A SPECIFIC TUBERCLE ANTIGEN, EXTRACTED FROM LIVING *MYCO. TUBERCULOSIS*

by

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During the past thirty years detailed investigations of the chemical composition of *Myco. tuberculosis* have been made, and many complex substances have been obtained. RAFFEL[1] has compiled a table from the work of ANDERSON, SEIBERT, LEDERER and others which shows the methods of isolating some of these materials, and SEIBERT[2] has reviewed attempts to prepare proteins with tuberculin-type activity from the bacillary cells. In spite of the large amount of chemical research carried out, no pure homogeneous fraction has been obtained from this organism which will produce a significant degree of immunity in experimental animals, nor has any pure fraction been shown to react with the complement-fixing antibodies in the sera of tuberculous patients. The use of urea for the extraction of biologically active material from heat-killed tubercle bacilli has been described by HAWORTH, STACEY AND KENT[3]. A re-investigation of the effect of urea has now been carried out but on living organisms to avoid the denaturing action of heat, and on young cultures of a virulent strain to avoid extracting dead or autolysed cells. This paper reports some of the biological and chemical properties of the extracted material and of its main component. Some of this work has been briefly reported elsewhere (ILAND, GILBERT, BALDWIN, JONES[4]).

EXPERIMENTAL

Cultivation of the organism. A strain of human-type tubercle bacillus was isolated from the sputum of a patient suffering from active pulmonary tuberculosis. Stock cultures were maintained by subculture on Lowenstein-Jensen medium. For large scale cultivation the organism was grown as a pellicle on the surface of a protein-free synthetic medium for four weeks at 37° C. A large batch of organisms, grown on the medium employed in the production of purified tuberculin protein (GREEN[5]), was also obtained from the Ministry of Agriculture Veterinary Laboratories at Weybridge (by courtesy of Dr H. H. GREEN).

Preparation of the crude urea extract. When the cultures were four weeks old the bacteria were distributed through the medium by gentle shaking and were then filtered off in a wire strainer. They were quickly transferred to a glass jar and treated with solid urea, with stirring, until an approximately equal volume of urea had been added. On the addition of the urea, the mass of cells immediately became moist, and was soon transformed into a thick smooth paste. The mixture was incubated at 37° C for four days with occasional stirring. After four days, the fluid was filtered rapidly through glass wool, and the opaque greenish filtrate centrifuged to remove most of the cells. (Crude urea extract, U.E.). The solution was then dialysed against continuous changes of Birmingham tap water at 0° C–4° C for five days to remove the urea, centrifuged for two to three hours (3,000 r.p.m. at 0° C) to remove any bacteria still in suspension, and vacuum dried from the frozen state to give a white powder (dialysed urea extract or D.U.E.).

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CHEMICAL PROPERTIES OF THE DIALYSED UREA EXTRACT

Electrophoresis. Solutions of D.U.E. were studied by the Tiselius electrophoresis technique, in phosphate buffer pH 7.7, ionic strength 0.1 and with a potential gradient of 4–5 volts per cm for one to two hours. The analysis showed that the product contained at least four components, the main component (*C*) comprising 65–70% of the whole, a small faster moving component (*D*) and two slower moving components (*A* and *B*). Fraction *A* appeared as a small spike just separating from the delta anomaly, while *B* was observed as a small shoulder on the main peak (component *C*). Similarly, at least four components were present in the D.U.E. extracted from the Weybridge batch of cells. Here the principal component accounted for 77% of the total material, and in addition, there were two fast moving components and a slower component which appeared as a shoulder on the main peak.

Spectrographic analysis. Solutions of the two samples were examined in a Hilger ultraviolet spectrophotometer. In both cases there was an absorption maximum at 260 mμ indicating the presence of nucleic acid (see Fig. 1).

