ISOLATION AND PARTIAL CHARACTERIZATION OF AN ANTIGEN OF *CORYNEBACTERIUM XEROSIS*

by

Jae Key Oh

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ABSTRACT

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A serologically active substance, extracted from sonicated cells of Corynebacterium xerosis ATCC 7094 with hot HCl, yielded one precipitin line by immunodiffusion against a homologous antiserum, but six protein bands by disc electrophoresis. Extracts of other species of corynebacteria failed to cross-react with the antiserum. The crude acid extract of C. xerosis was purified by DEAE-cellulose column chromatography, chemical and enzymatic treatments, Bio-Gel HTP adsorption, and gel filtration on a Sephadex G-200 column, and rechromatography on Sephadex G-200 column. The homogeneity and purity of the antigen for serological activity, protein, and carbohydrate was established by immunoelectrophoresis and disc electrophoresis.

The partially purified antigen from the chemical and enzymatic treatments was shown to be stable by the various chemicals. Biochemical analysis of the purified antigen showed that protein, carbohydrate and nucleic acid were present in the ratio of 31:1:1 respectively. The major amino acids of the antigen were alanine, glutamic acid, glycine, aspartic acid and arginine. The sugar of the antigen was identified as arabinose by paper chromatography. The molecular weight of the antigen, estimated by gel filtration method, was 145,000.

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INTRODUCTION

INTRODUCTION

In recent years the fractionation and analytical methods developed for the study of biologically active enzyme systems have been applied to the analysis of bacterial antigens.

Osmotic or enzymatic lysis, freeze-thawing, shaking, high-pressure and ultrasonic vibration have been useful methods for the extraction of antigens from whole cells. Gel immunodiffusion and immunoelectrophoretic analysis have provided techniques for the demonstration and identification of specific antigens. Ion exchange chromatography and gel filtration, which made it possible to separate biological macromolecules from complex mixtures without altering their integrity, have been widely used for enzyme systems but only recently applied to the study of bacterial antigens. Relatively few bacterial antigens have been isolated and characterized by biochemical analysis and their immunochemical specificity. This kind of study can serve as an additional criterion for bacterial classification.

Few reports are available on the serologically active substances isolated from disrupted whole cells of Gram-positive bacteria except for the group A streptococci, two species of staphylococci and some strains of lactobacilli. Little is known about antigens of the corynebacteria. A non-specific or group antigen has been found in Corynebacterium diphtheriae and various diphtheroids, which had failed

to show species differences by agglutination tests but revealed common antigenic components situated more deeply in the cells by complement-fixation tests(Bailey, 1925; Neill et al., 1931; Holdworth, 1952). An group specific polysaccharide and a type specific protein of C. diphtheriae was demonstrated by Wong and Tung(1938, 1939) and Cummins(1954) but immunochemical studies of these antigens have not been reported.

Recently, two serologically active substances of Corynebacterium hofmannii have been characterized (Banach and Hawirko, 1966). In the present study, an isolation, purification and partial characterization of an antigen of Corynebacterium xerosis is described.

HISTORICAL

HISTORICAL

Many antigens derived from Gram-positive bacteria which have been identified as proteins, polysaccharides, teichoic acids, or complex polymers are components of known cell wall structures and confer type, species, or group specificity (McCarty, and Moore, 1964). Recently immunochemical studies on antigens of a few Gram-positive bacteria have intensively been carried out.

Three serologically active substances isolated from hot acid extracts of whole cells of Streptococcus haemolyticus (Lancefield, 1928) were designated a group-specific carbohydrate(C), a type-specific protein(M), and a non-type-specific protein(P). The group-specific carbohydrate C substance and type-specific M protein have been identified as cell wall components (McCarty, 1952; Salton, 1953). A toxic cellular component of group A streptococci (Schwab et al., 1959) appeared to be a complex of polysaccharide and protein, the polysaccharide having the serological characteristics of the group-specific C substance. The group-specific carbohydrate C substance of the group A and A-variant streptococci are multibranched structures with a small amount glucosamine in the backbone and the basic structure of the rhamnose polysaccharide appeared to be the same in the two carbohydrates, but differ principally in the presence or absence of terminal N-acetyl-glucosaminide residues on the oligosaccharide side chains (McCarty and Lancefield, 1955; McCarty, 1956). McCarty (1958) found that the terminal N-acetyl glucosamine residues were essential for the precipitation reaction of the antigenic determinant of the

group A carbohydrate. More direct evidence was shown by the synthetic diazo antigens of p-aminophenyl-β-N-acetylglucosaminide coupled to various proteins cross-reacting with group A streptococcal antisera. More recently, McCarty (1964) also found that the ester-linked D-alanine was an important determinant in the specificity of group A streptococci.

The group D antigen of streptococci has been identified as a teichoic acid with a glycerophosphate backbone with glycosidically linked sugars and esterified D-alanine (Wicken and Baddiley, 1963). The major portion of this antigen appeared to be closely associated with the protoplast membrane of streptococci (Shockman and Slade, 1964). Separation of the antigen has been achieved by gel filtration using 0.15 M NaCl solvent and elution on a Sephadex G-200 column (Rasan, 1966).

A non-species-specific polysaccharide (NSS) was demonstrated by hemosensitization tests of culture filtrates of hemolytic streptococci (Harris and Harris, 1953). Rantz et al. (1956) also showed cross reactivity of culture filtrates of group A, C and G streptococci, enterococci, Staphylococcus aureus, pneumococci and Bacillus spp. by hemosensitization tests with normal human serum. The heterogenic antigen was shown to be heat stable, non-dialyzable and completely absent from Gram-negative bacteria. More recently, Chorpenning and Dodd (1966) obtained soluble antigens from species of Gram-positive bacteria which were serologically active with immune sera as well as normal sera. One of these heterogenic antigens seemed to be similar to the Rantz's streptococcal NSS.

A type-specific carbohydrate antigen (Juliannelle and Wieghard, 1934) and a group-specific polysaccharide antigen from avirulent staphylococci (Weighard and Julianelle, 1935) were isolated from staphylococcal whole cells by extraction with HCl. Verway (1940) used a milder acid treatment to demonstrate a type-specific protein antigen as well as the type-specific carbohydrate of type A staphylococci. A serologically active ribitol phosphate polymer from Staphylococcus aureus H was isolated by Armstrong et al. (1958) which contained a ratio of ribitol phosphate:acetylglucosamine:D-alanine of 1:0.99:0.49. Sanderson et al. (1961) showed that the determinant group was an α-N-acetylglucosaminyl ribitol of the ribitol phosphate polymer. Haukenes (1962) isolated a serologically active polysaccharide A substance from disrupted S. aureus cells. The antigen was purified by diethylaminoethyl(DEAE)-cellulose and Dowex-1 ion exchange chromatography and was found to be composed of a muco-peptide and a ribitol teicoic acid. Hisatsune et al. (1967) has described a serologically active cell wall peptide polymer of S. aureus.

Spores and vegetative cells of Bacillus spp. contain serologically active substances of taxonomic value (Lamanna, 1942). Davies (1951) found a species-specific antigen in spores of Bacillus polymyxa. A polypeptide and a polysaccharide antigen has been isolated from cells of Bacillus "M", a strain of Bacillus megaterium (Geux-Holzer and Tomsik, 1956). The polypeptide antigen proved to be identical to the poly-D-glutamic acid polymer of Bacillus anthracis.

The antigenic analysis of Corynebacterium diphtheriae using agglutination techniques showed the species could be divided into various serotypes (Havens, 1920; Smith, 1923; Eagleton and Baxter, 1923). Later, Bailey (1925) concluded that agglutination tests were not suited for revealing species differences between C. diphtheriae and other corynebacteria. However, the subspecies of C. diphtheriae; gravis, intermedius and mitis were shown by agglutination reaction to be antigenically diverse (Ewing, 1933). A common antigen which fixed complement in the presence of heterogeneous antisera was found in alcohol extracts of C. diphtheriae, certain diphtheroids, and the tubercle bacilli (Krah and Witebsky, 1930).

