same manner. The results are summarised in Table II.

TABLE II

pH buffer	Insoluble Fractions		Soluble Fractions	
	Weight g	C.P.A. activity	Weight g	C.P.A. activity
3 acetate	0.1	—	0.03	+++
4 acetate	0.22	++	0.02	±
5 acetate	0.40	++	0.09	+
6 acetate	0.31	++	0.10	—
7 acetate	0.19	+++	0.17	+
8 phosphate	0.29	+++	0.01	±
9 phosphate	0.30	+	0.07	—
10 phosphate	0.12	++	0.15	++

(ii) An aqueous urea extract of the active complex (100 ml) (before precipitation with ethanol) was dialysed in a cellophane sac against distilled water. The solution was centrifuged at intervals and the solids which separated was dried with alcohol and ether. The supernatant was returned to the dialysis sac after each separation.

Time	30	60	90	270	450	630	min
C.P.A.	++	±	+	±	±	±	
Weight	0.2	0.25	0.10	0.15	0.17	0.09	g

(A control specimen of the complex precipitated with ethanol had a C.P.A. activity ++).

Identical results were obtained when a solution of the complex in β-hydroxpropionamidine was dialysed. It appears that the active material is readily precipitated when the concentration of extracting agent falls below a critical high value.

Alkaline Hydrolysis of Fraction A. A specimen of fraction *A* (2.5 g : N, 9.4%), which had been thoroughly extracted with ether, was warmed with sodium hydroxide solution (40 ml of N) on a waterbath for 30 hours. A precipitate of unsaponified lipoid remained (1.1 g) and this was separated. The resulting solution was made acid with acetic acid and the precipitated material (0.1 g) which gave strong protein tests was removed. The supernatant so obtained was stirred with ethanol (4 vols.) and a precipitate (0.98 g : N, 4.7%) isolated which had strongly positive reactions indicative of the presence of a carbohydrate and deoxyribonucleic acid.

The mixture was fractionally separated by the addition dropwise of alcohol to an aqueous solution. Fractions were obtained as shown in Table III. Fraction F_1 was a deoxyribonucleic acid while the rest F_2 to F_6 consisted of a polysaccharide which appeared to be mainly homogeneous containing pentose and an amino sugar and which reacted with anti serum from patients with tuberculosis in a dilution of 1:3,000,000.

TABLE III

No.	Weight g	Ash %	$[\alpha]_D^{18}$ in water	Biuret	Molish	Bial	Dische
F1	0.177	47.7	± 0°	—	++	++	+++
F2	0.065	26.0	+ 30	—	++	+	+
F3	0.119	19.9	+ 28	—	++	+	—
F4	0.06	1.8	+ 27	—	++	+	—
F5	0.03	2.1	+ 29	—	++	+	—
F6	0.18	35.0	+ 26	—	++	+	—

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Examination of β-hydroxypropionamidine extract (fraction B). A specimen of fraction *B* (0.4 g) was exhaustively extracted with ether for 40 hours. A yield of lipoidal material was obtained corresponding 17% (by weight) of the original.

The "defatted" complex (0.26 g) was submitted to alkaline hydrolysis in the same way as fraction *A*. An unsaponifiable component (0.11 g) was isolated, 0.018 g of peptidal constituents and 0.1 g of a polysaccharide ($[\alpha]_D^{21} + 28°$).

The fat-free complex did not give a positive DISCHE test for deoxribonucleic acid until it has been heated with dilute acid for several minutes. A quantitative estimation of the nucleic acid content by this reagent[8] was performed: *e.g.* Fraction *B* 46.4 mg was heated at 100° C for 20 minutes with hydrochloric acid (2 ml of 0.5 *N*). The resulting solution was heated at 100° for 3 minutes with DISCHE reagent (2 ml) and the colour compared photometrically with that produced by a standard solution of thymus deoxyribonucleic acid. 2.8% of deoxyribonucleic acid was found to be present.

An acid-treated solution of the complex B when examined spectrophotometrically was found to have an absorption band at 2650 Å typical of that of a deoxyribonucleic acid.

DISCUSSION

We have found that certain urea-extracted complexes gave positive results in the C.P.A. test and that all the active complex could be extracted from the cells in the first two treatments with urea of β-hydroxypropionamidine.

