

THE ISOLATION AND PURIFICATION OF  
TWO ANTIGENS FROM CRUSHED CELLS OF  
BACILLUS MEGATERIUM ATCC 14581

by

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ABSTRACT

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A sonicated cell mass of Bacillus megaterium ATCC 14581, extracted with buffered salts pH 7.0, yielded four precipitin lines when assayed for serological activity by immunodiffusion against homologous high titer precipitating antisera; six precipitin lines were observed with immunoelectrophoresis. Passive hemagglutination gave a titer of 512.

Cell walls were isolated from a cell suspension of B. megaterium crushed by decompression rupture in the French pressure cell and purified by sucrose density gradient centrifugation followed by repeated washings in distilled water. Acid extracts of cell walls failed to produce precipitin lines when tested by immunodiffusion against homologous whole cell and cell wall sera.

Hot HCl extracts of crushed cells yielded one heavy and two faint precipitin lines. Alcohol precipitates of the acid extracts yielded two precipitating antigens, designated A and B. The major antigen, A, was eluted by chromatography on a Dowex-1-Cl anion exchange resin at a linear gradient concentration of 0.2 M ammonium formate. The minor antigen,

B, was eluted by gel-filtration on Sephadex G-100 in fractions 9 and 10.

Preliminary studies showed that antigen B is mainly carbohydrate in nature, with a small amount of protein and nucleic acid present, in an approximate ratio of 14:2:1 respectively.

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## INTRODUCTION

## INTRODUCTION

Recent advances in biochemical analysis have enabled research workers to localize, isolate, purify and characterize many types of microbial antigens. Gel-filtration and ion exchange chromatography on DEAE cellulose and Dowex resins have yielded purified antigen fractions from mycobacteria, staphylococci and several other bacterial species (Haukenes, 1962; Keeler and Pier, 1965; Hofstad, 1965; Lind, 1965). Agar gel diffusion and immunoelectrophoresis provide a method for the assay and the identification of multiple antigenic specificities. This kind of antigenic analysis can be used for the localization of structural antigenic components, as well as for the investigation of the nature of antigen-antibody reactions.

In this study, a method for the extraction of antigens from a crushed cell mass with large quantities of materials is described. Hot acid extraction and separation by column chromatography yield purified antigen fractions. The serological activity of the isolated components is measured by gel diffusion. Preliminary chemical analysis is carried out.

HISTORICAL

## HISTORICAL

Bacteria are composed of many different chemical constituents, some of which are antigenic in nature. The varied chemical nature of these antigens enables them to be differentiated and characterized by a combination of biochemical and serological techniques.

Bacterial antigens may be composed of proteins, polysaccharides or polysaccharide-phospholipid complexes (Carpenter, 1965). The antigenicity of these compounds is due to the presence of certain specific determinant groups. Thus, it can be seen that serological analyses can be a valuable tool to aid in classifying bacteria since related organisms would be expected to possess similar antigenic components.

The serological activity of cell walls, capsules, protoplasts and other isolated structures is, for the most part, highly specific. Vennes and Gerhardt (1956, 1959), using complement fixation tests, found that the polysaccharide components extracted from cell walls of B. megaterium KM were immunologically distinct from polypeptide antigens.

Methods used for the extraction of antigens from micro-organisms are designed to recover an antigenic fraction from large quantities of cells without altering its original antigenic specificity. Extraction of whole cells with hot HCl was introduced by Lancefield in 1928 as a method of isolating heat stable antigens from streptococci species. She found three antigens; two proteins and a species-specific carbohydrate (C) which was used as a basis for the serological grouping of Streptococci. However, Lancefield (1933) stated that this treatment may alter the structure of certain antigens.

Hot acid extraction of staphylococci by Julienne and Wieghard (1934) yielded a type-specific carbohydrate antigen A, which has been further investigated by Haukenes (1962). Maxted and Frazer (1964), using hot acid extraction of group D streptococci cells followed by ethanol treatment, found an alcohol-soluble, acetone-insoluble substance which combined with type-specific antibody but did not form a visible precipitate. Slade (1965) has also used hot trichloroacetic acid to extract the polysaccharide antigens from cell walls of various streptococci species.

Two antigens were isolated from Bacillus "M", a

strain of Bacillus megaterium, by boiling the organisms in water (Geux-Holzer and Tomcsik, 1956). A polysaccharide hapten was separated from a polypeptide antigen by ethanol fractionation. The polysaccharide was further purified by repeated ethanol precipitation and gave high titers when tested against homologous antisera. A unique modification of phase contrast microscopy was employed to show the relationship of the polysaccharide to the capsule and cell wall of Bacillus "M". The addition of anti-polypeptide serum to suspensions of whole cells produced a uniform halo surrounding the cell; whereas the addition of anti-polysaccharide serum resulted in a dense precipitate located at the septa and the polar caps. These workers concluded that the polysaccharide was present in both the cell wall and the capsular framework of Bacillus megaterium.

Gel diffusion methods of immunologic analysis are especially suitable for qualitative analysis of highly complex antigenic reactants as they allow the direct observation of the number of antigenic fractions present (Ouchterlony, 1958; Lind, 1965). Several modifications of Ouchterlony's original method (1949) have been devised, each providing a variation which enables one to exploit

fully the usefulness of immunodiffusion for serological analysis.

The antigenic analysis of culture filtrates of Mycobacterium tuberculosis has been carried out by Lind (1965) using the technique of gel diffusion. He stresses the importance of using a reference system to aid in the identification of different antigenic fractions.

The immunoelectrophoretic technique devised by Grabar and Williams (1953) is an important method by which the antigenic character of soluble precipitating antigens can be studied. Baillie and Norris (1964) used immunoelectrophoresis to follow changes in the types and levels of antigens present during spore formation in B. cereus. In the course of their studies, they found vegetative extracts to contain several antigens which were resistant to 80° C for 10 minutes.

Using Ouchterlony's technique as an assay system, Hofstad (1965) purified and characterized a major polysaccharide antigen from the 80/81 complex of S. aureus. This antigen proved to be the main precipitating antigen in the organism studied. A crude preparation was obtained by buffer extraction at an acidic pH followed by repeated

ethanol precipitation. The polysaccharide was further purified by chromatography on a DEAE cellulose anion exchange column, using a linear gradient of sodium chloride. The purified antigen was shown to consist of a polysaccharide and a polypeptide moiety that were firmly linked together. The carbohydrate portion of the antigen was identified as a glucosaminyl ribitol teichoic acid, similar in nature to the polysaccharide A antigen isolated from a strain of S. aureus by Haukenes (1962).

Haukenes considers polysaccharide A to be a teichoic acid-mucopeptide complex similar to that released from the cell walls of Bacillus megaterium KM by lysozyme treatment (Ghuysen, 1961, 1964). The teichoic acid portion of the B. megaterium complex was associated with a residue of glucose, N-acetyl hexosamine and amino acids. The polysaccharide could not be extracted by cold trichloroacetic acid treatment, the usual method used for the extraction of teichoic acids.

Teichoic acids are a class of carbohydrate associated with the cell walls and cytoplasmic membranes of several gram-positive species of bacteria. These polymers of ribitol phosphate or glycerol phosphate, to which sugars and

ester-linked alanine may be attached, were designated as teichoic acids by Armstrong et al (1958). Their chemical nature has been investigated by Baddiley et al (1961) and has been confirmed by studies on their enzymatic synthesis (Burger and Glaser, 1964; Glaser, 1964; Glaser and Burger, 1964).

Studies on ribitol teichoic acids by Armstrong et al (1958, 1959) indicate a basic structure consisting of ribitol units joined by phosphodiester linkages. The presence of ester-linked D-alanine was also characteristic. The glucosyl residues of the polymer were either glucose as in B. subtilis (Baddiley and Davison, 1961) or N-acetyl amino sugars as in S. aureus (Sanderson et al, 1961).

Glycerol teichoic acids yield glycerol, alanine, glycerophosphate and usually a sugar upon acid hydrolysis (Baddiley and Davison, 1961) and are found in the cytoplasmic membranes as well as in the cell wall of bacteria. They show a similar structure to that of ribitol teichoic acids (Kelemen and Baddiley, 1961). The presence of a glycerol teichoic acid in the cell wall of B. stearothermophilus has been reported by Wicken (1966). The release of teichoic acid occurred very slowly when cells were treated with

dilute trichloroacetic acid, similar to results reported for B. megaterium (Ghuysen, 1961). D-alanine, attached to the polymer through the glucose hydroxyl group was reported to be a major component of the polysaccharide.

