TYPE SPECIFICITY AND PURIFICATION OF THE CAPSULAR ANTIGEN OF A MAJOR TYPE OF *STAPHYLOCOCCUS AUREUS*

by

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A dissertation submitted to the faculty of the University of Utah in partial fulfillment of the requirements for the degree of

Doctor of Philosophy

Department of Microbiology

University of Utah

August 1970

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This Thesis for the Ph.D. Degree

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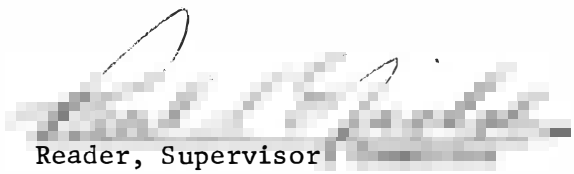

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ACKNOWLEDGMENTS

The author wishes to express his sincere appreciation to Dr. Bill B. Wiley for his counsel and direction in the development of this research and manuscript. He would like to acknowledge Dr. Stanley Marcus, Dr. Douglas W. Hill, Dr. Paul S. Nicholes, and Dr. Marvin Rogolsky for their helpful suggestions throughout the preparation of this thesis.

The author wishes to extend his appreciation to his wife, mother, and father for their patience, encouragement, and aid. Without their help, this thesis would not have been completed.

The investigator wishes to thank the University Research Committee for his University Research Fellowship which supported him during this work.

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ABSTRACT

The Smith diffuse variant and the wound mucoid strain of Staphylococcus aureus were shown to exhibit serologically distinct capsules. The Welwood and K-6 strains of S. aureus were tested to determine their capsular types. Both Welwood and K-6 were found to be representative of the Smith capsular type. An additional 13 isolates of S. aureus from mice were tested. Gel double-diffusion tests and immunoelectrophoresis of staphylococcal antigens disclosed the probable existence of at least two additional capsular types. Passive hemagglutination tests carried out with cells sensitized with 1 mg of antigen per ml showed a multiplicity of cross-reacting antigens. However, cells sensitized either with 0.1 or 0.05 mg of antigen per ml and reacted with antisera absorbed with 10 or 1 ug/ml of homologous antigen showed the presence of a specific antigen in extracts from each strain of S. aureus. Corroborative evidence for a multiplicity of capsular types was obtained by the specific capsular reaction. At least four capsular types of S. aureus were found. The prototypic strains for these antigens are the RLM or wound strain, the Smith diffuse strain, and mouse strains designated 36T and 43R. It was proposed to designate these types 1, 2, 3, and 4, respectively.

Three serologically active components in partially purified capsular material (PPCM) from the wound strain of S. aureus were separated by column chromatography on Sepharose 6B. Chemical analyses of the components following acid hydrolysis showed no significant

qualitative differences in their amino acid contents. The 73-80 ml pool showed 37% glucosamine and 36% reducing sugars. A significant difference was noticed in the glucosamine and reducing sugar values of the 73-80 ml pool as compared with the other pools. Immunoelectrophoretic analysis showed the 73-80 ml pool contained a single serologically active component which migrated anodally. Gel diffusion investigations confirmed the presence of the same serologically active component in PPCM by showing the presence of a reaction of identity between the PPCM and the 73-80 ml pool when they were reacted with specific antiserum. Antiserum absorption studies revealed that the 58-72 ml, 73-80 ml, and 81-100 ml pools all contained capsular antigen. The most active pool in the anticapsular antibody absorption tests was the 73-80 ml pool. This fraction was twice as active as the 58-72 ml pool and eight times more active than the 81-100 ml pool in the absorption of anticapsular antibodies.

INTRODUCTION

Since the description of encapsulation and the demonstration of a specific capsular reaction in Staphylococcus aureus strains, sixteen years ago (Price and Kneeland, 1954), the subject of encapsulation of the organism has abounded in controversy between contending groups of researchers. The first group of researchers (Price and Kneeland, 1954, 1956; Wiley, 1959, 1961, 1963, 1964) demonstrated encapsulation of S. aureus by using the specific capsular reaction (SCR). In this group, Wiley 1959, 1961, 1963 , presented experimental evidence which showed a possible relationship between encapsulation and virulence of S. aureus in embryonated eggs. The term SCR was used instead of "quellung" because no data were available to indicate that an increase in capsular size occurred when anticapsular antibodies and encapsulated S. aureus were mixed. The term specific capsular reaction refers to a microprecipitin reaction occurring at the cell surface (Tomcsik, 1956; Baker and Loosli, 1966). The second group of researchers (Koenig et al., 1962-1965) compared the surface antigens from a virulent and an avirulent strain of S. aureus, the Smith diffuse and compact strains of S. aureus. The results of their research indicated that the Smith diffuse strain of S. aureus contained a surface antigen not present in the compact organism. Lacking definitive proof of encapsulation, i.e., demonstration of an SCR, Koenig (1962) and Koenig et al. (1962, 1965) elected to refer to the antigen present on the Smith diffuse strain of S. aureus and absent from the Smith compact strain as a surface antigen.

The third group of researchers (Morse, 1960, 1962, 1963; Mudd, 1965; Mudd et al., 1965; and Hisatsune et al., 1966, 1967a, 1967b) believe that only one strain of S. aureus, the Smith strain of S. aureus, is encapsulated. Their criterion for encapsulation was the demonstration of capsules by the India ink technique which is less sensitive and lacks specificity. They in turn elected to disregard the evidence presented by the first group.

Work to be described in this thesis will establish that at least two serologically distinct capsular types of S. aureus exist. Evidence will also be presented to show that possibly a multiplicity of capsular types exist among S. aureus strains from animal and human sources. Finally data will be presented showing that the capsular antigen of a major type of encapsulated S. aureus, the RLM-wound strain, can be purified by means of column chromatography.

LITERATURE REVIEW

Reports of Encapsulation of Staphylococcus aureus

Staining Procedures. Gilbert (1931) isolated a strain of S. aureus from the pericardial and peritoneal fluid of a human with acute ulcerative gonococcal endocarditis. Gilbert's strain was mucoid and exhibited halos in wet mounts in India ink suggesting that the organism was encapsulated. When this mucoid strain was subcultured and refrigerated for one month, the replated culture showed a rough (R) to smooth (S) dissociation. Guinea pig passage of the R strain resulted in selection of the S strain. This was the first report in the literature of animal passage used for selection of encapsulated strains of S. aureus.

Lyons (1937) reported that a 3-8 hour old culture of toxigenic and non-toxigenic strain of S. aureus could produce capsules if grown in the proper broth. Toxigenic strains produced capsules in blood broth but not in serum. The non-toxigenic strain produced capsules in infusion broth but not in blood. A colloidal silver staining method and a carbol fushsin-methylene blue staining technique were employed to detect encapsulation. In 1939 Spink using the same staining procedures as Lyons (1937) could not show encapsulation of S. aureus isolated from patients.

Since none of the previous reports of encapsulation were confirmed because the strains of staphylococci were unobtainable or

because of the unreliability of the staining procedures for showing staphylococcal capsules (Spink, 1939), the subject of encapsulation remained controversial. In 1930 Dubos isolated a strain of S. aureus from a patient with osteomyelitis. It was characterized by Smith and Dubos (1956) as being coagulase positive and belonged to phage type 44A/42E. This strain of S. aureus can still be obtained from Dr. G. Koenig, Department of Microbiology, Vanderbilt University, Nashville, Tennessee. The Smith strain has been used by many authors to study encapsulation of S. aureus. Hunt and Moses (1958) isolated extremely virulent and avirulent S. aureus variants from the parent Smith culture. The virulent Smith strain grew as diffuse comet-shaped colonies in serum soft agar and the avirulent Smith strain grew as round, compact colonies. The LD_{50} of the Smith diffuse strain in mice was 580 organisms when injected with 5% hog gastric mucin (Hunt and Moses, 1958). Finkelstein and Sulkin (1957, 1958) observed that the compact variant was lysed by phage type 44A and agglutinated by a standard staphylococcal grouping antiserum. The diffuse variant was untypeable and was not agglutinated by any of the standard grouping sera. Morse (1962) observed halos surrounding the Smith strain of S. aureus when examined in India ink wet mounts. Isolated material from supernatant fluids, designated Smith surface antigen SSA, of the Smith strain of S. aureus was used to absorb an antiserum to remove agglutinins from the serum. The results of the test was used as supporting evidence that the SSA was a surface antigen. It was believed (Morse, 1962) that SSA was the antigen from the capsules observed in wet India

ink mounts of the Smith organism. Koenig (1962), using the Smith diffuse strain of S. aureus, did not observe halos in India ink wet mounts of the organism, nor was he able to observe capsules by the methods of Novelli (1953), Lyons (1937), or Butt et al. (1956). Intraperitoneal inoculation of the Smith diffuse organisms resulted in a higher mortality for mice than intravenous inoculation (Koenig et al., 1962b). This was explained on the basis that phagocytosis in the peritoneal cavity occurred later than intravascular phagocytosis. It was shown by Koenig and Melly (1965), using the gel diffusion, that the Smith diffuse strain possessed an antigenic component which was not present in the Smith compact strain. They also noted that the Smith diffuse strain was bound coagulase negative, whereas the Smith compact strain was bound coagulase positive. From these observations, they postulated that bound coagulase in the Smith diffuse strain was located beneath the antigenic structure absent from the Smith compact strain and, therefore, not detectable. In 1962, Lenhart et al. reported the observance of halos surrounding the cocci in India ink mounts of the Smith strain. Like Morse (1962), Lenhart et al. (1962) did not clearly indicate whether or not the Smith diffuse strain or the Smith compact strain was used. A comparison between the wound mucoid strain of S. aureus and the Smith strain of S. aureus used by Lenhart was reported by Mudd and DeCourcy (1965). Mudd (1965) and Mudd and DeCourcy (1965) compared the mucoid strain of Wiley (Wiley, 1959) and a variant isolated from a culture of the Smith strain by Lenhart (1962) for the presence of halos surrounding the cocci in India ink wet mounts.

Since the wound mucoid strain of S. aureus did not show halos in India ink wet mounts and Lenhart's variant of the Smith strain of S. aureus did, they concluded that the only prototypic encapsulated strain of S. aureus was Lenhart's variant of the Smith strain even though the wound strain underwent specific capsular reactions and Lenhart's variant of the Smith strain at this time had not been shown to do so. Culture of the Smith strain studied by Mudd (1965) and Mudd and DeCourcy (1965) and Morse (1962) were obtained upon request. After growing each strain in serum soft agar, it was apparent that both strains were compact type variants of the Smith strain. In 1968 Wiley and Maverakis published a full report on encapsulation of the Smith diffuse strain. This report will be reviewed in the following section.

The Specific Capsular Reaction. Price and Kneeland (1954, 1956) using specific immune serum were able to demonstrate capsular swelling with mucoid and viscid variants of S. aureus. The parent strain, RL, was isolated from a staphylococcal pneumonia patient. The organism was cultivated originally on rabbit blood agar, transferred through nutrient broth, and then passed through embryonated eggs with influenza virus. Subcultures from the eggs onto plating media yielded a colony that was watery in appearance but was difficult to scrape off. This was designated the RL mucoid variant (RLM). A viscid variant (RLV), lacking the watery appearance of the mucoid strain but retaining the adherent tendency resulted from the growth of the parent strain in allantoic fluid only. Formalized or heat-killed vaccines of the whole mucoid or viscid organisms were used to immunize rabbits. This

procedure was continued for a month to provide the immune serum for the capsular swelling reaction. Anti-mucoid or anti-viscid serum mixed with either viscid or mucoid organisms produced a positive specific capsular reaction. Price and Kneeland (1954) were neither able to explain why passage of the RL strain of S. aureus in embryonated eggs with influenza virus resulted in the isolation of the RLM strain nor were they able to repeat the experiment.

The wound strain of S. aureus was isolated from a sutured wound. This strain of S. aureus was grown in glycerol broth (Wiley, 1959) using the method of Bigger, Boland, and O'Meara (1927) for selecting mucoid strains of S. aureus. From the glycerol broth culture, the mucoid wound strain was isolated. Killed formalized suspensions of the wound mucoid strain were used to immunize rabbits or roosters by the method of Alexander et al. (1946). Hyperimmune rabbit or rooster serum and encapsulated wound mucoid staphylococci were mixed and observed under the oil immersion lens; the capsular region of the S. aureus was clearly delineated (Wiley, 1959). In 1961 Wiley showed that the RLM and wound strains of S. aureus had similar LD_{50}'s for embryonated eggs, phage sensitivity patterns, and were of the same capsular type. The wound mucoid strain of S. aureus was extensively studied by Wiley (1959, 1961, 1963) and by Wiley and Wonnacott (1962) and by Wiley and Maverakis (1968) and found to be the most commonly isolated encapsulated staphylococcus from clinical sources. Stamp and Hobbs (1967) seemingly unaware of the previous reports of Wiley (1959,

1961, 1963), confirmed the work of Price and Kneeland (1954, 1956) and reported the occurrence of antibodies specific for the RLM-wound strain in unimmunized rabbit sera. Wiley and Maverakis (1968) presented unequivocal evidence for the encapsulation of the Smith diffuse strain. The difference in virulence of the wound strain and the Smith diffuse strain was postulated to be due to the presence of anti-wound antibodies in the sera of normal mice.