A group-specific polysaccharide antigen of C. diphtheriae was demonstrated by precipitation and absorption tests (Wong and Tung, 1938). A year later, these workers showed that two polysaccharides, designated A and B, were present (Wong and Tung, 1939). There have been no subsequent studies on these group reactive carbohydrate antigens of C. diphtheriae.

Hoyle (1942) obtained species-specific antigens from alcoholic extracts of C. diphtheriae type mitis and C. hofmannii and a group-specific antigen common to the gravis, intermedius, and mitis strains of C. diphtheriae as well as to C. hofmannii. A thermostable group-specific polysaccharide and a thermolabile species-specific protein antigen of C. diphtheriae has been reported by Oeding (1950).

Hayes (1951) was the first to demonstrate homosensitizing

antigens of C. diphtheriae. These antigens also were found in saline extracts of various corynebacteria (Casille and Toucas, 1960).

The first separtaion of a group-specific polysaccharide compound from C. diphtheriae by modern methods was achieved by Holdworth (1952). The carbohydrate was isolated from the purified cell wall preparation and contained D-galactose, D-mannose, and D-arabinose in a molar ratio 2:1:3. Since the oligosaccharide had no inherent reducing power, he deduced that all terminal units were non-reducing galactose groups.

Cummins (1954) isolated two antigens from the cell walls of C. diphtheriae. Heat treated cell walls of intermedius and gravis subspecies agglutinated with the antiserum prepared against the mitis strain. Since the cross reaction did not occur with whole cells the antigen appeared to be situated deep with cell wall. The heatlabile type-specific protein antigen appeared to be present as a superficial layer. Cell wall suspensions of C. xerosis, C. renale, and C. hofmannii either did not agglutinate with antiserum to C. diphtheriae or did so at much lower titers than those given by the homologous cell wall suspensions.

The chemical composition and antigenic structure of the cell walls of corynebacteria and related organisms have been examined by Cummins and his collaborators (1955, 1956, 1958, and 1962) using agglutination tests with cell wall suspensions. A common antigenic component was identified in strains of corynebacteria which contained

arabinose and galactose as their principle cell wall sugars, and alanine, glutamic acid, and diaminopimelic acid as cell wall amino acids. This result suggested that these strains belong together in the same taxonomic group. The only exceptions were C. pyogenes and C. haemolyticum which did not contain arabinose and galactose as basic cell wall sugars and Cummins suggested that these species should be reclassified.

The techniques of immunodiffusion and immunoelectrophoresis have been used to study the identity, purity, specificity, and chemical structures of antigens. The classical serological methods of agglutination, precipitation and complement-fixation are useful for surveys and preliminary study, but cannot be applied directly to the examination of complex mixtures of bacterial antigens. The gel diffusion method originally described by Bechhold (1905), was applied to immunological analysis by Oudin (1946). The simple diffusion method proved to be applicable to quantitative as well as qualitative analysis. A double diffusion technique which showed a high resolving power was developed independently by Ouchterlony (1949) and Elek (1949), and has been particularly useful for comparative work on complex antigen-antibody systems and has largely replaced the Oudin method. The Ouchterlony technique has been employed for analysis of the polysaccharide antigen of Staphylococcus aureus (Haukenes, 1962) and Nocardia brasilienses (Estrada-Parra et al., 1965). A immunodiffusion analysis on extracts of whole cells of Staphylococcus aureus (Oeding and Haukenes, 1963) and extracts of cell walls of

corynebacteria, mycobacteria and nocardia (Cummins, 1965) showed the presence of multiple antigenic specificities.

Immunoelectrophoresis which combined the separation by electrophoresis and followed by gel diffusion was first described by Grabar and William (1953). A micro-technique by Scheidegger (1955) which was carried out on microscope slides simplified the procedure, shortened the time, and minimized the amount of antigen and antiserum required. Grabar (1960) used the micro-technique for the identification of bacterial antigens by immunoelectrophoretic analysis. Recently, immunoelectrophoretic analysis has been employed on ultrasonic extracts of young vegetative cells of Bacillus cereus (Baillie and Norris, 1964) and B. megaterium (Mastroeni et al., 1967) to show the presence of a large number of antigenic constituents.

In a recent study on the isolation and characterization of two group-specific antigens from C. hofmannii Banach and Hawirko (1966) obtained two peptide polymers designated antigen A and antigen B. The antigens were extracted from ultrasonic disintegrated cells with hot acid, purified by DEAE-cellulose column chromatography and separated by rechromatography on a Sephadex G-200 column. Biochemical analysis showed that the two antigens were composed of a major protein fraction with carbohydrate and a small amount of nucleic acid. The sugars of antigen A were identified as arabinose and glucose. No cross-reactivity was shown with other species of corynebacteria.

It is apparent that bacterial cells contain a large number

of antigenic substances and it is now possible to isolate and purify them by mild extractions and column chromatography on ion exchange resins and gel filtrations.

MATERIALS AND METHODS

MATERIALS AND METHODS

MEDIA

Trypticase soy broth (TSB, Baltimore Biological Laboratories) was used throughout these studies and for maintaining stock cultures.

CULTURES

The strains used were Corynebacterium xerosis ATCC 7094, Corynebacterium hofmannii ATCC 10700 and laboratory strains of Corynebacterium pyogenes, Corynebacterium equi and Corynebacterium diphtheriae type mitis and were transferred weekly in TSB.

The cell mass of C. xerosis was prepared from growth obtained from 12 liters of TSB incubated on a rotary shaker at 37 C for 18 hours. The cells were harvested by using a Sharples centrifuge, washed twice in saline, once in distilled water and stored at -15 C. Twelve liters of broth cultures were yielded approximately 60 g wet weight of cells.

PREPARATION OF ANTISERA

The vaccine of C. xerosis was prepared from cells of grown at 37 C for 18 hours in TSB, washed twice in saline and suspended in 0.2% formal saline to a density corresponding to a McFarland scale No. 3 in the Bechman Model C colorimeter at 38% light transmission using Klett tubes and red filter. The cell suspension was dialysed against 0.2% formal saline at 5 C for 48 hours and held at 5 C for a week. Sterility tests were carried out and the vaccine was stored

at 5 C.

Adult albino rabbits were used to produce antisera by the procedure of Kabat and Mayer (1961) except that a total of 41.5 ml of antigen was injected over a period of eight weeks. The immune serum was collected one week after the last injection and tested by the standard tube agglutination. The reciprocal agglutination titers of the sera were 512 to 1024. The antisera were stored at -15 C.

IMMUNODIFFUSION TESTS

A modification of Ouchterlony's plate technique (1949) was applied.

2% Noble agar (DIFCO) was coated with a thin film over the bottom of a petri dishes (inner diameter of 9 cm) and then layered with 20 ml of 0.8% Noble agar containing 1% sodium azide and 0.88% NaCl. A horizontal trough of 0.2× 10 cm was cut out in the agar gel plate and filled with undiluted antiserum. The wells were punched out at a distance of 1 cm on each side of the antiserum trough with a number one cork borer and filled with 0.03 ml of test antigens which had been dialysed against 0.02 M phosphate-buffered saline (PBS), pH 7.4. The plates were kept in a moist chamber at room temperature and observed daily for precipitin lines over a period of one week.

These technique were applied to all stages of the preparation of the test antigen and to a cross reactivity between the acid extracts of the other species and the antiserum of C. xerosis.

EXTRACTION OF ANTIGENS

The procedure was that of a modification of the Banach and Hawirko (1966).

