THE ISOLATION, PURIFICATION, AND PARTIAL CHARACTERIZATION OF AN ANTIGEN FROM *BACILLUS M*

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by

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ABSTRACT

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An antigen, the buffered salts Antigen, was extracted in buffered salts from cells of Bacillus M by sonication and purified by DEAE-cellulose chromatography, ammonium sulfate fractionation, rechromatography on DEAE-cellulose, and gel filtration on Sephadex G-150. Biochemical analysis showed that protein, carbohydrate, and nucleic acid were present in the ratio of 1:64:10 respectively. The antigen was shown to be homogeneous by disc electrophoresis. As serological activity was not affected by a myxobacter AL-1 proteolytic enzyme, and as the 280 mμ absorbing material in the sample could be dissociated from the serological activity, the protein, and perhaps the nucleic acid were considered to be extraneous material and not part of the antigenic complex.

A second antigen, the hot HCl Antigen, was extracted from crushed cells of Bacillus M with hot HCl, and partially purified by acid precipitation, ethanol fractionation, and DEAE-cellulose chromatography. Immunological homogeneity was established by immunoelectrophoresis, and 15 per cent carbohydrate was shown to be present.

A third antigen, the CM Antigen, was extracted from crushed cells with chloroform-methanol and shown to contain 18 per cent protein and 11 per cent carbohydrate.

Since the buffered salts Antigen, the hot HCl Antigen,

and the CM Antigen showed reactions of identity by immunodiffusion, the same hapten appears to be present in all three preparations. Evidence is given that the antigen was a heat stable polysaccharide, or lipopolysaccharide. The three extracted antigens failed to cross react with antisera to Bacillus cereus or Bacillus megaterium whole cells.

Chromatography of the buffered sonic extract on DEAE-cellulose revealed the presence of from 8 - 11 precipitinogens. Immunodiffusion cross reaction studies showed that two of these reacted with antiserum to whole cells of B. cereus, but not B. megaterium.

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INTRODUCTION

INTRODUCTION

The antigenic analyses of bacteria, and their constituent parts, capsules, cell walls, cytoplasmic membranes, spores, flagella, and even intracellular organelles have helped to elucidate the pattern of the bacterial anatomy. Similarly, specific immune sera prepared to vegetative cell and spore antigens have been used in the study of the spore germination process.

Antigen-antibody reactions can be rendered visible by fluorescent labelling of antibodies, and surface layers such as capsules and cell walls have been identified in situ. The processes of cell division and cell wall synthesis have also been studied by immunofluorescence.

Immunochemical analyses have proved to be useful in the serological grouping of the streptococci. Some information is also available about the antigens of the corynebacteria, mycobacteria, and nocardia. Relatively little is known about the antigens of the aerobic spore-foming bacilli, or other Gram-positive bacteria, with the exception of the group A streptococci, two species of staphylococci, and some strains of lactobacilli. On the other hand, extensive work has been carried out on the isolated lipopolysaccharides of the Gram-negative bacteria.

Antigens have been extracted from whole cells, crushed cells, various components such as cell walls, and

even culture filtrates. The usual methods of treatment have been with proteolytic enzymes, hydrochloric acid, formamide, trichloroacetic acid, phenol and water emulsion, and sonication in buffered salts. The antigens were purified by standard biochemical techniques and column chromatography, and assayed by gel diffusion tests. These have then been analysed to determine their chemical structure, the nature of the determinant group, and their relationship to the other components of the bacterial cell.

These studies describe the extraction of antigens of crushed cells Bacillus M. One antigen was isolated, purified and a preliminary biochemical analysis was carried out.

HISTORICAL

HISTORICAL

The cell walls of Gram-positive microorganisms consist mainly of a glycosaminopeptide backbone which confers rigidity to the wall. This backbone, usually termed the mucopeptide, is composed of a limited number of amino acids, some of which are in the D-configuration, and aminosugars. In addition to the mucopeptide, both carbohydrates and proteins are present, and the carbohydrates at least appear to be linked to the mucopeptide.

The vast majority of the antigens isolated from bacteria appear to be associated with the carbohydrates and proteins, and not with the mucopeptide backbone. Some antigens serve as serological determinants of group or type specificity; others, such as the streptococcal M protein, may play important roles in microbial pathogenesis and virulence (McCarty and Morse, 1964).

The antigenic properties of the streptococci are by far the best known of the Gram-positive bacteria. Lancefield (1928), using hot acid, extracted three serologically active substances from group A streptococci which were designated as a group specific carbohydrate (C), a type specific protein (M), and a non-type specific protein (P). She divided the streptococci into 13 groups based upon precipitin tests to the carbohydrate substance (Lancefield, 1941). Subsequent studies on the C and M substances (Salton, 1953; McCarty, 1952) revealed that they are associated with the cell wall.

On the other hand, the antigen of group N and group D streptococci occurs in the cytoplasmic membrane fraction, and has been identified as an intracellular teichoic acid (Elliot, 1962; Wicken et al., 1963).

The method used to solubilize the group specific carbohydrates determines to some extent their chemical composition. Fuller (1938) used hot formamide on whole cells of group A and the procedure has been applied to the cell walls of group C (Krause and McCarty, 1962), groups G and B (Curtis and Krause, 1964), and group F (Michel and Willers, 1964). Slade (1965) extracted the carbohydrate group antigens belonging to groups A, E, G, L and T with hot trichloroacetic acid (TCA). The extracts were then treated with phenol-water which had been used by Westphal (1952) to remove the cell wall liposaccharide. When hot formamide or hot acid were used, the carbohydrates were found to be free of mucopeptide components. However, when McCarty (1952) and Elliot (1960) applied Streptomyces albus cell wall dissolving enzymes to groups A and D, their preparations contained residual mucopeptide.

The cell wall antigens of two species of staphylococci have been studied extensively, and have been shown to be mainly teichoic acids. Julianelle and Wieghard (1934) described an antigenic "Polysaccharide A" from pathogenic staphylococci which precipitated Staphylococcus aureus but not Staphylococcus epidermidis antisera. Haukenes (1962)

later purified the polysaccharide from crushed cells by extraction and fractionation with ethanol and by column chromatography. The two main components of acid hydrolysis were found to be a mucopeptide and a ribitol teichoic acid, both compounds resembling the composition of the cell wall.

Protein antigens have also been found in cell walls of S. aureus as shown by Yoshida and Heden (1962) in agglutination tests. Subsequent studies indicated that this is a common antigen in the numerous serotypes of the species (Lenhart et al., 1963; Yoshida et al., 1963).

Wieghard and Julianelle (1935) isolated a group reactive antigen, polysaccharide B, from acid extracts of nonvirulent staphylococci which contained phosphorous and a reducing sugar. Morse (1963) extracted a polymer of glycerophosphate and glucose from cell walls of a strain of S. epidermidis which reacted with both homologous and heterologous S. epidermidis antisera. Acid extracts of four other strains of S. epidermidis yielded a reaction of identity with the antigen in gel diffusion studies. Reactions were not obtained with either S. aureus or streptococcal polyglycerophosphate antisera. At present, most of the strains of the staphylococci can be separated into two species, S. aureus and S. epidermidis, on the basis of their ribitol or glycerol cell wall teichoic acids.

The majority of the antigens of the lactobacilli appear to reside in polysaccharide components. Sharpe (1955),

on the basis of hot acid extracts, classified 70% of 442 strains of lactobacilli into six major serological groups and one sub-group which conformed closely to conventional taxonomy. The antigens responsible for groups B and C have been identified as polysaccharide components of cell walls (Knox, 1963; Glastonbury and Knox, 1963). Ribitol teichoic acids have been found in cell walls of group D (Archibald et al., 1961) and glycerol teichoic acids in the walls of groups A and E (Baddiley and Davison, 1961). Although serological analyses of these teichoic acids have not been carried out, it is likely that they will be shown to be antigenic determinants.

The corynebacteria, mycobacteria, and nocardia have been shown to have a similar pattern of cell wall composition, and to form a common serological group. Krah and Witebsky (1930) showed that alcoholic extracts of Corynebacterium diphtheriae, certain diphtheroids, and the tubercle bacilli share a common antigenic determinant. Wong and T'ung (1939) also described a group reactive carbohydrate antigen of C. diphtheriae. Recently, Cummins (1958, 1962) demonstrated that strains of corynebacteria, mycobacteria, and nocardia which have arabinose and galactose as their principle cell wall monosaccharides possess a common polysaccharide antigen. Cummins (1965) was able to confirm these results using the indirect immunofluorescent technique.

In addition to the group specific polysaccharides,

type specific protein antigens have been demonstrated in the corynebacteria. Cummins (1954) has described the occurrence of a superficial, thermolabile protein antigen in the walls of C. diphtheriae which was involved in agglutination reactions and which was type specific. Banach and Hawirko (1966) extracted two protein antigens, A and B, from crushed cells of C. hofmannii with hot acid, and purified them by column chromatography. Both antigens contained major protein components and antigen A was shown to contain arabinose and glucose. Cross reactivity was not detected with other Corynebacteria spp.

Compared to the other Gram-positive bacteria, little is known about the antigenic determinants of the aerobic spore-forming bacilli. Most of the serological investigations have been concerned with antigenic differences between vegetative cells and spores. Generally, the antigens have not been purified, and only preliminary biochemical analyses have been carried out.

Lamanna (1942) showed that the spores and vegetative cells of several Bacillus spp. possessed precipitinogens, and that those of the spores were of taxonomic value. He also found cross reactivity in agglutination tests of vegetative cell antigens. Davies (1951) studied the spore, somatic, and flagellar antigens of Bacillus polymyxa, and concluded that each was serologically distinct. A species specific antigen was found in the spores of the 39 strains

of B. polymyxa tested. Vennes and Gerhardt (1959) conducted an antigenic analysis of cell structures isolated from Bacillus megaterium. These workers detected distinct capsular, cell wall, and flagellar antigens, but suggested that the cell wall, and more specifically its polysaccharide, would be expected to contain the primary antigens. Norris and Wolf (1961) found distinctive spore, somatic O, and H antigens in several Bacillus spp. The multiplicity of spore precipitinogens was demonstrated, and interspecies cross reactions were shown to be of considerable taxonomic value.

