A Study of Protein-A of Staphylococcus Aureus of Bovine Origin.

Joseph Woodrow Pankey Jr
Louisiana State University and Agricultural & Mechanical College

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A STUDY OF PROTEIN A OF STAPHYLOCOCCUS AUREUS OF BOVINE ORIGIN

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy

in

The Department of Microbiology

by

Joseph Woodrow Pankey, Jr.
B.S., Louisiana Tech University, 1966
M.S., Louisiana Tech University, 1969
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GLOSSARY OF ABBREVIATIONS

DEAE  Diethylaminoethyl
DNAse  Deoxyribonuclease
Fab  Fraction of immunoglobulin G molecule which contains the antigen binding site after papain digestion
F(ab)_2  Fraction of immunoglobulin G which contains both antigen binding sites after pepsin digestion
Fc  Fraction of immunoglobulin G which crystallizes at 4 °C after papain digestion.
HF-4  Staphylococcus aureus, isolate Hill Farm-4
ID  Immunodiffusion
IEP  Immunelectrophoresis
IgG  Immunoglobulin G
NB+  Nutrient broth plus
NBS  Normal bovine serum
NHS  Normal human serum
NRS  Normal rabbit serum
SDS  Sodium dodecyl sulfate
S_{20w}  Sedimentation coefficient expressed in Svedburg units converted to conditions of infinite dilution in water at 20 °C
TBA  Tryptose blood agar
ABSTRACT

Staphylococcus aureus, designated Hill Farm-4, was isolated from a chronic case of bovine mastitis. Protein A was obtained from the organism by heat extraction. The heat extracted, crude protein A was further purified by HCl precipitation, ammonium sulfate precipitation and chromatography on Sephadex G-100 gel and DEAE-cellulose. The purified protein A (fraction A₆) had precipitin reactivity against normal human and rabbit serum. Fraction A₆ was serologically similar to the less pure fractions as indicated by precipitin lines of identity formed against anti-Hill Farm-4 serum in immunodiffusion.

Electrophoresis of fraction A₆ in polyacrylamide disc gel indicated that the preparation was heterogeneous as has been reported for heat extracted protein A preparations from other isolates. A molecular weight of 10,000 was determined by polyacrylamide disc gel electrophoresis in sodium dodecyl sulfate.

Amino acid analysis showed the presence of sixteen amino acids. Glycine, alanine, glutamic acid, lysine, and aspartic acid were present in the highest quantities.
INTRODUCTION

*Staphylococcus aureus* is ubiquitous and is the causative agent in specific cases of mastitis in most dairy herds. Mastitis caused by staphylococci is of particular importance to the dairy industry due to low efficacy of therapy with antibiotics presently available, the high economic losses sustained from reduced milk production, and shortened productive lives of affected cows.

Many factors contribute to the invasion and establishment of *S. aureus* in the bovine mammary parenchyma. The development of an effective immunization procedure would be of great value in preventing infections, and would provide for a better understanding of the factors mediating the pathogenicity of *S. aureus*.

The occurrence of a nonimmunological aggregation between a cell wall antigen, protein A, and certain classes of immunoglobulins, notably G (IgG), has been a deterrent to an understanding of these problems. Elucidation of the chemical, physical, and biological properties of protein A extracted from a bovine isolate of *S. aureus* could lead to a definitive explanation of the reaction. The primary objective of this investigation was to develop techniques for the purification and characterization of the cell wall antigen, protein A. The organism employed (*S. aureus*, Hill Farm isolate 4) was isolated from milk collected from a cow exhibiting chronic mastitis.
Protein A of *Staphylococcus aureus* of Human Origin

Protein A was isolated and characterized by Jensen (1958) as a cell wall component present in most human strains of *S. aureus*. The main criterion for identification of protein A is the development in vitro of a precipitin reaction between protein A and normal human γ globulin, or "normal" serum. Protein A will also precipitate normal rabbit and guinea pig γ globulins (Forsgren, 1968a, 1968b, Forsgren and Sjoquist, 1967, and Lofkvist, 1969). Grov, et al. (1970) have reported that protein A will not precipitate normal rabbit serum (NRS), however in a later study, Grov (1973) attached protein A to a Sepharose 4B column, and passed NRS through the column. A low concentration (0.5 - 1.0 mg/ml) of pure IgG was adsorbed to the protein A. The IgG isolated in this manner reacted weakly with protein A in the ring precipitin test. Grov surmised from this study that NRS has Fc reactive IgG, but in low concentration. Rabbit and guinea pig anti-Cowan I serum, antibodies to *S. aureus* human strain Cowan I, produced visible precipitin reactions with crude protein A. Immunelectrophoresis (IEP) preparations of rabbit and guinea pig anti-sera or normal human serum showed that crude protein A precipitated primarily with IgG.

Grov (1968) examined the serological activity of protein A with human IgG which had been treated with either papain or pepsin. Papain cleaves the IgG molecule into three fragments: two identical, serologically inactive antigen binding fractions (Fab) and a third fragment
which crystallizes at 4°C (Fc) (Porter, 1959). Pepsin digestion cleaves the IgG molecule to yield a large fraction composed essentially of both Fab fragments [F(ab)2], while the Fc portion is further digested into smaller peptides (Nisonoff et al., 1960). Grov (1968) used inhibition studies to show that protein A reacted with normal, papain-digested IgG, but not with normal, pepsin-digested IgG. Nor did pepsin-digested, normal, human IgG fractions inhibit precipitation of protein A with normal human serum. This suggested that protein A combines with the Fc region of the IgG molecule.

Protein A reacts with determinants present in the Fc-region (heavy chain) of IgG of several mammals, and provokes or initiates a series of phenomena generally, heretofore, considered the result of antigen-antibody-complement complexes (Stalenheim, 1971). Aggregates of human or guinea pig IgG and protein A will fix components of the guinea pig complement system (Sjoquist and Stalenheim, 1969). The Fc fragments of human myeloma protein will fix complement in the absence of antigenic material after treatment with protein A (Stalenheim and Sjoquist, 1970). Most aspects of the inflammatory response in tissue to S. aureus infection may be in part initiated by the activation of the complement system. The conformational change in the Fc region of IgG by protein A may be one way in which in vivo activation of complement occurs. The development of an Arthus reaction, which is complement mediated, occurred in rabbits as the result of simultaneous injection of human IgG and protein A (Stalenheim, 1971).

Kronvall, Messner, and Williams (1970) directed their studies toward elucidating the mechanism by which protein A and normal human IgG react and precipitate. Normal, pooled IgG and protein A-precipitating-myeloma globulin were acetylated or carbamylated and
subsequently tested for protein A reactivity. Acetylation rendered all preparations non-reactive with protein A. Carbamylation of normal IgG and the myeloma globulins changed the type of reactivity from one of precipitin to inhibition. The ability of IgG to precipitate with protein A was lost, without loss of reactivity. These authors suggest that precipitation with protein A depends on molecular configurations different from primary protein A reacting structures.

