THE ISOLATION AND IMMUNOCHEMICAL ANALYSIS OF TWO ANTIGENS OF *CORYNEBACTERIUM HOFMANNI*

by

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ABSTRACT

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A serologically active substance, extracted from sonicated cells of Corynebacterium hofmanni with hot HCl, produced two distinct precipitin lines by immunodiffusion tests against hyperimmune homologous serum. Extracts of other species of corynebacteria failed to cross-react with the antiserum. The active fraction of C. hofmanni was subjected to DEAE-cellulose column chromatography and the absorption peak, eluted at a KCl concentration of approximately 0.5 molar, contained the two precipitating antigens. Rechromatography on a Sephadex G-200 column resulted in a separation of these antigens. The major fraction is designated antigen A, the minor antigen B.

The homogeneity and purity of each antigen was established by immunoelectrophoresis and disc electrophoresis. Biochemical analysis showed that the two antigens were composed of a major protein fraction with carbohydrate and a small amount of nucleic acid, the approximate ratio of these constituents was 17:3:1 respectively. The protein and carbohydrate content of antigen A was less by 17.5% and 3.5% respectively than that of antigen B. Glutamic acid, aspartic acid, alanine, glycine, valine and leucine were the major amino acids of both antigens. The sugars of antigen A were identified as arabinose and glucose. The molecular weight, estimated by gradient centrifugation, was 16,500 for antigen A and 21,000 for antigen B.

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INTRODUCTION

INTRODUCTION

The antigenic complexity of bacteria has been demonstrated by the recent advances in immunochemistry. The techniques of immunodiffusion and immunoelectrophoresis have provided methods whereby the identity, purity, and specificity of antigens can be established. Furthermore, the criteria of antigenic homogeneity can be donfirmed by biophysical methods such as disc electrophoresis and ultracentrifugal analysis.

Serologically active substances have been derived from whole cells, crushed cells, various isolated components such as cell wall or even culture filtrates. The antigens are extracted, purified, and analysed to determine their biochemical structure, the nature of the determinant groups, and the combining sites which take part in the antigen-antibody reaction.

Little is known about the antigens of the corynebacteria and although Wong and Tung (1939) and Cummins (1954) identified a type specific protein and a group specific polysaccharide in the mitis strain of Corynebacterium diphtheriae, no subsequent analyses of these antigens have been reported. The present study describes the isolation, purification, and identification of two precipitating antigens of Corynebacterium hofmanni and presents data of their chemical composition.

HISTORICAL

HISTORICAL

The type, species, or group specific antigens of bacterial cells have been characterized as polysaccharides, proteins, lipids, nucleic acids, or a complex of these components (Boyd, 1956). The particular type of antigen obtained will, to a large extent, depend on the method of isolation and purification; drastic treatment is known to alter the antigenic structure (Lancefield, 1933).

A common method of obtaining antigens has been by hot acid extraction of bacterial cells as introduced by Lancefield (1928). She identified three serologically active substances from extracts of group A Streptococcus which were designated as a species-specific carbohydrate (C), a type-specific protein (M), and a non-type-specific protein (P). Lancefield divided the streptococci into 13 groups on the basis of precipitin tests using the carbohydrate (C) substance and group-specific antisera. Recently Maxted and Fraser (1965) obtained from the hot acid extracts of many strains of Streptococcus faecalis a type-specific polysaccharide which was used for serological typing.

A type-specific carbohydrate antigen was demonstrated in the hot acid extracts of intact staphylococci cells (Julanielle and Wieghard, 1933). Verwey (1940) was able to extract a type-specific protein from crushed cells of type A staphylococci in addition to the type-specific carbohydrate antigen of Julanielle and Wieghard by a milder acid treatment carried out at room temperature.

Alcoholic extracts of Corynebacterium diphtheriae, of certain

diphtheroids, and of the tubercle bacilli shared a common antigenic component (Krah and Witebsky, 1930). Hoyle (1942) used alcoholic extracts to obtain a species-specific antigen from C. diphtheriae type mitis and C. hofmanni. A group-specific antigen was shared by the mitis, gravis and intermedius strains of C. diphtheriae as well as C. hofmanni.

Culture filtrates of tubercle bacilli (Middlebrook and Dubos, 1948) and the hemolytic streptococci (Harris, 1953) were found to contain a hemosensitizing non-species-specific polysaccharide (NSS). These studies were extended to other groups of microorganisms by Rantz et al (1956) and Anzai et al (1960). They demonstrated this non-specific substance in various bacilli and other gram-positive organisms but showed it was completely lacking in gram-negative organisms. Also the NSS was shown to be heat stable, nondialyzable, and present in acid extracts of bacteria.

Hemosensitizing antigens were first reported in the corynebacteria by Hayes (1951). The ethanol precipitated polysaccharide fraction of C. diphtheriae was able to sensitize erythrocytes so that they agglutinated in the presence of specific antiserum. Saline extracts of various corynebacteria also contained antigens which sensitized erythrocytes (Caille and Toucas, 1960).

Various components of the bacterial cell such as cytoplasm, protoplasmic membrane, and cell wall have been serologically studied. Polysaccharide and protein fractions derived from the cytoplasm of

strains of mycobacteria, actinomyces and streptococci showed cross-reactivity with heterologous antisera while the reactivity of the cell wall antigen was limited to the homologous antisera (Kwapinski and Snyder, 1961). On the basis of the preceding, these authors suggest that the bacterial cell has originated from a serologically homogenous cytoplasmic body with antigenic differentiation being acquired during the evolution of the outer layers of the cell.

Most of the recent serological studies have been confined to cell walls of the bacteria. A serologically active group-specific polysaccharide was obtained from the hot acid extracts of cell walls of the three Cowan types of staphylococci (Stern and Elek, 1957). The agglutination of a cell wall suspension with specific antiserum was used by Cummins (1954) to show the presence of two antigens in the walls of C. diphtheriae. These antigens, a thermostable group-specific polysaccharide and a thermolabile species-specific protein, were similar to those reported by Oeding, (1950). The group polysaccharide antigen was detected only by agglutination of cell walls and so appears to be situated deep in the wall. This group antigen was found in C. diphtheriae and C. ovis but not in C. hofmanni, C. xerosis or C. renale. The chemical analysis of C. diphtheriae cell wall preparations by Holdsworth (1952) confirmed the polysaccharide nature of the group antigen.

