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CELLULAR LOCALIZATION OF FIVE EXOPROTEIN ANTIGENS OF STAPHYLOCOCCUS AUREUS

ISAAC ALBERT WAMOLA

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CELLULAR LOCALIZATION OF FIVE EXOPROTEIN ANTIGENS OF STAPHYLOCOCCUS AUREUS

by

ISAAC ALBERT WAMOLA

B.A., Hampton Institute, 1965
M.S., University of New Hampshire, 1967

A THESIS

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ABSTRACT

CELLULAR LOCALIZATION OF FIVE EXOPROTEIN ANTIGENS OF STAPHYLOCOCCUS AUREUS

by

ISAAC ALBERT WAMOLA

The cellular localization of five exoprotein antigens of *Staphylococcus aureus* was investigated with the objective of demonstrating what particular sub-cellular structures were associated with these exoproteins. Isolated cell walls, cytoplasmic membranes, mesosomes and cytoplasm were obtained and each examined for the presence of the exoprotein antigens using immunological, serological and enzymatic techniques to compare the purified exoproteins to the unknown antigenic components from the sub-cellular structures.

The cell wall proteins extracts were found to contain antigens similar to known exoprotein antigens (beta hemolysin and nuclease and enterotoxin B) besides the two other antigenic wall components (antigen A and techoic acid). The cytoplasmic membranes were demonstrated to contain alpha toxin, coagulase and nuclease, but the quantitation of these components remains to be established. Trace amounts of
alpha hemolysin and beta hemolysin were found to be localized in the mesosome and/or peri-plasmatic space proteins. The cytoplasmic fractions were shown to contain alpha toxin, nuclease and coagulase, but these, too, were present in low levels contributing only a small percentage of the cytoplasmic proteins.

The disc polyacrylamide gel electrophoresis of both cell wall proteins and the cytoplasmic membranes revealed a large number of protein bands. Such large number of bands of various patterns suggest several protein types that may be associated with these structures. Strain UNH 570 was shown to contain as much as 38.5% of its cell was dry weight as protein.

The results obtained in the investigation showed that the exoprotein antigens examined were found localized on sub-cellular structures distributed in a manner not clearly understood but seemed to suggest that they had moved by different routes from their site of synthesis to their place of location.
Cellular Localization of Five Exoprotein Antigens of *Staphylococcus aureus*

INTRODUCTION

Several exoenzymes and exotoxins that are protein antigens of *Staphylococcus aureus* have been isolated and purified sufficiently to permit study of their physicochemical properties as well as their immunological activity in animals. Yet none have been established with any degree of certainty as being solely extracellular. Furthermore, the classification of bacterial products as "extracellular" and "cell-bound" or "intracellular" serves only as an operational, or practical working definition. These classifications become physiologically meaningful only if it can be established with certainty how a protein found to be associated with the cell is oriented with respect to the cytoplasmic membrane, whether it is on the inside or outside of this permeability barrier.

Unfortunately, the criteria used to define enzymes as either "cell-bound" or "extracellular" do not in themselves suggest how large molecules move from their site of synthesis, in or on the cell, to the environment. Thus, in an effort to explain how such molecules are secreted, theories have been advanced which combine ideas concerning both the mechanisms of synthesis and liberation of exoproteins.
Knowledge concerning the relationship between the exoproteins found in the medium and the cell-associated protein antigens of \textit{S. aureus} may provide useful information on the role of these molecules as virulence factors that must be distributed from their site of synthesis to the environment.

This investigation concerns itself with the demonstration that some species of extracellular proteins occur cell-bound, localized in particular sub-cellular fractions of the cell structure, and that such components are antigenically related to the purified exoprotein antigens isolated from supernatants of actively growing cultures not exhibiting significant autolysis. The investigation required isolation and characterization of these individual sub-cellular components of the cells (cell wall, protoplast membranes, and cytoplasm) as purified material. The similarity of the protein antigens obtained from the sub-cellular fractions to the purified exoprotein antigens was established by use of gel immunodiffusion techniques, and determinations of enzymatic and biological activity.

Finally, attempts were made to characterize cell membrane and cell wall proteins using polyacrylamide gel electrophoresis with the hope of establishing which individual protein band, or bands, was responsible for the particular
biological activity associated with the sub-cellular fraction that was identical to the exoprotein antigens which were investigated. Using these techniques qualitative and semi-quantitative evidence concerning the cellular localization of these antigens was obtained.
LITERATURE REVIEW

In a review on microbial exoenzymes, Pollock (1962) described the theoretical and operational considerations used in defining the class of exoenzymes. He divided these enzymes into two groups with regard to the location of such enzymes in relation to the cell structure. He pointed out that cell-bound enzymes may refer to the fact that either the enzyme is intracellular, and found within the cytoplasmic membrane, or surface-bound, located on the outside part of the cytoplasmic membrane and bound to the cell wall, an area which includes the periplasmatic space. Surface-bound enzymes cannot be removed by repeated washing of cells as is the case with truly extracellular enzymes. It seems likely that all exoenzymes of bacteria exist in a cell-bound form at some stage during their synthesis and secretion as had been shown by studies with *Bacillus subtilis* which were described by Kushner and Pollock (1961) and also later by Lampen (1965) and Chesbro and Lampen (1968) using *Bacillus licheniformis*. These authors as part of their studies on the mechanism of synthesis and liberation of selected exoenzymes showed that presumably the same exoenzyme could be found cell-bound as well as recovered free in the medium.

Heppel (1967) reviewed selective release of enzymes from bacteria. He discussed how one enzyme may be extra-
cellular in one species of bacteria while cell-bound in another species. His discussion is particularly focused on the problem of location of selectively released enzymes that may not be bound to any particulate fraction of broken cells which can be identified and purified. These enzymes have been demonstrated by indirect evidence as being located near the cell surface of Enterobacteriaceae where they can be removed selectively by conversion of cells into spheroplasts, or by osmotic shock as a result of treatment with ethylenediaminetetraacetate (EDTA) in combination with an abrupt osmotic transition. Heppel stated that there exists ample evidence to support the fact that these enzymes may actually be extracellular in gram positive organisms while existing surface-bound in Escherichia coli. One example of these enzymes was demonstrated by the work of Malamy and Horecker (1961) who showed that alkaline phosphatase is almost completely released into sucrose medium when Escherichia coli cells are converted into spheroplasts. Alkaline phosphatase was shown to be localized internally in yeast by McLellan and Lampen (1965), who had previously demonstrated that acid phosphatase was external to the yeast cell (1963) and that yeast protoplasts secreted the newly synthesized enzyme into the medium. Neu and Heppel (1964a and 1964b) published evidence in two papers which supported cellular localization
of a family of hydrolytic enzymes near the cell surface of *E. coli* including ribonuclease and 5' nucleotidase. Cashel and Freese (1964) reported that alkaline phosphatase, a perienzyme in *E. coli*, was actually secreted into the medium from intact cells of *B. subtilis* following derepression by phosphate starvation. They showed that in this organism, levels up to 93% of the total enzyme activity appeared in the supernatant and only 7% remained with the washed cell extract after 5 hours in limiting medium.

Evidence for the localization of cell-bound enzymes in gram positive bacteria is clear. Investigations by Pollock (1961) and Kushner and Pollock (1961) showed that 85% of the penicillinase in penicillin-induced cultures of *B. subtilis* was cell-bound, but not free in the cytoplasm. It could be released in a soluble state by rupture of the cell envelope. They estimated 40% of the enzyme to be attached to the cell wall and up to 98% could be released by trypsin in the presence of a hypertonic sucrose solution from apparently intact cells. They showed that the cells were intact because only 5% of alpha glucosidase, which is known to be intracellular, was released under the same conditions. These authors established that most of the penicillinase activity was localized on the cell membranes when stable spheroplasts were made from their organism and that at least 30% of the
cell-bound fraction was fixed superficially to the cell because it was accessible to combination with antiserum. Thus, the results supported the hypothesis that cell-bound penicillinase was attached to the outside of the cytoplasmic membrane and may actually have been formed at its surface. This hypothesis stimulated a number of researches recently in an effort to explain synthesis and liberation of this enzyme and other similar exoenzymes (Collins, 1964; Lampen, 1965; Birnboim, 1966; Coles and Gross, 1967; Chesbro and Lampen, 1968; Sargent et al., 1968 and Holzman et al., 1969). It was Lampen who suggested that cell-bound penicillinase covalently linked to the cell membrane through a peptide chain which was probably cleaved by trypsin treatment.

In S. aureus, localization of exoenzymes and other exoproteins has been investigated for a limited number of exoproducts. This organism is known to secrete an impressive number of these products.

Duthie (1954) presented evidence for two forms of staphylococcal coagulase, one bound to cell wall, thus bound coagulase or clumping factor, and the other liberated as free coagulase in the medium. The free coagulase was responsible for the tube test and acted on the prothrombin of certain animal species to give a thrombin-like substance while bound coagulase acted directly on fibrinogen of certain animals to
cause clumping of the staphylococci. He showed that the two coagulases were antigenically separable and that each could initiate specific antibodies in rabbits. Also, none of the antisera against free coagulase had any inhibitory action on bound coagulase, which he preferred to call clumping factor.

Jacherts (1956) attempted to prove that both bound coagulase and free coagulase were identical in biological, immunological, and physical properties but his argument was refuted by Duthie and Haughton (1958) who contended that the bound coagulase preparations by Jacherts could have been contaminated with extracellular proteins. These might have adhered on washed cells to the extent of 0.1 unit of free coagulase per 100 ml crushed cells obtained from a fully grown digest broth culture and extracted at pH 2.0. This argument upheld Duthie's earlier observation that bound coagulase or clumping factor was distinct from free coagulase. The free coagulase was also found to show 2 components following electrophoretic purification.

Rogers (1954) made an observation that in *S. aureus*, whereas coagulase appears after inoculation of the culture without lag additional to that in the lag phase of growth, but increases at a slower rate than growth until it too ceases to increase before growth stops, hyaluronidase behaves
differently. The lag in the appearance of hyaluronidase activity after inoculation of the culture was longer than lag phase of growth and its rate of formation showed an increase after exponential growth had been established. The exponential increase in activity stopped sharply and sooner than growth. This observation about the lag in the formation of hyaluronidase led Rogers to believe that there had to be an accumulation of this enzyme inside the cells followed by release. Thus, experiments to demonstrate this cell associated hyaluronidase and coagulase and a method for the estimation of the amounts of cell-bound enzyme were undertaken. The results of these experiments indicated that delay in secretion as such did not play a major role in determining the lag in the production of hyaluronidase and that a constant amount of coagulase was associated throughout growth with the cells of the strain of \textit{S. aureus} used. Rogers and Spensley (1955) conducted experiments in which they demonstrated the ability of some macroanions (such as polyhydroquinone sulphate) to inhibit the liberation of hyaluronidase and coagulase in cultures of staphylococci. It was not possible for them to say with certainty that the polymers prevented the formation of these enzymes by the cells. But they established that 1 mg/ml final concentration of these macroanions in broth cultures failed to affect the growth
of the organism (*S. aureus* strain 524), although 50 μg/ml was sufficient to reduce hyaluronidase formation by about 50%. The inhibition was also affected by the buffer system used (phosphate or glycerophosphate). From these results, they concluded that since the macroanions did not affect enzymes responsible for the vital functions necessary for cell economy and growth, the macroanions must have remained on or near the surface of the treated organisms and did not penetrate the cell wall to reach the vital internal enzymes.

Other studies with compounds of this type and their influence on the liberation of penicillinase from *S. aureus* have recently been reported in two papers by Coles and Gross (1967a and 1967b). These authors reported that polyanions in the growth medium, such as dextran sulphate and polyvinylsulphonate, inhibited the formation of cell bound exopenicillinase without affecting the total amount of penicillinase in cell suspension by converting all cell bound enzyme into exopenicillinase. They postulated that the polyanions acted to prevent the appearance of ionically bound penicillinase rather than inhibiting formation of penicillinase because the polyanions had a strong affinity for the sites of the cell wall required for binding of the enzyme. Thus they thought that proteins such as exopenicillinase are formed outside the permeability barrier of the plasma membrane since these
polyanions are too big to penetrate the barrier and exert their inhibitory phenomenon. A report by Holzman et al (1969) seems to support this hypothesis, in that protoplasts were almost completely inhibited in their synthesis of penicillinase when treated with similar compounds but their total protein synthesis was not affected. This observation indicated to these authors that the ribosomes responsible for synthesis of penicillinase in *S. aureus* are located on the outside surface of the cell membrane. Therefore it seemed they had localized the site of synthesis of penicillinase, at least in this organism.

Hendricks and Altenbern (1968) studied the synthesis of staphylococcal alpha toxin in several strains of *S. aureus* and their results did not directly support a general explanation for alpha toxin synthesis based upon various aspects of lysogeny. They obtained evidences that the toxin was generated intracellularly and continually released into the medium. These authors noted that intracellular toxin, obtained by lysis of cells with lysostaphin, was synthesized at a rate that far outstripped the rate of general protein synthesis. In contrast, extracellular toxin appeared somewhat later than intracellular toxin and exhibited a relatively gradual increase throughout the course of the experiment. These authors suggested that the intracellular toxin synthesis
resembled an induction process, and that mitomycin C exerted a pronounced inhibitory effect on growth and prevented appreciable formation of either intracellular or extracellular alpha toxin.

Richmond (1963) reported 60% of penicillinase activity was cell-bound at the end of exponential growth of \textit{S. aureus}. His results as well as those of Lampen (1967) with cell-bound penicillinase from \textit{B. licheniformis} confirmed that purified exopenicillinase shows similar properties of those of the enzyme found cell-bound, thus cell-bound penicillinase may be an intermediate in the formation of the exopenicillinase.

McDonald (1964) presented evidence for a cell-bound proteinase system produced by a \textit{Staphylococcus lactis} strain grown in liquid medium containing sodium caseinate. He conducted investigations to determine the location of this proteinase in the cell. Results from cell fractionation indicated that the cell-bound proteinase in this strain was located at or near the cell membrane. The proteinase was not present in the cytoplasm fraction and, therefore, was not an intracellular enzyme in the classical sense. Furthermore, he showed that growing cells hydrolyzed sodium caseinate (a substrate with molecular weight about 70,000 which is unlikely to penetrate the cells) but only small amounts of extracellular enzyme were detected. This suggested to him that the
proteinase of this organism was a surface bound enzyme probably associated with the membrane fraction of the disrupted cells. Another enzyme which seems to be located in the same fraction was NADH oxidase and it too was found to be relatively lacking in the free state in the cytoplasm, as judged by the low catalase activity of this fraction.

While emphasis concerning antigens of S. aureus was laid on investigations employing extracellular products some or all of which may be related to the ability of this organism to harm the host, Rogers (1956) hypothesized that some of the extracellular active substances may also remain associated with the cell as a kind of capsule. Thus, he thought these substances would be important for initiating as well as for maintaining or spreading the organism from the site of entry.

Verwey (1940) demonstrated a type-specific, antigenic, protein B from staphylococcus cells which he obtained by his extraction technique. This method of extraction he believed was chemically milder than those previously employed and gave material which was in a state close to its native condition than earlier methods. He characterized the type specific substance as protein in nature by a number of qualitative chemical tests. He also showed that this substance was a precipitinogen not similar, by immunologic and chemical characterization, to the type specific carbohydrate fraction.
C described by Jullianelle and Wieghard (1934). This protein fraction B, type specific precipitinogen was shown to induce a high titre of precipitins in rabbits immunized intravenously with it alone and was responsible for the type specific antibodies in the rabbits. Further work immunizing other species of animals (mice and guinea pigs) confirmed the results obtained with rabbits. It may be noted here that Verwey's procedure for the fractionation of *S. aureus* involved disruption of dried organisms by a mechanical device, followed by precipitating colloidal material by acidifying with 0.1 N hydrochloric acid to pH 3.5, and finally centrifugation to obtain the supernatant from which the protein was concentrated by trichloracetic acid (TCA) precipitation. The precipitated protein fraction B was collected by high speed centrifugation and used for qualitative chemical analysis and immunological studies after the salts had been removed by dialysis.

Jensen (1958) discovered a precipitin antigen in some of the type strains he was using for serological typing of *S. aureus*. This antigen which he designated as "antigen A" was present in large quantities in a strain belonging to type Cowan 1 and was a powerful immunizing agent in rabbits. The antigen precipitated all normal human sera when tested in agar gels. He thought of this reaction as a natural specific immune response by humans to antigen A. However,
Forsgren and Sjöquist (1966, 1967) and Gustafson et al. (1967) and Grov et al. (1970) later reported the reaction was a non-specific type unrelated to the specific immune response against antigen A. Jensen showed that antigen A was widespread among Staphylococcus aureus strains, but his observation that the antigen was not secreted into the medium like exotoxins seems to have been disproved by more recent reports (Blackstock and Kelly, 1968; and Forsgren, 1969). Jensen's extraction procedure differed from that of Verwey (1940) in that it involved heat extraction of the antigen from whole cells by boiling in phosphate buffer solution at pH 5.9 followed by HCl precipitation at pH 3, and then by TCA and ethanol purification. Jensen et al. (1961) reported that the antigenic extract he designated as "Extract A" contained at least 3 components with antigen A being only one.