From the numerous results involving the examination of over a hundred fractions, it appears that the most active complexes were obtained by the action of both urea and β-hydroxypropionamidine. The two complexes thus obtained were separately subjected to further investigation. It was found that the C.P.A. activity was destroyed when either was exhaustively extracted with ether. Both of the fractions possessed acid-fast staining properties which were destroyed by the ether extraction. This property appears to be connected with the observation that active material could not be isolated from cells which had previously been completely defatted by organic solvents such as acetone, chloroform, ether etc.

When either fraction was shaken with buffer solutions between p_H 2 and 11, it was found that the most active material was insoluble between p 8_H and p_H 4. A C.P.A. active fraction was therefore prepared by removing inactive material from the urea fraction by extraction with a 2% aqueous solution of sodium acetate. The soluble inactive material was rejected and the composition of the insoluble material (designated fraction *A*) was further investigated.

Preliminary chemical examination of the active fraction showed that in addition to lipoids, it contained a carbohydrate, deoxyribonucleic acid and some protein. The staining reaction of the material suggested that it had acid fast properties. Initially the active fractions, dried by treatment with absolute ethanol and ether, were only sparingly soluble in water. It was found that the solubility could be maintained if metallic ions (particularly Ca^{++}) were eliminated before drying and that drying from the frozen state afforded a more soluble product. Electrophoresis in 1% solution of such a soluble product showed several peaks with one major peak at p_H 8 in 0.2 *M* phosphate buffer. This evidence coupled with behaviour of the active fractions when fractionally precipitated, suggests that the activity resided in a complex macromolecule.

Exhaustive extraction with ether of a urea-extracted complex removed its acid-fast staining properties and destroyed the C.P.A. activity. Evaporation of the ether extract furnished lipoidal material corresponding to 27% of the original material.

Alkaline hydrolysis of the defatted complex (9.4% N) was carried out at 100° C with *N* sodium hydroxide. The unsaponified residue (2.2% N) was centrifuged and the

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supernatant liquor neutralised with acetic acid. A small amount of protein was precipitated (*c.* 4%) and separated (centrifuge). To the supernatant was added, ethanol (4 vols.) and the resulting precipitate was dissolved in water, dialysed for 48 hours in a cellophane membrane and finally reprecipitated with ethanol and dried. The product (4.7% N) gave strongly positive MOLISCH AND DISCHE tests and was submitted to fractional precipitation from aqueous solution by addition of ethanol.

A polysaccharide ($[\alpha]_D^{20}$ + 28°, 16%) containing a pentose and an amino sugar, was thus isolated and it was shown to have properties closely resembling the "lipoid-bound" polysaccharide isolated from the intact organism[4]. Both polysaccharides reacted with tuberculous antiserum in the precipitin test, in a dilution of 1:3,000,000. A fraction composed largely of deoxyribonucleic acid was also separated in this fractionation.

A similar examination of a β-hydroxypropionamidine extracted complex (designated fraction *B*) was carried out. This was found to contain 17% of ether-soluble lipoid and, by a quantitative DISCHE estimation[2], *c.* 3% of deoxyribonucleic acid. The presence of the latter constituent was confirmed by the demonstration of an absorption band at 2650 Å. Alkaline hydrolysis of this complex also furnished the same lipoid-bound polysaccharide ($[\alpha]_D^{28}$ + 28°) as was in the urea complex and in the intact organisms. The general composition of the two active fractions can be summarised:

	Fraction *A* urea extract	Fraction *B* β-hydroxypropion-amidine extract
Ether soluble lipoid	27%	17%
Deoxyribonucleic acid	6	3
Specific polysaccharide	16	30
Unsaponifiable lipoid	40	40
Peptidic components	4	6

From these preliminary investigations and from an examination of other extracts, we have reached the conclusion that *M. tuberculosis* cells can be conveniently disrupted by the action of saturated urea solution at 37° on the heat killed cells. Among the water-soluble macromolecules are complexes of a polysaccharide and lipoids bound up with other molecules such as nucleic acids and peptides in the conjugated form. The polysaccharide-complex shows a fair degree of homogeneity in the TISELIUS electrophoresis apparatus and has interesting serological properties in the collodion particle agglutionation test[12]. After liberation from the complex by saponification, the polysaccharide reacts specifically in fair dilution with human tuberculous antiserum. It is unlikely that such a large complex is mainly an entity, despite its electrophoretic behaviour. We prefer to regard it as a conveniently prepared starting material for studying the antigenic behaviour of cell components of *M. tuberculosis* with special reference to those containing a specific polysaccharide.