Fifty g wet cell mass was suspended in 25 ml of 0.02 M phosphate buffer, pH 7.4 and disintegrated in a Raytheon ultrasonic oscillator at 10 KC/sec. for one hour. The crushed cell suspension was treated with four vol of 0.0625 N HCl (300 ml), held in a boiling water bath for 10 min, cooled rapidly on ice, adjusted to pH 7.4, and centrifuged at 27,000 x g for 20 min. The clear, supernatant was concentrated to be 1/20 of the original volume and dialysed against 0.02 M PBS, pH 7.4 at 5 C for six hours. The crude acid extract was stored at -15 C. A similar extraction procedure was followed using one g wet weight of cell mass of C. hofmannii, C. pyogenes, C. equi and C. diphtheriae type mitis. These extracts were tested for serological activity by immunodiffusion.

PURIFICATION OF ANTIGENS

a) DEAE-Cellulose Column Chromatography

The preparation of Diethylaminoethyl (DEAE)-cellulose (SIGMA) was that described by Peterson and Sober (1956). DEAE-cellulose (medium mesh) was washed with distilled water, hydrolysed once in 0.5 N NaOH for hours and washed with additional 0.5 N NaOH until no more color was removed. The hydrolysed material was further washed out in 20 vol distilled water until the pH was become neutral and equilibrated to

0.02 M phosphate buffer solution, pH 7.4 for 12 hours. The column (3.0 x 75 cm) was packed by pouring the prepared slurry and equilibrated by passing through two liters of 0.02 M phosphate buffer, pH 7.4 with a pressure of about 100 cm water.

The crude acid extract was subjected to ion-exchange chromatography using DEAE-cellulose as described by Banach and Hawirko (1966). Sixty ml of the sample from approximately 200 g of the wet cell mass was absorbed onto the DEAE-cellulose column and eluted using a continuous linear gradient of 0.0 to 1.0 M KCl in 0.02 M phosphate buffer, pH 7.4. The flow rate was regulated to about 20 ml per hour and 100 fractions of 20 ml were collected at 5 C. Each fraction was tested for precipitin lines by immunodiffusion; serologically active fractions were pooled and stored at -15 C.

b) Chemical and Enzymatic Fractionations of Antigen

The serologically active fractions from DEAE-cellulose chromatography were adjusted to pH 12, hydrolysed for 30 min and acidified to pH 4.7. The precipitate was collected by centrifugation at 2,000 x g for 20 min, dissolved in 50 ml of 0.02 M phosphate buffer, pH 7.4 and dialysed against 0.02 M PBS, pH 7.4 overnight.

The solution was treated with ribonuclease (50 ug/ml) at 37 C for 30 min, then with 3 ml of 2% protamine sulfate and 12 ml of 5% streptomycin at 5 C for 10 min. The supernatant was collected by centrifugation at 10,000 x g for 20 min and dialysed against five

liters of 0.02 M PBS, pH 7.4 for 16 hours.

Differential precipitation with ammonium sulfate (0.35-0.8 saturation) was carried out and the precipitate was redissolved in 30 ml of 0.01 M phosphate buffer solution and redialysed against 0.01 M PBS, pH 7.4 overnight.

The partially purified antigen was tested for chemical stability through over a period of one month. Three oxidizing agents (0.05% of potassium iodate, potassium ferricyanide and potassium periodate), two reducing agents (0.05% of cysteine and sodium glutathione), and four dissociating agents (6M urea, 0.05% sodium lauryl sulfate, 5% Dioxane, 10% Dioxane, and 0.05% EDTA) were used as selected chemicals. After treatment with chemicals, the pH range of the all systems was 6.5 to 7.9.

c) Gel Adsorption

The dehydrated material, Bio-Gel HTP (BIO-RAD) was suspended in 0.01 M phosphate buffer, pH 7.4. The column (2.5 x 20 cm) was prepared by pouring the material as a slurry and equilibrated it with the initial eluting buffer, 0.01 M phosphate buffer, pH 7.4, by passing 500 ml of the same buffer through the bed over a period of 24 hours.

A load of 5 mg of protein from antigen solution per ml bed vol was adsorbed onto the column and eluted with 150 ml of 0.03 M phosphate buffer, pH 7.4. Antigen was concentrated by centrifugation using

Sephadex G-25, suspended in 20 ml of 0.02 M phosphate buffer, pH 7.4 and collected by siphoning out (Flodin et al., 1960).

d) Gel Filtration on Sephadex G-200

The dry beads of Sephadex G-200 (40-120 u) (PHARMACIA) were hydrated in a solution of 0.02 M PBS, pH 7.4, containing 0.02% sodium azide and 0.002 M EDTA, and placed at 56 C overnight. The slurry was allowed to swell for two weeks at 5 C, then filtered through Whatman No. 1 paper and decanted to remove the smallest particles. Then the gel together with the excess of the buffer was placed in a vaccum flask and aspirated until bubbling ceased.

A chromatographic column (2.5 x 100 cm) (PHARMACIA) was filled with the same buffer, and a wide stem funnel was attached to the top using a rubber stopper. The hydrated gel was added and stirred constantly to settle the gel through the stem of the funnel into the column. A flow adaptor (PHARMACIA) was fitted and the column was equilibrated for several days with the buffer solution by upward flow elution (Rothstein, 1965). To regulate the operating pressure, the outlet tubing of the column was positioned at about 10 cm below the liquid level in the reservior, containing elution buffer solution.

The sample solution containing approximately 80 mg protein in 10 ml of the buffer was mixed with 10% sucrose, layed between the gel bed and the buffer head, and eluted with the buffer at a flow rate of approximately 9 ml per hour. Column effluents were collected

in 3 ml fractions and the optical density of each fraction was determined at 280 mμ in a Beckman DU spectrophotometer. All experiment were carried out at 5 C.

The fractions from each of the two absorption peaks were pooled, freeze-dried under vacuum, suspended in 10 ml of 0.02 M PBS, pH 7.4 and dialysed against the same buffer. The one peak was serologically active and was rechromatographed on Sephadex G-200, dialysed against six liters distilled water for 48 hours, freeze-dried, and stored in a dessicator at room temperature. This purified antigen was subjected to immunochemical analysis.

IMMUNOELECTROPHORESIS

Immunoelectrophoresis was carried out on microscope slides according to the method of Scheidigger (1955).

The supporting slides were coated with a thin film of 1.5% Agarose (NBC) which was dissolved in 0.05 M barbital buffer, pH 8.4 and then layed with 2.0 ml of agar. A trough of 0.2 × 6 cm was cut out with two surgical blades bound together and two starting wells were punched out at a distance of 0.3 cm on each side of the through with a No. 18 hypodermic needle. The slides were connected to the electrode vessels which contained 0.05 M barbital buffer of pH 8.4 in buffer tank by means of agarose wicks.

Five samples of purified antigen were filled in each of the wells with a No. 26 hypodermic needle. The agarose wicks were dampen-

ed with the buffer and the Shandon electrophoretic tank was connected to a Beckman Duostat D. C. power supply. Electrophoresis was carried out for 60 min at 10 volts/cm. After the run, undiluted antisera were placed in the horizontal trough and the reaction was allowed to develop at room temperature for 24 hours in moist chamber.

The slides were washed in 1% NaCl solution, in distilled water, dried, stained with saturated amino black solution for a few min, and destained with 7.5% acetic acid until clear (Parker et al., 1962).

POLYACRYLAMIDE GEL ELECTROPHORESIS

The method was that described by Davis (1964) using the Canalco Disc Electrophoresis Trial Kit (CIC).