In an attempt to determine the nature of the reactive sites in protein A, Sjoholm, Bjerken, and Sjoquist (1973) quantitatively nitrated the tyrosyl residues of protein A with tetranitromethane. Following nitration, protein A reactivity with Fc fragments was minimal, while immunological reactivity (Fab) with rabbit anti-serum was unaltered. The authors suggested that loss of Fc reactivity may be the result of direct changes in the tyrosyl groups or possibly conformational alterations of protein A following nitration.

Forsgren and Sjoquist (1969) extracted protein A from Staphylococcus aureus, Cowan I by heating to 100°C for 1 hour according to the technique of Jensen (1958). Protein A obtained by this method was heterogeneous when characterized by N-terminal amino acid determinations, gel filtration studies, and polyacrylamide disc electrophoresis. All amino acids were present except cysteine and tryptophan. Five to seven different N-terminal amino acids were identified in all preparations studied. Ultracentrifugation of protein A did produce a symmetrical peak, $S_{20w} = 1.6$, and a molecular weight of about 15,000. Conversely, Grov, Myklestad, and Oeding (1964) reported that protein A prepared by heat extraction was homogeneous with a molecular weight of 12,000. Their preparation was composed of about ten amino acids, and the
N-terminus was determined to be alanine.

More recently, Sjoquist, Meloun, and Hjelm (1972) reported that protein A extracted from *S. aureus*, strain Cowan I, by lysostaphin digestion was homogenous, exhibited a molecular weight of 42,000 when determined by ultracentrifugation, and contained sixteen amino acids. Sjoquist, Movitz, and Johansson (1972) reported that protein A is covalently linked to the peptidoglycan structure of the cell wall.

Reports on staphylococcal protein A have suggested that it is a constituent of the cell wall (Forsgren and Forsum, 1970; Nickerson, et al., 1970; Sjoquist, Movitz, and Johnasson, 1972; Yoshida, Mudd, and Lenhart, 1963) and also an extracellular product (Forsgren, 1969). The fact that protein A reacts directly with the Fc portion of the IgG of many mammals is of particular theoretical interest since many inflammatory events could be initiated by an IgG-protein A complex. Strains of *S. aureus* which possess large amounts of protein A appear to be more resistant to phagocytosis than other strains producing minimal amounts.

The production and elaboration of coagulase by staphylococci is accepted as the absolute criterion for pathogenicity and a close correlation is known to exist between coagulase production and deoxyribonuclease (DNAse) activity (Forsgren, 1970). Thus, the determination of DNAse production would likely be another valuable criterion for pathogenicity. Of 700 coagulase positive strains tested, all were DNAse positive, and 692 were positive for protein A, and of 100 coagulase-negative strains tested only two produced DNAse and protein A. Good correlation, therefore, exists between protein A production,
coagulase, and DNAase activity. Almost all strains regarded as potential pathogens produce protein A, and the presence of protein A may, possibly, be more directly related to the mechanism of pathogenesis than their ability to hydrolyze DNA or coagulate plasma (Forägren, 1970).

**Protein A of Staphylococcus aureus of Bovine Origin**

A paucity of reports exists concerning the nature of protein A from *S. aureus* isolates recovered from cases of bovine mastitis. Kronvall, Holmberg, and Ripa (1972) examined *S. aureus* strains from human infections and compared them with isolates from acute or chronic cases of bovine mastitis. Serological strains of *S. aureus* of human origin and strains isolated from acute mastitis cases produced high levels of protein A, whereas, strains isolated from chronic cases of mastitis produced significantly lower levels of protein A. The reasons are not yet known for the discrepancies in protein A content of staphylococcal strains causing acute or chronic bovine mastitis. Possibly acute mastitis is dependent upon protein A as a virulence factor.

Live and Ranu (1968) investigated the use of protein A as an antigen for sensitizing sheep red blood cells for use in the passive hemagglutination test. They reported that serum titers from dairy cows varied greatly. Cows with histories of staphylococcal mastitis demonstrated higher hemagglutination titers and immunodiffusion reactions were observed only with sera having high titer hemagglutination reactions.
MATERIALS AND METHODS

Bacterial Strain

The test organism, Staphylococcus aureus, was isolated from a chronic case of bovine mastitis, identified by standard methods (National Mastitis Council) and designated Hill Farm-4 (HF-4). Isolation was made on tryptose blood agar (TBA) (Difco Laboratories, Detroit, Mich.) containing 5% citrated bovine blood. HF-4 was used to obtain protein A and for immunization studies in rabbits.

Cultivation of S. aureus for Vaccine Production

HF-4 was grown in Kolle flasks containing 40 ml of Trypticase Soy Agar (BBL, Baltimore, Md.). Each agar flask was seeded with 5.0 ml of an 18 hour nutrient broth (Difco) culture of HF-4. The flasks were incubated at 37 C for 24 hours. The bacterial lawns were harvested with 40 ml sterile saline, (0.15 M NaCl) washed two more times with saline, and diluted with saline to yield approximately 5 X 10^9 cells/ml. Plate counts were done to determine the concentration of viable cells. The cells were killed by the addition of formalin to a concentration of 0.5% and held at 5 C for at least 24-48 hours. Streak plates were made from the formalized cell suspensions on TBA to determine sterility. The vaccine was stored at 5 C until used for immunization.

Immunization Procedures

Experimental Animals. New Zealand white rabbits, weighing 1.5-2.0 kg, were used throughout this study for the production of antiserum. The animals were housed in wire enclosures suspended above the floor and were fed approximately 4 ounces of Purina "rabbit
pellets" per day. Water was given ad libitum.

**Serum Collection and Preservation.** Prior to immunization each animal was bled via the central ear artery, and the collected blood was allowed to clot at room temperature for 1 hour before being rimmed. The rimmed clot was held overnight at 5°C and the serum was separated by centrifugation at 1200 rpm for 15 minutes in a refrigerated International centrifuge, model PR-2 (International Equipment Co., Needham, Mass.). The clarified serum was decanted and stored at -5°C until needed.

Normal bovine serum (NBS) was collected from a 48 hour old calf whose dam did not have any history of mastitis. The serum was prepared in a manner similar to that described above.

Normal human serum (NHS) was obtained from an individual with no history of *S. aureus* infections (Courtesy of Homer Memorial Hospital, Homer, La.)

**Immunization Schedule.** The immunization schedule of Cohen and Oeding (1962) was used for the production of anti-HF-4 serum in rabbits. Three series of injections of the whole cell vaccine were used. The first series consisted of 0.1, 0.2, and 0.4 ml aliquots of HF-4 vaccine injected on three successive days. After a five day rest period the second series was administered which included 0.4, 0.6, and 0.8 ml aliquot injections on three successive days. The third and final series of injections were made after a five day rest period and consisted of 0.8, 1.0, and 1.0 ml aliquots injected on three successive days. The rabbits were test bled four weeks after the final injection of the whole cell antigen.
Cultivation of S. aureus for Extraction

The S. aureus cells used for extraction of protein A were cultured in a medium designated nutrient broth plus (NB+). It consisted of Nutrient Broth (Difco) 16.0 g, NaCl 3.0 g, Na$_2$HPO$_4$·2 H$_2$O, 4.0 g, and glucose 2.0 g which were dissolved in 1000 ml distilled water. The pH was adjusted to 7.8 with N NaOH for 1000 ml quantities or with 10 N NaOH for 10 liter volumes.