Chemical Characterization of Capsular Material. Wiley and Wonnacott (1962) reported partial purification and partial chemical characterization of the capsular material from the wound mucoid strain of S. aureus. Chemical analysis indicated that cell-free capsular material contained glucosamine, four amino acids (glycine, alanine, glutamic acid, and lysine), glycerophosphate, and an unidentified compound, possibly ribitol phosphate. A nitrogen content of 8.02%, and organic phosphorus content of 3.98%, reducing sugar activity of 26.2%, and a hexosamine content of 26.9% were reported. Absorption experiments revealed that when the capsular material was added to sera, it removed protective antibodies and antibodies necessary for the specific capsular reaction. Gel diffusion tests showed the isolated partial purified capsular material (PPCM) to contain four antigens when tested in a 1% concentration against specific rabbit antiserum.

Hisatsune et al. (1966, 1967a, 1967b), reported the separation of various components from the capsular material of the wound strain of S. aureus. Their separation procedure consisted of repeated trichloracetic acid (TCA) extraction of capsular material. They used

exclusion chromatography and ion exchange chromatography to further separate various components in the TCA extracts. The four components observed in immunoelectrophoresis were designated precipitin 1 (P1), precipitin 2 (P2), precipitin 3 (P3), and precipitin 4 (P4). However, none of these components turned out to contain the capsular material as indicated by their report. The P1 component upon which most of their work was done was characterized as a peptide containing glutamic acid, lysine, alanine, and glycine. The other components were not chemically characterized and studied as extensively.

During the same year that Wiley and Wonnacott (1962) reported on the isolation and chemical characterization of capsular material, Morse (1962) reported on the isolation and properties of a surface antigen of Staphylococcus aureus. Lacking definitive proof of its location but with evidence that it was located on the cell surface, the isolated antigen was designated Smith surface antigen (SSA). The chemical characterization of SSA showed that it contained 8.49% nitrogen, .02% phosphorus, .03% sulfur, and 21.6% total acetyl groups. The reducing sugar content was 14.2%, hexosamine 25.0%, and amino acids, 28.9%. Morse (1962) reported SSA to contain 70% carbohydrate. However, the only carbohydrate content reported in his data were the reducing sugar and hexosamine values indicated above. In 1963-1964, Haskel and Hanessian reported the isolation and chemical characterization of staphylococcal polysaccharide antigen (SPA). The SPA was isolated from S. aureus strain 05068 by extraction with 0.1 N acetic acid at 100 C and was chemically characterized as 2 (N-acetylalanyl) amino -

2-deoxy-D-glucuronic acid residues linked by 1-4 type linkages.

As is evident from this review, a great deal more work will be required to completely characterize the capsular antigen of the major capsular type represented by the RLM-wound strain.

MATERIALS AND METHODS

Strains. S. aureus strain W (the wound mucoid strain) and strain Sd (the Smith diffuse strain) had been described previously (Wiley and Maverakis, 1968). Welwood and K-6 strains were received from R. Tompsett, Baylor University Medical Center, Dallas, Texas. The mouse strains, 36T, 43R, 47R, 50R, and 51R, were isolated on Mannitol Salt Agar plates (Difco) inoculated from throat and rectal swabs taken on DAL Swiss Webster mice. Single colonies were tested for mucosity after 48 hours of incubation at 37 C.

Media and Cultivation. Mannitol Salt Agar plates were used for primary isolation of S. aureus. For cloning and transferring cultures, buffered nutrient glycerol (BNG) or buffered nutrient dextrose (BND) agar plates were used (Wiley, 1961). When fermentation tests revealed that a given strain of S. aureus did not vigorously ferment glycerol, BND was used. BNG or BND was used in the coagulase test, for specific capsular reactions, and for cultivation of organisms used in the extraction procedures for preparation of capsular antigens. The semi-synthetic medium containing Casamino Acids, glycerol or dextrose in a broth was described in detail previously (Wiley, 1961). Incubation was carried out at 37 C.

Coagulase Tests. Tube coagulase tests were carried out as described previously (Wiley and Maverakis, 1968).

Acid Extraction of Antigens. The procedure described by Morse (1963) for isolation of Smith surface antigens was slightly modified.

Acid extracts were centrifuged prior to neutralization at 13,000 x g for 1 hour in a Sorvall centrifuge (model SS-4) with a GSA rotor and again at 13,000 x g for 1 hour for clarification after neutralization. Five volumes of acetone were then added to each supernatant fluid to precipitate the antigens, and the mixtures were stirred and refrigerated overnight at 4 C. The following day the supernatant fluids were decanted and the precipitate was dissolved in a minimal volume of distilled water. The resulting solutions were dialyzed against distilled water in the cold at 4 C for 3 days. The water was changed twice daily. The dialyzed material was lyophilized using a Virtis automatic freeze dryer, model 10-010 (Virtis Research Equipment, Gardner, New York) and stored over Drierite in a dessicator until needed.

Preparation of Partially Purified Capsular Material (PPCM). The method for isolation of capsular antigens from culture supernatant fluids was previously described (Wiley and Wonnacott, 1962); it was based on those employed for the isolation of capsular material of pneumococcus (Heidelberger et al., 1939). The capsular material used in column chromatography was dissolved in distilled water and centrifuged for 1 hour at 9750 x g by use of a Sorvall RC-2B centrifuge with SS 34 rotor. The supernatant fluid was removed and dialyzed against distilled water at 4 C for 3 days. The supernatant fluid from the dialysis sacks was removed and again centrifuged as mentioned above. The supernatant fluid was then lyophilized and stored over Drierite in a dessicator until needed.

Specific Capsular Reaction (SCR). The specific capsular reaction was carried out as previously described (Wiley and Maverakis, 1968).

Vaccines and Immunization of Animals. The vaccines were prepared as before (Wiley and Maverakis, 1968) and immunization of animals was carried out as before (Wiley and Maverakis, 1968).

Phosphate-buffered Saline (PBS). The composition of the PBS used throughout the work was as follows: anhydrous Na_2HPO_4, 1.58 g, anhydrous KH_2PO_4, 0.346 g; NaCl, 8.0 g; and water to make 1 liter. The pH was 7.2 to 7.3.

Absorption of Antisera. Antisera and antigen solutions were mixed in equal volumes, incubated at 37 C for 2 hours and refrigerated overnight. The mixture was centrifuged for 1 hour at 1,085 x g in a type A Sorvall centrifuge. The antigen solutions for the indirect hemagglutination test were prepared in PBS in concentrations of 10 ug/ml and 1 ug/ml. Purified antigen after column chromatography was dissolved in PBS in a concentration of 1.0 mg/0.1 ml.

Precipitin Test. A qualitative precipitin test was performed using undiluted rooster anti-wound staphylococcal antiserum and various dilutions of partially purified capsular material. The rooster serum was clarified by the method of Updyke and Conroy (1956). The capillary tubes were first dipped in the partially purified capsular material (CM) and the CM solution was allowed to rise by capillary action to the mid-mark. The capillary tubes were then dipped into the antiserum, allowing it to push the precipitinogen upwards and fill the remainder of the tube. The tubes were then incubated at 37 C for two hours and

refrigerated for three days. Controls consisting of antiserum and saline and partially purified capsular material and saline were included. The precipitin test was used in the early exclusion chromatographic studies to detect serologically active components of partially purified capsular material eluted from the column. The reactions were read at 24 hours and 74 hours.

Gel Diffusion Tests. The technique of Ouchterlony, described in Kabat and Mayer (1967) was followed. Unwashed agar (Difco) was used in a concentration of 1% with merthiolate at a final concentration of 1:10,000 added as a preservative. Plastic petri dishes (90 mm in diameter) were filled with 15 ml of molten agar per dish. After the agar had hardened, the wells were cut with a Feinberg agar-gel cutter (Colab Laboratories, Inc., Chicago Heights, Illinois) having a central well (10 mm in diameter) and six peripheral wells (7 mm in diameter). To the peripheral wells, a 1% antigen solution in PBS (pH 7.3) was added; to the center well, 0.2 ml of antiserum was added. The wells were filled only once. A micro gel diffusion test was used on the column purified antigens. The preparation of the agar was identical to that prepared for immunoelectrophoresis. The wells were 5 mm apart and consisted of a central well with six peripheral wells. The wells were 1 mm in diameter.

Immunoelectrophoresis. Colab gel contact troughs (no. 2576) were connected to a Vokam 2541 power supply (Colab Laboratories). A barbital buffer, ionicity 9.1, pH 8.5, was used (LKB, Stockholm 12, Sweden). A slide frame (LKB, no. 6801A) containing six glass slides (LKB, no.

6890-03) was filled with Noble agar (Difco). The proportions of agar-buffer-water in the solution were 1:25:75. The slide frame was placed on the gel contact troughs at an angle. A constant current of 1.2 milliamperes (ma) was applied across the terminals. Electrophoresis was carried out for 1 hour, after which troughs were cut in the agar and rabbit antiserum was added. The slides were then placed in a moist chamber for up to 72 hours at room temperature to allow development of the precipitin arcs.

Indirect Hemagglutination Test. Rabbit red blood cells were obtained from the central ear artery of the rabbit and mixed with Alsever's solution (Campbell et al., 1963). Samples of the rabbit blood were washed three times in cold PBS (pH 7.3). The cells were packed by centrifugation at 755 x g for 30 minutes in a type A Sorvall centrifuge and diluted to make a 5% suspension. The partially purified antigen (PPCM) extracts prepared by acid extraction of whole cells or by concentration from the culture supernatant fluids were diluted 1:1,000, 1:5,000, 1:10,000, and 1:20,000 in PBS (pH 7.3). Equal volumes of 5% cells and dilutions of extracts or crude capsular antigen were mixed. The mixtures were incubated in a water bath for 2 hours at 37 C. The test tubes were gently inverted every 15 minutes to resuspend the settled erythrocytes. At the end of 2 hours, the cells were centrifuged and washed three times with cold PBS (pH 7.3). The antiserum was diluted 1:4 through 1:512 in twofold dilutions; 0.12 ml of each dilution of antiserum was then added to each test tube, followed by 0.5 ml of saline and 0.1 ml of sensitized cells. The final dilutions were

considered to be sixfold greater than the initial dilutions, owing to the additional dilution by saline and sensitized cells. The tubes were incubated at 37 C for 2 hours, and the tubes were shaken every 30 minutes during incubation.

Determination of Mouse Mortality Ratios. Estimations of virulence of staphylococcal isolates were made by the method previously described (Wiley and Maverakis, 1968).

Ultraviolet Absorption. Ultraviolet absorption studies were carried out using a Beckman-DB spectrophotometer having a one cm light path (Beckman Instruments Co., Palo Alto, California). The nomograph of Warburg and Christian (1942) was used in the determination of nucleic acid concentrations.

Determination of Total Hexoses. (a) The anthrone reagents and procedure were based on the work of Scott and Melvin (1953) and reported by Kabat and Mayer (1967). To increase the sensitivity of the test, 1 ml of anthrone reagent and 0.05 ml of test solution were used in the test.

(b) The primary cysteine-sulfuric acid reaction for total hexoses was also investigated. The reagents and procedure employed were those of Dische (1949, 1955) and reported by Kabat and Mayer (1967).

Exclusion Chromatography. Gel filtration was used for separation of the various components of the partially purified capsular material. The columns were packed according to the Sephadex booklet, "Gel Filtration in Theory and Practice" and "Agarose Gels in Bead From" (Pharmacia Chemical, Inc.).

Sephedex G-100 and G-200 gels were suspended in a 1 M NaCl phosphate buffer of the following composition: NaCl 58 g, anhydrous Na_2HPO_4, 1.58 g, anhydrous KH_2PO_4, 0.346 g per liter, pH 7.2 to 7.3 and sodium azide .02% as preservative. The G-100 and G-200 gels were boiled on a boiling water bath for 5 hours to swell the gels and cooled to room temperature. The fine material was removed by decantation. After decantation, fresh buffer was added and the gel particles were resuspended by stirring. This procedure was repeated six times. The gel was then packed into a Sephadex column 2.5 cm in diameter. The gel bed was 40 cm. The final operating hydrostatic pressure was 10 cm. Buffer was allowed to flow through the column for three days. A 4 mg/ml sample of partially purified CM was placed on the top of the column and eluted with buffer at a flow rate of 7.5 ml/hr.

Sepharose 6B was purchased from Pharmacia Chemical, Inc., in slurry form. A 0.9 x 162 cm column was packed with Sepharose 6B. The gel was washed with the above buffer and packed in the column by the procedure described in the above two booklets. A second experiment was run using the same gel and a column 0.9 x 171 cm but, in this instance, the gel was washed with distilled H_2O containing .02% sodium azide as an eluting solvent. The final operating hydrostatic pressure was 33 cm. Both packed columns were calibrated with the following: D-glucosamine--M.W. 211 (Eastman Organic Chemicals, Rochester, New York), ovalbumin--M.W. 4.5×10^4 (Mann Research Lab, New York, New York), bovine albumin--M.W. 6.7×10^4 (Mann Research Lab, New York, New York), porcine globulin--M.W. 1.6×10^5 (Mann Research Lab, New

York, New York), equine apo ferritin--M.W. 4.8×10^5 (Mann Research Lab, New York, New York), and blue dextran--M.W. 2×10^6 (Pharmacia Chemicals, Inc.). The fractions were collected hourly using a Rinco fraction collector (Rinco Instrument Co., Greenville, Illinois). After determination of the average flow rate of the large column and with a knowledge of the distribution of various fractions in the collection tubes, fractions for analysis were collected in a bulk type operation by allowing the effluent to drop into a graduated cylinder for the number of hours necessary to collect the fractions desired. All fractions were dialyzed exhaustively and lyophilized.