The cell walls derived from most of the strains of corynebacteria, mycobacteria, and nocardia, analysed by Cummins and his co-workers

(1956, 1958, 1962 and 1965), were found to contain the sugars arabinose and galactose and the amino acids alanine, glutamic acid and diaminopimelic acid. The cell walls of the three genera which showed arabinose and galactose as their principal sugars shared a common antigen. The sole exception was C. hofmanni which did not contain the group antigen but had arabinose and galactose present in the cell wall.

Teichoic acids occur in the walls of many gram-positive bacteria. These acids have been identified by Baddiley (1962) as glycerophosphate or ribitol phosphate polymers possessing serological activity. Both glycerol and ribitol teichoic acids have been extracted from the walls of lactobacilli by Baddiley and Davison (1961). A ribitol teichoic acid was obtained from the walls of Bacillus subtilis (Armstrong, Baddiley and Buchanan, 1960). McCarty (1959) isolated a polyglycerophosphate antigen from crushed cells of many strains of the hemolytic streptococci, staphylococci and aerobic sporulating bacilli, but this antigen was not found in the gram-negative species examined or in pneumococci, clostridia or corynebacteria. In the case of lactobacilli, the presence and type of teichoic acid provided a useful means of classification of the species.

The type-specific M protein antigen of the Group A streptococci was found associated with the membrane from osmotically lysed protoplasts (Fox, 1963). This antigen was also present in a soluble form in the cytoplasm.

Intensive immunochemical studies have been recently carried out

on antigenic components of a few bacterial species. A serologically active polysaccharide A was extracted with cold buffer from disrupted cells of Staphylococcus aureus by Haukenes (1962). Isolation of this polysaccharide antigen was achieved by chromatography on diethylaminoethyl (DEAE)-cellulose and Dowex-1 ion-exchange columns. Chemical characterization of the polysaccharide revealed two structural units, a mucopeptide and a ribitol teichoic acid. A similar extraction and isolation procedure was used by Losengrad and Oeding (1963) to obtain a polysaccharide antigen from disrupted cells of strains of Staph. epidermidis. Keeler and Pier (1964) also used DEAE-cellulose column chromatography for the isolation of antigenic material from Nocardia asteroides.

Column chromatography is particularly useful for the fractionation of complex mixtures, the isolation of unstable substances and the separation of closely related compounds (Lederer, 1957). Two types of column chromatography are ion-exchange chromatography using cellulose ion-exchangers developed by Peterson and Sober (1956) and partition chromatography using a non-ionic dextran gel (Sephadex) developed by Porath and Flodin (1959) for the separation of materials of different molecular weights.

The techniques of immunodiffusion, immunoelectrophoresis, and polyacrylamide gel electrophoresis have been used to study the specificity, purity, active determinant groups, and chemical structure of antigens. The agar diffusion precipitin technique was introduced

by Oudin (1946) as a single diffusion method in one dimension; subsequently double diffusion in two dimensions was developed by Ouchterlony (1949) and by Elek (1949) and has largely replaced the single diffusion technique. The Ouchterlony method of immunodiffusion has been performed on extracts of cell walls of corynebacteria, mycobacteria and nocardia (Cummins, 1965) and extracts of whole cells of Staph. aureus (Oeding and Haukenes, 1963) to show the presence of multiple antigenic specificities. Haukenes (1962) established the purity of the polysaccharide A. antigen of Staph. aureus on the basis of immunodiffusion analysis and concluded that teichoic acid was responsible for its serological reactivity. The precipitin reaction by a group-specific polysaccharide from Nocardia brasilienses, which showed cross-reactivity with mycobacteria, was abolished by prior treatment with periodate indicating that the antigenic determinant responsible for the cross-reaction was a sugar identified as D-arabofuranose (Estrada-Parra, Zamora, and Bojalil, 1965).

Immunoelectrophoresis, a combination of electrophoretic and agar diffusion methods was first performed on single slabs of agar by Grabar and Williams (1953). A micromethod of immunoelectrophoresis, which was introduced by Scheidegger (1955), has simplified the technique, shortened the time, and reduced the amount of antiserum and antigen required for the procedures. This electrophoretic technique has been developed for the characterization and separation of antigens and

antibodies and for the determination of antigenic specificity (Grabar, 1959).

The utilization of polyacrylamide gels for zone electrophoresis was reported by Raymond and Weintraub (1959). The separation of macromolecules is achieved in such a gel matrix through dimensional as well as charge differences. Keeler and Pier (1964) were able to demonstrate the homogeneity of antigens isolated from Nocarida asteroides with polyacrylamide gel electrophoresis. This method has been used in the study of human serum proteins (Davis, 1964) and of the soluble protein components and esterases of the various strains of Group D streptococci (Lund, 1965).

Antigenic analysis of bacteria has been widely used in the taxonomic classification of species of bacteria such as in the genera Streptococcus and Salmonella. The first studies on C. diphtheriae, utilizing agglutination techniques, showed that the species could be divided into various serotypes (Havens, 1920; Smith, 1923; and Eagleton and Baxter, 1923). The antigenic structure of this organism also differed according to the varieties: gravis, intermedius or mitis (Ewing, 1933; Robinson and Peeney, 1936).

Species differences between C. diphtheriae and other corynebacteria were not revealed by agglutination tests (Bailey, 1925). However, the complement-fixation test proved more successful as Krah and Witebsky (1930) showed that alcoholic extracts of C. diphtheriae, of certain diphtheroids, and of the tubercle bacilli all fixed complement in the

presence of heterologous antisera. Hoyle (1942) using a similar extraction and serological procedure, described a group antigen common to the gravis, intermedius, and mitis strains of C. diphtheriae as well as C. hofmanni.

In recent studies on the chemical and antigenic analysis of the cell walls of corynebacteria and related organisms, Cummins (1958, 1962) demonstrated that all strains of corynebacteria, mycobacteria, and nocardia which had arabinose and galactose as their principal cell wall sugars shared a common antigenic component. This suggested that strains of these three genera could be grouped together in the same order. Cummins also thought that C. pyogenes and C. haemolyticum, which did not have arabinose and galactose as principal cell wall sugars, should be reclassified.

With the application of newer serological techniques, Cummins (1965) was able to confirm the results obtained with the corynebacteria, mycobacteria and nocardia by the indirect immunofluorescent technique. Slack et al,(1961), also using the fluorescent antibody technique, showed cross-reactivity between Group A Actinomyces and seven strains of C. acnes as well as 17 anaerobic diphtheroids. On this basis, these workers suggested that these organisms be included in the genera Actinomyces. However, additional species of Corynebacterium, Lactobacillus, Propionibacterium, Streptococcus and Nocardia did not fluoresce with any of the group-specific Actinomyces sera.