An isolated colony was picked from a TBA plate to 10 ml NB+ and incubated for 10 hours at 37 C and this was then used to inoculate 1000 ml of NB+. The 1000 ml seed culture was incubated at 37 C for 18 hours on a gyratory shaker at 37 C and used to inoculate 10 liters of NB+. The 10 liter batch cultures were grown on a New Brunswick Continuous Culture Apparatus, model CF 500 (New Brunswick Instruments, Inc. New Brunswick, N.J.), for 18 hours at 37 C with vigorous agitation (300 rpm) and aeration. The cells were subsequently collected by centrifugation at 40,000 rpm in a steam driven, cooled Sharples Super-Centrifuge (Sharples, Warminster, Pa.). The cells were weighed, washed once with saline and the pellet stored at -5 C.

Purification Methods

Sephadex Gel Preparation. Super fine grade Sephadex G-100 (Pharmacia Fine Chemicals, Inc., Piscataway, N. J.) was used in the purification process. Twenty grams of the gel were suspended in 700 ml of 0.1 M ammonium acetate buffer, pH 4.5 and swollen by heating in a boiling water bath for 5 hours. The swollen gel was cooled to room temperature and the fine particles were removed by siphoning the supernatant fluid by aspiration. Ammonium acetate buffer was added
to restore the volume to 700 ml and mixed for 15 minutes. The
swollen gel was allowed to settle, followed by aspiration of the
supernatant fluid. This step was repeated four to six times, or until
very few fine particles could be seen in the supernatant fluid after
15 minutes of settling.

After removing the fines, a slurry of the Sephadex gel was prepared
by the addition of 300 ml of ammonium acetate buffer. The slurry was
degassed with a vacuum pump and slowly poured down a glass rod into
a Pharmacia Type K26/40 column which contained approximately 50 ml of
ammonium acetate buffer. A flow adaptor was used at the base of the
column. The entire slurry was poured into the column and the attached
R-26 Pharmacia Eluant Reservoir. The gel was allowed to settle by
gravity; a flow adaptor, with a four-way valve (Pharmacia LV-4), was
attached, and the gel was subsequently packed by passing 500 ml of
ammonium acetate buffer through the column at a rate of about 6 ml/hour
with a constant head pressure maintained at 15 cm. Two ml (1 mg/ml)
of Blue Dextran 2000 was passed through the column to determine both
the packing efficiency and the void volume.

Sephadex Gel Chromatography. Following the ammonium sulfate
precipitation, fraction A4 (Figure 1) was chromatographed on Sephadex
G-100. The lyophilized sample (105 mg) was dissolved in 5 ml of 0.1 M,
ammonium acetate, pH 4.5 (Live and Ranu, 1968). This sample was
eluted with the ammonium acetate buffer and 5 ml fractions were col-
lected on a Gilson Model FC-80 Fractionator (Gilson Medical Electronics,
Inc., Middleton, Wisc.). These fractions were assayed for absorbance
at 280 nm in a Beckman DB-GT Spectrophotometer (Beckman Instruments,
Palo Alto, Calif.). Fractions collected under each peak were pooled and assayed by immunodiffusion against NHS to determine the presence of protein A activity. Approximately three void volumes were collected after the final peak and then 2 ml of Blue Dextran 2000 was applied to the column and eluted to ascertain the void volume.

**DEAE - Cellulose Column Preparation.** Diethylaminoethyl (DEAE) - cellulose, No. 71, 0.89 meg/gm, (Schleicher and Schuell, Inc., Keene, N. H.) was used in one purification step. Four grams of DEAE-cellulose powder was allowed to settle into 260 ml of deionized water. This wet powder was then mixed for 15 minutes, allowed to settle for 30 minutes, and the supernatant fluid, containing fines, was aspirated. This step was repeated three times, or until the fines were removed.

The wet cellulose was then suspended in 260 ml of 0.5 N NaOH, mixed for 15 minutes, allowed to settle for 60 minutes, and the supernatant fluid removed by aspiration. This step was repeated three times in order to remove the alkali soluble substances. The DEAE-cellulose was washed with deionized water until the pH returned to neutral.

The wet DEAE was treated once with 260 ml of 95% ethanol and washed four times in deionized water. The slurry was then treated four times with 260 ml of 0.5 N HCl by mixing 15 minutes, settling 60 minutes, and removing the supernatant fluid by suction. The DEAE-cellulose was washed with deionized water until a neutral pH was attained.

Next, the slurry was alternately washed four times with starting buffer (0.02 M, pH 7.2 phosphate buffer) and deionized water. Following the final wash, 0.02 M phosphate buffer was added to a volume of 40 ml. The cleaned DEAE-cellulose was degassed by evacuation with a vacuum pump. The degassed slurry was poured down a glass rod into a 0.9 x 30
cm column, Pharmacia Type K9/30, which contained about 5 ml of the 0.02 M phosphate buffer. The column was packed at room temperature. First, the resin was allowed to settle by gravity, and then about 300 ml starting buffer was passed through the column at a flow rate of about 25 ml/hour.

**DEAE - Cellulose Column Chromatography.** Fraction A5 (Figure 1) collected from the Sephadex G-100 column was dialyzed against three changes of deionized water over 24 hours, lyophilized, and dissolved in 4.0 ml of 0.02 M phosphate buffer, pH 7.2. A 3.5 ml aliquot of this preparation, containing 39.4 mg protein, was gently layered on the resin and allowed to sink into the bed by gravity. The sides of the column were washed twice with 1.0 ml of starting buffer. The protein was eluted by a stepwise addition of phosphate buffer pH 7.2: 0.02 M, 0.05 M, 0.1 M, 0.2 M, and pH 6.8, 0.5 M. Five ml fractions were collected and absorbance read at 280 nm. The next highest molarity of buffer was added after each peak had been eluted from the column.

**Assay Methods**

**Protein Determinations.** Protein determinations were done by a modification of the technique of Lowry et al. (1951) following each purification step. The sample to be tested was diluted in deionized water to approximately 0.1 - 0.3 mg protein/ml. The reagents used were as follows:

**Lowry Reagents for Protein Determination**

<table>
<thead>
<tr>
<th>Lowry A</th>
<th>Lowry B</th>
</tr>
</thead>
<tbody>
<tr>
<td>4 g NaOH</td>
<td>1.0 g sodium citrate</td>
</tr>
<tr>
<td>20 g Na₂CO₃</td>
<td>0.5 g CuSO₄ • 5H₂O</td>
</tr>
<tr>
<td>1000 ml deionized water</td>
<td>1000 ml deionized water</td>
</tr>
</tbody>
</table>
Lowry C  Lowry D
50 ml Lowry A : 1 ml Lowry B  1.0 ml Phenol Reagent
2 N Solution
1.0 ml deionized water

Reagents C and D were prepared freshly for each determination and
a standard curve was made with bovine serum albumin, fraction IV
(Sigma Chemical Co., St. Louis, Mo.) with triplicate samples containing 0.1, 0.2, 0.3 mg/ml.