Samples of antigen containing 100 µg dry weight were mixed with equal amounts of upper-gel in a total volume of 0.20 ml. Electrophoresis was carried out in Tris-glycine buffer, pH 9.5, for 60 to 90 min at 4 m. a. per gel column using the Canalco Model 150 V. power supply. After completion of the run, the gel columns (0.5 × 6.0 cm) were removed, immediately stained for 90 min with 0.0025% coomarssie brilliant green for protein and destained with 10% trichloroacetic acid. A modified periodic acid-Schiff (PAS) reaction was used for staining polysaccharide (Stewart-Tull, 1965). The gel predried on the glass plate was covered with a 1% sodium periodate solution, replaced with distilled water, and the sulfur dioxide gas was passed

into the water until the brown color had disappeared. The gel was stained with 1% pararosaniline hydrochloride which had been decolorized by passing SO_2 gas through the solution for 30 min, washed in SO_2 water and re-dried quickly at 80 C. The gel was a light purple and the carbohydrate band was dark pink.

BIOCHEMICAL ANALYSIS

a) PROTEIN

The procedure of Kunkel and Ward (1950) as outlined in Kwapinski (1965) was followed with gamma globulin (NBC) as the standard and read at 570 mμ.

b) CARBOHYDRATE

A modification of the Anthrone method of Scott and Melvin (1953) was used with dextrose as the standard. The sample was kept at 100 C for 15 min and read at 660 mμ.

c) PENTOSE

The procedure of Winzler (1955) as outlined in Kabat and Mayer (1961) was followed with arabinose as the standard and read at 670 mμ.

d) HEXOSAMINE

A modification of the procedure of Rondel and Morgan (1954) as outlined in Kabat and Mayer (1961) was followed with glucosamine HCl as the standard. Samples were hydrolysed in 2 N HCl at 100 C

for two hours and read at 540 mμ.

e) NUCLEIC ACID

The procedure of Warburg and Christian (1942), using DU Beckmam spectrophotometer to measure the ratio of optical density at 280 mμ and 260 mμ was followed for the nucleic acid determination.

f) DEOXYRIBONUCLEIC ACID

A modification of the procedure of Burton (1956) as outlined in Kwapinski (1965) was followed with thymus DNA as standard. The samples were extracted with 5% trichloroacetic acid and the results were read at 600 mμ.

g) PHOSPHOROUS

The procedure of Fiske and Subbarow (1926) as outlined in Kabat and Mayer (1961) was followed with KH_2PO_4 as the standard and read at 660 mμ.

h) NITROGEN

Nitrogen was determined by micro-kieldahl method as outlined in Kabat and Mayer (1961).

f) AMINO ACIDS

A Technicon Auto Analyser was used for determination of amino acids. The samples were hydrolysed in 6 N HCl for 18 hours, dried in a desiccator overnight, dissolved in N/10 HCl, and analysed.

j) PAPER CHROMATOGRAPHY

Reducing sugars and hexosamine were identified by a descending chromatography on Whatman No. 1 paper (46 x 75 cm) using phenol water (80:20 by volume) as the solvent. 0.9 mg samples were hydrolyzed in 0.3 ml of 2 N H_2SO_4 at 100 C for 2 hours, neutralized with $Ba(OH)_2$, centrifuged to remove barium sulfate, and evaporated to dryness. The hydrolysed samples were dissolved in water and applied to the paper. Each standard sugar was spotted separately and a mixture of all the sugars was also spotted. Elution was carried out for 24 hours, the paper was dried, and reducing sugars were identified by spraying with aniline hydrogen phthalate reagent (Partridge, 1949). For the identification of hexosamine, dried paper was dipped quickly in silver nitrate solution and dried in fume chamber.

MOLECULAR WEIGHT DETERMINATION BY GEL FILTRATION

A modification of the Sephadex-gel-filtration method of Andrews (1964, 1965) was used for the estimation of molecular weight of the antigen. The method followed was the same procedure as described in previous gel filtration on Sephadex G-200. 0.02% Blue Dextran 2000 (PHARMACIA), which was excluded from the gel interior, was employed to determine the void volume (Vo), the volume exterior to the gel phase.

Standards were ovalbumin (OA) (SIGMA CHEM. CO., 2 x crystallized) mol wt 45,000*; bovine serum albumin (BSA) (NBC, crystallized) mol wt

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* The mol wt values estimated by ultracentrifugal method

67,000*; yeast alcohol dehydrogenese (YAD) (SIGMA CHEM. CO., 2 x crystallized) mol wt 126,000**; gamma globlin (GG) (NBC, human fraction II) mol wt 160,000; and ferritin (FN) (MRL, horse spleen, 2 x crystallized) mol wt 790,000*. Reference proteins were used without purification and experiments were done at 5 C. Five mg of each BSA and GG, 3 mg of OA and YAD, 2 mg of FN, and 5 mg of antigen were dissolved in 5 ml of the equilibration buffer and the solution was applied to the bottom of a column using a sample applicator. When a half of the sample solution had entered into the gel column, the column efluates were collected with a fraction collector in 3 ml fractions. The variation in the volume of eluate fractions was ± 3% (v/v) respectively.

The fractions were monitoried at 280 mμ and 230 mμ with Beckman DU spectrophotometer. The elution volume (V_e) of a given solute zone was taken in all cases as the volume of the concentration maximum in the zone. The V_e/V_o ratios were plotted against the log mol wt of the individual reference proteins to obtain a calibration curve. The antigen was fitted to the standard curve and the mol wt estimated.

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* The mol wt values estimated by ultracentrifugal method

** The mol wt values estimated by gel-filtration method

RESULTS

RESULTS

Immunodiffusion of the crude acid extract obtained from the crushed cells of C. xerosis yielded only one precipitin line with the homologous antiserum, but six protein bands by disc electrophoresis (Fig. 6 a). The precipitin line remained present after each stage of the purification process (Fig. 1). No precipitin line was obtained with crude acid extracts of C. hofmannii, C. pyogenes, C. equi and C. diphtheriae type mitis with the C. xerosis antiserum.

The serologically active fractions of the crude acid extract showed by immunodiffusion were eluted from DEAE-cellulose column at 0.3 M KCl concentration in tubes No. 42 to 47 (Fig. 2) and showed three protein bands by disc electrophoresis. The elutes precipitated at pH 4.7, treated with ribonuclease and salted out by ammonium sulfate precipitation remained serologically active. The next stage of purification by elution from the Bio-Gel HPT column with 0.03 M phosphate buffer, pH 7.4, showed two bands by disc electrophoresis. The final purification by Sephadex G-200 column chromatography (Figs. 3 and 4) gave an eluate which showed one protein band by disc electrophoresis (Fig. 6 b).

The serological active fraction from the second stage of purification was stable against the reducing agents (0.05% of cysteine and sodium glutathione) and the dissociating agents (6 M urea, 0.05% sodium lauryl sulfate, 5% Dioxane, 10% Dioxane and 0.05% EDTA) for a period of one month).

The yield from 200 g of wet cell mass was approximately 32 mg purified antigen. The antigen was relatively soluble in water and appeared as a pale brown colored powder in dry state.

Micro-immunoelectrophoresis of the antigen showed a single slow migrating arc of precipitation (Fig, 5). Disc electrophoresis of the crude acid extract yielded at least six protein bands, whereas the purified antigen revealed only one protein band at a distance of 10 mm behind the tracking band (Fig. 6 a and b). The periodic acid-Schiff (PAS) method for carbohydrate showed a dark band on the same position as the protein band, and a faint band at a distance of 4 mm behind the first band (Fig. 7 a and b).

The biochemical analysis of the antigen for total protein, carbohydrate, pentose, hexosamine, nucleic acid, deoxyribonucleic acid, phosphorous, and nitrogen is shown in Table 1.

Paper chromatography of sugars and hexosamines of the antigen showed that arabinose was present and possibly glucosamine.

The amino acid composition of the antigen obtained by the Technicon Automatic Amino Acid Analyser is shown in Table 2. The major amino acids were alanine and glutamic acid and relatively high levels of aspartic acid, glycine, leucine, and arginine.

The molecular weight of the antigen by gel filtration method was estimated to be 145,000(Figs. 8 and 9).