In recent years, milder extraction and isolation procedures involving column chromatography on ion-exchange resins and dextran gels has made it possible to obtain purified antigens. Immunochemical and biochemical analysis has given an insight into the complexity of these serologically active substances.

MATERIALS AND METHODS

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CULTURES

Corynebacterium hofmanni (ATCC-10700) was used throughout the studies and laboratory strains of *Corynebacterium pyogenes*, *Corynebacterium equi*, *Corynebacterium xerosis*, *Corynebacterium diphtheriae* type *mitis* and *Staphylococcus aureus* were used for testing cross-reactivity.

Stock cultures were maintained in trypticase soy broth (BBL) at 37°C and transferred weekly. Cells were grown in 500 ml. volumes of trypticase soy broth on rotary shakers for 24 hours at 37°C and collected by centrifugation at 8,000 x g for 20 minutes in a Servall RC-2 centrifuge. Broth cultures of *C. hofmanni* were pooled in 12 liter carboys, harvested with a Sharples centrifuge, washed twice in saline and once in distilled water, and then stored at -15°C.

Whole Cell Antigens

A twenty-four hour broth culture of *C. hofmanni* was washed twice in 0.85% saline and suspended in 0.2% formol saline. The cells were filtered through glass wool to remove clumps and further diluted with 0.2% formol saline to a density corresponding to a McFarland scale No.3 on the Beckman Model C. colorimeter (38% light transmission using Klett tubes and red filter). The cell suspension was dialysed against 0.2% formol saline for 48 hours at 5°C and tested for sterility.

The suspension was stored at 5°C and was employed for the production of antibody and for agglutination tests.

Preparation of Antiserum

Six adult albino rabbits were immunized by the procedure of Kabat and Mayer (1961) except that a total of 22.5 ml of cell suspension was administered over a period of six weeks. Pre-immune and immune sera were tested by the standard tube agglutination test. Immune sera which showed a reciprocal dilution titre of 2048 were collected and stored at -15°C for further study.

Bacterial Extracts

Extracts, for serological and biochemical analysis, were prepared from frozen stocks of bacteria.

Twenty gram lots of C. hofmanni wet cell mass were mixed with a few ml of distilled water and disintigrated in a Raytheon ultrasonic oscillator (10 Kc/sec.) at 7°C for one hour. The crushed cell mass was suspended in 5 x the volume of N/16 HCl (100 ml.) and placed in a boiling water bath for 15 minutes. The mixture was cooled on ice, adjusted to pH 7.2 with 1.0 N NaOH, and centrifuged at 15,000 x g for 20 minutes. The clear, yellow, supernatant was placed in dialysis tubing, concentrated against moving air for seven hours and then equilibrated overnight at 5°C against 0.02 M phosphate-buffered saline of pH 7.4. The crude extract was stored at -15°C. A similar extraction procedure was followed using one gram wet weight of cell mass of C. pyogenes, C. equi, C. xerosis, C. diphtheriae type mitis and Staph. aureus. These extracts were used undiluted in the agar diffusion tests.

Immunodiffusion Tests

A modified technique of Ouchterlony (1949) was followed.

Petri dishes (inner diameter of 9 cm.) were coated with a thin layer of 2% Noble agar (DIFCO) and then layered with 20.0 ml. of 0.8% Noble agar containing 1% sodium azide and 0.88% sodium chloride. A horizontal trough (0.2 x 16 cm.) was cut in the agar plate and the wells were punched out at a distance of 10 mm. on each side of the trough with a number one cork borer. Antigens previously dialysed against 0.02 M phosphate-buffered saline of pH 7.2 were placed in each of the wells and the horizontal trough was filled with undiluted antiserum. The plates were kept at 25°C in a closed moist chamber for eight to ten days. Each plate was filled with saline and photographed by dark-field illumination.

The acid extracts of the other species were tested for cross-reactivity against C. hofmanni antiserum.

Purification of Antigens

The crude acid extract was further treated by ion-exchange chromatography. Diethylaminoethyl (DEAE)-cellulose column chromatography was performed as described by Haukenes (1962) with some modifications.

Sigma DEAE-cellulose was suspended in 0.5 N NaOH for 48 to 72 hours, washed in large volumes of distilled water several times,

adjusted to pH 7.4 with 2 N phosphoric acid and allowed to equilibrate to 0.02 M phosphate buffer of pH 7.4 for 12 hours. Columns of 2.5 x 38 cm. were packed to a pressure of about 75 cm. water and again equilibrated with 0.02 M phosphate buffer of pH 7.4.

Fifteen ml. of extract (obtained from approximately 10 grams of C. hofmanni cell mass) was applied to the DEAE-cellulose column. Elution was performed with 0.02 M phosphate buffer, pH 7.4 and then with a continuous gradient using an increasing molarity of KCl. The flow rate was regulated to about 18 to 20 ml. per hour and one hundred fractions of ten ml. were collected at 5°C.

The optical density of each fraction was measured at 280 mμ in the Unicam SP-700 spectrophotometer. Each fraction was also tested for carbohydrate content by the Anthrone method of Scott and Melvin (1953). The fractions from each of the four absorption peaks were pooled, dialysed against moving air at room temperature for seven hours and against two liters of 0.02 M phosphate-buffered saline of pH 7.4 overnight, and stored at -15°C. Samples from each concentrate were tested by immunodiffusion.

Separation of Antigens

The third DEAE absorption peak, which gave two precipitin lines, was subjected to Sephadex gel column chromatography.

Sephadex G-200 (Pharmacia, Upsala, Sweden) was allowed to swell in 0.02 M phosphate buffer of pH 7.4 and to settle by gravity

in a 2 x 50 cm. column. Two ml. of the third DEAE pool, derived from five grams of wet weight cells, was placed on the column and at a flow rate of 8 to 10 ml. per hour one hundred fractions of 2.5 ml. volumes were collected during elution with the buffer at 5°C.

The optical density of each fraction was measured at 280 mμ using the Unicam SP-700 spectrophotometer. The fractions from each of the two absorption peaks were pooled, concentrated by dialysis against air for seven hours, equilibrated by dialysis against 0.02 M phosphate-buffered saline of pH 7.4 and then tested by immunodiffusion. The two active pools were then dialysed against air to concentrate the solutions and against 2.5 liters of distilled water for 48 hours to remove buffer salts, dried by flash evaporation, and stored in a dessicator at room temperature.