Five ml of Lowry C solution was added to 1.0 ml of the sample,
mixed on a Vortex mixer and allowed to stand for 10 minutes. Next,
0.5 ml of Lowry D solution was added, quickly mixed on a Vortex
mixer and incubated for 2 hours at room temperature. The samples were
read on a Klett-Summerson Colorimeter (Klett Manufacturing Co., Inc.,
New York, N. Y.) using filter No. 66 (660 nm). The protein concentra-
tion of each sample was calculated from the standard curve.

**Immunodiffusion: Preparation of Agar and Slides.** The procedures
used for conducting the immunodiffusion (ID) assay were a modification
of those published in the Gelman Pamphlet No. 75824-A (Gelman
Instrument Co., Ann Arbor, Mich.).

One bottle (454 g) of Special Agar-Noble (Difco) was washed
three times in 4 liters of deionized, glass distilled water. The wet
agar was then washed once with 1000 ml of reagent grade acetone, the
acetone was siphoned off and the agar air dried under a dust proof
cover. A 1.5% concentration of acetone dried agar was made in 0.10 M
NaCl, dissolved at 100 C, and held in an 80 C water bath until dispensed.

Slides used in the ID assay were cleaned immediately prior to
use. Slides were washed with Alconox soap in warm water, rinsed
thoroughly with deionized water, immersed in dichromate cleaning solution for 60 minutes, rinsed in copious amounts of deionized water, and stored in acetone. The slides were removed from the acetone, air dried and placed in a Gelman immuno slide frame. An adhesive agar film was layered over each slide in the following manner: two to three drops of molten 0.1% noble agar were pipetted to each row of three slides and then spread evenly across the surface of each slide with a microporous wick. The film was allowed to dry for at least 15 minutes before the frame was placed on a leveling table. Twelve ml of molten 1.5% noble agar was pipetted over each row of three slides.

**Immunodiffusion Assay.** Three sets of gel punch patterns were used: Gelman die No. 72410, circular patterns, die 72409, parallel rows, and die 71639, center troughs. When the circular punch pattern was used, the wells were 5.0 mm apart. The parallel well punch pattern, with a center trough, gave wells 2.5 mm from the 2.0 mm diameter trough. All wells were 2.0 mm in diameter.

The precipitation of NHS by protein A present in the various fractions obtained during the purification procedure was assayed by a modification of the technique described by Lofkvist and Sjoquist (1963). The various fractions, A1 through A6, and NHS were diluted in saline. The protein concentration for the six fractions was 3.75 mg/ml in all assays performed against NHS. Lofkvist and Sjoquist (1963) used 1 mg protein/ml in their assays. In this study inconsistent results were obtained when 1.0 ml samples were used, therefore, 3.75 mg/ml was used. Two-fold dilutions of each fraction were made in the saline and 10 µl volumes of each dilution were carefully introduced into representative wells using a 50 µl Hamilton syringe
(Hamilton Co., Whittier, Calif.). The center well was filled with 150 µl of a 1:2 dilution of NHS. The ID slides were incubated in a humidity chamber at room temperature for 24 hours and then incubated at 5°C for an additional 24 hours before precipitin lines were read.

Total and specific activities were calculated by the method reported by Lofkvist and Sjoquist (1963). The total activity is determined by the equation: dilution X volume of undiluted protein A. Specific activity is expressed as dilution coefficient/mg protein per ml.

For photographic purposes the slides were stained by a modification of the protocol outlined by the Gelman pamphlet. The slides were rinsed for 6 hours in 1% NaCl, followed by a second rinse of 16 hours with 1% NaCl, and finally rinsed in deionized water for 1 hour. Slides were stained for 5 minutes with 1% Buffalo Black NBR (Allied Chemical Co., Morristown, N. J.) dissolved in rinsing solution: absolute methanol: concentrated acetic acid: deionized water, at a ratio of 45:10:45. The stained slides were washed four times, for 10 minutes each, in the rinsing solution.

Additional Immunodiffusion Assays. Various fractions obtained during the purification process were tested for precipitation activity against normal bovine serum (NBS) and normal rabbit serum (NRS) by the ID assay described above. The titers of the different fractions against rabbit anti-HF-4 were determined by the ID technique.

Purification Steps

The protocol employed for the extraction and partial purification of protein is pictured in Figure 1.
Heat Extraction. Frozen HF-4 cells were thawed at 5 C and then heat extracted according to the method described by Jensen (1958). Cells were suspended in 0.15 M phosphate buffer, pH 5.9. The cell suspension was placed in a boiling water bath for 60 minutes, chilled to 5 C in an ice bath, and then sedimented at 2,750 x g (6,500 rpm) for 30 minutes in Sorvall RC-2B centrifuge (GSA centrifuge head, Ivan Sorvall, Inc., Newton, Conn). The supernatant fluid was collected and recentrifuged at 6,500 for an additional 30 minutes and subsequently designated fraction A1.

Acid Precipitation. The yellow colored supernatant fluid was decanted and 0.1 N HCl was added dropwise, with slow mixing, until a pH of 3.5 was reached. This step was performed at 5 C. The resulting precipitate was sedimented by centrifugation at about 10,000 x g for 60 minutes in a Sorvall RC-2B centrifuge. The sediment was dissolved in 0.1 M phosphate buffer, pH 5.9 (fraction A3). The supernatant fluid was decanted and the pH was adjusted to 7.0 with N NaOH (fraction A2), and was used for further purification.

Ammonium Sulfate Fractionation. Solid ammonium sulfate was added slowly to fraction A2 to achieve 80% saturation (561 g/l) and gently stirred for one hour at 5 C. The resulting precipitate was collected by centrifugation, at 10,000 x g, for 60 minutes and was then dissolved in deionized water, dialyzed against three changes of deionized water over a 24 hour period at 5 C, and then lyophilized (fraction A4).

Sephadex Gel Chromatography. Lyophilized fraction A4 was dissolved in 5 ml 0.1 M ammonium acetate buffer pH 4.5. Fraction A4 was applied to a Sephadex G-100 column equilibrated with the same
buffer, and eluted at a flow rate of 6 ml per hour. Five ml fractions were collected dropwise with a Gilson Micro-Fractionator, Model FC-80. Each fraction was assayed at 280 nm in a Beckman DB-GT Spectrophotometer. Fractions under peaks which demonstrated 280 nm absorbancy were pooled and assayed by the immunodiffusion assay against NHS. The peak with activity was used for further purification (fraction A₅).

**DEAE - Cellulose Chromatography.** Fraction A₅ was dialyzed against three changes of deionized water over 24 hours and lyophilized. The sample was dissolved in 3.5 ml of 0.02 M phosphate buffer, pH 7.2, and applied to a DEAE column (0.9 x 10 cm), equilibrated with the same buffer. The sample, which contained 30 mg of protein, was eluted stepwise with pH 7.2 phosphate buffer: 0.02 M, 0.05 M, 0.1 M, 0.2 M, and pH 6.8, 0.5 M phosphate buffer. Five ml fractions were collected on a Model FC-80 Gilson Micro-Fractionator at a flow rate of about 25 ml/hour. Absorbancy was monitored at 280 nm with a Beckman DB-GT Spectrophotometer. The buffer was changed to the next highest molarity after each peak was eluted from the column. The fractions collected under each peak were pooled and assayed by the immunodiffusion technique against NHS. The peak containing precipitin activity was designated fraction A₆ and this fraction was considered to be protein A as purified according to the methods of Live and Ranu (1968).