Several studies have shown that teichoic acids constitute an important antigenic component of several genera of gram-positive bacteria as these polymers are known to confer species-specificity (McCarty, 1959; Juergens et al, 1960; Haukenes, 1962). The group D antigen of streptococci has been identified as an intracellular teichoic acid present in the cytoplasmic membrane (McCarty, 1959). The addition of teichoic acid to high titer sera prior to agglutination tests inhibits agglutination of staphylococcal cell walls (Juergens et al, 1960, 1963; Sanderson et al, 1961). The antigenic determinant of the teichoic acid isolated from S. aureus 80/81 strain was shown to be  $\beta$ -linked N-acetyl glucosamine (McCarty and Morse, 1964). Also, the passive hemagglutination of tanned red blood cells sensitized with teichoic acid from the same strain was inhibited by the addition of  $\beta$ -N-acetyl glucosamine derivatives to antisera prior to testing (Nathenson and Strominger, 1962).

From the work previously discussed, it can be seen that a variety of sophisticated biochemical techniques are currently being used in the field of immunochemistry for the isolation, purification and characterization of microbial antigens. Both Haukenes (1962) and Hofstad (1965) have used anion exchange chromatography to isolate and purify antigenic teichoic acid complexes from buffered salts extracts of crushed S. aureus cells. Keeler and Pier (1965) used DEAE cellulose columns to purify extracellular antigens of Nocardia asteroides. Gel-filtration techniques have also been used to purify precipitinogenic material found in culture filtrates of Mycobacterium tuberculosis (Lind, 1965). Chromatography on Sephadex G-50, G-100 and G-200 resulted in a partial separation of six antigen factors, three of which were stable to 100<sup>o</sup> C for 15 min. In all the above systems, agar gel diffusion was used to assay for serological activity of the isolated fractions.

MATERIALS AND METHODS

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Cultures

The strain Bacillus megaterium ATCC 14581 was used throughout the studies. Bacillus megaterium strain "M" (received from Dr. Tomcsik), Bacillus subtilis ATCC 9945A, Bacillus cereus ATCC 10206, Bacillus anthracis ATCC 14578, and laboratory strains of Bacillus pumilis, Bacillus mycoides, Bacillus fusiformis and Bacillus polymyxa were used to test cross reactivity. The cultures were maintained in brain heart infusion, BHI, (B.B.L.) media and subcultured at frequent intervals.

Mass Cultures

B. megaterium ATCC 14581 was grown in 600 ml BHI broth in 2-liter Fernbach flasks on a rotary shaker at 30° C for 12 hours. The cells were harvested at 0° C in a Servall RC<sub>2</sub> centrifuge at 7,000 x g for 20 minutes, washed twice with 0.1 M phosphate buffer pH 7.0 and stored at -15° C.

### Whole Cell Antigens

B. megaterium, Bacillus "M", B. anthracis and B. cereus were grown on BHI agar in Roux bottles at 28° C for 20 hours. The cells were suspended in 10 ml of 0.85% saline, washed twice in saline and resuspended in 0.2% formal saline to give a density of 38% light transmission on the Beckman model C colorimeter, corresponding to MacFarland tube no. 3. The cells were inactivated by storage at 4° C for 4 to 7 days with the exception of B. anthracis which was heated at 100° C for 45 min. The vaccines were tested for sterility and stored at 5° C.

### Cell Wall Antigen

Purified cell walls, obtained by the French pressure cell technique, were suspended in 0.2% formal saline and prepared as described for whole cell antigens.

### Antisera Production

Male albino rabbits were used for the production of high titer antisera. Sera were collected by cardiac puncture and stored at -15° C.

A whole cell suspension was injected according to the method of Kabat and Mayer (1961). Each animal received a total of 140 to 200 mg antigen over a period of 5 to 7 weeks.

A cell wall suspension was injected according to the method of Slavin (1950) using calcium alginate as adjuvant. A total of 30 mg cell wall suspension was given over a period of 9 weeks.

#### Buffered Sonic Extract

A cell mass of 30 grams wet weight B. megaterium ATCC 14581 was suspended in 15 ml of 0.1 M phosphate buffer pH 7.0. The slurry of 45 ml was sonicated at maximum current for 1 hour and centrifuged at 10,000 x g at 0° C for 30 min. The supernatant was labeled "buffered sonic extract" and stored at -15° C.

#### Hot HCl Extract

A crushed cell mass, as used for the buffered extract, was treated with 4 volumes of 0.1 N HCl at 90-95° C for 25 min, cooled on ice to room temperature, adjusted to pH 7.0-7.2 with 1 N NaOH and centrifuged at 12,000 x g in the Servall RC<sub>2</sub> for 20 min. The clear yellowish supernatant

was concentrated to approximately 1/10 volume by dialysis against moving air at room temperature, dialysed for 12 hours against 6 liters of 0.02 M phosphate buffer pH 7.0 at 4° C and stored at -15° C. This was labeled "hot acid extract".

#### Alcohol Precipitate of HCl Extract

Twenty ml of hot acid extract were adjusted to pH 5.0 with 1/10 the volume of a 20% sodium acetate-HAc solution, allowed to stand at room temperature for 5 to 10 min and centrifuged at 20,000 x g at 0° C for 20 min. The supernatant was treated with 2.5 times the volume of 95% ethanol at -15° C and stored at -15° C overnight. The white flocculant alcohol precipitate was collected by centrifugation at 12,000 x g for 20 min, suspended in 10 ml 0.02 M phosphate buffer pH 7.0 and recentrifuged at 10,000 x g for 10 min. The supernatant was labeled "ethanol precipitate" and, together with the insoluble residue, was stored at -15° C.

## Column Chromatography

### (A) Sephadex G-100

The ethanol precipitate was eluted by gel-filtration on a Sephadex G-100 column. A column 2.5 cm x 35 cm was packed and washed with 0.02 M phosphate buffer pH 7.0. Two ml of ethanol precipitate were applied to the column and eluted with the equilibrating buffer at room temperature at a flow rate of 20 ml per hour. Fifty 4-ml fractions were collected and assayed for absorption at 260 m $\mu$  and 280 m $\mu$  on the Unicam SP 700 or the Gilford spectrophotometer. Fractions were assayed for the presence of carbohydrate by a modification of the anthrone test of Scott and Melvin (1953). Appropriate fractions of the absorption peaks were tested for serological activity by immunodiffusion and passive hemagglutination.

### (B) Dowex-1-Cl

The alcohol precipitate was eluted by ion exchange chromatography on Dowex-1-Cl, according to the method of Haukenes (1962). Dowex-1-Cl resin, in the formate form, was packed into a 2.5 cm x 18 cm column and equilibrated with

0.005 M ammonium formate pH 5.0 at 4° C. Two ml of the ethanol precipitate, previously dialysed against the starting buffer, were applied to the column and eluted with 200 ml of 0.005 M ammonium formate into 9.5 ml fractions. A linear gradient from 0.005 M ammonium formate to 2.0 M ammonium formate was then run and 100 fractions collected. Fractions were analysed for absorption at 280 m $\mu$  on the Gilford spectrophotometer. The fractions comprising the main absorption peaks were pooled, dialysed against 0.02 M phosphate buffer pH 7.0 for 12 hours and tested for serological activity by immunodiffusion.

### Cell Walls

#### (A) French Pressure Cell

Approximately 30 grams wet weight of cell mass were suspended in distilled water to a final volume of 40 ml. This slurry was passed three times through a French pressure cell (Aminco) at 4° C, 20,000 lbs/sq. inch pressure. The crushed cell suspension was centrifuged at 1,000 x g for 20 min; the cell walls were recovered from the supernatant, washed once in 0.1 M phosphate buffer, twice in

1.0 M KCl and resuspended in 10 ml 1.0 M KCl. The crude walls were further purified by a modification of the sucrose density gradient technique of Yoshida et al (1961). Five ml of the suspension were layered on a 40 ml sucrose-KCl linear gradient, and spun at 460 x g at 4° C for 1 hour on a no. 215 swinging head in the International model H centrifuge. The cell walls were recovered from the cloudy layer of the gradient, washed three times with distilled water and stored at -15° C. Purity of cell wall preparations was confirmed by scanning on the Beckman DB spectrophotometer and on the phase contrast microscope.

