VIRULENT AND AVIRULENT *STAPHYLOCOCCUS AUREUS*

by

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ABSTRACT

Inhibition of indirect hemagglutination studies showed that the variant of the wound mucoid staphylococcus produced less capsular material than the encapsulated wound mucoid staphylococcus when grown under normal atmospheric conditions or when grown in an increased CO_2 atmospheric condition. Although the variant did not exhibit a specific capsular reaction, it elaborated capsular material. Therefore, "non-encapsulated" staphylococci may produce capsular material. A comparison of LD_{50} values for embryonated hens' eggs of the wound mucoid and the nonencapsulated variant of the strain showed that the nonencapsulated variant was less virulent.

There are at least two serologically distinct capsular types of coagulase positive Staphylococcus aureus. Until now, unequivocal evidence for encapsulation of the Smith diffuse variant was lacking. However, the data presented in this thesis provide definitive evidence of encapsulation of the Smith strain. A marked difference in LD_{50} values for the two serologically distinct capsular types of S. aureus was demonstrated. The paradoxical behavior of these two strains suggested that the mouse was resistant to one and was susceptible to the other. A survey of the carriage incidence in mice for staphylococci and staphylococcal capsular antibodies disclosed the presence of staphylococci and capsular antibodies in these animals. The capsular antibodies detected were reactive against only one of the

capsular types of S. aureus. None of the sera from the mice surveyed possessed capsular antibodies against the Smith diffuse variant of S. aureus, but the average incidence for the capsular antibodies against the wound mucoid strain of S. aureus was 46%. It was postulated that the susceptibility of the mice to the Smith diffuse variant of S. aureus was caused by the absence of protective, type-specific capsular antibodies. Conversely, the resistance of the mice to the wound mucoid strain of S. aureus may have been a result of their possession of type-specific capsular antibodies.

INTRODUCTION

Although less has been said in the past two or three years concerning the problem of staphylococcal disease, especially as it relates to hospital acquired infection, the problem is still with us. The combination of constant use of antibiotics and the reduced resistance of hospital patients furnishes an ideal situation for the proliferation and emergence of highly virulent and resistant staphylococci. The ecological niche provided by the hospital environment results in wide dissemination of the new highly adaptable antibiotic-resistant strains by person-to-person contact. The problem of post operative wound infections is far from being solved and only barely controlled at present. Public health workers fully anticipate a return to the high incidence of hospital acquired infections which characterized the decade of the 1950's. Already reports are appearing in the literature of staphylococci which are resistant to some of the new synthetic penicillins such as methicillin. At present the per cent of staphylococci resistant to new antibiotics is small; but through wide-spread use of these drugs, the forces of mutation and selection could shift the balance in favor of the strains resistant to new antibiotics.

Experimental evidence indicates that resistance to staphylococcal infections can be increased by specific antibodies or by stimulation of

nonspecific mechanisms. In the case of many bacteria, substances which play an important role in the virulence of the organism also stimulate protection in the host. This thesis will review literature concerning staphylococcal toxins, enzymes, and surface antigens such as cell walls and capsules and present the results of experiments designed to indicate that capsules play an important role in staphylococcal virulence and that capsular antibodies may play a role in the host's resistance to staphylococcal infections. An attempt was made to evaluate experimentally their role in staphylococcal virulence and their value as immunizing agents.

LITERATURE REVIEW

I. TOXINS AND ENZYMES

The role of anti-alpha toxins in staphylococcal immunity has been studied. Alpha-toxin or staphylotoxin has been purified and chemically characterized as a protein (Bernheimer, 1965). Whitby (1936), Dolman (1932), Murray (1935), and Parish et al. (1934) observed similar anti-alpha-toxin titers in normal human sera and sera from patients with prior staphylococcal infections. Forssman (1935, 1936a, 1936b, 1937, 1938) immunized rabbits with formalized whole S. aureus cells. Protective antibodies were at their highest titer seventeen days after the last injection. The antibody titer declined rapidly and again rose to high titer on the 57th day following the last injection. Serum from immunized rabbits protected unimmunized rabbits; yet, no anti-alpha toxin was detected in immune serum. He concluded from his studies that antitoxins were not significant in staphylococcal immunity. This conclusion was substantiated by Smith (1937), Lord Stamp (1961), and Koenig (1962), and Koenig et al. (1962a, 1962b).

Hyaluronidase apparently does not play an important role in the virulence of S. aureus. Normal human sera and sera of other animals have been found to contain antibodies and nonspecific inhibitors of staphylococcal hyaluronidase (Kulonen, 1951). There was no correlation between the virulence of S. aureus strains and the increased

production of hyaluronidase (Boe, 1944); however, hyaluronidase may play a role in long-standing infections (Kulonen, 1951).

Panton and Valentine (1932) identified a lytic substance in the supernatant of a culture of S. aureus as leucocidin. It caused cytotoxic effects on human and rabbit polymorphonuclear leukocytes and macrophages. Woodin (1959, 1960, 1961a, 1961b) observed that leucocidin was composed of two protein components. Immunization with purified leucocidin resulted in little protection (Mudd, Gladstone, and Lenhart, 1965).

There seems to be no correlation between quantitative coagulase production and virulence (Koenig, 1962; McCabe, 1966). Coagulase-negative mutants isolated from coagulase-positive parent strains produced similar infections (Kapral and Li, 1960); and in rare instances, coagulase-negative staphylococci have been isolated from human infections (Quinn and Fisher, 1965). Coagulase may act to form a fibrin barrier which temporarily interfers with phagocytosis (Hale and Smith, 1945; Smith et al., 1947). Rabbits immunized with coagulase survive longer than unimmunized animals but eventually die (Boake, 1956).

II. VACCINES

A. Labile Vaccines

Fisher (1957) found a labile staphylococcal antigen in the culture supernatant fluid of S. aureus that immunized mice against staphylococcal challenge. When nonformalized culture supernatant fluids diluted 1:50 were injected intraperitoneally as the immunizing agent, twelve

mice survived out of thirty-two challenged. The staphylococci were mixed with a saline-human fibrinogen-human plasma mixture prior to challenge. The antigenicity of the protective antigen in the supernatant fluid was destroyed by formalization (Fisher, 1957). North (1958) reported that the Newman strain of S. aureus was a poor producer of alpha-hemolysin but was capable of elaborating a protective antigen. This finding was confirmed by Fisher (1962) and further supported his previous observation that the culture supernatant fluid of the Wood 46 strain of S. aureus contained a protective antigen. In 1965, Robson and Fisher partially purified the protective antigen. The protective fraction contained no detectable catalase, coagulase, fibrinolysin, hyaluronidase, alpha-toxin, leucocidin, phosphatase, protease, RNase, or staphylokinase. A positive Feulgin reaction was observed. An increased amount of antigen could be recovered from the crude culture supernatant material when protease inhibitors were added to the medium. Loss of antigenicity of the protective antigen occurred when the fractionated material was stored at 28-32°C or when the material was stored in the refrigerator for one month. Small quantities of the partially purified material (5-10 ug) injected subcutaneously over the back of mice protected them against challenge two weeks after the last injection. Antihemolysin titers in the unimmunized and immunized mice were the same.

B. Teichoic Acid and Ammonium Sulfate Precipitated Vaccines

Type specific carbohydrates designated A and B were first isolated from staphylococci by Julianelle and Wieghard (1935). Polysaccharide A

was extracted from a staphylococcal strain isolated from a patient with staphylococcal septicemia and polysaccharide B was extracted from a staphylococcal strain isolated from the conjunctiva of a normal individual. The two polysaccharides differed in their per cent of reducing sugars and in their optical rotation. Injection of the whole organisms into rabbits elicited precipitins which reacted with the type specific polysaccharides (Julianelle and Wieghard, 1935; Wieghard and Julianelle, 1935). However, pure polysaccharides were not antigenic when injected into rabbits. Intradermal injection of polysaccharide A resulted in an "immediate wheal and erythema" reaction in patients with staphylococcal infections (Julianelle and Hartman, 1936). Sera from patients with staphylococcal infections had precipitating antibodies to polysaccharide A, but normal human sera were nonreactive (Julianelle and Hartman, 1936). A number of years passed and in 1958 a group of newly described polymers, teichoic acids, were isolated from the cell walls of Gram positive bacteria (Armstrong et al., 1958). Experimental evidence showed that the cell walls of Staphylococcus epidermidis contained glycerol teichoic acid (Davison and Baddiley, 1963; Ellwood et al., 1963); whereas, the cell walls of S. aureus contained ribitol teichoic acid. Although the strains of staphylococci that Julianelle and Wieghard isolated were lost, it was shown by Haukenes (1962a, 1962b) that the extraction method of Julianelle and Wieghard extracted teichoic acid and that polysaccharide A was serologically and chemically similar to ribitol teichoic acid. Injection

of ribitol teichoic acid and polysaccharide A into rabbits and mice failed to elicit antibody production (Haukenes, 1962a, 1962b). Intradermal injection of teichoic acid from strain Copenhagen resulted in an "immediate wheal and erythema" skin reaction similar to polysaccharide A isolated by Julianelle and Wieghard (Julianelle and Hartman, 1936; Strominger, 1962). Sera from five human volunteers showed antibodies to teichoic acid prior to immunization and a significant increase in teichoic acid antibodies after immunization (Torii et al., 1964). Phagocytosis promoting antibodies in human serum may be removed by absorption with teichoic acid (Mudd et al., 1963). Teichoic acid may be obtained from cell wall fragments of S. aureus by enzymatic digestion and extraction with trichloracetic acid or by trichloracetic acid extraction only (Mandelstam and Strominger, 1961; Morse, 1962b; Sanderson et al., 1962; Davison and Baddiley, 1963). The cell walls of S. aureus are composed of ribitol teichoic acid and a mucopolypeptide, acetylglucosamine-acetylmuramic acid and peptide chains cross linked by polyglycine bridges (Strominger, 1965). The purified mucopeptide fraction was not immunogenic (Morse, 1965); but recently, serological activity of a peptide polymer was reported (Hisatsune et al., 1966). The capsular material isolated from the Smith diffuse strain of S. aureus may contain teichoic acid (Ekstedt, 1965, 1966). Teichoic acid has been found in the cytoplasmic matrix, cell wall, and possibly in the capsular material of the Smith diffuse strain of S. aureus. Ribitol teichoic acid was structurally characterized as