Baillie and Norris (1964) examined ultrasonic extracts of young vegetative cells of Bacillus cereus by immunoelectrophoresis and detected seven heat resistant antigens (80°C for 10 min.),and at least eight heat labile antigens. During spore formation, the majority of thermolabile antigens disappeared, and new antigens were detected. Baillie (1967) studied the antigenic composition of cell extracts, cell walls, protoplasts, and flagella of B. cereus by immunoelectrophoresis. The vegetative cell extracts contained 15 heat resistant antigens (80°C for 10 min.), ten of which gave a positive result when stained for polysaccharide. The cell walls showed five heat stable antigens, three of which stained for polysaccharide. Three of the cell wall antigens were present in both spore and vegetative cell extracts.

As is the case with other Gram-positive micro-organisms, teichoic acids,and teichoic acid-like polymers have been found in cell walls and membranes of Bacillus spp. such as Bacillus subtilis (Armstrong et al., 1960), Bacillus megaterium KM (Ghuysen, 1961, 1964), Bacillus stereothermophilus (Wicken, 1966), Bacillus lichenformis (Burger and Glasser, 1966), and Bacillus coagulans (Forrester and Wicken, 1966). These studies were based on biochemical analyses of cell wall hydrolysates, but it is probable that the teichoic acids are antigenic.

A non-species specific antigen was first described by Rantz (1956) using hemagglutination and hemolytic tests. The antigen is found in many Gram-positive bacteria and has been shown to be an intracellular glycerol teichoic acid (Gorzynski et al., 1960). Anzai et al., (1960) have demonstrated that this antigen is also found in members of the genus Bacillus. Of the six species of bacilli tested, all strains of B. megaterium, B. cereus, B. cereus var. mycoides, B. subtilis, and Bacillus spp. strain medusa reacted against serum containing Rantz antibodies to S. aureus by hemagglutination tests. Only strains of Bacillus brevis failed to react with the Rantz antiserum. Similarly, Neter et al. (1959) showed that common antigens are present in cells of B. subtilis and S. aureus.

Engers (1966) extracted two antigens from crushed cells of B. megaterium with hot acid and purified them by

acid precipitation, ethanol fractionation, and column chromatography. One of the antigens was a polysaccharide, and alanine appeared to be the only major amino acid present, as determined by paper chromatography. He suggested the possibility of this antigen being a teichoic acid. The hot acid extract failed to cross react with Bacillus M, B. cereus, or B. subtilis antisera when examined by gel diffusion. On the other hand, cross reactions were obtained to all three by passive hemagglutination tests.

Recently, Chorpenning and Dodd (1966) have demonstrated a water soluble antigen extractable from many Gram-positive bacteria which modifies red blood cells so that they react with homologous bacterial antisera, cross react with some normal human sera, and with antisera to other Gram-positive species. The antigen was found in all eight species of bacilli tested, as well as in selected strains of streptococci and staphylococci. The antigen was extracted and appeared to be a complex nitrogen containing polysaccharide with uronic acid as a possible constituent.

A polysaccharide antigen has been isolated from Bacillus anthracis, and studied extensively. Ivánovics (1940a,b,c) first obtained the antigen from virulent and avirulent strains after cell lysis with acriflavine and determined that it was composed of equimolar amounts of D-galactose and N-acetylglucosamine. An identical polysaccharide was extracted from ammonium carbonate lysates

of the organism (Smith et al.,1953a,b; Smith and Zwartouw, 1954, 1956) and was shown to contain 38 - 43% galactose, 38 - 43% glucosamine, 14.5% acetyl, 4.0% nitrogen,and 0.3% α-carboxlamino nitrogen. The molecular weight was estimated at 27,000. Glycine, aspartic acid, alanine, glutamic acid, α, ε-diaminopimilic acid, and muramic acid were subsequently detected accounting for the excess nitrogen (Smith et al., 1956). As the polysaccharide could not be completely separated from the mucopeptide, and as the anthrax antiserum containing polysaccharide antibody reacted with the cell walls but not with the capsule, it was inferred that it was a cell wall component. Ivánovics (1940b), and Heidelberger et al. (1958) have demonstrated by precipitin tests that the polysaccharide cross reacts with Type 14 pneumococcal antiserum.

Another polysaccharide antigen has been prepared from culture filtrates and from lysates of the anthrax bacillus which contained D-galactose and N-acetylglucosamine in a molar ratio of 2:1 (Cave-Browne-Cave et al., 1954). However, precipitin tests with this antigen failed to yield a positive reaction with Type 14 pneumococci antisera (Heidelberger et al. 1958).

The strain Bacillus M used in these studies was first described by Tomcsik (1951) and was originally considered to be a variant of B. anthracis. It has now been identified as a variant of B. megaterium, and is referred to as Bacillus M

(Geux-Holzer and Tomcsik, 1956).

Bacillus M forms an extensive capsule when grown on a solid medium, and this was utilized by Tomcsik (1951) in his classical phase contrast studies of capsular structure. His work revealed that the capsule of Bacillus M is complex, consisting of a polysaccharide framework, with the spaces being filled out by polypeptide.

Geux-Holzer and Tomcsik (1956) obtained an aqueous extract of the polypeptide and polysaccharide antigens of the capsular material of Bacillus M by boiling, and separated and purified them by fractional ethanol precipitation. The polypeptide proved to be identical to the poly-D-glutamic acid polymer of B. anthracis, and it inhibited the capsular reactions of both B. anthracis and Bacillus M antisera. The polysaccharide absorbed the antibody in the homologous Bacillus M antisera which was responsible for the framework reaction of the capsule. Biochemical analysis of the polysaccharide yielded 4% nitrogen, 0.5% phosphorous, and 40% carbohydrate. Galactosamine and glucosamine were detected by paper chromatography.

These workers isolated a mucoprotein antigen from capsulated and non-capsulated forms of Bacillus M with lysozyme. The purified antigen gave identical serological reactions with the polysaccharide antiserum, and was considered to be a less degraded form of the cellular substance participating in building up the cell wall and the

capsular structure. Biochemical analysis revealed 7.6% nitrogen, 4.7% phosphorous, 4.3% acetyl, and 18% reducing substance. Upon paper chromatography, galactosamine, glucosamine, diaminopimelic acid, alanine, glutamic acid, and two unknown ninhydrin positive substances were found.

It is clear that much work remains to be done in elucidating the antigenic nature of the Bacillus spp. Workers in the field have shown that there are distinct antigens present in the vegetative cell components, as well as in spores. The spore precipitinogens appear to show great cross reactivity and should be of taxonomic value. Vegetative cell precipitinogens appear to be more type specific than species specific. On the other hand the considerable cross reactivity of vegetative cells examined by hemagglutination tests may be associated with the teichoic acids present in the cells. Bacillary antigens should be extracted, purified, and analysed as to their chemical structure, and their role in the bacterial cell. Such work may aid in the understanding of the Gram-positivity of bacteria, and should be of considerable taxonomic importance.

MATERIALS AND METHODS

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CULTURE

Bacillus M (received from Dr. Tomcsik) was used throughout these studies. The culture was maintained on Brain Heart Infusion (BHI) agar slants and subcultured at frequent intervals.

GROWTH

Cells were inoculated into 5 ml portions of BHI broth and grown for 12 hours. These 5 ml cultures were first transferred into 100 ml volumes of BHI broth. After incubation for 12 hours, they were transferred again into one liter volumes of BHI broth in two liter Fernbach flasks and incubated for 24 hours. All the cultures were incubated on a rotary shaker at 28°C. The cells from 12 l. cultures were harvested by Sharples centrifugation, washed twice in 0.1 M phosphate buffer, pH 7.2, and stored at -15°C. The yield from 12 l. cultures was 80-100 g., wet weight cells.

BUFFERS

Unless otherwise designated, 0.02 M sodium phosphate buffer, pH 7.2, was used throughout the course of these investigations.

WHOLE CELL ANTIGENS

Twelve hour cultures of *Bacillus M* were inoculated onto 200 ml of BHI agar in Roux bottles and incubated for

12 hours at 28°C. The cells were suspended in 0.85% saline, pH 7.0, washed three times, and resuspended in 0.3% formalin saline. The suspension was standardized to give 38% light transmission on a Beckman model C colorimeter, red filter, corresponding to MacFarland tube No. 3, (Kabat and Meyer, 1961). The vaccine was held at 5°C for 7 days and tested for sterility by the streak plate method.

PRODUCTION OF ANTISERA

Adult male albino rabbits were immunized by the procedure of Kabat and Meyer (1961) except that a total of 21 ml of whole cell antigen were administered over a period of 5 weeks. The blood was collected by cardiac puncture, incubated for 2 hours at 37°C, and stored overnight at 5°C. The serum was collected and stored at -15°C.

Standard tube agglutination tests showed no titre in the pre-immune sera, and a reciprocal dilution titre of 800 in the immune sera.

SEROLOGICAL ACTIVITY

a) Immunodiffusion

The double diffusion plate technique of Ouchterlony (1949) was used, except that a horizontal trough was used as the antiserum reservoir. Petri plates (9 cm) were coated with a thin layer of 2% Noble agar* containing 1%

*Difco

sodium azide and 0.88% sodium chloride. A horizontal trough (2 mm x 75 mm) was cut 6 mm from the antigen wells and filled with 0.4 ml of undiluted antiserum. Antigen wells were cut with a No. 1 cork borer (diameter 4 mm) and filled with 0.03 ml of antigen. The plates were placed in a moist chamber at 25°C for 3 days to allow precipitin lines to develop.

b) Immunoelectrophoresis

Immunoelectrophoresis was performed on microscope slides according to the method of Shleidigger (1955).

