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Isolation of polysaccharides from protoplasm of Mycobacterium tuberculosis variety H37Ra| antigenicity of isolates in skins of sensitized guinea pigs

Robert Henry List

The University of Montana

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ISOLATION OF POLYSACCHARIDES FROM PROTOPLASM OF MYCOBACTERIUM TUBERCULOSIS VARIETY H37Ra; ANTIGENICITY OF ISOLATES IN SKINS OF SENSITIZED GUINEA PIGS

By

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B. S., Idaho State University, 1953

Presented in partial fulfillment of the requirements for the degree of

Master of Science

UNIVERSITY OF MONTANA

1970

Approved by:

[Signatures]

Chairman, Board of Examiners

Dean, Graduate School

Date June 9, 1970
Acknowledgments

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Appreciation is also expressed for the interest, suggestions and other assistance offered by my advisor, Dr. Carl L. Larson at the University of Montana.

I wish to thank Dr. Jon Rudbach for his guidance and supervision of the ultracentrifugal analysis.

And last but certainly not least, I wish to revere Mr. William Bickel, chemist, for the analyses which played an important key role in this work.
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CHAPTER I

INTRODUCTION

The importance of the tuberculin-type reaction as a primary diagnostic procedure for detection of tuberculosis has been known since the time of Koch. Literature describing the use of culture filtrates of tubercle bacillus or other mycobacteria for eliciting the reaction is indeed voluminous.

Much remains to be discovered regarding the problem of specificity of the tuberculin reaction, as is shown by even a brief survey of the subject of delayed sensitivity reactions. For example, cross reactions occur when sensitized humans or animals are skin tested with antigens derived from closely related varieties of mycobacteria or with antigens from other species (1, 14, 19, 26, 36, 37, 41, 54, 57, 58, 59, 60, 64, 70, 71, 75, 78, 81).

Tuberculin Test and Animals

Infections with mycobacteria other than tubercle bacilli may cause a positive tuberculin response in cattle (36, 42, 54, 60). For example, Purified Protein Derivative (PPD) derived from Mycobacterium tuberculosis gives rise to noticeable reactions in cattle sensitized with Mycobacterium avium (54). The economic importance of obtaining a species- or variety-specific skin-test antigen capable of differentiating infections or sensitization by M. tuberculosis from sensitization by Johne's bacillus in testing cattle (42) is obvious. Not only is variety or species non-specificity presenting problems in interpretation
of delayed hypersensitivity but also the presence of immediate- and mixed-type Arthus reactions may cause confusion in analysis (22-25). It is not always possible to determine the cause of a particular reaction by size alone, and there are instances of pulmonary tuberculosis proved by culture and/or microscopy in which repeatedly negative skin tests to PPD occur (43).

Since the discovery of old tuberculin and its value as a diagnostic agent, many workers have labored with problems of analysis and purification of this product. Physicochemical and biological analysis of mycobacterial culture filtrates are becoming sophisticated and complex, yet the ultimate goal of obtaining skin-test antigens capable of yielding clear-cut differentiation of mycobacterial infections appears distant.

What is the chemical makeup of that component specifically responsible for the reaction? Is it a pure protein, polysaccharide, lipid, nucleic acid, etc., or a certain combination in specific proportions of some or all of these substances (2, 3, 8, 13, 32, 34, 53, 74)?

Mycobacterial Components Responsible for Provoking Tuberculin Reaction.

Protein

It is generally accepted that tuberculin protein is responsible for provoking tuberculin reactions. Seibert's (65-69) long-term experimentation involving fractionation of culture filtrate and immunological analysis of these fractions resulted in the ultimate production of PPD and these studies undoubtedly established the significant role of tuberculin protein in skin-test reactions. However, Glenchur et al. (32) postulated the impurities in PPD may have a potentiating effect. In
their experience with bacillary extracts of the H37Ra strain of
*M. tuberculosis,* some fractions having high nitrogen contents did not
provok delayed reactions in tuberculin sensitive rabbits. They sug-
gested that "the active principle" in tuberculin may be a property of
"molecular structure." *Yamamura, Someya et al.* (72, 73, 88) have
described a tuberculin-active peptide (TAP) and its potency was reported
to be almost equivalent to that of PPD. *Beam, Stottmeier and Kubica* (10)
also isolated tuberculin active peptides (with molecular weights of the
same magnitude as reported by the Japanese workers, i.e., 5,000-10,000)
from protoplasm of mycobacteria and these peptides exhibited species
specificity.

**Nucleic Acid**

No tuberculin potency was evident with nucleic acid according to
Seibert (68).

**Lipopolysaccharides and Polysaccharides**

**Lipopolysaccharides.** Little is actually stated in the literature
concerning the role of mycobacterial lipopolysaccharide in eliciting
delayed reactions in sensitized subjects. *Choucroun* (as cited by Holborow
and *Loewi* (38)) suggested that it provoked delayed reactions but
Kourilsky (45) considered that the lesions represented a prolonged
reaction of this antigen with circulating antibody.

*Choucroun’s* (16) paraffin oil extract, a carbohydrate-lipid complex
(PmKo), from heat-killed tubercle bacilli was tested by Dannenberg et al.
(18) by intradermal injections into sensitized rabbits. The dermal
response to PmKo was high in one strain of inbred rabbits but the authors
attributed the reaction to toxicity and a possible combination of delayed hypersensitivity and "other factors." The presence of bacilli in the product was not ruled out; this antigen also contained 1.05% nitrogen. According to these workers, PmKo, as a test antigen, has definite limitations.

Polysaccharides. Polysaccharides derived from culture filtrates of \textit{M. tuberculosis} have been shown to be serologically active but are generally considered to be haptens according to Holborow and Loewi (38). Stacey (74) concluded his article on "Mycobacterial Polysaccharides" with this statement: "Polysaccharides do not appear to elicit skin reactions and Seibert's work on the purified derivative (PPD) of tuberculosis places the emphasis on proteins as being of the greatest importance in skin sensitivity. Work on lipopolysaccharides is, however, of potential importance in this field. There is still a great deal of work to be done on the chemistry of \textit{Mycobacterium tuberculosis} cells and cell products. Attention should perhaps now be directed to the chemical and biological properties of mucopolysaccharide, mucoprotein and mucolipid constituents."