Chemical analysis. Qualitative tests indicated the presence of protein, carbohydrate, ribonucleic acid and deoxyribonucleic acid. Batch No. 6 gave the following data: Ash, 3.2%; Nitrogen, 9.7%; ether extracted material, 5.4%: deoxypentosenucleic acid, 2.4%; pentosenucleic acid, 2.5%; phosphoprotein phosphorus, 0.1%; (SCHMIDT AND THANNHAUSER[6]).

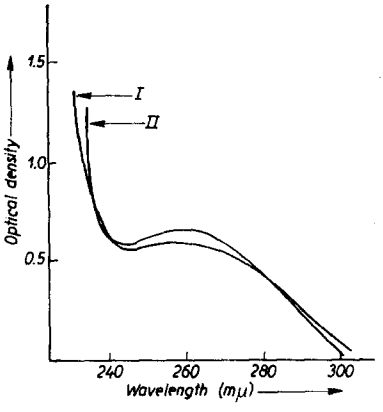

Fig. 1. Absorption spectra of dialysed urea extract (I) and Weybridge dialysed urea extract (II)

Fractionation of dialysed urea extract. The dialysed urea extract was fractionated in order to isolate the main electrophoretic component (*C*). Freeze-dried D.U.E. was extracted with ammonium sulphate solution (39.4% w/v; NH_3 to pH 7) at 0° C. The residue left after extraction with three portions of ammonium sulphate was called U_c since it corresponded to the component *C* of the electrophoretic analysis of D.U.E. The supernatant from the first ammonium sulphate extraction was dialysed against tap water to remove the ammonium sulphate and then treated with lanthanum acetate until precipitation was complete. This process precipitated a nucleoprotein fraction (U_2) which was removed by centrifuging. The lanthanum was then removed as the insoluble carbonate, by the addition of sodium carbonate solution until the pH reached 7, and recentrifuging. The supernatant from the lanthanum treatment contained a small amount of material, U_3. Fractions U_2 and U_3 were dialysed against tap water. Solutions of all the fractions, except U_c, were stable, and could be kept for periods of 14 days or more at 0° C with the addition of a preservative (merthiolate) to suppress bacterial growth. The fractions were freeze-dried after dialysis against 0.15 *M* NaCl or phosphate buffer pH 7.7, ionic strength 0.1. They were not, however, completely soluble after drying in this way and were usually freshly prepared as required.

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CHEMICAL PROPERTIES OF THE U_c PROTEIN

Electrophoresis. A freshly prepared 2% solution of U_c was dialysed against tap water at 0° C to remove ammonium sulphate and then against phosphate buffer pH 7.7 ionic strength 0.1. Electrophoretic analysis (Fig. 2) showed that the fraction consisted of a major component ($U_c/1$) and minor faster moving components ($U_c/2$). The mobility of $U_c/1$ determined from successive photographs of the descending boundary was $5.8–6.5 \cdot 10^{-5}$ cm²/volt/sec. The faster components moved with approximately twice this mobility.

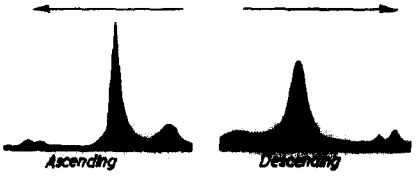

Fig. 2. Electrophoresis of U_c in phosphate buffer, pH 7.7, ionic strength 0.1 (time 52 minutes)

Spectrographic absorption. The ultraviolet absorption spectra of the fractions of D.U.E. were determined (see Fig. 3).

Chemical analysis. Qualitative chemical tests indicated that the U_c fraction contained protein and carbohydrate. The carbohydrate was not detectable by a Molisch test but was indicated by the carbazole reaction (6% estimated as glucose) (SEIBERT[7]). The nitrogen content of the material (13.6% by Kjeldahl) suggested that the major part of the fraction was protein. Quantitative biuret estimations on 2 mg samples gave 105% and 97% protein (against human globulin as standard).