Immunoelectrophoresis

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

Agarose (NBC) was dissolved in 0.05 M barbital buffer of pH 8.4 to make a 1.5% concentration. The antiserum trough (1 x 60 mm.) was cut with two surgical blades bound together and two starting wells were made 3.0 mm. from the sides of the trough with a number 18 hypodermic needle.

Samples from the Sephadex pools were placed in each of the wells with a number 26 hypodermic needle. Contact with the electrode vessels, which contained 0.05 M barbital buffer of pH 8.4, was made

by means of agarose wicks. Electrophoresis of the antigens was carried out for 60 minutes at 70 v. and 50 v. using a Beckman Duostat D. C. power supply. Upon completion of the run, undiluted antiserum was placed in the trough and the reaction was allowed to develop for 24 hours at room temperature in a moist chamber.

The slides were washed, dried and then stained with amido-black (Parker, Stackiw and Wilt, 1962). Photographs were taken by dark-field illumination.

Disc Electrophoresis

Acrylamide gel electrophoresis was performed as described by Davis (1964) using the Canalco Disc Electrophoresis Trial Kit supplied by Canal Industrial Corporation, Bethesda, Maryland.

Two hundred µg. samples, mixed with equal amounts of upper-gel in a total volume of 0.30 ml., were layered on the spacer-gel. Electrophoresis was carried out for 60 minutes using the Canalco Model 150 v. power supply. After the run, the gel columns (0.5 x 6 cm.) were removed, stained in acidified aniline black, and electrophoretically destained with acetic acid. Results were photographed.

Spectrum Analysis

An aqueous solution of antigens A and B, both at a concentration of 200 µg./ml. were scanned in the Unicam SP-700 spectrophotometer.

Biochemical Analysis

A Klett-Summerson photoelectric colorimeter was used in the

protein, carbohydrate, pentose, and hexosamine determination. The protein and carbohydrate contents were read at 660 mμ., the pentose and hexosamine at 560 mμ.

Protein

The procedure of Lowry, Rosebrough, Farr, and Randall (1951) was followed with crystalline bovine albumin as the standard.

Carbohydrate

A modification of the procedure of Scott and Melvin (1953) was followed with dextrose as the standard. The tubes containing the samples and Anthrone reagent were vigorously shaken, placed in a boiling water bath for 15 minutes and immediately cooled.

Pentose

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

Hexosamine

A modification of the procedure of Elson and Morgan (1954) as outlined in Kabat and Mayer (1961) was followed with glucosamine hydrochloride as the standard. Samples were hydrolysed in 2 N HCl at 100°C for two hours and were used for hexosamine determination.

Nucleic acids

Nucleic acid was determined spectrophotometrically by the procedure of Warburg and Christian (1942) using the Unicam SP-700

spectrophotometer to measure the ratio of optical density at 280 mμ. and 260 mμ.

Nitrogen

Nitrogen was determined by calculating the total μmoles of nitrogen obtained with the Technicon Amino Acid Analyser.

Amino acids

Amino acids were determined with a Technicon Auto Analyser. Samples of 2.0 mg. were hydrolysed in 5.0 ml. of 6 N HCl for 16 hours, dried in a desiccator overnight, and dissolved in 0.5 ml. of N/10 HCl. Volumes of 0.30 ml. were analysed.

Chromatography

For the identification of sugars 12.0 mg. of sample was hydrolysed in 1.0 ml. of 2 N H_2SO_4 in sealed vials at 100°C for two hours. The cooled hydrolysate was neutralized with saturated $Ba(OH)_2$, centrifuged to remove barium sulphate, and evaporated to dryness by flash evaporation. The dried hydrolysate was redissolved in 0.25 ml. of distilled water.

Standards (10 μl. of 50 mg./ml. solution) and the hydrolysed sample were applied to Whatman No.1 paper (46 x 75 cm.) and run in 80% phenol down the long axis of the paper until the solvent reached the edge. The paper was dried, dipped in aniline phthalate reagent mixture (as described by Cummins and Harris, 1956) and heated at 80°C for 20 minutes. Arabinose and glucose appeared red and greenish-

red respectively with the indicator. A chromatogram of Antigen B was not carried out due to insufficient material.

For the identification of amino acids, 2 mg. samples were hydrolyzed in 6 N HCl at 121°C for two hours. Hydrolysates were then evaporated to dryness in a boiling water bath several times and finally redissolved in 0.2 ml. of distilled water. The separation procedure was similar to the above except that the chromatographic paper was buffered by spraying with a buffer mixture of pH 12 (McFarren, 1951). The chromatographic paper was spotted with standard amino acids (10 µl. of 2 mg./ml. solution) and hydrolysed sample (50 µl. of 2 mg./0.2 ml. solution). After completion of the run, the paper was dried, stained with ninhydrin solution (as prepared by McFarren, 1951), and placed in a hot air oven at 80°C for ten minutes.

Molecular Weight Determination of Antigens

The molecular weights of the antigens were determined by the sucrose density gradient method of Martin and Ames (1961) with some modifications. Linear sucrose gradients were made in lusteroid centrifuge tubes from 3% to 20% (W/V) sucrose in 0.10 M phosphate buffer of pH 7.5. Each sample was suspended in a total volume of 0.2 ml: 0.2 mg. of Antigen A, 0.10 mg. of antigen B, and 1 mg. of reference protein (crystalline bovine hemoglobin, Sigma) and layered on separate gradients. The gradient tubes were centrifuged at 39,000 r.p.m. for 12 hours at 5°C in a model-L Spinco centrifuge using S W 39 swinging bucket rotor. The rotor was accelerated slowly to prevent backlash and

then the speed control was immediately set at 39,000 r.p.m. At the end of the run, the rotor was allowed to decelerate with the brakes off.

Two drops fractions were collected by puncturing the bottom of the tube with a needle and these were assayed for protein concentration by the Lowry test. The standard hemoglobin fractions were examined at 405 mμ. in the Unicam SP-700 spectrophotometer.

The molecular weights of the antigens were calculated from the molecular weight of the reference protein (68,000, Merck Index - 7th edition) and the distance which antigens A and B and reference protein travelled in the sucrose density gradient. The equation used for calculation was:

$$\frac{S_1}{S_2} = \left(\frac{MW_1}{MW_2}\right)^{2/3}$$

where S_1 and S_2 equals the distances travelled from the meniscus by substances of known and unknown molecular weights respectively, and MW_1 and MW_2 are equal to the molecular weights of these unknown and known substances (Schachman, 1959).