(B) Sonication

Thirty grams of wet cell mass were suspended in 0.1 M phosphate buffer pH 7.0 to a total volume of 45 ml. The cells were disintegrated by sonication in the Raytheon sonic oscillator (10 KC) at maximum current for 15-20 min. The disrupted cells were centrifuged at 12,000 x g for 30 min, resuspended in 1.0 M KCl, homogenized and centrifuged at 1,000 x g for 20 min. The cell walls were recovered from the supernatant, washed 8-10 times in distilled water, lyophilized and stored at -15° C. Purity was assessed by

paper chromatography of the acid hydrolysates and by phase contrast microscopy.

#### Trichloroacetic Acid Extract

A 10 ml aqueous suspension of cell walls, prepared by the French pressure cell method, was adjusted to pH 3 with 50% trichloroacetic acid (TCA) and placed at 4° C for 3 hours. The precipitate was removed by centrifugation at 10,000 x g at room temperature for 20 min; the supernatant was dialysed against saline at 4° C for 12 hours and stored at -15° C.

#### Hot HCl Extract

One hundred mg (dry weight) of cell walls, prepared by sonication, were extracted in HCl at 95° C for 25 min as described above. The extract was concentrated against moving air, dialysed against 0.02 M phosphate buffer pH 7.0 overnight and stored at -15° C.

## Serological Activity

### (A) Agglutination Tests

The standard tube agglutination test was used to determine the serological activity of antisera. Halving dilutions of antisera in 0.4 ml volumes were added to an equal volume of antigen suspension, incubated at 37° C for 2 hours and at 4° C for 18 hours. Agglutination was read macroscopically with the aid of a Fisher-Kahn viewer and the titer recorded as the reciprocal of the highest dilution of serum giving a visible aggregate.

### (B) Immunodiffusion

The double diffusion plate technique of Ouchterlony (1949) was followed, except that a horizontal trough was used as the antiserum reservoir. Special Noble agar (Difco) was layered over the bottom of a 9-cm plastic petri plate and covered with a second layer of 20 ml 0.8% agar in saline with 1% NaN<sub>3</sub>. A horizontal trough (2 mm x 75 mm) was cut 7 mm from the antigen wells and filled with 0.30 ml undiluted antiserum. The wells were cut with a No. 1 cork borer and filled with 0.03 ml of antigen. The plates were

placed in a moist chamber at 25<sup>o</sup> C for 4 days and photographed by direct contact printing on Ilford single weight glossy paper.

(C) Immuno-electrophoresis

The method of Baillie and Norris (1964) was used, a modification of the technique introduced by Graber and Williams (1953).

The supporting glass plate (12 cm x 12 cm) was coated with a thin layer of 1% Agarose (N.B.C.), then layered with 20 ml 1% Agarose in 0.02 M barbital buffer pH 7.5 with 1% NaN<sub>3</sub>. Horizontal troughs (0.2 cm x 9 cm) were cut 1.5 cm apart. Wells, 3.5 mm in diameter, were cut approximately 7 mm from the troughs and filled with test antigen. The gel was connected to 0.02 M barbital buffer pH 7.5 by Agarose wicks. The Shandon electrophoresis tank was connected to a Heathkit regulated power supply and the gel subjected to a potential gradient of 10 to 12 volts/cm for 3 hours at 4<sup>o</sup> C. Following the electrophoresis, the trough was filled with homologous whole cell antisera and the reaction allowed to develop in a moist chamber at 25<sup>o</sup> C for 1 to 3 days.

The agar gels were washed, dried and stained with

amido black according to Parker et al (1962) and photographed by direct contact printing.

(D) Passive Hemagglutination

Sheep cells were collected in Alsever's solution and washed 3 times in 0.1 M phosphate buffered saline (PBS) pH 6.5. One ml of a 2.5% sheep cell suspension was added to a 1:20 antigen dilution in 3 ml of 0.1 M PBS pH 7.2 and incubated in a water bath at 37° C for 2 hours. Serum, previously absorbed with washed sheep cells, was diluted with 0.1 M PBS pH 7.2 in a series of halving dilutions in a volume of 0.4 ml. The titration was carried out by adding approximately 0.05 ml of sensitized cells to each tube of each series of serum dilutions. The tubes were incubated in a 37° C water bath for 2 hours and placed at 4° C for 18 hours. Controls of sensitized cells in PBS and unsensitized cells with the test sera were included. Titers were recorded as the reciprocal of the highest dilution of serum showing visible aggregates of red blood cells.

### Chemical Analyses

Protein content was determined by the Folin method of Lowry et al (1951) with egg albumin as a standard. Optical density was read at 660  $m\mu$  on a Klett-Summerson colorimeter.

Nucleic acid was assayed spectrophotometrically using the method of Warburg and Christian (1942). The optical density at 260  $m\mu$  and 280  $m\mu$  was determined on the Unicam SP 700 spectrophotometer.

Carbohydrate was estimated by a modification of the anthrone method of Scott and Melvin (1953), with dextrose as a standard. In this test, 1.5 ml sample were added to 3 ml of anthrone reagent and boiled for 3 min. The tubes were cooled to room temperature and read at 660  $m\mu$  on the Klett.

### Spectrum Analyses

Aqueous solutions of antigens A and B, at a concentration of 200  $\mu\text{g/ml}$ , as well as a 1/300 dilution of the ethanol precipitate were scanned from 200  $m\mu$  to 320  $m\mu$  on the Unicam SP 700 spectrophotometer.

### Paper Chromatography

Ascending chromatography was run on Whatman no. 1 paper in a 15 cm x 30 cm circular tank.

Amino acids were chromatographed by the method of McFarren (1951). Samples were hydrolysed in 2 N HCl in sealed ampoules at 105° C for 2 hours. The HCl was removed by repeated evaporation and the sample redissolved in 0.4 ml H<sub>2</sub>O. Ten  $\lambda$  of sample and standards (20  $\lambda$ ) were spotted on the base line and run for 9 hours with phenol:H<sub>2</sub>O (80:20) as the solvent. The chromatogram, which was buffered to pH 12.0, was dried and developed with 0.4% ninhydrin according to McFarren.

Reducing sugars were detected by the aniline hydrogen phthalate reagent of Partridge (1949). Samples to be chromatographed were hydrolysed in 1.0 ml of 2 N H<sub>2</sub>SO<sub>4</sub> in a sealed ampoule at 105° C for 2 hours. The hydrolysate was then neutralized with saturated Ba(OH)<sub>2</sub>; the barium sulphate removed by centrifugation. The sample was concentrated against moving air in a test tube, and chromatographed in the phenol:H<sub>2</sub>O (80:20) solvent. Aqueous solutions of sugars at a concentration of 50 mg/ml were used as standards. The

dried chromatogram was sprayed with the phthalate reagent and developed at  $95^{\circ}$  C for 10 min. Pentoses could be differentiated from hexoses by their difference in colour.

## RESULTS

## RESULTS

A buffered salts extract of sonicated cells of Bacillus megaterium ATCC 14581 yielded at least four precipitin lines when examined by gel diffusion against homologous whole cell serum (Fig. 1, center well). Immunoelectrophoresis of the buffered salts extract showed six distinct precipitin lines (Fig. 2, well 1). Passive hemagglutination tests of the extract against the homologous serum gave a titer of 512 (Table I). The protein concentration was determined to be 43 mg/ml.

A hot acid extract of the crushed cell suspension showed three lines by gel diffusion; one heavy broad line appearing between two lighter lines. Immunoelectrophoresis showed three lines displaced toward the anode of the gel (Fig. 2, well 2), which appeared to be identical to those observed by gel diffusion. The protein concentration of the clear yellow extract was 13 mg/ml.

The hot acid extract gave no cross reactivity when tested by gel diffusion against Bacillus "M", B. cereus ATCC 10206 and B. subtilis ATCC 9945A antisera. On the other hand, passive hemagglutination showed a titer of 512

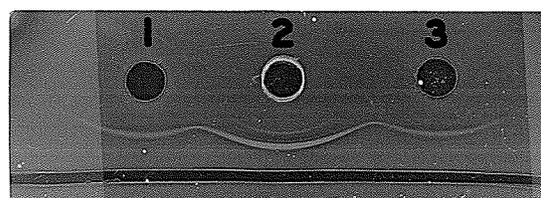


Figure 1. Immunodiffusion with B. megaterium antiserum in the horizontal trough. Wells 1 and 3 - 1/5 dilution ethanol precipitate; well 2 - buffered sonic extract.

Table I

Passive hemagglutination of antigenic fractions  
against B. megaterium whole cell antiserum.