This investigation has been extended by some of our colleagues[5].

ACKNOWLEDGEMENTS

This work was carried out with the close interest of the late Professor Sir NORMAN HAWORTH, F.R.S.

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The authors wish to thank Messrs. Glaxo Laboratories Ltd., for material and financial assistance and Drs. UNGAR AND EMERY for the preparation of the collodion particle suspensions and for helpful discussions. They also express their indebtedness to Dr H. H. GREEN, Director of the Weybridge Veterinary Research Station, and his colleagues for the generous provision of *M. tuberculosis* cells.

SUMMARY

Moist killed *M. tuberculosis* cells have been extracted by a series of basic compounds. Using saturated solutions of urea particularly a complex was isolated which contained a specific polysaccharide, deoxyribonucleic acid, peptide and lipoid components. The complex possessed some serological activity with human tuberculous serum when examined by the collodion particle agglutination test. It forms a suitable starting point for research on serologically important complexes from *M. tuberculosis*.

RÉSUMÉ

Extraction a été faite des cellules humides *M. tuberculosis*, sterilisées par vapeur, par une serie de substances basiques. Surtout, avec les solutions saturées d'urée, on a isolé un complexe contenant un polysaccharide specifique, l'acide desoxyribonucléique, un protéide et des lipides.

En ajoutant le complexe au serum tuberculeux humain, il a montré un peu d'activité quand examiné par l'essai "collodion particle agglutination". Ce complexe se prête à l'étude des complexes d'importance sérologique dans *M. tuberculosis*.

ZUSAMMENFASSUNG

Nasse, getötete Tuberkel-Zellen wurden durch eine Reihe basischer Verbindungen ausgezogen. Besonders mit gesättigten Harnstofflösung, wurde eine Verbindung isoliert, die ein spezifisches Polysaccharid, eine Deoxyribonukleinsäure, ein Peptid und ein Lipid enthielt. Der zusammengesetzte Stoff besass etwas serologische Aktivität gegenüber menschlichem tuberkulosem Serum wenn es durch die Agglutinationprüfung der Kollodiumpartikel geprüft wurde. Es bildet einen guten Ausgangspunkt für die Erforschung serologisch wichtiger Verbindungen aus *M. tuberculosis*.

REFERENCES

[1] A. R. CANNON AND C. E. MARSHALL, *J. Immunol.*, 38 (1940) 365.
[2] Z. DISCHE, *Mikrochemie*, 8 (1930) 4.
[3] K. GOODNER, *Science*, 94 (1941) 241.
[4] W. N. HAWORTH, M. STACEY, AND P. W. KENT, *J. Chem. Soc.*, (1948) 1211.
[5] C. N. ILAND, G. A. GILBERT, A. S. JONES, AND R. W. BALDWIN, *5th Int. Cong. Microbiol Brazil*, 1950.
[6] G. MIDDLEBROOK AND R. J. DUBOS, *J. exptl Med.*, 88 (1948) 521.
[7] W. T. J. MORGAN, *Biochem. J.*, 31 (1937) 2003.
[8] J. T. RIORDAN, *Proc. Soc. Exptl Biol. Med.*, 49 (1942) 622.
[9] F. R. SABIN, C. A. DOAN, AND C. E. FORKNER, *J. exptl Med.*, 52 (1930) suppl. 3.
[10] F. B. SEIBERT, *Bact. Rev.*, 5 (1941) 69.
[11] M. STACEY, AND P. W. KENT, *Adv. Carbohyd. Chem.* Academic Press, New York, 3 (1948) 311.
[12] J. UNGAR, *Proc. 4th Int. Congr. Microbiol.* Copenhagen (1947) p. 158.

Received January 18th, 1951