

INDIRECT VISUALIZATION OF STAPHYLOCOCCUS AUREUS PROTEIN A*

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Protein A of *Staphylococcus aureus* reacts directly with the Fc portion of γ G-globulins of many mammalian species (1-3). Studies of protein A by Jensen (4), Löfkvist and Sjöquist (5), and Grov and Rude (6) indicated that it appeared to be a constituent of the staphylococcal bacterial cell wall. A direct reaction between the Fc portion of γ G-globulin and such a common bacterial cell wall component is of considerable theoretical interest. It is possible that many events occurring in staphylococcal inflammatory reactions—activation of complement, chemotaxis and erythema—might be initiated through this sort of nonspecific γ G-protein A reaction (7-9). The present study was designed to localize precisely by indirect ultrastructural studies where protein A could be visualized on the staphylococcal cell wall as it reacted with labeled myeloma γ G-globulin. The topographical localization of protein A was demonstrated to be in the outermost portions of the staphylococcal cell wall, the first material in contact with its host environment.

Staphylococci are known to react with fluorescein-isothiocyanate-labeled human antisera used in diagnostic tests for bacterial diseases (10-14). This interference was originally attributed to antigenic similarities between staphylococci and other bacteria or to universal natural antibodies to staphylococci. It now appears to be primarily a function of cell wall protein A (15, 16).

Materials and Methods

Bacteria.—*Staphylococcus aureus* Cowan I¹ was used in these studies as a representative protein A producing strain. *S. aureus* Wood 46² was used as a control non-protein A producer (17). The bacteria were routinely cultured in Penassay broth or agar. For study of protein A production under altered growth conditions, bacteria were cultured at 44°C on Penassay agar (18) or at 37°C on mannitol-salt agar (19). In certain studies the bacteria were incubated in 2% trypsin at pH 8.1 for 30 min or in 5% formaldehyde (16) for 24 hr in attempts to remove protein A. In all studies, the bacteria were washed twice in phosphate-buffered saline (PBS), pH

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7.4, 0.15 M, and suspended to an optical density of 0.6 at 620 m μ to give a concentration of 10⁹ bacteria/ml.

Reagent Immunoglobulins.—Three separate IgG preparations were used: IgG₁ myeloma globulin, IgG₃ myeloma globulin, and rabbit IgG globulin hyperimmune to *S. aureus*. The myeloma proteins were isolated by starch block electrophoresis (20) and the rabbit globulin was separated by 10–40% sucrose density gradient centrifugation (21). Certain immunoglobulin preparations were labeled with horseradish peroxidase Sigma Type II, RZ 1.5, by the technique of Avrameas (22). A γ G₁ myeloma protein producing a strong precipitin reaction with protein A was used as the primary or positively reacting reagent (23). Since previous studies had shown *absence of reactivity* for protein A in γ G₃ myeloma proteins (23), the γ G₃ myeloma protein served as an ultrastructural control for the specificity of reactions observed.

Preparation of Samples for Electron Microscope Study.—1 ml of bacterial suspension was incubated at room temperature for 20 min with 1 ml of an immunoglobulin solution ranging in concentration from 10 to 100 μ g/ml. After incubation, the bacteria were washed twice in PBS. Bacterial pellets were fixed 1 hr at 4°C with 2.5% glutaraldehyde in 0.2 M cacodylate buffer and washed with cacodylate buffer. This was followed by fixation in 2% OsO₄ in cacodylate buffer for 2 hr. The bacteria were dehydrated and embedded in Epon-12 by methods previously described (24). Sectioned samples were examined with a Philips 200 electron microscope.

Light Microscopic Studies.—For light microscopic studies bacteria were suspended in saline and samples transferred to glass slides. These were air-dried and heat-fixed. The smears were next covered with peroxidase-labeled immunoglobulin solution and incubated for 10 min at room temperature. The excess was washed off with PBS and the peroxidase label was developed by incubation for 20 min at 4°C with Karnovsky reagent (25). The final step was a 15 sec exposure to 1% crystal violet. The slides were examined with the oil immersion objective (900 \times). Brown staining due to peroxidase indicated the presence of protein A. Purple staining showed protein A to be absent.