\textbf{Tuberculin Reactivity of a Carbohydrate Component of Unheated BCG Culture Filtrate}

Baer and Chaparas (8) indicated that a carbohydrate and a protein fraction obtained from an unheated culture filtrate of BCG had equal ability to provoke delayed reactions in guinea pigs sensitized with heat-killed BCG. The action of two proteolytic enzymes caused little alteration in the reactivity of the carbohydrate fraction but almost completely eliminated the reactivity of the protein moiety. The carbohydrate
contained 0.4% nitrogen which they attributed to some enzyme protein. In a later paper (9) they reported that this polysaccharide can give a "true delayed skin reaction." Guinea pigs were sensitized with doses of 1 to 500 mg BCG without stimulating anti-polysaccharide antibody, although anti-protein antibody was invariably present.

Recently, Godfrey, Baer and Chaparas (33) demonstrated inhibition of macrophage migration by a skin-reactive polysaccharide from BCG culture filtrate.

**Delayed Hypersensitivity to Polysaccharides derived from Organisms Other than Mycobacteria**

Campbell (12) isolated from *Ascaris lumbricoides* a pure nitrogen-free polysaccharide capable of eliciting a delayed skin reaction in rabbits sensitized to this worm. Knight and Marcus (44) observed dermal reactions in guinea pigs infected with *Histoplasma capsulatum* or *Blastomyces dermatitidis* following injections of 10 gamma (µg) of polysaccharide from these organisms. In their report, it was not made clear as to whether these reactions were considered to be of delayed or immediate type, although there was one reference to a reading 24 hours after injection. However, upon analysis, these preparations contained 4 to 5% nitrogen. Coccidioidin "polysaccharides" had also been shown to provoke delayed skin reactions in specifically sensitized guinea pigs but treatment of these materials with proteolytic enzymes depressed their skin-test activity. These antigens contained 6.1% nitrogen. *Aspergillosis fumigatus* skin-test antigens studied by Azuma and co-workers (4) included a polysaccharide fraction which contained no
nitrogen. This fraction was useful for precipitation antigens but produced no skin reactivity.

Protoplastum Compared to Culture Filtrate as Source Material of Skin-test Antigen

The crude culture filtrate and, for that matter, PPD could very likely represent a complex mixture of cell debris from the entire organism (32, 66-69). Intense tuberculin reactivity and a degree of specificity of the reaction has been demonstrated with mycobacterial protoplasts (46-50). These results have been confirmed later by Counts and Kubica (17). Consequently, an important fractionation step had been achieved as differential centrifugations removed the cell wall portion of disrupted mycobacteria. Thus protoplasm, in the author's opinion, would conceivably represent a less complex system for investigation than that of PPD.
CHAPTER II

STATEMENT OF THE PROBLEM

Two significant findings were gleaned from the literature: (i) the apparent tuberculin-type reactivity of a mycobacterial polysaccharide and (ii) the activity and specificity of mycobacterial protoplasms as skin-test antigens.

Subsequently, the original goal of this project was an attempt to isolate from *M. tuberculosis* H37Ra protoplasm pure polysaccharide(s) with particular ability to induce tuberculin (delayed) reactions, and on a smaller scale to study anaphylactic (immediate) type reactions in sensitized guinea pigs.

I attempted (i) to separate and isolate a variety of fractions from protoplasm of *Mycobacterium tuberculosis*, variety H37Ra, by physico-chemical means; (ii) to determine the chemical composition of the fractions in terms of total carbohydrate and nitrogen content; (iii) to compare relative tuberculin skin-test reactivity of the high carbohydrate-containing components (polysaccharides), PPD, and the protein-rich fractions derived from protoplasm; and (iv) to determine if protoplasmic polysaccharides would produce anaphylaxis in sensitized guinea pigs.
CHAPTER III

METHODS AND MATERIALS

(1) Preparation of Protoplasm

Several lots of \textit{M. tuberculosis} variety H37Ra grown for 2 weeks at 37°C on Sauton's liquid medium were harvested on gauze over a wire strainer and washed at least twice with cold sterile distilled water. The excess moisture was removed by vacuum filtration in a Buchner funnel containing two layers of Whatman number 1 filter paper. Purity of the washed cells was checked with heavy inoculations on duplicate nutrient agar and blood-agar plates which were incubated at 37°C for at least a week.

The wet cell mass was suspended in cold water to a concentration of approximately 200 mg wet weight per ml and disrupted in the Sorvall-Ribi Refrigerated Cell Fractionator at 35,000 psi. The resulting disruption product was then centrifuged at 10,000 G for 1 hour. The supernatant (protoplasm) was similarly recentrifuged in an SS-34 Sorvall rotor 27,000 G to remove possible intact organisms and cell wall fragments. The protoplasm was either (i) immediately shell-frozen in glass bottles rotated in an alcohol-dry ice bath and then lyophilized or (ii) placed into plastic bottles and stored frozen (-24°C) until further use.

(2) Fractionation of Protoplasm

A. Ammonium sulfate precipitation

Fractionations of protoplasm in aqueous solutions (25-50 mg dry wt/ml H$_2$O) were made with ammonium sulfate slowly added to the solution...
in an ice bath on a magnetic stirrer. The salt was added stepwise to 20, 40, 60, and 100% saturation (70.6 grams ammonium sulfate plus 100 ml water = 100% saturation). After attainment of each salt concentration, the mixture was stirred 20 min and centrifuged 30 min at 27,000 G. The supernatant was then treated to attain the next higher salt concentration. The pellets were taken up with 5- to 10-ml volumes of water and dialyzed.