**Physical and Chemical Methods**

Polyacrylamide disc gel electrophoresis was conducted as described by Davis (1964) and Ornstein (1964). The fractions tested were placed in 40% sucrose instead of 3% acrylamide, at the following protein concentrations: fraction A₁-476 ng, and fraction A₆-375 ng. A
Beckman-Spinco Constat Regulated Power Supply (Beckman Instruments, Inc., Palo Alto, Calif.) was used to provide 4 mA/gel. Bromphenol blue was employed as the tracking dye. Electrophoresis was performed for 60 minutes and the gels were then stained with 1% amido black 10B (in 7% acetic acid) for 10 minutes in 20 x 125 mm screw cap tubes. The gels were destained in 7% acetic acid by shaking overnight on a reciprocating shaker, and the acetic acid was changed several times during the destaining process.

Sodium Dodecyl Sulfate Gel Electrophoresis. Sodium dodecyl sulfate (SDS) gel electrophoresis was carried out on fraction A6 (375 ng) according to the methods of Dunker and Ruecker (1969). In order to estimate the molecular weight of the protein A, three standards (60 ng each) were used in different gels run in parallel. Lysozyme (Sigma Chemical Co., St. Louis, Mo.), molecular weight (mw) 14,300 and ovalbumin (Pharmacia), mw 43,000 were run by cielectrophoresis and yeast alcohol dehydrogenase (Nutritional Biochemical Co., Inc., Cleveland, Ohio), mw 37,000 was electrophoresed in a separate gel. A Beckman power supply was employed to provide 6 mA/tube, and bromphenol blue was again used as the indicator marker.

Following a 5 hour electrophoresis run the gels were stained with 0.02% Coomassie brilliant blue R250 (in 50% methanol and concentrated acetic acid, 454:46) overnight, and then destained with methanol: acetic acid:distilled water (50:75:875) on a reciprocating shaker for approximately 24 hours. The destaining solution was changed frequently.

Amino Acid Analysis. Duplicate protein A samples (1 mg) were hydrolyzed with 2 ml of constant boiling HCl at 110 C in sealed
evacuated hydrolysis tubes for 48 hours.

The samples were analyzed by the methods of Meachum, Colvin, and Braymer (1971) using a Spinco 120C amino acid analyzer (Beckman Instruments Co., Palo Alto, Calif.).
Figure 1. Outline of procedures used to purify protein A from \textit{Staphylococcus aureus}, HF-4.
Staphylococcus aureus, HF-4
206 g cell/ 405 ml 0.15 phosphate buffer
pH 5.9

1. heat extraction 100 C, 60 minutes
2. centrifuge 2,750 x g, 30 minutes

Sediment
Discard

Supernatant fluid (fraction A₁)
405 ml
1. adjust to pH 3.5 at 5 C
2. centrifuge 10,000 x g, 30 minutes

Supernatant fluid (fraction A₂)
1. adjust to pH 7.0
2. dialyze against deionized water
3. lyophilize
4. dissolve with 50 ml phosphate buffer, 0.1 M pH 7.0
5. 80% ammonium sulfate precipitation
   60 minutes at 5 C
6. centrifuge 10,000 x g, 30 minutes

Sediment
(fraction A₃)

Precipitate
1. dissolve in deionized water
2. dialyze against deionized water
3. lyophilize, dissolve in 5.0 ml, 0.1 M ammonium acetate, pH 4.5
4. chromatograph on Sephadex G-100 column
   Fractions under 280 nm absorbance peaks
   1. ID against NHS
   2. peak with precipitin activity dialyzed against deionized water
   3. lyophilized, dissolved in 3.5 ml 0.02 M phosphate buffer, pH 7.2 (fraction A₅)

   DEAE - cellulose
   1. DEAE - cellulose
   2. stepwise elution with phosphate buffer
   3. assayed against NHS by ID
   4. peak with activity dialyzed against deionized water
   5. lyophilized,
   6. dissolved with 8.0 ml of 0.02 M phosphate buffer, pH 7.0 (fraction A₆)
RESULTS AND DISCUSSION

Extraction and Purification Procedures

The yellowish supernatant fluid, fraction A₁, obtained after centrifugation of the heat extracted S. aureus, HF-4 cells, had a pH of 6.8. The protein concentration was 4.76 mg/ml (Table 1) and exhibited a specific activity of 0.27 (Table 2) as determined by the ID assay against NHS.

Fraction A₂, the supernatant fluid collected after HCl precipitation, showed a protein concentration of 7.5 mg/ml (Table 1) and a specific activity of 0.53 (Table 2). The recovery of protein was 19.4% with a two-fold purification. Lofkvist and Sjoquist (1963) reported that treatment of cells, disintegrated by an X-press, with 0.1 N HCl produced an eight-fold purification and 50-60% recovery. The discrepancy between their observation and the results of this investigation may be explained by the different extraction techniques used and the possible differences between bovine protein A and that of human strains of S. aureus. Staphylococcus aureus, HF-4, cells were heat extracted in 0.15 M phosphate buffer, whereas Lofkvist and Sjoquist disintegrated S. aureus, Cowan I, and extracted with distilled water at 5°C prior to treatment of the supernatant fluid with HCl.

The ammonium sulfate precipitate (fraction A₄) exhibited a protein concentration of 21.0 mg/ml (Table 1) and a specific activity of 1.07 (Table 2). This step yielded a two-fold purification of the acid treated supernatant fluid which agrees with the two-fold purification observed by Lofkvist and Sjoquist (1963). However, a recovery
of only 28% was obtained in this study while Lofkvist and Sjoquist reported approximately 80% recovery of the protein by ammonium sulfate precipitation. This difference in recovery could be explained by the difference in protein concentration of the material which was precipitated. The HF-4 fraction A2 had a protein concentration of 7.5 mg/ml and the fraction precipitated by ammonium sulfate by Lofkvist and Sjoquist (1963) had a concentration of about 30 mg/ml. Within certain limits, the higher the protein concentration the greater the efficiency of recovery after ammonium sulfate precipitation. The elution pattern of fraction A4 when passed through Sephadex G-100 with 0.1 M ammonium acetate, pH 4.5 is presented in Figure 2. Two peaks were eluted. The first and major peak had a shoulder following the apex of the 280 nm absorbance peak. Fractions composing the second peak had 280 nm absorbance reading of less than 1.0, and ID assay of these pooled fractions in the second peak against NHS showed no precipitin reaction. Fractions of the initial peak did produce a precipitin reaction against NHS by ID and these pooled fractions were designated fraction A5. Fraction A5 had a specific activity of 2.13 (Table 2). A two-fold purification and approximately a 38% recovery was obtained by this purification step. The ammonium sulfate precipitate of Lofkvist and Sjoquist (1963) separated into four peaks when chromatographed through Sephadex G-100 with 0.1 M ammonium acetate, pH 4.4. Protein A activity was localized in the first two peaks eluted. These two investigators used a flow rate of 30 ml/hour as opposed to a flow rate of 6 ml/hour used in this study. The slower flow rate may account for the splitting of the first peak, producing appearance of a shoulder. This shoulder
Table 1. Protein determination data for the purification fractions