RESULTS

Ultrastructural Studies.—The Wood 46 and Cowan I strains were indistinguishable in electron micrographs of bacteria incubated in PBS used as controls. Incubation with myeloma globulin of subclass IgG₁ clearly differentiated the two strains. The Cowan I strain and Wood 46 strain appeared identical in control preparations (Fig. 1). The Cowan I strain, however, became finely coated with a fluffy appearing layer of the myeloma globulin during incubation with IgG myeloma protein at 100 μ g/ml (Fig. 2). There was no coating of Wood 46 bacteria under these conditions (Fig. 3). This coat was distributed over the entire surface of the Cowan I bacterium and appeared to be present on the entire

FIG. 1. Cowan I *Staphylococcus aureus* incubated in phosphate-buffered saline. Note the somewhat less electron dense outer layer of the cell wall and the electron dense inner cell wall. \times 155,400.

FIG. 2. Cowan I *Staphylococcus aureus* incubated in phosphate-buffered saline with IgG₁ myeloma protein 100 μ g/ml. Note the fluffy layer of myeloma protein distributed over the entire surface of the bacterium. \times 116,550.

FIG. 3. Wood 46 *Staphylococcus aureus* incubated in phosphate-buffered saline with IgG₁ myeloma protein 100 μ g/ml. There is no evidence of association of myeloma protein with the bacterial surface. \times 168,300.