RESULTS

RESULTS

Serological activity of hot acid extracts from crushed cells of C. hofmanni, as measured by agar plate diffusion, gave two precipitin lines; one diffuse line appeared closer to the trough containing the antiserum, the other sharp line further from the trough (Figure 1, upper well 4). Precipitin lines were not observed with the hot acid extracts of C. xerosis, C. pyogenes, C. equi, C. diphtheriae type mitis, and Staph. aureus tested against C. hofmanni antiserum.

The acid extracts were separated, by DEAE-cellulose column chromatography, into four absorption peaks, (Figure 2). Samples from each of the four peaks gave carbohydrate values of 75%,< 2%, 7%,and 15% respectively as measured by the Anthrone test. Immunodiffusion tests against homologous serum showed two heavy precipitin lines with samples from the third absorption peak which had been eluted at the KCl concentration of 0.5 molar (Figure 1, upper well 2 and lower well 3). The precipitin lines corresponded in identity to those formed by the hot acid extract. Faint lines were observed in the first, second, and fourth absorption peaks and were not further identified (Figure 1, lower wells 1, 2 and 4).

Material from the third DEAE absorption peak, eluted on a Sephadex G-200 column, gave two absorption peaks designated as A and B (Figure 3). Two sharp precipitin lines were observed by immunodiffusion tests. Samples from peak A gave one heavy line while a lighter line was observed from peak B (Figure 1, upper wells 3 and 1 respectively); the lines corresponded in identity to the

diffuse and sharp lines obtained with the hot acid extract and the third DEAE peak. Hereafter, the active material obtained from Sephadex peaks A and B are referred to as antigens A and B respectively.

The yield from 10 grams of wet weight cell mass was 16 mg. of antigen A and 7.2 mg. of antigen B. The antigens were soluble in water and appeared as a brown, granular material in the dry state.

Spectrum analysis of both antigens (200 µg./ml. aq.soln.) were characterized by a higher extinction value at 260 mµ. than at 280 mµ. with a maximum absorption at 264 mµ. (Figure 4). The absorption spectrum of antigen A was lower and was therefore composed of less UV absorbing material.

The homogeneity of each antigen was established by micro-immunoelectrophoresis which showed slow-moving single arcs of precipitation (Figure 5). Precipitin line formed by antigen A appeared closer to the antiserum trough than the precipitin line obtained with antigen B. Under higher voltage the migratory rate was increased but the arcs of precipitation were drawn out.

The purity of antigen A was verified by disc electrophoresis on polyacrylamide gel; a single dark band at a distance of 7 mm. behind the tracking band was obtained (Figure 6). No band was observed with antigen B.

The biochemical analysis for total protein, carbohydrate, pentose, hexosamine, nucleic acid, and nitrogen are shown in Table 1.

Paper chromatography of amino acids showed that glycine and alanine were present in each of the antigens. Chromatography of sugars of antigen A showed that arabinose and glucose were present.

The analytical results obtained by the Technicon Amino Acid Analyser are reported in Table 2. The major amino acids were glutamic acid, aspartic acid, alanine, glycine, valine, and leucine. One unidentified peak, which was eluted between the aspartic and threonine peaks, was obtained with antigen A.

The molecular weights of antigen A and B, by the sucrose density gradient method, were estimated to be 16,500 and 21,000 respectively.

TABLE 1

BIOCHEMICAL ANALYSIS OF THE SEPHADEX ABSORPTION PEAKS

COMPONENTS	ANTIGENS A	B
PROTEIN	55*	72.5
CARBOHYDRATE	6.7	10.2
PENTOSE	1.0	2.6
HEXOSAMINE	0.7	2.5
NUCLEIC ACID	3.4	3.9
NITROGEN	8.7	9.0

* percentage of dry weight.

TABLE 2

AMINO ACID ANALYSIS

AMINO ACIDS	ANTIGENS A.	B.
GLUTAMIC	.764*	.687
ASPARTIC	.706	.629
ALANINE	.629	.663
GLYCINE	.433	.492
VALINE	.402	.445
LEUCINE	.396	.454
LYSINE	.348	.292
THREONINE	.280	.338
ISOLEUCINE	.271	.331
SERINE	.246	.283
ARGININE	.222	.271
PROLINE	.222	.254
PHENYLALANINE	.171	.180
HISTIDINE	.139	.115
TYROSINE	.100	.121
METHIONINE	.021	.032
CYSTEINE	tr.	tr.
AMMONIA	.793	.793
TOTAL NITROGEN	7.431	7.693

* µmoles nitrogen

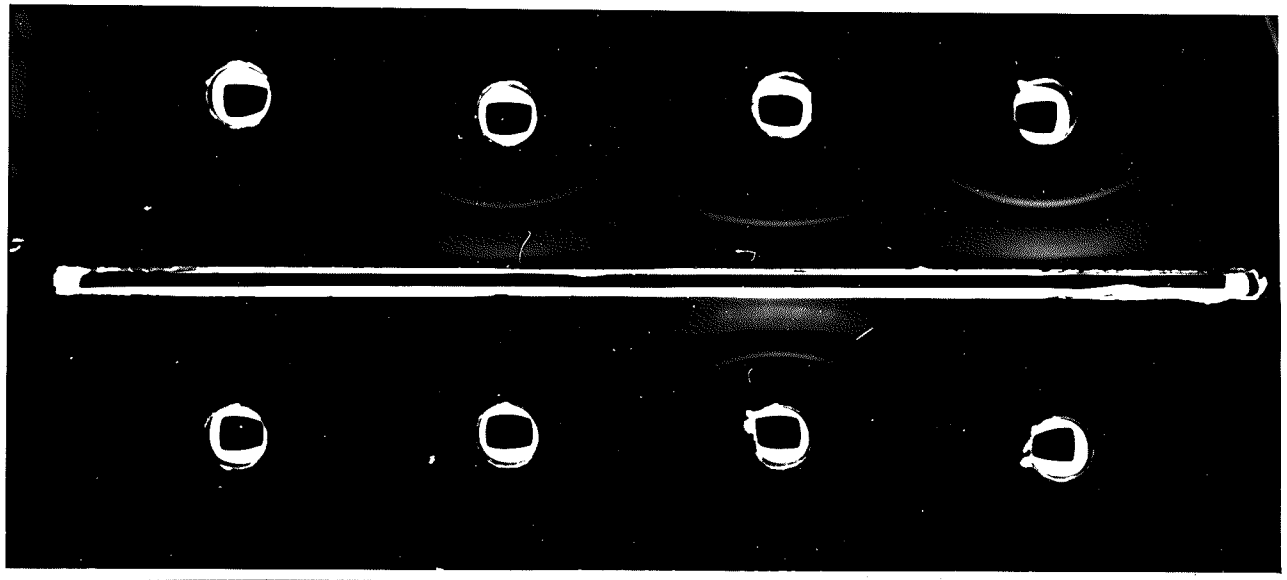

FIGURE 1. Immunodiffusion with C. hofmanni antiserum in horizontal trough. Lower wells contained samples from DEAE absorption peaks 1, 2, 3 and 4 respectively; upper well 4, hot acid extract; upper well 2, 3rd. DEAE absorption peak; upper well 3, Sephadex fraction A; upper well 1, Sephadex fraction B.