Fractionation of the U_c protein

Since this fraction had been shown by electrophoresis to contain two components, attempts were made to purify it further. Fractions were precipitated from standard volumes of a solution of U_c by adding increasing amounts of ammonium sulphate pH 7.0, at 0° C. The precipitates were dissolved in 0.15 *M* NaCl, dialysed against the same solution at 0° C and then made up to a standard volume. The nitrogen content

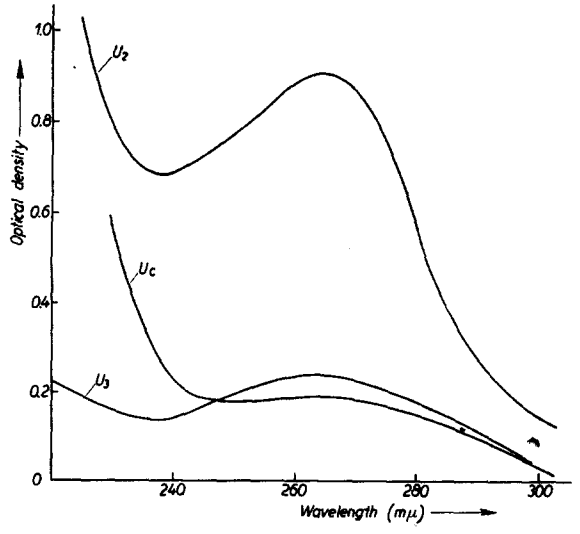

Fig. 3. Absorption spectra of fractions of dialysed urea extract (concentration = 0.13 mg/ml)

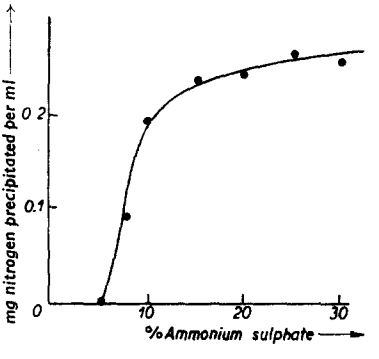

Fig. 4. Precipitation of U_c from ammonium sulphate solution

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(FALCONER AND TAYLOR[8]) and the antigen dilution titre, against tuberculosis rabbit sera, of the fractions were determined. The precipitation curve (Fig. 4) shows that most of the protein in the U_c was precipitated between narrow limits (5% to 12% w/v of ammonium sulphate) and that all the material was precipitated by 30% (w/v) ammonium sulphate. The antigen dilution titre indicated that all the fractions possessed some activity and that the activity of any fraction was proportional to its nitrogen content.

A sample of U_c prepared by extracting D.U.E. three times, at 0° C with 30% (w/v) ammonium sulphate solution pH 7.0, and dialysing against tap water, was analysed electrophoretically in phosphate buffer, pH 7.7, ionic strength 0.1. The analysis showed that the U_c had not been freed by this method of preparation from the small fast moving components. Samples of the fast components, and of the slower main component (93%), were isolated electrophoretically and their ultraviolet absorption spectra determined. The faster moving components ($U_c/2$) gave an absorption curve with a maximum at 2500 A to 2600 A indicating the presence of nucleic acid, while the curve of the main component ($U_c/1$) had a maximum at 2,700 A to 2,800 A, typical of protein. Both fractions had a positive carbazole reaction. Since $U_c/1$ was effectively free from nucleic acid, this indicates that U_c contains carbohydrate other than that due to nucleic acid. An attempt was made to fractionate U_c with ethanol at low temperature but the method was not successful and no fractionation was achieved.

Properties of the other fractions of D.U.E.

Fraction U_2 was shown to consist of protein, deoxyribonucleic acid and carbohydrate (Millon, Molisch and Dische), and contained nitrogen, 10.2% and phosphorus 2.9% (N/P 3.5). Fraction U_3 gave negative Millon, biuret and ninhydrin reactions and contained 2.63% nitrogen. The ultraviolet absorption spectra of U_2 and U_3 are shown in Fig. 3; and both have a maximum at 2,600 A indicating the presence of nucleic acid ($\leqslant$ 10% in the case of U_3).