Slides were coated with a thin layer of 1.5% Agarose* dissolved in 0.05 M barbital buffer, ph 8.4, and then with 2.0 ml of the same solution. The antiserum trough (2 mm x 60 mm) was cut and two starting wells were made 3 mm from the sides of the trough with a No. 18 hypodermic needle. The slides were connected to 0.05 M barbital buffer, ph 8.4, in a Shandon electrophoresis tank by means of Agarose wicks dampened with the buffer, and the tank was connected to a Beckman Duostat D.C. power supply. Electrophoresis was carried out for 60 min. at 10 volts/cm. After completion of the run, undiluted antiserum was placed in the trough and the reaction allowed to develop for 24 hours in a 25°C moist chamber.

The slides were washed in 0.85% saline, dried, stained with amido black solution for one hour, and destained with

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*National Biochemicals Co.

5% acetic acid until clear.

COLUMN CHROMATOGRAPHY

a) DEAE-Cellulose

Diethylaminoethyl cellulose* (DEAE-cellulose) was washed twice in 1 N NaOH, several times in distilled water, and then in phosphate buffer until a final pH of 7.2 was obtained. The slurry was poured into a column, and the column packed and equilibrated by passing through 500 ml of phosphate buffer with a hydrostatic pressure of 100 cm. The sample was placed on the column and eluted with a continuous linear gradient of KCl. All experiments were performed at 5°C. The details of individual runs are shown in later sections.

b) Sephadex G-150

Sephadex G-150** (bead form) was added slowly to phosphate buffer, allowed to swell for 3 days at 5°C, and then the fines decanted. A 2.5 cm x 100 cm column was half filled with phosphate buffer, and a thick slurry of the Sephadex was poured into the column. After the gel had settled, 500 ml of phosphate buffer was passed through the column to equilibrate it. At all times, during the packing and subsequent runs, a 10 cm hydrostatic pressure head was maintained to prevent overpacking and slow flow rates.

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*Sigma ** Pharmacia

Column temperature was maintained at 5°C.

The sample solution was placed on the column and eluted with phosphate buffer at a column flow rate of 15 ml per hour. Fractions of 3 ml were collected.

The column was used for both preparative and analytical purposes.

c) Column Fractions

Column fractions were collected on either a Gilson or Analco automatic fraction collector, and assayed for protein on a Unicam SP-700 spectrophotometer at 280 mμ (1 cm light path) and for serological activity by immunodiffusion. Unless otherwise stated, the serologically active fractions were pooled and concentrated by ammonium sulfate precipitation (0.70 saturation). The precipitate was dissolved in 5 ml of phosphate buffer, dialyzed for two 6 hour periods at 5°C against 6 l. of phosphate buffer, and stored at -15°C.

AMMONIUM SULFATE FRACTIONATION

Forward ammonium sulfate fractionation was carried out at 5°C. The fractions between 0.45 and 0.70 saturation were collected by centrifugation, redissolved in 15 ml of phosphate buffers dialysed for two 6 hour periods against phosphate buffer, and stored at -15°C.

EXTRACTION OF ANTIGENS

Antigens were extracted from crushed cells by three methods. The first method was by extensive sonication in

buffered salts. The extract is referred to as the buffered sonic extract. One antigen, referred to as the buffered salts Antigen, was extensively purified.

The second method was by extraction with hot HCl. The extract is referred to as the Hot HCl Extract. One antigen, referred to as the hot HCl Antigen was partially purified.

The third method was by extraction with chloroform-methanol. This antigen is referred to as the CM Antigen.

a) Buffered Sonic Extraction

Four fractions of cells, approximately 25g. wet weight each were suspended in 20 ml of 0.1 M phosphate buffer, ph 7.2, and sonicated for one hour at maximum current in a Raytheon sonic oscillator at 10 Kc/sec. The fractions were pooled and centrifuged at 78,000 x g for one hour at 5°C in the L-30 roter on a model L Spinco ultracentrifuge. The red viscous supernatant was collected and dialyzed at 5°C for two 6 hour periods against 6 l of phosphate buffer.

The Spinco supernatant was applied to a 3.3 cm x 55 cm DEAE-cellulose column and eluted to a final concentration of 0.27 M KCl. One liter each of phosphate buffer and 0.27 M KCl was used. Fractions of 25 ml were collected and assayed.

Ammonium sulfate fractionation was carried out on the serologically active DEAE-cellulose fractions obtained from 240-300 g. of cells. The 0.45-0.70 saturation fraction was redissolved, dialyzed, and chromatographed on a 2.5 cm x

35 cm DEAE-cellulose column to a final concentration of 0.25 M KCl. Volumes of 400 ml of phosphate buffer and 0.25 M KCl were used. The 10 ml fractions were assayed, and the serologically active fractions concentrated to 5 ml by ammonium sulfate precipitation.

The sample was then subjected to gel-filtration on Sephadex G-150. The 3 ml fractions were assayed, and the serologically active fractions concentrated to 5 ml by ammonium sulfate precipitation.

This buffered salts Antigen was subjected to immunological, biophysical, and biochemical analyses.

b) Hot HCL Extraction

A hot HCl extraction was performed on crushed cells according to the method of Engers (1966), with some modifications.

A cell mass of 40 g. wet weight was suspended in 40 ml of 0.1M phosphate buffer, pH 7.2, and sonicated for one hour. The crushed cell suspension was treated with 4 volumes of 0.1 N HCl at 90-95°C for 25 min., cooled on ice to 25°C, adjusted to pH 7.0, and centrifuged at 12,000 x g at 0°C for 20 min. The supernatant was dialyzed against 6 l phosphate buffer for 4 hours at 5°C, against moving air at 25°C until reduced to 1/10 the original volume, and again against 6 l of phosphate buffer at 5°C.

The Hot HCl Extract was adjusted to pH 4.8 with a

20% sodium acetate-acetic acid (pH 4.8) solution, allowed to stir at 25°C for 10 min. and centrifuged at 20,000 x g for 20 min. at 0°C. The precipitate was called the hot HCl-HAc precipitate.

The supernatant was treated with 2.5 volumes of 95% ethanol for 12 hours at -15°C. The precipitate was collected by centrifugation at 20,000 x g for 20 min., and redissolved in 10 ml of 0.05 M KCl in phosphate buffer, pH 7.0.

The dissolved ethanol precipitate was chromatographed on a 2.5 cm x 35 cm DEAE-cellulose column which had been previously equilibrated in 0.05 M KCl in phosphate buffer, pH 7.0. Elution was to a final concentration of 0.20 M KCl. Volumes of 500 ml each of starting buffer and 0.20 M KCl were used. The 10 ml fractions were assayed, and the serologically active fractions concentrated to 5 ml by ammonium sulfate precipitation. The antigen is referred to as the hot HCl Antigen.

c) Chloroform-Methanol Extraction

Approximately 10 g. wet weight of cells were suspended in 10 ml of 0.1 M phosphate buffer, ph 7.2, and sonicated for 30 min. The crushed cell suspension was treated with 5 volumes of chloroform-methanol (2:1, v/v) for 18 hours at 5°C. The extract was centrifuged at 30,000 x g for 15 min. and the precipitate was collected. The supernatant was filtered through Whatman No. I filter paper, and evaporated to dryness

under vacuum.

The yellow residue was redissolved in phosphate buffer, centrifuged to remove the insoluble fraction, and the supernatant assayed by immunodiffusion. This supernatant is referred to as the CM Antigen.

SURVEY OF ANTIGENS

a) Chemical Fractionation

A crushed cell suspension was extracted with chloroform-methanol as described above. The chloroform-methanol precipitate was further fractionated according to the method of Prescott et al. (1966).

Briefly, the precipitate was redissolved in 0.25 N NaOH, treated with cold ethyl alcohol-ether (3:1, v/v), and centrifuged. The supernatant was acidified to pH 5.0 with acetic acid, the precipitate collected by centrifugation, redissolved in phosphate buffer, and labelled Protein I. The supernatant fluid was evaporated to dryness, redissolved in phosphate buffer, and labelled Polysaccharide I.

The alcohol-ether precipitate was redissolved in water and extracted with 15% trichloroacetic acid (TCA). After centrifugation, the supernatant fluid was again extracted with alcohol-ether, the precipitate and supernatant becoming Polysaccharide II and III respectively.

The TCA precipitate was dissolved in phosphate buffer and centrifuged, the supernatant becoming Protein II. The

insoluble fraction was redissolved in 0.5 N NaOH, the pH adjusted to 7.0, and treated with phenol to give a 10% saturation. The sample was centrifuged, and the supernatant labelled Phenol Extract.

All fractions were dissolved in phosphate buffer and assayed by immunodiffusion.

b) DEAE-Cellulose Chromatography of Buffered Sonic Extract

A buffered sonic extract was chromatographed on DEAE-cellulose to separate the different antigenic fractions. In one experiment, the column was eluted to a final concentration of 1.40 M KCl, and in another experiment to 3.00 M KCl. The 10 ml fractions were assayed for protein and serological activity. The number of antigens present in each tube tested was judged according to the number of distinct precipitin lines observed. Antigens from adjacent tubes were judged to be the same if synonymous lines of identity could be detected between them.

CROSS REACTION STUDIES

Five antigenic fractions, buffered sonic extract, buffered salts Antigen, hot HCl Antigen, hot HCl-HAc precipitate, and CM Antigen were tested by immunodiffusion for cross reactivity against antisera to Bacillus megaterium ATCC 14581 and Bacillus cereus ATCC 10266. The standard tube agglutination reciprocal dilution titre of the B. megaterium antisera was 512.

DISC ELECTROPHORESIS

Acrylamide gel electrophoresis was performed as described by Davis (1964) using the Canalco Disc Electrophoresis Trial Kit*.

The Buffered Salts Antigen (80 µg. protein) was mixed with equal amounts of the upper-gel (containing twice the amount of acrylamide) and layered on the spacer-gel. Electrophoresis was carried out until the marker bank reached the bottom of the separating-gel. The Canalco Model 150 V. power supply was used at 5 m.a. per sample. After the run, the gel columns (0.5 cm x 6 cm) were removed, immediately stained for one hour with amido black, and then electrophoretically destained with 7.5% acetic acid.