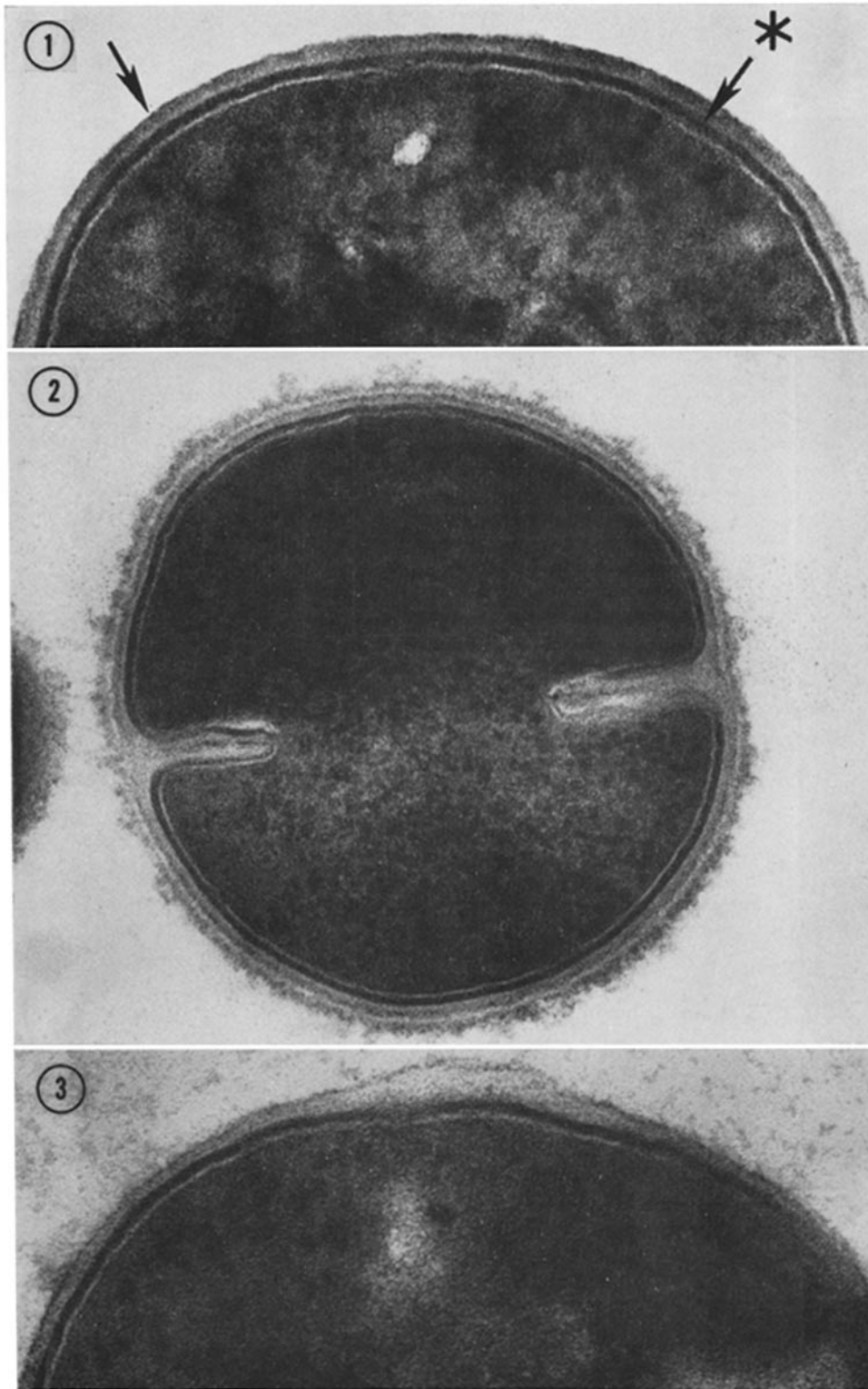


FIG. 1-3
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population of bacterial cells examined. A dividing bacterium (Fig. 2) had no myeloma associated with the incompletely formed septum.

The amount of coating achieved was to some degree related to the amount of myeloma added. Amounts of myeloma protein added ranged from 10 to 100 $\mu\text{g}/\text{ml}$. A difference in coating of the staphylococcal cell with myeloma protein could be detected when 100 $\mu\text{g}/\text{ml}$ concentrations were compared with 50 $\mu\text{g}/\text{ml}$ concentrations (Figs. 4 and 5). Coating was apparent using only 10 $\mu\text{g}/\text{ml}$, but this was much less dense than that achieved with higher concentrations of myeloma protein.

IgG₃ globulins have been shown to be unreactive with protein A in precipitation studies and are unable to inhibit the reaction between protein A and the other subclasses of IgG globulin (23). For this reason, IgG₃ myeloma and IgG₃ myeloma labeled with horseradish peroxidase were used to determine if protein A present in Cowan I bacteria reacted with this protein added to the incubation mixture. When isolated IgG₃ myeloma globulin was incubated with the two strains, neither the Wood 46 nor the Cowan I organisms showed any coating of the outer surface.

A number of treatment schemes were evaluated for their effect on the staphylococcal protein A–myeloma protein reaction. The first modification was a change of the bacterial growth incubation temperature from 37°C to 44°C (18). This appeared to have no effect on protein A production. A fluffy coat was present when γG_1 myeloma protein was added and this did not seem to be any less dense than that observed after growth at 37°C. Therefore, elevation of temperature did not appear to alter production of protein A.

Growth on mannitol-salt agar at 37°C (19) produced a distinct decrement in protein A as indirectly visualized during these experiments. Cowan I organisms grown on this media showed a coating that was somewhat unevenly distributed and appeared considerably less dense than that present when growth had taken place on nutrient agar.

Two methods showed some promise for removing or inactivating protein A. Prolonged treatment of bacterial cells with a strong formalin solution (16) eliminated reaction between Cowan I bacteria and myeloma protein. After 24 hr in 5% formaldehyde, the bacteria showed no coating on addition of previously reactive γG_1 myeloma protein (Fig. 6). Proteolytic treatment of the bacteria with

FIG. 4. Cowan I *Staphylococcus aureus* incubated with IgG₁ myeloma protein 100 $\mu\text{g}/\text{ml}$. $\times 120,000$.