Properties of the urea extracted bacterial cells

Treatment with urea as described above did not disrupt the bacteria or alter their morphology. Urea killed the organisms as was shown by the failure of the treated cells to infect guinea pigs after subcutaneous injection. Their acid-fast staining property was retained although beading was common. Further extraction of the cells with a saturated solution of urea produced a fraction which on electrophoresis had a pattern similar to that obtained from the original D.U.E. More material could also be obtained from the extracted cells with tap water, but these fractions have not been investigated further.

BIOLOGICAL PROPERTIES

Immune sera. These were prepared by the intravenous injection of rabbits.

Each animal was given three 10 mg doses on consecutive days, and the injections were repeated at weekly intervals for three weeks. The animals were bled 7 to 10 days after the last injection. When living tubercle bacilli were injected one 10 mg dose was given per week for three weeks. Human sera were obtained from the freshly drawn blood of patients with active pulmonary tuberculosis, and from healthy adults who were Mantoux positive.

Precipitin tests. These were carried out using the antigen dilution technique.

The fraction under test was dissolved in 0.15 *M* sodium chloride, and progressive dilutions

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were mixed with a constant volume of antiserum. The antisera were usually diluted with an equal volume of 0.15 *M* sodium chloride before use. The mixtures of antigen and antiserum were incubated at 37° C for one hour and then left in a refrigerator for 48 hours. The usual controls were set up with each test.

The results are given in Table I.

TABLE I

PRECIPITIN TITRES OF D.U.E. AND OF ITS FRACTIONS

Rabbit No.	*Condition*	*Precipitin titre with fraction*		
		D.U.E.	U_C	U_3
N3	Normal	0	0	0
N5	Normal	0	0	0
B	Tuberculous	500,000	20,000	+
B1	Tuberculous	1,000,000	50,000	++
B5	Tuberculous	500,000	—	+
B6	Tuberculous	100,000	—	—
B7	Tuberculous	100,000	—	—
A1	Injected	100,000	40,000	50,000
A2	with D.U.E.	50,000	—	—
A3	with D.U.E.	10,000	—	—
D	with D.U.E.	40,000	—	+
C	Injected	1,000,000	200,000	++
C1	with U_c	100,000	—	—
C2	Protein	800,000	—	—

0 = no reaction; — = test not carried out; + = positive reaction; ++ = strongly positive reaction

Complement fixation tests. This technique was used when testing human sera.

The sera were inactivated by heating for 30 minutes at 56° C, and a constant amount of serum was then allowed to react with a range of dilutions of the antigen, in the presence of 2 minimum haemolytic doses of guinea pig complement.

Results are given in Table II.

TABLE II

COMPLEMENT FIXATION WITH HUMAN SERA

No. of sera	*Condition*	*Antigen*	*Reaction*	
			Positive (+ highest titre)	*Negative*
8	Tuberculous	U.E.	8 (1/150)	0
6	Normal	U.E.	0	6
10	Tuberculous	D.U.E.	9 (1/500)	1
4	Normal	D.U.E.	0	4
10	Tuberculous	U_c	9 (1/1000)	1
4	Normal	U_c	0	4

Skin tests. Solutions of the material under test were injected intradermally into tuberculous guinea pigs or into Mantoux-positive healthy humans. The results are given in Table III.

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TABLE III

SKIN REACTIONS WITH OLD TUBERCULIN AND WITH D.U.E.