N-acetyl glucosaminyl ribitol units by Baddiley (1962) with ester linked D-alanine. The specificity of the polymer depends on the alpha- or beta-linkage of N-acetyl glucosamine to the ribitol. Strains of S. aureus may have either alpha- or beta-linked N-acetyl glucosamine or a combination of both. Strain Copenhagen contained 15% of 4-O-alpha-N acetyl glucosaminyl and 85% of 4-O-beta-N acetyl glucosaminyl linkages. Strain H contained 95-100% of 4-O-beta-N acetyl glucosaminyl linkages, and strain 3538 contained 95-100% of 4-O-alpha-N acetyl glucosaminyl linkages. If both alpha and beta linkages are present and comprise at least 10% of the cell wall fraction, then antibodies are produced against both types of linkage (Nathenson and Strominger, 1962). Ribitol teichoic was shown to be the type specific antigen of S. aureus (Juergens et al., 1963). Antiteichoic antibodies could be produced in rabbits by using whole cells, cell walls, or crude extracts but not with pure teichoic acid preparations (Haukenes, 1962a).

Protection of adult mice by two different staphylococcal antigens was demonstrated by Ekstedt (1963a, 1963b, 1963c, 1965, 1966). Mice were immunized with an ammonium sulfate precipitated vaccine prepared from the supernatant fluid of disintegrated Smith diffuse S. aureus cells. Trypsinizing or boiling the vaccine destroyed the protection elicited against homologous organisms suspended in saline. However, active immunization was observed with trypsin digested or boiled vaccines when the challenge organisms were suspended in 5% hog gastric mucine. The ammonium sulfate fraction contained 20% nucleic acid and

0.63% hexoses. No amino acid composition was reported. The ammonium sulfate fraction had an isoelectric point of pH 5.5 to 5.6 and showed five precipitin lines in gel diffusion and immunoelectrophoresis (Ekstedt, 1963, 1965). Three of the antigens have been identified as teichoic acid, Jensen antigen A, and Verwey protein antigen (fraction B). The remaining two antigens have not been identified. The teichoic acid fraction conferred immunity against the Smith diffuse strain of S. aureus when the challenge organisms were suspended in 5% hog gastric mucin. Ekstedt (1966) believed teichoic acid to be present in the polysaccharide fraction which had been isolated from the Smith diffuse organism by Morse (1962a). A protein or protein complex conferred immunity against the Smith diffuse staphylococcus when the challenge organisms were suspended in saline. The Verwey fraction and the Jensen antigen A fractions did not stimulate protection in mice (Ekstedt, 1966). However, Lord Stamp (1964) had shown that the Verwey fraction stimulated protection in rabbits. Two possible mechanisms were presented to explain the protection produced by the two protective antigens. The first mechanism proposed that antibody to the teichoic acid acted as an opsonin resulting in phagocytosis of the Smith diffuse organisms; whereas, the trypsin sensitive, heat labile protective antigen elicited antibodies which took part in intracellular destruction of the phagocytized Smith diffuse staphylococcus. The second mechanism proposed that teichoic acid elicited specific protective antibodies and the heat-labile trypsin-sensitive antigen stimulated nonspecific defense mechanisms.

C. Capsular Vaccines

Numerous investigators have defined encapsulation and presented methods for detecting capsules. The most widely used, the most specific, and artifact free method has been the specific capsular reaction. Suggestive evidence presented in 1931 by Gilbert and in 1937 by Lyons indicated that Staphylococcus aureus may be encapsulated. Price and Kneeland (1954, 1956) showed that S. aureus was encapsulated.

Gilbert (1931) isolated a mucoid strain of S. aureus from the pericardial and peritoneal fluid of a person with acute ulcerative gonococcal endocarditis. Suggestive evidence of encapsulation was obtained when this staphylococcal strain was mixed with India ink and observed under oil immersion. A clear area measuring three microns surrounded the organism. The mucosity of the colony on agar media also indicated the possibility of encapsulation. A smooth (S) to rough (R) dissociation was observed when the cultures were stored in the refrigerator for a month. Occasionally S variants of staphylococci were isolated from R cultures by guinea pig passage. Intraperitoneal injection of 8 to 10 mls of a twenty-four hour broth culture of the rough from was avirulent for guinea pigs; whereas, 0.25 ml of a twenty-four hour broth culture of the smooth form killed guinea pigs.

Lyons (1937) also obtained suggestive evidence of encapsulation of S. aureus using a colloidal silver and a carbol fuchsin-methylene blue staining techniques. The S. aureus strains he used were isolated

from human pus, human blood, a septic arthritic patient and a case of osteomyeltic bacteremia. These S. aureus strains resembled encapsulated diplococci when stained by the above techniques. Cultures grown in human serum appeared to lose their capsules. Nonencapsulated S. aureus grown in infusion broth were encapsulated in young actively multiplying three-hour-old cultures. The capsule disappeared on prolonged incubation. Antibodies prepared by injecting heat-killed encapsulated S. aureus into rabbits specifically and effectively sensitized encapsulated S. aureus, thus favoring phagocytosis of the organisms (Lyons, 1937). He concluded that the antibacterial antibody was a type-specific antibody to the capsular antigen. Spink (1939) and Klienburger-Nobel (1948) were unable to confirm his findings. In addition, Klienburger-Nobel (1948) was unable to demonstrate capsules, using the specific capsular reaction.

Price and Kneeland (1954, 1956) immunized rabbits with a mucoid strain of S. aureus designated RL-mucoid (RLM) variant. A specific capsular reaction was observed when homologous antiserum and staphylococci were mixed and viewed under oil immersion. The parent strain was originally cultivated on rabbit blood agar, transferred through nutrient broth, and then passed through embryonated eggs with influenza virus. Subculture from the egg onto plating media yielded a colony that was watery in appearance but was difficult to scrape off. This was designated the mucoid colony. A viscid strain, lacking the watery appearance of the mucoid strain but retaining the adherent property

resulted from the growth of the parent strain in allantoic fluid only. Formalized or heat-killed vaccines of the whole mucoid or viscid organisms were injected intravenously into rabbits for several days followed by a rest period. This procedure was continued for a month to provide the immune serum for the capsular swelling reaction. Antimucoid or antiviscid serum mixed with either viscid or mucoid cells produced a positive capsular reaction.

Wiley (1959) confirmed the work of Price and Kneeland (1954, 1956). When specific immune serum and encapsulated staphylococci were mixed and observed under oil immersion, the capsular region of S. aureus was clearly delineated. Embryonated hens' eggs were used to estimate the LD_{50} of encapsulated S. aureus. As little as 0.04 ml of undiluted human globulin or hyperimmune rabbit or rooster serum passively protected the host against 20 LD_{50} doses. The passive protective properties of hyperimmune rabbit or rooster serum and human globulin was attributed to anticapsular antibodies. The results of staphylococcal challenge of embryonated hens' eggs were reported by Wiley (1961, 1962). If the embryonated hens' eggs were capable of detecting differences in virulence between encapsulated and nonencapsulated S. aureus, they should be able to reflect virulence differences between S. aureus and S. epidermidis strains. Injection of 5,000,000 S. epidermidis failed to produce a 50% lethal end point (Wiley, 1961, 1962). Experimental evidence showed a correlation between LD_{50} differences and capsular size of S. aureus (Wiley, 1961, 1962). Viable count/

packed cell volume ratios, passive hemagglutination inhibition, and egg virulence were studied to determine if capsular size was related to virulence of similar encapsulated types of S. aureus. Strains of S. aureus with high, moderate, and low virulence were used. A correlation between capsular size, egg virulence, release of capsular material and the amount of coagulase produced were observed (Wiley, 1963a, 1968, unpublished data). A highly encapsulated wound strain of S. aureus and a nonencapsulated variant of the wound strain were studied. The nonencapsulated variant and the encapsulated strain of S. aureus elaborated similar quantities of coagulase and alpha-toxin. However, the LD_{50} for the encapsulated S. aureus was 32 organisms; whereas, injection of 1,000 nonencapsulated organisms failed to produce a 50% kill (Wiley and Maverakis, unpublished data).