ANTIGEN	TITER
Buffer Salts Extract	512
Hot Acid Extract	512
Ethanol Precipitate	512
Sephadex G-100 Fractions	
9	32
10	64
11	256
12	256
13	128
14	32
15	64
16	0

with homologous antisera (Table I) and with Bacillus "M" antisera; a titer of 128 and 32 was obtained with B. cereus and B. subtilis antisera respectively.

The ethanol precipitate of the extract gave two lines by gel diffusion, which showed a pattern of identity with the heavy line and the faint line closest to the antiserum reservoir of the hot acid extract. The lines also exhibited a synonymous reaction of identity with those of the buffered sonic extract (Fig. 1). Immunoelectrophoresis of the ethanol precipitate showed two precipitin arcs displaced toward the anode (Fig. 2, well 3). Passive hemagglutination gave a titer of 512 with homologous serum (Table I). The white ethanol precipitate gave an almost colorless solution in buffer and contained approximately 2 mg/ml protein.

The alcohol precipitate was eluted by gel-filtration on Sephadex G-100 into two absorption peaks as assayed at 260 m $\mu$  (Fig. 3). The major peak showed a very high absorbance, but no serological activity as tested by gel diffusion. The minor peak (tubes 6-15) contained 33% of the total carbohydrate present, as measured by the anthrone test (Fig. 3). Immunodiffusion tests on each tube yielded two precipitin lines; a faint line in tubes 9 to 17 and a sharp

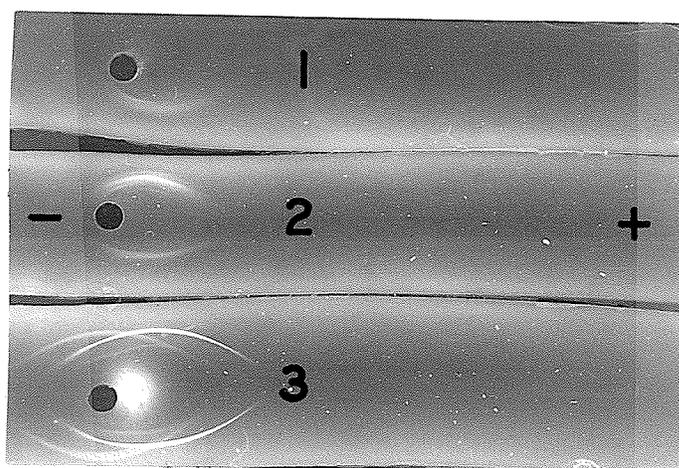
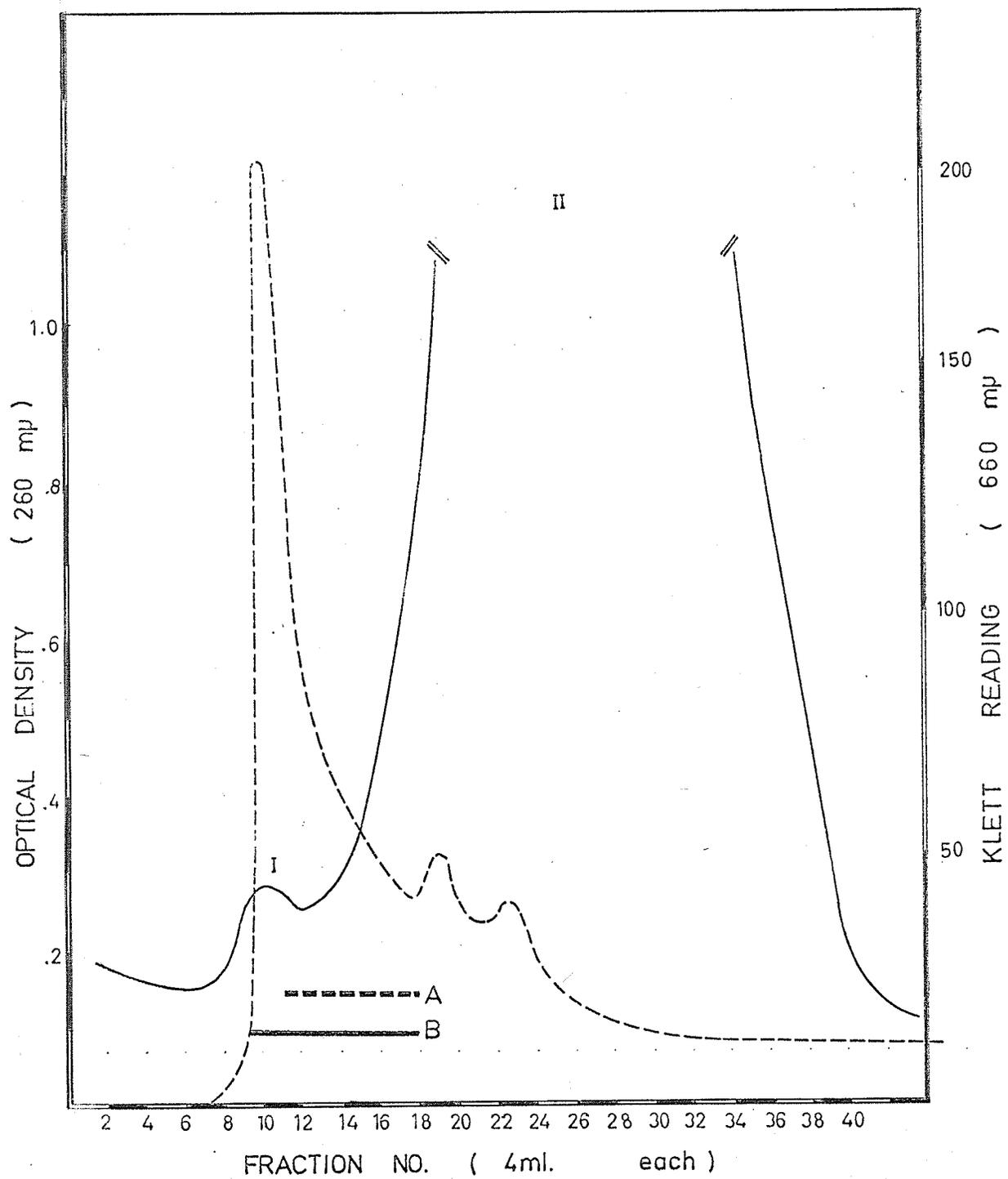


Figure 2. Immunoelectrophoresis with B. megaterium antiserum in horizontal troughs. Well 1 - ethanol precipitate; well 2 - hot acid extract; well 3 - buffered sonic extract.

Figure 3

Chromatography of ethanol precipitate on  
Sephadex G-100.

—————	absorption at 260 m $\mu$
-----	anthrone at 660 m $\mu$
-----	antigen A
—————	antigen B



dense line in tubes 11 to 17 (Fig. 4). The two lines showed a pattern of identity with those of the ethanol precipitate. The major antigen, which appeared in tubes 11 to 17, was designated antigen A; the minor antigen, which appeared in tubes 9 to 17, was designated antigen B. Serological activity of the eluates was also assayed by the hemagglutination test (Table I); tubes 9 to 15 showed titers ranging from 32 to 256, with the maximum titer of 256 present in tubes 11 and 12.

The ethanol precipitate, eluted from a Dowex-1-Cl anion exchange column with a linear gradient of 0.005 M to 2.0 M ammonium formate pH 5.0, showed four absorption peaks when assayed at 280 m $\mu$  (Fig. 5). Peak I, eluted with the void volume of the column, appeared to contain a large amount of protein as compared with the other three peaks. A sample from peak I, tested by gel diffusion, gave one line which corresponded to major antigen A of the ethanol precipitate (Fig. 6). Peak II, eluted from the column at a linear ammonium formate concentration of approximately 0.4 M also gave one line corresponding to antigen A. Peaks III and IV, eluted at concentrations of 0.70 M and 2.0 M ammonium formate respectively, did not form any