Subject	*Condition*	*Size in millimetres of skin reaction to*			
		U.E. 0.1 ml of 1/10	*D.U.E. 0.1 ml of 1/10*	*D.U.E. 0.001 mg*	*O.T. 1/10 0.1 ml*
Guinea Pig 51	Tuberculous	15 × 10	14 × 10		15 × 10
52	Tuberculous	15 × 15	15 × 17	—	14 × 9
53	Tuberculous	15 × 15	16 × 13	—	14 × 10
54	Tuberculous	16 × 17	11 × 13	—	15 × 15
55	Tuberculous	24 × 15	— —	—	20 × 24
56	Tuberculous	25 × 15	10 × 13	—	23 × 25
58	Normal	4 × 3	0	—	0
59	Normal	3 × 3	0	—	0
60	Normal	3 × 2	0	—	0
Humans	*Normal*	*0.1 ml of 1/1.000*			*0.1 ml of 1/1,000*
CI	(Mantoux positive)	10 × 10	—	15 × 22	10 × 15
UB	(Mantoux positive)	12 × 10	—	17 × 22	11 × 12
LG	(Mantoux positive)	14 × 12	—	14 × 21	12 × 12
JC	(Mantoux positive)	12 × 15	—	13 × 13	13 × 10

O.T. = Old Tuberculin (Burroughs Wellcome and Co.)

Absorption experiments. Rabbit antisera were treated with sufficient U_3 to precipitate all the corresponding antibody, and the resultant sera tested against U_c and D.U.E. to see if further precipitation occurred. A similar experiment was carried out using U_c for the initial precipitation (Table IV).

TABLE IV

PRECIPITIN ABSORPTION TESTS

Sera	*Absorbed with*	*Absorbed sera test*		
		U_c	U_3	*D.U.E.*
Anti-D.U.E.	U_c	—	—	—
	U_3	+	—	+
Anti-U_c	U_c	—	—	—
	U_3	+	—	+

Sensitisation. Four guinea pigs were given three subcutaneous injections of the crude urea extract (3 ml) at weekly intervals. Twenty days after the last injection the animals were tested by intradermal injection of Old Tuberculin, the crude urea extract and dialysed urea extract. Skin reactions averaging 15 × 15 millimetres were obtained showing that sensitisation had occurred. The freeze-dried D.U.E. was also able to sensitise guinea pigs, but three subcutaneous doses of 20 mg at weekly intervals were required.

Antigenicity. Both the D.U.E. and the purified U_c protein were antigenic and gave precipitin reactions with the homologous material (see Table I).

Immunisation. Four groups of twelve mice from a homogeneous stock were treated as follows: the first group were left as controls, the second and third received 0.1 mg

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of freeze-dried D.U.E. intraperitoneally, and the fourth group was given 0.01 mg of live virulent tubercle bacilli. Fourteen days later all four groups received 1.0 mg of virulent tubercle by intravenous injection. The results are shown in Table V and reveal a slight protective effect from the D.U.E. injections. In an experiment with guinea pigs, inoculation with D.U.E. did not increase the survival time of the treated animals.

TABLE V

IMMUNISATION OF MICE WITH D.U.E.

Method of treatment	*Number of Mice*	*Survivors (25th day)*	*Mean Survival*	*Increase*
1. Controls	12	—	16.5	—
2. D.U.E.	12	5	20.9	4.4
3. D.U.E.	12	5	21	4.5
4. Live Tb.	12	1	17.3	0.8

Test for significance between 2 and 3 and the control: $t = 2.45$; $n = 22$; $p = 0.02$.

DISCUSSION

The experiments described in this paper demonstrate that treatment with solid urea, extracts from *Myco. tuberculosis*, a complex mixture (D.U.E.) which can bring about a typical tuberculin skin reaction in tuberculous rabbits and Mantoux-positive humans, and gives a positive result in the complement fixation test with the sera of tuberculous humans. D.U.E. does not give a precipitin reaction with serum from normal rabbits, but does so with serum from tuberculous rabbits and from rabbits injected with either D.U.E. or dead *Myco. tuberculosis* cells. Guinea pigs injected with D.U.E. show typical skin reactions with old tuberculin.