Wiley (1960, 1963b) reported that anticapsular antibodies were common in normal human, rabbit, and rooster sera. Of a group of 109 healthy humans, 80.7 per cent had anticapsular antibodies in their serum. Forty-seven of these persons were nasal carriers of S. aureus and twenty-one of the staphylococcal isolates were encapsulated. A group of 27 strains of S. aureus were isolated from post operative wound infections, boils, and skin abscesses (Wiley, 1964). Twenty-five exhibited a positive specific capsular reaction when mixed with rabbit antistaphylococcal serum, twelve were positive with acute phase human serum, and thirteen were positive with convalescent phase serum. Nine of the patients' sera passively protected embryonated hens' eggs against

challenge with autologous organisms. A second group of S. aureus strains isolated from hospital personnel did not differ significantly in virulence from the strains isolated from post operative wound infections, boils and skin abscesses (Wiley, 1964).

Isolation and partial chemical characterization of the capsular material was reported by Wiley and Wonnacott (1962). Absorption experiments revealed that the capsular material removed protective antibodies and antibodies necessary for the specific capsular reaction. Chemical analysis indicated that cell-free capsular material was composed of glucosamine, four amino acids (glycine, alanine, glutamic and lysine) and an unidentified compound.

Partially purified capsular material elicited protection when injected into mice and challenged 12 to 120 hours later. Since maximum antibody response did not occur during this time interval, it was believed that nonspecific resistance was stimulated by the partially purified capsular material. It seemed to stimulate cellular protective mechanisms rather than humoral mechanisms. A leucocytosis was observed in two hours followed by a leucopenia eight hours later and then a reoccurring leucocytosis lasting for at least 48 hours. No agglutinating antibodies or antibodies active in the passive hemagglutination test were detected. The possibility that 500 ug of capsular material may have caused immunological paralysis was considered (Wiley, 1965). Immunization of mice with 100 ug to 1 mg amounts of capsular material mixed with Freund's incomplete adjuvant failed to stimulate

agglutinating or precipitating antibodies. The mice were bled four days later after the immunizing dose (personal communication). A passive hemagglutination inhibition test was devised to detect non-agglutinating or nonprecipitating antibodies. The sera from immunized mice were mixed with mouse erythrocytes sensitized with partially purified capsular material and incubated. Rabbit antistaphylococcal serum was then added and the mixture was again incubated. A positive test was indicated by no hemagglutination and a negative by hemagglutination. The presence of staphylococcal antibodies in normal mouse sera could have been due to environmental contact between the host and S. aureus resulting in immunization of the host.

D. Surface Components of Possible Capsular Nature

The Smith strain of S. aureus was isolated from a patient with osteomyelitis (Dubos, 1930) and briefly characterized by Smith and Dubos (1956) as being coagulase positive by the tube test and belonging to phage type 44A/42E. Hunt and Moses (1958) isolated an extremely virulent and an avirulent S. aureus from the parent Smith culture. The virulent Smith strain grew as a diffuse comet shaped colony in serum soft agar, and the avirulent Smith strain grew as a round, compact colony in serum soft agar. Finkelstein and Sulkin (1957, 1958) observed that the compact variant was lysed by phage type 44A and agglutinated by a standard staphylococcal grouping antiserum. The diffuse variant was untypeable and was not agglutinated by any of the standard grouping sera. Intraperitoneal inoculations of the Smith

diffuse organisms resulted in a higher mortality ratio for mice than intravenous inoculations (Koenig et al., 1962b). This was explained on the basis that phagocytosis in the peritoneal cavity occurred later than intravascular phagocytosis. It was shown by Koenig and Melly (1965), using the gel precipitation technique, that the Smith diffuse type possessed an antigenic component which was not present in the Smith compact type. They also noted that the diffuse S. aureus was clumping factor negative; whereas, the compact S. aureus was clumping factor positive. From these observations, he postulated the location of the bound coagulase in the Smith diffuse strain as beneath the antigenic structure absent from the compact type and, therefore, not detectable. In 1962, Morse isolated and partially purified a polysaccharide fraction from the Smith diffuse strain. This polysaccharide fraction stimulated protection in mice when challenged with suspensions of the Smith diffuse variant. The polysaccharide fraction was designated SSA (Smith surface antigen) by Morse (1962a) and chemically characterized as being composed of 2-deoxy-2 amino-glucuronic acid units by Perkins (1963). A second group of investigators, Fisher et al. (1963), isolated a polysaccharide fraction from a highly virulent strain of S. aureus (05068) and designated the chemical compound as SPA (staphylococcal polysaccharide antigen). SPA was chemically characterized as being composed of D-glucosaminuronic acid and L-alanine with O- and N- acetyl substitution (Haskell and Hanessian, 1963, 1964). Both SSA and SPA were chemically and immunologically similar (Morse, 1963). The absorption of Smith

staphylococcal antiserum by SSA removed agglutinins and opsonizing antibodies (Morse, 1962a). Microscopic examination of the Smith diffuse strain of S. aureus in India ink preparation (Morse, 1962a) and electron photomicrographs (Koenig and Melly, 1965) showed an envelope structure surrounding the organisms. The above experiments indicated that SSA was a surface component of the Smith diffuse strain of S. aureus. However, the specific capsular reaction was negative (Morse, 1962a; Koenig, 1962).

MATERIALS AND METHODS

I. STRAINS

The strains of S. aureus used in these studies were the encapsulated wound mucoid strain supplied by Dr. B. B. Wiley, and a variant of that strain obtained by testing colonies and selecting one that was butyrous rather than mucoid. The wound variant did not exhibit a specific capsular reaction. Dr. M. G. Koenig and Dr. S. I. Morse supplied the compact and diffuse Smith strains used. All these strains were grown in serum soft agar to observe their colonial morphology (Finkelstein and Sulkin, 1958).

II. CAPSULAR MATERIAL

The wound strain of S. aureus capsular material was kindly furnished by Dr. B. B. Wiley and prepared according to his procedure (Wiley, 1961).

III. MEDIA

For cloning and transferring cultures, a buffered nutrient glycerol agar (BNG agar) was used (Wiley, 1961). It contained the following ingredients: Nutrient broth, 8.0 g (Difco); glycerol, 20 ml; sodium monohydrogen phosphate (anhydrous), 1.62 g; potassium dihydrogen phosphate (anhydrous), 0.24 g; agar, 20 g (Difco); and

distilled water to make 1000 ml. The BNG agar was adjusted to pH 7.4 with 1 N NaOH and autoclaved.

The staphylococci were grown in a semisynthetic medium, Casamino acids-glycerol broth, to minimize cross reactions because all of the components in the medium were dialyzable (Wiley, 1961). This broth contained the following: Casamino acids, Technical, 8.60 g (Difco); potassium monohydrogen phosphate (anhydrous), 1.63 g; potassium dihydrogen phosphate, 0.454 g; glycerol, 20 ml; phenol red, 0.01 g; and distilled water to make 1000 ml. The medium was adjusted to pH 7.4 and autoclaved. When the sterile medium had cooled, sterile solutions of the following were added aseptically: 10 ml of 2 per cent solution of magnesium sulfate; thiamine hydrochloride, 10 ugm; pyrodoxine 1 ugm; and nicotinic acid, 200 ugm. The pH was again checked, and if needed, the pH was adjusted to 7.4 with sterile 1 N NaOH.

For the preparation of vaccines, buffered nutrient glycerol broth (BNG broth), buffered nutrient glycerol agar (BNG agar) and the semisynthetic medium previously described were used. The semisynthetic medium was slightly modified for the growth of the Smith strain. Dextrose (1%) was substituted for the glycerol.

Mannitol salt agar (Difco) was used for primary isolation of S. aureus from rectal and throat swabs. Single colonies were selected from the Mannitol salt agar and streaked onto BNG agar plates. The organisms from the colonies were Gram stained and tested for coagulase production.

The serum soft agar used for cloning the Smith diffuse and Smith compact strains contained the following: tryptone, 1% (Difco); yeast extract, 0.5% (Difco); glucose, 0.57 gm (Difco); K_2HPO_4, 0.5%; agar, 0.15% (Difco); and distilled water to make a liter. Five ml quantities of the sterilized basal medium was pipetted into test tubes and stored in the refrigerator. As needed, 0.05 ml of human or rabbit serum was added to each tube.