MOLECULAR WEIGHT DETERMINATION

Two separate methods were tried in an effort to determine the molecular weight (M.W.) of the buffered salts Antigen.

a) Gel-Filtration

The molecular weight of the antigen was estimated using Sephadex G-150 as described by Andrews (1964). Three proteins were used to calibrate the column, yeast alcohol dehydrogenase** (M.W. 150,000), horse heart cytochrome C**

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*Canal Industrial Corporation, Bethesda, Maryland.

**Sigma

type III (M.W. 12,270), and bovine hemoglobin* (M.W. 64,500). These were dissolved in phosphate buffer at concentrations of 7.5, 1.0, and 5.0 mg/ml respectively. A volume of 1.5 ml of the protein solution was passed through the column and the fractions were assayed at 280 mμ and 415 mμ (Fig. 1). The elution volume (Ve) of each peak was plotted against the log molecular weight of the proteins to obtain a calibration curve (Fig. 2).

The antigen Ve obtained during the purification procedure was fitted to the curve and the molecular weight estimated.

b) Density Gradient Centrifugation

The sucrose density gradient method of Martin and Ames (1961) was used, with some modifications. Linear sucrose gradients were made in lusteroid centrifuge tubes from 10% - 30% (w/v) in one experiment, and from 4% - 20% (w/v) in another. Bovine hemoglobin* was used as the reference proteins. The gradient tubes were centrifuged at 139,000 x g for 12 hours at 5°C in a model-L Spinco ultracentrifuge using the SW-39 swinging bucket rotor. At the end of the run, two drop fractions were collected after puncturing the bottom of the tube with a needle. The hemoglobin was assayed by absorbtion at 415 mμ, and the antigen by immunodiffusion.

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*Sigma

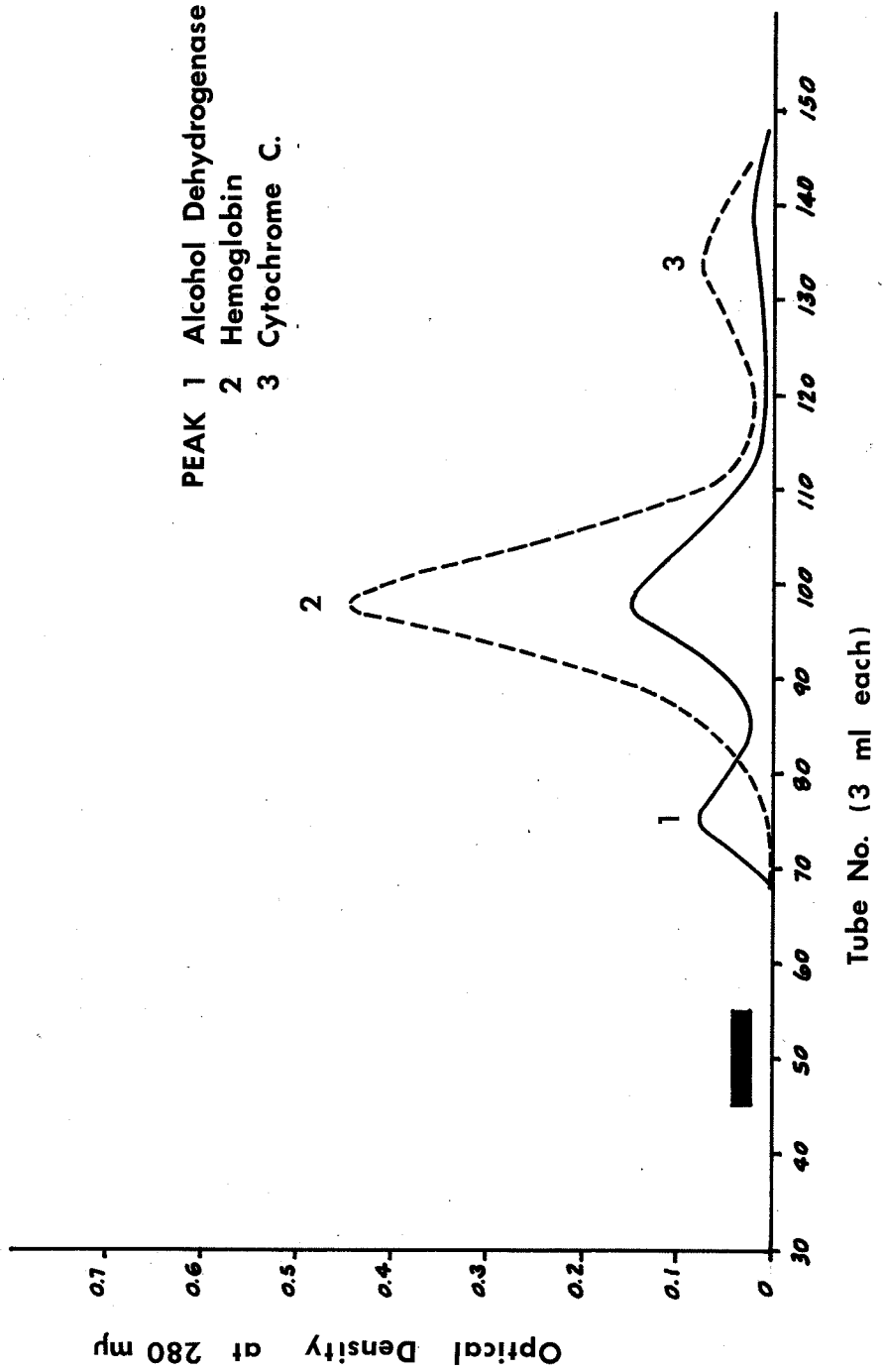

Figure 1. Calibration of Sephadex G-150 Column.

—— Absorbance at 280 m
- - - Absorbance at 415 m
▬▬ Antigenic activity.

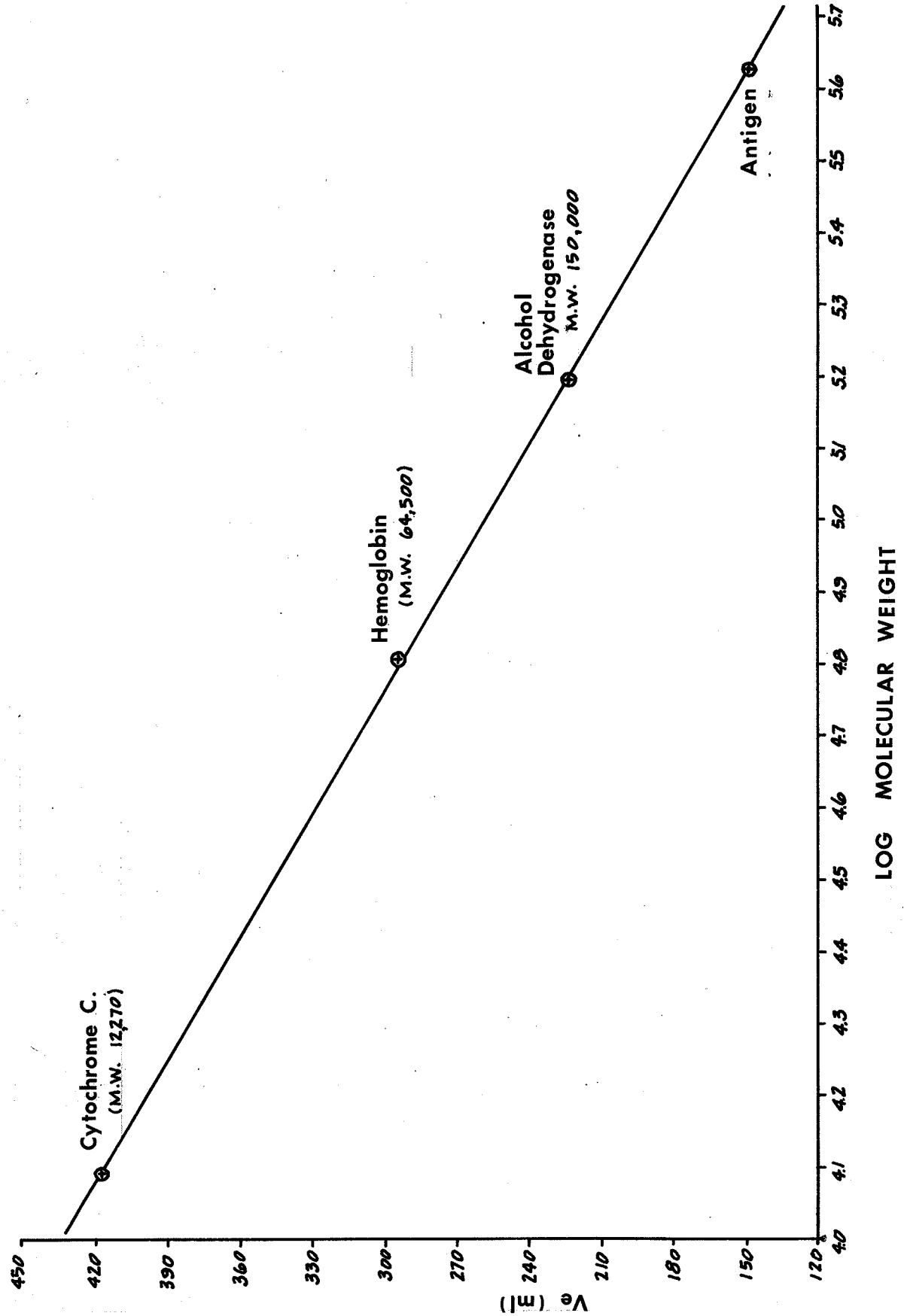

Figure 2. Calibration Curve of Sephadex G-150 Column.

PROTEOLYTIC ENZYME TREATMENT OF BUFFERED SALTS ANTIGEN

The buffered salts Antigen was treated with a proteolytic enzyme (received from Dr. Wolfe) isolated from a myxobacter, strain AL-1. The enzyme hydrolyses proteins and bacterial cell walls by attacking peptide bonds, (Ensign and Wolfe, 1965).