The need to compare the two antigenic extracts from Staphylococcus aureus arose in order to establish if the two different techniques gave products which were similar. Both antigenic extracts were considered common antigens for coagulase-positive strains of Staphylococcus aureus. Such an investigation was initiated by Löfkvist and Sjöquist (1962). They found that Jensen's antigen A from Staphylococcus aureus contained a protein that was serologically identical with an antigen in Verwey's protein fraction B. They observed a reaction of identity between the
sample they had received from Dr. Jensen and both of their preparations obtained by Jensen's and Verwey's methods respectively. Also, both preparations contained more than one precipitable antigen, although this was most pronounced in the fraction obtained with the Verwey's technique. Further purification of Jensen's antigen A was done by Løfkvist and Sjöquist (1963) and these authors described a separation procedure for several staphylococcal antigens contained in the Jensen's antigen A extracts. Using a Sephadex G 100 column after precipitation with 80% ammonium sulphate, these authors separated Jensen's antigen A extract solution into four peaks, the last of which tended to split into two. Antigen A was localized in and between effluent collected for the first and the second peaks of protein absorption monitored at 280 μm. Two more antigens which formed precipitates with rabbit immune serum were found in the third and in the front of the fourth peaks of absorption. One takes note here that the method for obtaining antigen A extract did not involve heat extraction as described by Jensen. These authors used a gentler procedure, comparable to Verwey's method, except for the ammonium sulphate precipitation step which in addition served to concentrate and purify the antigen about 3-fold. These authors, along with several later authors, (Forsgren et al., 1966; Lenhart et al., 1963; Grov, 1968; and
Live and Ranu, 1968) demonstrated the heterogeneity of extract A on precipitation in gels with sera from immunized rabbits. Only one fraction from gel chromatography on Sephadex G 100 and subsequently purified by electrophoresis was precipitated with normal human serum which indicated identity with antigen A as described by Jensen.

Lenhart et al. (1963) reported immunologic and chemical properties of a non-type specific protein agglutinogen of _S. aureus_. The agglutinogen was distributed in the 13 internationally recognized serotypes. In another paper by the same group of investigators, Yoshida et al. (1963), they showed that the antigen is a protein of molecular weight of ca. 13,000, and it is a principal agglutination factor of the bacterial cells. They demonstrated serologic identity between the agglutinogen and Jensen's antigen A. Their material had agglutinin combining activity and showed three lines of precipitation on Ouchterlony plates with rabbit anti-staphylococcal sera prepared by injecting animals with living staphylococcal cells. These authors had shown that the protein antigen which was both an agglutinogen and a precipitinogen was an integral part of the cell walls of _S. aureus_ since it could be recovered from undigested cell walls. The isolated cell walls contained about 30% of their weight as protein. They claimed that the protein obtained from the
bacterial cell walls must be the principle staphylococcal surface component responsible for agglutination. The presence of protein A on cell walls was also confirmed by Grov and Rude (1967), Löfkvist (1966), Live and Ranu (1968).

In an effort to clarify the immunochemical nature of antigen A, it had to be extracted and purified to a degree which allowed chemical analysis to be performed. One such study was done by Grov et al. (1964). They showed that while their purified antigen A preparation seemed to be homogenous on immunoelectrophoresis, gel double diffusion on agar showed 2 precipitin lines with antiserum against Cowan 1, and it contained a sensitizing substance, using a tanned cell haemagglutination technique. The sensitizing substance was not identical to the precipitinogen, however.

The amino acid analysis by Grov et al. (1964) showed the antigen to contain only 10 amino acids which by their quantitative determinations accounted for 99% of the weight of the purified antigen. This was contradicted by the investigation of Forsgren and Sjöquist (1969) who reported evidence to indicate that their preparation, unlike that of previous authors, seemed to be very heterogeneous as judged from N-terminal amino acid determinations, gel filtrations and electrophoresis in polyacrylamide gel. In addition, the amino acid analysis of their product showed all the common
amino acids present except for tryptophan and half cystine. The amino acids determined by these authors, accounted for 87.5-92.2% of the weight of the preparation. Their preparation, which had been purified by electrophoresis in polyacrylamide gel, gave a weak precipitation line with normal rabbit $\gamma$-G-globulin and two lines with immune $\gamma$-G-globulin. One of these lines was weak and gave a reaction of identity with the one obtained using normal rabbit $\gamma$-G-globulin. Since after digestion of the immune globulin with pepsin the strong line of anti-protein A $\gamma$-G-globulin remained and the weak line of both $\gamma$-G-globulin preparations disappeared, these authors interpreted their results to mean that the weak line represented the reaction of protein A with the Fc-part of the $\gamma$-G-globulin and that the strong line remaining after pepsin digestion represented the protein A reaction with the $\text{F(ab')}_2$-part. This interpretation of the results confirmed earlier experiments by Forsgren (1968) in which the authors described a reaction between protein A and the H and L-chains from $\gamma_2$-globulin and fragments of $\gamma_1\gamma$ and $\gamma_2$-globulin obtained by pepsin and papain degradation. Analysis of these results had led to the conclusion that protein A non-specifically binds $\gamma$-globulin at the Fc fragment. This binding site is believed not to contain an active site reacting with the antigenic determinant as does the Fab fragment. Other
pseudo-immune reactions of antigen A had been reported earlier in separate studies (Forsgren and Sjöquist, 1966 and Gustafson et al., 1967).

A concise review on current research concerning protein A was published in a pamphlet by Forsgren (1968). An investigation concerning research with protein A not covered in Forsgren's review was concerned with efforts to identify the antigens contained in both Jensen's antigen A and Verwey's protein fraction B extracts as described by original techniques in relationship to some of the better characterized exoprotein antigens of \textit{S. aureus}. Blackstock and Kelly (1968) compared protein A and the clumping factor (bound coagulase) of \textit{S. aureus}. These authors felt that the two substances shared common characteristics, such as being protein antigenic cell surface components which were released into the culture medium as growth proceeds. Also, certain mutants of \textit{S. aureus} seem to lose both of these proteins at the same time which may indicate that they are linked. However, their results with a number of immunodiffusion techniques indicated that staphylococcal clumping factor and antigen A are not identical substances despite their similar properties.

A careful study of published physico-chemical characteristics for protein A (Forsgren, 1969) and nuclease (Taniuchi and Anfinsen, 1967) indicated that these antigens exhibited
certain resemblances. They both show similar isoelectric points and their molecular weights are rather close. A survey on production of both these antigens indicate that better than 90% of the Staphylococcus aureus population produce nuclease just as over 85% produce protein A. It therefore seemed possible that a comparison of these antigens might provide a link between the exoprotein antigen nuclease and the largely cell-bound protein A. Similar exoprotein antigens of this organism could also be represented cell-bound in these protein extracts.

Localization of antigens has been aided by the use of the fluorescence microscopy technique particularly in histological examination of tissue cells. A classical review on the development of this technique is that of Coons and Kaplan (1950). The application of immunofluorescent staining to bacteria has been covered in a review by Bautner (1961). A review of the use of the fluorescent antibody (FA) techniques in diagnostic bacteriology was made by Cherry and Moody (1965). These authors cautioned investigators on the use of this technique which is based on the assumption that in order for bacterial species to give immunofluorescence an exposed antigen or hapten must be required at the surface of the bacterial cell.
It needs to be mentioned, too, a few specific studies in which this technique was used to offer evidence for surface located antigens, such as those of DeRepentigny and Frappier (1958) using *Hemophilus pertussis* liquid cultures, those of Thomas *et al.* (1957) using *Salmonella typhosa* and finally, those of Emmart, *et al.* (1958) for localization of streptococcal hyaluronidase. Fluorescence antibody technique was used by Friedman and White (1965) for immunofluorescent identification of cell-associated staphylococcal enterotoxin B by making smears of a culture of strain S₆ with the globulin fraction of antiserum against purified enterotoxin B produced in burros. The isolated globulin fraction was labelled with fluorescein isothiocyanate (FITC) and was absorbed 3 times with cells of non-enterotoxic strains of *S. aureus*. Thus, it was presumed that the antiserum was monovalent and reaction with the antigen contributed by intact cells was a proof that the antigenic sites were characteristic of cell surface based constituents of the bacteria. Genigeorgis and Sadler (1966, 1969) used a similar method to demonstrate staphylococcal enterotoxin B in culture media and in cells using a modified method but employing smears reacted with FITC-conjugated antienterotoxin B. Their method involved use of membrane filters to retain the cells and formed precipitate which were subsequently washed and transferred to slides by impression.
smears. These authors could demonstrate enterotoxin B at concentrations as low as 1 µg of toxin per ml. Stark and Middaugh (1969) also presented evidence for immunofluorescent detection of enterotoxin B in food and a culture medium which seems to indicate that cell-associated enterotoxin B and free enterotoxin in food and culture medium could be rapidly and quantitatively demonstrated by using fluorescence antibody technique.

It is clear from the foregoing studies concerning localization of staphylococcal enterotoxin B on cells, that considerable research has been directed in recent years toward identifying antigenic sites on this organism. A word of caution regarding the interpretation of these results seems to be offered by the work of Lind (1968) who published evidence which suggested that non-specific absorption of FITC-labelled serum globulins to S. aureus was indeed a major obstacle with the FA technique. Lind (1967) had encountered a non-immunological affinity of certain strains of S. aureus for serum globulins that interfered with his study on identification of Neisseria gonorrhoeae by means of fluorescent antibody technique. Certain strains of S. aureus were stained brilliantly by rabbit antigonococcal sera labelled with FITC and exhibited a striking morphological similarity to gonococci. At first it seemed that there might be an antigenic relationship
between *S. aureus* and *N. gonorrhoeae*, a possibility which was disproved by experiments to explain this phenomenon. Instead, his work, as well as previously published evidence, pointed to protein A from *S. aureus* as the substance responsible for this non-specific reaction. Protein A has been shown to occur in a large number of *S. aureus* strains and had clearly been demonstrated to non-specifically precipitate with 45% of pooled normal human and rabbit γ-G-globulins (Forsgren and Sjöquist, 1966, 1967). Lind called strains of *S. aureus* that displayed this phenomenon "reactive strains" exemplified by Cowan type 1 already shown to have a high content of protein A as compared to "non-reactive strains" which included Wood 46 (protein A negative). Acting on a suggestion by Haukenes (1967) that cells grown in mannitol agar with a high concentration of NaCl had a reduced protein layer content in their cell wall, Lind was able to eliminate most of the fluorescence from his reactive strains when he grew the cells accordingly. He concluded that it was the protein A production by cells which had been presumably cut down. Stark and Middaugh (1969) observed similar reduction in fluorescence and enterotoxin production with increase in NaCl concentration during the first 12 hours of growth. All results cited above claiming cellular localization of enterotoxin B do not mention anywhere any specific attempts to absorb their
antiserum preparation with antigen A although some absorption with non-enterotoxigenic strains of *S. aureus* was attempted. One other precaution on the interpretation of FA technique localized antigen was suggested by Cohen *et al.* (1961) in respect to antibodies against *S. aureus* present in non-immunized rabbits other than the already recognized antigen A antibodies. They warned that FITC-labelled globulin portion from normal serum from pathogen free rabbits demonstrated brilliant staining with coagulase positive staphylococci.

Elkins, *et al.* (1970) used immunofluorescence to examine typical *S. aureus* strain and L-form variants for clumping factor. They were able to establish that antisera which were relatively high in clumping-inhibiting antibodies, but low in agglutinins, gave no fluorescence with coagulase-negative, clumping factor negative variants and L-forms. This indicated that clumping factor, probably a cell wall component of *S. aureus*, is absent in the penicillin-induced stable L-forms. This study seemed to support the idea that variants of *S. aureus* which are negative for both coagulase and clumping factor possess few, if any, surface antigens characteristic of typical coagulase-positive staphylococci. This statement concerning L-forms disagrees with the results obtained by Forsgren (1969) who showed that protein A occurred
in his L-forms although in much lower amounts than in cocal organisms.

Limited literature exists specifically reporting investigations designed to localize various biological activities of *S. aureus* in sub-cellular fractions beside what has been discussed with cell associated antigens. One investigation that seems to have been directly planned for this aspect was conducted by Popovici, et al. (1968). This group of workers reported on cellular localization of various biological activities of Wood 46 strain of *S. aureus*. They disrupted washed cells of this organism to obtain various sub-cellular components which they separated by centrifugation. They then looked for the various biological activities in each of the components. They characterized each of the sub-cellular components by the centrifugal force used to sediment them, supported by morphological interpretations which they made from electron micrographs taken of the sub-cellular suspensions analyzed. These authors claimed to have demonstrated coagulase and hemolytic activity in cell wall fragments and in the membrane-mesosome fractions. Their demonstration of alpha hemolysis was based on the fact that material from a membrane-mesosome fraction (described as fraction 2) caused complete hemolysis of a suspension of rabbit erythrocytes although this test is not specific enough
to justify the claim since rabbit erythrocytes are known to
be susceptible to other hemolysins including delta hemolysin.
Also, the means by which each fraction was obtained do not
rule out cross contamination with each other. However, it
still may be considered that these authors localized on
sub-cellular components at least two antigenic proteins that
are considered extracellular products. A dermonecrotic and
bactericidal activity was also present in fraction 3(c) which
was composed of ribosome and polyribosome like particles.

The use of staphylolytic enzymes for isolation of
sub-cellular fraction from staphylococci by enzymatic treat­
ment has made the task simpler than mechanical methods used
before. A number of such staphylolytic enzymes are now
available from a number of bacterial genera including
Staphylococcus, Pseudomonas, Streptomyces and Chalaropsis.
Some of these have been purified and characterized (Hash,
1963; Schindler and Schuhart, 1964; Browder, et al., 1965,
protoplasts using such staphylolytic enzymes and the chemical
analysis of the resultant membranes proved that the membrane
preparations were free from wall contamination. Their mem­
brane preparations from protoplasts were comparable, but not
identical, to L-form membranes from the same strains of S.
aureus. One notes here that published data on similar
analyses of cell membranes from protoplasts varies among authors. Of particular significant differences were the two major components of membranes, i.e. protein and lipid contents. Ward and Perkins (1968) reported 62-71% protein and 22-25% lipids in their membrane preparations from two strains while Coles and Gross (1968) reported 34.4% protein and 34.5% lipids from protoplast membranes from their strain of S. aureus. These results may indicate that chemical analyses as check for the purity of sub-cellular fractions may still leave some doubt. Even harder to quantitate for cross contaminations are the cytoplasm and cell wall fractions.

The problem of membrane solubilization also must be solved before any meaningful work can be done with identification and characterization of membrane proteins. Currently, reports in the literature indicate most investigators have dealt with this problem using hydrogen bond-breaking agents. Among these agents, Rottem and Razin (1967) used phenol-acetic acid-water (2:1:0.5 w/v/v/) to dissolve their bacterial membranes. Sodium dodecyl sulphate (SDS) was used by Agarman and Razin (1969) to dissolve membranes prior to studying the antigenic properties of Mycoplasma membrane proteins. Many synthetic detergents seem to accomplish this task (Thirkell et al., 1969). It has also been pointed that another problem encountered here is the difficult job of removing the agent
once solubilization has been achieved. One is never sure if
the proteins are left in a comparable state to that which
they were in the native membranes. Until these questions are
solved, the exact meaning of the large number of protein
bands that have been demonstrated with membrane proteins
using polyacrylamide gel electrophoresis will not be completely
understood.

Reports that culture conditions may affect changes in
the membranous structure of *S. aureus* (Kats and Tordzhyan,
1968; White and Frerman, 1968) should also caution investi­
gators who are engaged in attempts to identify or define
membrane composition and other similar sub-cellular fractions
of the cell. Thus, methodology involved in obtaining cell
fraction, along with conditions under which the organisms
are grown, will continue to control what may be cellular
localized and in turn what relationship these may bear to the
extracellular antigenic products of *S. aureus*. 
MATERIALS AND METHODS

Organisms and Cultural Methods

Six strains of *Staphylococcus aureus* were used in these studies. Each strain was chosen on the basis of characteristics of interest reported in the literature or revealed in earlier studies (Wamola, 1967). *Staphylococcus aureus* UNH 570 is a strain with high virulence for mice and cattle originally isolated in this laboratory from a bovine mastitis case. Strains UNH 10 and UNH 15 were also isolated from bovine mastitis but are both low in virulence for mice and cattle. Cowal 1 and Wood 46 strains were included in this study as Jensen's antigen A positive and negative strains respectively. Strain 243 was used because it is positive for enterotoxin B production. Cowan 1 and 243 are low virulence strains for mice while Wood 46 is a high virulence strain.

All strains were maintained on Trypticase Soy agar (Baltimore Biological Laboratories, Baltimore, Maryland) slants and transferred to Tryptose Blood Agar base (Difco Laboratories, Detroit, Michigan) plates made with 5% whole sheep blood for isolation of single colonies prior to use in all experiments. The colonies showing hemolytic patterns typical of the original sub-culture were inoculated into broth tubes which were used in turn as inocula for media in the
larger vessels necessary for the production of large amounts of cells used in most experiments.

The medium for the production of cells had the following composition: 20 g N-Z Amine Type A (Sheffield Chemical Company, Norwich, N. Y.); 10 g yeast extract (Baltimore Biological Laboratories, Baltimore, Maryland); 6 g K₂HPO₄; 10 g KCl; 5 g NaCl; 1 g (NH₄)₂SO₄ and distilled water 1 liter. The components were dissolved by agitation and the medium adjusted to pH 7.2. The medium was dispensed in either 1 or 2 liter amounts in 4 liter Erlenmeyer flasks provided with rubber stoppers with a central hole. Other portions of the same medium were put in broth tubes in 10 ml amounts, and in 500 ml Erlenmeyer flasks in 40 ml amounts. All portions of the medium were sterilized by autoclaving for 15 minutes at 121 C.