FIG. 5. Cowan I *Staphylococcus aureus* incubated with IgG₁ myeloma protein 50 $\mu\text{g}/\text{ml}$. $\times 120,000$.

FIG. 6. Cowan I *Staphylococcus aureus* pretreated with 5% formaldehyde, and incubated with IgG₁ myeloma protein 100 $\mu\text{g}/\text{ml}$. $\times 120,000$.

FIG. 7. Cowan I *Staphylococcus aureus* pretreated with 2% trypsin and incubated with IgG₁ myeloma protein 100 $\mu\text{g}/\text{ml}$. $\times 120,000$.

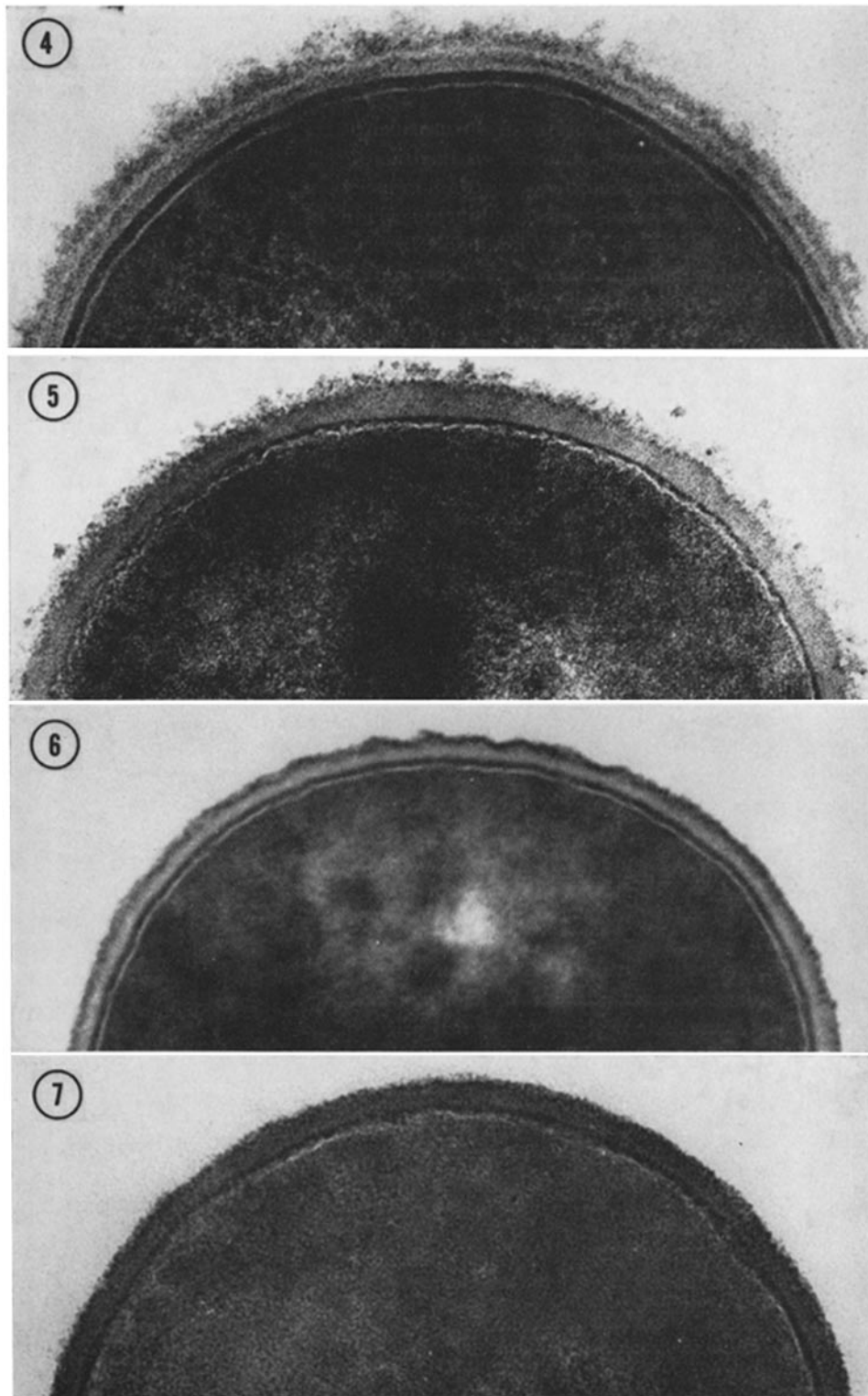


FIG. 4-7
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a 2% trypsin solution at pH 8.1 for 30 min achieved similar results. After such treatment, most bacteria showed no absorbed γG_1 myeloma protein (Fig. 7).

Light Microscopic Studies.—Peroxidase-labeled myeloma protein was also used in light microscopic studies. The procedure of incubation with peroxidase-labeled γG_1 myeloma protein, developing of the enzyme labeling with the Karnovsky reaction, and counter-staining with crystal violet nicely differentiated Wood 46 and Cowan I. The Wood 46 organisms were all purple, i.e., negative for protein A. Cowan I organisms were uniformly coated with a brown product of the Karnovsky reaction. An occasional Cowan I organism was stained purple. Since no organisms of this strain appeared to be lacking a brown stain prior to counter-staining, it seemed likely that the occasional negative bacterium represented artifact or incomplete treatment. An attempt was made to use this procedure for determining the presence of protein A in a number of bacterial strains. There was no clear separation into strains with protein A and those devoid of protein A. Many strains had both positive and negative bacteria. The most clear cut results were with Cowan I and Wood 46 strains.

DISCUSSION

Clarification of the reaction between IgG globulins and protein A has led to development of a specific tool for further study of protein A. The localization of the protein A-reactive site to the IgG Fc fragment (1, 3) and the reactivity with only three of the four IgG subgroups (23) has been of considerable use in determining the ultrastructural localization of protein A on the bacterial cell wall. This nonimmunologic specificity makes it possible to use a pure myeloma protein of IgG subclass 1, 2, or 4 as a reagent specific for protein A (23). Using such an isolated IgG₁ myeloma protein, it was shown that protein A in the intact cell wall reacts with gamma globulin as does isolated and purified protein A.