IV. PREPARATION OF SUPERNATANT FLUIDS

Staphylococci were streaked onto BNG agar plates and incubated for 18 to 24 hours at 37°C. After incubation the staphylococci were harvested from the plates by scraping the surface of the BNG agar with a wire loop. The growth was transferred aseptically to a test tube containing 1 ml of BNG broth and a sterile glass stirring rod used to partially break up the clumps of organisms. From the first test tube one ml of the suspension was pipetted into a second having 9 ml of BNG broth and 6 glass beads. The test tube was then fitted with a vaccine screw cap. The final dispersion of the staphylococci was accomplished by drawing the mixture into a sterile Luer-Lok syringe fitted with a 20 gauge needle and by expelling the mixture back into the test tube. This mixing procedure was repeated twenty times. A small quantity, approximately 0.1 ml, of this mixture was added to 15.9 ml of BNG broth to give an optical density difference of 0.32, as compared with a BNG broth blank read at 515 mu in a Bausch and Lomb Spectronic "20" colorimeter. One ml of the diluted material was

transferred into a 250 ml flask containing 100 ml of semisynthetic broth. Half of the flasks were incubated at 37°C in an atmosphere containing increased carbon dioxide (candle jar) and the other flasks were incubated at 37°C under normal atmospheric conditions for 24 hours. All flasks were incubated on a reciprocating shaker (Eberbach and Son Co., Ann Arbor, Michigan) and the pH was maintained at 7.0 to 7.4 by addition of sterile 1 N KOH several times daily. The cultivation procedure is diagramed in Figure 1. Ten ml samples were withdrawn from each flask at 0, 2, 4, 6, 8, 12, and 24 hours. The samples were immediately centrifuged in a refrigerated centrifuge for thirty minutes at 1,500 X g, the supernates saved and the cells discarded. Each supernatant fluid was adjusted to pH 7.0 and frozen at -20°C until needed.

V. SPECIFIC CAPSULAR REACTION

The specific capsular reaction was carried out by placing one loopful of staphylococcal antiserum on a vaseline-rimmed coverslip and mixing a loopful of a 6- to 8-hour BNG broth culture of staphylococci with it. A loopful of 1% aqueous methylene blue was also added. An alternative procedure was carried out with an 18-hour BNG agar culture of staphylococci. A small portion of a single colony was picked and mixed with the staphylococcal antiserum and 1% aqueous methylene blue. All specific capsular reactions were observed microscopically under oil immersion magnification at 1, 2, and 24 hours after being set up.

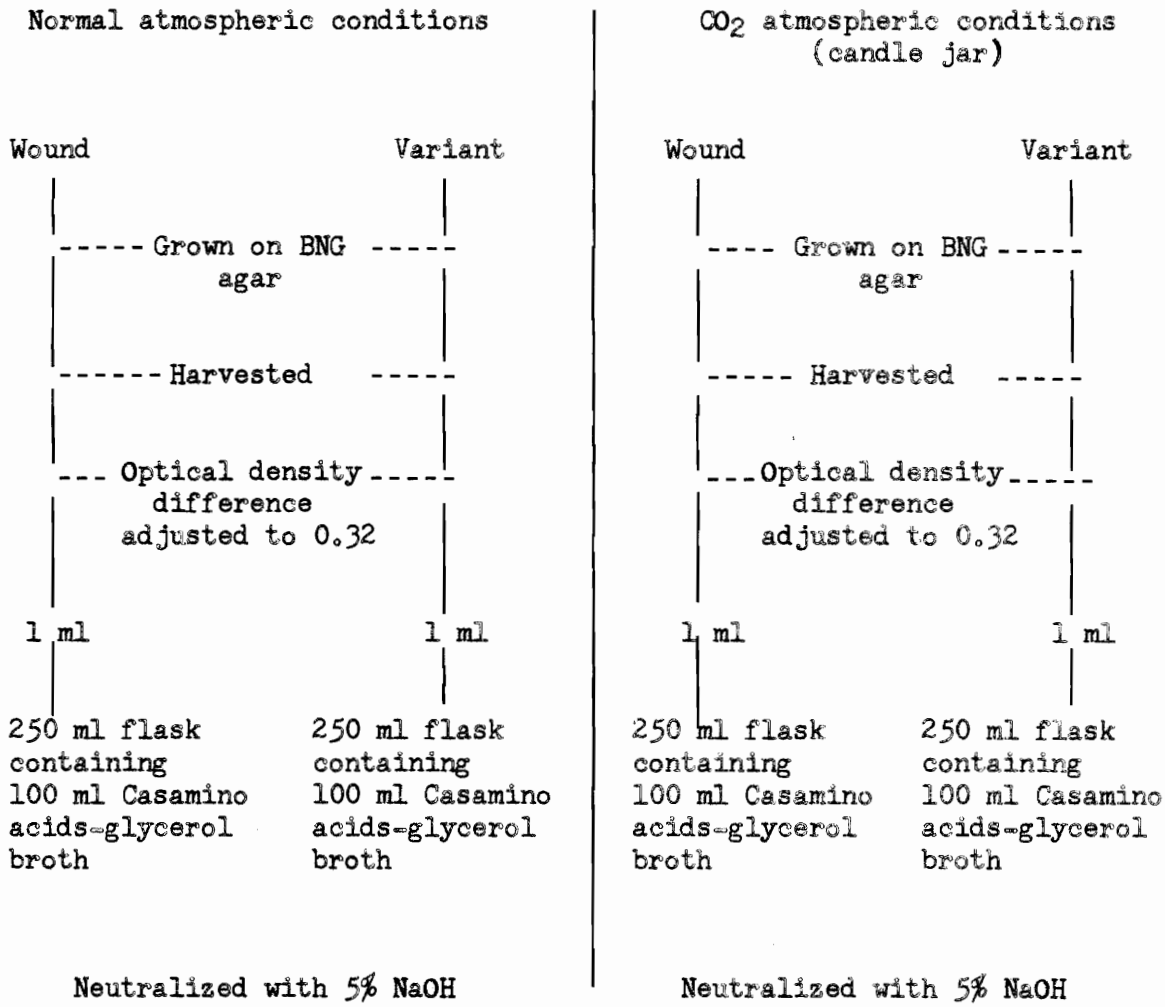

FIGURE 1. Procedure used for the cultivation of the staphylococci.

VI. COAGULASE TESTS

Tube coagulase tests were carried out by mixing equal parts of human plasma diluted 1:3 with sterile saline buffered at pH 7.2 and a 24-hour broth culture of the staphylococci. For quantitative determination of free coagulase the method of Yotis and Ekstedt (1959) was followed and human plasma diluted 1:3 with saline was used in all coagulase tests.

VII. QUANTITATION OF ALPHA HEMOLYSIN

The method of McFarlan (1938) modified by Jackson (1955) was used to quantitate the amount of alpha hemolysin in the broth supernatant fluid. The organisms were grown at 37°C for 48 hours in an atmosphere of 30% carbon dioxide. Fresh 2% rabbit cells were used in the titrations.

VIII. PREPARATION OF MUCIN

Wilson's granular mucin Type 1701-W, lot number 123810, was prepared for use by the method of Miller (1935). The mucin was blended in a Waring blender. A 5% suspension was employed for virulence enhancement.

IX. PREPARATION OF VACCINES AND INJECTION OF ANIMALS

A starter flask of fifty mls of semisynthetic medium was inoculated with a loopful of staphylococci that had been previously cloned and grown on BNG agar for 18 to 24 hours. The flask was incubated at 37°C on a reciprocating shaker (Eberbach Corporation, Ann Arbor,

Michigan). When growth had begun in the starter flask, the contents were aseptically transferred into 1 liter of semisynthetic medium. The pH was maintained at 7.0 to 7.4 by addition of sterile 1 N KOH as necessary. After cessation of growth, the organisms in the culture were examined microscopically for culture purity. Slides were stained by the Gram method. Formalin was then added to a final concentration of 0.5% and the cultures once again returned to the shaker for 24 hours. Each culture was tested for sterility by plating one ml onto a sheep-blood agar plate. The staphylococci were harvested from the semisynthetic broth by centrifugation at 4,080 X g, using a Servall centrifuge, model SS-4, and a GSA rotor. The pelleted organisms were washed in buffered saline (pH 7.2) three times and suspended in the same solution. This preparation constituted the stock vaccine. The suspensions were adjusted as needed to an optical density difference of 0.67, as compared with a water blank, at 515 mu in a Bausch and Lomb Spectronic "20" colorimeter. The suspension was then diluted 1:20.

Equal volumes of Freund's incomplete adjuvant and diluted cells were mixed and sonically vibrated with a Branson sonifier, model S-75 with an average output of 75 watts (Branson Sonic Power, Danbury, Connecticut) until a thick white emulsion was obtained. One-half ml of the suspension was injected into each hind foot pad of a 2- to 3-kg albino rabbit. After one week, additional injections were given subcutaneously at weekly intervals, doubling the concentration of antigen

each time. The animals received from 8 to 12 injections. A similar procedure was used for the immunization of New Hampshire red roosters. Antigen preparations were injected into the breast muscle. The animals were bled by cardiac puncture when their serum showed a specific capsular reaction titer of 1:32 or greater. The sera were stored in the freezer until needed.

X. PREPARATION OF STAPHYLOCOCCAL SUSPENSIONS FOR LD_{50} DETERMINATIONS

Much of the difficulty encountered in determining accurate plate counts of encapsulated staphylococci lies in adherent properties of the organisms caused by their mucosity. In this study sonic treatment of the suspensions was used to break up the clumps of bacteria. A Branson sonifier, equipped with a 1/8 inch (0.3-cm) micro tip stephorn, was used. It was found that suspensions sonified for two minutes with the power input at a setting of 2 and an amperage reading of 3.5 to 4.0 amperes gave the highest viable counts.