The enzyme was dissolved in 0.025 M Tris buffer, pH 9.0, at a concentration of 0.1 mg/ml. A volume of 1 ml of the enzyme solution was mixed with an equal volume of antigen solution (0.02 mg/ml). As a control, 1 ml of the antigen solution was mixed with 1 ml of water. Both sample and control were incubated for 1 hour at 37°C, and assayed by immunodiffusion.

The enzyme was in an active state, as in a similar experiment, performed at the same time and under the same conditions, the serological activity of a Corynebacterium hofmani antigen was destroyed by the enzyme, (Hawirko and Oh, unpublished results).

BIOCHEMICAL ANALYSIS

A Klett-Summerson photoelectric colorimeter was used in the protein and carbohydrate determination. The samples were read at 660 mμ.

Protein content was determined by the Folin method of Lowry et al. (1951).

Nucleic acid was assayed by the method of Warburg

and Christian (1941). The optical density at 260 mμ and 280 mμ were determined on a Unicam SP-700.

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 of sample were added to 3 ml of anthrone reagent, boiled for 3 min., and immediately cooled.

RESULTS

RESULTS

BUFFERED SONIC EXTRACT

The buffered sonic extract yielded at least four precipitin lines, two major and two minor, when examined by immunodiffusion against homologous whole cell antiserum. The buffered salts Antigen showed a reaction of identity with one of the major lines.

The buffered salts Antigen was obtained from the buffered sonic extract and purified by DEAE-cellulose chromatography (Fig. 3), ammonium sulfate fractionation, rechromatography on DEAE-cellulose (Fig. 4), and gel filtration on Sephadex- G-150 (Fig. 5). The antigen was eluted from DEAE-cellulose at 0.15 M KCl concentration, from Sephadex G-150 almost with the void volume, and was salted out by ammonium sulfate in the fraction between 0.45 and 0.70 saturation. The yield from 240-300 g. wet weight cells was approximately 125 mg. The antigen was soluble in water and appeared as a colorless, translucent substance in the dry state.

Immunodiffusion of the buffered salts Antigen yielded only one precipitin line. Disc electrophoresis of the sample after gel filtration revealed only one protein band (Fig. 6).

Biochemical analyses showed that protein, carbohydrate, and nucleic acid were present in 1, 64, and 10

per cents respectively.

The molecular weight of the antigen as estimated by gel filtration was shown to be greater than, or equal to, 150,000. The density gradient method of molecular weight determination proved to be unsuccessful as the antigen merely floated on the surface of the gradient.

Treatment of the antigen with the myxobacter AL-1 proteolytic enzyme did not result in any loss of serological activity.

The antigen was shown to be of a very stable nature as serological activity was retained after 12 months at -15°C, four months at 4°C, and two days at 25°C. These values are not maxima, but indicate the limits of the times tested.

HOT HCL EXTRACT

The hot HCl extract yielded at least three precipitin lines when examined by immunodiffusion.

The HCl Antigen was partially purified by pH precipitation with sodium acetate-acetic acid (pH 4.8), ethanol fractionation, and DEAE-cellulose chromatography (Fig. 7),the antigen being eluted at 0.13 M KCl concentration.

The hot HCl-HAc precipitate yielded two distinct precipitin lines by gel diffusion. The serologically active ethanol and DEAE-cellulose fractions yielded only one line, and neither showed a reaction of identity with the hot HCl-

HAc precipitate. Immunoelectrophoresis of the hot HCl Antigen yielded one line which had migrated towards the anode (Fig. 8). Disc electrophoresis revealed at least six protein bands.

Biochemical analysis for total carbohydrate showed that approximately 15 per cent carbohydrate was present. The antigen exhibited similar physical characteristics as described for the buffered salts Antigen. It was salted out by ammonium sulfate in the 0.45-0.70 saturation fraction, and was stable over the temperature range from -15 - 95°C.

CHLOROFORM-METHANOL EXTRACT

The CM extract (CM Antigen) yielded only one precipitin line when examined by immunodiffusion. Biochemical analysis showed the presence of 18 per cent protein and 11 per cent carbohydrate.

REACTIONS OF IDENTITY

The buffered salts Antigen, HCl Antigen, and CM Antigen showed reactions of identity with each other when examined by immunodiffusion (Fig. 9).

SURVEY OF ANTIGENS

Chromatography of the buffered sonic extract on DEAE-cellulose (Fig. 10) revealed from 8 - 11 different antigens present in the extract.

Upon chemical fractionation of crushed cells,

serological activity was not detected except in the CM extract and the phenol extract which yielded one precipitin line each by immunodiffusion.

CROSS REACTION STUDIES

The buffered sonic extract yielded two precipitin lines, one major and one minor, when tested by immunodiffusion against B. cereus ATCC 10266 antisera. The hot HCl Antigen, CM Antigen, and the purified buffered salts Antigen failed to react with the heterologous sera.

Reactions were not observed with the B. megaterium antisera and any of the antigenic fractions.

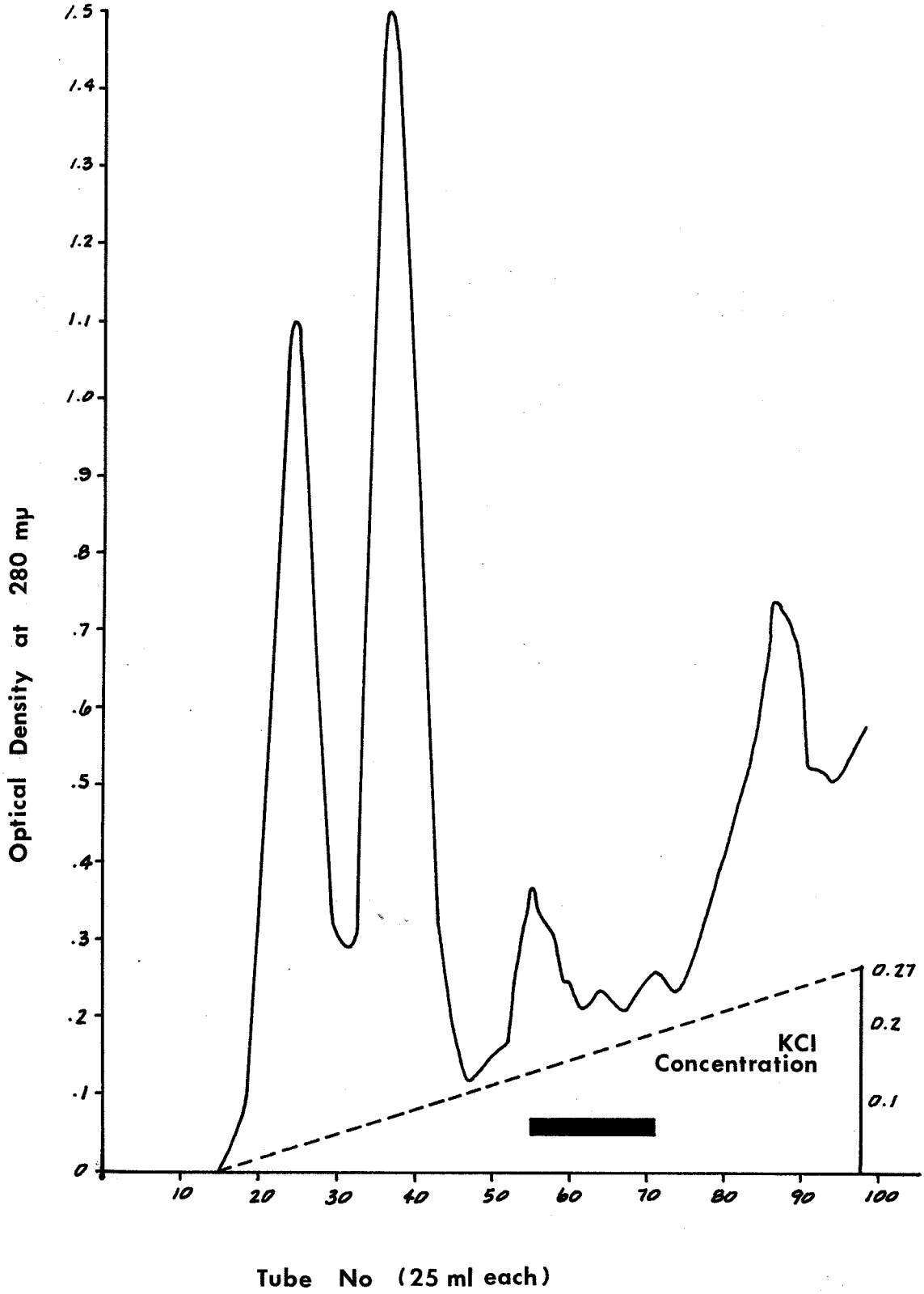

Figure 3. Chromatography of Spinco supernatant on DEAE-cellulose.

▬▬▬ Antigenic Activity.