The studies summarized above allow several conclusions to be drawn about protein A in the staphylococcal cell wall. First, protein A seems to be present over the entire surface of the cell. There are evidently a larger number of sites than can be individually recognized with the resolving power of the electron microscope. All individual bacteria of the Cowan I population demonstrated protein A. No evidence for heterogeneity among this latter bacterial strain was obtained.

The absence of protein A reactivity associated with the forming bacterial wall septum observed in many experiments may reflect very late incorporation of protein A in the outer cell wall components of staphylococci. However, the labeling IgG myeloma protein may not have been readily accessible to the septal structures under the conditions employed. The difference in density of coating which was noted with amounts of myeloma protein is of some interest. Inability fully to saturate the protein A-reactive sites using concentrations up to 1 mg/ml of myeloma protein suggests a large number of sites on the bacterial surface.

The reaction between myeloma globulin and the intact staphylococcus cell wall has some important implications. Such evidence substantiates the suggestion of Lind (16) that protein A was the element responsible for the positive result given by some staphylococci in fluorescent antibody tests designed to be specific for other organisms. It may also explain difficulty in interpreting tests where the staphylococcal organism is used as antigen. Such routine tests as agglutination and complement fixation which work satisfactorily for other microbial species are understandably more difficult to interpret when it is recognized that the staphylococcus will react with the test serum whether or not anti-staphylococcal antibodies are present. Considerable difficulty in development of serologic typing of many strains of staphylococci would also be expected, since the three protein A-reactive IgG subgroups comprise over 90% of the total IgG globulin in human sera.