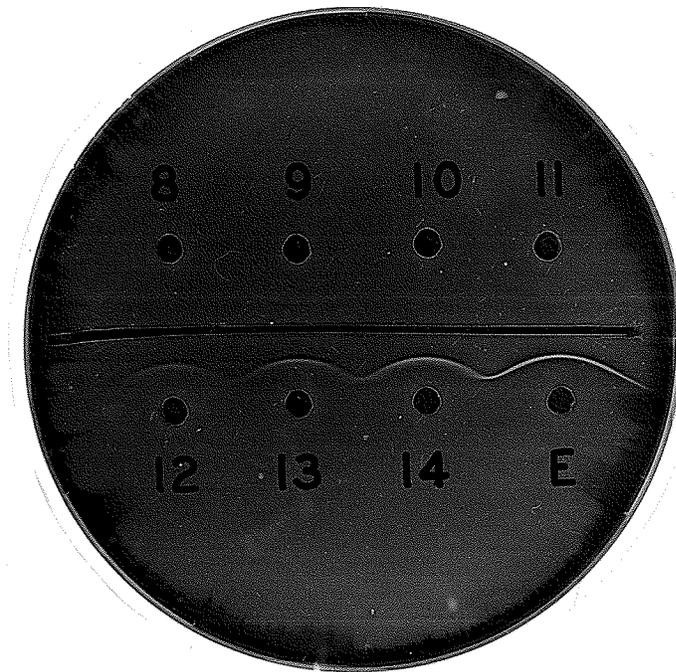
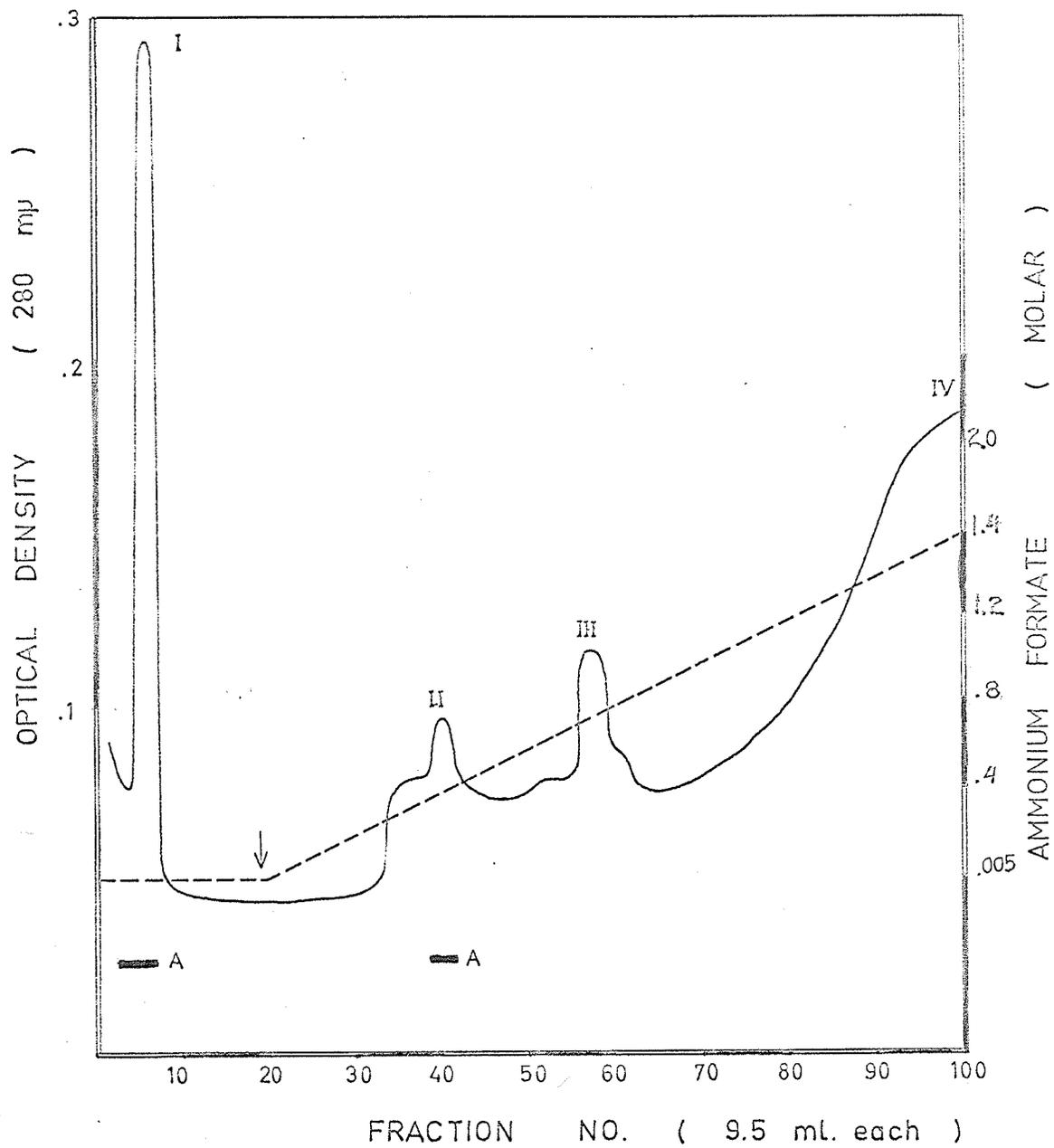


Figure 4. Immunodiffusion with B. megaterium antiserum in the horizontal trough. Wells 8 to 14 - Sephadex G-100 fractions 8 to 14; well E - ethanol precipitate.

Figure 5

Chromatography of ethanol precipitate on  
Dowex-1-Cl.

———— absorption at 280 m $\mu$   
----- effluent molarity  
———— antigen A



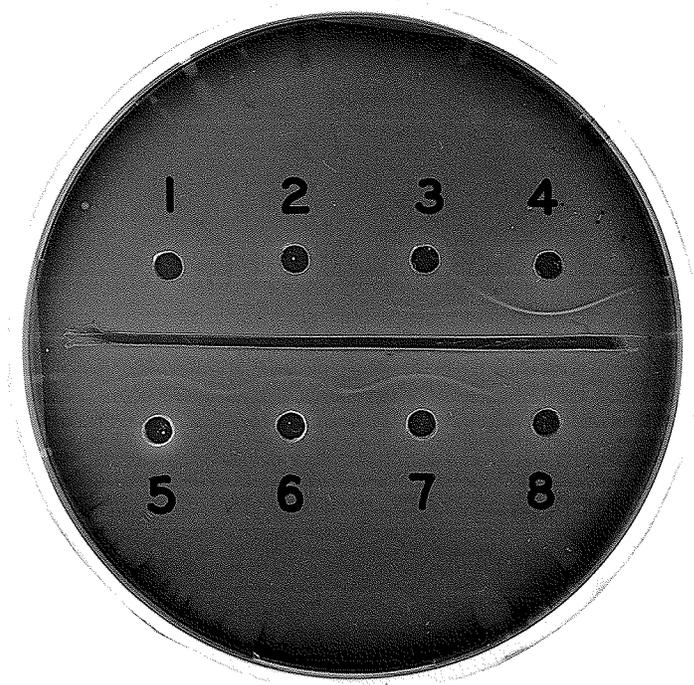


Figure 6. Immunodiffusion with B. megaterium antiserum in the horizontal trough. Well 1 - 1/5 dilution Dowex peak I; well 2 - 1/5 dilution Dowex peak III; well 3 - Sephadex G-100 fractions 9 and 10; well 4 - ethanol precipitate; well 5 - Dowex peak I; well 6 - Dowex peak III; well 7 - 1/5 dilution ethanol precipitate; well 8 - 1/5 dilution hot acid extract.

precipitin lines. Antigen B was not detected in any of the Dowex column eluates.

Dowex peak II (tubes 38 to 42) yielded approximately 10 mg of antigen A. Similarly, tubes 9 and 10 from the Sephadex G-100 column yielded 8.3 mg of antigen B. The two antigens formed a thin transparent cellophane-like film in the dry state.

The ultraviolet absorption spectrum of antigens A and B, recorded in Fig. 7, showed very low extinction values when examined in aqueous solution. Each antigen was estimated to contain 2% nucleic acid material.