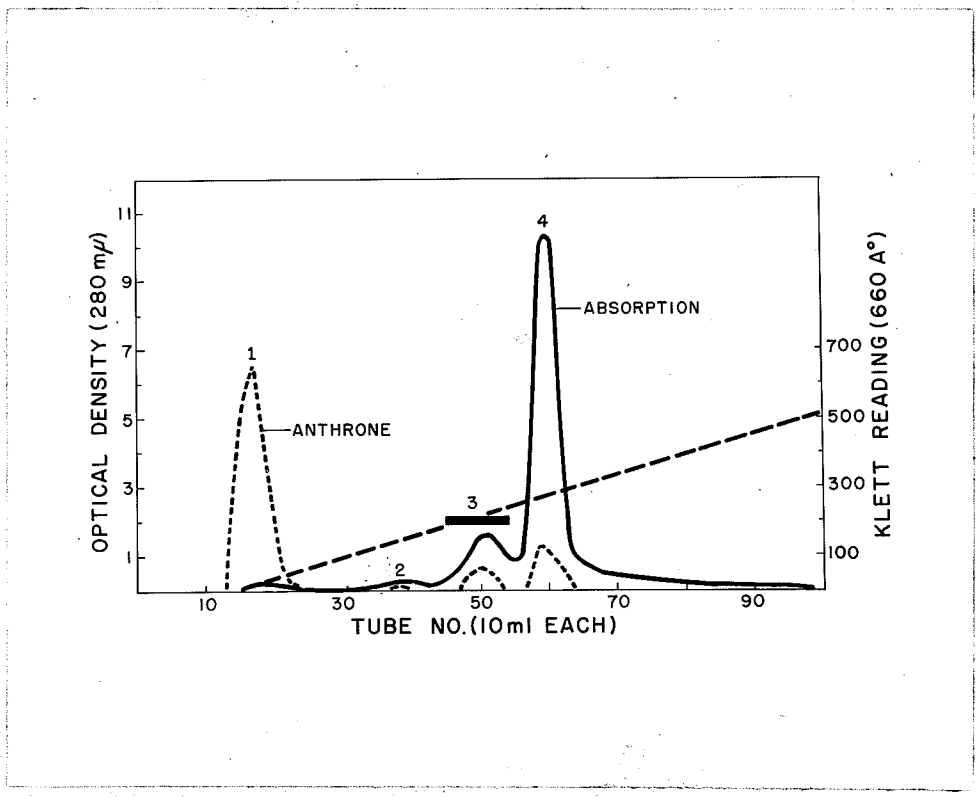

FIGURE 2. Chromatography of acid extract of C. hofmanni on DEAE-cellulose.

———— absorption at 280 mμ.

- - - - - - - anthrone at 660 mμ. Å

— — — linear KCl gradient (0.0 M to 1.2 M).

▬▬▬ precipitate by immunodiffusion.

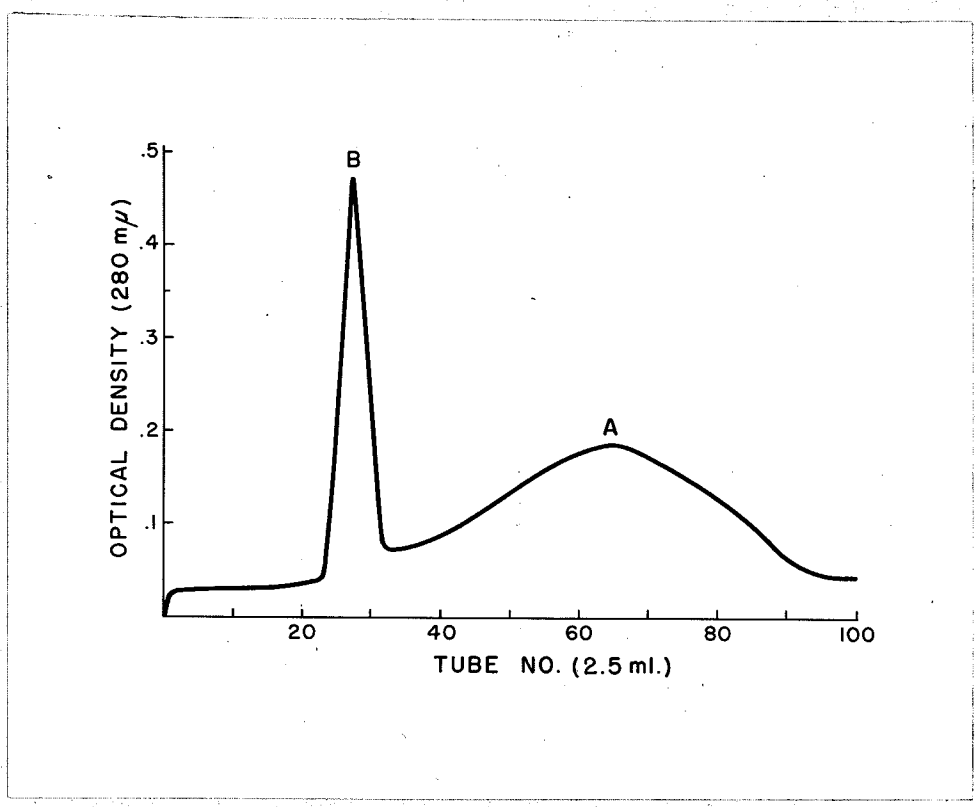

FIGURE 3. Chromatography of the 3rd DEAE absorption peak on Sephadex G-200.