Calculation of Kav. The constant, Kav, is the partition coefficient available when the stationary phase is considered to be the whole gel phase. The Kav value is determined by the molecular dimension of the substance under study and is expressed by the following formula:

$$Kav = \frac{V_e - V_o}{V_t - V_o}$$

V_e = elution volume

V_o = void volume

V_t = total volume of the gel

Hydrolysis. For chemical analysis of samples, hydrolysis was carried out using 3 N HCl plus an equal volume of sample containing 1 mg/ml. The samples were put into sealed ampoules and boiled for 1 hour in a water bath. The time of hydrolysis was based on the previous studies of Wiley and Wonnacott (1962) and allowed for maximal liberation of reducing sugars, hexosamines, and amino acids from the sample. The ampoules were cooled and opened and transferred to a 1 ml volumetric

flask. A drop of phenolphthalein was added and samples were then neutralized by the addition of .05 N NaOH. For analysis of the hexosamines and amino acids, the samples were placed in a vacuum dessicator containing NaOH pellets and H_2SO_4 and evaporated to dryness. The samples were transferred with distilled water or "machine buffer" to volumetric flasks depending on whether or not the samples were to be analyzed for hexosamines or amino acids.

Ninhydrin Test. The formula for the ninhydrin reagents was taken from Beckman Technical Bulletin (T B6082A, March 1962, Palo Alto, California) and was based on the research of Spackman, Stein, and Moore (1958). The ninhydrin spot test was run by placing one drop of ninhydrin reagent and one drop of hydrolyzed sample on a slide. The mixture was then heated in a drying oven for 10 minutes at 100 C. The modified ninhydrin test was run in the following manner: An equal volume of samples (0.2 ml) plus 3 N HCl (.2 ml) were placed in screw capped tubes and hydrolyzed for 1 hour. Then 0.2 ml of an acetate buffer, 4 M pH 5.5 (Kabat and Mayer, 1967), and 0.2 ml of ninhydrin solution was added and the screw caps replaced. The tubes were returned to the boiling water bath for an additional twenty minutes. The tubes were cooled to room temperature and 3 ml of 50% ethanol added. The color development was read at 570 mu.

Amino Acid Analyses. Qualitative and quantitative amino acid analyses were made using a Beckman Model 120 B amino acid analyzer (Beckman Instruments, Inc., Palo Alto, California). Reagents were prepared according to the Beckman Model 120 B instruction manual.

Interpretation of chromatograms and calculations were performed according to the instruction manual. A Beckman amino acid calibration mixture was used for the standardization of the machine runs.

Hexosamine Determination. (a) A spot test devised to detect small quantities of glucosamine in column volumes was employed by using 1/4 of the required sample and 1/4 of the reagents required in the Elson-Morgan method (Kabat and Mayer, 1967). The test provided a means of conserving sample and reagents. The test could detect 3 ug of glucosamine.

(b) The Elson-Morgan method, modified by Kabat and Mayer (1967), was used for qualitative and quantitative analysis of the pooled volumes eluted from the column. The samples contained from 100 to 300 ug of the material to be analyzed. Where possible, samples were run in triplicate. The test was read in a Bausch and Lomb Spectronic-20 spectrophotometer at 540 mu. The test could detect 10 ug of glucosamine.

Reducing Sugar Determinations. The method of Schales and Schales for determination of reducing sugars was used. The samples contained from 160 to 420 ug and the test could detect 30 ug of reducing sugar. Where possible, samples were run in triplicate. The loss of color was read in a Bausch and Lomb Spectronic-20 spectrophotometer at 540 mu.

RESULTS

It was decided to first examine two strains of S. aureus well-known to workers in the staphylococcal field to ascertain whether or not they are encapsulated and to which capsular type they belong. These strains, designated Welwood and K-6, were received from R. Tompsett, Baylor University Medical Center, Dallas, Texas, and were known to be mouse-virulent. A high-titered rooster antiserum against S. aureus Sd and a rabbit antiserum against S. aureus W were used in the specific capsular reactions carried out with these strains. Table 1 shows the results of the specific capsular reactions. Both K-6 and Welwood proved to be representative of S. aureus Sd and exhibited mortality ratios in white mice similar to that of the diffuse variant of the Smith strain.

It was postulated that mice might owe their susceptibility to Sd staphylococci to lack of contact with the organism or absence of protective antibodies; a survey of mice for carriage of S. aureus was undertaken. One hundred mice were surveyed to assess their carriage of S. aureus. Particular attention was focused on possibly encapsulated strains occurring naturally in mice. Both throat and rectal swabs were taken. All swabs were streaked directly on Mannitol Salt Agar. Mannitol fermenting colonies were picked off and examined directly in wet mounts of India ink. Mucosity of the colonies was also noted at the time of picking. All isolates were tested for free coagulase and clumping factor. None of the isolates reacted with the

TABLE 1

Specific Capsular Reactions of Four Strains of S. aureus

Strain	Antiserum prepared against:[a]	
	Sd	W
Sd	+	-
K-6	+	-
Welwood	+	-
W	-	+

[a]Positive reaction (+), negative reaction (-).

antiserum against the Sd variant strain of S. aureus in the specific capsular reaction. Since we were interested in discovering possibly different capsular types, we selected strains of greatest mucosity showing evidence of a halo in India ink mounts and negative-specific capsular reactions with anti-W and anti-Sd sera to be used for immunization of rabbits. Formalinized vaccines were used to immunize the animals. Antigens for immunoelectrophoresis were prepared by the technique described in Materials and Methods.

The acid extracts and partially purified capsular material (PPCM) were made up to a concentration of 1% in PBS for use in immunoelectrophoresis. The results with acid extracts and PPCM were similar; hence, only the results with acid extract will be presented. Two antisera were quite specific and did not react with acid extracts prepared from heterologous strains. These were the antisera prepared against the Sd and the 43R strains. The latter strain was isolated from the rectum of a mouse. The immunoelectrophoretic patterns of the acid extracts prepared from the Sd and 43R strains, when reacted with their homologous antisera, are shown in Figure 1. The acid extract of Sd gave a single precipitin arc with homologous antiserum, whereas the acid extracts from the W, 36T, or 43R strains did not react with Sd antiserum (Fig. 1, a and b). The acid extract from the 43R strain gave two precipitin arcs with homologous antiserum, whereas the acid extracts from Sd, W, and 36T did not react with 43R antiserum (Fig. 1, c and d). Immunoelectrophoresis indicated that acid extracts from the Sd and 43R strains were serologically distinct from each other and any of the other strains.

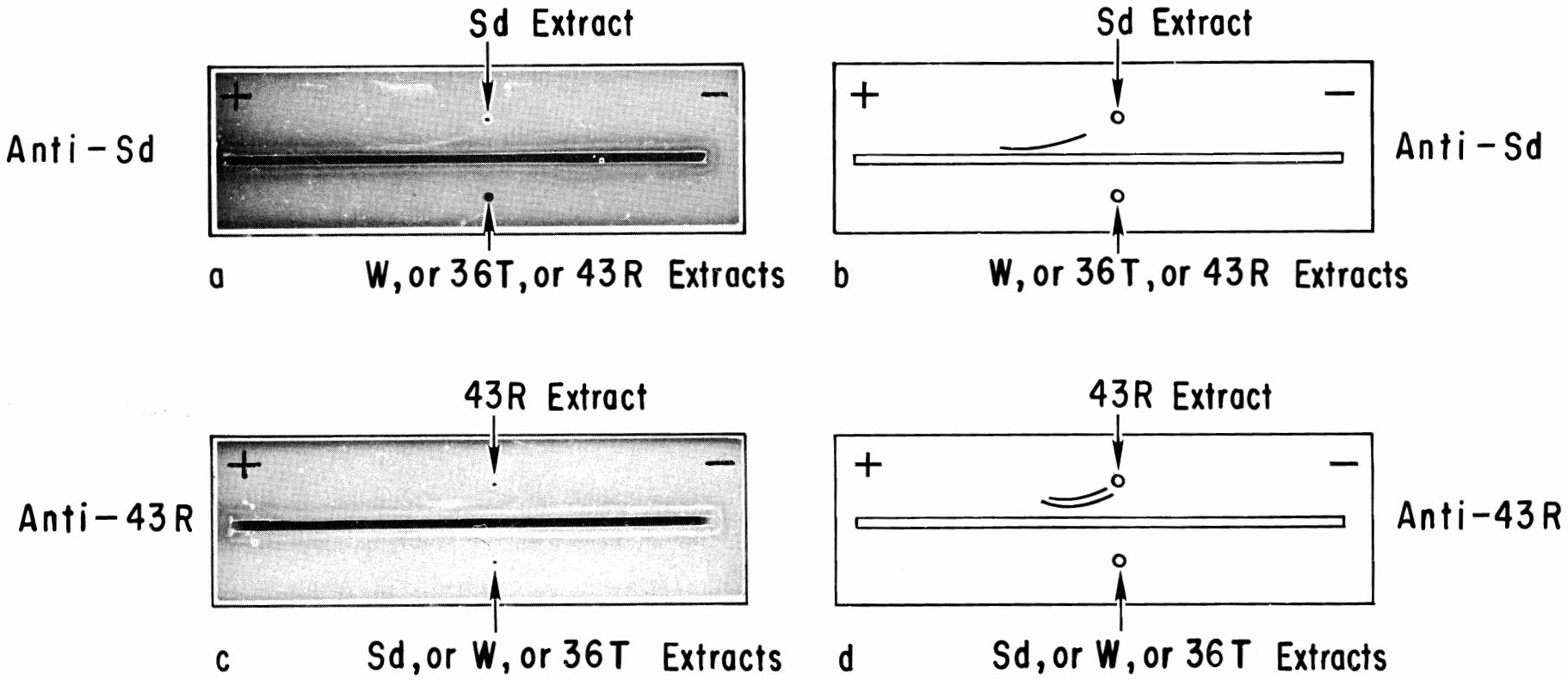

Figure 1. Immunoelectrophoresis of antigen extracts. Immunoelectrophoresis of acid extracts of encapsulated staphylococcal strains. Development of precipitin arcs was allowed to proceed for 72 hours at room temperature, after which the photographs (a and c) were made; (b and d) diagrammatic representations of the reactions, shown for purposes of clarity.

The situation with the other strains was more complicated. The original strain with which so much of our work has been done is designated the W strain. Another strain included here and designated the 36T strain originated in the throat of a mouse. Figure 2 shows the results of immunoelectrophoresis of acid extracts of these strains when reacted with their homologous antisera. The acid extract of the W strain produced three precipitin arcs when reacted with homologous antiserum. Neither 36T nor 43R acid extracts exhibited any reactivity with W antiserum (Fig. 2, a and b). The acid extract prepared from the 36T strain yielded two precipitin arcs when reacted with homologous antiserum. The acid extracts of the Sd and W strains did not react with 36T antiserum (Fig. 2, c and d). Figure 3 (a and b) depicts the three precipitin arcs exhibited by W acid extract when reacted with homologous antiserum. The acid extract of the Sd strain cross-reacted with W antiserum to produce a weak precipitin arc. The acid extract of 43R yielded a weak precipitin arc when reacted with 36T antiserum (Fig. 3, c and d). It was considered possible that the cross-reaction between the Sd acid extract and W antiserum and that between 43R acid extract and 36T antiserum could have resulted from the presence prior to immunization of natural antibodies against antigens in the acid extracts. Such a possibility was not remote, since previous work in our laboratory had disclosed the frequent presence of natural antibodies to staphylococci in normal rabbits (Wiley, 1961). The results of immunoelectrophoretic tests carried out with the preimmunization serum from the rabbits used to prepare the W antiserum and 36T antiserum, respectively,

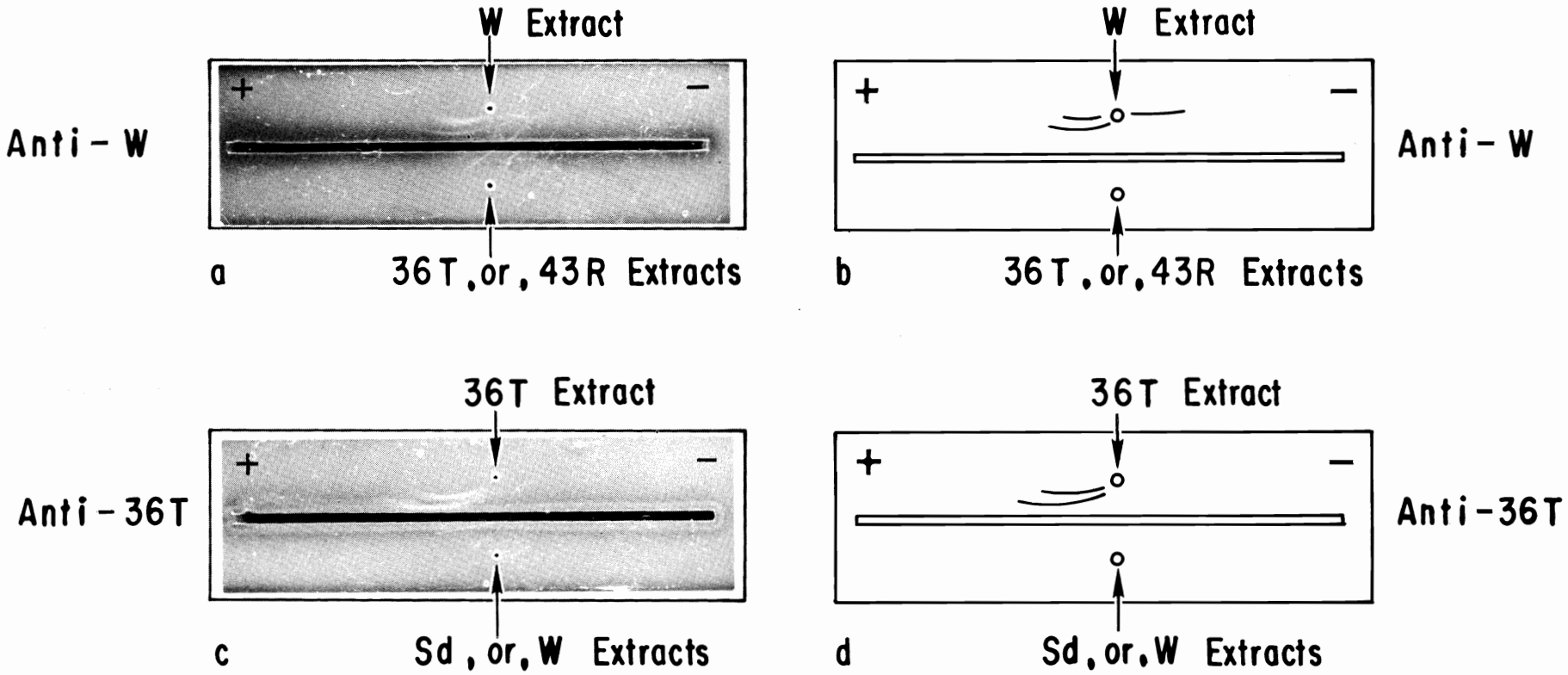

Figure 2. Immunoelectrophoresis of antigen extracts. Immunoelectrophoresis of acid extracts of encapsulated staphylococcal strains. Development of precipitin arcs was allowed to proceed for 72 hours at room temperature, after which the photographs (a and c) were made; (b and d) diagrammatic representations of the reactions.