B. Acetic acid precipitations

The acid-soluble carbohydrate fractions were obtained by the procedure of Baer and Chaparas (7). The dried material (protoplasm or its subfraction at 25 mg dry wt/ml) was suspended in ice cold 0.15 M phosphate buffer containing 0.5 M NaCl at pH 7.2. A 1% solution of acetic acid was added with stirring to adjust the solution to pH 4. This mixture was stored overnight at 4 C and then centrifuged at 27,000 G for 30 min. The supernatant (acid sup) was carefully decanted from the pellet.

C. Phenol extractions

Before phenol extraction of protoplasm was attempted, the protoplasm was pre-extracted to remove portions of various waxes and lipids which could conceivably shield the polysaccharide portion of the protoplasm from the phenol. Previous phenol extractions of either untreated mycobacterial protoplasm or cell walls produced disappointingly low yields of carbohydrate. The extractions prior to the aqueous phenol treatment were performed as follows: Overnight continuous extractions in a Soxhlet apparatus with acetone, followed by several chloroform extractions in a separatory funnel. The resultant residue was then subjected to phenol extractions by Westphal's (83) method: Equal volumes of liquid phenol (88%) and aqueous protoplasm (50 mg/ml) were stirred
in a 67°C water bath for 30 min. The phenol-aqueous mixture was placed in 100-ml glass centrifuge tubes, cooled in an ice bath for 30 min, and centrifuged for 1 hr at 1,400 G in a refrigerated International centrifuge. The upper aqueous phase was carefully removed and dialyzed.

D. Ethyl alcohol (30%) precipitation

In some cases, further fractionation of the acid sup or of the aqueous phases of the phenol extract was obtained with a 30% ethyl alcohol precipitation at 4°C as described by Seibert and Glenn (69). The above materials were dissolved (10 mg/ml) in 0.01 M phosphate buffer, pH 7.0. Absolute alcohol was slowly added with stirring until a 30% concentration was reached. The mixture was stirred for 1 hr and was then centrifuged in the No. 30 Spinco head at 73,300 G for 30 min. Pellet and supernatant were separated and dialyzed.

E. Trichloroacetic acid extractions.

A simplified version of the trichloroacetic acid (TCA) extraction technique of Webster et al. (82) was used. Extractions were made by constantly stirring in an ice bath equal volumes of protoplasm (50 mg dry wt/ml of water) and 0.5 M TCA for 3 hr. The precipitated solids were separated from the supernatant (extract) by centrifugation (1,400 G for 1 hr) and the two components were dialyzed and lyophilized. Analytical and differential runs of the TCA extracts were made in the Spinco Models E and L, respectively, at 20°C.

(3) Dialysis and lyophilization of protoplasmic fractions

After fractionation, all samples were dialyzed at 4°C against approximately a 4-liter volume of double demineralized water for 3–4
days and a minimum of three changes of water. The dialyzed materials were lyophilized.

(4) Purified Protein Derivative (PPD)

Second strength PPD from Parke, Davis and Company, Detroit, Michigan, was used as a control.

(5) Guinea Pig Skin Tests

The guinea pig was chosen as test animal because of its "par excellence" in exhibiting tuberculin-type hypersensitivity (84). Female guinea pigs weighing approximately 30 g were either of the Hartley albino strain, grown locally by R. C. Rosecrans, Hamilton, Montana, or of mixed strains supplied by D. Patterson, Hamilton, Montana. The latter were predominantly albinos, but an occasional animal possessed small black or brown markings.

A. Methods of sensitizing guinea pigs

(1) H37Ra in incomplete Freund's adjuvant. The incomplete adjuvant consisted of 65% Drakeol 6-VR (Pennsylvania Refining Co., Butler, Penn.) and 35% Arlacel A (Atlas Chemical Industries, Inc.) by volume. Dried H37Ra cells were suspended in water (10 mg/ml) and churned in a Sorvall Omnimixer for about 5 min to facilitate thorough dispersion and some disruption of the intact cells for release of internal antigenic material. These dispersed cells were heated at 67 C for 35 min (unless stated otherwise) and emulsified with an equal volume of incomplete Freund's adjuvant. Guinea pigs were each inoculated with 10 mg (dry weight) H37Ra in each axillary and inguinal area with
0.5 ml doses of the complete adjuvant. In each experiment, four untreated animals were held for normal controls.

(ii) H37Ra in mineral oil. For sensitization, each animal received an intramuscular injection (left hip) of 5 mg dried heat-killed H37Ra in 0.5 ml mineral oil (Drakeol 6-VR). Similar doses (of 5 mg of the dry cells in oil) were given to each guinea pig in the same general area 1 week following the initial dose. Three non-injected animals served as normal controls.

B. Methods of challenge

Intradermal injections of 0.1 ml of the test antigens in all experiments were given approximately 7 weeks after the initial sensitizing injections. The antigens were prepared with either 5- or 10-fold serial dilutions in saline. The highest dose used was either 1 or 10 µg. Non-sensitized control animals were subjected to the highest dose of each test antigen.

C. Interpreting reactions of provoking doses

(i) Delayed hypersensitivity. The area of erythema was measured in mm² and induration with a "Schnelltester" (H. C. Kroplin, Schuchtern, Hessen, Germany) in millimeters at 24 and/or 48 hr after challenge. Lesions less than 5 mm in diameter and less than 1 mm in thickness were considered negative.

(ii) Immediate hypersensitivity

(a) Active cutaneous anaphylaxis. As described by Ovary (56), two ml of 0.6% Evans Blue in saline were injected i.v. into the guinea pigs, immediately followed with intradermal injections of
antigens. A dark blue area approximating 10 to 15 mm in diameter and generally appearing within 5 min constituted a positive reaction.

(b) Systemic anaphylaxis. Two mg of antigen in 1 ml saline were given i.v. to each guinea pig.

D. Guinea pig test sera

In all of the experiments, the animals were bled prior to sensitization and again just before eliciting skin reactions. In most experiments the animals were also bled again a week or two after the skin tests.

E. Anesthesia

Animals were anesthetized with 0.2 to 0.3 ml sodium nembutal intraperitoneally 30 min prior to bleedings and injections to enable a one-man task. One exception was made, however: i.v. injection of Evans Blue dye required an assistant for holding guinea pigs and anesthesia was not necessary.