A typical run for cell production was conducted by the following procedure: 2-3 colonies from an overnight blood agar plate were inoculated into 10 ml of production medium in a 16 x 150 mm test tube and incubated at 37 C for 18 hours. The broth culture was used to inoculate 40 ml medium contained in a 500 ml Erlenmeyer flask and the incubation continued for 5 hours at 37 C on a rotary shaker (G 10 Gyrotery Shaker, New Brunswick Scientific Company, New Brunswick, New Jersey) at setting #6 on the dial. At the
end of this time, the larger volume of medium in the 4 liter flask, which had been pre-warmed to 37 C, was seeded with this culture as a 2.5% inoculum. The flask was promptly evacuated, the air replaced with 80% O_2 - 20% CO_2 and placed on the shaker to continue growth. After 3 hours, the atmosphere in the flask was changed by introducing a fresh mixture of the gases, and growth was continued for a total period of 18 hours, which put the culture into stationary phase before the cells were harvested. Prior to the harvesting procedure, which was by centrifugation at 9000 xg for 15 minutes, the culture was placed in a 56 C water bath for 1 hour to inactivate autolysins and kill the organisms. The cells were collected by centrifugation and washed a total of 3 times, twice with cold saline and once with buffer.

**Preparation of cell wall antigens**

*Jensen's antigen A extraction*. The method of Löfkvist and Sjöquist (1962) was followed for the preparation of Jensen's antigen A extract. Washed cells of Cowan 1, grown and harvested as described above, were suspended in 0.15 M phosphate buffer, pH 5.9. The cells were boiled for 1 hour followed by rapid cooling to 4 C. The boiled cells were removed by centrifugation (5000 xg for 20 min) at 5 C. The supernatant was adjusted to pH 3.0 with 0.1 N HCl and the
precipitate formed was collected by centrifugation. The pellet was suspended in the phosphate buffer and ethanol was added to the clear solution to a final concentration of 70%. The precipitate resulting was spun down and redissolved in the buffer. The protein from this solution was precipitated by adding trichloroacetic acid (TCA), final concentration of 2%, and the precipitate that formed after standing for 1 hour was collected by centrifugation. The precipitate was dissolved in the buffer and the ethanol precipitation step repeated 3 times. Finally, the precipitate was dissolved in distilled water and dialyzed against several changes of distilled water for 2-3 days at 5 C. The solution was then lyophilized and stored dry at -20 C for the immunodiffusion studies after the protein content had been estimated by the Lowry et al. (1951) method.

The relative production of Jensen's antigen A by different S. aureus strains was surveyed by the acid extraction procedure of James and Brewer (1968) using the classically prepared, partially purified, antigen A from Cowan 1 as a reference against normal human serum. A similar method was used for the recovery of antigen A in the supernatants of all the strains used after the supernatants had been concentrated by TCA precipitation.
Verwey's Protein Fraction B Extraction

The method of Verwey (1940), slightly modified, was used for the extraction of Verwey's protein fraction B. Several batches of washed cells from each strain were grown and harvested as described above. The cells were suspended in a small volume of distilled water and lyophilized. The dried cells were pooled, weighed and resuspended in a known volume of 0.1 M phosphate buffer, pH 7.0. The cells were mixed with glass beads (Minnesota Mining and Manufacturing Company, Superbrite No. 0.15 mm diameter) and disrupted in an Omnimixer homogenizer (Ivan Sorvall, Inc., Norwalk, Conn.) for 50 minutes according to the methods of Sharon and Jeanloz (1964). The beads were removed by passing the mixture through a Buchner funnel and washing several times with cold distilled water. The suspension was centrifuged at 1500 xg for 30 minutes to remove beads and unbroken cells. The supernatant which contained disrupted cells was acidified with 0.1 N HCl to pH 5.2 and centrifuged at 1500 xg for 30 minutes. The precipitate was discarded and the supernatant further acidified to pH 3.5. The precipitate was again removed by centrifugation and discarded. To the remaining clear, straw colored supernatant 50% trichloroacetic acid was added to produce a final concentration of 11-14%. Turbidity developed slowly at 5 C and coalesced into a precipitate at this
temperature after standing overnight. This precipitate was removed by centrifugation at about 13000 xg for 30 minutes and was resuspended in distilled water. The solution was brought to pH 7.5 with NaOH and dialyzed against distilled water overnight at 5 C to remove salts. The product was lyophilized and the resultant white fluffy powder was made into a small amount of solution of known volume. The protein content was determined by the Lowry method. These solutions were used for immunodiffusion studies and in polyacrylamide gel-electrophoresis experiments.

Other Methods for Extraction of Cell Wall Proteins

Purified cell walls were obtained by mechanical disruption of washed cells according to the method of Sharon and Jeanloz (1964). The walls were lyophilized and stored at -20 C in a desiccator. Three methods were used to obtain protein extracts from these preparations.

The first method utilized 100 mg of cell walls that were suspended in 10 ml distilled water. To reduce nucleic acid contamination they were treated with RNase and DNase (Worthington Biochemical Corporation, Freehold, New Jersey) each at a concentration of 200 ug/ml suspension with addition of a small amount of MgCl₂ as co-factor for DNase. The suspension was incubated for 30 minutes at 37 C and the cell
walls were sedimented by centrifugation (12000 xg for 20 minutes). The walls were washed 3 times with distilled water to remove the added enzymes and the pellet containing the cell walls was finally suspended in 0.05 M tris-saline buffer, pH 7.5. The cell walls were hydrolyzed with lysostaphin (a gift from Dr. W. A. Zygmunt of Mead Johnson, Evansville, Indiana) at a concentration of 10 units per ml according to the assay procedure described by the supplier. The treatment with lysostaphin was done at 37 C for 2 hours. Cell walls unhydrolyzed at the end of this period were removed by centrifugation. Most of the cell walls were digested into a soluble fraction containing the proteins and lysostaphin. This digest was used in immunodiffusion and polyacrylamide gel electrophoresis studies designated as lysostaphin solubilized cell wall (Lns).

The second method for solubilizing cell wall proteins also employed (100 mg suspended in 10 ml) cell wall preparations similar to those described in the first method. A sample of the clean walls was taken, acid hydrolyzed, and amino acid analysis performed on it (Beckman automatic analyzer, Beckman/Spinco, model 120). A second sample was treated sequentially with 3 proteolytic enzymes under suitable conditions (Trysin and chymotrypsin incubated separately for 12 hours under toluene and chloroform at 45 C with Ca++ added
as co-factor; pronase was added to wall suspension adjusted
to pH 7.5 and incubated at 37 C for 2 hours) to remove as
much as possible of the protein layer on the cell walls.
Trypsin and chymotrypsin, each 100 ug/ml, was obtained from
Worthington Biochemical Corporation and pronase, 100 ug/ml,
Grade B, from Calbiochem, Los Angeles, California). These
walls were hydrolyzed with 6 N HCl for 24 hours, and subjected
to amino acid analysis designated as "deproteinized walls" (DP)
to provide data presumably on the amino acids of the pepti-
doglycan. The rest of the cell walls were treated with
phenol-acetic acid-water (2:1:0.5 v/v/v/) by mixing one
volume of clean cell wall suspension with 2 volumes of the
phenol-acetic acid-water solution. The insoluble residue
(PA-R) was removed by centrifugation. The PA-R fraction was
washed 3 times, acid hydrolized, and subjected to amino acid
analysis to establish if residual protein amino acids were
left after this treatment by comparison with the amino acid
analysis of clean walls, deproteinized walls and PA-R fraction.
The three amino acid analyses were used to establish the
percentage of protein removed by the treatment used to obtain
the phenol-acetic acid-water soluble protein (PA-S) by calcu-
lations based on amino nitrogen content. The PA-S was used
in the polyacrylamide gel electrophoresis studies on cell
wall proteins.
The third procedure for getting total cell wall proteins combined the treatment of clean walls with lysostaphin and TCA precipitation of the soluble fraction to concentrate the proteins. Enough 3 M TCA was added to yield a final concentration of 0.3 M TCA in the lysostaphin soluble wall fraction. The precipitate was removed by centrifugation and re-dissolved in a small volume of buffer. The final solution was brought to pH 7-8 with NaOH and used in gel immunodiffusion studies.

Preparation of Purified Exoprotein Antigens of *S. aureus*

**Alpha hemolysin.** Purified alpha hemolysin was obtained from Dr. A. R. Buckelew, Jr. (Buckelew, 1968). The toxin was stored as an ammonium sulphate precipitate. Samples of 10 ml were taken and spun down at 23500 xg for 30 min. The pellet was resuspended in 2 ml of physiological saline and both the hemolytic units (HU) and protein content were determined before dilutions were made for experiments. All solutions were kept frozen until used.

**Micrococcal nuclease.** Purified nuclease was obtained from Worthington Biochemical Corporation, Freehold, New Jersey. Small amounts were weighed and suspended in 0.05 M Trischloride buffer, pH 7.0. The protein content was
determined by the Lowry method. Portions for immunodiffusion studies were made and stored frozen until used.

**Beta hemolysin.** Cellulose phosphate purified beta hemolysin from strain 234 was obtained from Dr. W. R. Chesbro of the University of New Hampshire. The toxin was stored frozen in glycerol and dilutions were made with 0.05 M Tris-chloride buffer, pH 7.0 to known protein levels for immunodiffusion.

**Enterotoxins.** Purified enterotoxin B preparation was obtained from Dr. E. J. Shantz (U. S. Army Laboratories, Fort Detrick, Maryland). Enterotoxins A and B preparations were also obtained from Dr. W. R. Chesbro. The protein contents of the preparations were determined and appropriate dilutions for immunodiffusion were made.

**Coagulase.** Electrophoretically purified coagulase was a gift from Dr. M. Tager (Emory University, Atlanta, Georgia). The purified enzyme had 0.27 mg protein per mg of powder and specific activity of a clotting titer of 7,447, as given by Dr. Tager.

**Teichoic acid.** Although teichoic acid is not an exoprotein antigen, it too was obtained in a purified form for this investigation. It was a gift from Miss Ann Brown (Brown, 1968).
Preparation of Antisera Against Exoprotein Antigens in Animals.

Anti-alpha antiserum. Three rabbits about 2 months old were used for the production of anti-alpha hemolysin antiserum. They were first bled to determine if they showed any anti-alpha hemolysin activity by neutralization test and immunodiffusion before immunization was begun. The vaccine was prepared from detoxified alpha hemolysin of 64,000 hemolytic units (HU), about 6.5 HU per ug protein, and inactivated with formalin. The vaccine preparation was mixed with an equal amount of a mixture of one part Freund's incomplete adjuvant and one part staphylococcal DNA. The mixture was homogenized for 10 seconds in the Omni-mixer operating at 50 volts (Ivan Sorvall, Inc., Norwalk, Conn.). Each rabbit received a 1.0 ml intraperitoneal and a 0.25 ml intramuscular injection of the stable emulsion suspension of the vaccine on each quarter. The animals were rested for 28 days before test bleeding to determine anti-alpha hemolysin titers in gel immunodiffusion plates against homologous alpha toxin. When it was established that the animals had produced antibodies against alpha toxin, they were bled by cardiac puncture and the serum separated from the blood was stored frozen with 1:10,000 merthiolate as a preservative. Neutralization titers for the sera were performed by standard
procedures with the kind help of Dr. C. H. Bartley of this Department.

**Anti-nuclease antiserum.** Nuclease preparations from Worthington Biochemical Corporation and purified nuclease from Strain UNH 15 prepared by Dr. W. R. Chesbro were used either alone or in combination to immunize groups of rabbits. The animals that showed antibodies against the nuclease were bled and the serum separated from the blood was stored frozen with merthiolate.

**Anti-beta toxin antiserum.** The purified beta hemolysin prepared by Dr. Chesbro was used to immunize rabbits. The immune serum obtained from these animals was stored frozen in vials with a preservative.

**Anti-enterotoxins antisera.** Specific rabbit anti-enterotoxin B was received from Dr. E. J. Shantz along with its homologous antigen. Rabbit anti-enterotoxin A and B sera were raised against Dr. Chesbro's enterotoxin A and B preparations. The animals were bled and the sera separated from the blood was stored frozen with merthiolate as preservative.

**Anti-leucocidin antiserum.** Rabbit serum samples with anti F and anti S antibodies were a gift from Dr. G. P.
Gladstone, of the Sir William Dunn School of Pathology, Oxford University, Oxford, England.

**General Antiserum Against Staphylococcus aureus.**

Rabbit heterologous antiserum against washed heat killed cells of *S. aureus* (strains UNH 570 and UNH 10) was prepared by immunizing the animals with a series of injections of washed heat killed cells. Blood was collected from these animals and the serum was separated and stored frozen until used.

Several bovine heterologous anti-staphylococcus antisera were kindly supplied by Dr. C. H. Bartley who prepared the sera by immunizing cattle with staphylococcal cell-toxoid vaccines of various preparations. The antisera exhibited vaccines of various preparations. The antisera exhibited a high neutralization titer for alpha toxin when compared to the international antistaphylococcal antitoxin serum (U. S. Standard Antistaphylococcal Serum, Division of Biological Standards, NIH, Bethesda, Maryland).

Both bovine heterologous antisera and rabbit heterologous antisera were used whenever a specific antiserum for a purified exoprotein antigen was not available with the purified antigen serving as a reference for identification in immunodiffusion.
Formation of Staphylococcal Protoplasts and Membranes from Strain UNH 570: Cultural Conditions.

Staphylococcus aureus strain UNH 570, cultured in slightly modified conditions from those used for cell production, was used to provide high yields of actively growing cells for protoplast formation. The medium described for cell production was used in 950 ml amounts in 4 liter Erlenmeyer flasks. Starter culture, in a 10 ml broth tube, was grown for 18 hours and used to inoculate 40 ml of the same sterile medium in a 500 ml Erlenmeyer flask. The culture was incubated on a gyrotory shaker at 37 C for five hours. The entire culture was then used to inoculate the 950 ml fresh, sterile medium prewarmed at 37 C. Immediately after inoculation the air in the flask was replaced with 80% O₂ and 20% CO₂. The culture was then placed on the incubator shaker at a setting of six at 37 C for 3 hours before the gases were replaced with fresh oxygen and carbon dioxide. Incubation was continued for an additional 4 hours which made the total incubation time for the growing culture 12 hours. The young, actively growing cells in early stationary phase were immediately harvested by centrifugation and washed 3 times with cold 0.05 M tris-chloride buffer, pH 7.5. Finally, the cells were suspended in the buffer in one-tenth the original culture volume and an aliquot sample withdrawn for dry weight determination.
Preparation of Protoplasts for Membranes. Washed cell suspensions of the type described above were sedimented and resuspended in hypertonic solutions composed of 24% NaCl in 0.05 M tris-chloride buffer, pH 7.5, for protoplast formation with lysostaphin as described by Schuhardt and Klesius (1968) and Schuhardt et al. (1969). Lysostaphin (10 units/ml) was added in an amount equal to 0.5-1% of the dry weight of the cocci. The cells-enzyme mixtures were incubated in a 37°C water bath with occasional shaking for 2 hours. Protoplast formation was confirmed with phase microscopic examination and loss of gram positive reactivity. The protoplasts were sedimented by centrifugation (8000 xg for 20 minutes). The supernatant, which contained excess enzyme, hydrolyzed cell walls, and any extruded mesosomes, was saved for further investigation after dialyzing against distilled water to remove the salts. The protoplasts were washed 3 times in the hypertonic buffer and finally lysed in 0.05 M tris-saline buffer, pH 7.5 in which 0.01 M MgCl₂ was added to prevent fragmentation of the membranes (Ghosh and Carroll, 1968). The lysis of the protoplasts was enhanced by homogenizing for a 1 minute period in a Sorvall Omni-mixer operating at 50 volts. The homogenate was centrifuged at 25000 xg for 1 hour at 5°C to separate the cytoplasm (supernatant) from the membranes (pellet) after the homogenate had been treated with
RNase and DNase 100 units/ml each (Worthington Biochemical Corporation, Freehold, N.J.) for 30 minutes at 37 °C. The sedimented membranes were washed 3 times with 0.01 M Mg Cl₂ water, pH 6.8, and finally resuspended in a known volume. The membrane preparation was stored frozen and an aliquot sample used for the chemical analysis and the polyacrylamide gel electrophoresis studies.

**Chemical Composition of the Protoplast Membranes.**

The dry weight of membranes as well as the dry weight of cell suspensions was determined by transferring in duplicate known volumes of samples of washed membranes and washed cells into clean, tared aluminum dishes. The samples were heated overnight in an oven at 110 °C followed by cooling in a desiccator before weighing. The average differences in weights between tare weight and dried sample-aluminum dish after subtracting the average weight contributed by an equivalent amount of blank containing buffers used to suspend the membrane of the cells, gave the dry weight of either the cell membrane or the cells respectively. The percentage of the cell weight that is contributed by membranes can be calculated using the figures obtained above.

Ribonucleic acid (RNA) and deoxyribonucleic acid (DNA) contamination of the membranes was estimated after the
extraction of the membranes for these substances by the method of Bodman and Walker (1969) which involved extraction of membranes in 0.5 N HClO₄ for 20 minutes, cooling and centrifuging at 3000 xg for 15 minutes. The supernatant fluid was used for determination of RNA by the orcinol reaction procedure of Cerriotti (1955) and for DNA by the diphenylamine reaction method of Burton (1956).

The protein content of the membranes was quantitated by the method of Lowry et al. (1951) using a standard curve prepared from lysozyme in 0.05 M NaCl. The extraction of lipids was by the chloroform-methanol solvent (2:1v/v) procedure of Bligh and Dyer (1959). The lipids extracted from a known weight of membranes were saved for the estimation of phosphorus content of the membranes after the extract had been filtered through filter paper (#589 white, Schleicher and Schuell, Keene, New Hampshire). The residue of the membranes left after extraction of the lipids was dried overnight at 110 C, cooled in a desiccator and finally weighed. The differences in the weights between the original membrane weight and that of the residue was used to determine what percentage of the membrane weight is lipid content.