Modification or elimination of the protein A-immunoglobulin reaction presents some problems. Removal of protein A without affecting other cell wall components would be desirable. Selective culture conditions seem to offer greatest promise in this regard. Neither of the modifications tested here seemed to meet optimal requirements. Mannitol-salt medium cultivation did show some reproducible ability as seen in both ultrastructural and light microscopy to reduce the reaction quantitatively, however, elevation of the culture temperature seemed of no value.

The two methods that did eliminate reaction with immunoglobulin-G, i.e. trypsin digestion or formaldehyde treatment, have several drawbacks. Strong formaldehyde treatment effects cross-links between free amino groups of proteins, and the treated bacterial cell wall may not resemble the native state so that precipitinogens or agglutinogens would be masked. Proteolysis with trypsin would be expected to remove considerable exposed protein from the cell wall. Since protein antigens are significant in serologic tests, bacteria treated with trypsin may not be close enough to the native state to be used in such tests.

The light microscopic studies undertaken during this study were less valuable than had been hoped. The procedure depends upon deposition of the product of the Karnovsky reaction in a coat thick enough to shield the cell wall from crystal violet. This shielding could not occur if the bacteria were damaged and crystal violet staining would result. Quantitation of protein A on the bacteria using this technique was not possible. The range of staining observed indicates that protein A is probably present in varying amounts in *S. aureus*. It may be, however, that there is a material similar to protein A present in all *S. aureus*, but reactivity of this material with IgG is variable.

The ultrastructural localization of protein A on the outermost portion of the cell wall of *S. aureus* is of considerable theoretical interest. Since most γ G molecules (γ G₁, γ G₂, and γ G₄) can react with protein A through sites on Fc, it is conceivable that activation of the complement sequence and subsequent

chemotaxis and anphylotoxin release are mediated by this reaction between bacteria and host in the preantibody phase of staphylococcal infections (9).

SUMMARY

Specific but nonimmunologic reaction between staphylococcal protein A and the Fc portion of gamma globulin provided the basis for ultrastructural studies to determine the localization of protein A, using intact staphylococci and labeled myeloma gamma G-globulin.

Protein A appeared to be part of the outermost layer of the staphylococcal cell wall. Strains with protein A demonstrated a coating of myeloma globulin over the entire bacterial surface. There was no coating of strains without protein A.

Identification of protein A on the surface of the staphylococcal cell wall provides evidence that this may be the first material in contact with host environment. It probably accounts for apparent cross-reactions of staphylococci with antibodies to many antigens. More importantly, even in the nonimmune host protein A immunoglobulin reactivity may initiate complement activation and inflammatory reactions including chemotaxis and pus formation.