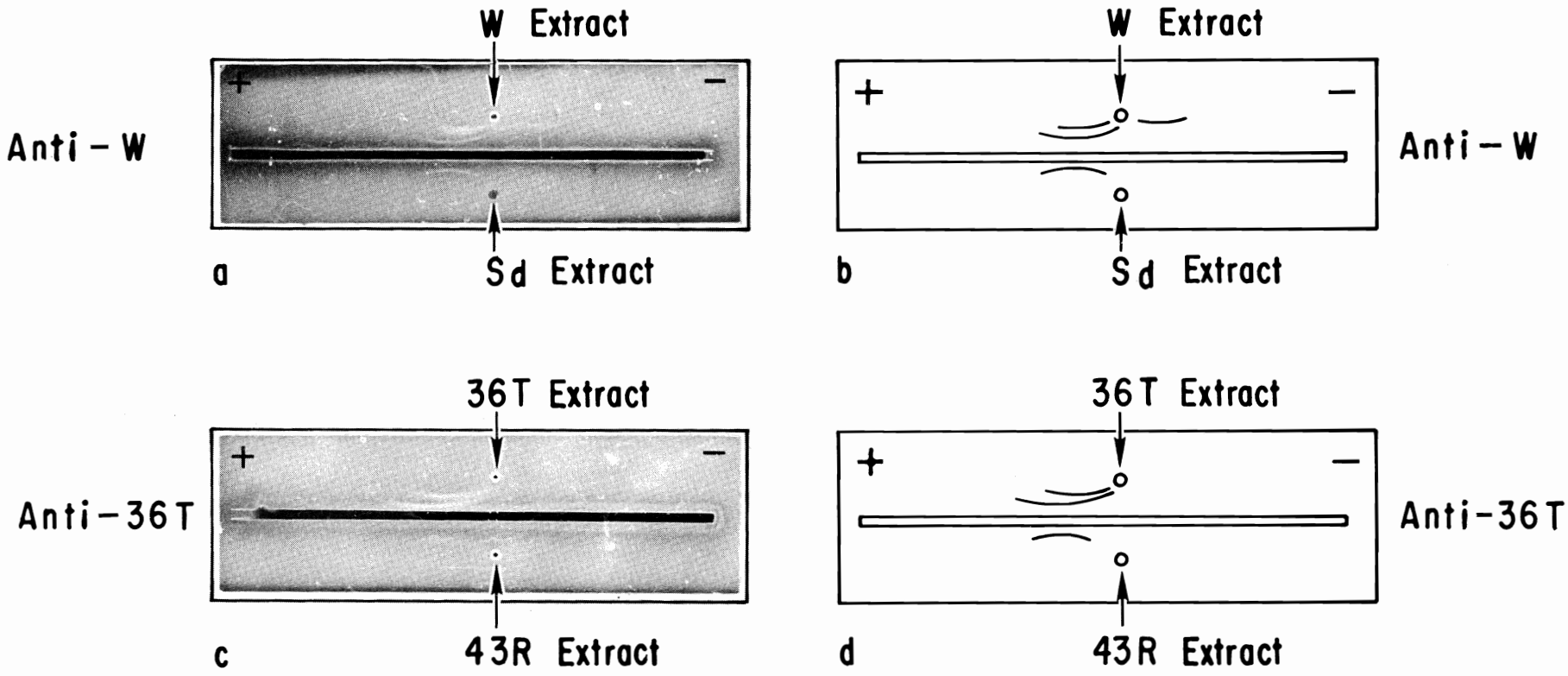

Figure 3. Immunoelectrophoresis of antigen extracts. Immunoelectrophoresis of acid extracts of encapsulated staphylococcal strains. Development of precipitin arcs was allowed to proceed for 72 hours at room temperature, after which the photographs (a and c) were made; (b and d) diagrammatic representations of the reactions.

were negative; i.e., 43R acid extract and Sd acid extract failed to react with the preimmunization serum, thus invalidating any hypothesis explaining cross-reactivity as being due to natural cross-reacting antibodies. It was necessary to postulate that a cross-reacting antigen was present in acid extracts of the Sd strain which reacted with an antibody in the W antiserum. Similarly, a cross-reacting antigen in the acid extract of 43R reacted with the antiserum against 36T. These cross-reactions appeared to be unidirectional, since W acid extract did not react with antiserum to Sd organisms, nor did 36T acid extract react with antiserum to 43R organisms. It could be postulated, then, that Sd organisms possibly contained more of the cross-reacting antigen than W organisms and that 43R organisms possibly contained more of the antigen than 36T organisms. That the W and 36T strains did produce the cross-reacting antigen was evidenced by the ability of these strains to stimulate antibodies in rabbits which would cross-react with the acid extracts of Sd and 43R, respectively.

A summarization of the various cross-reactions just described is shown in Table 2. It is apparent that, except for the two cross-reactions described, the acid extracts and antiserum for each strain were specific.

The antisera, acid extracts, and PPCM preparations used in the immunoelectrophoresis tests were then examined by means of the passive hemagglutination test. To avoid unnecessary absorption of antisera with rabbit erythrocytes to remove isoagglutinins, the antisera were first tested against unsensitized rabbit cells for the presence of

TABLE 2

Summarization of Immunoelectrophoresis Reactions

Antigen extracted from:	Antiserum prepared against:			
	W	Sd	36T	43R
W	3[a]	-[b]	-	-
Sd	1	1	-	-
36T	-	-	2	-
43R	-	-	1	2

[a]Indicates the numbers of precipitin arcs.

[b]Indicates the absence of precipitin arcs.

isoagglutinins. All antisera showed an isoagglutinin titer no higher than 1:2 against the cells of the rabbit being used. The erythrocytes from this rabbit were used as the source of blood for the hemagglutination tests. The antisera were diluted 1:4 to eliminate any positives resulting from isoagglutination. The final dilution of the antiserum after the addition of 0.5 ml of saline and 0.1 ml of 5% cells was 1:24 in the first tube. In the passive hemagglutination tests, cells sensitized with antigen 1:1,000 or 1:5,000 showed marked cross-reactivity when reacted with unabsorbed or absorbed antisera. All sera agglutinated these sensitized erythrocytes equally (Table 3). Antigen solutions diluted 1:10,000 or 1:20,000 were then used for sensitization of cells. It was hoped that sensitization of erythrocytes with the least amount of antigen required would eliminate binding to the cells of cross-reacting antigens. Rabbit erythrocytes were sensitized with antigen solution diluted 1:10,000 (Table 4). The antibody titers are reported as the reciprocal of the final antiserum dilution. The results of this experiment showed that the W and 36T antigen-antibody systems were not affected by absorption of antisera with 10 of 1 ug of homologous or heterologous antigens per ml. Specificity of the absorption was shown in the Sd and 43R antigen systems when antisera were absorbed with homologous antigens.

Anti-Sd or anti-43R antiserum could be absorbed with 10 ug of Sd or 43 R antigen per ml, respectively, resulting in a significant reduction in the homologous hemagglutination titers, i.e., fourfold reduction.

TABLE 3

Passive Hemagglutination with Homologous and Heterologous Unabsorbed Antisera

Cells sensitized with antigen 1:1,000 prepared from:	Antiserum prepared against:			
	W	Sd	43R	36T
W	1,536[a]	1,536	1,536	1,536
Sd	768	768	384	768
43R	768	768	768	768
36T	384	384	768	768

[a]Titers reported as the reciprocal of the highest dilution of antiserum producing any discernible degree of hemagglutination.

TABLE 4

Absorption of Capsular Antibodies from Homologous Antisera with Homologous or Heterologous Antigens

Cells sensitized with homologous antigen 1:10,000 prepared from:	Unabsorbed antiserum titer[a]	Antiserum titer after absorption with:[a]							
		W antigen		Sd antigen		43R antigen		36T antigen	
		10 ug/ml	1 ug/ml	10 ug/ml	1 ug/ml	10 ug/ml	1 ug/ml	10 ug/ml	1 ug/ml
W	192	192	192	192	192	384	384	384	192
Sd	384	192	384	0[b]	24[b]	384	384	768	384
43R	384	192	384	192	192	96[b]	192	192	384
36T	384	192	384	192	192	192	192	192	192

[a]Reciprocal of final antiserum dilution.

[b]Significant reduction in titer.

Since W- and 36T-sensitized cells cross-reacted extensively when reacted with homologous or heterologous antisera, the sensitizing antigens were diluted to 1:20,000. Table 5 shows hemagglutination titers before and after absorption when cells were sensitized with antigen diluted to 1:20,000. Both W and 36T antigen-antibody systems showed at least fourfold reductions in titers when antisera were absorbed with 10 or 1 ug of homologous antigens per ml. The 43R antigen-antibody system still showed a significant reduction in titer, but the results with the Smith diffuse antigen-antibody system were uninterpretable, owing to the very weak reactions. The final and most definitive test used to disclose the serological specificity of the four strains of S. aureus described here was the specific capsular reaction carried out with each strain and its homologous antiserum (Table 6). The serological specificity of the Sd, W, and 36T strains is clearly shown. A group of cross-reacting strains comprised of 43R, 47R, 50R, and 51R represented a separate group distinguishable from all the others, but not from each other, by the specific capsular reaction.

Although this work disclosed the existence of multiple capsular types of S. aureus, the major capsular type in terms of occurrence was that of the wound strain (Wiley, 1959, 1961, 1962, 1963, 1968). Any further work on encapsulated strains of S. aureus required better knowledge of the chemical make-up of the capsular antigen and its state of purity. It was, therefore, decided to attempt to further purify the capsular antigen of the major capsular type. The first step in attempting further purification of the partially purified capsular material

TABLE 5

Absorption of Capsular Antibodies from Homologous Antisera with Homologous or Heterologous Antigens

Cells sensitized with homologous antigen 1:20,000 prepared from:	Unabsorbed antiserum titer[a]	Antiserum titer after absorption with:[a]							
		W antigen		Sd antigen		43R antigen		36T antigen	
		10 ug/ml	1 ug/ml	10 ug/ml	1 ug/ml	10 ug/ml	1 ug/ml	10 ug/ml	1 ug/ml
W	384	24[b]	96[b]	192	384	384	384	384	384
Sd	U[c]	U	U	U	U	U	U	U	U
43R	384	192	192	192	192	48[b]	96[b]	192	192
36T	384	192	384	192	384	192	192	96[b]	96[b]

[a]Reciprocal of final antiserum dilution.

[b]Significant reduction in titer.

[c]Uninterpretable.