XI. DETERMINATION OF LD_{50}

Adult male Swiss albino mice weighing approximately 25 to 30 g were used in all experiments. The staphylococci were grown on BNG agar plates for a period of 18 to 20 hours, scraped from the plates and diluted with BNG broth to an optical density difference of 0.33 to 0.41, as compared with a BNG broth blank at 515 mu in a Bausch and Lomb Spectronic-20 colorimeter. These staphylococci were sonically vibrated as previously indicated and kept chilled in ice. They were

then diluted in BNG broth dilution blanks with glass beads and mixed on a Vari Whirl Mixer (Van Waters & Rogers, Inc., Salt Lake City, Utah). The mice were divided into five groups of fifteen mice per group and injected by the intraperitoneal route with 0.50 ml of mucin (5%, W/V), followed immediately by 0.25 ml of the appropriate dilution of S. aureus. At least five dilutions were used. The mice were observed daily for 14 days. The method of Miller and Tainter (1944) was used to estimate the LD_{50} and the standard deviation.

Egg inoculations were carried out according to the procedure reported by Wiley (1961). Twelve-day-old embryonated eggs were divided into seven groups of twelve eggs per group and candled to locate a large vein in the chorioallantoic membrane. The site was marked, swabed with iodine and then with alcohol. A small triangular window was cut through the shell over the vein, using a Moto-tool drill (Dremel Mfg. Co., Racine, Wisconson) fitted with a 1 7/8 inch damascus separating disk (W. M. Dixon, Newark, New Jersey). The shell was removed with a needle and a drop of sterile mineral oil was placed on the shell membrane to make it transparent. Eggs with nicked shell membranes or eggs that bled after inoculation were not used. A 0.25 ml Luer-Lok syringe was fitted with a 26 or 27 gauge needle and an inoculum of 0.05 ml of serially diluted S. aureus suspensions were inoculated into the blood stream of the embryonated hen's egg. The cultures were grown, sonified, and diluted in the same manner as those used in the mouse inoculations. The LD_{50} end

points were calculated by the method of Miller and Tainter (1944) from deaths occurring between 9 and 120 hours.

XII. SENSITIZATION OF ERYTHROCYTES

Twenty-five mls of blood was obtained from a rabbit by cardiac puncture using a 50 ml Luer-Lok syringe fitted with an 18 gauge needle. The blood was immediately transferred into 25 mls of sterile 4.2 per cent sodium citrate and stored in the refrigerator at 4°C until needed. Aliquots of the citrated blood were washed three times in cold sterile saline. The cells were packed by centrifugation at 1,060 X g for a period of ten minutes and diluted to a five per cent suspension (Figure 2). Citrated blood more than three days old was discarded and fresh blood obtained. The partially purified capsular material was diluted 1:1,000, 1:2,000, 1:4,000, 1:8,000 and 1:10,000 in saline. Three mls of the five per cent cells and three mls of each of the dilutions of capsular material were mixed. The mixture was incubated in a water bath for two hours at 37°C. The test tubes were gently inverted every five minutes to resuspend the settled rabbit erythrocyte. At the end of the two-hour incubation period the cells were centrifuged and washed three times with cold sterile saline. The packed cells were diluted to 3 mls with cold sterile saline. The sensitized cells were immediately used.

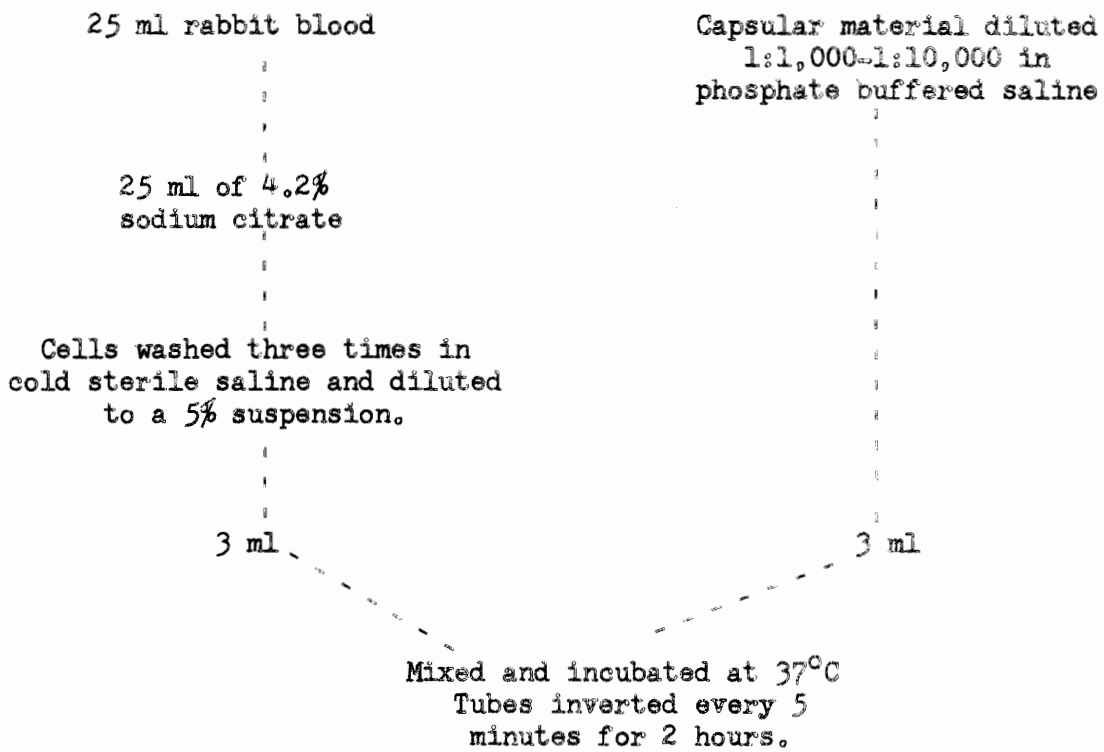

FIGURE 2. Procedure used to sensitize rabbit erythrocytes with capsular material.

XIII. INDIRECT HEMAGGLUTINATION

The antiserum was diluted 1:2 through 1:512 by two-fold dilutions. Then 0.12 ml of each dilution of antiserum was added to each test tube followed by 0.5 ml of saline and 0.05 ml of sensitized cells. Up to this point all the procedures were carried out in the cold. The tubes were incubated at 37°C for two hours; during this time, the tubes were shaken every fifteen minutes. Overnight refrigeration followed and the results were read the next day. The last tube showing a 2+ reaction was considered one unit of hemagglutinating antibody. The three controls were as follows: (1) 0.62 ml saline plus 0.05 ml of sensitized cells; (2) 0.12 ml of 1:2 antibody, 0.5 ml saline, and 0.05 ml sensitized cells; (3) 0.12 ml of 1:2 antibody, 0.5 ml saline, and 0.05 ml unsensitized cells (Figure 3).

XIV. INHIBITION OF INDIRECT HEMAGGLUTINATION

Four units of hemagglutinating antibody contained in 0.12 ml were used in all inhibition of indirect hemagglutination tests. The four hemagglutinating units of antibody were mixed with varying dilutions of staphylococcal culture supernatant fluid increasing in 0.05 ml increments from 0.1 ml to 0.5 ml. All volumes were made up to 0.5 ml with phosphate buffered saline pH 7.2. These antiserum-culture supernatant fluid mixtures were incubated for one hour at 37°C, then 0.05 ml of sensitized rabbit erythrocytes were added. The tubes were again incubated at 37°C for two hours. During the second incubation the tubes were gently shaken every fifteen minutes until

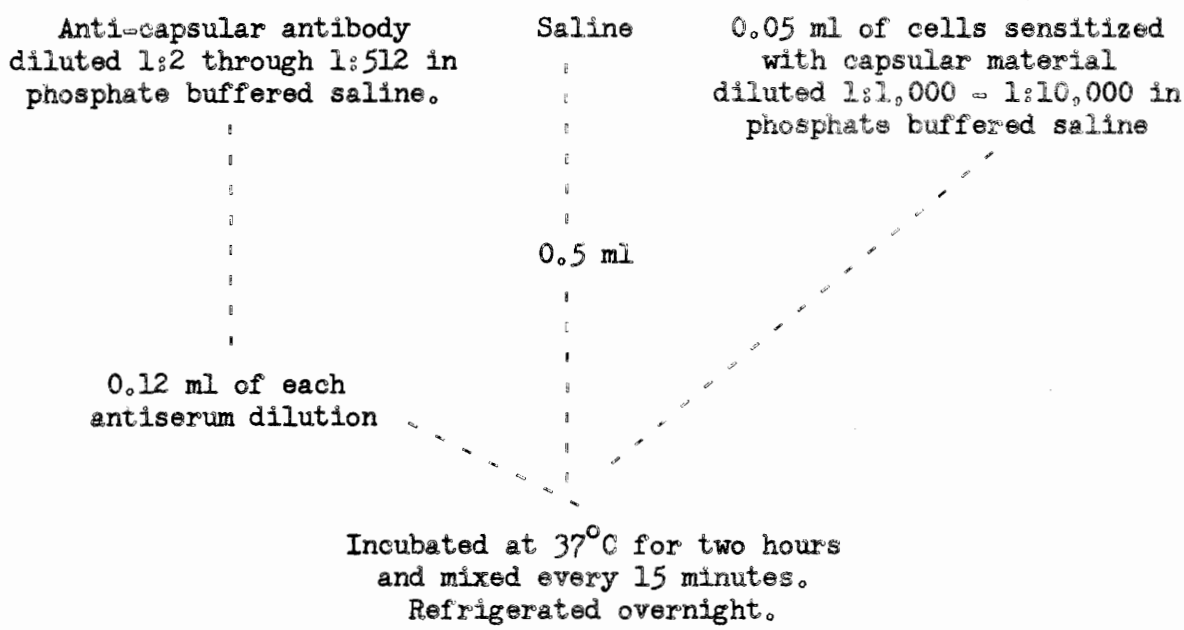

FIGURE 3. Procedure used for indirect hemagglutination experiments.

the contents were thoroughly mixed. At the end of the two hours the tubes were refrigerated overnight at 6°C and read the following morning. The controls were the same as in the indirect hemagglutination test. For a standard a known amount of partially purified capsular material was used instead of the culture supernatant fluid to determine the amount of partially purified capsular material necessary to inhibit hemagglutination. The least amount of partially purified capsular material that gave a 2+ inhibition of hemagglutination was used in the calculation of the ugm of capsular material in staphylococcal supernatant fluids.