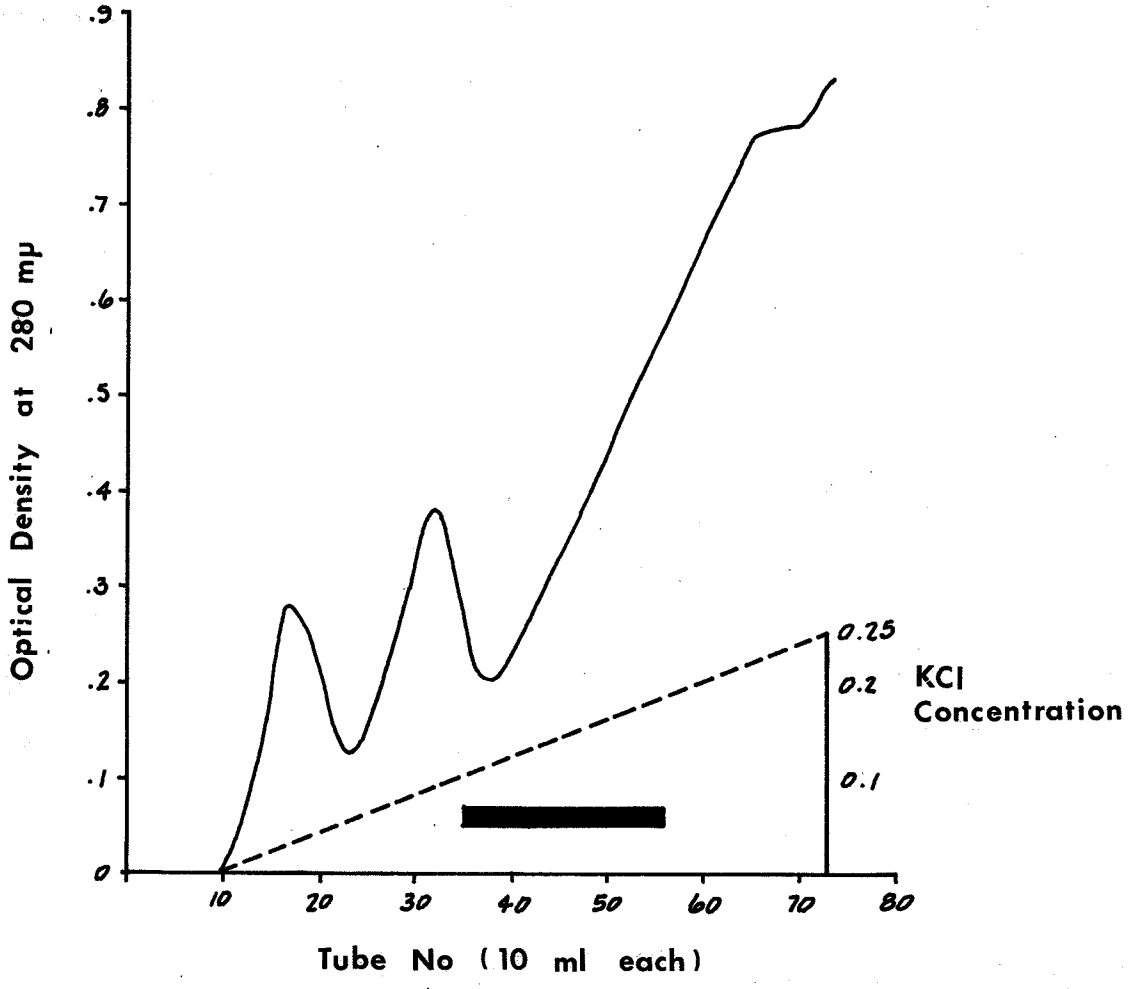

Figure 4. Chromatography of Ammonium Sulfate Fraction (0.45-0.70) Saturation on DEAE-cellulose.

▬▬▬ Antigenic Activity.

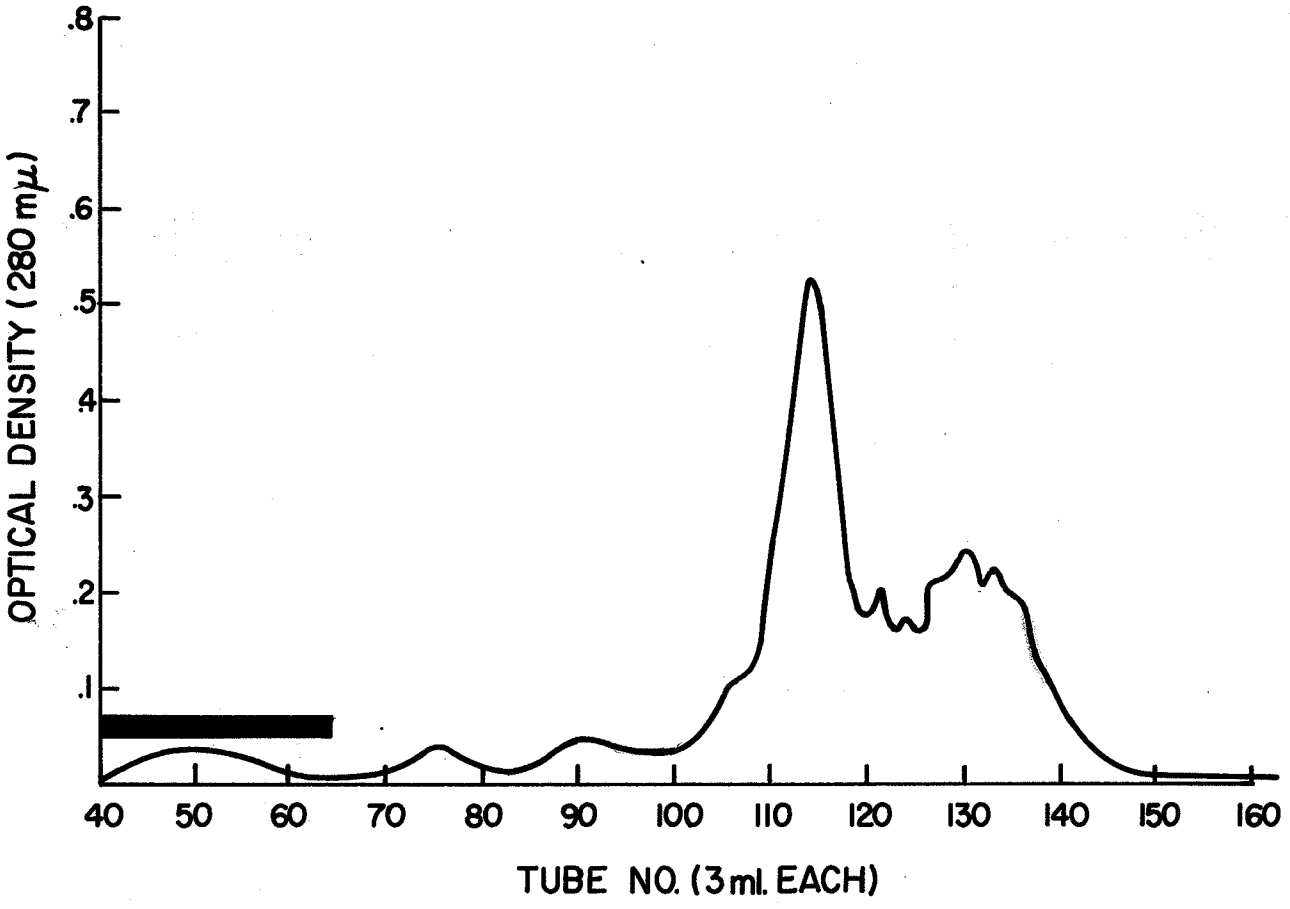

Figure 5. Chromatography of DEAE-cellulose Concentrate on Sephadex G-150.

Antigenic Activity.

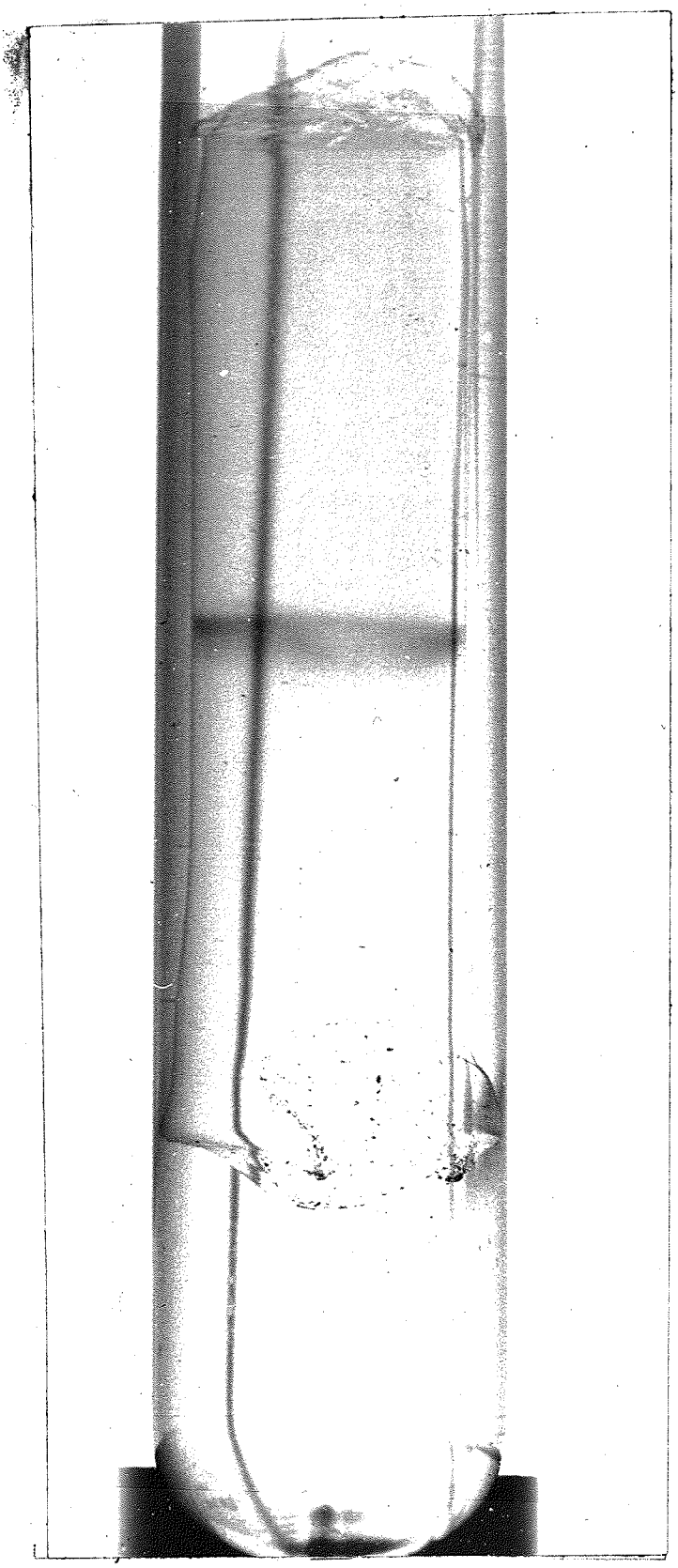

Figure 6. Disc Electrophoresis of Buffered Salts Antigen.