(6) Immunological Analysis

The Ouchterlony immunodiffusion tests were performed in 0.5% "Oxoid Ionagar No. 2" (Consolidated Laboratories, Inc., Chicago Heights, Illinois) containing 0.85% NaCl and 0.01% merthiolate.

(7) Chemical Analysis

A. Total hexose

The determination by the anthrone method is modified from that described by Scott and Melvin (65).
B. Total carbohydrate
The method of Dische (20) was used, with glucose as the standard.

C. Nitrogen
The assays were made with method of Johnson (40), as modified by Umbreit et al. (80).

D. Total fatty acid
The composition was determined by the method of Haskins (35).

E. Phosphorus
The content was analyzed by the procedure of Dryer, Tommes and Routh (21).
CHAPTER IV

EXPERIMENTAL PROCEDURES AND RESULTS

Fractionation of Protoplasm and Purification of Polysaccharides

Initially, pilot experiments were attempted for fractionation of crude protoplasm by Sephadex G-25 filtration. However, difficulties were encountered when very fine Sephadex particles filtered through the column and distorted chemical analysis, particularly carbohydrate determinations. Fractional ammonium sulfate precipitations appeared to be satisfactory for our purpose and this proved to be a comparatively simple technique.

The 5-step ammonium sulfate-precipitated fractions obtained by 20% to 100% saturation of H37Ra protoplasm showed no significant separation of protein and carbohydrate, although the proportions of these two substances in the precipitate varied according to the salt concentration used. Similar results were obtained by Pickett et al. (61). Thus, an acetic acid precipitation (Seibert method) (67) was done on each of the five precipitates as an additional fractionation step. The results are shown in Fig. 1. The supernatants in every case contained a greater proportion of total carbohydrate than did the sediment. Fraction C, the pellet obtained by 60% saturation, contained the greatest amount of carbohydrate (86.6%) and a relatively low concentration of nitrogen (3.21%).

Further purification of supernatant C was carried out with a 30% ethyl alcohol precipitation using the Seibert (67) technique. The
Fig. 1. Fractional ammonium sulfate precipitations of H37Ra protoplasm further separated with addition of 1% acetic acid to pH 4. The supernatant (S) and pellet (P) resulted from differential centrifugations of the acidified fractions.

The plain bars represent per cent of total carbohydrate and the slashed bars show the nitrogen percentage.
supernatant subsequently proved to contain a very pure carbohydrate (100%) with nitrogen, fatty acids and phosphorus essentially absent. This product was designated as Fraction C ETOH precipitate (CAEP) (Table 1 and Fig. 2A, Procedure I).

A portion of H37Ea protoplasm was extracted to isolate polysaccharide, according to the method of Baer and Chaparas (7). Analysis of the supernatant (acid sup), which remained after the system was adjusted to pH 4 by drop-wise addition of 1% acetic acid indicated that it contained 75.9% total carbohydrate and 4.5% nitrogen. The precipitate formed after alcohol concentration contained 100% total carbohydrate and no detectable nitrogen. This product was labeled acid-ETOH precipitate (AEP) (Fig. 2A, Proc. II).

Another relatively pure polysaccharide containing 100% carbohydrate and only 0.31% nitrogen was obtained from crude protoplasm by a single TCA extraction (82). This fraction was designated TCA extract (Fig. 2A, Proc. III). This procedure was unique in its simplicity and high productivity: an average yield of 10% polysaccharide was obtained from the crude protoplasm. The recoveries of carbohydrate from supernatants of TCA extracts were many-fold greater than obtained by any of the other techniques described.

**Phenol extractions.** The phenol extraction method of Westphal (83) of H37Ra protoplasm was carried out after previous acetone-chloroform extractions were made of the protoplasm to remove portions of waxes and lipids. The aqueous phase resulting from phenol extraction contained 3.85% nitrogen and 71.8% total carbohydrate. Another step for
<table>
<thead>
<tr>
<th>Column I</th>
<th>Column II</th>
<th>Column III</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>AMMONIUM SULFATE ACID-ETOH PRECIPITATE (CAEP)</strong></td>
<td><strong>ACID-ETOH PRECIPITATE (AEP)</strong></td>
<td><strong>TRICHLOROACETIC ACID EXTRACTION (TCA Ext)</strong></td>
</tr>
<tr>
<td>Crude protoplasm (NH₄)₂SO₄, Precipitation Fraction C (60% saturation)</td>
<td>Crude protoplasm</td>
<td>Crude protoplasm</td>
</tr>
<tr>
<td>27,000 G, 30 min</td>
<td>Dissolved in 0.015 M phos. buffered saline pH 7.2 1% acetic acid added to pH 4</td>
<td>Dissolved in H₂O Precipitated in 0.25 N TCA</td>
</tr>
<tr>
<td><strong>SUPERNATANT</strong></td>
<td>27,000 G, 30 min</td>
<td>1,400 G, 1 hr</td>
</tr>
<tr>
<td>Dialyzed and lyophilized</td>
<td><strong>PELLET</strong></td>
<td><strong>PELLET</strong></td>
</tr>
<tr>
<td>Dissolved in 0.015 M phos. buffered saline, pH 7.2 1% acetic acid added to pH 4</td>
<td><strong>SUPERNATANT</strong></td>
<td>144,000 G, 3 hr</td>
</tr>
<tr>
<td>27,000 G, 30 min</td>
<td><strong>PELLET</strong></td>
<td><strong>HSS</strong></td>
</tr>
<tr>
<td>Dialyzed and lyophilized</td>
<td><strong>SUPERNATANT</strong></td>
<td><strong>PELLET</strong></td>
</tr>
<tr>
<td><strong>SUPERNATANT</strong> in 0.015 M phos. buffer pH 7.0 30% ETOH</td>
<td></td>
<td>(AEP)</td>
</tr>
<tr>
<td>73,300 G, 30 min</td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>SUPERNATANT</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>PELLET (CAEP)</strong></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figure 2A. Procedures for obtaining various polysaccharides from H37Ra protoplasm.
IV