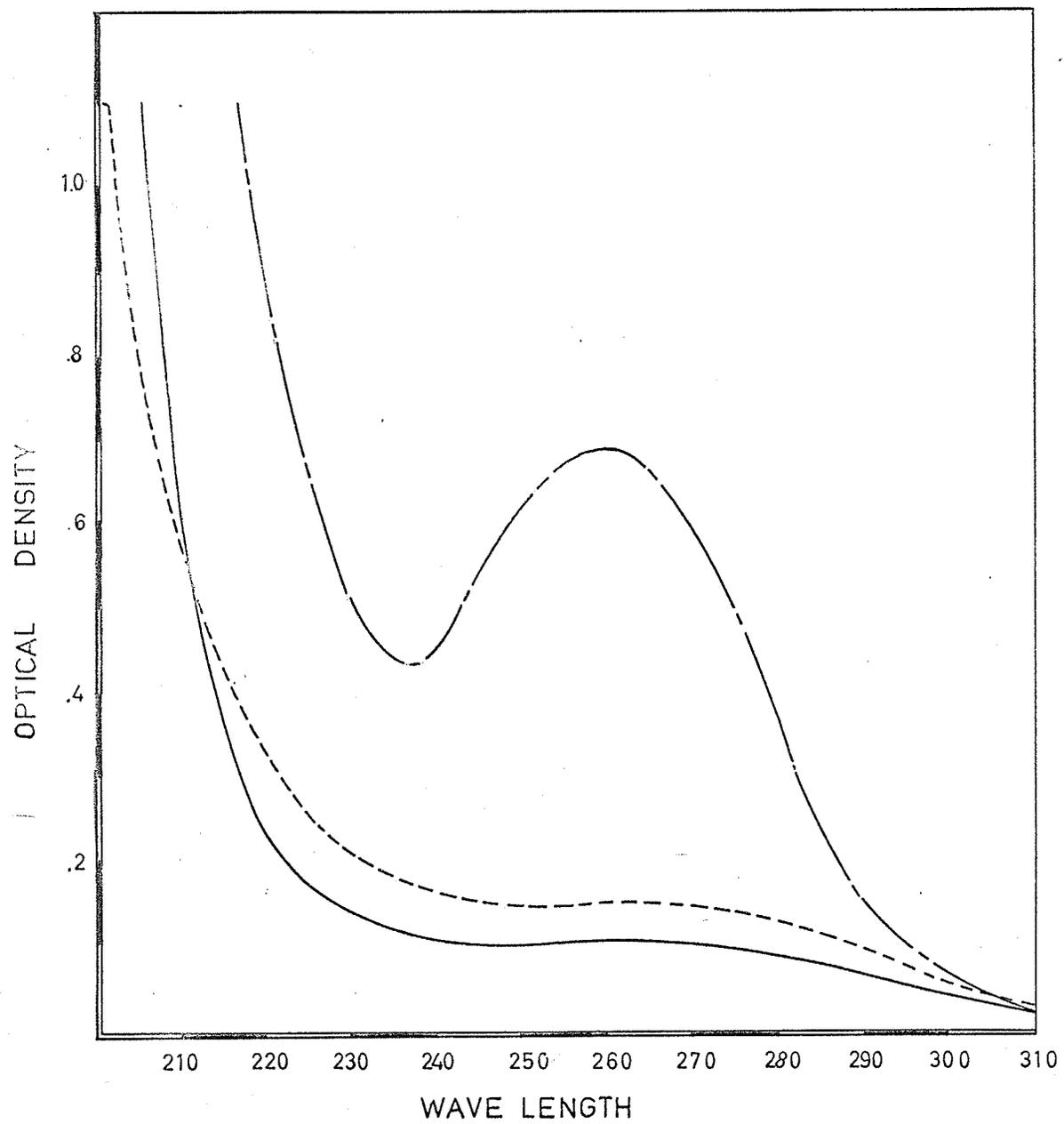
Preliminary studies on the biochemical analysis of antigen B showed approximately 5% protein, as determined by the Folin method; 35% reducing content, as assayed by the anthrone reagent; and alanine to be the only major amino acid, as detected by paper chromatography.

Cell walls, obtained by disruption of a cell mass of B. megaterium in a French pressure cell, were purified by sucrose density gradient centrifugation and examined by phase contrast microscopy, methylene blue wet mount (Fig. 8). The cell walls appeared dark blue, while any intact cells, approximately one cell per field, appeared deep red in

Figure 7

Ultraviolet absorption spectra of antigens A and B  
as compared with ethanol precipitate.

----- antigen A  
———— antigen B  
— — — ethanol precipitate



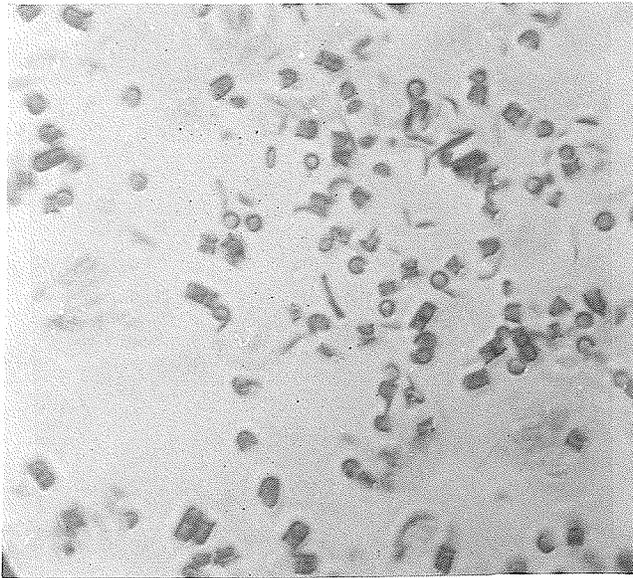


Figure 8. Cell wall fragments of Bacillus megaterium ATCC 14581 ruptured by the French pressure cell (x 1,200).

color. The cell wall fragments were large, almost entire, whereas walls obtained by sonication were highly fragmented.

The ultraviolet absorption spectrum of a cell wall suspension (Fig. 9) showed a low extinction value at 260 m $\mu$ , indicating the absence of nucleic acid material; the cytoplasmic fraction gave a very high extinction value and was calculated to contain 9 mg/ml nucleic acid.

Standard agglutination tests of whole cell suspensions gave titers of 128 with cell wall antisera and 512 with whole cell antisera (Table II). Agglutination tests of cell wall suspensions gave titers ranging from 0 to 64 with cell wall antisera and 64 to 512 with whole cell antisera.

Acid extracts with cold TCA or hot HCl of purified cell walls, as well as alcohol precipitates of these acid extracts, failed to form precipitin lines when tested by gel diffusion.

Figure 9

Ultraviolet absorption spectrum of isolated cell walls as compared with cytoplasmic fraction.