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Volume</th>
<th>Protein concentration</th>
<th>Total protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>A1</td>
<td>405</td>
<td>4.76</td>
<td>1927.8</td>
</tr>
<tr>
<td>A2</td>
<td>50</td>
<td>7.5</td>
<td>375.0</td>
</tr>
<tr>
<td>A4</td>
<td>5</td>
<td>21</td>
<td>105.0</td>
</tr>
<tr>
<td>A5</td>
<td>3.5</td>
<td>11.25</td>
<td>39.4</td>
</tr>
<tr>
<td>A6</td>
<td>8</td>
<td>3.75</td>
<td>30.0</td>
</tr>
<tr>
<td>Fraction&lt;sup&gt;a&lt;/sup&gt;</td>
<td>Titer&lt;sup&gt;b&lt;/sup&gt;</td>
<td>Total activity&lt;sup&gt;c&lt;/sup&gt;</td>
<td>Specific activity&lt;sup&gt;d&lt;/sup&gt;</td>
</tr>
<tr>
<td>---------------------</td>
<td>----------------</td>
<td>--------------------------</td>
<td>---------------------------</td>
</tr>
<tr>
<td>A&lt;sub&gt;1&lt;/sub&gt;</td>
<td>0</td>
<td>405</td>
<td>0.27</td>
</tr>
<tr>
<td>A&lt;sub&gt;2&lt;/sub&gt;</td>
<td>2</td>
<td>100</td>
<td>0.53</td>
</tr>
<tr>
<td>A&lt;sub&gt;4&lt;/sub&gt;</td>
<td>4</td>
<td>20</td>
<td>1.07</td>
</tr>
<tr>
<td>A&lt;sub&gt;5&lt;/sub&gt;</td>
<td>8</td>
<td>28</td>
<td>2.13</td>
</tr>
<tr>
<td>A&lt;sub&gt;6&lt;/sub&gt;</td>
<td>16</td>
<td>128</td>
<td>4.27</td>
</tr>
</tbody>
</table>

<sup>a</sup>Each fraction was diluted with 0.15 M NaCl. Undiluted fractions contained 3.75 mg/ml protein.

<sup>b</sup>Reciprocal of the greatest dilution of the fraction which exhibited a visible precipitin reaction with normal human serum.

<sup>c</sup>Total activity = dilution x volume of undiluted protein A.

<sup>d</sup>Specific activity = total activity per total volume of undiluted protein A x mg protein per ml.
region might correspond to the second peak observed by Lofkvist and Sjoquist (1963) which also had a protein A activity against NHS as did the shoulder fraction on the elution pattern seen in Figure 2. Lofkvist and Sjoquist (1963) reported that this step produced a three-fold purification and almost quantitative recovery, whereas Live and Ranu (1968) observed a 1.5-fold purification by gel filtration through Sephadex G-100 and also had a near quantitative recovery. The two-fold purification obtained in this study agrees with that noted by Lofkvist and Sjoquist (1963) and Live and Ranu (1968), but only a 37.5% recovery was obtained which is much lower than these two groups obtained. The size of the Sephadex column used in this study may be one explanation of the low yield. The column may have been too large for the load of fraction A4 used.

Fraction A5 was subsequently chromatographed on DEAE-cellulose according to the technique of Live and Ranu (1968). The pattern obtained by stepwise elution is illustrated in Figure 3. The only peak which demonstrated protein A activity against NHS was eluted with 0.02 M phosphate buffer, pH 7.2. The fractions under this peak were pooled, concentrated by lyophilization and dissolved in 8 ml of 0.02 M phosphate buffer, pH 7.2 (fraction A6). Fraction A6 had a specific activity of 4.27 (Table 2) and represented approximately a 16-fold purification when compared to the activity of crude protein A (fraction A1) and two-fold purification of fraction A5. Recovery was about 76%. This material was considered purified protein A and used for further characterization studies.

Live and Ranu (1968) reported that DEAE-cellulose chromatography
Figure 2. Absorbance at 280 nm of fractions eluted from a Sephadex G-100 column with 0.1 M ammonium acetate, pH 4.5.

--- Fractions precipitated by NHS and further purified.
Figure 3. Absorbance at 280 nm of fractions collected from DEAE-cellulose column by stepwise elution with pH 7.0 phosphate buffer: 0.02 M, 0.05 M, 0.1 M, 0.2 M, and pH 6.8, 0.5 M. ——— Fractions precipitated by NHS.
of the material eluted from Sephadex G-100 produced a 1.5-fold purification with about 90% recovery. The 76% recovery obtained in this study is in close agreement with these data. The 1.5-fold purification reported by Live and Ranu is close to the 2.0-fold purification of fraction A5 obtained in this study by DEAE-cellulose chromatography.

**Serological Studies**

The results of the immunodiffusion assay performed with the different fractions (3.75 mg/ml) against a 1:2 dilution of NHS in saline are presented in Table 2. The precipitin activities of the different fractions (A1 - A6) against NHS increased with each purification step. These results are in good agreement the specific activity data obtained by Lofkvist and Sjoquist (1963). Figure 4 illustrates the precipitin reaction of fraction A6 against 1:2 NHS which, when assayed, had a titer of 16. Six fractions collected during the purification steps were tested by immunodiffusion against antiserum, with anti-HF-4 activity, to determine their serological activity. Figure 5 presents an Ouchterlony two dimensional diffusion with anti-HF-4 in the center well and the six fractions in the satellite wells. A reaction of identity was formed between all six fractions. A reaction of partial identity developed between fraction A3 and fractions A2 and A4. Fraction A3, the HCl precipitate, which was not used for further purification, contains about 50% of the total protein A obtained by heat extraction (Lofkvist and Sjoquist, 1963). Apparently protein A along with other antigenic determinants were removed by the acid precipitation step. The darker color stain of fraction A3 may be explained by the higher protein concentration used
with this fraction for immunodiffusion.

Several of the fractions show more than one precipitin line with NRS (Figure 6). The precipitin line which developed between fraction A3 and fractions A2 and A4 may represent different size aggregates of protein A. Bjork, Peterson, and Sjöquist (1972) have reported that heat extraction causes dissociation of protein A into different molecular weight entities. Subsequent purification steps of this study could have separated the different entities which are now demonstrable by separate, two or more, precipitin lines between each fraction and NRS. The precipitin lines observed on this assay represent a reaction between each of the fractions and the Fc portion of IgG, whereas precipitin reaction observed in Figure 5 represent a composite of protein A Fc reaction and specific antibody activity (Fab) directed against antigenic determinants on the different fractions.