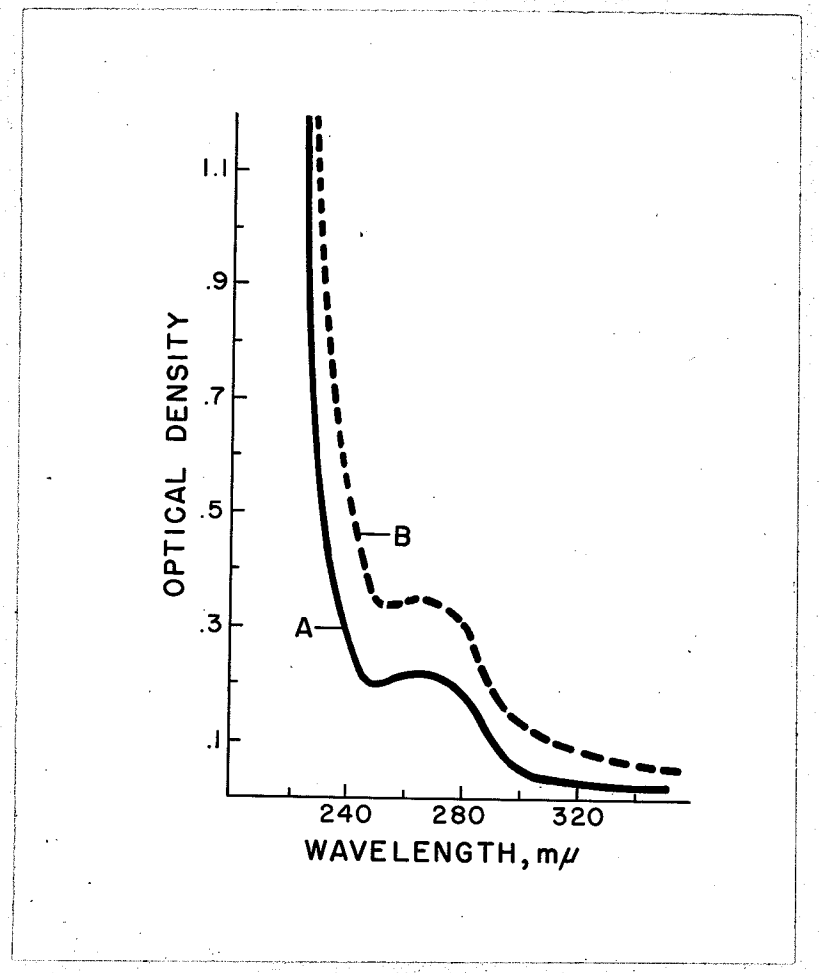

FIGURE 4. Ultraviolet absorption spectra of Sephadex fractions A and B (200 μg./ml. aq.soln.)

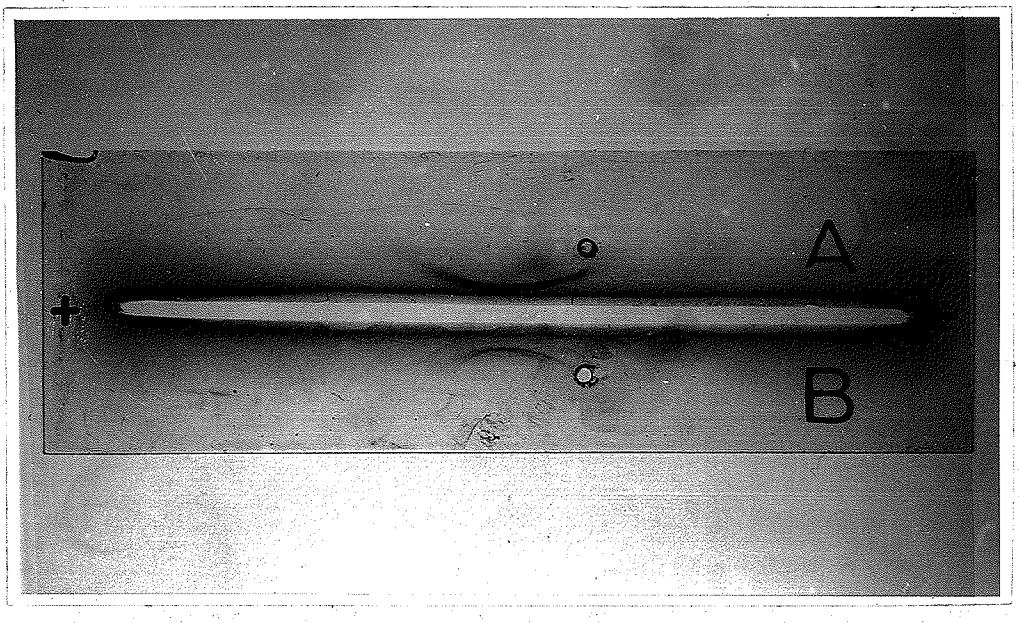

FIGURE 5. Immunoelectrophoresis at 50 v. for 60 min. Upper well contained antigen A; lower well, antigen B; trough, C. hofmanni antiserum.

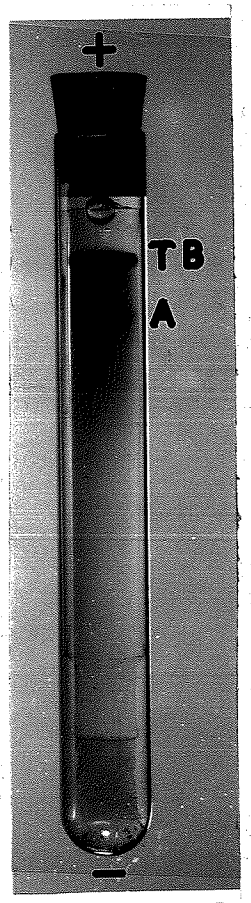

FIGURE 6. Polyacrylamide gel disc electrophoresis of antigen A. Lower dark band, antigen A; tracking band (T.B.), control.

DISCUSSION

DISCUSSION

Two precipitating antigens were obtained by column fractionation of the hot acid extract from crushed cells of C. hofmanni. Haukenes (1962) previously used DEAE-cellulose column chromatography to isolate the polysaccharide A antigen of Staph. aureus. Both of the antigens in this study were eluted from the third absorption peak of the DEAE column indicating that they possess a similar negative charge.

The separation of the two antigens from the third DEAE peak was first attempted by precipitation with cold alcohol and by chromatography using various sizes of Sephadex G-75 columns, but only partial separation was accomplished. Differential chromatography was finally achieved on a Sephadex G-200 column (2 x 50 cm.) indicating that antigens A and B have a similar molecular size.