———— cell walls  
----- cytoplasmic fraction

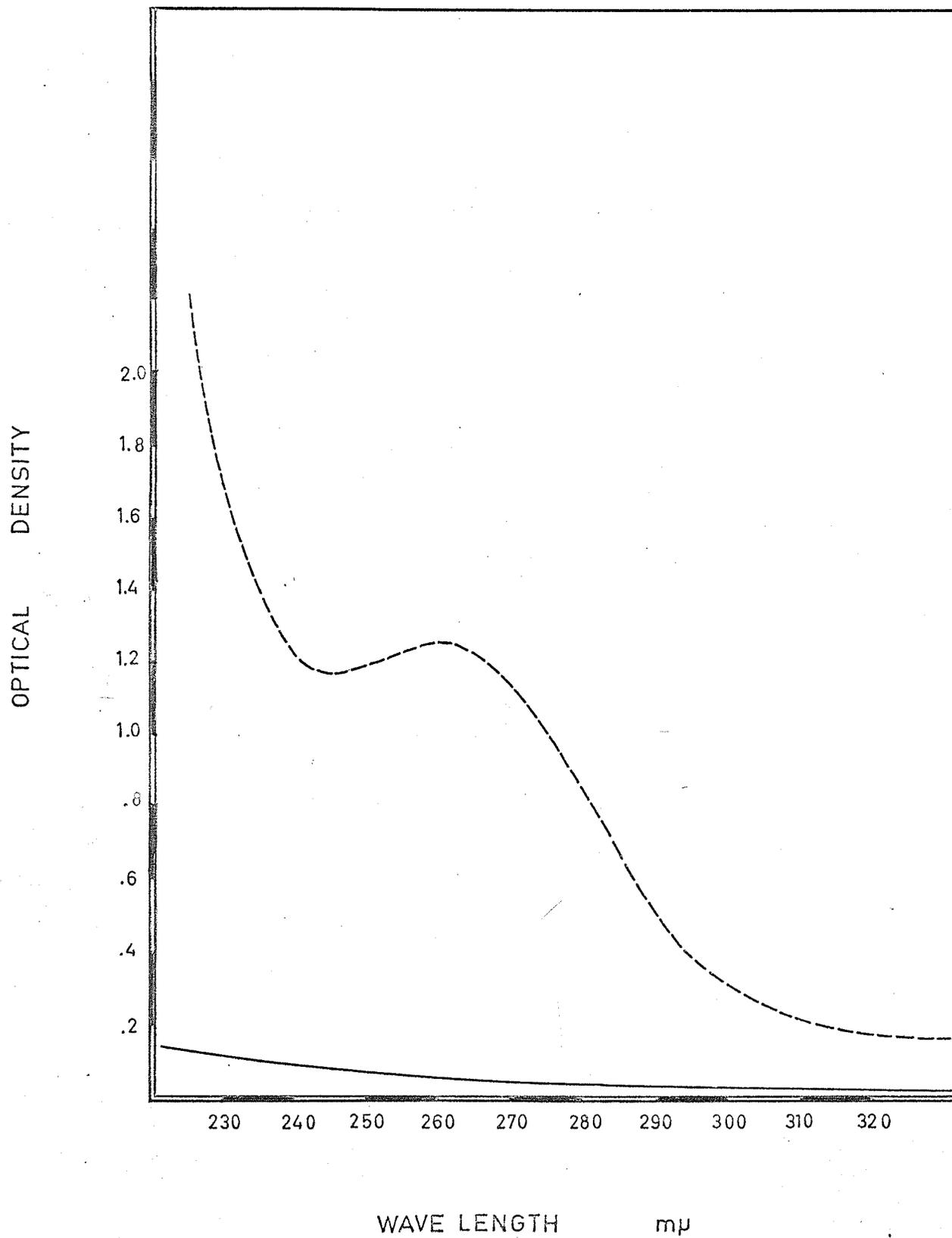


Table II

Agglutination titers with whole cell and cell wall suspensions of B. megaterium.

ANTIGEN	ANTISERUM			
	Whole Cell		Cell Wall	
	1	2	3	4
Whole Cells	512	1024	128	128
Cell Walls	512	64	64	0

## DISCUSSION

## DISCUSSION

The serological activity of the buffered sonic extract, as measured by immunodiffusion, showed four precipitin lines; whereas six antigens were revealed by immunoelectrophoresis. It appears that the isolation and identification of antigens requires separation on the basis of ionic charge by immunoelectrophoresis.

The pattern of gel diffusion in this study differed from that used by Ouchterlony (1949) in that the antiserum was placed in a horizontal trough with wells containing antigen placed on either side. This arrangement provided a more constant ratio of antibody to antigen, and the resulting precipitin lines were clear-cut with the patterns of identity more easily established.

Precipitation of the hot acid extract with ethanol at pH 7.0 yielded two precipitating antigens, A and B, but a large amount of contaminating protein was also present. The precipitate which formed when the acid extract was adjusted to pH 5.0 prior to ethanol treatment removed most of the contaminating protein, since the pH 5.0 ethanol precipitate contained only 2 mg protein per ml.

One of the antigens present in the hot acid extract could not be detected in either the ethanol precipitate or in the supernatant. Similarly, Maxted and Frazer (1964) found that ethanol precipitation of a hot acid extract of group D streptococci removed an ethanol-soluble antigen which combined with specific antibody but did not form a precipitate, ie. it was haptenic in nature. It may be that the ethanol treatment alters a portion of the antigen.

When the ethanol precipitate was resuspended in buffered salts, a portion remained insoluble. This residue gave a hemagglutination titer of 512 and showed two diffuse precipitin lines when tested by gel diffusion. Kelemen (1961) notes that teichoic acids, obtained by a similar procedure to that of antigens A and B, also yield an insoluble residue when precipitated with ethanol.

The ethanol precipitate was subjected to gel-filtration on Sephadex G-100 but only partial separation of the two precipitating antigens was achieved, presumably because their similar molecular weights failed to permit separation on the basis of a molecular sieve principle. The two antigens were eluted in the first minor absorption peak while most of the nucleic acid material was recovered in the

second large absorption peak, well-separated from the antigen fractions. This indicated that the ultraviolet absorbing material could be separated from the antigens and was not an integral part of them.

A carbohydrate assay of the eluates showed that 33% of the total amount eluted from the column was present in the same fraction as the antigen. This confirmed the fact that the antigens were likely carbohydrate in nature. It is of interest to note that the antigens were eluted from the column at a position similar to that of the glycerol teichoic acid isolated from B. licheniformis using an identical column (Burger and Glaser, 1964).

Elution of the ethanol precipitate from a DEAE cellulose anion exchange column using a linear NaCl gradient yielded two broad absorption peaks. The fractions from these peaks, as well as all the other fractions, were pooled and concentrated but no serological activity was detected when examined by immunodiffusion.

Separation was achieved when the ethanol precipitate was eluted from a Dowex-1-Cl column by a method similar to that used by Haukenes (1962) for isolating the polysaccharide antigen A from crude ethanol precipitates of a Staphylococcus

aureus buffered salts extract.

The major antigen A was present in the first absorption peak which was eluted with the void volume of the column. Since antigen A was also detected in peak II, eluted from the column after the gradient had started, it appears that the column may have been overloaded. Antigen B was not detected in any of the Dowex eluates; either antigen B was denatured by the ammonium formate treatment or it was not eluted in sufficient amounts to be detected.

Antigen A appears to constitute a major soluble antigenic component of B. megaterium since it shows a pattern of identity with the major heavy precipitin line found in each step of the purification procedure.

The minor antigen B consists of 2% nucleic acid, 5% protein and 35% reducing substance, while alanine was the only amino acid detected by paper chromatography. The nucleic acid and protein content may be due to contamination but this remains to be tested. Also, ribitol or glycerol, the major components of cell wall and intracellular teichoic acids, would not be detected by the anthrone reagent; this may explain the lack of stoichiometry found for antigen B. Larger amounts of antigen are required for the

identification of sugars by paper chromatography.

Since antigens A and B are stable to treatment with hot 0.1 N HCl at 95° C for 25 min and are precipitated with ethanol at pH 5.0, it appears that they are carbohydrate in nature. The antigens possess a similar negative charge as well as similar, but not identical, molecular weights as shown by immunoelectrophoresis and gel-filtration.

Ultraviolet spectrum analysis showed the purified cell walls of B. megaterium to be relatively free of nucleic acid material, since very low extinction values at 260 m $\mu$  were recorded. Varying concentrations of walls were examined to ensure that any absorption due to contamination was not masked by the high light-scattering properties of the walls (Barkulis and Jones, 1957).

The acid extraction procedures which were used failed to recover any heat stable precipitating antigen from the walls. These results are in accord with those of Ghuysen (1961, 1964) who states that a teichoic acid-like carbohydrate polymer could only be extracted from the cell walls of B. megaterium after prolonged acid treatment, which would most likely destroy any of its antigenic properties.

The low titers obtained with agglutination tests of

cell wall suspensions, coupled with the absence of precipitin lines by immunodiffusion of cell wall acid extracts, suggests that cell walls of B. megaterium do not contain antigens A and B. These results suggest also that the cell walls of this species are not a major reactive antigenic fraction of the intact organism. In studies of B. megaterium cell walls by the immunofluorescent labeling technique (Chung et al, 1964), it was necessary to pretreat the cells with trypsin and ribonuclease in order to obtain a uniform fluorescein-antibody label of the cell. This would lead one to believe that the major reactive antigens of B. megaterium are cellular in nature, possibly located adjacent to the cell wall. Such is the case with the intracellular teichoic acids which are associated with the cytoplasmic membrane in certain gram-positive species, including B. megaterium (Baillie and Holmes, 1966).

The method used for extracting antigens A and B, ie. acid extraction followed by ethanol precipitation, is similar to that used for the extraction of teichoic acids (Baddiley and Davison, 1961; Haukenes, 1962; Wolin et al, 1966). Teichoic acids from several strains of S. aureus produce a heavy main precipitin line with homologous sera

(Haukenes, 1962; Hofstad, 1965) and also show passive hemagglutination activity (Morse, 1962). According to McCarty and Morse (1964), these glycerol teichoic acids are predominantly found in the soluble supernatant fraction following disruption and fractionation of the bacterial cell and are usually present in no more than trace amounts in isolated cell walls. They also point out evidence which indicates that glycerol teichoic acids induce the formation of antibodies only when the intact cell is used as an antigen. The distinctive feature of the group D streptococci intracellular teichoic acid polymer is the presence of 30 - 40% glucose in the form of glucosyl residues (Wicken et al, 1963). All these observations regarding the nature of glycerol teichoic acids are analogous to those of antigens A and B isolated in this study.