Immunodiffusion of fractions A3 and A4 against anti-HF-4 showed a line of identity which further substantiates the supposition that these fractions are homogeneous antigenically and also shows multiple lines of precipitation which may represent heterogenous aggregate size (Figure 7). Others have reported that more than one antigenic determinant is present during the purification steps (Lofkvist and Sjöquist, 1963). These investigators observed four to five precipitin lines between the ammonium sulfate precipitated fraction and anti-S. aureus, Cowan I serum. Grov et al. (1964) reported that a double precipitin line developed between the protein A preparation and anti-Cowan I serum in agar precipitation. However, fraction A5 developed only a single precipitin line against anti-HF-4 serum, but did have two
precipitin lines against NRS as was also seen with fraction A₅.

Physical and Chemical Characterization

Polyacrylamide disc electrophoresis of fraction A₆ (375 ng) resulted in six visible bands, one band stained darker than the other five (Figure 8). Disc electrophoresis was also performed with fraction A₁ in order to compare the crude protein A (fraction A₁) and purified protein A (fraction A₆). Eight protein bands were observed on the gel in which fraction A₁ was electrophoresed (Figure 8). These results indicate that some of the contaminating proteins associated with crude protein A were removed by the purification procedures.

An attempt was made to assay an unstained gel after electrophoresis in an effort to determine which of the protein bands had protein A activity. The disc gel was placed in molten 1.5% noble agar (60°C), parallel troughs were cut on each side of the gel, and filled with 150 µl of either 1:2 NHS or 1:2 anti-HF-4 serum. No precipitin lines developed between the gel and either serum sample. One possible explanation for no discernable reaction may be the low concentrations of antigenic material present in the disc gel. As observed during the immunodiffusion assay, concentrations in excess of 1 mg/ml were needed in order to obtain satisfactory precipitin reactions against NHS.

The multiple protein bands observed on the polyacrylamide gel resemble gel patterns for protein A reported by Forsgren and Sjoquist (1969). Analytical polyacrylamide electrophoresis of the material obtained from DEAE-Sephadex chromatography yielded a broad band with
Figure 4. Immunodiffusion assay of fraction A\textsubscript{6} with a 1:2 dilution of NHS. Highest dilution giving a precipitin reaction was 1:16. Each well contained 10 \(\mu\)l of diluted antigen and the center trough contained 150 \(\mu\)l of 1:2 NHS in saline. 0-3.75 mg/ml, 2-fold dilutions.
Figure 5. Immunodiffusion reactions of A\textsubscript{1} 4.76 mg/ml, A\textsubscript{2} 2.5 mg/ml, A\textsubscript{3} 10.1 mg/ml, A\textsubscript{4} 4.5 mg/ml, A\textsubscript{5} 5.5 mg/ml, and A\textsubscript{6} 3.75 mg/ml against a 1:2 dilution of rabbit anti-\textit{S. aureus}, HE-4. Clockwise from A\textsubscript{1} are fractions A\textsubscript{2}, A\textsubscript{3}, A\textsubscript{4}, A\textsubscript{5}, and A\textsubscript{6}. Each satellite well was filled with 10 \( \mu l \) of antigen and the center well was filled with 10 \( \mu l \) of antibody.
Figure 6. Immunodiffusion reactions of $A_1$ 4.76 mg/ml, $A_2$ 2.5 mg/ml, $A_3$ 10.1 mg/ml, $A_4$ 4.5 mg/ml, $A_5$ 5.5 mg/ml, and $A_6$ 3.75 mg/ml against a 1:2 dilution of NRS in saline. Clockwise from $A_1$ are fractions $A_2$, $A_3$, $A_4$, $A_5$, and $A_6$. Each satellite well contained 10 μl of antigen and the center well contained 10 μl NRS.
Figure 7. Immunodiffusion of fractions A₃ 10.1 mg/ml and A₄ 4.5 mg/ml against a 1:2 saline dilution of anti-\textit{S. aureus}, HF-4.
Figure 8. Polyacrylamide disc electrophoresis and sodium dodecyl sulfate system patterns for the various fractions of protein A. A. fraction A₁. B. fraction A₆. C, D, and E are in an SDS system. C. fraction A₆. D. yeast alcohol dehydrogenase. E₁ ovalbumin, and E₂ lysozyme.
1-3 faint bands. These workers also used heat extraction to obtain the crude protein A from *S. aureus* cells.

Bjork, et al. (1972) reported on certain physiochemical properties of protein A extracted from *S. aureus*, Cowan I, by lysostaphin digestion, heat extraction, and protein A extracted with lysostaphin and then heated for 2 hours to simulate heat extraction.

Lysostaphin possess three enzymatic specificities: amidase activity which will cleave the amide bond between muramic acid and the N-terminal L-alanine in the tetrapeptide, peptidase activity which will hydrolyze the pentaglycyl bridge that connects adjacent tetrapeptides; and N-acetylglucosaminidase activity which will cleave the glucosidic linkage between muramic acid and N-acetylglucosamine (Sjoquist, Meloun, and Hjelm, 1972) Lysostaphin extraction of protein A from *S. aureus* cell walls is considered to be a more gentle method by which to extract protein A than heat extraction (Sjoquist, Meloun, and Hjelm, 1972).

Lysostaphin extracted protein A, designated protein A-1 by Bjork and co-workers (1972), proved to be homogeneous with a molecular weight of about 42,000. The heat treated (100°C, 2 hours) protein A-1 appeared to break into smaller fragments with a maximum molecular weight of 34,000 when subjected to gel chromatography in 6 M guanidine-HCl. All of the eluted protein fractions retained reactivity with anti-protein A antibodies, which suggested that smaller fragments with serological activity were produced by heating.

Protein A prepared by heat extraction from *S. aureus*, Cowan I, was characterized by Bjork et al. (1972). After purification on DEAE-Sephadex, the active material was chromatographed on Sepharose
6B in 6 M guanidine - HCl. The fractions were tested by the ID assay against both anti-protein A and normal human gamma globulin. The most concentrated peak had a molecular weight of about 29,500. Heat extracted protein A eluted as a broad peak which suggested a heterogeneous product. The use of heat extraction as the first step by which to free protein A from the cell wall may explain the presence of a heterogeneous product. The use of heat extraction could also explain the presence of multiple bands when A6 was electrophoresed by polyacrylamide disc electrophoresis.

Polyacrylamide gel electrophoresis of fraction A6 in 0.1% SDS system produced a single, faint band (Figure 8). Three standards: lysozyme, ovalbumin, and yeast alcohol dehydrogenase were run in parallel with fraction A6. A molecular weight of 10,000 was calculated for fraction A6 when the molecular weight of each standard was applied to a logarithmic plot for each of their relative mobilities (Figure 9).