TABLE 6

Specific Capsular Reactions of Staphylococci

Strains	Antisera						
	W	Sd	36T	43R	47R	50R	51R
W	+	-	-	-	-	-	-
Sd	-	+	-	-	-	-	-
36T	-	-	+	-	-	-	-
43R	-	-	-	+	+	+	+
47R	-	-	-	+	+	+	+
50R	-	-	-	+	+	+	+
51R	-	-	-	+	+	+	+

(PPCM) was to subject PPCM to a simple clarification step. A 1% solution of PPCM was centrifuged at 9,750 x g for 1 hour in a Sorvall RC-2B centrifuge. The supernatant fluid was separated from any insoluble material sedimented by the centrifugation and the supernatant fluid subjected to extensive dialysis. After dialysis the clarified PPCM was lyophilized and dried to a constant weight over Drierite. The resulting product was a white amorphous powder which was completely soluble at a concentration of 12 mg/ml in distilled water or phosphate buffered 1 M NaCl solution. Due to the unknown resolving capacity of the Sephadex G-100 and G-200 columns the initial column chromatographic studies were carried out with PPCM at a concentration of 4 mg/ml. In the initial chromatographic studies two Sephadex gels, G-100 or G-200, were used to obtain some estimate of the molecular size of the clarified PPCM, to attempt to further purify the clarified PPCM, and to indicate directions for future research on the clarified PPCM of the wound mucoid strain of S. aureus. A sample containing 4 mg/ml of PPCM suspended in 1 ml of 1 M NaCl phosphate buffer, pH 7.2, was placed on the top of the column. After the solution of clarified PPCM had penetrated into the gel, 2 ml of buffer were used to rinse the gel bed. One ml samples were collected and tested for precipitinogen activity. The precipitin test was capable of detecting 125 ug of capsular material. The precipitin reactions were graded from 1+ - 4+. In the experiments using either Sephadex G-100 or G-200, the precipitinogen peak occurred in the void volume. This indicated that no detectable separation of the components of the clarified PPCM had occurred or that the most

active serological component of the clarified PPCM was excluded by the gel. It was decided to use a gel with a higher and wider fractionation range as well as a longer gel bed in the next experiments. Sepharose 6B was used because of its characteristic fractionation range (up to about 4×10^6). A 0.9 cm x 162 cm column was packed with Sepharose 6B and used to attempt to further purify the clarified PPCM from the wound mucoid strain of S. aureus. An average flow rate of 3.0 ml per hour was maintained by a hydrostatic pressure of 33 cm. A buffer consisting of 1 M sodium chloride plus phosphate buffer pH 7 and .02% sodium azide was used. A total volume of 231 ml was collected. Blue dextran with a known average M.W. of 2×10^6 and the following proteins of known M.W. were used in 10 mg quantities to calibrate the Sepharose 6B column: ovalbumin (M.W. 45,000), bovine albumin (M.W. 67,000), porcine globulin (M.W. 160,000), and equine apo-ferritin (M.W. 480,000). The elution volume for each of the above proteins was found by testing 3 ml fractions from the column for their absorbance at 280 mu. The results of this experiment can be found in Table 7. The partition constant, Kav, plotted for each protein as a function of M.W. is shown in Figure 4. One can estimate the molecular weight of a similar unknown macromolecule by determining its elution volume, calculating its Kav, and then reading the molecular weight directly off the curve in Figure 4 (Andrews, 1964; Male, 1967).

A 12.0 mg sample of clarified partially purified capsular material in 1.0 ml of column buffer was then added to the top of the column. Each 3 ml sample was tested for precipitin activity, primary amino

TABLE 7

Separating Capacity of Sepharose 6B Using
1 M NaCl Phosphate Buffer[a]

Test substance	Elution volume ml	Kav
Ovalbumin	136	.84
Bovine albumin	130	.76
Porcine globulin	114	.60
Horse apo ferritin	95	.39
Blue dextran	56	

[a]Total volume of the gel bed = 153 ml.

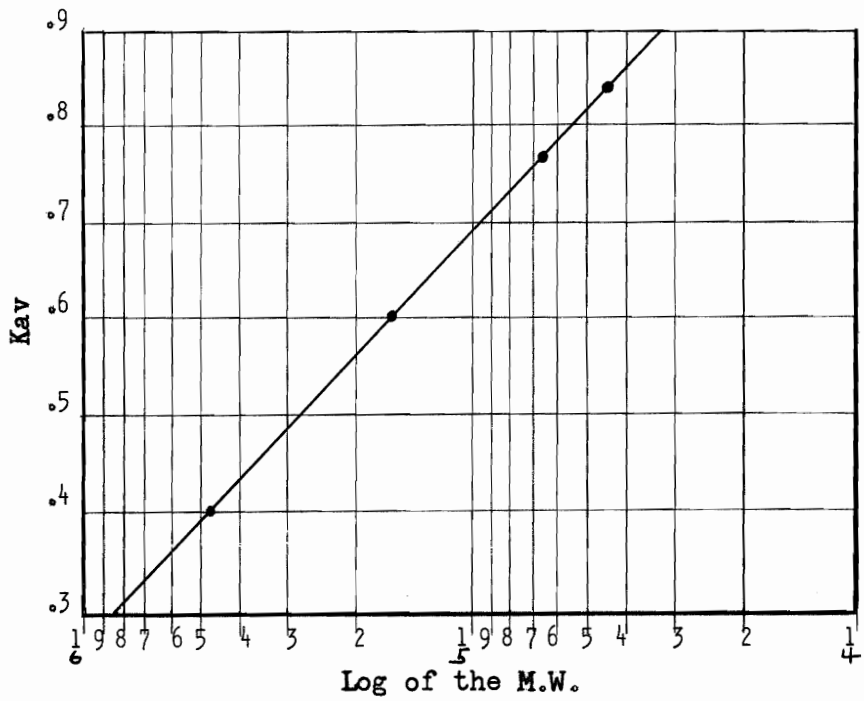

Figure 4. A graph of the partition coefficients (Kavs) for the various proteins versus log of their M.W. The data in Table 7 were used to make the graph.

groups by the ninhydrin spot test, and absorbance at 260-380 mu. Although the absorbancy of the material was measured between 250-380 mu, selecting 10 mu increments, only the absorbance in the 260-280 mu range will be presented. The absorbance at 260-280 mu is shown in Figure 5. The precipitin reactivity and the ninhydrin test results are also shown in Figure 5. The 3.0 ml samples were then pooled according to their precipitin activity and dialyzed against distilled water at 4 C for three days. The distilled water was changed three times daily. The pools were then lyophilized and weighed. The total weight of material recovered was twice as high as the initial weight placed on the column, indicating that the fractions of partially purified capsular material still contained salts from the column buffer. The pools were then washed with 95% ethanol to remove the remaining salts. After this step in the procedure, an 89% recovery of the initial weight (12 mg) of clarified PPCM material was noted.

At this time, to conserve antiserum, various chemical tests were tested to determine their applicability for detection of the clarified PPCM fractions eluted from the column. Among the tests used were the anthrone, primary cysteine-sulfuric acid, a modified glucosamine, and modified ninhydrin tests (Kabat and Mayer, 1967). In modifying the anthrone test to increase its sensitivity, the standard tested against, N-acetyl-glucosamine, showed unreproducible results. The second test, the primary cysteine-sulfuric acid test, was unreactive when clarified PPCM was used. Therefore, neither the anthrone nor the primary cysteine-sulfuric acid tests were used.

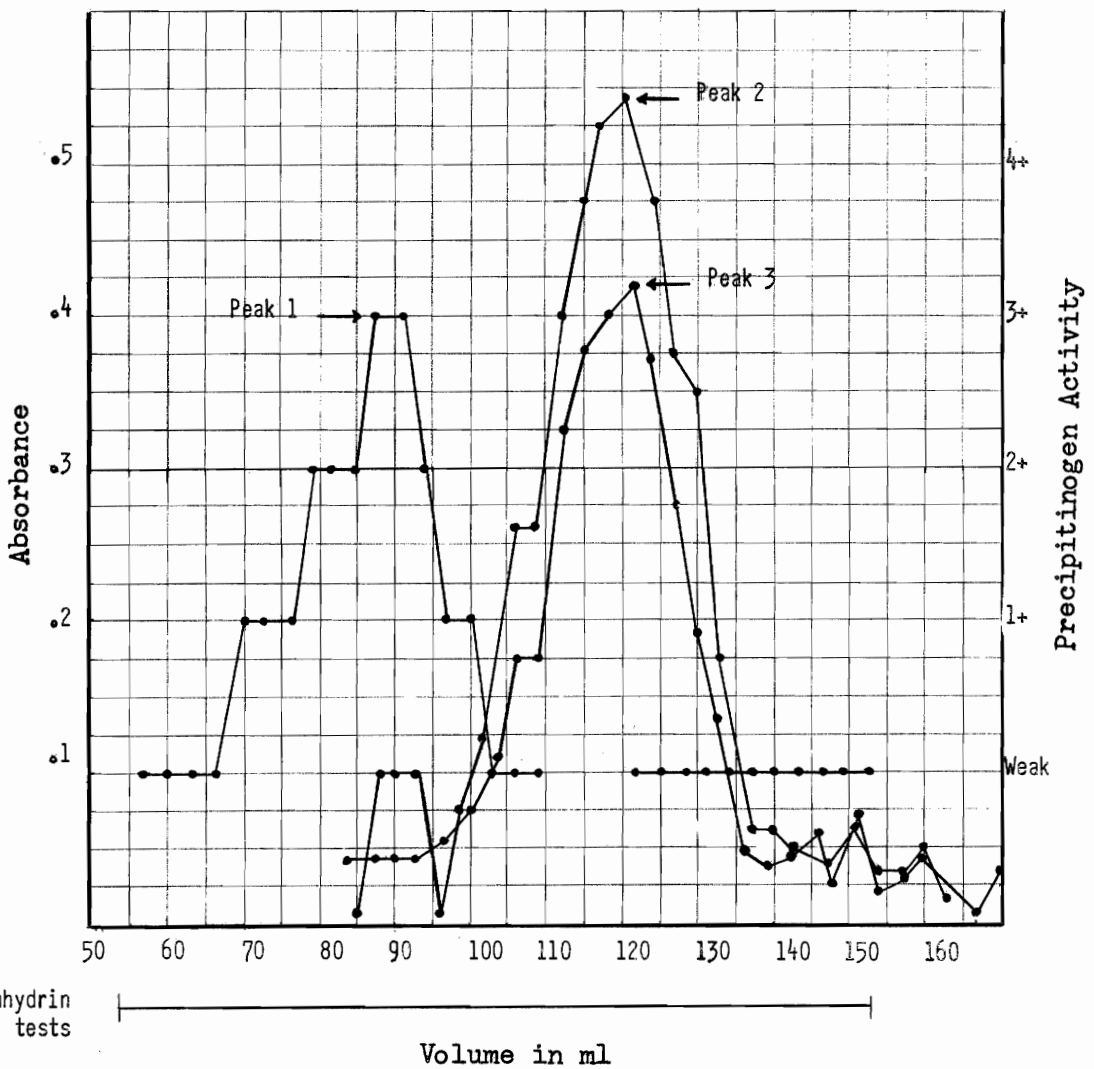

Figure 5. Elution pattern of 3 ml fraction of PPCM from the column. Peak 1 represents precipitinogen activity. Peak 2 represents absorbance read at 260 mu and peak 3 represents absorbance read at 280 mu. The occurrence of primary amino groups in the fractions as detected by the modified ninhydrin spot test is indicated by the straight line in brackets.

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However, the two remaining tests, the modified glucosamine and modified ninhydrin tests, seemed applicable to the detection of various fractions of the clarified PPCM. To facilitate the recovery of the fractions and to avoid cumbersome and time consuming dialysis of the fractions a second Sepharose 6B column was packed and equilibrated with distilled water. This time the gel bed was slightly lengthened and the top fitted with a 3-way valve to allow continuous application of samples without disturbing the flow rate. An eluent of distilled water containing .02% sodium azide was used to elute the various fractions from the column. This new column was calibrated as before with proteins of known molecular weight. The Kavs of these proteins were calculated and the results are shown in Table 8. A plot of Kav as a function of molecular weight is shown in Figure 6 for this column. The new column was then washed for 1 week with distilled water before 12 mg of clarified PPCM was added to the top of the column. The flow rate of this column was 1.6 ml/hour. The fractions collected were tested by the modified ninhydrin test, a modified glucosamine test, and absorbance tests at 260 and 280 mu. Figure 7 shows the results of the modified ninhydrin and of the modified glucosamine test. The 1 ml fractions between 57-104 ml showed a positive ninhydrin and glucosamine test, whereas the 1 ml fractions between 131-170 ml showed only a positive ninhydrin test.

Figure 8, curve 1, shows the absorbancy of the fractions at 260 mu, and Figure 8, curve 2, shows their absorbancy at 280 mu. The maximum absorbance at 260 and 280 mu occurred in the 76 ml fraction.

TABLE 8

Separating Capacity of Sepharose 6B Using Distilled H_2O Containing .02% Sodium Azide[a]

Test substance	Elution volume ml	Kav
Ovalbumin	134	.76
Bovine albumin	130	.73
Porcine globulin	121	.65
Horse apo ferritin	113	.55
Blue dextran	54	

[a]Total volume of gel bed = 159.

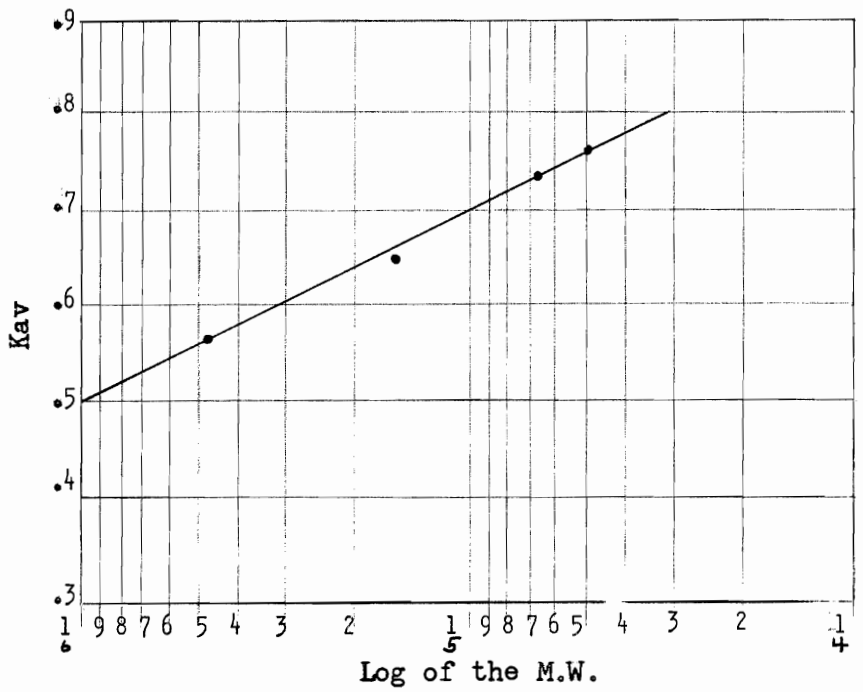


Figure 6. A graph of the partition coefficients (Kavs) for the various proteins versus log of their M.W. The data in Table 8 were used to make the graph.