The extracted lipid was used for phosphorus determination along with standard samples made from a stock phosphate solution by digestion in 10 N H₂SO₄ for one and half hours
in a 150-160 C oven according to the modified method of Bartlet (1959). Two drops of 70% HClO₄ were added and the digestion continued for another 30 minutes. The color was developed by adding 4.6 ml of 0.22% ammonium molybdate and 0.2 ml of the Fiske-Subba Row reagent followed by thorough mixing and heating for 7 minutes in a boiling water bath. The optical density of the color was read at 830 mu with a spectronic 20 spectrophotometer equipped with a red-sensitive phototube. The standard curve for phosphorus was prepared from a stock solution containing 0.351 g of KH₂PO₄ per liter (M.W. 136.091) which was diluted 1:10 so that the working solution contained a calculated phosphorus content of 8 ug per ml.

The carbohydrate content of membrane samples was determined using the phenol-H₂SO₄ method for polysaccharides and monosaccharides described by Dubois et al. (1956) with glucose as standard. Hexosamines, as indicator of membrane contamination with cell walls, were determined by the colorimetric method of Elson and Morgan (1933) as modified by Horowitz et al. (1950) using D-glucosamine-HCl as a standard.

Production of Anti-membrane Antisera in Rabbits.

Four rabbits, about 2 months old, were used for the production of anti-membrane antisera. A membrane preparation,
pooled from two batches containing 130 mg of protein in 7.5 ml suspension, was mixed with an equal volume of Freund's incomplete adjuvant (Difco Laboratories, Detroit, Michigan). The mixture was homogenized for 10 seconds at high speed in a Sorvall Omni-mixer. The stable emulsion was injected in the animals, which had been established earlier by test bleeding to have no antibodies against purified exoprotein antigens from *Staphylococcus aureus* using gel immunodiffusion. Each rabbit received, in each quarter by intramuscular injection, 0.4 ml and by the intraperitoneal route, 1.0 ml of the stable emulsion. The remainder of the adjuvant-membrane mixture was stored at 5°C with merthiolate (1:10,000) added. The animals were allowed to rest for 3 weeks before receiving a 1.0 ml booster of the same mixture by intraperitoneal injection, thus each animal received a total 3.6 ml or 31 mg protein. The animals were again rested for a week before test bleeding to determine antibodies against membranes and purified exoprotein antigens. After 2½ weeks from the time the animals received a booster shot, all the rabbits were bled to death by cardiac puncture and the collected sera stored frozen in vials with merthiolate (1:10,000 final concentration). The sera were used for immunodiffusion and neutralization experiments.
Preparation of Mesosomes and Cytoplasmic Fractions.

**Mesosome Fraction from S. aureus strain UNH 570.**

During formation of protoplasts from cells of *S. aureus* strain UNH 570 in hypertonic solution (24% NaCl-tris chloride buffer, pH 7.5), the mesosomes which were extruded into the protection medium plus the peri-plasmic space substances were collected as the supernatant after sedimenting the protoplasts by centrifugation (8000 xg for 20 minutes). This supernatant contained hydrolyzed cell wall particles, lysostaphin, mesosomes, peri-plasmic substances and salts from the buffer. This fraction was dialyzed against several changes of distilled water in the cold to remove the salts. It was hoped that the mesosomes extruded would be in the retentate, which was centrifuged at 12000 xg for 20 minutes to remove any particulate matter and the soluble portion was lyophilized. The dried powder from this product was dissolved in distilled water and the protein content measured by the Lowry method. The solution was checked by immunodiffusion for antigens with purified exoprotein antigens as references against specific antisera and for hemolysis on washed 5% blood agar plates.

**Preparation of the Cytoplasmic Fraction**

Cells from 1 liter of a culture of UNH 570, grown under conditions identical to those described for protoplast
formation, were used for the preparation of the cytoplasmic fraction. The washed cells were suspended in 0.01 M Na$_2$HPO$_4$ buffer, pH 7.0 (about one-tenth culture volume) and an aliquot was withdrawn for dry weight determination. The rest of the cells were mixed with washed glass beads and mechanically disrupted by the method of Sharon and Jeanloz (1964). The beads and unbroken cells were separated by centrifugation after passage through a Buchner funnel. The supernatant collected after centrifugation at 12000 xg for 20 minutes, containing the membrane-cytoplasmic fraction, was treated with 1 mg of DNase and 3 mg RNase at 37 C for 30 minutes with added MgCl$_2$. The membranes were sedimented by rapid centrifugation (25000 xg for one hour) and the cytoplasm, along with the added nucleases, were collected as the supernatant. The membrane sediment was washed 3 times and saved for analysis while the large volume of cytoplasm (about 500 ml) was concentrated by placing in a dialyzing bag covered with aquacide I and II powder (Calbiochem, Los Angeles, California). The volume was reduced to 18 ml overnight at 5 C.

Dry weight of the non-dialyzable material was determined in manner similar to that described earlier for membranes and cells, along with the saved, washed membranes. The protein contents of both the cytoplasm and the membrane fractions were measured by the Lowry method. The phospholipid
extraction by the chloroform:methanol (2:1 v/v) procedure was undertaken with the cytoplasm fraction to establish the degree of contamination with membranes. Using the same conditions, this extraction was done on the membrane pellet after suspending it in 0.01 M MgCl₂, pH 6.8. The lipid extracts from known dry weights of cytoplasm and membranes were used for the phosphorus determination by the modified method of Bartlet (1959) described earlier.


Fluorescein isothiocyanate (FITC) obtained from Blatimore Biological Laboratories was conjugated with the globulin fraction of antisera by method of Clark and Shepard (1963). The labelled conjugate was purified according to a method modified from that of Goldstein (1962), using BioGel P6 (Bio-Rad Laboratories, Richmond, California) and DEAE-cellulose (Whatman CE32, W and R Bolston, Ltd., England) column chromatography, the latter column being eluted with increasing concentrations of NaCl in 0.0175 M phosphate buffer, pH 6.3. The optimum FITC-protein ratio (F:P) was determined from the optical density at 280 and 495 nm in a spectronic 600 (Bausch and Lomb, Rochester, New York) as described by Goldman (1968).
The direct staining method of Coons and Kaplan (1950) was used, employing viable washed cells of the different strains. The cells were heat fixed on clean slides, then mixed with a drop of FITC-labelled antiserum. The slides were incubated in a moist chamber for 30 minutes at 37°C to allow the antigen-antibody reaction to occur. The excess serum was removed by rinsing with 0.025 M carbonate-bicarbonate buffer, pH 9.0, phosphate buffered saline, pH 7.2; and finally with distilled water. The fresh, moist slides were examined under a fluorescent microscope, Reichert Zetopan research microscope (Reichert, Vienna, Austria) equipped with suitable light filters (KG2, BG12 and GG9). Ten percent glycerol solution on the cover slip served as the immersion medium.

The indirect method was attempted with commercially prepared FITC-conjugated sheep anti-rabbit serum (Sycco, Sylvana, Millburn, New Jersey) which was further purified using DEAE chromatography to obtain the portion with F:P ratio most suitable for maximal brightness. The cells were first reacted with immune rabbit antistaphylococcal antiserum prior to adding the conjugated antiserum. The same staining conditions used in the indirect method were employed and the fresh moist slides were examined under the fluorescent microscope.
**Enzymatic Measurement of Cell-Associated Nuclease.**

Growth conditions and the medium were slightly modified to give both high yield of cells and extracellular enzymes. The medium was prepared in the following manner:

30 g N-Z amine type A, and 30 g yeast extract were dissolved in 200 ml of distilled water by boiling for 5 minutes. The solution was cooled and placed in a dialyzer bag. The dialyzer bag was then placed in a 4 liter flask containing 12 g K$_2$HPO$_4$, 7.5 g NaCl, and 7.5 g KCl dissolved in 3 liters of distilled water. Ten ml of chloroform was added to prevent any microbial growth while the dialysis bag was stirred at 5 C for 48 hours. The dialysis bag was removed at the end of this period and discarded with its contents. Undissolved chloroform was removed by decanting the diffusate into another flask where the volume was finally adjusted to 3 liters and the pH to 7.2. This medium was distributed in vessels as described for cell production runs and autoclaved at 121 C for 15 minutes. The cultures were grown in the conditions described for cultures used for protoplast formation. An aliquot was withdrawn from culture when 12 hours old and immediately frozen for determination of total nuclease activity in the culture. The rest of the culture was centrifuged to separate cells from the supernatant and an aliquot of the supernatant was saved and immediately frozen.
for determination of nuclease activity. The cells were washed 3 times in cold saline and finally suspended in one-tenth volume of total culture. An aliquot sample of cells was taken and immediately frozen for measurement of nuclease activity associated with cells. Finally two aliquots of the cells were withdrawn and one lysed with a staphylolytic enzyme partially purified from *Pseudomonas aeruginosa* (Burke and Pattee, 1967) while the other was disrupted mechanically with the Nossal disintegrator using a total shaking time of 3 minutes. Both lysate samples were used for determination of nuclease activity in lysed cells. All samples that had been collected in this operation were divided into 2 portions, one of these was boiled for 10 minutes while the other was held on an ice water bath. After cooling both portions of all samples were assayed for nuclease activity at the same time and the operation lasted no more than 2-3 days with precautions taken to keep the samples either frozen or at ice-bath temperature at all times during this operation.

The nuclease assay method used is the modified assay system of Alexander et al. (1961) using heat-denatured, calf thymus DNA as substrate. Appropriate dilutions of samples were introduced in 0.025 ml to the assay mixture of 0.475 ml composed of 0.200 ml of DNA solution (2 mg/ml), 0.250 ml of 0.05 M tris-chloride buffer, pH 8.6; and 0.025 ml of 0.2
M CaCl solution. The enzyme-substrate mixture was allowed to incubate at 37 °C for exactly 30 minutes and the reaction was immediately stopped by addition of 0.050 ml of 10% citric acid with the aid of a 100 ul syringe and the samples were promptly chilled in an ice-water bath. Unhydrolyzed DNA was precipitated by addition of 0.5 ml of 7% HClO₄ followed in one minute by 3 ml of cold distilled water. Immediately, the tubes of all samples were centrifuged at about 1000 xg for 10 minutes in the refrigerated Sorvall centrifuge. The supernatant was carefully removed by decantation into clean glass tubes and the optical density measured using the spectronic 600 (Bausch and Lomb) against distilled water as zero absorbance. Included and treated in identical manner was the assay mixture blank which gave an O.D. less than 0.05 against water. Also with all material suspected of 260 endogenous absorbing contamination, such as whole culture, supernatant and cells, individual blanks were prepared and treated the same except instead of incubation for 30 minutes at 37 °C they were stopped immediately after introduction of the samples. A unit of nuclease was here defined as that amount of enzyme yielding on O.D. of 1.0 in 30 minutes under these test conditions.

Immunodiffusion in Agar Gel Plates.

The Ouchterlony double diffusion technique in agar gel plates was employed. Noble agar (Difco) was used at a
concentration of 1.3% in Veronal buffer, pH 8.2, with 0.01% sodium azide added to inhibit growth of bacterial contamination. Five ml of molten agar was put in the petri dishes (55 x 14 mm) which had been coated with a 0.1% solution of agar and allowed to dry. The plates were dried overnight at 30 C. Patterns of wells capable of holding 0.05 ml were made in the agar with a cutter by positioning the plate on the desired pattern drawn on paper such that wells were spaced 5-8mm apart. The agar was removed from these wells by suction using a capillary pipette. The wells were loaded with antigens of known protein content and the antisera were placed in adjacent wells. The plates were incubated for 24-48 hours at 30 C before immunoprecipitation lines were examined over a diffused fluorescent light and the antigen-antibody reaction interpreted as to their relation to the unknown antigen under investigation. The plates were dried, stained and de-stained following the procedure outlined in LKB operation manual #6800A (LKB-Produkter Ab, Stockholm, Sweden).

**Immunoelectrophoresis of Jensen's Antigen A Extracts**

Clean microscope slides were coated with 0.1% solution of agarose (Fisher Scientific Co.) in 0.05% glycerine and left to dry for 15 minutes. Agarose, 1.3%, with 0.01%
sodium azide added in phosphate buffer, pH 6.8 and ionic strength of 0.05, was layered on the slides placed on a frame (about 10 ml/3 slides). The agarose was allowed to cool in a humid chamber for at least 30 minutes before a central well for holding the antigen and side troughs for the antisera were cut using LKB equipment. The agar was lifted from the central well by suction while the troughs were left intact until electrophoresis had been completed. Into the central well, 0.01 ml of Jensen's antigen A extract (10 ug protein) was introduced mixed with 5% sucrose. The slides, mounted on a holder, were placed on the electrophoresis apparatus (Savant Instrument, Inc., Hicksville, N. Y.) which had been pre-cooled by circulating ice-water. Cold phosphate buffer, used to prepare the agarose, was placed in troughs inside the apparatus and contact with the slides was established by filter paper wicks dipped into the buffer. The power supply (HV 1000A power supply, Savant Instrument, Inc.) was turned on and electrophoresis conducted with a direct current of 300V for 20 minutes. The power was turned off and the troughs on both sides cleared of agar. Normal pooled human serum was introduced in one trough and anti-beta toxin antiserum in the other. In another slide purified beta-hemolysin was electrophoresed under identical conditions. The slides, put in a moist chamber, were incubated for 24-48
hours at 30 C. The precipitin lines were examined and the record of the precipitin lines preserved by drying down the agar, staining, and de-staining according to the LKB operation manual.

The Preer Technique for Demonstrating Antibodies Present in Antisera in Low Concentrations.

Quantitative double diffusion agar as described by Preer (1956) was used to demonstrate antibodies in anti-membrane antisera against purified nuclease (Worthington Biochemical Corporation). The Preer Technique was used to demonstrate the presence of alpha hemolysin in the membranes by absorption with anti-alpha hemolysin serum prior to reacting with a known amount of purified alpha toxin. Micro blood collecting tubes of 1.5 mm bore were cut about 5 cm long. The tubes were flame sealed on one end and 3 marks scratched on the outside 1 cm apart measured from the sealed end (each division thus holding approximately 0.020 ml). The tubes were coated with 0.1% agar containing 0.01% sodium azide. The tubes were dried and dilutions of specific antiserum introduced in 0.02 ml volume. The antiserum was layered with an equal volume of 0.6% Noble agar in 0.01 M tris-chloride containing 0.01% sodium azide. The agar was allowed to solidify and the antigen dilution introduced in 0.20 ml volume. The
tubes were plugged with melted parafilm to avoid evaporation and incubation was made at 30 C for several days. Measurements of precipitin lines were made after 3-4 days and described as P-values, defined as the ratio of the distance of the precipitate from the antigen-agar interface to the total length of the agar. To avoid any changes in the P-value due to migration of precipitin lines in a non-equivalent system, all measurements were made on same day (usually after 3 days incubation).

A suitable dilution of alpha toxin (0.36 ug per 20 ul) which gave the best precipitin line with a 1:4 dilution of anti alpha toxin anti-serum was chosen for the study of alpha hemolysin in the membranes. About 0.1 ml of membrane preparation (5.5 mg protein/ml) was absorbed with 0.1 ml of 1:2 dilution of antiserum, thus giving a final dilution of the antibody 1:4. The absorption mixture was incubated in a 37 C water bath for 2 hours. Twenty ul of the absorption mixture was placed in the Preer tubes and layered with equal volume of neutral agar. This absorbed antisera was reacted with antigen (0.36 ug/20 ul of alpha toxin) as described above. A control set of samples in which buffer was substituted for the membrane and treated in identical manner. A shift in the P-value greater than twice the differences between duplicate samples (about 0.025 p-units), which was equivalent to twice
the standard deviation, was considered significant and directly attributed to the alpha toxin in the membrane which was responsible for the reduction of antibodies in the serum.

Anti-membrane antisera from 4 rabbits were checked for anti-nuclease activity against purified nuclease using this system. Since it had been possible with this system to detect as little as 0.95 ug of nuclease against a 1:4 dilution of its hemologous antisemum it was felt that using the anti-membrane serum undiluted against such low levels of purified nuclease, precipitin lines would indicate presence of anti-nuclease activity in the antimembrane sera, even though present in very low concentration.

Toxin Neutralization for Assay of Anti-membrane Antisera.

The ability of anti-membrane serum to neutralize purified alpha toxin was demonstrated and compared to the commercially available standard antitoxin serum (U. S. Standard staphylococcal antitoxin, NIH Biological Laboratories, Bethesda, Maryland) by the following procedure: alpha toxin (7.3 ug/ml) was diluted by doubling series in 0.01 M phosphate buffered saline, pH 7.0 and titered against 1 International Unit (I.U.) of standard antitoxin using washed 2% rabbit erythrocytes as an indicator system with 50% hemolysis as end point (Cooper et al., 1964). The dilution of alpha toxin
that neutralized 1 unit of antitoxin was used against anti-
membrane antiserum to establish how many anti alpha toxin
units the sera from animals immunized with membrane had
generated against staphylococcal alpha toxin.

An estimation of content of alpha toxin in the mem-
brane preparation was carried out by an absorption technique
using the standard International antitoxin serum. One unit
(I.U.) in 0.5 ml antiserum was absorbed at 37 C for 2 hours
with a membrane preparation containing 0.5 mg protein in 0.1
ml. The membrane material was removed by centrifugation and
the absorbed serum diluted in doubling dilution. An amount
of alpha toxin just adequate to neutralize I.U. antiserum
was added (1.825 ug) and 1 ml of 2% washed rabbit erythrocytes
as indicator of hemolysis was introduced. The tubes were
incubated at 37 C for 1 hour. A control containing no mem-
brane was treated in same condition. The 50% hemolysis end
point was read at 541 mu in a spectronic 600 (Bausch and Lomb)
after the incubation period and the titer was calculated. The
difference between the control tube and the absorbed anti-
serum was attributed to alpha toxin present in the membrane
which was responsible for the absorption of antibodies in the
antiserum, thus lowering the anti-alpha capacity necessary to
neutralize the fixed amount of alpha toxin used in both the
test and control samples. The percentage of the membrane
protein which is alpha toxin was calculated and estimated from the data obtained.