XV. CALCULATIONS

The following formulas were used to calculate the minimal amount of capsular material necessary to prevent hemagglutination and the amount of capsular material in 1 ml of staphylococcal culture supernatant fluid.

$$CMS = CM \times v$$

$$S = \frac{D \times 1\ ml \times CMS}{V}$$

CM = known amount of capsular material

CMS = standard amount of capsular material that caused inhibition of indirect hemagglutination

D = dilution

S = ugm of capsular material in 1 ml of supernatant fluid

V = volume of supernatant fluid

v = volume of known amount of capsular material used.

RESULTS

This work was undertaken to study the relationship between encapsulation and virulence of strains of Staphylococcus aureus and to determine possible resistance mechanisms active in the host. The amount of soluble capsular material elaborated by an encapsulated wound mucoid strain and a nonencapsulated variant of the wound mucoid strain was determined. The LD_{50} for these strains for embryonated eggs was calculated. This could give some indication of the possible relationship between degree of encapsulation and the virulence of the staphylococci. This thesis is also concerned with the paradoxical behavior of two encapsulated strains of S. aureus, one that is virulent for laboratory mice and one that is not. The virulent strain, the Smith diffuse variant, is commonly accepted as being encapsulated, but some investigators have failed to elicit a specific capsular reaction with this strain (Koenig, 1962; Morse, 1960, 1962a). The avirulent strain, the wound strain isolated by Wiley (1961), exhibited a specific capsular reaction. Although the wound strain of Wiley was less virulent for mice than the diffuse variant of Smith, it gave a positive specific capsular reaction, which in the opinion of some investigators, is the most definitive method for demonstrating capsules of S. aureus. This thesis is an attempt to

explain the possible paradox involved when one considers that both strains of S. aureus are encapsulated, but only the Smith diffuse variant is virulent for mice. However, both strains are virulent for embryonated eggs and evidence bearing on this part will be presented here.

Seven antisera prepared by injecting rabbits with the wound mucoid strain of S. aureus were tested to determine their hemagglutination titers. The antiserum showing the highest hemagglutination titer was selected to be used in all inhibition of indirect hemagglutination tests. The least amount of capsular material necessary to sensitize the rabbit erythrocytes was determined. All indirect hemagglutination and inhibition of indirect hemagglutination tests were carried out in triplicate. Sensitization of erythrocytes with the least amount of capsular material would prevent the possibility of excess capsular material on the erythrocytes. Table 1 shows the hemagglutination titer of the antiserum used and the least amount of capsular material necessary for sensitization of rabbit erythrocytes. It can be seen that rabbit erythrocytes can be sensitized with a 1:10,000 dilution of the purified capsular material (CM) and that four units of hemagglutinating antibody were contained in a 1:64 dilution of the serum.

Estimation of the amount of partially purified capsular material necessary to prevent hemagglutination was determined. This test was done with each inhibition of indirect hemagglutination test of the

TABLE 1

Indirect Hemagglutination
(Sensitization of Cells and Determination of Antibody Titer)

CM* dilution	Antiserum dilutions** 2	4	8	16	32	64	128	256	512	Controls*** 1	2	3
1,000	3+****	3+	3+	3+	3+	3+	3+	3+	3+	-	+	-
2,000	3+	3+	3+	3+	3+	3+	3+	3+	3+	-	+	-
4,000	3+	3+	3+	3+	3+	3+	3+	3+	3+	-	+	-
8,000	3+	3+	3+	3+	3+	3+	3+	3+	3+	-	+	-
10,000	3+	3+	3+	3+	3+	3+	3+	2+	±	-	+	-

* Reciprocal of the dilutions of capsular material used to sensitize the rabbit erythrocytes.

** Reciprocal of the dilution of antiserum.

*** Control 1 contained 0.62 ml saline plus 0.05 ml of sensitized cells; control 2 contained 0.12 ml of a 1:10 dilution of antibody, 0.5 ml saline, and 0.05 ml sensitized cells; control 3 contained 0.12 ml of a 1:10 dilution of antibody, 0.5 ml saline and 0.05 ml unsensitized cells.

**** 3+ = a carpet of cells folded inward at the edges
2+ = a complete carpet of cells covering the bottom of the tube
1+ = a partial carpet of cells with some unagglutinated cells
- = no agglutinated cells

supernatant fluid and served as a standard. From Table 2, the minimal amount of CM necessary to prevent hemagglutination was estimated. The average of the four calculations was 2.3 ugm. Therefore, 2.3 ugm of partially purified capsular material was the least amount capable of preventing hemagglutination by four hemagglutinating units of antibody.

The supernatants of the samples taken at 2, 4, 8, 16, and 24 hours were tested to determine the amount of capsular material elaborated by the wound mucoid strain of S. aureus and the nonencapsulated variant of the same strain. The results of these tests are shown in Table 3. It can be seen that there was an increased amount of capsular material in the supernatant fluid with an increased incubation period of the organism. A graphic representation of the average amount of CM is shown in Figure 4. The wound mucoid S. aureus produced 1,288 ugm/ml of capsular material in 24 hours when grown under increased CO_2; whereas, under normal atmospheric conditions 1,141 ugm/ml of capsular material was produced by the wound strain. The nonmucoid variant of the mucoid wound strain produced less capsular material than the wound mucoid strain regardless of the conditions under which it was grown.

Egg LD_{50} values (Table 4) were determined for virulence comparisons of encapsulated and nonencapsulated organisms. Although both the supernatant fluids of the encapsulated wound mucoid strain and the nonencapsulated variant of the same strain contained capsular

TABLE 2

Inhibition of Indirect Hemagglutination by Capsular Material (Standard)

CM* ugm/ml	Degree of inhibition of indirect hemagglutination — Volume of capsular material in milliliters									Controls**		
	0.50	0.45	0.40	0.35	0.30	0.25	0.20	0.15	0.10	1	2	3
50.00	4+***	4+	4+	4+	4+	4+	3+	3+	3+	-	+	-
25.00	4+	4+	4+	3+	3+	2+	2+	2+	2+	-	+	-
12.50	3+	3+	3+	3+	3+	+	+	-	-	-	+	-
10.00	3+	3+	3+	2+	+	+	-	-	-	-	+	-
6.25	3+	3+	2+	+	-	-	-	-	-	-	+	-
5.00	2+	2+	+	-	-	-	-	-	-	-	+	-

* Capsular material

** Control 1 contained 0.62 ml saline plus 0.05 ml of sensitized cells; control 2 contained 0.12 ml of a 1:10 dilution of antibody, 0.5 ml saline, and 0.05 ml sensitized cells; control 3 contained 0.12 ml of a 1:10 dilution of antibody, 0.5 ml saline and 0.05 ml unsensitized cells.

*** 4+ = No agglutinated cells
3+ = A large button of unagglutinated cells and a few agglutinated cells at the edges
2+ = A large button of unagglutinated cells and a zone of agglutinated cells
1+ = A small button of unagglutinated cells and a large zone of agglutinated cells
\- = A carpet of agglutinated cells folded inward at the edges

TABLE 3

Inhibition of Indirect Hemagglutination by
Culture Supernatant Fluids

Strain	Age of culture in hours				
	2	4	8	12	24
Wound mucoid grown	0*	62	192	460	1196
under normal	0	74	180	460	1073
atmospheric conditions	0	66	192	460	1153
Average		67	188	460	1141
Wound mucoid grown	0	41	240	805	1380
under increased CO_2	0	53	240	736	1196
atmospheric conditions	0	51	240	690	1288
Average		46	240	744	1288
Wound variant grown	0	59	160	394	987
under normal	0	59	160	402	1073
atmospheric conditions	0	50	160	362	987
Average		56	160	386	1016
Wound variant grown	0	44	167	345	789
under increased CO_2	0	50	167	358	854
atmospheric conditions	0	49	167	368	862
Average		48	167	357	835

* ug of capsular material measured in culture supernatant fluids

FIGURE 4

Determination of Capsular Material in Culture Supernatant Fluids of S. aureus by Inhibition of Indirect Hemagglutination