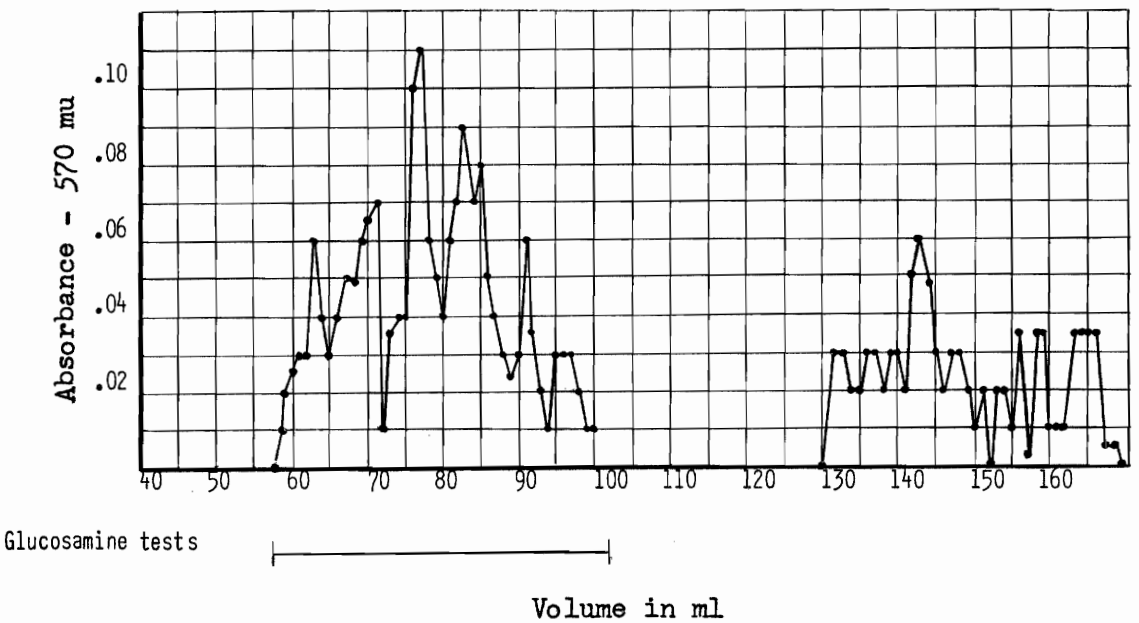


Figure 7. Elution pattern of PPCM as shown by modified ninhydrin and modified glucosamine tests carried out on 1 ml fractions in distilled H_2O containing .02% sodium azide. Positive glucosamine tests are indicated by the straight line in brackets.

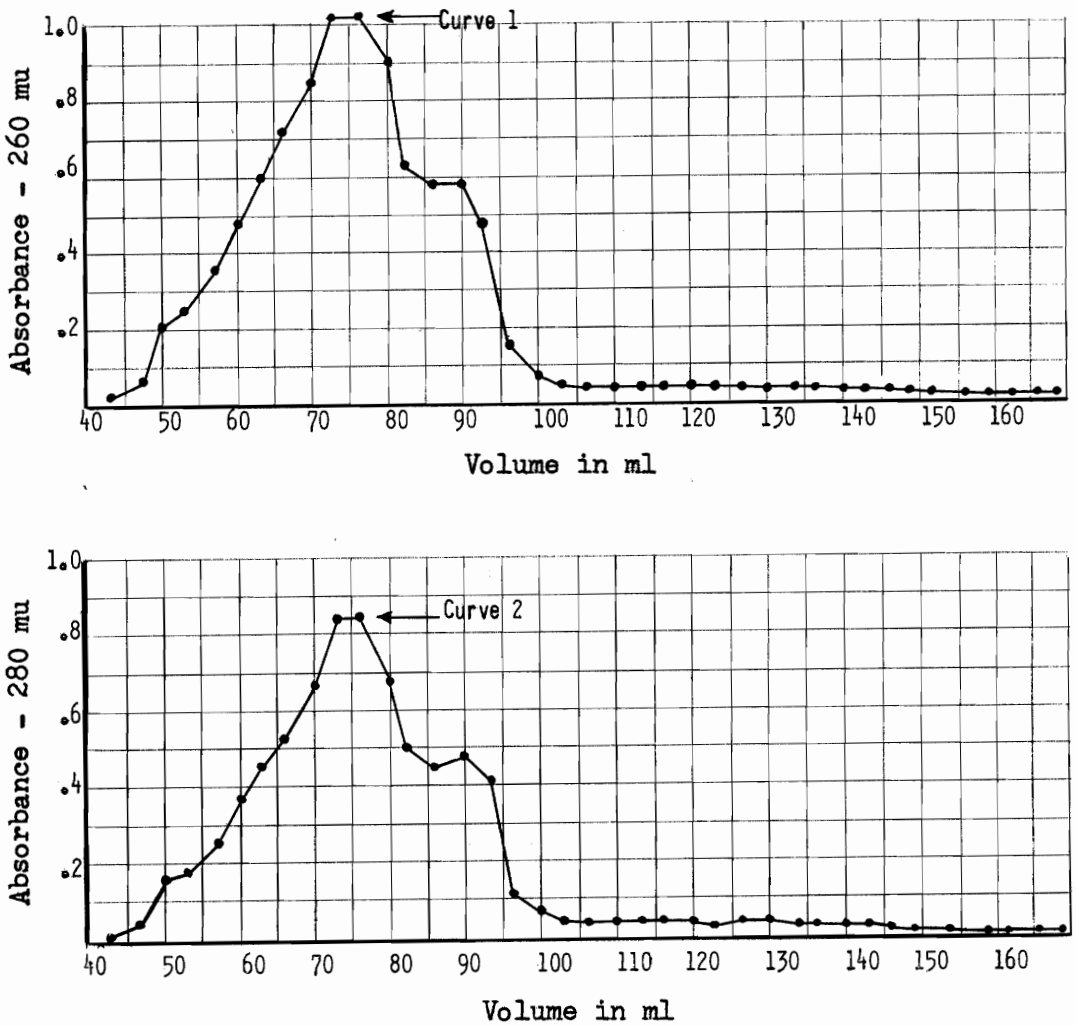


Figure 8. Elution pattern as shown by absorbance at 260 and 280 mu of 3 ml fractions in distilled H_2O containing .02% sodium azide. Curve 1 is the elution pattern when absorbance was measured at 260 mu. Curve 2 is the elution pattern when absorbance was measured at 280 mu.

The fractions were pooled according to the results of the ninhydrin, glucosamine, and absorbance tests as follows: 58-72 ml, 73-80 ml, 81-100 ml, 101-127 ml, 128-147 ml, and 148-200 ml. Additional runs were then collected to determine total recovery of partially purified capsular material from the column on a weight basis. The percent recovery is shown in Table 9. It was shown that there was a total recovery of 92.7%. It was felt that a 7.3% loss of material was within experimental error and assured us that only small amounts of clarified PPCM may have remained on the column or were lost in the recovery procedure.

Hydrolyzed clarified PPCM and hydrolyzed pools from the column pools were then analyzed qualitatively and quantitatively for amino acids using a Beckman Model 120 B amino acid analyzer (Palo Alto, California). Tables 10-12 show the amino acid analysis of hydrolyzed clarified PPCM and the amino acid analyses of the hydrolyzed pools from the column. Since no known standards for taurine, phosphoethanolamine, and glycerophosphoethanolamine were available, the quantities of these compounds represented in the table are estimates. A total of 125 ug was used for each analysis. The percent recovery was good considering that chemical substances such as nucleic acids and ribitol phosphate were not considered in the recovery figures in Tables 10, 11, and 12. The major amino acids detected in amounts greater than ten millimicromoles in the various pools were: 58-72 ml pool, lysine, aspartic acid, threonine, serine, glutamic acid, glycine, alanine, leucine, 73-80 ml pool, lysine, aspartic acid, threonine, serine, glutamic acid, glycine,

TABLE 9

Weight of Various Pools After Drying

Pools ml	Weight mg[a]	Weight as % of total weight pool[b]
58-72	2.08	17.4
73-80	1.68	13.9
81-100	4.05	33.8
101-127	1.30	10.8
128-147	1.25	10.4
148-200	0.76	6.3
Total	11.12	92.6

[a]The accuracy of the balance used was ± 34 ug.

[b]12.00 mg was added to the top of the column and eluted with distilled water containing .02% sodium azide.

TABLE 10

Amino Acid Analyses of Clarified PPCM and Indicated Pools From the Column

Amino acid	PPCM[a]	58-72 ml pool[b]	73-80 ml pool
	muM[c]	muM	muM
lysine	24	38	53
histidine	8	5	7
aspartic acid	7	30	31
threonine	2	18	23
serine	7	106	106
glutamic acid	11	52	57
glycine	101	84	77
alanine	24	42	36
valine	1	6	19
methionine	11	7	8
isoleucine	6	3	4
leucine	37	27	6
tyrosine	7	6	7
phenylalanine	6	5	6
taurine	7	7	7
"phosphoethanolamine"	1	12	1
"glycerophosphoethanolamine"	1	15	2
ammonia	196	16	2
glucosamine	191	167	254

[a]Clarified partially purified capsular material. Analysis carried out on 125 ug quantities of clarified PPCM.

[b]Analysis carried out on 125 ug quantities of the pool.

[c]muM represents millimicromoles.

TABLE 11

Amino Acid Analyses of the Indicated Pools From the Column

Amino acid	81-100 ml pool[a]	101-127 ml pool	128-147 ml pool
	muM[b]	muM	muM
lysine	19	48	71
histidine	3	10	1
aspartic acid	17	32	48
threonine	11	23	36
serine	46	129	180
glutamic acid	38	74	115
glycine	62	111	128
alanine	30	43	57
valine	11	15	19
methionine	1	2	2
isoleucine	1	1	2
leucine	18	15	14
tyrosine	6	7	6
phenylalanine	5	6	5
taurine	7	5	6
"phosphoethanolamine"	6	--	--
"glycerophosphoethanolamine"	8	3	--
ammonia	265	54	178
∅-glucosamine	156	20	3

[a]Analysis carried out on 125 ug quantities of the pool.

[b]muM represents millimicromoles.

TABLE 12

Amino Acid Analysis of the Indicated Pool From the Column

Amino Acid	148-200 ml pool[a]
	muM[b]
lysine	65
histidine	10
aspartic acid	45
threonine	28
serine	184
glutamic acid	114
glycine	145
alanine	58
valine	26
methionine	1
isoleucine	3
leucine	14
tyrosine	6
phenylalanine	5
taurine	7
"phosphoethanolamine"	--
"glycerophosphoethanolamine"	6
ammonia	83
d-glucosamine	17

[a]Analysis carried out on 125 ug quantities of the pool.

[b]muM represents millimicromoles.

alanine, and valine; 81-100 ml pool, lysine, aspartic acid, threonine, serine, glutamic acid, glycine, alanine, valine, leucine; 101-127 ml pool, serine and glutamic acid; 128-147 ml pool, lysine, serine, glutamic acid, and glycine. The amino acids detected represented amino acids found as common constituents of protein. The 101-127 ml pool, the 128-147 ml pool, and the 148-200 ml pool were combined in equal concentrations and designated the 101-200 ml pool. As shown in Figure 9, the absorbance maximum at 260 mu suggested the presence of nucleic acids in the pools. The percent nucleic acids in the various pools calculated using a nomograph of Warburg and Christian (1942) was 10.4% in the 58-72 ml pool, 5.5% in the 73-80 ml pool, 9.7% in the 81-100 ml pool, and 4.2% in the 101-200 ml pool.

The next analyses carried out were glucosamine and reducing sugar (Schales and Schales, 1945) determinations in PPCM. The results were similar to those observed on PPCM by Wiley and Wonnacott (1962). The glucosamine determinations on PPCM are shown in Table 13 and the reducing sugar determinations are shown in Table 14. In Tables 13 and 14 the percent glucosamine and reducing sugar are almost equal. Therefore, all the reducing sugar can be accounted for as glucosamine.