TABLE 1

BIOCHEMICAL ANALYSIS OF THE PURIFIED ANTIGEN

COMPONENTS	PERCENTAGE OF DRY WEIGHT
PROTEIN	82
CARBOHYDRATE	2.6
PENTOSE	0.8
HEXOSAMINE	0.5
NUCLEIC ACID	2.4
DEOXYRIBONUCLEIC ACID	0.14
PHOSPHOROUS	4.1
NITROGEN	9.94

TABLE 2

AMINO ACID ANALYSIS

AMINO ACIDS	RESIDUES/1000 RESIDUES
ALANINE	260
GLUTAMIC ACID	138
GLYCINE	104
ARGININE	93
ASPARTIC ACID	71
LEUCINE	61
VALINE	53
PROLINE	41
LYSINE	37
ISOLEUCINE	33
THREONINE	31
SERINE	31
PHENYLALANINE	16
ORNITHINE*	15
AMMONIA	17.7**

* The ornithine probably comes from breakdown of arginine during hydrolysis.

** μ moles nitrogen / 0.75 mg of antigen.

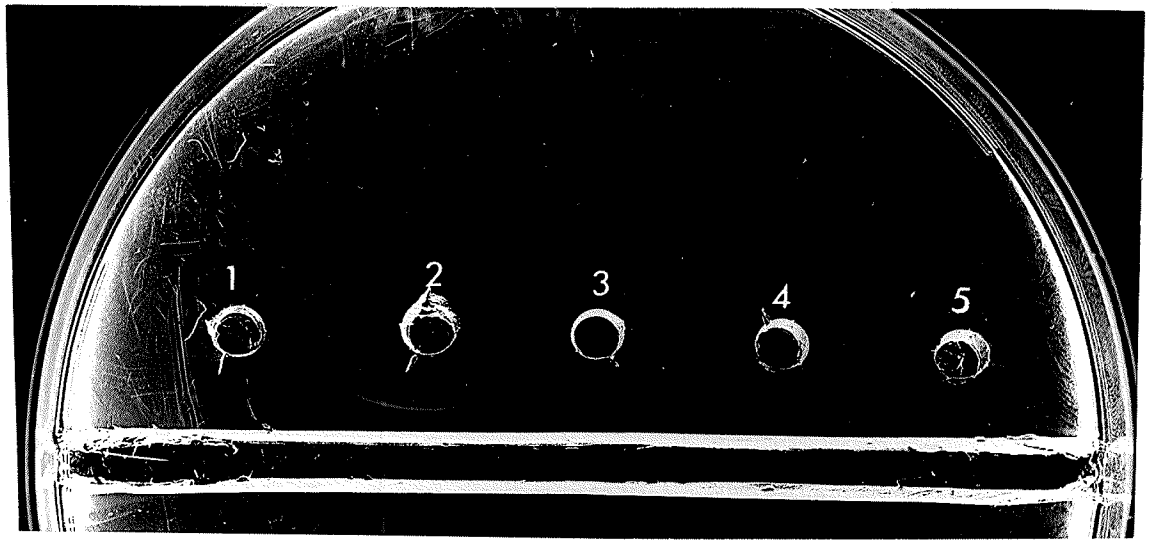

Figure 1. Immunodiffusion with C. xerosis Antiserum against Various Antigenic Fractions. Well 1, Control; Well 2, Buffered Sonic Extract; Well 3, Crude Acid Extract; Well 4, Purified Antigen.

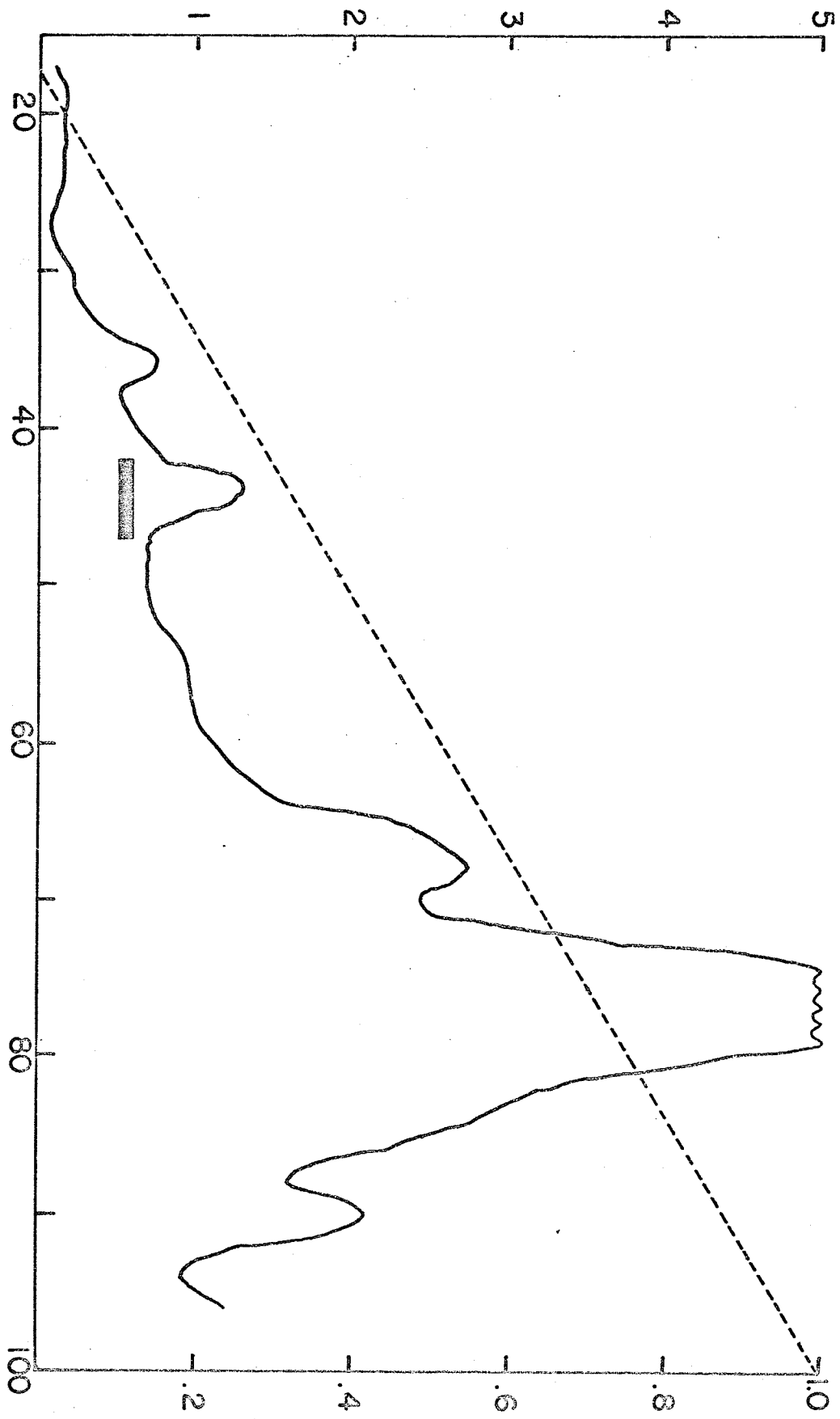

Figure 2.
Chromatography of Crude Acid Extract of C. xerosis on DEAE-cellulose Column.