**Polyacrylamide Gel Electrophoresis with Membrane and Cell Wall Proteins.**

Membrane preparations and cell wall protein extracts were subjected to polyacrylamide gel electrophoresis using a Canalco Research Disc Electrophoresis equipment, model 1200 (Canalco- Rockville, Maryland). The 7.5% polyacrylamide gel (Canalco) in 35% (v/v) acetic acid and 5 M urea was prepared by the method of Takayama et al. (1964). Clean glass columns (5 x 65 mm) were placed upright on one end standing in vaccine bottle caps, and 1.4 ml of the separating gel was introduced in the tube in the other end by long-nosed pipette. Polymerization was induced by N', N', N', N'-tetramethylethylene diamine (TEMED, Canalco) and ammonium persulfate (E-C Apparatus Corporation, Philadelphia, Pa.). The tubes were incubated at 37 C for 1 hour to allow polymerization to complete.

Membranes of known protein content were dissolved by adding 2 volumes of phenol-acetic acid-water (2:1:0.5) to 1 volume of membrane suspension according to the method of Rottem and Razin (1967). The membrane samples to be analyzed (100 to 300 ug of protein) were mixed with 0.05 ml of 40%
sucrose in 35% (v/v) acetic acid. Both chambers were filled with 10% (v/v) acetic acid with the lower chamber electrode serving as cathode. Electrophoresis was performed for 2 hours at a constant current of 5 ma per tube with a Spinco Duostat regulated D. C. power supply (Beckman Instruments, Inc., Palo Alto, California). The cell wall proteins obtained by both the action of lysostaphin on isolated walls (LnS) and by extracting with phenol-acetic acid-water (PA-S) were also subjected to electrophoresis in the same conditions described for membrane proteins. The gels were stained with 0.5% (v/v) amido black in 7% (v/v) acetic acid for 30-60 minutes, rinsed with tap water and finally destained electrophoretically in 7% acetic acid by direct current of 10 ma per tube.

Gels identical to those stained for each run of the membrane proteins were sliced (about 1-2 mm) with a gel cutter and the slices were placed in individual tubes containing 0.5 ml solution of 0.01% sodium deodecyl sulphate (SDS), 0.2% bovine serum albumin fraction V (BSA) and 0.01% sodium azide. The protein was allowed to elute overnight at room temperature before the gels were removed. The protein was precipitated by adding enough 3 M TCA to a final concentration of about 0.3 M TCA. The precipitate formed was collected by centrifugation and made up into 0.1 ml solution containing
0.01% SDS, 0.81% veranol buffer, pH 8.6. This solution then was adjusted to pH 7-8 (if necessary with 1 N NaOH) and 0.05 ml amounts were used in the center well of Ouchterlony gel diffusion agar plates along with known antigens and their specific antiserum in the adjacent peripheral wells. A control solution with no gel piece eluted in it was treated under the same conditions and checked for the ability of the BSA to precipitate antisera used in the experiment.

Verwey's protein fraction B extracts from 3 strains were run in 7.5% polyacrylamide gel electrophoresis using 0.1 M tris-EDTA-boric acid buffer, pH 8.9. The gels were prepared and run according to the method of Agarman and Razin (1969) except for the inclusion of 5 M urea in the gels. These gels were stained in the manner described and the pattern of the protein bands compared.
RESULTS

**Jensen's Antigen A and Verwey's Protein Fraction B Extracts**

The protein extract obtained by the classical Jensen's method from strain Cowan 1 was shown, by double gel diffusion technique, to give a maximum of 3 precipitin lines against antiserum prepared in rabbits immunized with washed, heat killed cells of *S. aureus* UNH 10. (Fig. 1). It can be seen that only a broad line was obtained with this material against commercially available pooled normal human serum, a reaction which has been described as non-specific between antigen A and $\gamma$-G-globulin. Evidence suggesting lines of identity between two of the lines in Jensen's A extract with teichoic acid and beta hemolysin were obtained (Fig. 2). Also, hemolytic activity was observed with the extracts using blood agar plates which seemed to support evidence for the presence of beta hemolysin.

The protein material obtained by the Verwey's extraction procedure (Verwey's protein fraction B) from 5 strains of *S. aureus* was demonstrated to contain at least 5 precipitin lines against rabbit antistaphylococcal serum. Four of these precipitin lines had identity with purified teichoic acid, beta hemolysin, nuclease and Jensen's antigen A (absent in Wood 46). Figures 1, 2, 3 and 4 illustrate these results.
Fig. 1. Immunodiffusion of Jensen's antigen A and Verwey's protein B extracts against normal human serum and rabbit antistaphylococcal antiserum. Center well contained in 50 ul rabbit anti UNH 10 antiserum (anti 10). Peripheral wells contained in 50 ul: normal human serum (H); 25 ug of Jensen's antigen A extract from Cowan 1(A); 150 ug of Verwey's protein B extract from Cowan 1(C1); 200 ug of Verwey's protein B extract from Wood 46 (46).
Fig. 2. Immunodiffusion of Jensen's antigen A extract, Verwey's protein B extract and teichoic acid against rabbit antistaphylococcal antiserum. Center well contained in 50 ul of rabbit anti UNH 10 antiserum (7). Peripheral wells contained in 50 ul: 25 ug of Jensen's antigen A extract (1); 150 ug of Verwey's protein B extract from Wood 46 (2), 50 ug of teichoic acid (3 and 5) 150 ug of Verwey's protein B extract from Cowan 1 (6).
Fig. 3. Immunodiffusion of Verwey's protein B extract, Jensen's antigen A extract and purified beta hemolysin against bovine antistaphylococcal antiserum. Center well contained in 50 ul bovine antistaphylococcal antiserum (6). Other wells contained in 50 ul: 175 ug of Verwey's protein B extract from Wood 46 (1); 5 ug of beta hemolysin (2); 50 ug of Verwey's protein B extract from stain 243 (3); 25 ug of Jensen's antigen A extract from Cowan 1 (4); 50 ug of Verwey's protein B extract from UNH 15.
Fig. 4. Immunodiffusion of Verwey's protein B extracts and purified nuclease against rabbit antinuclease antiserum. Center well contained in 50 ul of rabbit antinuclease antiserum (8). Peripheral wells contained in 50 ul: 13 ug of purified nuclease (1, 3 and 6); 150 ug of Verwey's protein B extract from UNH 570 (2); 175 ug of Verwey's protein B extract from Wood 46 (4); 150 ug of Verwey's protein B extract from Cowan 1 (5); 25 ug of Verwey's protein B extract from strain 243.
Although Verwey's protein B from strains Cowan 1 and Wood 46 do not show a visible line against antinuclease (Fig. 4), the same extracts were shown to contain nuclease activity by the enzymatic assay technique. The extracts were compared with purified exoprotein antigens against specific antisera, general heterologous rabbit antistaphylococcal sera, and bovine heterologous antistaphylococcal serum prepared by immunizing cattle with a staphylococcus cell-toxoid vaccine. Examination of the precipitin lines (Fig. 2) indicates a strong line of identity exists between Jensen's antigen A and Verwey's protein B extracts from Cowan 1 which is completely lacking in the Verwey's protein B extract from Wood 46. The two other components in Jensen's antigen A extract are present in both Wood 46 and Cowan 1, and may be identical to teichoic acid and beta hemolysin respectively (Figures 1 and 2).

Verwey's protein B extract from strain 243 yielded a precipitin line that showed a partial fusion with purified enterotoxin B when tested against antienterotoxin B antiserum (Fig. 5). None of the other strains examined showed this reaction with the enterotoxin specific antiserum.

All attempts to identify the fifth component in these Verwey's protein extracts were unsuccessful, using as references purified exoprotein antigens such as alpha
Fig. 5. Immunodiffusion of Verwey's protein B extract from strain 243 and purified enterotoxin B against rabbit antienterotoxin B antiserum. Wells contained in 50 ul: antienterotoxin B antiserum (anti E); 70 ug left of E and 35 ug right of E Verwey's protein B extract from strain 243 (243); 5 ug purified enterotoxin B (E).
hemolysin, coagulase, and enterotoxin A against both specific and general antiserum; and anti-leucocidin sera with specific anti-F and anti-S antibodies did not react with any of the extracts, indicating absence of leucocidin in these preparations.

However, the presence of protein A in cell wall extracts as a major antigenic component was evident in cell wall protein studies as can be seen in Fig. 6.

Studies with Cell Wall Proteins: Amino Acid Analysis and Polyacrylamide Gel Electrophoresis

Isolated cell walls were prepared from all six strains of \textit{S. aureus} under investigation and stored lyophilized in a desiccator. Nucleic acid content of the walls was reduced with RNase and DNase before they were used for protein studies. Hydrolysis of such clean walls with 6N HCl at 110 C for 24 hours and subsequent amino acid analysis gave a typical spectrum of protein amino acids except for low cystine content, whereas after treatment of the same walls with three proteolytic enzymes (trypsin, chymotrypsin, and pronase), prior to acid hydrolysis they showed only the amino acids normally attributed to the peptidoglycan structure. Tables 1 and 2 show data for amino acid analyses of two strains (UNH 570 and UNH 10). Both nuclease treated
Fig. 6. Immunodiffusion plates showing distribution of cell wall proteins in Verwey's protein B extracts from two strains of *S. aureus*.

Top plate. Center well contained in 50 ul, 175 ug of protein B from Wood 46 (7). Peripheral wells contained in 50 ul: antinuclease antiserum (1); 13 ug nuclease (2); anti beta hemolysin antiserum (3); 5 ug beta hemolysin (4); normal human serum (5); 25 ug partially purified Jensen's antigen A from Cowan 1 (6).

Bottom plate. Center well contained in 50 ul, 150 ug protein B from Cowan 1 (7). Peripheral wells contained in 50 ul: antinuclease antiserum (1); 13 ug nuclease (2); anti beta hemolysin antiserum (3); 5 ug beta hemolysin (4); normal human serum (5); 25 ug partially purified Jensen's antigen A from Cowan 1 (6).
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<th>Amino acid</th>
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<th>DP&lt;sup&gt;a&lt;/sup&gt; Walls (u moles/0.5 mg sample)</th>
<th>Residue Ratios&lt;sup&gt;b&lt;/sup&gt;</th>
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</tr>
<tr>
<td>Tyrosine</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Deproteinized cell walls (see text).

<sup>b</sup> Residue ratios calculated on the basis of one residue of glutamic acid per peptidoglycan tetrapeptide in the deproteinized cell walls.
Table 2

Amino acid analysis of clean walls and deproteinized walls obtained from *Staphylococcus aureus* strain UNH 10.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Clean Walls&lt;sup&gt;a&lt;/sup&gt; (u moles/0.5 mg sample)</th>
<th>DP Walls&lt;sup&gt;b&lt;/sup&gt; (u moles/0.5 mg sample)</th>
<th>Residue Ratios&lt;sup&gt;c&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lysine</td>
<td>0.089</td>
<td>0.022</td>
<td>1</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.012</td>
<td>0.001</td>
<td></td>
</tr>
<tr>
<td>Arginine</td>
<td>0.018</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Aspartic Acid</td>
<td>0.064</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Threonine</td>
<td>0.036</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Serine</td>
<td>0.045</td>
<td>0.007</td>
<td></td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.121</td>
<td>0.021</td>
<td>1</td>
</tr>
<tr>
<td>Proline</td>
<td>0.111</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Glycine</td>
<td>0.335</td>
<td>0.094</td>
<td>4.5</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.180</td>
<td>0.044</td>
<td>2</td>
</tr>
<tr>
<td>Half Cystine</td>
<td>0.000</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Valine</td>
<td>0.050</td>
<td>0.003</td>
<td></td>
</tr>
<tr>
<td>Methionine</td>
<td>0.006</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.056</td>
<td>0.008</td>
<td></td>
</tr>
<tr>
<td>Leucine</td>
<td>0.067</td>
<td>0.005</td>
<td></td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.017</td>
<td>0.000</td>
<td></td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.080</td>
<td>0.006</td>
<td></td>
</tr>
</tbody>
</table>

<sup>a</sup> Cell walls treated with DNase and RNase (see text).

<sup>b</sup> Deproteinized cell walls (see text).

<sup>c</sup> Residue ratios calculated on the basis of residue of glutamic acid per peptidoglycan tetrapeptide in the deproteinized cell walls.
walls, designated as "clean walls", and the proteolytic enzyme treated cell walls, designated as "deproteinized walls" (DP) walls are represented in these tables. The data for DP walls show amino acid residue ratios similar to those published for *S. aureus* (Mandelstram and Strominger, 1961) which indicates that the preparations showed typical peptidoglycan amino acids, essentially free of proteins amino acids.

Methods used for isolating crude cell walls are quite well-developed but those employed for obtaining proteins from such undigested cell walls are varied and still experimental. Thus, three approaches were attempted in this investigation and the proteins so obtained compared. One method used for obtaining cell wall proteins free of particulate cell walls employed extraction of the clean cell walls with phenol-acetic acid-water (2:1:0.5, w/v/v). Although this procedure was effective in obtaining some proteins in soluble form as was indicated by amino acid analysis data on the phenol-acetic acid-water soluble (PA-S) fraction, it was shown (Table 3) that small amounts of protein still remained associated with the phenol-acetic acid-water residue (PA-R) after this treatment which was confirmed by amino acid analysis performed on the PA-R. The data giving the amino acid contents (μ moles) were compared between clean walls, DP walls and residue (PA-R) walls as indicated in the table. Thus,
A comparison of amino acid analysis and calculated amino N of clean walls, deproteinized walls and PA-R walls from *Staphylococcus aureus* strain UNH 570.

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Clean Walls&lt;sup&gt;b&lt;/sup&gt;</th>
<th>Clean walls&lt;sup&gt;a&lt;/sup&gt;</th>
<th>DP Walls&lt;sup&gt;c&lt;/sup&gt;</th>
<th>DP walls&lt;sup&gt;a&lt;/sup&gt;</th>
<th>PA-R Walls&lt;sup&gt;d&lt;/sup&gt;</th>
<th>PA-R walls&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>u moles amino acid</td>
<td>u moles amino N&lt;sup&gt;a&lt;/sup&gt;</td>
<td>u moles amino acid</td>
<td>u moles amino N&lt;sup&gt;a&lt;/sup&gt;</td>
<td>u moles amino acid</td>
<td>u moles amino N&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>Lysine</td>
<td>0.154</td>
<td>0.308</td>
<td>0.062</td>
<td>0.124</td>
<td>0.099</td>
<td>0.198</td>
</tr>
<tr>
<td>Histidine</td>
<td>0.027</td>
<td>0.027</td>
<td>0.000</td>
<td>0.000</td>
<td>0.008</td>
<td>0.008</td>
</tr>
<tr>
<td>Arginine</td>
<td>0.053</td>
<td>0.106</td>
<td>0.000</td>
<td>0.000</td>
<td>0.014</td>
<td>0.028</td>
</tr>
<tr>
<td>Aspartic acid</td>
<td>0.175</td>
<td>0.175</td>
<td>0.001</td>
<td>0.001</td>
<td>0.059</td>
<td>0.059</td>
</tr>
<tr>
<td>Threonine</td>
<td>0.077</td>
<td>0.077</td>
<td>0.001</td>
<td>0.001</td>
<td>0.024</td>
<td>0.024</td>
</tr>
<tr>
<td>Serine</td>
<td>0.074</td>
<td>0.074</td>
<td>0.003</td>
<td>0.003</td>
<td>0.026</td>
<td>0.026</td>
</tr>
<tr>
<td>Glutamic acid</td>
<td>0.273</td>
<td>0.273</td>
<td>0.065</td>
<td>0.065</td>
<td>0.128</td>
<td>0.128</td>
</tr>
<tr>
<td>Proline</td>
<td>0.043</td>
<td>0.043</td>
<td>0.000</td>
<td>0.000</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Glycine</td>
<td>0.631</td>
<td>0.631</td>
<td>0.310</td>
<td>0.310</td>
<td>0.401</td>
<td>0.401</td>
</tr>
<tr>
<td>Alanine</td>
<td>0.350</td>
<td>0.350</td>
<td>0.148</td>
<td>0.148</td>
<td>0.212</td>
<td>0.212</td>
</tr>
<tr>
<td>Half Cystine</td>
<td>0.007</td>
<td>0.007</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
<td>0.000</td>
</tr>
<tr>
<td>Valine</td>
<td>0.089</td>
<td>0.089</td>
<td>0.000</td>
<td>0.000</td>
<td>0.026</td>
<td>0.026</td>
</tr>
<tr>
<td>Methionine</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Isoleucine</td>
<td>0.079</td>
<td>0.079</td>
<td>0.002</td>
<td>0.002</td>
<td>0.025</td>
<td>0.025</td>
</tr>
<tr>
<td>Leucine</td>
<td>0.101</td>
<td>0.101</td>
<td>0.004</td>
<td>0.004</td>
<td>0.031</td>
<td>0.031</td>
</tr>
<tr>
<td>Tyrosine</td>
<td>0.052</td>
<td>0.052</td>
<td>0.000</td>
<td>0.000</td>
<td>0.001</td>
<td>0.001</td>
</tr>
<tr>
<td>Phenylalanine</td>
<td>0.049</td>
<td>0.049</td>
<td>0.000</td>
<td>0.000</td>
<td>0.001</td>
<td>0.001</td>
</tr>
</tbody>
</table>

a  Amino nitrogen calculated on basis of u moles/0.5 mg sample of a given amino acid multiplied by the number of amino groups of the amino acid.
b  Cell walls treated with DNase and RNase (see text).
c  Deproteinized cell walls (see text).
d  Phenol-acetic acid-water insoluble residue of cell walls (see text).
calculations of amino-nitrogen as indicator of protein left after extraction with phenol-acetic acid-water based on the data of Table 3 showed that after subtraction of the amino-nitrogen contributed by the pure peptidoglycan metrix amino acids (see equation 1) the residual protein in PA-R (see equation 2), accounted for at least 28% of the cell wall protein, which was not extracted by this treatment.