**ETCH PRECIPITATED AQUEOUS PHENOL EXTRACT (APE)**

Crude protoplasm

Soxhlet extraction with acetone overnight

**SUPERNATANT**

**PELLET**

CHCl₃ extraction in separatory funnel 3-4 times

**SUPERNATANT**

**PELLET**

Suspended in H₂O (50 mg/ml)

Extracted with equal volume of 88% phenol 67°C, 30 min

1,400 G, 1 hr

**PHENOL PHASE AND PELLET**

**SUPERNATANT**

(Aqueous phase)
dialyzed and lyophilized

Suspended in 0.01 M phos. buffer, pH 7

73,000 G, 30 min

**SUPERNATANT**

**PELLET**

(APE)

---

Figure 2B. Procedure for obtaining a polysaccharide from H37Ra protoplasm using phenol and alcohol.
<table>
<thead>
<tr>
<th>Preparation</th>
<th>Nitrogen</th>
<th>Phosphorus</th>
<th>Hexose</th>
<th>Total carbohydrate</th>
<th>Hexosamine</th>
<th>FAA and FAE&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Crude protoplasm</td>
<td>9.24</td>
<td>2.49</td>
<td>14.9</td>
<td>21.3</td>
<td>0.67</td>
<td>22.1</td>
</tr>
<tr>
<td>Fraction C acid-ETOH precipitate</td>
<td>0</td>
<td>&lt;0.1</td>
<td>99</td>
<td>100</td>
<td>0.94</td>
<td>0</td>
</tr>
<tr>
<td>Acid &lt;sup&gt;-&lt;/sup&gt; precipitate</td>
<td>0</td>
<td>&lt;0.1</td>
<td>100</td>
<td>100</td>
<td>1.13</td>
<td>0</td>
</tr>
<tr>
<td>TCA extract</td>
<td>0.31</td>
<td>0.17</td>
<td>94.4</td>
<td>100</td>
<td>0.94</td>
<td>1.68</td>
</tr>
<tr>
<td>Aqueous-phenol- ETOH precipitate</td>
<td>1.23</td>
<td>4.92</td>
<td>78.0</td>
<td>89.3</td>
<td>0.88</td>
<td>3.69</td>
</tr>
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</table>

<sup>a</sup> Fatty acid amides and fatty acid esters.
purification of this was attempted by precipitation with 30% alcohol. Alcohol precipitation of the phenol extract yielded a product containing a fairly high carbohydrate content (89.3%) but contaminated with fatty acid and esters (3.69%), phosphorus (4.9%) and nitrogen (1.23%). This rather complex procedure is outlined in Fig. 2B, Proc. IV. The phenol extract was not skin tested in preliminary experiments because it contained a greater proportion of impurities than polysaccharides derived by other procedures described (Table 1).

Skin tests in guinea pigs. In the first three experiments, each of the test animals was sensitized with 10 mg of H37Ra in incomplete Freund's adjuvant and tested intradermally approximately 7 weeks later.

Experiment #1. Role of Polysaccharides (acid-ETOH precipitates, TCA extract) in Challenge. The components tabulated in Table 1 were tested (with the exception of the phenol extract) in the skins of sensitized guinea pigs. H37Ra cell walls were also included in this experiment.

As shown on Table 2, polysaccharides failed to elicit measurable reactions. The TCA extract, which contained 0.31% nitrogen, elicited a mild reaction at this site of injection of 10 μg. The high nitrogen-containing crude protoplasm was the most active material studied in this test, having 5-fold greater reactivity than the next most active preparation, cell walls. Unfortunately, skin thickness was not measured in this experiment.
TABLE 2. Delayed skin reactions of guinea pigs sensitized 7 weeks previously with H37Ra in incomplete Freund's adjuvant

<table>
<thead>
<tr>
<th>Preparation(^a)</th>
<th>No. animals tested</th>
<th>Mean area of erythema in (\text{mm}^2) at 48 hr</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Doses ((\mu)g)</td>
</tr>
<tr>
<td></td>
<td></td>
<td>10</td>
</tr>
<tr>
<td>CAEP(^b)</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>AEP(^c)</td>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>TCA extract</td>
<td>10</td>
<td>30</td>
</tr>
<tr>
<td>Crude protoplasm</td>
<td>20</td>
<td>284</td>
</tr>
<tr>
<td>Cell walls</td>
<td>20</td>
<td>50</td>
</tr>
</tbody>
</table>

\(^a\) Four nonsensitized controls subjected to the highest dose of each preparation were negative.

\(^b\) Polysaccharide produced by 60\% ammonium sulfate--acid--ETOH precipitation.

\(^c\) Polysaccharide produced by acid--ETOH precipitation.
Experiment #2. Two Lots of TCA Extracts Compared in Ability to Elicit Delayed Responses. In the second skin-test experiment, an additional TCA extract of protoplasm derived from another culture of H37Ra cells was used. These additional protoplasmic products of the second TCA extraction will be designated as Lot 2 (L2).

The two lots were tested simultaneously in sensitized guinea pigs. The crude protoplasms, high nitrogen-containing TCA residues PPD and EAP were also tested for their skin-test reactivity (Table 3). It should be noted here that the results in Table 3, and in succeeding tables, cannot be directly compared to the results given in Table 2 as data in Table 2 did not include induration of the lesions. The most reactive component in Experiment #2 was PPD as was anticipated because of its high nitrogen content (15%). The 1 μg dose of PPD gave a 3-to 4-fold greater reaction than similar doses of high nitrogen-containing crude protoplasms. In this experiment, no clear-cut correlation could be made in the increase or decrease of the size of the lesions at 24- and 48-hours. The slight activity of the EAP was somewhat enhanced in one particular animal which was unusually sensitive to all preparations used in this test.

Experiment #3. Further Separation and Purification of TCA Extracts by High-Speed Centrifugation. Immunodiffusion analysis.