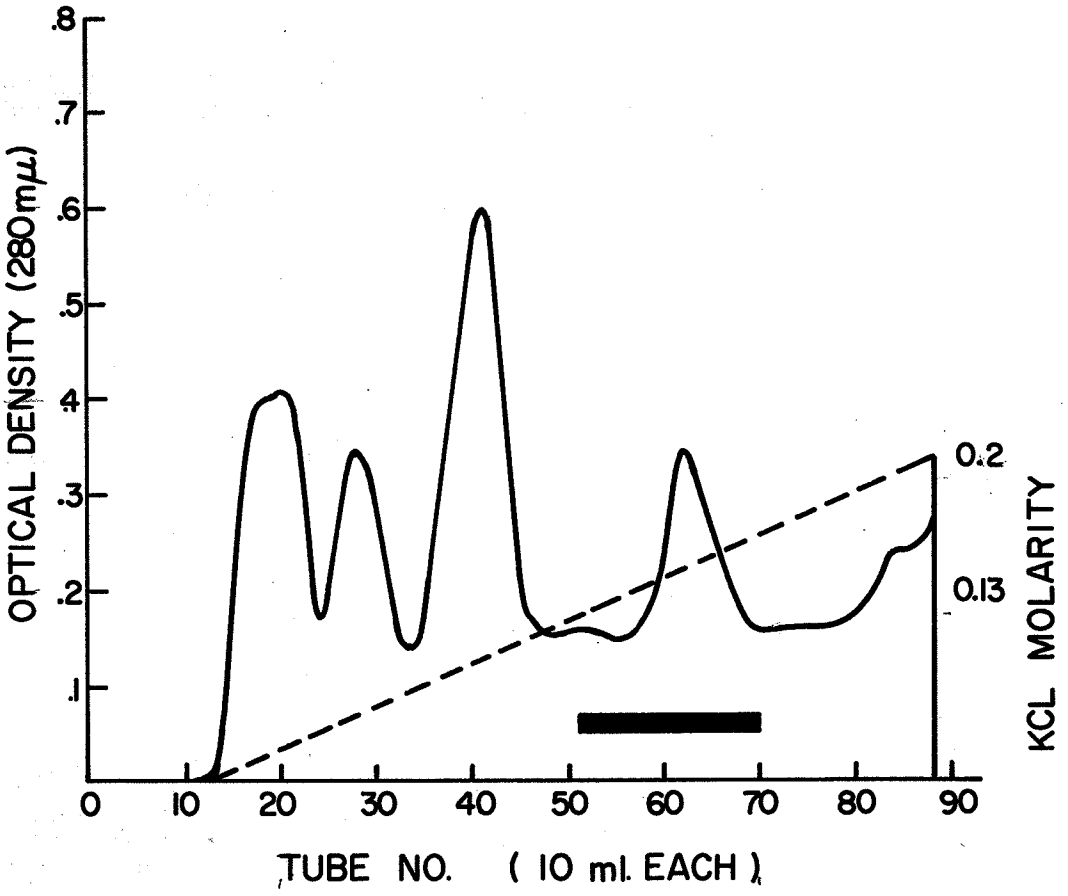

Figure 7. Chromatography of Hot HCl Antigen on DEAE-cellulose.
▬▬ Antigenic Activity.

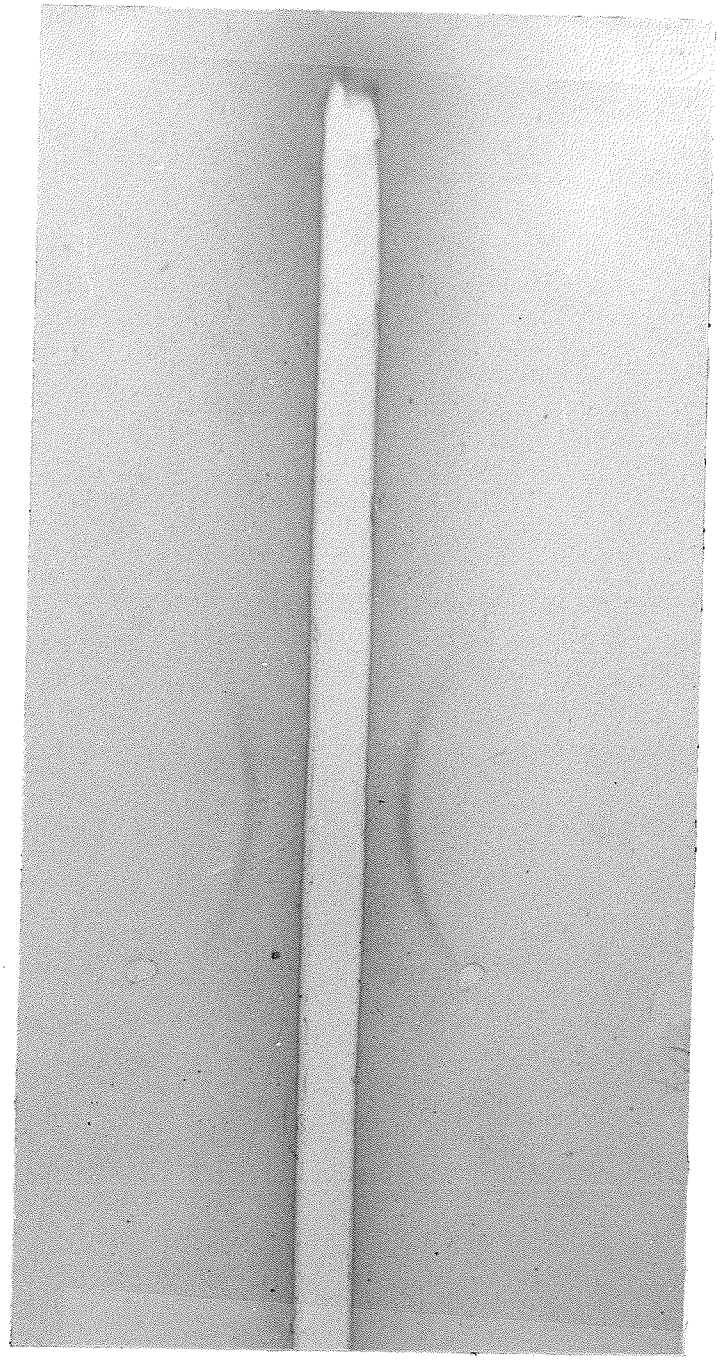

Figure 6. Immunoelectrophoresis of Hot HCl Antigen.

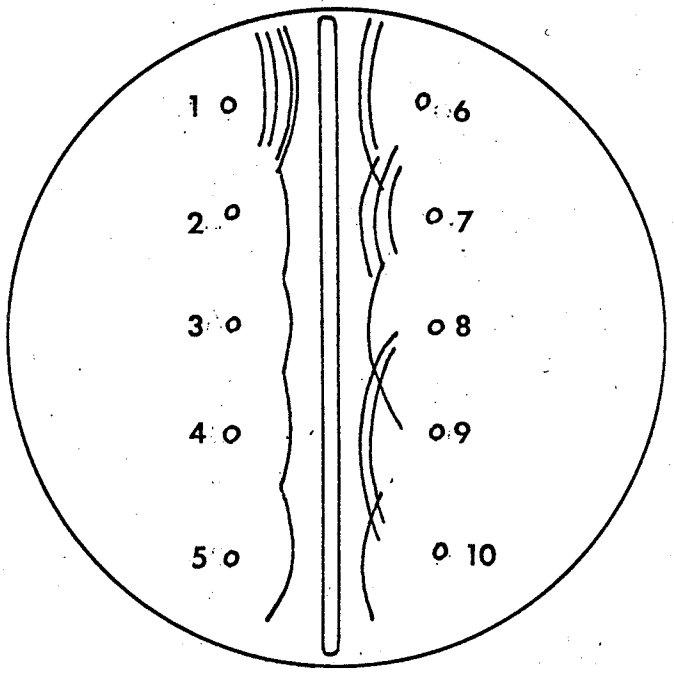

Figure 9. Immunodiffusion Reactions of Identity.

1. Buffered sonic extract.
2. Buffered salts Antigen.
3. Hot HCl Antigen.
4. CM Antigen.
5. Buffered salts Antigen.
6. Hot HCl-HAc precipitate.
7. Hot HCl extract.
8. Hot HCl Antigen.
9. Hot HCl-HAc precipitate.
10. Phenol extract.