Glucosamine and reducing sugar analyses on the various Sepharose 6B column fractions are shown in Tables 15 and 16. It is worth noting that the 73-80 ml pool exhibited the highest reducing sugar and glucosamine content. Although the 58-73 ml pool and the 81-100 ml pool both contained significant amounts of reducing sugar and glucosamine, their

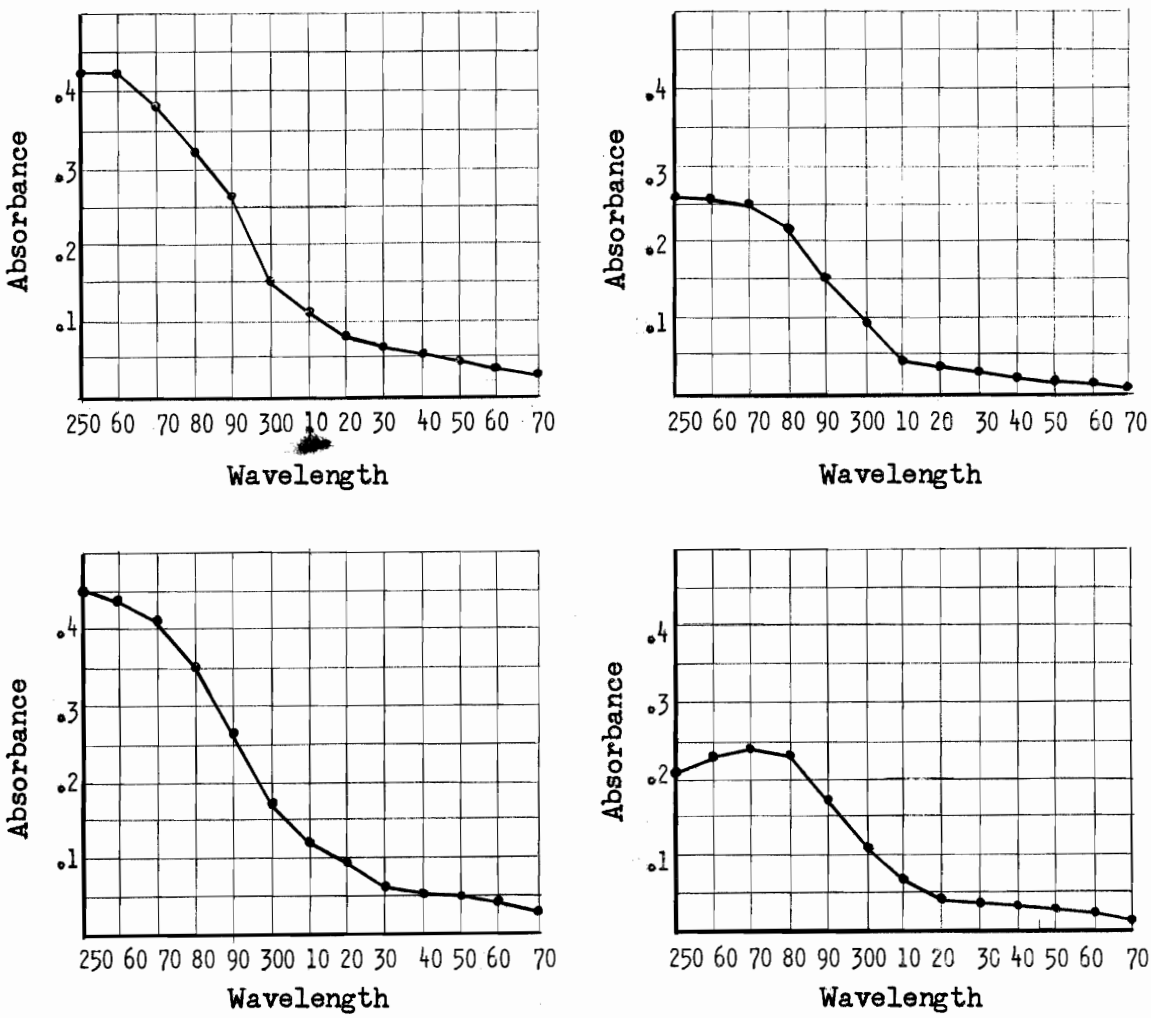


Figure 9. Absorbance at 250-370 mu of pools from the column. Curve 1 shows the absorbance of the 58-72 ml pool. Curve 2 shows the absorbance of the 73-80 ml pool. Curve 3 shows the absorbance of the 81-100 ml pool. Curve 4 shows the absorbance of the 101-200 ml pool.

TABLE 13

Glucosamine Determinations on Clarified PPCM

Sample	ug tested	ug of glucosamine	% glucosamine[a]	Average %
PPCM[b]	100	26	26	
	200	55	27	26
	300	73	24	

[a]Determined as glucosamine hydrochloride.

[b]Clarified partially purified capsular material.

TABLE 14

Reducing Sugar Determination on Clarified PPCM

Sample	ug tested	ug of reducing sugar	% reducing sugar[a]	Average %
PPCM[b]	160	45	27	
	160	45	27	27
	332	86	26	

[a]Determined as glucose.

[b]Clarified partially purified capsular material.

TABLE 15

Glucosamine Determinations on Pools From the Column

Pools in ml	ug tested	ug of glucosamine	% glucosamine[a]	Average %
58-72	100	26	26	
	200	53	27	27
	300	82	27	
73-80	200	73	36	
	200	74	37	37
	200	74	37	
81-100	300	71	24	
	300	72	24	24
	300	73	23	
101-200	157	<10 ug	<12	<12
	157	<10 ug	<12	~~12~~

[a]Determined as glucosamine hydrochloride.

TABLE 16

Reducing Sugar Determination on Pools From the Column

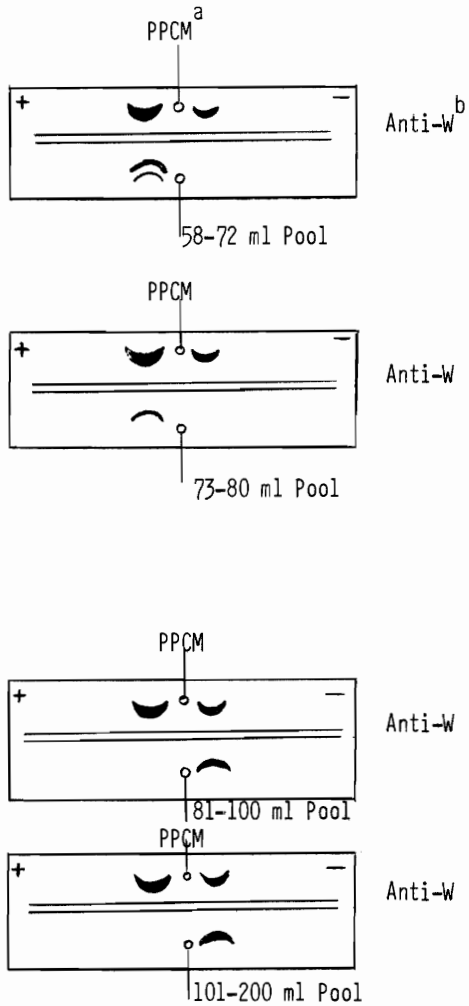
Pools in ml	ug tested	ug of reducing sugar	% reducing sugar[a]	Average %
58-72	500	130	27	27
	500	130	27	
73-80	200	76	38	36
	200	70	35	
81-100	420	99	24	24
	420	99	24	
101-200	157	<30	<24	<24
	157	<30	<24	

[a]Determined as glucose.

content of these components was lower than that of the 73-80 ml pool. The 101-200 ml pool was essentially devoid of glucosamine and contained only small amounts of reducing sugar compared to the other fractions. The glucosamine content and reducing sugar value of the fractions approximated each other, thus providing evidence that it was unlikely that reducing sugars other than glucosamine were present.

In order to ascertain whether or not the column fractions represented immunologically homogeneous fractions, they were subjected to immunoelectrophoresis. The results of the immunoelectrophoresis of each of the fractions are shown in Figure 10. The 58-72 ml pool exhibited 2 anodal precipitin arcs indicating it was not immunologically homogeneous. The 73-80 ml pool exhibited a single precipitin arc migrating toward the anode and, thus, appeared to represent an immunologically homogeneous fraction. The 81-100 ml pool exhibited a single precipitin arc which migrated toward the cathode. The 101-200 ml pool also exhibited a single precipitin arc which migrated toward the cathode. The 58-72 ml pool exhibited two precipitin arcs. These possibly could have been resolved into their component parts if, instead of pooling the 58-72 ml volume, two pools comprised of the 58-65 ml volume and the 66-72 ml volume had been made instead. The rationale behind this proposal was based on examination of Figure 7, which shows that the 58-72 ml pool consisted of two distinct absorbance peaks.

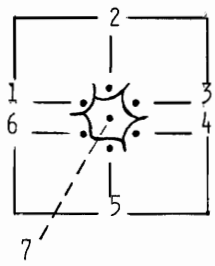
The gel diffusion experiment corroborated the immunoelectrophoretic analysis. Figure 11 shows the results of the gel diffusion experiment. Clarified PPCM and the 58-72 ml pool reacted with an



[a]Clarified partially purified capsular material from the wound strain of Staphylococcus aureus.

[b]Rabbit anti-Staphylococcus aureus (wound strain) antiserum.

Figure 10. Immunoelectrophoresis of pools from the column. Schematic drawing of precipitin arcs observed when partially purified capsular material and pools from the column were subjected to immunoelectrophoresis. Development of precipitin arcs were allowed to proceed for 24 hours at room temperature and the slides were refrigerated for an additional 48 hours at 4 C.



1. Clarified PPCM
2. 58-72 ml pool
3. 73-80 ml pool
4. Clarified PPCM
5. 81-100 ml pool
6. 101-200 ml pool
7. Rabbit anti-Staphylococcus aureus (wound strain) antiserum

Figure 11. Gel diffusion patterns of various pools from the column. Schematic drawing of precipitin arcs observed when clarified partially purified capsular material and various pools from the column were subjected to gel diffusion. Development of precipitin arcs was allowed to proceed for 24 hours at room temperature and then the slide was refrigerated for an additional 48 hours at 4 C.

anti-wound rabbit serum to produce a reaction of identity. The 73-80 ml pool reacted with the antiserum and exhibited a reaction of partial identity with the 58-72 ml pool and with PPCM. This could be explained on the basis that both clarified PPCM and the 58-72 ml pool contained a serologically active component present in the 73-80 ml pool; but, both the clarified PPCM and the 58-72 ml pool apparently contained an additional antigen. The 81-100 ml pool and the 101-200 ml pool also showed a reaction of identity when tested against the anti-W rabbit antiserum.

The final and most definitive experiment was reduction of the SCR of a specific antiserum by absorption with the various pools. Specific capsular reaction tests using the various pools are the only definitive tests which will indicate whether the fractions contain capsular antigen. The results of such an experiment are shown in Table 17. When the absorption by the 73-80 ml pool was compared with the absorption by the other pools, it could be seen that the most serologically active substance was present in this pool. Complete absorption of the antibodies necessary for the elicitation of the SCR was obtained when 0.2 ml volume of antiserum diluted 1-16 and an equal volume of a solution containing 62 ug of the 73-80 ml pool were combined. The next most serologically active fraction was the 58-72 ml pool. The 81-100 ml pool showed only slight absorption of anti-capsular antibodies, while the 101-200 ml pool showed no absorption of anti-capsular antibodies. It was interesting to note that ability to absorb anti-capsular antibodies correlated well with the glucosamine and reducing sugar content and was the most effective absorbent in the absorption tests. In

TABLE 17

Absorption of Anticapsular Antibodies by Clarified PPCM[a] and Various Pools From the Column

Absorbing antigen or pool	ug of antigen or pool						
	800	500	250	125	62	31	15
PPCM	-[b]	+	+	+	+	+	+
58-72 ml pool	ND[c]	-	-	-	+	+	+
73-80 ml pool	ND	-	-	-	-	+	+
81-100 ml pool	ND	-	+	+	+	+	+
101-200 ml pool	ND	+	+	+	+	+	+

[a]Clarified partially purified capsular material.

[b]A negative reaction (-) indicates absorption of anticapsular antibodies has occurred; therefore, no specific capsular reaction was visible. A positive reaction (+) indicates that the material had not absorbed the antibodies; and therefore, a specific capsular reaction was observable.

[c]ND = not done.

regard to the antibody absorption capacity of the 73-80 ml pool, it can be said that 13 fold increase in purity of the capsular antigen had occurred or that a 13 fold increase in its anti-capsular absorption capacity occurred. This was evident when the absorption capacity of the 73-80 ml pool for anti-capsular antibodies was compared with that of clarified PPCM.

DISCUSSION

Various investigators have isolated mouse-virulent strains of S. aureus that grow in a diffuse manner in serum-soft agar (Alami and Kelly, 1960; Koenig and Melly, 1965). Gel diffusion tests have shown the presence of an antigen in the Sd strain which was absent from the Smith compact strain of S. aureus (Koenig and Melly, 1965). Experimental data from our laboratory had shown definitively that the Sd strain was encapsulated and that the capsule was serologically different from that of the wound strain (Wiley and Maverakis, 1968). A staphylococcal polysaccharide antigen (SPA) from S. aureus strain 05068 was chemically analyzed and found to be a polymer of 2-amino-2-deoxy-D-glucuronic acid, a heretofore undescribed component of bacterial capsules (Haskell and Hanessian, 1963, 1964). Gel diffusion tests with an antigen called Smith surface antigen (SSA) by Morse (1963) and extracts from Welwood and K-6 have shown that SSA was extractable from these strains. The present report showed that the Sd, Welwood, and K-6 strains were of the same capsular type as shown by the specific capsular reaction tests. Immunoelectrophoresis of extracts prepared by the method of Morse (1963) and Wiley and Wonnacott (1962), from the W, Sd, 43R, and 36T strains, showed the presence of antigens specific for each strain. The observation that anti-W serum cross-reacted with Sd extract and anti-Sd serum did not cross-react with W extract was explained on the basis that these reactions

were one-way cross-reactions similar to those observed by Pease and Laughton (1965) with mycoplasma antigens.