Ultracentrifugal analysis was introduced in Experiment #3. One per cent TCA extract in 0.1 M Tris buffer, pH 8, was centrifuged in a Spinco Model E analytical rotor. At 22,000 G (17,250 rev/min) a fast-moving component(s) appeared which rapidly sedimented. Five exposures
TABLE 3. Results of skin tests of guinea pigs sensitized 7 weeks previously with H37Ra in Freund's incomplete adjuvant. A comparison of 24- and 48-hr recordings

<table>
<thead>
<tr>
<th>Preparation</th>
<th>No. animals tested</th>
<th>Mean volume mm$^3$</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Hours after injection</td>
<td>Doses (µg)</td>
<td>10</td>
<td>1.0</td>
</tr>
<tr>
<td>TCA extract</td>
<td>8</td>
<td>24</td>
<td>262</td>
<td>40</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>273</td>
<td>21</td>
<td>0</td>
</tr>
<tr>
<td>Crude protoplasm</td>
<td>8</td>
<td>24</td>
<td>387</td>
<td>89</td>
<td>28</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>564</td>
<td>131</td>
<td>26</td>
</tr>
<tr>
<td>EAP</td>
<td>8</td>
<td>24</td>
<td>97</td>
<td>28</td>
<td>8</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>140</td>
<td>13</td>
<td>0</td>
</tr>
<tr>
<td>PPD</td>
<td>18</td>
<td>24</td>
<td>---</td>
<td>498</td>
<td>149</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>---</td>
<td>360</td>
<td>115</td>
</tr>
<tr>
<td>Lot 2</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>TCA extract</td>
<td>10</td>
<td>24</td>
<td>210</td>
<td>42</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>146</td>
<td>28</td>
<td>0</td>
</tr>
<tr>
<td>TCA residue</td>
<td>10</td>
<td>24</td>
<td>313</td>
<td>115</td>
<td>43</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>336</td>
<td>81</td>
<td>23</td>
</tr>
<tr>
<td>Crude protoplasm</td>
<td>10</td>
<td>24</td>
<td>693</td>
<td>183</td>
<td>86</td>
</tr>
<tr>
<td></td>
<td></td>
<td>48</td>
<td>521</td>
<td>141</td>
<td>33</td>
</tr>
</tbody>
</table>

a Four non-sensitized controls subjected to highest dose of each antigen were negative.
b Average area of erythema in mm$^2$ x induration in mm.
c Two deaths as a result of faulty cardiac puncture or anesthesia.
at 8-min intervals were made and the patterns are shown in Fig. 3A. The sedimentation coefficient was estimated to be about 100S. When the speed was increased to 187,100 G (50,740 rev/min), a rounded slow-moving peak resulted. During this run, five 16-min exposures were made (Fig. 3B) and the sedimentation coefficient was calculated to be approximately 1.8S.

Another portion of the 1% TCA extract in 0.1 M Tris buffer was centrifuged at 144,700 G (40,000 rev/min) in a No. 40 Spinco head for 3 hr to separate the slow- and fast-moving components. Here an assumption had been made that, since the fast-moving component of the solution did rapidly sediment at 22,000 G, a 3-hr centrifugation at 144,700 G should have been adequate for separating these fractions. After centrifugation, the high-speed supernatant (HSS) was carefully separated from the pellet (HSP) (Fig. 2A, Proc. III).

Skin test in guinea pigs. Experiment 4. In this test, 20 animals were injected with H37Ra cells in Freund's incomplete adjuvant, 10 with living and 10 with killed cells. The skin test data of HSS and HSP, the TCA extract and PPD are given in Table 4.

The most striking result in the centrifugal separation of the two major components is that the activity remained in the HSS (0.56% N). The HSP (no measurable N) was completely negative even at the highest dose levels in all 19 sensitized animals. The HSS was also somewhat more potent in eliciting reactions than the crude TCA extract (0.31% N) from which it was derived. PPD was again the most active of all components tested.
Fig. 3. Ultracentrifuge patterns of 1.0% TCA extract in 0.1 M Tris buffer pH 8.
A. Represents a 22,000 G centrifugation with 5 exposures at 8-min intervals.
B. Shows a 187,100 G run with 5 exposures at 16-min intervals.
TABLE 4. Delayed skin reactions of guinea pigs sensitized 7 weeks previously with living or killed H37Ra in incomplete Freund's adjuvant induced by H37Ra TCA products and PPD

<table>
<thead>
<tr>
<th>Preparation</th>
<th>Method of sensitization:</th>
<th>Mean volume mm$^3$ at 48 hr</th>
<th>Chemistry</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Doses (µg)</td>
<td>N</td>
</tr>
<tr>
<td></td>
<td>K</td>
<td>0.1</td>
<td>0.1</td>
</tr>
<tr>
<td>TCA (HSP)</td>
<td>K</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>TCA (HSS)</td>
<td>K</td>
<td>371</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>587</td>
<td>0</td>
</tr>
<tr>
<td>TCA (crude)</td>
<td>K</td>
<td>211</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>284</td>
<td>0</td>
</tr>
<tr>
<td>PPD</td>
<td>K</td>
<td>---</td>
<td>421</td>
</tr>
<tr>
<td></td>
<td>L</td>
<td>---</td>
<td>438</td>
</tr>
</tbody>
</table>

- Four control animals subjected to highest dose of each antigen were negative.
- 10 animals sensitized with killed and 10 with live cells.
- Chemistry according to F. Seibert and J. T. Glenn (69).
Guinea pigs sensitized with living organisms showed greater reactivity to all provoking doses than those animals injected with the corresponding killed organisms.

**Immunodiffusion analysis. Experiment 3.** The crude protoplasm, its TCA extract, HSS and HSP were subjected to an immunodiffusion test. All antigens (10 mg/ml saline) were tested against sera (4X concentrated) from guinea pigs sensitized 7 weeks previously with H37Ra in Freund's incomplete adjuvant. No precipitation bands were apparent between the HSP well and the center reservoir containing immune serum (Fig. 4). Several bands, however, were detected near the nitrogen-containing HSS, which were identical with some bands in the individual TCA extractions. These antigens (10 mg/ml) were tested against concentrated pooled normal guinea pig sera obtained prior to sensitization of the same lot of animals. There were no positive reactions.