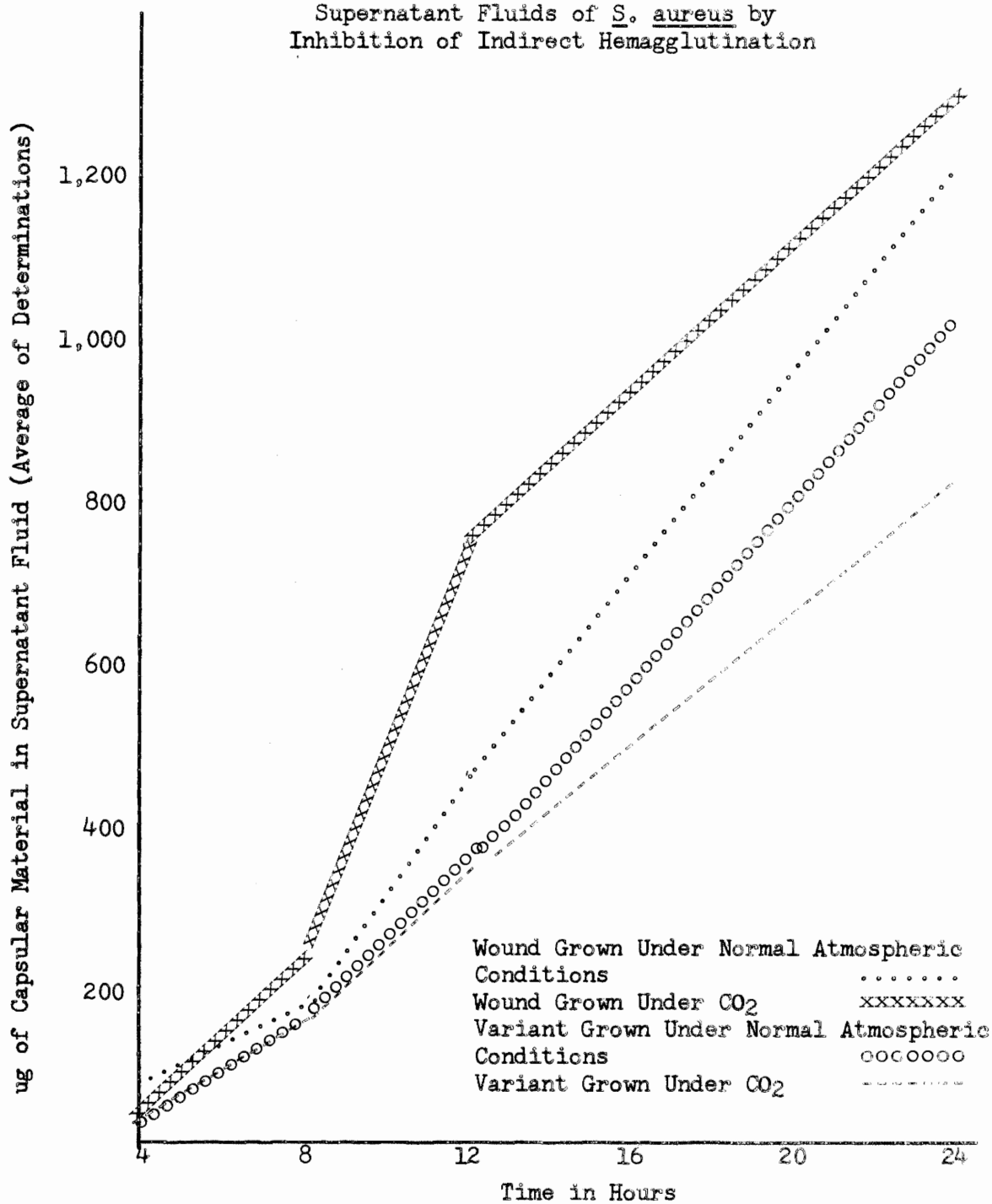

TABLE 4

Estimation of LD_{50} in 12-Day-Old Embryonated Hens' Eggs with the Encapsulated Wound Mucoid and the Nonencapsulated Variant of the Same Strain Injected iv

Strain	Number of organisms	
	LD_{50}*	95% confidence limits*
Wound mucoid	33	10-105
Wound variant	2512	1097-5755

* The LD_{50} values and the 95% confidence limits are expressed as the number of organisms producing a 50% kill.

material, there was a significant difference in their LD_{50} values for embryonated hens' eggs. A possible explanation for this finding was that the amount of capsular material in the supernatant fluids was not correlated with the amount of capsular material bound to the encapsulated wound mucoid strain.

Figure 5 illustrates a typical specific capsular reaction of the Smith diffuse strain after 18 hours of growth on a BNG agar plate. Homologous antiserum was prepared in roosters. Figure 5 depicts a negative reaction obtained with heterologous antiserum. The optimal conditions for demonstration of a positive specific capsular reaction differ for the wound strain and the Smith strain. The Smith strain shows capsules optimally from an 18-hour plate culture, and the wound strain shows capsules optimally in a 6- to 8-hour broth culture.

Table 5 reveals that each of the encapsulated strains has a serologically distinct capsule. The compact strain referred to in this paper is a nonencapsulated variant of the Smith diffuse strain of S. aureus.

The susceptibility of mice to the two encapsulated staphylococci and the lethality of mucin for mice were studied. Experiments in this laboratory indicated that high concentrations of mucin alone were lethal for mice. The effect of injecting 0.5 ml of 6, 5, 4, and 3% (W/V) mucin ip as an enhancer of staphylococcal virulence was investigated. The injection of 0.5 ml of 6% ip, but not 5% (W/V) mucin or less, killed four out of ten mice. The latter concentration

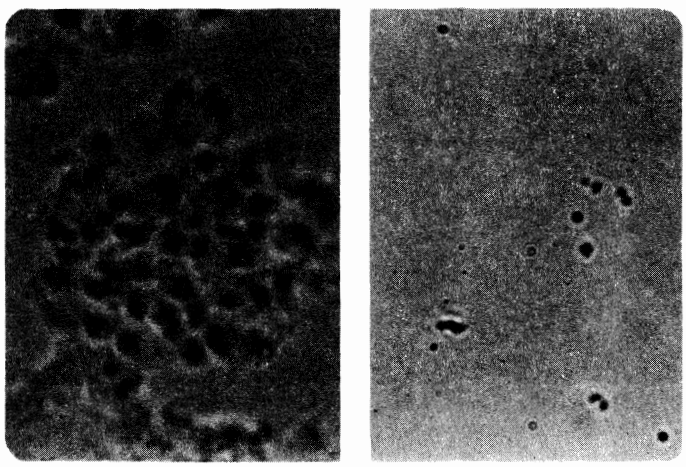

FIGURE 5. Specific capsular reactions of staphylococci: (a) positive specific capsular reaction of the Smith diffuse variant; (b) negative reaction obtained by using heterologous antiserum and Smith diffuse variant.

TABLE 5

Differentiation of Two Capsular Types by the Specific Capsular Reaction

Strain	Antiserum prepared against	
	Encapsulated wound	Smith diffuse
Wound mucoid	+	-
Wound variant	-	-
Smith diffuse	-	+
Smith compact	-	-

was used. To obtain an accurate viable count of the number of staphylococci injected, sonic treatment of staphylococcal suspensions was employed. In Table 6, LD_{50} values and their 95% confidence limits are expressed as the number of organisms producing a 50% kill. From these LD_{50} estimations given in Table 6 it is clear that a significant difference exists in the virulence of these two encapsulated strains for mice.

The virulent form of the Smith strain grows as a diffuse variant in serum soft agar. It was believed that organisms growing in this fashion do so because the serum lacks antibody to the staphylococci (Finkelstein and Sulkin, 1958; Hunt and Moses, 1958). The absence of antibody to a surface antigen of the Smith strain could explain the lowered resistance in mice to the Smith diffuse strain; and, conversely, the presence of antibody to such a surface component of the staphylococcus could account for the increased resistance in the host. The capsular antigen could be just such a surface component. If animals carried encapsulated staphylococci in their throat or rectum, then they could respond to the antigens of the microbe by producing antibodies.

To explore this line of reasoning, the carriage incidence of staphylococci in mice was assessed. Rectal and throat swabs were obtained from a group of mice and were then streaked on Mannitol salt agar plates. All mannitol-fermenting colonies were seeded to BNG broth for coagulase testing. Only those isolates that were

TABLE 6

Estimations of LD_{50} in 25- to 30-g Mice for the Wound Mucoid and Smith Diffuse Staphylococci Injected ip

Strain	Number of organisms	
	LD_{50}*	95% confidence limits*
Smith diffuse	12	4-32
Wound mucoid	550,000	350,000-850,000

* The LD_{50}'s values and the 95% confidence limits are expressed as the number of organisms producing a 50% kill.

coagulase-positive were considered to be S. aureus. The results of this survey are given in Table 7. The results of the survey showed an incidence of staphylococcal carriage of 61.5%. Of 60 mice, 37 harbored coagulase-positive staphylococci. A total of 45 coagulase-positive strains were isolated (some mice carried staphylococci in both the throat and rectum). Also, it was noted that two of the staphylococcal strains isolated from the mice were naturally encapsulated, and these strains reacted with an antiserum prepared against the wound strain.