1. Total wall protein amino-nitrogen = (amino N of clean walls) - (amino N of DP walls)

2. % wall protein residue in PA-R = \(\left(\frac{\text{amino N of PA-R}}{\text{amino N of DP walls}}\right) \times 100\) (100)

\(\frac{(\text{Total wall protein amino N})^{-1}}{100}\)

The figure obtained from equation 2 suggested that this portion of wall protein may be linked to the peptidoglycan by a more than hydrogen bonding since these agents, which break hydrogen bonds, were not effective in removing this portion. However, it seemed evident from the results that the phenol-acetic acid-water solution protein (PA-S) was about 70% of the total cell wall protein. This fraction was utilized in the polyacrylamide gel electrophoresis studies designed to compare protein bands of the product obtained by the three methods employed.

The two other methods used for obtaining cell wall proteins were the classical Verwey's protein B extraction
procedure and a method involving solubilization of clean walls by a staphyloolytic enzyme (lysostaphin), whose product was designated lysostaphin soluble (LnS) wall proteins.

The results of polyacrylamide gel electrophoresis of the PA-S and LnS wall proteins from strain UNH 570, all run in gels containing 7.5% polyacrylamide, 5M-urea and 35% acetic acid, showed differing protein band patterns. As can be seen on Fig. 7, LnS tubes on the right show 8 bands which differed slightly from each other when stacking gel was used (extreme right) as compared to when no stacking gel was used. The band in the front presumably is lysostaphin itself, since it was uniquely found in LnS wall protein samples. On the same plate are two tubes displaying protein band patterns for PA-S protein (left hand side of the plate) also run in similar gels. These gels show 14 bands, with a substantial amount of amido black staining material remaining at the origin (top). This material may be a protein-complex that was not disso­ciated by the phenol-acetic acid-water treatment. No such material was evident on gels with LnS, in particular where a stacking gel was incorporated.

A comparison between Verwey's protein B extracts from 3 strains revealed band patterns strikingly different as can be seen on Fig. 8. Cowan 1 (tube on the right) showed 10 bands with two extremely heavy bands, one in the leading
Fig. 7. Polyacrylamide gel electrophoresis of cell wall proteins. Right side; 2 tubes containing protein obtained by lysostaphin treatment of cell walls (LnS), with tube far right run in gel which had stacking gel incorporated on top. Left side; 2 tubes containing proteins obtained by phenol-acetic acid-water treatment (PAS) of clean walls. Tube on far left loaded with estimated 130 ug PAS protein and the tube next to it loaded with similar amounts but gel run on a different day. Gels containing 7.5% polyacrylamide, 5 M urea and 35% acetic acid were run in 10% acetic acid for 2 hours at a constant current of 5 ma per tube.
Fig. 8. Polyacrylamide gel electrophoresis showing protein band patterns for Verwey's protein B extracts obtained from 3 strains. Right tube, extract from Cowan 1 275 ug; center tube, extract from Wood 46 140 ug; and left tube, extract from UNH 570 190 ug. Gels containing 7.5% polyacrylamide in 5 M urea were run in 0.1 M tris-boric acid-EDTA-buffer, pH 8.9 for 2 hours at a constant current of 5 ma per tube.
edge and the other about the middle of the gel. Wood 46 (center tube) showed 8 bands, two heavy ones distributed such that one was in the leading front while the other seemed to be trailing at the back very close to the origin. UNH 570 (tube on the left) had 8 bands also, with only one heavy band in the leading front. All extracts were run under the same conditions, at the same time; thus any variations in band pattern can be attributed to differences in the protein present in the extracts. The intensity differences of the bands between tubes reflects the differing amounts of protein loaded on the gels which were for Cowan 1, 275 ug; for Wood 46, 140 ug and for UNH 570, 190 ug as estimated by Lowry protein determinations. But, it can be noted that the bands relatively correspond to each other in all the gels except for 2 or 3 bands situated about two-thirds down the gels which are lacking in Wood 46 preparation (center tube). It is also evident that within each tube there are marked differences in the intensity of the bands which reflects varying amounts and/or types of individual proteins that are responsible for the band patterns. For example, in Cowan 1 (right tube), at least 5 bands stain heavily while the other 5 bands are stained relatively lightly. Since the band patterns revealed in these gels may represent the maximum number or types of proteins in the preparation, it was not surprising
that the same preparations had been shown by immunodiffusion experiments to contain at least 5 precipitin lines against rabbit antistaphylococcal antiserum. Thus, some of these bands may reflect more than one form of immunologically similar protein.

**Demonstrations of Cell-Located Exoprotein Antigens**

**Fluorescent Antibody Technique.** The fluorescent antibody direct method was employed with general immune rabbit antisera against *S. aureus*, specific antinuclease antiserum and normal rabbit serum all conjugated with fluorescein isothiocyanate (FITC) at a suitable fluorescein:protein (F:P) ratio. All sera showed a general non-specific fluorescence with several washed *S. aureus* strains. This non-specific absorption of the serum globulins by the cells was indicative of the presence of protein A in the majority of the strains of *S. aureus*. This protein A was subsequently confirmed to be present on strains investigated, as a cell wall component except for the Wood 46 strain. Jensen's antigen A was recovered from supernatants of two strains only in trace amounts. Also it was shown that the non-specific reaction between *S. aureus* cells and the conjugated γ-globulin fraction from the sera was not evident when *Escherichia coli* and *Bacillus licheniformis* cells were substituted and examined after incubation with the same conjugated sera.
Attempts to eliminate the non-specific reaction between the sera and S. aureus cells by treatment of the cells with 5% HCHO at various temperatures and times helped reduce the non-specific fluorescence only to a limited degree. However, such treatment also reduced any specific reaction to an extent that most of the fluorescence was eliminated. Consequently, the FA technique as a means of cellular localization of antigenic sites that may be present on the cell was deemed unsuitable for this investigation.

The indirect method was also found to be of little help since the commercial preparation of FITC-conjugated antiserum seemed to possess poor F:P ratios for maximal brightness. This handicap, coupled with the problem of non-specific reaction encountered in the direct method, led to the abandoning of the indirect method, too.

Enzymatic Demonstration of Nuclease Associated with Cells

A survey to demonstrate the distribution of nuclease activity in cultures of S. aureus was conducted with the hope of producing evidence for the amounts, and sub-cellular location, of the enzyme that occurred cell bound. Such figures could be compared with the portion of the enzyme secreted in the medium to elucidate its distribution at a particular growth phase of the organism. Stationary cultures were
studied for this experiment. The results presented in Table 4 showed a very interesting feature. It can be seen that, except for two strains (Wood 46 and UNH 10), 25-70% of the activity of the enzyme detected in whole cultures, unboiled, could not be accounted for as extracellular enzyme in the supernatant. Yet it cannot be explained from these results why very little or none of the enzyme activity was associated with whole cells or cell lysates obtained by either enzymatic lysis or mechanical lysis of the cells using a Nossal disintegrator. Also pooled cell washings did not contain significant amounts of the enzyme activity.

Preliminary studies with crude supernatant nuclease and what effect sub-cellular components might have on the expression of the enzyme activity when mixed and incubated with the enzyme before assay suggested that certain cell components, especially the cell wall and cell membranes, were inhibitory to the expression of the enzyme's activity. Further studies were initiated using purified nuclease and purified cell wall preparations. It was established that DP cell wall preparations were more inhibitory than cell walls only cleaned with DNase and RNase. Results shown in Table 5 give typical inhibition patterns obtained with cell wall preparations from the six strains investigated.
## Table 4

A survey of nuclease production in strains of *S. aureus* harvested in the stationary phase of growth

<table>
<thead>
<tr>
<th>Sample description</th>
<th>UNH 570</th>
<th>Wood 46</th>
<th>UNH 15</th>
<th>243</th>
<th>UNH 10</th>
<th>Cowan 1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Culture unboiled</td>
<td>800</td>
<td>1360</td>
<td>3080</td>
<td>1880</td>
<td>1160</td>
<td>204</td>
</tr>
<tr>
<td>Culture boiled</td>
<td>400</td>
<td>472</td>
<td>1280</td>
<td>1160</td>
<td>560</td>
<td>72</td>
</tr>
<tr>
<td>Supernatant unboiled</td>
<td>612</td>
<td>1312</td>
<td>1800</td>
<td>1380</td>
<td>1152</td>
<td>64</td>
</tr>
<tr>
<td>Supernatant boiled</td>
<td>429</td>
<td>520</td>
<td>1000</td>
<td>1280</td>
<td>560</td>
<td>30</td>
</tr>
<tr>
<td>Cells unboiled</td>
<td>0.31</td>
<td>0.56</td>
<td>5.44</td>
<td>1.32</td>
<td>0.60</td>
<td>0.24</td>
</tr>
<tr>
<td>Cells boiled</td>
<td>0.32</td>
<td>0.40</td>
<td>0.30</td>
<td>1.30</td>
<td>1.40</td>
<td>0.20</td>
</tr>
<tr>
<td>Cell Lysate unboiled</td>
<td>0</td>
<td>0</td>
<td>0.04</td>
<td>0</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Cell Lysate boiled</td>
<td>0</td>
<td>0</td>
<td>0.02</td>
<td>0</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Mechanically disrupted cells unboiled</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>NA</td>
<td>0</td>
</tr>
<tr>
<td>Mechanically disrupted cells boiled</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>NA</td>
<td>0</td>
</tr>
</tbody>
</table>

a  See material and methods for definition of one unit of the enzyme  
b  Not assayed  
c  Enzymatic lysis of cells with a staphylolytic enzyme.
Table 5

Inhibitory effect of cell wall preparations on the expression of nuclease activity when mixed and incubated with the enzyme before assay.

<table>
<thead>
<tr>
<th>Staphylococcus aureus strains</th>
<th>Preparations of nuclease activity recovered&lt;sup&gt;a&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clean Walls&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Wood 46</td>
<td>10.4</td>
</tr>
<tr>
<td>UNH 570</td>
<td>27.3</td>
</tr>
<tr>
<td>UNH 10</td>
<td>35.0</td>
</tr>
<tr>
<td>UNH 15</td>
<td>35.0</td>
</tr>
<tr>
<td>243</td>
<td>9.0</td>
</tr>
<tr>
<td>Cowan 1</td>
<td>32.7</td>
</tr>
</tbody>
</table>

<sup>a</sup> Based on the enzyme activity measured after the enzyme solution was mixed with an equal volume of the diluent used to suspend the walls and incubated overnight at 5 C, together with the wall containing samples, as 100%.

<sup>b</sup> Enzyme solution incubated with an equal volume of cell wall suspensions (1 mg/ml).

<sup>c</sup> Deproteinized cell walls.
It was apparent that either the peptidoglycan was binding the nuclease in a way that made it not accessible to the substrate or the peptidoglycan was removing the co-factor necessary for the maximum expression of the enzyme activity, thus interfering with the assay system. The latter assumption was most likely because the enzyme requires Ca^{++} in the assay system and also in consideration of the reports by Cutinelli, et al. (1967, 1969) that the peptidoglycan may act as an ion exchange matrix to bind cations. The results of the experiment designed to prove this assumption are presented in Table 6. They demonstrated that saturation of sites on the peptidoglycan for binding Ca^{++} by incubating the cell wall preparations with CaCl_{2} before incubating with nuclease, made the walls less inhibitory to the enzyme than when the walls were not incubated with CaCl_{2}.

Furthermore, it was shown that the inhibitory ability of the cell wall preparations improved as the walls were enzymatically reduced to the basic peptidoglycan structure. It was found (Fig. 9) that the binding capacity was dependent on the concentration of the cell wall material added. The graph was not linear but indicated more calcium binding power at concentrations less than 200 ug of cell walls per ml.
Table 6

Effect of CaCl$_2$ incubation on cell wall inhibition capacity for nuclease activity.

<table>
<thead>
<tr>
<th>Strains</th>
<th>Percentages of enzyme activity recovered$^a$</th>
<th>Cell walls treated with CaCl$_2$$^b$</th>
<th>Cell walls treated with CaCl$_2$</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Clean walls     DP walls$^c$</td>
<td>Clean walls     DP walls</td>
<td></td>
</tr>
<tr>
<td>UNH 570</td>
<td>110            87.8</td>
<td>32.5            7.3</td>
<td></td>
</tr>
<tr>
<td>UNH 10</td>
<td>90             82.5</td>
<td>37.5            10.0</td>
<td></td>
</tr>
</tbody>
</table>

$^a$ Based on the enzyme activity measured after the enzyme solution was mixed with an equal volume of the diluent used to suspend the walls and incubated overnight at 5°C, together with the wall containing samples, as 100%.

$^b$ Cell wall preparations stripped of teichoic acid were incubated overnight with 0.1 M CaCl$_2$ to saturate Ca$^{++}$ binding sites before incubation with purified nuclease solutions followed by the enzyme assay.

$^c$ Deproteinized cell walls.
Fig. 9. Inhibition of nuclease activity by increasing concentration of cell wall after incubation for 5 hours in ice water bath. Symbols: - DP walls; clean walls. —
Effects of Cell Wall on Purified Hemolysins

The observation that the expression of nuclease activity was inhibited by the cell walls because of their capacity to bind cations made it worthwhile to examine what effects these cell wall preparations might have on the purified hemolysins. If the mechanism of inhibition was by binding cations, the expression of hemolysis by beta hemolysin, which is known to require Mg$^{++}$ for its activity on red blood cells, would be inhibited whereas alpha hemolysin which has no such requirements would not be affected.

The results obtained with the experiment confirmed this mechanism and showed that beta hemolysin was inhibited more than 80% in the expression of hemolytic activity on sheep erythrocytes while alpha hemolysin under similar conditions, but using rabbit erythrocytes instead, was not affected in its expression of hemolytic activity. It was shown that when beta hemolysin, with a titer of 6400 hemolytic units (HU) was mixed with an equal volume of cell walls, incubated prior to assay it gave a titer of only 800 HU. On the contrary, alpha hemolysin of 1280 HU treated in identical conditions, 640 HU were recovered which was the expected titer. Thus, these experiments supported earlier experiments with nuclease in which the cell walls inhibited its activity by binding Ca$^{++}$. It was then concluded that, in
the case of beta hemolysin, Mg\(^{++}\) necessary for its activity was removed.

**Analysis of Protoplast Membranes and Anti-Membrane Sera**

Protoplasts were enzymatically prepared by exposing washed cells of strain UNH 570 to lysostaphin. They were lysed to obtain protoplast membranes which accounted for about 9% of the dry weight of the cocci. The membranes were chemically analyzed and the results are given in Table 7. These analyses compared favorably to those recently published by Ward and Perkins (1968) for the protoplast and L-form membranes from 2 strains of *S. aureus* and L-form derived from same strains. Ward and Perkins results are shown in Table 7 for comparison. It can be seen that higher values were obtained with UNH 570 for carbohydrate and phosphorus contents than those quoted from these authors. The values for RNA appear low because, unlike these authors who treated their preparations with DNase only, the membranes reported here were treated with both RNase and DNase. The values for hexosamines were included in the analysis as a check for cross contamination of the membranes by cell walls. The percentages for both protein and lipid contents of the membrane were very similar to those quoted.
Table 7

Chemical analysis of staphylococcal protoplast membranes expressed as % of dry weight of the membrane

<table>
<thead>
<tr>
<th>Strains and Batch #</th>
<th>Protein</th>
<th>Lipid</th>
<th>Carbohydrate</th>
<th>Hexosamine</th>
<th>Phosphorus</th>
<th>RNA</th>
<th>DNA</th>
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<tr>
<td><strong>S. aureus</strong></td>
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<td></td>
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<tr>
<td>UNH 570</td>
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<tr>
<td>Batch #1</td>
<td>62.5</td>
<td>24.6</td>
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<td>0.94</td>
<td>1.00</td>
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<tr>
<td>Results adopted from Ward and Perkins (1968)</td>
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<tr>
<td><strong>S. aureus 100</strong></td>
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<td></td>
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</tr>
<tr>
<td>Protoplast</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Batch #1</td>
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<td>Protoplasts</td>
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<td>1.14</td>
<td>4.47</td>
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<tr>
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<td>1.08</td>
<td>4.78</td>
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<td>1.18</td>
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<td><strong>L-Form HL</strong></td>
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</tr>
<tr>
<td>Batch #1</td>
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<tr>
<td>3</td>
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<td>3.13</td>
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</table>
The membranes analyzed above were used to immunize 4 rabbits and the sera separated from the blood collected from the animals six weeks after initial immunization. These sera were examined for antibodies against purified exoprotein antigens. All animals showed antibodies against purified alpha toxin and coagulase and serum from one rabbit (Fig. 9) showed a precipitin line against nuclease by the Ouchterlony immuno-diffusion technique. The results can be seen on Figures 10, 11, 12 and 13. The sera from 3 out of 4 animals showed an antinuclease response when examined by the Preer technique.