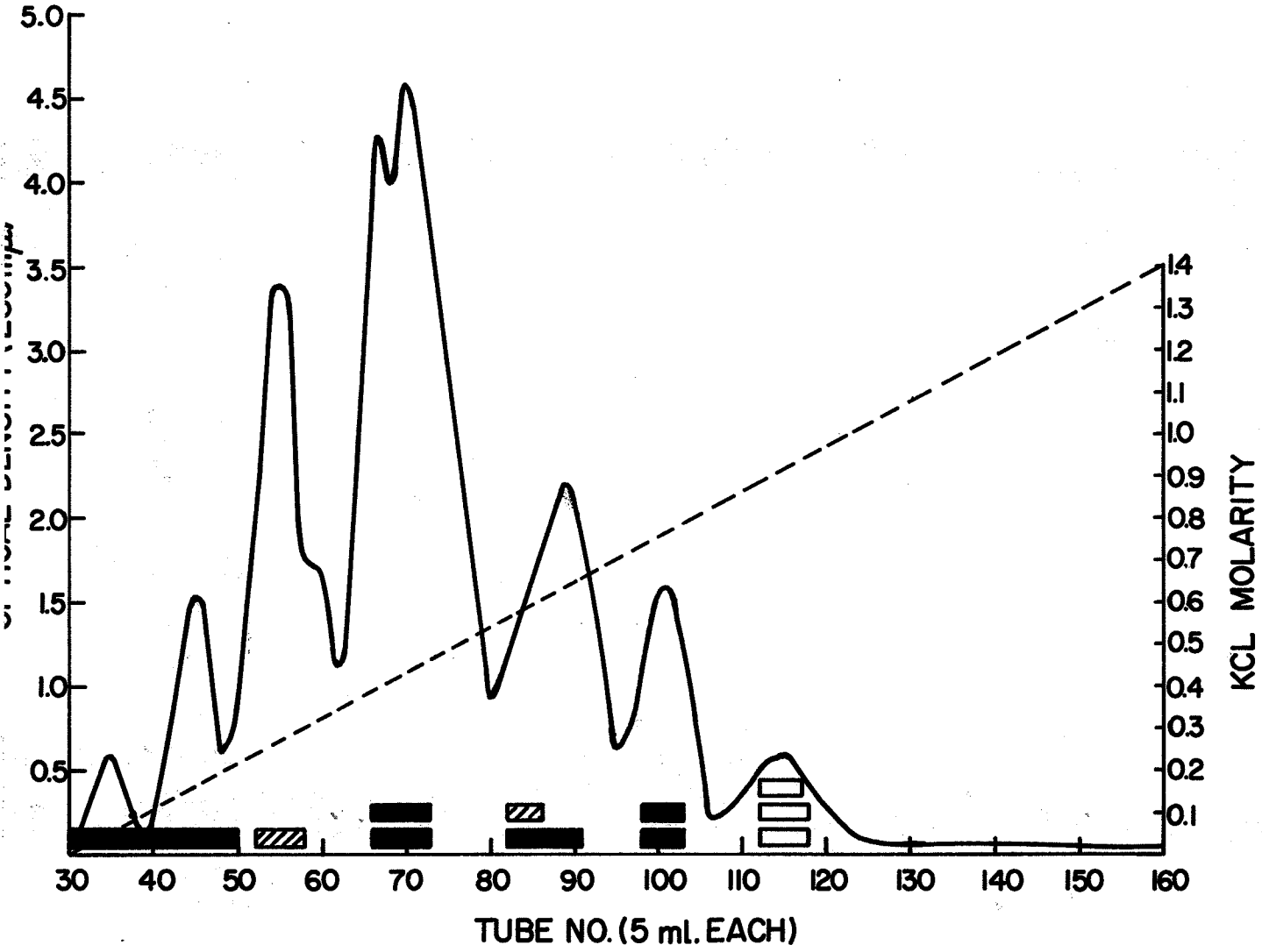

Figure 10. Survey of Antigens: Chromatography of Spinco Supernatant on DEAE-cellulose.

Antigenic Activity (1.4 M KCl Column only)
Antigenic Activity (3.0 M KCl Column only)
Antigenic Activity (Common to both columns)

DISCUSSION

DISCUSSION

Since the buffered salts Antigen, the hot HCl Antigen, and the CM Antigen all yielded a reaction of identity when examined by immunodiffusion, it appears that the three fractions contained the same hapten. The purified antigen showed a reaction of identity when one of the major lines of the buffered sonic extract, indicating that it is one of the principal soluble antigens of Bacillus M.

Further evidence to support the identity of the buffered salts Antigen and the hot HCl Antigen is as follows. The antigens both appear to possess a similar negative charge. The buffered salts Antigen was eluted from a DEAE-cellulose column, pH 7.2, at a 0.15 M KCl concentration, and the hot HCl Antigen was eluted from a similar column, pH 7.0, at a 0.13 M KCl concentration. At a pH of 7.0 the hot HCl Antigen would be expected to possess a slightly less net negative charge, and as a result might be eluted at a lower KCl concentration. Also, both antigens migrated towards the anode in the disc electrophoresis and immunoelectrophoresis experiments.

The finding that the buffered salts Antigen contained 64 per cent polysaccharide left little doubt that this was an integral part of the structure. Although the hot HCl Antigen and the CM Antigen were shown to contain 15 per cent and 11 per cent polysaccharide respectively, these variations

in content do not preclude the possibility that the haptens are identical since these fractions were only partially purified. It was shown by disc electrophoresis that the hot HCl Antigen contained at least six proteins and the crude chloroform-methanol extract also undoubtedly contained extraneous material.

The occurrence of lipid as an important constituent of the buffered salts Antigen was first suggested by the observation that the ammonium sulfate precipitates of it somewhat resembled that of a fat globule. In an attempt to corroborate this, a lipid solvent, chloroform-methanol, was used to extract sonicated cells. The extract (CM Antigen) was found to be serologically active and to give a reaction of identity with the buffered salts Antigen. In addition, the buoyance of the buffered salts Antigen on the surface of a four per cent sucrose gradient, even after 12 hours at 139,000 x g., indicated a very low density such as would be expected from a lipid. Larrabee et al. (1965) obtained a similar result with a lipopolysaccharide antigen isolated from Pasteurella pestis and serum lipoproteins also behave in the same way.

Therefore, as a result of the immunodiffusion reactions of identity, the similar negative charge, and the presence of polysaccharide, it is suggested that the hapten present in the three antigenic fractions is identical.

Only one protein band was revealed by disc electro-

phoresis of the buffered salts Antigen, indicating that the sample was at least 90 per cent pure with respect to protein. One per cent protein and ten per cent nucleic acid were shown to be present by the Lowry and Warburg-Christian determinations.

It was assumed that this protein band was a part of the antigen. However in a subsequent molecular study using Sephadex gel filtration, the antigenic activity was present in eluates from tubes 40-60 (Fig. 5), but 280 mμ absorbing material was not detectable. On the other hand, eluates from tubes 110-120 contained a 280 mμ absorbing peak, but no antigenic activity, and this was the area of the greatest protein elution peak obtained during the purification procedure. Andrews (1964) had previously shown that proteins are eluted from Sephadex columns of this type in approximately the same elution volume. In view of this, it appears likely that the protein band detected by disc electrophoresis was simply extraneous protein which had become bound to the antigen in some manner and was carried over during the purification. Although the antigen may contain a protein deficient in amino acids with conjugated double bonds so that it would not absorb at 280 mμ, the Lowry test showed only one per cent protein, and this test measures peptide bonds as well. This may also explain the high nucleic acid content of the sample before the molecular weight study, as these compounds also absorb at 280 mμ.

Additional evidence of the non-protein nature was provided by the action of the myxobacter AL-1 proteolytic enzyme of the buffered salts Antigen. Ensign and Wolfe (1965) have demonstrated that the enzyme hydrolyzes peptide bonds in both proteins and bacterial cell walls, and that about one-third of the peptide bonds of such proteins as bovine serum albumin are attached. Since the enzyme did not affect the serological activity of the antigen it seems reasonable to assume that the protein was not part of the antigen, or at least of its determinant group.

It appears that the antigen is a heat stable polysaccharide, or lipopolysaccharide, and that protein is not part of the complex. If the antigen is lipopolysaccharide, it is likely not a cell wall component, as it is known that the cell walls of Bacillus species contain little or no lipid (Salton, 1964). As membranes are rich in lipid and also contain nucleic acid, the antigen may be associated with the membrane. Baillie (1967) detected many heat stable polysaccharide antigens in B. cereus which were not associated with the cell walls.

This antigen is probably different from the polysaccharide antigen extracted from Bacillus M by Geux-Holzer and Tomcsik (1956) as their antigen contained glucosamine and galactosamine, and peptide bonds would be involved. As has been noted above, the antigenic activity of the buffered salts Antigen was not associated with

peptide bonds. The antigen is also probably different from the polysaccharide of Chorpenning and Dodd (1966) as their antigen was denatured by boiling.

The molecular weight determination by gel filtration showed that the buffered salts Antigen was eluted from the Sephadex G-150 column approximately 15 ml behind the void volume. This indicates a molecular weight of about 150,000, the fractionation range of the gel. However, the small degree of retention in the area of the void volume is not critical as the calibration curve (Fig. 2) is not linear in this area (Andrews, 1964). It therefore seems more likely that the molecular weight is greater than 150,000. From recent studies it is known that the teichoic acid of Hawkenes (1962), the polysaccharide of Engers (1966), and the lipopolysaccharide of Larrabee (1965) behaved in a similar manner on Sephadex G-100 and G-200 columns.

The procedure for the purification of the buffered salts Antigen is fairly long and tedious. It could be simplified by extraction with hot HCl, treatment with chloroform-methanol, followed by column chromatography. However, it would be difficult to obtain appreciable amounts of antigen by this method and the drastic hydrolysis treatment may alter the structure (Lancefield, 1933). A less cumbersome and milder method would be the preliminary fractional precipitation of the sonicated cells by ammonium sulfate followed by column chromatography. This would

reduce the initial large volume of the buffered sonic extract and enable the worker to use larger masses of cells to obtain a greater yield of antigen. It would be of interest to compare the effect of harsh and mild methods on the chemical composition of the antigen after purification.

In the studies reported here, at least two common precipitinogens were shared by Bacillus M and B. cereus. As 8-11 different antigens were detected upon DEAE-cellulose chromatography of the buffered sonic extract, it should be possible to separate the antigens responsible for the cross reactions. Purification and characterization of these antigens, and their detection in other bacteria might be of taxonomic value. It is surprising that common precipitinogens were not detected in B. megaterium, as Bacillus M is considered to be a variant of this species. Tomcsik (1951) had originally considered Bacillus M to be an induced mutatant of B. anthracis. Since Bacillus M is known to be morphologically similar to B. cereus and since some workers consider B. cereus to be a strain of B. anthracis, perhaps Bacillus M should be reclassified as a variant of either B. cereus or B. anthracis, rather than B. megaterium. These results are in accord with those of Engers (1966) who also failed to detect cross reacting precipitinogens in Bacillus M and B. megaterium.

Additional work is needed to establish the precise chemical nature of the buffered salts Antigen, and its

relationship to the vegetative cell. If lipid and nucleic acid can unequivocally be confirmed to be an integral part of the antigenic complex, this would be a pertinent finding as antigens containing these constituents have not been reported in Bacillus spp.

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