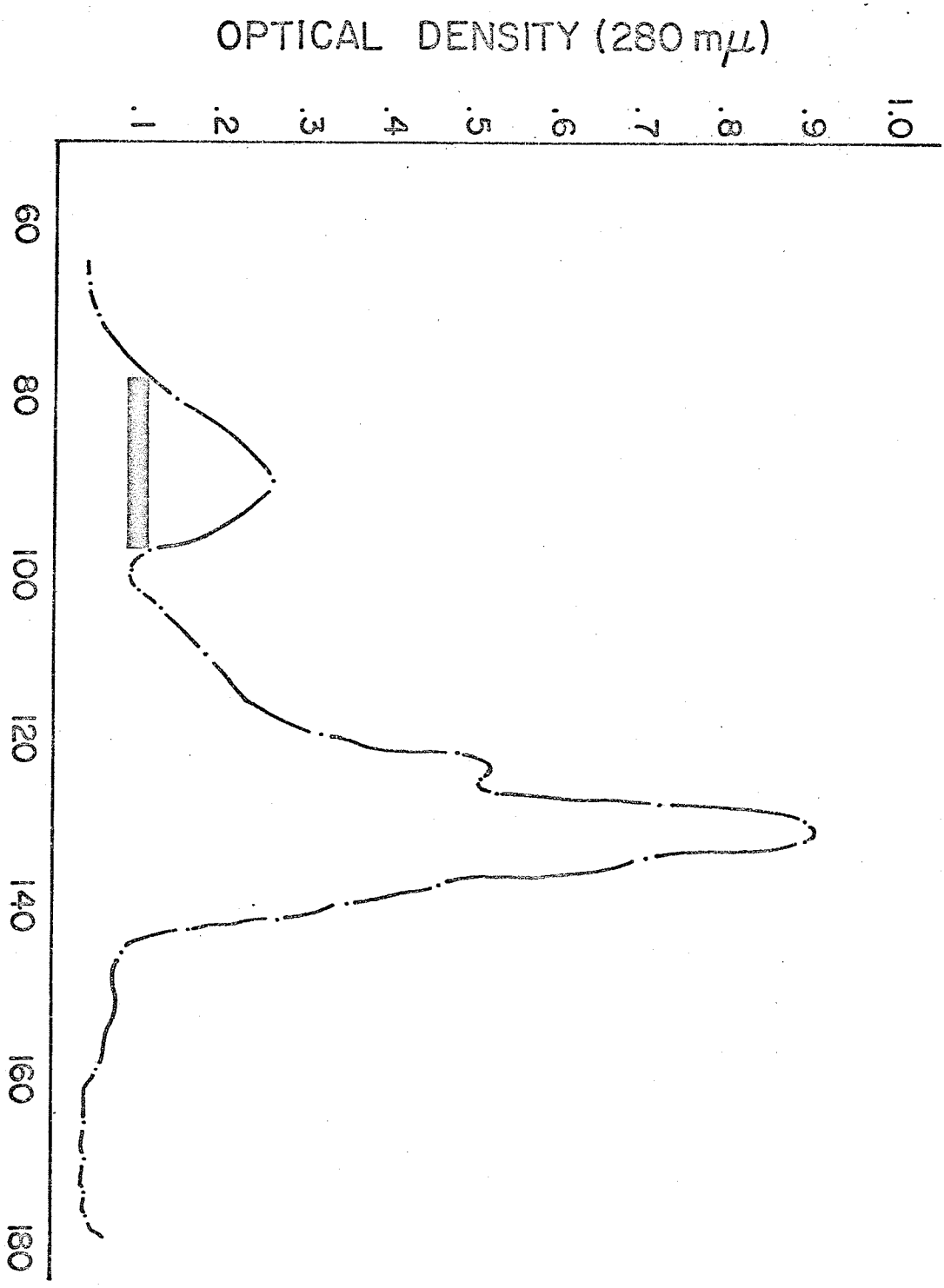

Figure 3. Chromatography of Partially Purified Antigen on Sephadex G-200 Column.
Antigenic Activity

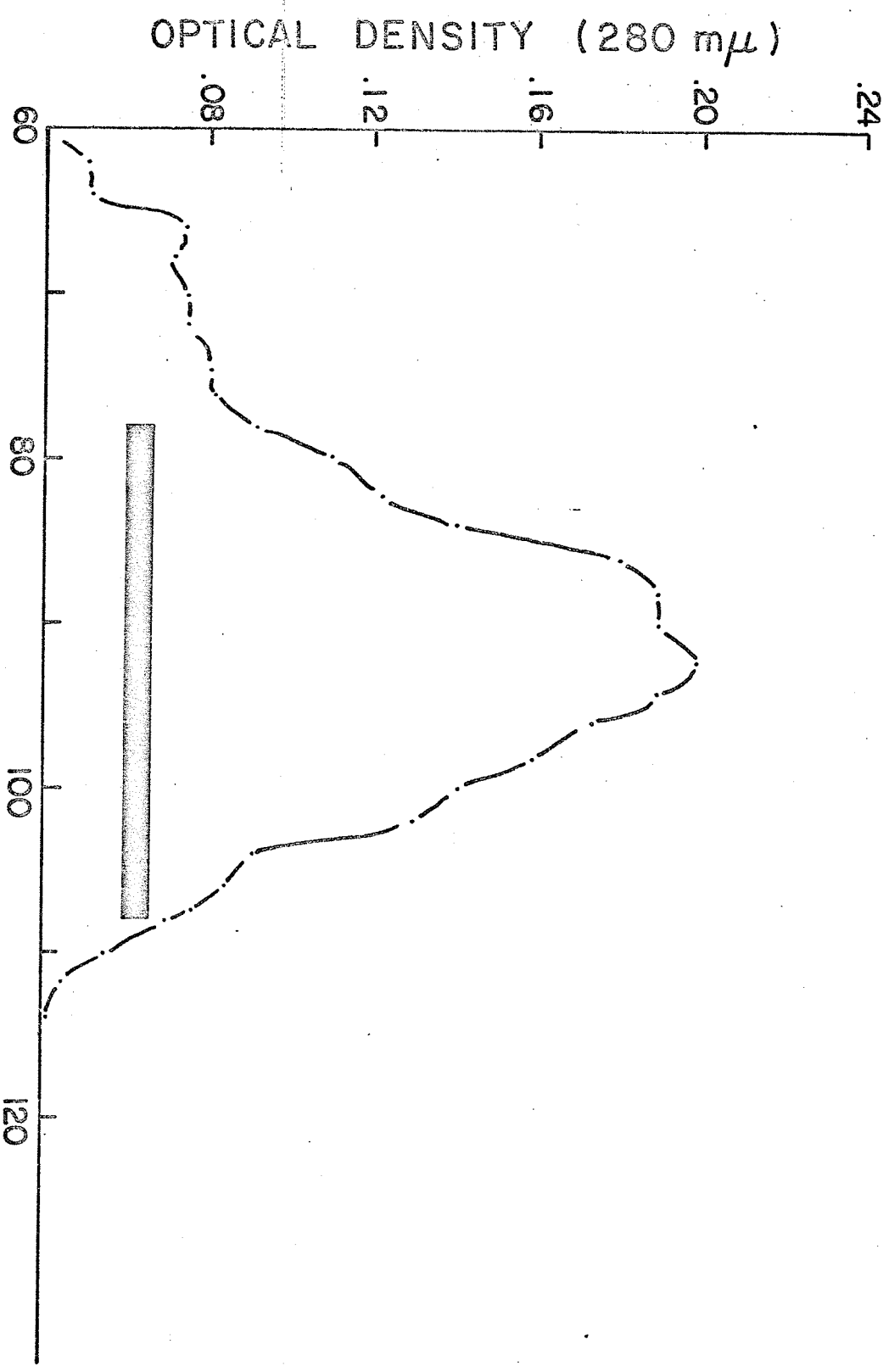

Figure 4. Rechromatography of the 1st Sephadex G-200 Peak on Sephadex G-200 Column.