**Experiment 4. Delayed and Immediate (cutaneous anaphylaxis) Sensitivity in Animals Sensitized with H37Ra Cells in Oil.**

Guinea pigs in this test were sensitized with dried H37Ra (5 mg) in mineral oil. An additional 5 mg booster dose was injected 1 week after the initial dose. Three animals not subjected to the antigen were the normal controls.

**Skin test antigens.** Test antigens HSS and HSP from two different lots of TCA extracts were compared for cutaneous anaphylactic and tuberculin activity. In addition, the aqueous phenol extract APE (Fig. 2B, Proc. IV), the crude polysaccharide (L2) not previously tested, polysaccharide (acid-alcohol precipitate) AEP (Fig. 2A, Proc. II), the residue of the TCA extraction, crude protoplasm and PPD were
Fig. 4. Zones of precipitation produced by products of H37Ra protoplasm. Center reservoir SS, sera from sensitized guinea pigs; 1, high-speed pellet (HSP); 2, high-speed supernatant (HSS) of TCA extract; 3, TCA extract; and 4, crude protoplasm.
also included in this test.

Dilutions of each preparation in saline were given intracutaneously in doses of 10, 2, 0.4, 0.08, 0.016 and 0.003 μg. Because of its relatively high reactivity, only 2 μg of PPD was injected as the highest dose. The results are given in Table 5.

Delayed hypersensitivity. As before, the polysaccharides were essentially inert in skins of sensitized guinea pigs (Table 5). The relatively impure phenol extract (1.23% N and 39.3% total carbohydrate) did not produce a lesion. L2 AEP was mildly active in one animal for some reason. The strongest reactions resulted with injected PPD and milder reactions were expressed with TCA extract residue and crude protoplasm.

Active cutaneous anaphylaxis. All animals, including the non-sensitized normals, were given 2 ml 0.6% Evans Blue dye i.v. 1 to 2 hr prior to the i.q. challenge. Both lots of HSS, the residue to TCA extraction and the crude protoplasm were positive in the 10- and 2-μg dose ranges. No positive reactions were detected with the lower dose; the cut-off point of activity was strikingly abrupt and there was no diminution or quantitative response in the lower doses. The reactions exhibited solid dark blue areas of approximately 12 mm in diameter. All positive reactions became apparent within 5-10 min (Table 5).

Anaphylactic shock. The polysaccharides AEP, HSP, HSS and the residue of TCA extraction were tested in guinea pigs for anaphylactic shock; all were negative. However, a trace of activity was attributed to HSS which caused 3 of the 5 animals challenged to cough or sneeze briefly a few minutes after challenge.
TABLE 5. Delayed (48-hr tuberculin) and immediate (anaphylactic) type reactions in skins of guinea pigs sensitized 7 weeks previously with H37Ra in mineral oil

<table>
<thead>
<tr>
<th>Skin test antigena</th>
<th>No. animals tested</th>
<th>Tuberculin reactionb Doses (μg)</th>
<th>Immediate reactionc</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10  2.0  0.4  0.08</td>
<td>10  2.0  0.4</td>
</tr>
<tr>
<td>HSP (40,000 rev/min pellet)</td>
<td>5</td>
<td>0   0   0    0</td>
<td>0    0    0</td>
</tr>
<tr>
<td>L2 HSP (40,000 rev/min pellet)</td>
<td>5</td>
<td>0   0   0    0</td>
<td>0    0    0</td>
</tr>
<tr>
<td>HSS (40,000 rev/min sup)</td>
<td>5</td>
<td>240 68 13    0</td>
<td>5    5    0</td>
</tr>
<tr>
<td>L2 HSS (40,000 rev/min sup)</td>
<td>5</td>
<td>199 96 0     0</td>
<td>3    3    0</td>
</tr>
<tr>
<td>AEP (acid-alcohol ppt)</td>
<td>5</td>
<td>trace trace 0</td>
<td>0    0    0</td>
</tr>
<tr>
<td>APE (aq. phenol ext)</td>
<td>5</td>
<td>0   0   0    0</td>
<td>0    0    0</td>
</tr>
<tr>
<td>TCA res</td>
<td>5</td>
<td>238 114 7    0</td>
<td>4    4    0</td>
</tr>
<tr>
<td>Crude protoplasm</td>
<td>5</td>
<td>320 201 34   0</td>
<td>4    4    0</td>
</tr>
<tr>
<td>PPD</td>
<td>5</td>
<td>-   284 108 37</td>
<td>0    0    0</td>
</tr>
</tbody>
</table>

a No delayed or immediate reactions in 3 normal animals subjected to highest dose of each antigen.
b Volume mm$^3$ at 48 hr.

c Number of animals positive to active cutaneous reactions in 5-10 min. The reaction was characterized by a dark blue area approximating 12 mm in diameter.
In general, the intensity of tuberculin-type reactivity and relative nitrogen content of the test antigen appears to be directly correlated which is in accord with the findings of others (4, 5, 16, 17, 53, 68). For example, PPD, which contained the highest per cent of nitrogen of all products tested, also produced the greatest degree of delayed-type hypersensitivity in skins of guinea pigs. Carbohydrate-rich, nitrogen-poor samples were relatively inert and preparations containing measurable amounts of nitrogen subsequently induced skin erythema.

The extent of immediate hypersensitivity, in general, also corresponded quantitatively to nitrogen content, with the exception of PPD which was unexpectedly negative.