An attempt to quantitate the amount of alpha toxin in the membranes was made using the Preer technique and rabbit specific anti-alpha antiserum calibrated with known amounts of purified alpha toxin. With the Preer technique, the P-value obtained when a known amount of alpha toxin (0.36 ug in 20 ul) was reacted with a fixed antiserum dilution did not significantly differ from the P-value of same antiserum dilution absorbed with 0.055 mg membrane proteins prior to reacting with the 0.36 ug of purified alpha toxin which was equivalent to the alpha toxin reacted with the unabsorbed antiserum. Table 8 gives the results of two experimental series comparing the P-value obtained between control antiserum dilutions and a constant amount of alpha toxin antigen with the membrane-absorbed antiserum reacted with the same
Fig. 10. Immunodiffusion of rabbit anti-membrane antiserum against purified exoprotein antigens. Center well contained 50 ul of rabbit A antiserum (7). Peripheral wells contained in 50 ul: 3.6 ug alpha toxin (1); 5 ug beta toxin (2); 5.6 ug coagulase (3); 13 ug nuclease (4); 5 ug enterotoxin B (5); 25 ug partially purified Jensen's antigen A extract from Cowan 1 (6).
Fig. 11. Immunodiffusion of rabbit anti-membrane antiserum against purified exoprotein antigens. Center well contained 50 ul of rabbit B antiserum (7). Peripheral wells contained in 50 ul: 3.6 ug alpha toxin (1); 5 ug beta toxin (2); 5.6 ug coagulase (3); 13 ug nuclease (4); 5 ug enterotoxin B (5); 25 ug partially purified Jensen's antigen A extract from Cowan 1 (6).
Fig. 12. Immunodiffusion of rabbit anti-membrane antiserum against purified exoprotein antigens. Center well contained 50 ul of rabbit C antiserum (7). Peripheral wells contained in 50 ul: 3.6 ug alpha toxin (1); 5 ug beta toxin (2); 5.6 ug coagulase (3); 13 ug nuclease (4); 5 ug enterotoxin B (5); 25 ug partially purified Jensen's antigen A extract from Cowan 1 (6).
Fig. 13. Immunodiffusion of rabbit anti-membrane antiserum against purified exoprotein antigens. Center well contained 50 μl of rabbit D antiserum (7). Peripheral wells contained in 50 μl: 3.6 μg alpha toxin (1); 5 μg beta toxin (2); 5.6 μg coagulase (3); 13 μg nuclease (4); 5 μg enterotoxin B (5); 25 μg partially purified Jensen's antigen A extract from Cowan 1 (6).
Table 8

P-value obtained for dilutions of antialpha antiserum and membrane-absorbed antialpha antiserum reacted with 0.36 ug alpha toxin^a^.

<table>
<thead>
<tr>
<th>Antiserum dilutions</th>
<th>P-value</th>
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<tr>
<td></td>
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<td>Series 1</td>
<td>Series 2</td>
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<tr>
<td>Undilute</td>
<td>0.280</td>
<td>0.316</td>
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<tr>
<td>1:2</td>
<td>0.357</td>
<td>0.457</td>
<td></td>
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<tr>
<td>1:4</td>
<td>0.477</td>
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<td></td>
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<tr>
<td>1:8</td>
<td>0.654</td>
<td>0.639</td>
<td></td>
</tr>
<tr>
<td>1:4 absorbed^b</td>
<td>0.534</td>
<td>0.462</td>
<td></td>
</tr>
</tbody>
</table>

^a^ Amount of alpha toxin shown to form a non-migrating band of precipitin with 1:4 dilution of the antiserum.

^b^ One tenth ml of antialpha antiserum diluted 1:2 was absorbed with an equal volume of membrane suspension containing 0.055 mg of protein, prior to introducing 20 ul of the mixture into Preer tubes to react with 0.36 ug alpha toxin as in all other dilutions. Thus, just by dilution, the antiserum was brought to 1:4 in strength and the P-values should be compared to those of control 1:4 dilution.
amount of alpha toxin. Figures obtained for the 1:4 dilutions of antiserum giving the P-values in both series of experiments and for the absorbed antiserum were similar within the observed experimental error in each set of readings. The calculated standard deviations for this set of readings was 0.025 P-units, thus any significant shift in P-value had to be greater than twice the standard deviation (0.050 P-units). This was not the case and therefore the shift in P-value was considered not sufficient to permit the quantitation of alpha toxin in the membranes. It had also been observed that at least 0.18 ug of the purified alpha would give a band when reacted with a 1:4 dilution of the antiserum. Thus at least an amount of alpha toxin equivalent to 0.18 ug of protein or 0.33% of the membrane used would have been adequate to neutralize the antiserum or shift the P-value in the experiment.

The toxin neutralization method was employed to compare the anti-alpha toxin activity of the antimembrane sera to that of standard antitoxin serum. Alpha toxin (1.825 ug), which was just adequate to neutralize one international unit (IU), was titered against the anti-membrane sera and it was demonstrated that all the animals generated antibodies equivalent to 1-4 IU of anti-alpha antibody. It was evident that although the animals received equal amounts of
membrane protein for immunization, their response in terms of antibodies generated was varied. Thus some animals had fairly higher levels of anti-alpha and anti-nuclease antibodies than others.

An absorption technique employing standard international antitoxin serum and membrane preparation as a means of quantitating the amount of alpha toxin in the membrane by checking for inhibition of the hemolysis end point of a known amount of alpha toxin (1.825 ug) which was just adequate to neutralize 1 unit (International Unit) of the antiserum. The differences between the absorbed antiserum and the control antiserum both reacted with 1.825 ug of purified alpha toxin was 2 hemolytic units which suggested that alpha toxin in the membranes contributed this difference. Calculations based on approximately 20 hemolytic units per ug of purified alpha toxin (Bernheimer (1965), our own alpha toxin had lost 90% of its activity) indicated that alpha toxin in the membrane accounted for approximately 0.5% of the total membrane protein used for absorption. This figure was derived on the assumption that the alpha toxin in the membrane was serologically active and that its specific activity was similar to the alpha toxin used as a standard. Observations made when the membranes were examined for hemolysin on washed 5% rabbit blood agar plates that the antigen was hemolytically
inactive, tend to support the argument that the alpha toxin in the membrane was serologically masked. Thus, the results obtained with neutralization experiment could not be expected to give an accurate estimation of alpha toxin content in the membranes. Furthermore, semi-quantitation of alpha toxin in the membranes was complicated by the particulate nature of the membranes which could explain lack of hemolysis on blood agar plates.

Attempts to quantitate both coagulase and nuclease in the membranes were not made with the absorption technique. The Preer technique was deemed not suitable for the nuclease-antinuclease reaction because of the broad type of precipitin band formed by this reaction. Such precipitin lines would be difficult to measure with the accuracy necessary for the calculation of P-values. The lack of specific antiserum for coagulase ruled out any attempts of quantitation of amounts of this component present in the membranes.

**Polyacrylamide Gel Electrophoresis of Membrane Proteins**

The presence of more than one antigen in the membranes which was evident in studies with anti-membrane antiserum seemed to suggest a number of protein species in the membranes that might be separated into several bands using the disc polyacrylamide gel electrophoresis technique. The
problem of membrane solubilization was solved by the use of phenol-acetic acid-water (2:1:0.5) and 1% sodium dodecyl sulphate (SDS). When 7.5% polyacrylamide gel containing 5 M urea and 35% acetic acid was used to separate the membrane proteins dissolved in phenol-acetic acid-water, consistently, a pattern of 13-15 bands of proteins was demonstrated. Some portion of the material staining with amido black was observed to remain at the origin indicating that as much as 50% of the membrane proteins applied on the gel did not penetrate it. Typical patterns of these membrane proteins in gel run in 10% acetic acid for 2 hours, are shown on Figure 14 (right hand side). On the same figure (left hand side), the same membrane proteins solubilized with SDS and run in 0.1 M tris-EDTA-boric acid buffer, pH 8.9 are shown. Only 4 bands of proteins, with one of them rather diffused, are evident. When the polyacrylamide gel concentration was increased to 15%, it did not improve the problem of diffusion nor did it increase the number of bands detected in the SDS preparation. However, it was noted that no material staining with amido black remained at the origin similar to that shown for membranes solubilized in phenol-acetic acid-water.

Since it looked as if phenol-acetic acid-water solubilization of membrane proteins gave the maximum number of
Fig. 14. Polyacrylamide gel electrophoresis of membrane protein band patterns. Right hand tube shows membrane preparation (210 ug protein) solubilized in phenol-acetic acid-water (2: 1: 0.5) and run in 10% acetic acid for 2 hours. Left hand tube shows membrane preparation (130 ug protein) solubilized in sodium dodecyl sulphate (SDS). The gel was run in tris-boric acid-EDTA buffer, pH 8.9 for 2 hours.
protein bands, it was decided to attempt to identify which band or bands was responsible for the alpha toxin, coagulase and nuclease activities to which animals immunized with membranes responded to by forming antibodies. The membrane proteins separated into bands by this technique were eluted individually from gel pieces, and each eluted protein was checked for antigenic activities serologically. It was demonstrated that only the material obtained from the band at the origin of the gels gave a line of identity with alpha toxin against anti-alpha toxin serum. This reaction is presented in Figure 15 which shows a continuous precipitin line of identity between the material from the origin of the gel placed in the center well against the anti-alpha toxin serum on the peripheral well #2 and its homologous purified alpha toxin on the adjacent well #1. No lines were observed between all other fractions of proteins eluted from other gel pieces against the various specific antisera tested along with their homologous purified exoproteins serving as reference antigens. This may indicate that if these antigens were part of membrane proteins, their concentration may be less than that of alpha toxin, and therefore beyond the sensitivity of the Ouchterlony immunodiffusion technique. All antisera used in this experiment had to be treated with 1% SDS (Agarman and Razin, 1969) before reacted with membranes
Fig. 15. Immunodiffusion of membrane protein eluted from polyacrylamide gel, alpha toxin, coagulase, and nuclease against antialpha toxin antiserum, antinuclease antiserum and bovine antistaphylococcal antiserum. Center well contained 50 ul of membrane protein eluted from band at the origin of the gel. Peripheral wells contained in 50 ul: 3.6 ug alpha toxin (1); antialpha antiserum (2); 10 ug coagulase (3); bovine antistaphylococcal antiserum (4); 16 ug nuclease (5); antinuclease antiserum (6).
dissolved in SDS. Controls which contained bovine serum albumin (BSA) dissolved in SDS and checked against the various antisera did not show any precipitin lines. Whole membranes solubilized with SDS and diffused against anti-membrane sera also treated with SDS gave precipitin lines which were diffused as illustrated in Fig. 16.

Mesosome, Periplasmic Space and Cytoplasmic Antigenic Exoproteins.

The mesosomes and any periplasmic space proteins, that were contained in the supernatant fractions which were saved during protoplast formation using lysostaphin, were demonstrated by the Lowry protein determination to contain protein in an amount equivalent to about 5% of the dry weight of the cocci used. It was recognized here that this portion also contained the added lysostaphin protein but as can be seen in Table 9, the amount of added protein was only a small fraction of the total protein recovered in the mesosome fraction. Besides this, it was also expected that the cell wall proteins which were released by the action of lysostaphin were in this fraction. From the earlier results, both nuclease and beta hemolysin at the least were expected to be among the cell wall proteins.
Fig. 16. Immunodiffusion of membranes solubilized with 1% SDS and alpha toxin against rabbit anti-membrane antisera and rabbit antialpha toxin antiserum treated with 1% SDS. Center well contained 130 ug membrane protein. Peripheral wells contained in 50 ul: rabbit A antiserum (1); rabbit B antiserum (2); rabbit C antiserum (3); rabbit D antiserum (4); 3.6 ug alpha toxin (5); rabbit antialpha toxin antiserum (6).
**Table 9**

Mesosomes and Peri-plasmatic space proteins from *S. aureus* strain UNH 570

<table>
<thead>
<tr>
<th>Batch #</th>
<th>Cells dry wt. in mg</th>
<th>Added lysostaphin in mg</th>
<th>Recovered protein in mesosome fraction in mg</th>
<th>as % of dry wt. of cells</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>900</td>
<td>6</td>
<td>50.5</td>
<td>5.6</td>
</tr>
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<td>2</td>
<td>3400</td>
<td>10</td>
<td>155.8</td>
<td>4.6</td>
</tr>
</tbody>
</table>
Studies with this fraction showed that both alpha and beta hemolysins were detected with this material using 5% blood agar plates when as little as 50 µg of proteins from the fraction were placed in a well cut in blood agar. Immunodiffusion showed a very faint precipitin line similar to alpha toxin (Fig. 17) whereas no precipitin lines could be shown with other antisera against other exoprotein antigens when as much as 1 mg protein per ml were loaded in wells of Ouchterlony plates. The apparent lack of nuclease and beta-hemolysin serologically in this fraction was an unexpected surprise. However, it was noted earlier these antigens exist in small amounts in the walls of this strain UNH 570. The exposure of this fraction to high salt concentration (24% NaCl-tris chloride buffer) may also have played a part in destroying these antigens such that they could no longer participate in the antigen-antibody reactions.

The cytoplasmic material was obtained by mechanical disruption of washed cells from strain UNH 570 followed by differential centrifugation (25,000 xg for 1 hour) to separate it from the membranes. The material was concentrated by osmoadialysis to a total volume of 18 ml derived from 1 liter of culture. The non-dialyzable material had a dry weight which accounted for about 50.2% of the dry weight of the cocci used. Its protein content was only 15.5% of the
Fig. 17. Immunodiffusion of mesosome fraction compared to alpha toxin, and beta hemolysin against antialpha toxin antiserum, antibeta hemolysin antiserum and antinuclease antiserum. Center well contained 50 ug protein from the mesosome fraction (M). Peripheral wells contained in 50 ul: 3.6 ug alpha toxin (x); antialpha toxin antiserum (anti x); 5 ug beta toxin (B); antibeta hemolysin antiserum (anti B); antinuclease antiserum (anti N).
dry weight as compared to membranes obtained at the same time which had 61.7% of their dry weight as protein. As a check for the degree of contamination of the cytoplasmic fraction by membranes, both membrane and cytoplasm fractions were extracted for phospholipids and the extracted lipid was used for the determination of the phosphorus content. Membranes had as much as 2.4% of their weight as lipid phosphorus while less than 0.1% of the cytoplasm dry weight was lipid phosphorus (Table 10). These results indicated little cross-contamination of the fractions. Both figures for protein and phosphorus content of the membrane agree with those presented in Table 7 where the membranes from this same strain were obtained by the formation of protoplasts using a staphyloolytic enzyme.

This cytoplasmic fraction was serologically and by hemolysis checked for biological activities that may be similar to exoprotein antigens. It was shown that bovine heterologous anti-staphylococcal serum showed 3 precipitin lines against this material. It also showed a line against anti-alpha toxin (Fig. 18) which was not identical to either purified alpha toxin or crude delta hemolysin as judged by absorption of the anti-alpha toxin antiserum with purified alpha toxin and reacting it against cytoplasmic fraction, alpha toxin and boiled supernatant (Fig. 19). However, the
Table 10

Chemical analysis of cytoplasm and cytoplasmic membrane fractions obtained by mechanically lysed cells of *S. aureus* strain UNH 570

The results given are based on 3.5 g dry weight of cells used.

<table>
<thead>
<tr>
<th>Fraction</th>
<th>Analysis</th>
<th>Weight in mg</th>
<th>% of the fraction</th>
</tr>
</thead>
<tbody>
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<td>Cytoplasm</td>
<td>Dry weight</td>
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<td></td>
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<tr>
<td></td>
<td>Phosphorus</td>
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<td></td>
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</tr>
<tr>
<td></td>
<td>Phosphorus</td>
<td>0.715</td>
<td>2.38</td>
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<sup>a</sup> 50.2% as percentage of the dry weight of cocci after treatment with RNase and DNase and concentration with aquacide I and II.
Fig. 18. Immunodiffusion of alpha toxin cytoplasmic fraction and crude supernatant from UNH 570 against antialpha antiserum. Center well contained 50 ul of antialpha antiserum (1). Peripheral wells contained in 50 ul: 0.18 mg cytoplasmic fraction (2 and 5); 3.6 ug alpha toxin (3); 250 ug crude supernatant un-concentrated from UNH 570 (4).
Fig. 19. Immunodiffusion of purified alpha toxin, cytoplasmic fraction and boiled supernatant from strain UNH 15 against antialpha toxin antiserum absorbed with the purified alpha toxin. Wells contained in 50 ul: antialpha toxin antiserum absorbed with equal volume of purified alpha toxin (1); 0.8 mg cytoplasmic fraction (2); 3.6 ug alpha toxin (3); estimated 200 ug boiled supernatant from strain UNH 15 of source of crude delta toxin (4).
cytoplasm showed a typical alpha hemolysis on washed 5% blood agar plates made from both rabbit or sheep erythrocytes. Using the tube method of doubling dilution titer for hemolytic end point with 1% washed rabbit erythrocytes as indicator of 50% hemolysis end point, this material had about 200 hemolytic units per ml. This titer was compared to purified alpha toxin (20 hemolytic units per ug) in the same system and it was equivalent to 10 ug of alpha toxin which was less than 0.1% of the total protein recovered from the cytoplasm.

The cytoplasm showed a very faint line of precipitin by immunodiffusion plates against anti-nuclease serum which fused in identity with the purified nuclease used as reference. To confirm the presence of nuclease in this cytoplasmic fraction, an enzymatic assay was conducted. It was demonstrated that a very low level of nuclease activity was present in the cytoplasm, about 80 units per ml or approximately 1240 units for the total cytoplasm recovered. This nuclease activity in the cytoplasm was compared to about 600,000 units per liter recovered from the supernatant from the same strain under similar conditions. Thus, about 0.2% of the extracellularly secreted enzyme activity was apparent in the cytoplasmic fraction, if other enzymes in the cytoplasm capable of attacking the substrate, denatured calf thymus DNA under the assay conditions, are not considered
here. This observation indicated that both alpha hemolysin and nuclease may only be present in this fraction as contaminants from membrane since they were recovered in amounts less than 1% of the total protein in the cytoplasmic fraction.
DISCUSSION

Extraction of Cell Wall Proteins of Staphylococcus aureus.