Antigenic Activity

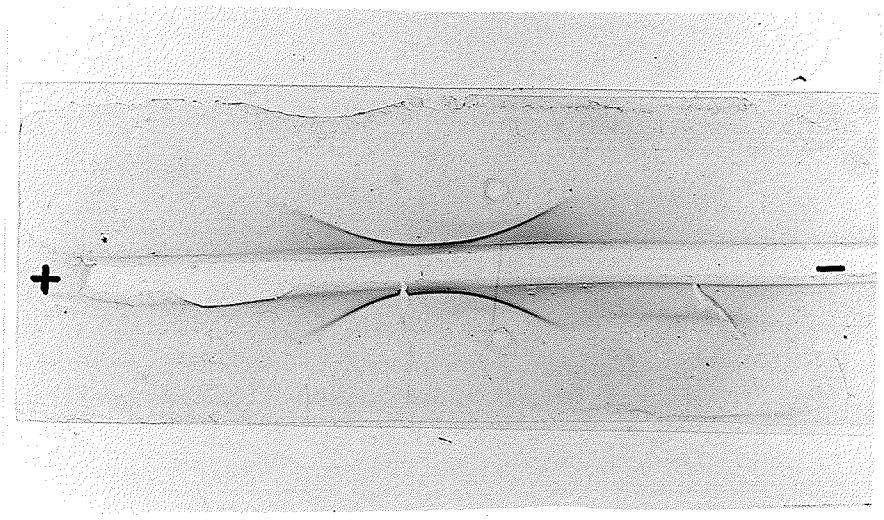

Figure 5. Immunoelectrophoresis of Purified Antigen

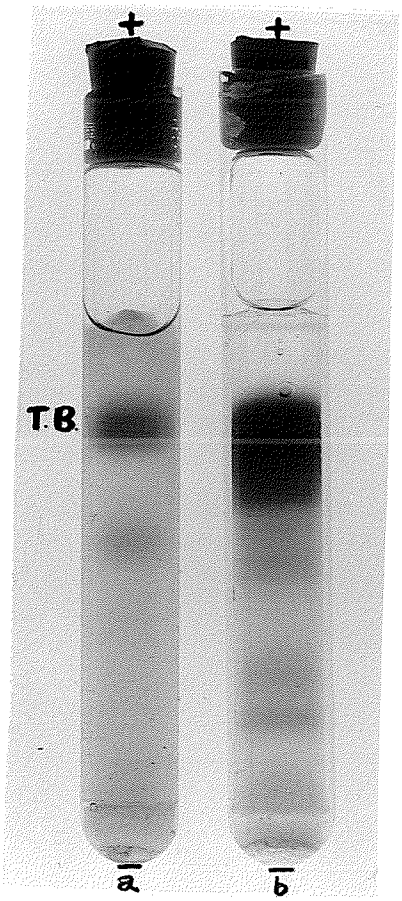

Figure 6. Polyacrylamide Gel Electrophoresis stained for Protein of Crude Acid Extract(b) and Purified Antigen(a).
T.B.; Tracking Band

Figure 7. Polyacrylamide Electrophoresis of Purified antigen stained for Protein(a) and Carbohydrate(b)

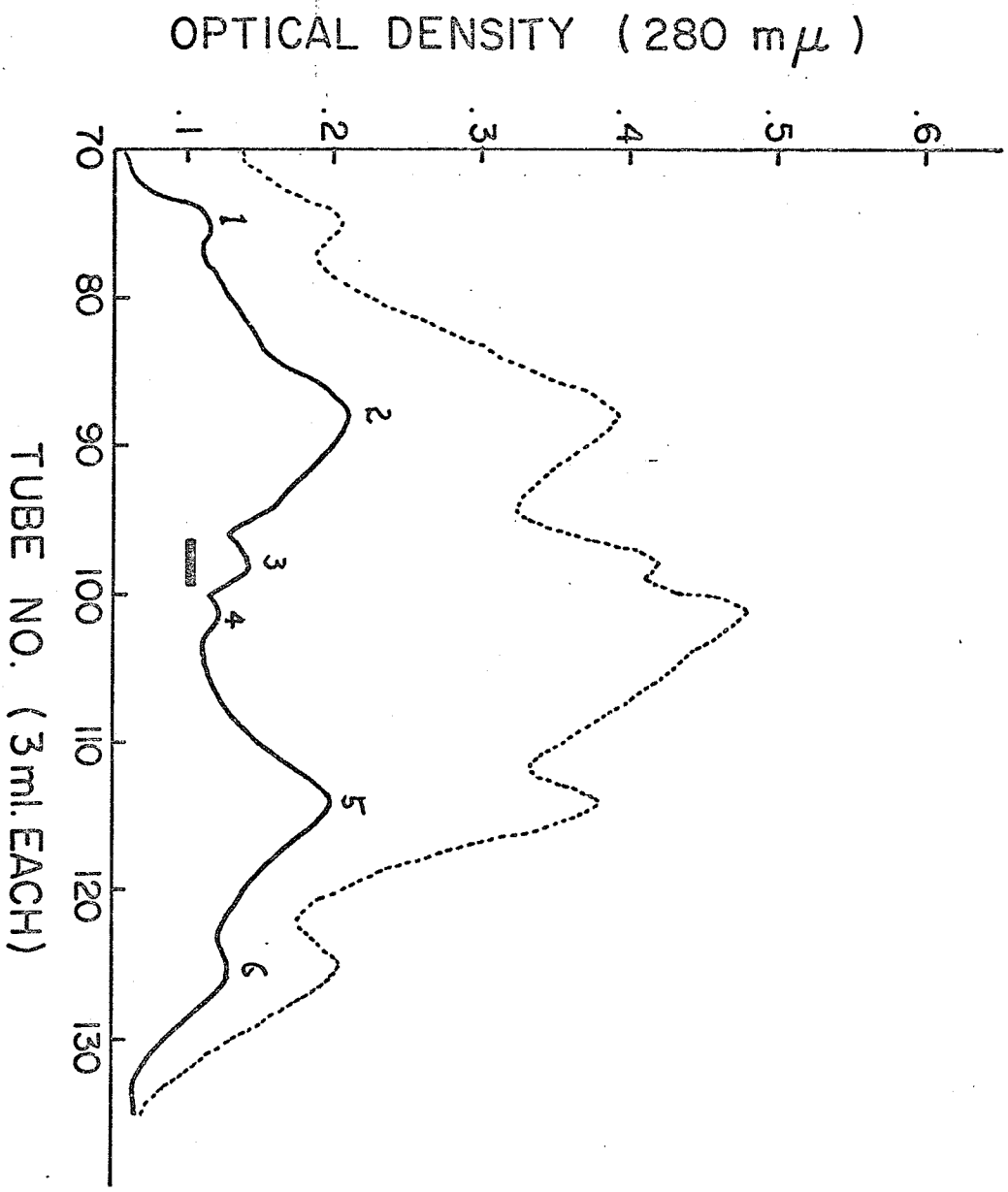

Figure 8. Elution Pattern of Antigen and Reference proteins on Sephadex G-200 Column.