Tillet and Frances (79) in 1929 demonstrated the immediate-type reaction (30-60 min) with their pneumococcal polysaccharides which were type specific. They also produced delayed-type hypersensitivity to pneumococcal protein fractions which, however, were not type specific. More recently (1970), a pneumococcal polysaccharide was involved in both induction and elicitation to delayed hypersensitivity (30). Foshay (29) in 1936 indicated that a "bacterial-specific intradermal antiserum reaction" is due to an antigen-antibody reaction involving only the species-specific Francisella tularensis polysaccharide. He suggested that the same or a similar mechanism may be responsible for the identical type of bacterial-specific reaction in certain other infectious diseases. Perhaps specificity in polysaccharides of myco-
bacteria could also play a role in species-specific immediate hypersensitivity (23, 52, 85, 86).

In this study, methods of isolating and extracting polysaccharides of mycobacteria were quite variable. Some physicochemical methods described by others were followed but no attempt was made to compare data of biological activities of their respective products with the data obtained in this investigation. In these other experiments, culture filtrates were generally the initial raw materials for fractionation, whereas in this study protoplasm was used which could conceivably represent a different medium free of most cell wall materials. However, some cell-wall contamination cannot be conclusively ruled out.

Criteria for selecting samples for continuing purification and analysis were simply choosing those components with the highest total carbohydrate and the least nitrogen. As work progressed, the TCA-extraction method proved to be the simplest and most productive in isolating relatively pure polysaccharides from crude protoplasm: isolation of the low nitrogen-, high carbohydrate-containing component was accomplished in one simple maneuver. Consequently, emphasis was placed onto the TCA extracts during the last stages of this project. However, the fact that the crude TCA extract contained approximately 0.3% nitrogen presumably accounted for the moderate tuberculin activity in sensitized guinea pig skins. Analytical ultracentrifugal analysis of the TCA extract further indicated two major components or composites thereof, one of which (HSP) could be rapidly sedimented at a relatively low speed, 22,000 G. The component(s) in the supernatant which
scarceiy moved at 187,000 G was calculated to be about 1.8S. Subsequent chemical data on these two isolated components show that delayed-type hypersensitivity is associated with the nitrogen-containing portion, which is in the supernatant (HSS) of the high-speed run.

In immunodiffusion analysis, no reaction (precipitation band) was apparent with the nitrogen-free component, HSP, under the conditions of this particular test. According to Janicki (personal communication) who analyzed our components (H37Ra crude protoplasm, TCA extract, HSP and HSS), failure to develop a precipitation band to HSP was due to the quality of the antiserum used. Janicki's laboratory also had difficulties making anti-polysaccharide II antibody. By immunoelectrophoresis he found polysaccharide II to be the component in HSP. A measurable amount of polysaccharide I in the HSS fraction was also detected by him using the immunoelectrophoretic technique. They observed that rabbits sensitized with killed H37Rv or BCG in incomplete Freund's adjuvant formed this antibody and that guinea pigs fail to do so (39).

The nitrogen-containing component is obviously one of low molecular weight. It may be presumptuous to state categorically that nitrogen content has a direct relationship to tuberculoprotein reactivity. Other nitrogenous substances may be represented here. Perhaps there is a peptide since the findings of Yamamura and Someya (72, 73, 86) indicated "tuberculin active" peptides (TAP) capable of eliciting a tuberculin reaction almost to that of standard PPD. The molecular weights of their peptides were estimated to be 5,000-10,000. Counts and Kubica's (17) purified protoplasmic peptide (PPP) (molecular weight < 10,000) from M. tuberculosis was also obtained in the supernatant with high-speed
centrifugations (final run 3 hr at 144,700 G). A high degree of
sensitivity and also specificity was found in sensitized guinea pigs
with their PPP.

Not all the carbohydrate-rich protoplasmic fractions derived in
our laboratory have been processed and tested. Many carbohydrate-rich
segments from the ammonium sulfate fractionations have not been
investigated and these portions could be further purified and analyzed
for biological activity in some future survey (Fig. 1).

Methods of extracting polysaccharides from cellular material could
be degrading to a polysaccharide and the degree of oxidation and
hydrolysis may be undetected or difficult to measure (55). Some mechan­
ical and physical methods could possibly be ideal, if these techniques
prove to be productive of pure isolates and if the activity is not so
harsh and extreme as to modify or destroy macromolecules and subse­
quently biological activity (2, 55, 63). However, in some cases,
chromatography has not been entirely successful in separating protein
and polysaccharides or highly sensitive or specific antigens from proto­
plasm (17, 61).

In this survey, I was not concerned about effect of surfactants,
such as Tween 80, as dispersing agents in suspension mediums and dilu­
et of skin-test antigens. Counts and Kubica (17) found that extracts
obtained from protoplasmic extracts of tubercle bacilli disrupted in
buffer containing Tween 80 was much more potent as skin-test antigen
than the extract from bacilli disrupted in buffer without Tween 80.
The pH was not strictly regulated at all times described in the present report and it is possible that activity of certain antigens may have been affected. However, the polysaccharide components appeared, superficially at least, to be completely water soluble.
CHAPTER VI

SUMMARY

(1) Various fractionating and purifying techniques were employed in isolating polysaccharides from protoplasm of Mycobacterium tuberculosis, variety H37Ra. These carbohydrate-rich components were tested primarily for delayed-, and also for immediate-, type reactions in guinea pigs sensitized with H37Ra. The simplest and most productive procedure was found to be 0.25 M TCA protein precipitations leaving almost unadulterated carbohydrate in the supernatant. On the other hand, the phenol-alcohol procedure, the most complex and time-consuming method used, produced a relatively impure product.

(2) Tuberculin and immediate reactivities in skins of sensitized guinea pigs were roughly proportional to nitrogen content of the antigen. However, some positive or negative activity could not be explained on the basis of quantitative nitrogen alone.

(3) With the Ouchterlony technique, using concentrated sera from sensitized guinea pigs, no precipitation band was apparent with the nitrogen-free fraction, HSP (144,700 g or 40,000 rev/min) pellet of the TCA extract. This fraction represented carbohydrate of relatively high molecular weight which was completely inactive in sensitized guinea pigs. With this immunological test, several zones of precipitation were formed by crude or associated fractions of the TCA extract containing measurable amounts of nitrogen.
LITERATURE CITED