The results in this thesis do not correspond to those reported by Morse (1963). In every instance of this work, except where an extract of Sd was employed, the results reported in this thesis showed multiple precipitin arcs with the extracts. Usually there were two arcs, but in the case of the W extract, we obtained three precipitin arcs. One of the anti-Sd sera employed consistently gave a single arc when reacted with the Sd extract, but a second anti-Sd serum gave two precipitin arcs with Sd extract. The precipitin arc detected when extracts of the Sd strain reacted against anti-W could not have represented the capsular antigen of the Sd strain because results of Wiley and Maverakis (1968) showed previously, and quite definitively, that capsules of the two strains are serologically distinct. The possibility that the rabbit used in the preparation of the anti-W serum possessed naturally occurring antibodies reactive with the Sd extract was ruled out by our failure to detect a precipitin arc when reacting Sd extract and a preimmunization serum. The best explanation seems to be that acid extraction of staphylococci by the method of Morse (1963) extracts multiple antigens in many instances. It is worth pointing out that among the reasons for differences in the results presented in this thesis and those of Morse (1963) were that the antigen was employed at concentrations 10 times stronger than his, and immunoelectrophoresis was used, a procedure which was potentially more sensitive and capable of better resolution of multiple antigens. It is wise to sound a word

of caution here, that interpretation of results in immunoelectrophoresis or double diffusion should be made cautiously. When multiple arcs are present, it would be impossible to ascribe one of them as being due to capsular antigen. The results presented in this thesis would have been difficult to interpret without the specific capsular reaction which were used as a definitive test to detect capsular antigens.

Among the most sensitive methods for detecting antibodies is the passive hemagglutination test. It is capable of detecting from 0.003 to 0.006 ug of antibody nitrogen per ml, whereas the qualitative precipitin test in gels is capable of detecting 3 to 5 ug of antibody nitrogen per ml (Humphrey and White, 1964). The passive hemagglutination test was used to detect antibodies to antigen in extracts of staphylococci (Hayes, 1951; Keogh, North, and Warburton, 1948; Rountree and Barbour, 1952). The results of passive hemagglutination tests reported here disclosed that an acid extract of staphylococci as well as crude capsular material were capable of sensitizing erythrocytes and rendering them agglutinable by antiserum against S. aureus. When large amounts of the extracts or crude capsular material were used, there was little evidence of strain-specific antigens in hemagglutination tests. When the sensitizing antigens were diluted to 1:10,000 or 1:20,000, however, and the antisera were absorbed with homologous and heterologous antigen diluted to 1:100,000 or 1:1,000,000, strain specificity was evident, for homologous antigen reduced the titer of the antiserum fourfold or more. Since it was evident in most instances

that multiple antigens were present in the antigen-extracts, the cross-reactivity evident in hemagglutination tests carried out with antigen diluted 1:1,000 was not surprising.

Rantz et al. (1956), isolated a non-species specific antigen from gram-positive bacteria that was capable of sensitizing erythrocytes to subsequent agglutination by antiserum. Grov et al. (1964), isolated a substance they termed protein A from S. aureus strains and showed that it was capable of sensitizing tanned sheep cells to subsequent agglutination by antisera against S. aureus. Most normal human sera also were capable of agglutinating tanned sheep erythrocytes sensitized with protein A. The point we made here is that extracts of staphylococci may indeed contain a variety of antigenic moieties such as teichoic acid, protein A, Rantz antigen, or capsular antigen. When multiple precipitin arcs are detected, it is difficult to identify each of the antigens producing the precipitin reactions, unless purified antigens are available for purposes of identification. It seems likely that Rantz antigen could be present in our antigen extracts and this could explain the marked cross-reactivity of the antisera in the hemagglutination test when antigen diluted 1:1,000 or 1:5,000 was used. When the hemagglutination tests were carried out with antigen diluted 1:10,000 or 1:20,000 and absorption tests were set up, specificity of the hemagglutination reaction was shown by failure of heterologous antigen to diminish the homologous hemagglutination titers. In all instances, absorption of homologous antiserum by homologous antigen at a concentration of 1 ug/ml or 10 ug/ml resulted in at least a fourfold

reduction in the homologous hemagglutination titer. The final conclusion that at least four capsular types of S. aureus exist was based on the results of specific capsular reactions carried out with rabbit antiserum. Two of the capsular types represented by strains 36T and 43R have not been described before to our knowledge.

Analytical serological methods suitable for quantitative determination of antibody require that antigen be monospecific. A reasonable approach seemed to be to attempt purification of capsular antigen from the wound strain of S. aureus since partial purification and chemical characterization of the capsular material from the wound strain had been reported by Wiley and Wonnacott (1962). In the partial chemical characterization of staphylococcal capsular material a glucosamine content of 26.9% was reported. Paper chromatograms provided evidence for the presence of glycerophosphate and an unidentified component which could have been a ribitol compound. Glucosamine has been isolated from capsules of Group A streptococcal and pneumococcal capsules (Kabat and Mayer, 1967). Glycerophosphate and ribitol phosphate compounds have been reported as chemical constituents of teichoic acid (Julionelle et al., 1935, 1936; Armstrong et al., 1958; Davidson and Baddiley, 1963; Ellwood et al., 1963; Haukenes, 1962a, 1962b; Mandelstam and Strominger, 1961; Sanderson et al., 1962; Davidson and Baddiley, 1963; Strominger, 1956). Four amino acids were identified which commonly occur in cell walls of S. aureus (Wiley and Wonnacott, 1962). The molar ratio of the amino acids they reported was glutamic acid 1, lysine 1, alanine 2, and glycine 6.

Other strains of encapsulated S. aureus have also been used in studies of the chemical characterization of capsular antigens. The Smith diffuse strain of S. aureus was shown by Wiley and Maverakis (1968) to possess capsules serologically distinct from those of the wound strain of S. aureus. Chemical analysis of the capsular material of the Smith strain disclosed the presence of 14.2% reducing sugar, 25% hexosamine, 28.9% amino acids, and a nitrogen content of 8.49% (Morse, 1962). Haskell and Hanessian (1963, 1964) also reported chemical characterization of a capsular antigen from a strain of S. aureus, designated 05068 by them, but believed to be closely related to the Smith strain. The main chemical constituents of the capsule was shown to be 2-acetamido-2-deoxy-D-glucuronic acid and 2 (N acetyl-alanyl) amino 2-deoxy-D-glucuronic acid.

Hisatsune et al. (1966, 1967a, 1967b) attempted to further purify the capsular material of the wound strain of S. aureus. They isolated four components from the capsular material of the wound strain designating them as P1, P2, P3, and P4. They failed, however, to find the specific capsular antigen among the four components they isolated because none of the fractions isolated absorbed anti-capsular antibodies from a rabbit antistaphylococcal serum.

Since the original procedure of Wiley and Wonnacott (1962) was successful in yielding a fraction that absorbed the specific capsular antibodies, their procedure was used in the initial stages of the preparation of the PPCM described in this thesis. Centrifugation was used to clarify the PPCM as a further purification procedure. Initial

exclusion chromatography on Sephadex G-100 and G-200 indicated that serologically active capsular fractions were present in the void volume so further experiments with the above gels were discontinued and a column containing Sepharose 6B was prepared. Various tests were used to detect fractions of clarified PPCM eluted from the columns. Absorbance studies in the 240-760 mu absorbance range were carried out to determine whether or not the clarified PPCM could be detected in column fractions. Initial absorbance studies of clarified PPCM from the wound strain of S. aureus suggested the presence of nucleic acids due to the absorbance peak at 260 mu. The second test used to detect the various fractions of the clarified PPCM eluted from the column was the precipitin test. The column buffer used was a phosphate buffered 1 M NaCl solution. Since the precipitinogen eluted from the column would be in a 1 M NaCl phosphate buffer, anti-wound rooster serum was used (Goodman et al., 1951). The low sensitivity of the precipitin test and the expenditure of valuable antiserum discouraged its use in routine detection of clarified PPCM fraction eluted from the column. The third test used in these experiments was the ninhydrin spot test. The ninhydrin spot test was discontinued because of its extreme sensitivity which did not allow for the detection of separate fractions.

The Sepharose 6B column was calibrated with proteins of known molecular weight and their partition coefficients (Kav's) calculated. A plot of Kav versus logarithm of the molecular weight of a protein yielded a straight line over the range of Kav's of the proteins used in the calibration of the column. The Kav of the fraction of PPCM

yielding the highest precipitinogen activity was 0.35 which, when read off the calibration curve in Figure 5, gave an estimated molecular weight of 6.8×10^5 for this particular fraction. One must consider this estimate with a certain degree of caution since location of the peak was carried out by using an antiserum against whole staphylococcal cells; hence, any antigen present in the PPCM other than capsular antigen could also have yielded a positive precipitin reaction. It was, therefore, apparent that this estimation of molecular weight need not necessarily represent the molecular weight of the capsular antigen.

Work with the Sepharose 6B column, employing the phosphate buffered 1 M NaCl eluent, was discontinued because of the necessity of employing extensive dialysis to remove NaCl from the column fractions. It was also apparent that the dry weights of the column fractions did not represent true weights of the capsular fractions when the results of capsular antibody absorption tests were read because none of the fractions from the column appeared to be consistently capable of absorbing the anti-capsular antibodies. A new column was prepared using distilled water containing .02% sodium azide as an eluent. This column was calibrated as before. Two tests were used to detect eluate from the new column. These were the modified glucosamine spot test and the modified ninhydrin test. The modified glucosamine spot test was not used to detect the peaks of the various fractions, but was used as a specific test to detect glucosamine in fractions located by the ninhydrin test. The most practical chemical test for detecting the reactivity in various fractions was the modified

ninhydrin test, which could detect 1-20 ug of amino-N.

Although the ninhydrin test is a non-specific test too in that it is positive with any primary amino group or ammonia (Kabat and Mayer, 1967), it was apparent from subsequent antibody absorption tests that peaks located by the modified ninhydrin test were more sharply defined than those located by the precipitin test. When the Kav of the column fraction showing the greatest antibody absorption ability was calculated, it was too low to be plotted on the calibration line in Figure 7. Since this column fraction had been located more specifically and tested specifically for antibody absorption ability, the accuracy of the molecular weight estimation made from Figure 5 was seriously doubted. The explanation for this was that the line in Figure 7 was determined by using proteins of known molecular weights. Beyond the upper and lower M.W. range of the proteins, the line may not be linear; therefore, the Kav's beyond the upper and lower limits in Figure 7 cannot be used to estimate M.W. The second possibility, and the best explanation as to why Figure 7 cannot be used to estimate the M.W. of capsular material, was that the capsular material most probably was not a protein but a polysaccharide of high molecular weight. Polysaccharides are not partitioned like proteins (Andrews, 1964, Male, 1967); therefore, polysaccharides that have a partition coefficient in this range should have been used to calibrate the column. Since each 1 ml eluate could not be tested in routine operation for the presence of capsular material, it was necessary to determine what eluates could be pooled. It was decided to pool the eluate volumes representing the

highest peaks separately while volumes representing either side of the highest peak were also pooled separately. Amino acid analysis was then carried out on the various pools as well as unfractionated clarified PPCM. The clarified PPCM contained 17 amino acids. Any amino acid present in less than 10 muM's (millimicromoles) was considered to be a trace amount. The following amino acids were present in greater than 10 muM concentrations: glutamic acid, lysine, glycine, alanine, and leucine. The molar ratio of glycine to glutamic acid was higher than originally observed by Wiley and Wonnacott (1962). Glycerophosphate and an unidentified compound, possibly ribitol phosphate, was detected by Wiley and Wonnacott (1962). These same compounds may have been present in the clarified PPCM. Although no standards were available for glycerophosphoethanolamine and phosphoethanolamine, compounds with their chromatographic characteristics were detected in all the pools except the 128-200 ml pool. The following were the principal amino acids in all the column pools: lysine, aspartic acid, threonine, serine, glutamic acid, glycine, alanine, leucine, and valine. The 73-80 ml pool was the only exception in that it contained smaller amounts of leucine than the other fractions. Lysine, glutamic acid, glycine, and alanine had been reported by others to be common cell wall amino acids (Salton, 1964). Serine, aspartic acid, and threonine had been n ted before by others, but their position in the wall has not been accounted for in proposed schemes of cell wall structure in S. aureus (Salton, 1964). No muramic acid was detected on the chromatographic chart. This peak would have been

located between serine and glutamic acid. Since no muramic acid was detected, the glucosamine detected was not considered to represent cell wall glucosamine.

Immunoelectrophoresis, gel diffusion, and antiserum absorption studies were carried out to determine the serological characteristics of the PPCM in the pools. Molecular size may have been a determining factor in the ability of the various pools to absorb the anti-capsular antibodies as indicated by the relative absorption effectiveness of the 58-72 ml pool and relative absorption ineffectiveness of the 81-100 ml pool. There is, however, evidence in Tables 15 and 17 that glucosamine content and reducing sugar value correlate with absorption effectiveness because the most effective pool in the absorption test was the 73-80 ml pool.

Although the 81-100 ml pool did not have a significantly lower glucosamine content than that of the 58-72 ml pool, it was shown by immunoelectrophoresis to migrate as an anion, whereas the 58-72 ml pool migrated as a cation. Furthermore, the 58-72 ml pool consisted of two distinct moieties migrating to the anode. Although the 58-72 ml pool and the 81-100 ml pool exhibited a similar glucosamine content, the 81-100 ml pool was much less effective in the absorption of capsular antibodies. Since the 81-100 ml pool migrated in an entirely different direction, the fact that the two pools exhibited a similar content of glucosamine was considered to be coincidental.

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