The hot acid extract did not cross react with antisera from other *Bacillus* species when tested by immunodiffusion. However, it did cross react when tested by passive hemagglutination. Since antigens A and B, purified by ethanol precipitation, do not cross react when tested by hemagglutination or immunodiffusion, it appears that they

are type-specific. The third antigen present in the hot acid extract may therefore be responsible for the hemagglutination titer and may be similar to a heat stable polysaccharide-like heterogenic antigen found in several *Bacillus* species (Chorpenning and Dodd, 1966).

Further immunochemical studies of antigens A and B should be of some value in the elucidation of the nature of polysaccharide antigens found in the *Bacillus* species, as well as in the confirmation of the presence of antigenic teichoic acids in this organism.

## REFERENCES

## REFERENCES

- Armstrong, J. J., Baddiley, J., Buchanan, J. G., Carss, B. and Greenberg, G. R. (1958). Isolation and structure of ribitol phosphate derivatives (teichoic acids) from bacterial cell walls. *J. Chem. Soc.* 4344.
- Armstrong, J. J., Baddiley, J., Buchanan, J. G., Davison, A. L., Kelemen, M. V. and Neuhaus, F. C. (1959). Teichoic acids from bacterial walls. Composition of teichoic acids from a number of bacterial walls. *Nature*. 184:247.
- Baddiley, J. and Davison, A. L. (1961). The occurrence and location of teichoic acids in lactobacilli. *J. Gen. Microbiol.* 24:295.
- Baillie, A. and Norris, J. R. (1964). Antigen changes during spore formation in Bacillus cereus. *J. Bacteriol.* 87:1221.
- Baillie, R. D. and Holmes, W. H. (1966). Function of teichoic acid. *Biochem. J.* 99:28p.

- Barkulis, S. S. and Jones, M. F. (1957). Studies of streptococcal cell walls. I. Isolation, chemical composition and preparation of M protein. J. Bacteriol. 74:207.
- Burger, M. M. and Glaser, L. (1964). The synthesis of teichoic acids. I. Polyglycerophosphate. J. Biol. Chem. 10:3168.
- Carpenter, P. L. (1965). 'Immunology and Serology', 2nd edition, p. 43. W. B. Saunders Co., Philadelphia.
- Chorpenning, F. W. and Dodd, M. C. (1966). Heterogenic antigens of gram-positive bacteria. J. Bacteriol. 91:1440.
- Chung, A., Hawirko, R. Z. and Isaac, P. K. (1964). Cell wall replication. I. Cell wall growth of Bacillus cereus and Bacillus megaterium. Can. J. Microbiol. 10:43.
- Geux-Holzer, S. and Tomcsik, J. (1956). The isolation and chemical nature of capsular and cell wall haptens in a Bacillus species. J. Gen. Microbiol. 14:14.

Ghuysen, J. M. (1961). Complexe acide teichoïque-mucopeptide des parois cellulaires de Bacillus megaterium KM  
Biochim. Biophys. Acta. 50:413.

Ghuysen, J. M. (1964). Alkaline degradation of the phospho-  
mucopolysaccharide from cell walls of Bacillus  
megaterium KM. Biochim. Biophys. Acta. 83:133.

Glaser, L. (1964). The synthesis of teichoic acids.  
II. Polyribitol phosphate. J. Biol. Chem. 10:3178.

Glaser, L. and Burger, M. M. (1964). The synthesis of  
teichoic acids. III. Glucosylation of polyglycero-  
phosphate. J. Biol. Chem. 10:3187.

Grabar, W. B. and Williams, C. A. (1953). Méthode permettant  
l'étude conjuguée des propriétés électrophorétiques et  
immunochimiques d'un mélange de protéines.  
Application au sérum sanguin. Biochim. Biophys. Acta.  
10:193.

Haukenes, G. (1962). Immunochemical studies on  
polysaccharide A of Staphylococcus aureus.  
I. Purification on DEAE cellulose columns. Acta.  
Pathol. Microbiol. Scand. 55:110.

- Haukenes, G. (1962). Immunochemical studies on polysaccharide A of Staphylococcus aureus. II. Further studies on purification methods. Acta. Pathol. Microbiol. Scand. 55:117.
- Haukenes, G. (1962). Immunochemical studies on polysaccharide A of Staphylococcus aureus. VI. Antigenic properties. Acta. Pathol. Microbiol. Scand. 55:450.
- Hofstad, T. (1965). Studies on the antigenic structure of the 80/81 complex of Staphylococcus aureus. III. Purification and chemical characterization of a major polysaccharide precipitinogen. Acta. Pathol. Microbiol. Scand. 63:59.
- Julianelle, L. A. and Wieghard, C. W. (1934). Immunological specificity of carbohydrates derived from staphylococci. Proc. Soc. Exptl. Biol. Med. 31:947.
- Juergens, W. G., Sanderson, A. R. and Strominger, J. L. (1960). Chemical basis for the immunological specificity of a strain of Staphylococcus aureus. Bull. Soc. Chim. Biol. 42:1669.

- Juergens, W. G., Sanderson, A. R. and Strominger, J. L. (1963). Chemical basis for an immunological specificity of a strain of Staphylococcus aureus. J. Exptl. Med. 117:925.
- Kabat, E. A. and Mayer, M. M. (1961). 'Experimental Immunochemistry', 2nd edition, p. 871. Charles C. Thomas Co., Springfield, Illinois.
- Keeler, R. F. and Pier, A. C. (1965). Extracellular antigens of Nocardia asteroides. II. Fractionation and chemical characterization. Amer. Rev. Resp. Dis. 91:400.
- Kelemen, M. and Baddiley, J. (1961). Structure of the intracellular glycerol teichoic acid from Lactobacillus casei ATCC 7469. Biochem. J. 80:246.
- Lancefield, R. C. (1928). The antigenic complex of Streptococcus haemolyticus. I. Demonstration of a type-specific substance in extracts of Streptococcus haemolyticus. J. Exptl. Med. 47:91.

- Lancefield, R. C (1933). A serological differentiation of human and other groups of hemolytic streptococci. J. Exptl. Med. 57:571.
- Lind, A. (1965). Immunologic analysis of mycobacterial antigens. Amer. Rev. Resp. Dis. 92:54.
- Lowry, O. H., Rosebrough, N. J., Farr, A. L. and Randall, R. J. (1951). Protein measurement with the Folin phenol reagent. J. Biol. Chem. 193:265.
- Maxted, W. R. and Frazer, C. A. M. (1964). The serological typing of Streptococcus faecalis. Zentr. Bakteriolog. Parasitenk. Abt. 1. Orig. 196:76.
- McCarty, M. (1959). The occurrence of polyglycerophosphate as an antigenic component of various gram-positive bacterial species. J. Exptl. Med. 109:361.
- McCarty, M. and Morse, S. I. (1964). Cell wall antigens of gram-positive bacteria. In 'Advances in Immunology', Vol. 4, p. 249. Academic Press, New York.
- McFarren, E. F. (1951). Buffered filter paper chromatography of the amino acids. Anal. Chem. 23:168.

- Morse, S. I. (1962). Studies on the chemistry and immunology of cell walls of Staphylococcus aureus. J. Exptl. Med. 116:229.
- Nathenson, S. G. and Strominger, J. L. (1962). Enzymatic synthesis and immunochemistry of N-acetylglucosaminyl ribitol linkages in the teichoic acids of Staphylococcus aureus strains. J. Biol. Chem. 237:3839.
- Ouchterlony, O. (1949). Antigen-antibody reaction in gels. Acta. Pathol. Microbiol. Scand. 26:507.
- Ouchterlony, O. (1958). Diffusion-in-gel methods for immunological analysis. Progr. Allerg. 5:1.
- Parker, W. L., Stackiw, W. and Wilt, J. C. (1962). C-Reactive protein in virus infection. J. Can. Med. Assoc. 87:791.
- Partridge, S. M. (1949). Aniline hydrogen phthalate as a spray reagent for chromatography of sugars. Nature. 164:443.

- Wicken, A. J., Elliott, S. D. and Baddiley, J. (1963). The identity of streptococcal group D antigen with teichoic acid. *J. Gen. Microbiol.* 31:231.
- Wicken, A. J. (1966). The glycerol teichoic acid from the cell wall of Bacillus stearothermophilus B 65. *Biochem. J.* 99:108.
- Wolin, M. J., Archibald, A. R. and Baddiley, J. (1966). Changes in wall teichoic acid resulting from mutations of Staphylococcus aureus. *Nature.* 209:484.
- Yoshida, A., Heden, C. G , Cedergren, B. and Edebo, L. (1961). A method for the preparation of undigested bacterial cell walls. *J. Biochem. Microbiol. Tech. Eng.* 3:151.