In view of these observations, a survey of the incidence of capsular antibodies in Swiss albino mice was undertaken. From the same group of mice used above, blood samples were obtained and the serum was tested for the presence of anticapsular antibodies against the wound mucoid strain of S. aureus and against the strain of S. aureus carried by the mouse from which the blood was obtained. The results of these tests are presented in Table 8. Of the 60 sera tested, 33 gave a positive specific capsular reaction with the wound strain. This number represented 55% of the mice. None of the sera demonstrated a positive specific capsular reaction when tested against the S. aureus obtained from the same mouse as the serum. This finding, while unexpected, may be understood on the basis that the strains carried by the mice might not have been encapsulated and, hence, could not undergo a specific capsular reaction.

TABLE 7

Carriage of *Staphylococcus aureus* in 60 Mice

Site of carriage	Coagulase positive S. aureus carried* by: Number of animals	Per cent
Throat	19	31.6
Rectum	10	16.6
Throat & rectum	8 (16)**	13.3
Total	37	61.5

* Total number of strains isolated was 45.

** Sixteen isolates from a total of eight animals.

TABLE 8

Incidence of Specific Capsular Reactions in Mouse Sera

Test strain	Number positive	Per cent
Wound mucoid	33/60	55
Autologous S. aureus	0/60	0

Another survey was conducted on a group of 40 mice to ascertain whether antibodies against the encapsulated Smith strain of S. aureus and the encapsulated wound strain of S. aureus could be present simultaneously. The results of these specific capsular reactions are given in Table 9; 32.5% of the mice possessed capsular antibodies reactive with the wound strain, but none of the mice possessed capsular antibodies reactive with the Smith diffuse strain. The latter observation was not caused by the inability of the mice to make capsular antibodies against the Smith diffuse strain. All mice that survived the virulence tests of the Smith diffuse strain were injected with living Smith diffuse organisms. The first injection consisted of 500 staphylococci suspended in BNG broth. After 1 week, the mice were injected with 50,000 Smith diffuse organisms. The final injection was two weeks after the first, and a dose of 500,000 organisms was given to each mouse. The mice were bled seven days later, and their sera were pooled. The pool from these mice exhibited positive specific capsular reactions with the Smith diffuse strain.

TABLE 9

Incidence of Specific Capsular Reactions in Mouse Sera*

Test strain	Number positive	Per cent
Smith diffuse	0/40	0
Wound mucoid	13/40	32.5

* Another survey for anticapsular antibodies in 40 additional mouse sera.

DISCUSSION

The term specific capsular reaction has been used in this thesis instead of capsular swelling reaction or "quellung" because no data were available to indicate that an increase in capsular size occurred when specific antiserum and encapsulated staphylococci were combined. Tomcsik (1956) believed that the term "quellung" or capsular swelling was a misnomer. His observation suggested that the majority of encapsulated organisms showed no detectable swelling of the capsule when exposed to specific antiserum. Therefore, he proposed the term specific capsular reaction. Recently Baker et al. (1966), using the electron microscope, noticed that the capsular reaction phenomenon, at least in the case of Diplococcus pneumoniae type 1, was a true swelling of the capsule. When specific antiserum and organisms were mixed, the antigen-antibody reaction occurred only at the surface of the capsule. However, the cell wall, cytoplasmic matrix, and nuclear material were not affected. Price and Kneeland (1954, 1956) elicited a specific capsular reaction with a strain of S. aureus (RLM) and called the reaction capsular swelling. Wiley (1959, 1960, 1961) substantiated their findings but termed the phenomenon specific capsular reaction instead of capsular swelling or "quellung."

The present study showed that erythrocytes sensitized with capsular material prepared from the wound strain of S. aureus were

agglutinated by homologous staphylococcal antiserum. Gel double-diffusion tests showed three distinct precipitation lines when the partially purified capsular material was diluted 1:1,000 in buffered saline pH 7.2 and only one precipitation line when the partially purified capsular material was diluted 1:10,000. However, since the partially purified capsular material used for the sensitization of rabbit erythrocytes was diluted 1:10,000 in buffered saline and since it showed only one precipitation line at this concentration in gel double-diffusion tests, it was justifiable to ascribe the sensitization of rabbit erythrocytes as probably due to the capsular antigen. The specificity of the hemagglutination reaction was clearly shown by inhibition of hemagglutination by microgram quantities of the partially purified capsular material prepared from the wound strain of S. aureus. Capsular material from the encapsulated wound strain and a nonencapsulated variant of it was detected in culture supernatant fluids by inhibition of indirect hemagglutination. The encapsulated wound strain retained capsular material on its surface as shown by the specific capsular reaction while the nonencapsulated variant did not. The increased virulence of the encapsulated wound strain for embryonated hens' eggs as compared to the nonencapsulated variant derived from it was believed to be due to a better retention of capsular material. Similar differences in virulence between the Smith diffuse and the Smith compact strains of S. aureus for embryonated hens' eggs were reported by Lyles et al. (1965). Using freshly isolated strains of

S. aureus Wiley (unpublished data) showed a correlation between virulence for embryonated hens' eggs, production of coagulase, and amount of capsular material in the supernatant fluid. The strains that were the most virulent had the largest capsules and produced the greatest quantities of coagulase and capsular material.

In the present work the specific capsular reaction was used exclusively to demonstrate capsules on the Smith diffuse variant and to demonstrate their absence on the compact variant of the Smith strain of S. aureus. Morse (1962a), Koenig (1962) and Mudd and DeCourcy (1965) were unable to elicit a specific capsular reaction using the Smith strain, but workers in this laboratory succeeded in this endeavor by employing both rooster and rabbit antistaphylococcal serum. Morse used the Smith diffuse strain of S. aureus in all of his experiments (personal communication). India ink preparations of this strain showed that the organism was surrounded by an envelope structure (Morse, 1962a). Mudd and DeCourcy (1965) reported that the encapsulated Smith strain of S. aureus grew as a compact colony in normal rabbit serum. He reported that this staphylococcus was also surrounded by an envelope structure as demonstrated by the India ink method. Although this thesis does not dispute the validity of these observations, his data tend to obscure the basic question, namely, whether or not the virulent form of the Smith strain is encapsulated and grows as a diffuse colony in serum or plasma soft agar. Growth of the Smith strain of S. aureus in normal rabbit serum soft agar as a compact colony has

usually been interpreted (Koenig, 1962; Koenig et al., 1962a, 1962b; Koenig and Melly, 1965) as an indication of nonencapsulation and characteristic of the avirulent form of the Smith strain of S. aureus, but Mudd and DeCourcy (1965) reported that their culture of the encapsulated Smith strain of S. aureus grew as a compact colony in normal rabbit serum soft agar.

Electron photomicrographs of the Smith diffuse variant of S. aureus showed the presence of a capsule (Koenig and Melly, 1965). However, Koenig (1962) was unable to find any difference in the diffuse or compact Smith strains of S. aureus when these strains were examined in India ink preparations. To use wet India ink preparations as the only criterion for the demonstration of capsules of S. aureus could lead to confusion. It should be re-emphasized that the most definitive method for the demonstration of capsules of S. aureus is the specific capsular reaction.

In his research Mudd (1965) maintained that the encapsulated Smith strain of S. aureus should be considered as the prototype of encapsulated S. aureus strains and that the wound strain isolated in this laboratory should be considered as a representative of a phenomenon which he called the extracellular peripheral precipitation reaction (EPPR). The latter term, EPPR, seems to be only a circumlocution used to describe the specific capsular reaction. Mudd and DeCourcy (1965) would exclude the wound strain of S. aureus as being truly encapsulated because they would restrict true encapsulation to virulent naturally

occurring strains. The wound strain in its encapsulated state was obtained by the method employed by Bigger, Boland and O'Mera (1927). Its virulence in embryonated hens' eggs is indisputable (Wiley, 1961). Naturally occurring encapsulated S. aureus of the wound mucoid type have been isolated from rabbits, roosters, mice, and humans. Each isolate exhibited a positive specific capsular reaction upon initial isolation (Wiley, 1961, 1963). Wiley (1961) devised and employed a virulence test in embryonated hens' eggs which is well suited to the testing of the virulence of S. aureus isolates. In this test, the wound strain behaved as a virulent strain with an LD_{50} of less than 500 organisms. The finding of anticapsular antibodies that react with the wound strain in normal rabbits, roosters, mice, and humans (Wiley, 1961, 1963) further supports the validity of this position. Apparently, the wound strain is a major capsular type of S. aureus, but not the only one. Evidence presented in this thesis indicates, for the first time, that the Smith diffuse variant is also encapsulated, as shown by the specific capsular reaction. The wound mucoid strain and the RLM strain of Price and Kneeland represent members of a major capsular type against which antibodies are widely distributed (Wiley, 1961, 1963).

No detectable differences were found between the wound mucoid strain and the Smith diffuse variant, regarding the amount of hemolysin present in cultures. Differences in the amount of coagulase formed by each strain were not great enough to explain the difference in

virulence observed. Apparently, the best explanation is that the mice were resistant to the wound strain because they carried it, and they had elaborated antibodies against it. Our tests did not reveal that mice carried the Smith capsular type of S. aureus, nor could we detect anticapsular antibodies against the latter.

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