Peaks: 1. Ferritin 2. γ-Globulin
3. Antigen 4. Alcohol Dehydrogenase
5. Serum Albumin(bovine) 6. Ovalbumin

——— Absorbance at 280 mu
- - - - - - - Absorbance at 230 mu
▬▬ Precipitate by Immunodiffusion

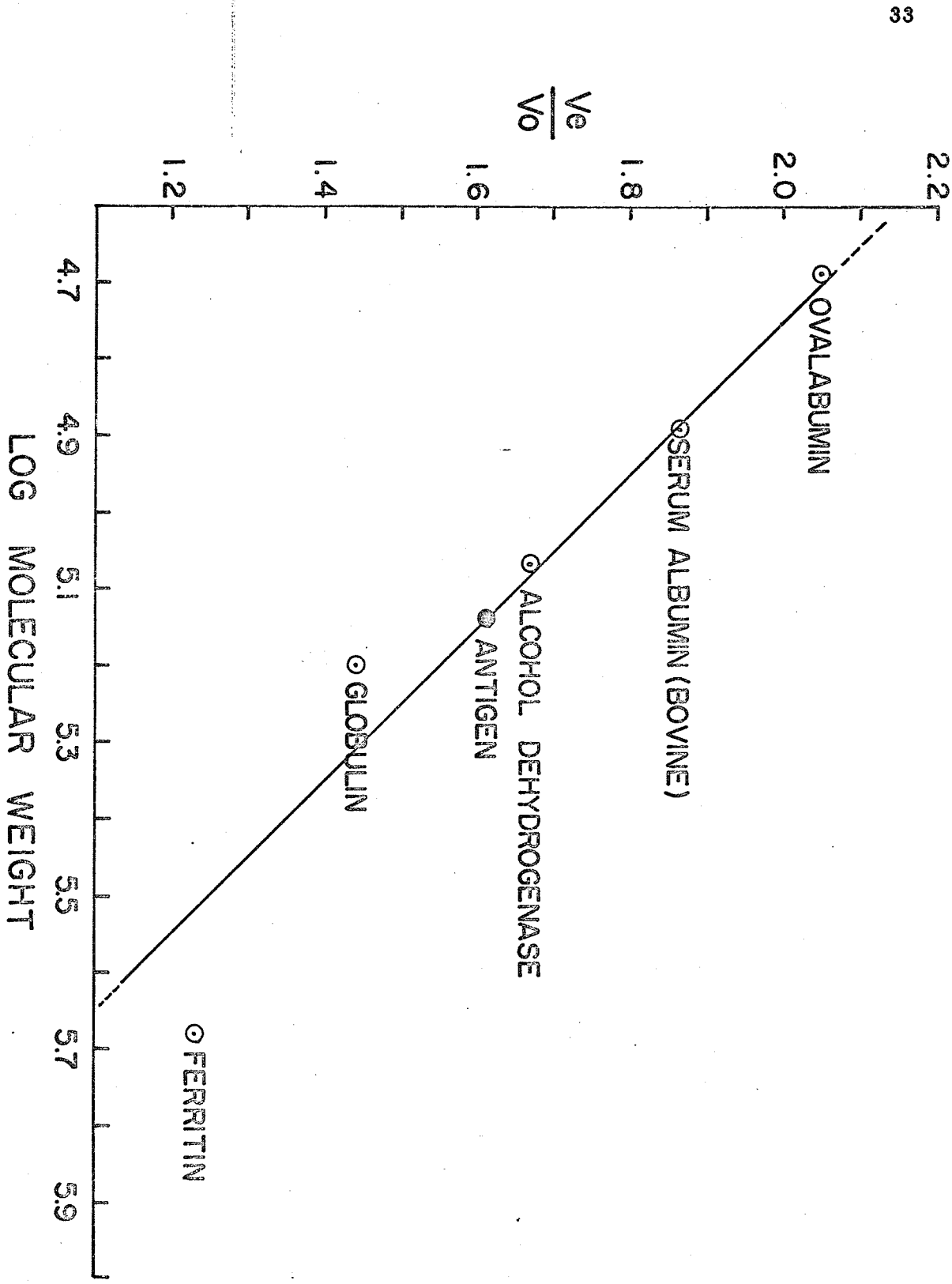

Figure 9. Calibration Curve Between Elution Volume and Log of Mol Wt for Estimation of the Molecular Weights of Antigen on Sephadex G-200 Column.

DISCUSSION

DISCUSSION

In the first attempt of purification of the antigen present in the crude acid extract which gave six bands by disc electrophoresis, the method followed was that used for isolating the precipitating antigens of C. hofmannii (Banach and Hawirko, 1966). After DEAE-cellulose chromatography and Sephadex G-200 gel filtration the antigen gave two or three bands of protein by disc electrophoresis and was eluted on the near void volume of Sephadex column indicating a large molecular weight complex. Further purification was by treatment with ribonuclease, precipitation with ammonium sulfate, followed by Bio-Gel PHT adsorption. The antigen still showed two bands and it was only after a second filtration on Sephadex G-200 that the serologically active fraction gave one band by disc electrophoresis.

The antigen appears to possess heat stable and acid resistant properties and since it was eluted from DEAE-cellulose column it appears to have negative charge. Only one precipitin line was observed by immunodiffusion throughout all stages of purification.

The electrophoretic mobility of the antigen in agar gel was relatively slow and when immunoelectrophoresis was applied with higher voltage (7 m a) or a longer time, the precipitin line resulted in larger arcs of moderate curvature. This suggests that the antigen may consist of a electrophoretically heterogenous component with minute graduated differences (Grabar and Burtin, 1964).

Disc electrophoresis on polyacrylamide gel stained by coomarssie brilliant green showed one band indicating that the antigen was over 90 per cent pure with respect to protein. When stained by a modified periodic acid-Schiff technique for carbohydrates in the gel (Stewart-Tull, 1965) in addition to the major carbohydrate a second faint band appeared which was difficult to interpret.

Biochemical analysis of the antigen showed 82 per cent protein by ninhydrin method. The three other methods for protein, the phenol method of Lowry (1951), modified biuret method of Robinson and Hogden (1940), and the measurement of ultraviolet absorption spectra gave variable results which may be due to the constituent amino acids and the structure of peptides occurring in the antigen. The relatively small amount of carbohydrate detected by the anthrone test suggests that the antigen is a complex peptide. The nucleic acid present is not considered to be significant. Although the lipid content was not tested, the phosphorous analysis suggests a lipid component may be present. Also a trial staining of lipid and lipoprotein (Uriel and Grabar, 1956) of the precipitin line indicated the presence of lipid. The chemical composition of the antigen appears to be similar to that of cell membrane of group A streptococci which was shown to be chemically distinct from the cell wall and was composed of 72 per cent protein, 26 percent lipid, and 2 per cent carbohydrate (Freimer, 1963).

The sucrose density gradient method of estimating molecular weight can give rise to errors since there are three independent parameters in

the Svedberg equation, the sedimentation coefficient, the partial specific volume, and the diffusion coefficient (Martin and Ames, 1961). The molecular weight determination of antigen attempted by this method proved to be unsuccessful. Molecular weight estimation by gel filtration involves the principle of a comparison between the gel filtration behaviour of a test sample with that of compounds which conform to an appropriate relationship. This method has proved to be the most useful because of its simplicity in operation, wide application and equivalence with preparative procedure (Andrews, 1964). Although anomalous behaviour can be expected from glycoprotein, basic protein, and those capable of undergoing association-dissociation reactions, compounds with a carbohydrate content of only 3 - 4 per cent such as ovalbumin appear to have little effect on molecular size, as compared with typical globular proteins (Whitaker, 1963; Squire, 1964; Andrews, 1965). The method adapted in this study was a column design for reverse-flow on gel filtration on as described by Rothstein (1965) and allowed milder conditions and better separation. Immunodiffusion performed with antiserum placed in a central well according to the technique of Ouchterlony (1949), showed that the antigen had similar diffusion coefficient to immunogloblin and this concurs with the molecular weight determined by gel filtration method.

The antigen extracted from C. xerosis appears to be species-specific since the hot acid extracts from other corynebacteria species failed to give precipitin lines by immunodiffusion. Similarly Cummins

(1954) reported the presence of a type-specific protein in several strains of C. diphtheriae but not in C. xerosis, C. hofmannii or C. renale, but he did not carry out immunochemical analysis.

McCarty (1964) showed that an ester-linked D-alanine was an important determinant in the specifity of group A streptococci antigen. More recently, Jones and Lewis (1966) identified the determinant group of cellular antigens of C. diphtheriae as alanine by the antibody-combining sites using immunofluorescent techniques. Furthermore, the antigen of C. xerosis in this study was shown to be highly stable to various chemicals and was rapidly inactivated by periodate oxidation, so that the determinant groups of antigen may be presumably a small component such as a single amino acid, sugar, or a combination of them.

Further studies of the antigen are needed to establish the nature of determinant group and the relationship to the cell structure.

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