Various molecular weights and sedimentation coefficient values have been assigned to protein A in the current literature. Lofkvist and Sjoquist (1963) first reported a sedimentation coefficient value for protein A extracted from S. aureus, Cowan I, cells which were disintegrated and extracted with cold distilled water. This extract was purified by HCl precipitation, ammonium sulfate precipitation, Sephadex G-100 chromatography and finally electrophoresed on a Pevikon block. An S20,w = 2.65 at 5 mg/ml was calculated for this preparation. Yoshida, Mudd, and Lenhart (1963) purified protein A from S. aureus, international serotype 13, after extraction of cell wall preparations with pancreatic deoxyribonuclease. Their preparation
had a molecular weight of 13,200 and an $S_{20,w} = 1.65$ at 10 mg/ml. Grov (1967) heat extracted *S. aureus*, Cowan I, as did Forgren and Sjoquist (1969) and obtained molecular weight estimations of 12,000 and 15,000, respectively. Recently Bjork, Peterson, and Sjoquist (1972) reported that protein A, extracted from *S. aureus*, Cowan I, by the mild lysostaphin digestion yielded a preparation with a molecular weight of about 42,000. Thus, reported molecular weights for protein A range from 12,000 to 42,000. Possible explanations for these discrepancies are, first, the strain of *S. aureus* used by Yoshida et al. (1963) may have a protein A which differs from that of strain Cowan I used by the other investigators, secondly, different techniques used for extraction and purification have been utilized by the different groups. These different techniques may release polypeptide units of various lengths and weights, all of which possess a reactive site for the Fc region of IgG. This latter possibility has been supported by the work of Bjork et al. (1972), who heat treated lysostaphin extracted protein A and observed both lower molecular weight fractions and a more heterogeneous product which retained Fc reactivity.

Amino acid analysis of fraction Aα showed that 16 amino acids were present in this preparation (Table 4). These 16 amino acids were the same as those reported by Sjoquist, Meloun, and Hjelm (1972) to be present in protein A extracted from *S. aureus*, Cowan I, by lysostaphin digestion. Forsgren and Sjoquist (1969) heat extracted *S. aureus*, Cowan I, and their protein A preparation contained the same 16 amino acids found in fraction Aα of HF-4. Previously, Grov et al. (1964) reported finding only 10 amino acids in their protein A
preparation obtained by purification of heat extracted *S. aureus*, Cowan I. Differences in the relative proportions for the various amino acids in each of these three analyses were noted, but of the five amino acids present in the highest concentrations four were common in all four reports: aspartic acid, alanine, glutamic acid, and lysine. Fraction A₆ contained much more glycine than found by the other three investigators. Since *S. aureus*, HF-4, a bovine isolate obtained from mastitic milk, was the source of fraction A₆, this may account for the discrepancy. Also, heat extraction of HF-4 was used in this study and Sjoquist et al. (1972) used lysostaphin. Techniques used for the initial extraction of crude protein A from the *S. aureus* cell may also explain amino acid analyses differences.
Figure 9. Molecular weight estimation by polyacrylamide gel electrophoresis in 0.1% sodium dodecyl sulfate. A. ovalbumin, mw 43,000, B. yeast alcohol dehydrogenase, mw 37,000, C. lysozyme, mw 14,300, D. fraction A₆, mw 10,000. Relative mobilities determinations based on a value of 1.0 for lysozyme.
Log Molecular Weight

Relative Mobilities
Table 3. Amino acid analysis of fraction Ag hydrolyzed for 48 hours in 6 N HCl at 110 °C

<table>
<thead>
<tr>
<th>Amino Acid</th>
<th>µ Moles</th>
<th>No. of residues&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>0.0368</td>
<td>11.39</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.0020</td>
<td>0.62</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.0041</td>
<td>1.27</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.0326</td>
<td>10.09</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.0082</td>
<td>2.54</td>
</tr>
<tr>
<td>Serine</td>
<td>0.0012</td>
<td>0.37</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.0381</td>
<td>11.80</td>
</tr>
<tr>
<td>Proline</td>
<td>0.0081</td>
<td>2.51</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.1320</td>
<td>40.87</td>
</tr>
<tr>
<td>Alanine</td>
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<td>20.12</td>
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<tr>
<td>Valine</td>
<td>0.0158</td>
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</tr>
<tr>
<td>Methionine</td>
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<tr>
<td>Isoleucine</td>
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</tr>
<tr>
<td>Leucine</td>
<td>0.0102</td>
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</tr>
<tr>
<td>Tyrosine</td>
<td>0.0010</td>
<td>0.31</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.0245</td>
<td>7.58</td>
</tr>
</tbody>
</table>

<sup>a</sup>Calculations based on a molecular weight of 10,000.
SUMMARY

A bovine isolate of Staphylococcus aureus, designated Hill Farm 4, was used as the source of a cell wall antigen, protein A. The staphylococcal cells were heat treated and the crude protein A extracted was purified by HCl precipitation, ammonium sulfate precipitation, column chromatography with Sephadex G-100 gel and DEAE-cellulose. Fractions obtained after the various purification steps were found to produce a precipitin reaction against normal human serum (NHS), which is the criterion for determining protein A reactivity. Fraction A6, considered to be purified protein A, exhibited a titer of 16 against NHS and represented about a 16-fold purification compared to crude protein A, fraction A1.

Each of the six fractions also had precipitin activity with normal rabbit serum (NRS) but not with normal bovine serum (NBS) taken from a 2-day-old Jersey calf. Reactions of identity were observed between all fractions when tested by immunodiffusion against both NRS and anti-HF-4 serum.

Further characterization of fraction A6 by polyacrylamide disc electrophoresis indicated that this fraction contained at least five protein entities when stained with amido black. Electrophoresis of fraction A6 after sodium dodecyl sulfate treatment produced a single, faint band. A molecular weight determination made on fraction A6 using lysozyme, ovalbumin, and alcohol dehydrogenase as standards was determined to be approximately 10,000.

Amino acid analysis of fraction A6 showed that sixteen amino
acids were present. The five amino acid residues in highest concentration were glycine, 40.9, alanine, 20.1, glutamic acid, 11.8, lysine, 11.4, and aspartic acid, 10.1 residues.

Discrepancies between these findings and data reported for staphylococcal protein A may be partially explained by different strains of S. aureus examined, or different extraction and purification procedures employed.
LITERATURE CITED


VITA

Joseph Woodrow Pankey, Jr. was born in Ruston, Louisiana, on September 27, 1944. He attended elementary school in Winnboro, Louisiana, and graduated from Winnboro High School in 1962. In the fall of 1962 he enrolled in Louisiana Tech University, Ruston, Louisiana where he received his B.S. and M.S. degrees in Bacteriology, in 1966 and 1969, respectively. In June, 1969, he entered graduate school at Louisiana State University, Baton Rouge, Louisiana. He is a candidate for the Ph.D. degree in the Microbiology Department in December, 1973.

He is married to the former Phyllis E. Brister and they have a two year old son, Joseph Marvin Pankey.
Candidate: Joseph Woodrow Pankey, Jr.

Major Field: Microbiology

Title of Thesis: A Study of Protein A of Staphylococcus aureus of Bovine Origin

Approved:

R. J. Stehel
Major Professor and Chairman

James G. Traughan
Dean of the Graduate School

EXAMINING COMMITTEE:

A. D. Lawson

J. D. Russell

M. D. Sterkley

Arthur R. Cohen

Date of Examination:

November 16, 1973