The precipitin lines obtained with antigens A and B were much sharper, but still showed complete identity to those of the hot acid extract (Figure 2). This would indicate that the extraneous material had been removed and that the antigens were unaltered by the separation and purification procedures. When immunodiffusion was performed with antiserum placed in a central well according to the technique of Ouchterlony (1949), the results were difficult to interpret. When the antiserum was placed in a horizontal trough, however, the precipitin lines were more easily visualized and their identity was clearly established. This may be due to the greater amount of antibody available and perhaps to the linear direction of diffusion which stabilized the concentration of the antibody.

The single arcs of precipitation obtained by immunoelectrophoresis of antigens A and B, corresponded in terms of relative distances from the antiserum to those obtained by immunodiffusion. The application of a higher voltage (250 volts) or an increase in the time of electrophoresis resulted in longer arcs of moderate curvature suggesting that the antigens consist of electrophoretically hterogenous components with minute graduated differences in electrophoretic mobility (Crowle, 1961).

Disc electrophoresis tests on polyacrylamide gel showed that antigen A is relatively pure with respect to protein content. Faint traces of several other bands, which appeared in the photograph, were not evident on visual examination of the column. These residual bands may be due to diffusion which occurred as a result of setting and disturbances of the column during the time elapsed prior to photography. However, it may be that the faint bands do indicate impurities; the minute amounts present would likely be within the experimental error of this work. Also the method does not detect carbohydrate or nucleic acids which may be present. The failure of antigen B to migrate in the acrylomide gel could be due to the migration of the antigen towards the opposite pole, the use of an inadequate buffer system, or some other unexplained reason.

The sucrose density gradient method of estimating molecular weights is subject to error since it is based on the assumption that the protein consists of spherical molecules and that it has a partial specific volume of 0.725 cm^3 per gram (Martin and Ames, 1961). However, it was confirmed that antigen A was lighter than antigen B by the Sephadex gel column elution of antigen A in the latter fractions and the more rapid rate of diffusion in agar gels.

Biochemical analysis of the two antigens showed the presence of a major protein fraction with carbohydrate and a smaller amount of nucleic acid. Since the nucleic acid may be an impurity, further purification should be attempted by chromatography on a Dowex-1 ion-exchange column which Haukenes (1962) used for the removal of nucleic acid from the polysaccharide A of Staph. aureus.

The amino acid composition of antigens A and B, obtained by the Technicon Analyser, was almost identical except for one unknown acid present in antigen A which remains to be tested. The unidentified component may be a diaminopimelic acid since it carries an acidic charge and also appears to be strongly related to aspartic acid from which diaminopimelic acid is synthesized.

Although paper chromatography of amino acids for each of the antigens showed distinct spots only for alanine and glycine, these results may not necessarily conflict with those of the Technicon Analyser since the paper chromatograms contained much ninhydrin-positive material which was not resolved by uni-dimensional elution. Further resolution could be obtained by two-dimensional chromatography.

The amino acid composition of the cell walls of several corynebacteria species is similar to that of antigens A and B (Cummins, 1956 and 1962). The cell walls, after treatment with proteolytic enzymes, were characterized by paper chromatography. Alanine, glutamic acid and diaminopimelic acids were the major components and some species also contained significant amounts of lysine, serine, glycine, aspartic acid, valine, and leucine. These latter amino acids were not considered by Cummins (1956) to be part of the cell wall complex proper but to represent residues of protein layers after

incomplete digestion with proteolytic enzymes. By analogy our antigens might be polymers associated with the basal mucopeptide structure of the cell wall.

The antigens extracted from C. hofmanni appear to be species-specific since the immunodiffusion tests with hot acid extracts from other corynebacterial species and Staph. aureus failed to give precipitin lines. However, several strains and other species need to be tested. A type-specific protein was obtained from the acid extracts of Streptococci (Lancefield, 1933) and Staphylococci (Verwey, 1940). Previously Cummins (1954) demonstrated the presence of two distinct antigens in some species of corynebacteria: a type-specific protein in C. diphtheriae and a group-specific polysaccharide in several strains of C. diphtheriae and in one strain of C. ovis but not in C. hofmanni, C. xerosis or C. renale.

Species-specific antigens were isolated from the cell walls while group-specific antigens were obtained from the cytoplasm of Mycobacterium, Actinomyces, Streptococcus and Diplococcus by Kwapinski and Snyder (1961). These workers proposed that species and type specificity are defined by certain components of the cell wall, whereas the constituents of the cytoplasm carry common antigenic groups. This concept was also reached by Cummins and Harris (1956) from their investigations of the walls of gram-positive bacteria. The species specificity of antigens A and B also suggests that these antigens may be derived from the cell wall.

The presence of common antigens in various species of corynebacteria with related genera has been reported by various workers. Kwapinski (1956) showed an antigenic relationship between M. tuberculosis and C. diphtheriae, while Slack, Winger and Moore (1961) found cross-reactivity between several strains of C. acnes and Group A. Actinomyces; eight other species of corynebacteria including C. hofmanni (ATCC-10700) did not cross-react with the actinomyces species. An antigenic fraction derived from N. asteroides (Keeler and Pier, 1964) closely resembles the chemical composition of antigens A and B except for the absence of hexosamine and the presence of four additional sugars. A common cell wall polysaccharide antigen had been obtained from strains of Mycobacterium, Nocardia, and Corynebacterium by Cummins (1962) which suggested a taxonomic relationship on basis of cell wall structure.

Two studies on the type of determinant group of corynebacterial antigens have been recently reported. Cummins (1962, 1965) showed that strains of mycobacteria, corynebacteria and nocardia which shared the group antigen contain arabinose and galactose as their principal cell wall sugars. He considered that the specificity of this antigen was conferred by arabinose or a small polymer containing arabinose. Immunofluorescent techniques were used by Jones and Lewis (1966) to demonstrate the importance of alanine in the antibody-combining sites of cellular antigens of C. diphtheriae. Therefore, it appears that the species-specificity and the determinant groups

of antigen A and B may be conferred by a small component such as amino acid, sugar, or amino-sugar.

Antigens A and B appear to be similar in terms of chemical structure, electrophoretic mobility and serological reactivity. The method of extraction and separation required the antigens to be thermostable, acid resistant, and of different molecular size. These antigens may be of value in the taxonomic classification of C.hofmanni and related species.

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