The observation that undigested Staphylococcus aureus cell walls may contain as much as 30% of their dry weight as protein (Yoshida et al. 1963), and the evidence obtained in the present study that the walls of S. aureus 570 contained 38.5% protein, made it important to investigate the protein antigens of the cell walls and establish their relationship to the exoprotein antigens. Some studies have been done concerning the immunochemistry of the cell wall and teichoic acid (Morse, 1962). The numerous studies cited in the literature review have discussed the nature of Jensen's antigen A, or protein A, of this organism. It was shown that as much as 0.9% of the cell wall dry weight may be antigen A (Forsgren, 1968). In the present investigation it was found that, even with a strain of S. aureus, such as Wood 46, which does not demonstrably contain antigen A by the classical methods of extraction of Verwey (1940) and Jensen (1958), some protein antigens can be extracted from its undigested cell walls. And it was further established, as other authors (Löfkvist and Sjöquist, 1962, 1963; Yoshida et al. 1963) have also found, that mechanical disintegration of S. aureus cells by the modified procedure of Verwey (1940) yielded a mixture
of proteins in the extract. This protein mixture contained antigen A in all the strains investigated except for Wood 46. It was evident from the results that the concentration of protein A varied among the strains with Cowan 1 showing the highest yields.

Forsgren (1968) reported that the action of lysozyme released protein A without lysing the cells but made no mention if other proteins of the cell wall were also released by this treatment. However, this observation seemed to confirm that the protein was located outside the plasma membrane and left no doubts that it is a cell wall constituent. The present investigation also obtained evidence that treatment of isolated, purified cell wall preparations with either lysostaphin or phenol-acetic acid-water (2: 1: 0.5) released proteins which were separable into several bands by polyacrylamide gel electrophoresis. Such band patterns for three strains extracted by the Verwey's method revealed mobility and concentration differences that were thought to reflect some strain differences, although there were similarities among the three strains.

An observation made in the present investigation that the phenol-acetic acid-water treatment of clean cell walls released some proteins (PA-S) but left other proteins residual in the PA-R suggested that there may exist differences between
the ways PA-S and PA-R proteins are bonded to other cell wall constituents. One would speculate that covalent bonding may exist between PA-R proteins and the peptidoglycan rather than hydrogen-bonding which should have been attacked by the hydrogen-bond breaking agents used. Conversely, PA-S proteins may be linked to the peptidoglycan matrix by hydrogen-bonding and non-polar or London type-bonds.

**Antigenic Composition of the Cell Wall Protein Extracts Obtained by Jensen's and Verwey's Techniques.**

The protein extracts obtained by both the Jensen's and Verwey's methods of extraction have been shown to be heterogeneous both by serological and chemical criteria (Grov et al. 1964; Grov 1968 and Forsgren 1969). The task of this investigation, in part, was to obtain evidence concerning relationships that may exist between the antigenic components of these extracts and some of the better known exoprotein antigens of *S. aureus*. The results obtained showed that a maximum of three precipitin lines occurred between Jensen's extract from Cowan 1 and heterologous rabbit immune serum prepared against washed, heat killed cells of *S. aureus* (strains UNH 10 or UNH 570). It was further established that two of these precipitin lines showed reactions of identity with beta hemolysin and teichoic acid, leaving the third line
as specific for antigen A itself. Thus, it had been demonstrated that such extracts contained a known extracellular protein (beta hemolysin). Protein A can also be considered as an exoprotein since it is secreted in the medium in amounts up to 30% of that which can be extracted from the cell wall (Forsgren, 1969).

Teichoic acid was established earlier to be a structural cell wall polymer which is also released into the medium (Morse, 1962). It is yet to be elucidated how protein A or any other protein constituents of the cell wall are attached to the peptidoglycan. A knowledge of the nature of this attachment would help in understanding why the extraction procedure designed for obtaining antigen A also extracts the other two antigens, one of which (teichoic acid) has already been established to be a cell wall component.

The suggestion by James and Brewer (1968) that both teichoic acid and a protein (Jensen's protein A being a major constituent) overlying the glycopeptide were responsible for establishing the ionic structure of the cell surface of \textit{S. aureus}, may indicate that these antigens are closely linked in their attachment to the peptidoglycan.

The protein extracts made from five strains by the Verwey's method were shown to form a maximum of five precipitin lines against rabbit antistaphylococcal serum. These
precipitin lines were shown to correspond to beta hemolysin, nuclease, teichoic acid, and antigen A (antigen A was lacking in Wood 46 extracts). Strain 243 had a line of partial identity with enterotoxin B obtained from Dr. E. J. Shantz. This line was completely lacking in other strains investigated which are known to be non-enterotoxigenic. The fifth line apparent in the immunodiffusion precipitates was not identified with any of the purified exoprotein antigens that were available to us, which included alpha hemolysin, coagulase, and enterotoxin A. Antileucocidin antiserum did not react with any of the extracts, which excluded the possibility that the unidentified antigen was leucocidin.

Two observations were made with these extracts. First, that the heat extraction method of Jensen for obtaining proteins from *S. aureus* seemed to destroy some of the antigens. This may account for the fewer number of precipitin lines observed as compared to the Verwey's method. This is evident when one examines extracts obtained from the same strain (Cowan 1) by the two separate methods. Second, these extracts contained antigenic components that were serologically identical or similar to the purified exoprotein antigens which suggested a direct relationship between the exoproduct, definable as such by Pollock's classification (Pollock, 1962), and the antigens still associated with particular sub-cellular
structures. Furthermore, it was observed that there was a direct relationship between what the cells were known to secrete into the medium and what remained associated with the sub-cellular fractions. For example, strain 243, which was the only positive strain for enterotoxin B, was the only strain that contained enterotoxin B in cell wall extracts. On the other hand, strain Wood 46, which has no antigen A in its wall extracts, did not show any antigen A in the supernatant. Also, strain Wood 46, which produces only low levels of beta hemolysin in the medium, and strain Cowan 1, which secretes low levels of nuclease, were both shown to contain in their cell wall protein extracts low levels of beta hemolysin and nuclease respectively. In fact, these small amounts were detected in these strains by the more sensitive enzymatic methods rather than the serological methods used, exemplified in the nuclease assay for Wood 46 and Cowan 1 extracts which gave 260 units and 80 units per 4 mg protein extract from each of the respective strains. Thus these units of nuclease activity indicated very small amounts of the enzyme as compared to the purified enzyme which has a specific activity of 35000 units/mg. This observation seems to support the speculation that the secreted exoproteins monitored in the medium corresponded with how much of that protein was present in the cell wall.
The presence of protein A in cell wall extracts as a major antigenic component was evident in this study as can be seen in Fig. 6. It was also suggested by the non-specific binding of the FITC-conjugated γ-globulin to the washed cells of *S. aureus* that made the use of fluorescent antibody (FA) technique difficult. It became obvious here that the validity of cellular localization of antigens of *S. aureus* based on evidence built on the fluorescence due to FITC-labelled specific antiserum reaction with antigenic sites on the cell, such as was presented for enterotoxin B (Friedman and White 1965, Genigeorgis and Sadler 1966, 1969 and Stark *et al.* 1969), is difficult to support unless special efforts have been made to ensure that antigen A present on the cells does not interfere with the reaction. Furthermore, it was noted, that in most strains, the presence of protein A in excess of all other protein antigens, made the task of obtaining levels of equivalence for the antigen-antibody reaction by the immunodiffusion technique exceedingly difficult because one almost always has a great excess of protein A when trying to locate the minor constituents of the extracts. As a result, the unbalanced system that is created results in a massive precipitation of antigen and antibody that obscures other precipitin reactions. This difficulty was eliminated, of course, when specific antisera were employed. Also if it
became necessary the individual components of the extracts could be separated and purified.

One experiment designed to separate protein A and beta-hemolysin in Jensen's antigen A extracts by immunoelectrophoresis was a failure. This was somewhat interesting because it suggested that these antigens possessed similar electrophoretic mobilities under the conditions of the experiment. A study of some of the physiochemical properties of the two antigens revealed that they have similar isoelectric points and molecular weights. Protein A was estimated to have its isoelectric point between 7.4 and 8.6 (Yoshida et al., 1963) while that of beta-hemolysin is 8.6 (Chesbro, unpublished data). Protein A has molecular weight of 15,000 (Forsgren, 1968) and beta-hemolysin, from strain 243, was calculated to be 15,500 by Dr. Chesbro. These two properties, usually taken advantage of in immunoelectrophoresis separation of antigens, seemed to offer an explanation why it was difficult to achieve a separation in this experiment. This question would have been clearly answered if purified beta from Cowan 1 was available for a study on these physicochemical properties. Thus, it was not feasible to attempt separation of the large number of antigens in the Verwey's protein B extracts which migrate similarly when this technique is employed. However, disc polyacrylamide gel electrophoresis
technique, capitalizing mostly on charge of the proteins, seemed to separate the extracts into several protein bands.

**Cell Wall Inhibition of Nuclease and Beta-hemolysin Activities**

The apparent indication that all the nuclease activity of the cultures of *S. aureus* was not accounted for as being extracellular suggested that some of the enzyme was cell-bound. But evidence was obtained in this investigation indicating that the cell wall preparations had the ability to inhibit expression of both nuclease and B-hemolysin activities. This inhibition phenomenon was due to the affinity of the cell walls for cations, which served to explain why alpha-hemolysin activity was not effected. These results were an indirect confirmation of the studies of Cutinelle et al. (1967,1969) which reported the cation-binding capacity of both cell walls and cell membranes. However, this phenomenon was of great interest here because of the possible explanation it seemed to offer regarding the role of cell walls as a self protective mechanism of the cells for preventing these molecules from re-entering the cell, and possibly damaging it, once they have been released into the environment. The observation that both nuclease and beta-hemolysin exist serologically active in cell wall protein extracts ruled out the speculation that these antigens become immunologically
inactivated once bound to the cell wall. No explanation could be offered here regarding the role of cell wall on keeping alpha toxin from re-entering the cell beyond the wall barrier, in fact, it was demonstrated that most of the alpha toxin and coagulase cellurally localized in strain UNH 570 was associated with the cytoplasmic membrane, presumably membrane-bound or forming part of the membrane structure. Reports in the literature indicating that bound-coagulase exists in an antigenically different form from free coagulase (Duthie 1954, Duthie et al. 1958) were not confirmed here nor was the possible effect of cell wall preparations on coagulase activity investigated.

**Cell Membranes: Chemical Composition and Antigen Analyses**

The cell membranes prepared from strain UNH 570 revealed a chemical composition that was largely lipid and protein. The protein content was 60-62% of the membranes dry weight. Gross contamination with lysostaphin used to obtain protoplasts was not expected to contribute significantly to the protein content since only 0.5-1% of the cell's dry weight was added as the enzyme. The lipid content was about 25% of the membrane weight. The membranes resembled those of two strains of *S. aureus* reported by Ward and Perkins (1968) in respect to these two major components. The treatment of the membranes with DNase and RNase, each at a
concentration of 100 µg/ml, made it difficult to estimate how much of the RNA and DNA were constituents of the membrane as had been indicated by some investigators (Vennes and Gerhardt, 1956; Yudkin and Davis, 1965; Gross and Coles, 1968). The RNA content could not be accurately compared to the results of Ward and Perkins because they used DNase, known to be contaminated with RNase, to clean their membrane preparation. It may be noticed that the carbohydrate content of 2–3% for these membranes was about twice that reported by the quoted authors, but about equal to the content for the L-form HL derived from one of their strains. However, these percentages were considered reasonable because the nucleic acids present (about 2%) may have contributed to the carbohydrate content. The low figure for hexosamines (0.43–0.51%) suggested negligible contamination with cell wall components. Thus, these membranes were considered to be sufficiently purified for the study on immunological response in animals.

Rabbits immunized with these membranes alone were shown to contain antibodies against alpha toxin, coagulase, and nuclease, which suggested that membrane proteins contained these exoprotein antigens since the sera collected prior to immunization did not contain these antibodies. Examination of membrane proteins revealed that alpha toxin represented less than 0.5% of the total membrane proteins. This
demonstration suggested that alpha toxin was either membrane-bound or was part of the membrane proteins. This could mean that either alpha toxin molecules became bound to the membrane as they were moved from their site of synthesis to the environment, or alternatively, that their site of synthesis was located on or in the cell-membranes. However, using the same serological technique employed to demonstrate alpha toxin, coagulase and nuclease were not directly demonstrable in the membrane fraction, which argues that these components were present in very small amounts.

It was also noted that the alpha toxin detected in membrane proteins was associated with the material that failed to separate into bands and remained at the origin of the 7.5% polyacrylamide gel used. Similar material had been observed to remain in the origin of gels containing 7.5% polyacrylamide, 5 M urea and 35% acetic acid by other authors (Theodore et al. 1969). These authors thought of it as being a characteristic pattern for staphylococcal membranes solubilized in phenol-acetic acid-water. It therefore seemed to suggest that this material may be a protein-complex similar to a glycoprotein which may account for the 2-3% carbohydrates in the membrane. Purified alpha toxin was demonstrated to penetrate similar gels.
Although several bands of proteins were resolved with these membranes which may indicate presence of several individual proteins, the failure to identify any of the band or bands with the exoprotein antigens investigated may be attributed to the low sensitivity of the immunodiffusion methods used since only small amounts of the membrane protein could be applied on the gels (Macy et al. 1968), which then separated into individual bands represented very small amounts of protein.

Mesosome Fraction Proteins

Mesosome fraction, and/or periplasmatic space, proteins released during protoplast formation contained a hemolytic substance that on blood agar plates gave a reaction that was typical of alpha toxin hemolysis. The protein content of this fraction was about 5% of the dry weight of the cells. The alpha toxin associated with this fraction seemed to suggest that the extruded mesosomes were, in this respect, similar to the cytoplasmic membranes.

Coles and Gross (1967) suggestion that proteins, such as exopenicillinase from S. aureus, may be formed outside the permeability barrier of the plasma membrane seems less likely to be the case in an explanation for the presence of alpha toxin, nuclease and coagulase found in the membrane
fraction because traces of alpha toxin, nuclease and coagu-
lace were detected in the cytoplasmic fraction. But the
presence of alpha toxin in the mesosomal fraction may support
the concept that mesosomes are the structures involved in the
formation and externalization of exoprotein (Lampen, 1965).

**Cytoplasmic Protein Antigens**

The overall protein content from the cytoplasmic
fraction after concentration by osmo-dialysis against aqua-
cide was about 15% of its dry weight, yet this fraction was
about 50% of the dry weight of the cocci. It may be noted
here that the nucleic acids which had been acted upon by
RNase and DNase added were presumably lost along with the low
molecular nucleotides during the concentration process. Also,
the low protein content suggested that osmo-dialysis technique
may have lost some proteins that might have been present in
the cytoplasm. Immunodiffusion and enzymatic analysis of
the cytoplasmic fraction indicated that alpha toxin, nuclease,
and coagulase were present at low levels. Semi-quantitative
estimation showed that these represented less than 1% of the
total protein which made it possible that they may have been
contributed by membrane contamination. The cytoplasmic pro-
tein material gave a precipitin line with rabbit antistaphylo-
coccal serum which could not be eliminated by absorption of
the antiserum with alpha toxin prior to reacting with the cytoplasm material. Other experiments showed that the cytoplasm had a component which was highly hemolytic on blood agar, with zones on rabbit erythrocytes, typical of alpha or delta clearing. However, it was noted here that the antiserum was not homologous to the cytoplasm alpha toxin which may suggest differences in antigenic determinants.

**Distribution and Significance of Cellular Localized Exoprotein Antigens of S. aureus**

The demonstration of exoprotein antigens associated with sub-cellular components provided interesting information. It was shown that at least four exoprotein antigens were localized as cell wall proteins while three of such antigens were found in the membrane fraction. Since exprotein antigens may be factors of virulence in *S. aureus* it seemed logical and important that, while some portions of these are secreted into the medium, where they may harm the host, some portions of the antigen should remain associated with the organism as cell-bound antigen where they may be important in initiating and spreading the organism from the site of entry (Roger 1956). This concept was made reasonable by the fact that antigens demonstrated in the sub-cellular components showed identity to those purified from media. The sub-cellular distribution of exoprotein antigens are summarized in Table 11.
It was believed from the results presented that protein A may be the largest component in the cell wall protein antigens which may be very important to the organism as an antiphagocytosis agent reported by Dosset et al. (1969), and a staphylococcal agglutinogen reported by Lenhart et al. (1963; Yoshida et al. (1963); Lofkvist (1966); Kronvall (1970); and Grov et al. (1970). The findings of Hendricks and Altenbern (1968) that alpha toxin was continually synthesized intracellularly and released into the medium, as well as those of other authors involving coagulase and penicillinase, may lend support to the concept that all these antigens may be released at different rates in the course of growth of the organism, and must be considered both cell-bound and extracellular throughout the growth of the organism. All these have been shown to be cell-bound but the mechanism by which they are released into the medium requires research to show how the process is controlled by the cell. Answers to these questions may shed light on the problem of whether cell-bound enzymes are precursors for the extracellular enzymes or if the cell-bound and extracellular enzymes move along different routes after synthesis.

The experiments with exopenicillinase synthesis in Staphylococcus aureus have supported the concept that the synthesis of this enzyme does not require the preformed membrane bound-
enzyme (Coles and Gross, 1969). This may suggest that the membrane-bound penicillinase (may be, other membrane-bound enzymes) may have moved along a different route from that which the extracellular enzymes have moved regardless of origin of their synthesis. The association of penicillinase production in S. aureus with an extrachromosomal genetic element or plasmid DNA has suggested that the synthesis differences that may exist between cell-bound enzyme and the exoenzyme are relatively unimportant (Novick and Richmond, 1965; Richmond, 1967; and Rush et al. 1969). It revealed that the mechanisms by which the enzyme is moved from its site of synthesis may be important in determining if it would be either cell-bound or extracellular enzyme provided sites of its attachment are available. Such sites may in turn be controlled by the isoelectric charge of the protein.

The results presented have suggested a distribution of the extracellular proteins in sub-cellular components which supports a speculation that the exoproteins are moved from their site of synthesis by different routes. This was illustrated by the fact that some were found in all components (nuclease) while others (antigen A, beta hemolysin and coagulase) were localized only in limited number of sub-cellular components.
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