These important biological properties made it advisable to attempt a separation of the complex mixture. Simple precipitation with ammonium sulphate was effective in isolating a component (U_c), representing 60–70% of the D.U.E. The U_c fraction was relatively homogeneous according to electrophoresis, and consisted largely of protein. It elicited typical tuberculin skin reactions, showed complement fixation with tuberculous human sera, and precipitin reactions with sera from tuberculous animals. A comparison of the proteins isolated from unheated tuberculin and those extracted by urea is obviously desirable.

The 30–40% residue of the D.U.E. after removal of U_c can be separated by treatment with lanthanum salts into a principal fraction (U_2) which is largely nucleic acid and protein, and a minor fraction (U_3) which contains carbohydrate. This small fraction is intensely reactive in the precipitin reaction against the sera of tuberculous rabbits and guinea pigs.

Attempts to determine the serological activity of U_2 were inconclusive due to precipitation occurring in some of the control normal sera, probably through traces of lanthanum remaining in the preparation.

Because the substance U_3 gave such a strong precipitin reaction, it was thought that the activity of U_c in the precipitin test might have been due to contamination of U_c with U_3. The absorption experiments described were therefore carried out. Anti-D.U.E. sera containing antibody to U_3 was found to have this antibody removed by absorption with U_c a result which is consistent with either U_3 being present in U_c or

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with U_c containing the same determinant group as U_3. In addition, antibodies to U_3 obtained by injecting U_c into rabbits were removed by absorption with U_3 but in this case the serum continued to react with U_c after absorption with U_3. U_c thus produces more than one antibody on injection.

D.U.E. does not seem to give any appreciable protection against infection by *Myco. tuberculosis* in animals, but preliminary experiments have indicated that some protection is conferred by injection of the residual urea extracted cells.

We wish to thank Professor M. STACEY, of the Chemistry Department, Birmingham, for his advice and encouragement. Two of us (C.N.I. and R.W.B.) received personal grants from the Medical Research Council of England and the Council also gave a grant for expenses.

SUMMARY

1. Extraction of living cultures of virulent *Myco. tuberculosis* with solid urea results in the production of a complex mixture of proteins, polysaccharide and nucleic acid.

2. The crude mixture has the biological properties of an unheated tuberculin and reacts in the complement fixation test with the sera of tuberculous humans.

3. By various methods of fractionation a main protein component representing 60–70% of the total extract has been isolated. This main component still contains a small amount of nucleic acid and polysaccharide. Attempts to purify it further have failed.

4. The biological properties of the crude extract are all possessed by the purified protein.

RÉSUMÉ

1. L'extraction à l'urée solide de cellules vivantes de *Myco. tuberculosis* donne lieu à un mélange de protéines, polysaccharide et acide nucléique.

2. Le mélange brut possède les propriétés biologiques d'une tuberculine non-chauffée et, dans le test de fixation de complément, il réagit avec les sérums de sujets tuberculeux.

3. Par diverses méthodes de fractionnement un constituant protéinique principal qui représente le 60–70% de l'extrait total a été isolé. Ce constituant principal contient encore de faibles quantités d'acide nucléique et de polysaccharide. Des tentatives de le purifier d'avantage ont échoué.

4. La protéine purifiée possède toutes les propriétés biologiques de l'extrait brut.

ZUSAMMENFASSUNG

1. Die Extraktion lebender Kulturen des virulenten *Myco. tuberculosis* mit festem Harnstoff ergab eine komplexe Mischung von Proteinen, Polysaccharid und Nukleinsäure.

2. Die ungereinigte Mischung hat die biologischen Eigenschaften eines nicht erhitzten Tuberkulins und reagiert im Komplementbindungstest mit den Sera tuberkulöser Menschen.

3. Mit verschiedenen Fraktionsmethoden wurde eine Hauptproteinkomponente isoliert, die 60–70% des gesamten Extraktes darstellt. Diese Hauptkomponente enthält noch einen kleinen Anteil Nukleinsäure und Polysaccharid. Versuche es weiter zu reinigen scheiterten.

4. Das gereinigte Protein besitzt alle biologischen Eigenschaften des rohen Extraktes.

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Received July 16th, 1952