BIBLIOGRAPHY

1. Forsgren, A., and J. Sjöquist. 1966. "Protein A" from *Staphylococcus aureus*. I. Pseudo-immune reaction with human gamma globulin. *J. Immunol.* **97**:822.
2. Forsgren, A., and J. Sjöquist. 1967. "Protein A" from *Staphylococcus aureus*. III. Reaction with rabbit gamma globulin. *J. Immunol.* **99**:19.
3. Kronvall, G., and D. Frommel. 1969. Definition of staphylococcal protein A reactivity for human immunoglobulin G subunits. *Immunochemistry*. In press.
4. Jensen, K. 1958. A normally occurring staphylococcal antibody in human serum. *Acta Pathol. Microbiol. Scand.* **44**:421.
5. Lofkvist, T., and J. Sjöquist. 1962. Chemical and serological analysis of antigen preparations from *Staphylococcus aureus*. *Acta Pathol. Microbiol. Scand.* **56**:295.
6. Grov, A., and S. Rude. 1967. Immunochemical characterization of staphylococcal cell walls. *Acta Pathol. Microbiol. Scand.* **71**:409.
7. Gustafson, G. T., J. Sjöquist, and G. Stalenheim. 1967. The protein A from *Staphylococcus aureus*. Arthus-like reaction produced in rabbits by interaction of protein A and human γ -globulin. *J. Immunol.* **98**:1178.
8. Martin, R. R., J. G. Crowder, and A. White. 1967. Human reactions to staphylococcal antigens. A possible role of leukocyte lysosomal enzymes. *J. Immunol.* **99**:269.
9. Sjöquist, J., and G. Stalenheim. 1969. Protein A from *Staphylococcus aureus*. IX. Complement-fixing activity of protein A-IgG complexes. *J. Immunol.* **103**:467.
10. Yoshida, A., S. Mudd, and N. A. Lenhart. 1963. The common protein agglutinin of *Staphylococcus aureus*. II. Purification chemical characterization and serologic comparison with Jensen's antigen. *J. Immunol.* **91**:777.
11. Odeing, P., A. Grov, and B. Mykleslad. 1964. Immunochemical studies on antigen

- preparations from *Staphylococcus aureus*. II. Precipitating and erythrocyte-sensitizing properties of protein A (antigen A) and related substances. *Acta Pathol. Microbiol. Scand.* **62**:117.
12. Pittman, B., and M. D. Moody. 1960. Staining of *Staphylococcus aureus* with fluorescein-labeled globulin from non-immunized and streptococcus-immune animals. *Bacteriol. Proc.* **60**:140.
 13. Kendrick, P. L., G. Eldering, and W. C. Eveland. 1961. Fluorescent antibody techniques for identification of *Bordetella pertussis*. *Amer. J. Dis. Child.* **101**:149.
 14. Bergman, S., A. Forsgren, and B. Swahn. 1966. Effect of normally occurring rabbit antibodies on fluorescent antibody reaction. *J. Bacteriol.* **91**:1664.
 15. Lind, I. 1968. Non-specific adsorption of FITC-labeled serum globulins to *Staphylococcus aureus*. *Acta Pathol. Microbiol. Scand.* **73**:624.
 16. Lind, I., and B. Mansa. 1968. Further investigation of specific and non-specific adsorption of serum globulins to *Staphylococcus aureus*. *Acta Pathol. Microbiol. Scand.* **73**:637.
 17. Daugharty, H., R. R. Martin, and A. White. 1967. Antibodies against staphylococcal teichoic acids and type-specific antigens in man. *J. Immunol.* **98**:1123.
 18. James, A. M., and J. E. Brewer. 1968. A protein component of the cell surface of *Staphylococcus aureus*. *Biochem. J.* **108**:257.
 19. Hankenes, G. 1967. Serological typing of *Staphylococcus aureus*. *Acta Pathol. Microbiol. Scand.* **70**:590.
 20. Kunkel, H. G. 1954. Zone electrophoresis. *In* Methods of Biochemical Analysis. Interscience Publishers, Inc. New York. 41.
 21. Kunkel, H. G. 1960. Macroglobulins and high molecular weight antibodies. *In* The Plasma Proteins. New York, Academic Press, Inc. 294.
 22. Avrameas, S. 1969. Coupling of enzymes to proteins with glutaraldehyde. *Immunochemistry.* **6**:43.
 23. Kronvall, G., and R. C. Williams. 1969. Differences in anti-protein A activity among IgG subgroups. *J. Immunol.* **103**:828.
 24. White, J. G. 1968. Fine structure alterations induced in platelets by adenosine diphosphate. *Blood J. Hematol.* **31**:604.
 25. Karnovsky, M. J. 1963. Vesicular transport of exogenous peroxidase across capillary endothelium into the T system of muscle. *J. Cell Biol.* **17**:49.