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CHAPTER I

INTRODUCTION

Study of the virulence of Staphylococcus aureus is complicated by its ability to produce a variety of metabolic substances, most of which have been associated at one time or another with its pathogenicity (Dubos, 1956; Blair, 1939, 1958; Elek, 1959). Solution of the problem of staphylococcal virulence may well depend on future purification and characterization of these substances. Furthermore, the lack of a susceptible, small experimental animal presents an immediate obstacle to the investigation of staphylococcal host-parasite relationships. Mice are relatively resistant to the ordinary strains of staphylococci encountered in human infections. Discovery of strains of unusually high virulence for mice could be an important step in the elucidation of staphylococcal host-parasite relationships. The present study includes an examination of mouse virulence of two such recently isolated strains of S. aureus, which differ from ordinary staphylococci in that their cells fail to clump in plasma or fibrinogen, although they are coagulase producers. The production of coagulase by the great majority of staphylococci isolated from human infections led to its association with the pathogenicity of the organism, and to the extensive investigation of its nature and its possible role in the virulence of the organism.

Coagulase

There is, at present, general agreement that coagulase production is the most reliable criterion of the potential pathogenicity of staphylococci. Only coagulase-positive staphylococci are isolated regularly from lesions. There is almost complete correlation between the ability of a staphylococcal strain to clot the citrated plasma of a given animal species and its capacity to produce disease in that species. Apparently coagulase-negative staphylococci fail to invade, or they are readily eliminated from the tissues of man and animals.

Loeb (1903) noted that staphylococci from clinical cases coagulated citrated goose plasma and he concluded that "Staphylococcus pyogenes aureus has a specific influence in causing coagulation of the blood," probably by means of an enzyme preformed in cultures. Earlier workers had observed the clotting of horse plasma by a variety of microorganisms, including certain infusoria, yeasts, and bacteria (Salvioli, 1894; Elek, 1959). Salvioli (1894) reported on the loss of blood coagulability when sterile filtrates of pyogenic staphylococci were injected intravenously into dogs. He also indicated that the same activity was effected by an alcohol-precipitable substance which was present in the filtrates of staphylococci but not in those of Proteus, vibrios or streptococci. Much (1908) reported that only the orange pigmented "aureus" strains, which he recovered from clinical cases, and not the white "albus" or yellow "citreus" strains of staphylococci, nor other bacterial species, clotted citrated human and horse plasmas but not sheep plasma. The name "staphylocoagulase" was given by Gratia (1920) to the enzyme-like staphylococcal product responsible for the coagulation of plasma. Daranyi

(1926) was the first to call attention to the usefulness of the plasma clotting activity of coagulase as an index of staphylococcal pathogenicity. Although it was first thought that living organisms were essential for the demonstration of coagulase, later studies clearly proved that bacteria-free, culture filtrates possessed this activity (Waltson, 1935; Lominski, 1944; Smith and Hale, 1944; Tager and Hales, 1947; Duthie, 1952, 1954a,b; Duthie and Haughton, 1958). Coagulation of citrated plasma by microorganisms other than staphylococci has been explained by their utilization of the citrate in the plasma (Harper and Conway, 1948; Evans et al., 1952; Mushin and Kerr, 1954; Wood, 1959).

When comparisons were made (Duthie, 1954a) of human plasma and those of different animal apecies (sheep, horse, cow, dog, guinea pig, mouse, and rabbit), only three human, horse, and rabbit) of the eight were clotted by coagulase. The observations of a number of investigators indicated that human and rabbit plasmas are the most susceptible to coagulase action. Horse, dog, pig, goat, calf, donkey, and goose plasmas are also regularly clotted by coagulase, whereas variable results are observed with sheep and ox plasmas (Elek, 1959). Guinea pig plasma is susceptible to coagulase action at 20 C but it is resistant at 37 C (Smith and Hale, 1944; Kaplan and Spink, 1948; Gerheim and Ferguson, 1949). Failure of fowl and mouse plasmas to be clotted by coagulase-positive staphylococcus has been noted (Smith and Hale, 1944; Gerheim and Ferguson, 1949). However, rare clotting of mouse plasma by staphylococci has been reported by Gorrill (1951).

Calcium is not required for coagulase action since its ionization was suppressed with citrates, oxalate, and fluoride (Much, 1908; Miale,

1949b). Heparin, chlorazol-fast pink azo dye, hydroquinone and cobra venom also fail to inhibit the plasma clotting activity of coagulase (Waltson, 1935; Miale, 1949b; Elek, 1959).

The most widely accepted hypothesis for the mode of action of coagulase involves a component of plasma which has been referred to variously as coagulase activator, accessory factor, coagulase globulin and coagulase-reacting factor or CRF (Smith and Hale, 1944; Tager, 1948a; Miale, 1949a; Duthie, 1952; Duthie and Lorenz, 1952). Tager (1956a) postulated the following sequence of reactions:

1. coagulase + coagulase-reacting factor (CRF) $\longrightarrow$ coagulase-CRF
2. coagulase-CRF + fibrinogen $\longrightarrow$ fibrin.

Another explanation of the coagulase clotting mechanism suggests that it is a proteolytic enzyme which activates prothrombin (Eagle, 1937). Waltson (1935) proposed that coagulase acts directly on fibrinogen. Recently Murray and Gohdes (1959) expressed the opinion that coagulase is a complete enzyme, capable of converting purified and commercial preparations of fibrinogen to fibrin; that the coagulase-reacting factor merely functions as an accelerator of the reaction which could be represented as follows:

$$\text{coagulase} + \text{fibrinogen} \xrightarrow{\text{CRF}} \text{fibrin}.$$

Little is known of the nature and kinetics of the reaction between coagulase and the coagulase-reacting factor which results in the active clotting agent. Smith and Hale (1944) and later Duthie and Lorenz (1952) agreed that when the two components, coagulase and its activator, are incubated together there is a progressive increase in the clotting activity of the mixture. More recently, Haughton and Duthie (1959a)

demonstrated that the active fibrinogen-clotting agent, i.e., activated coagulase, resembles human thrombin in certain respects. These authors presented evidence that coagulase activator is not permanently altered in the reaction, whereas coagulase is destroyed. Therefore they regarded the reaction as enzymatic in nature with coagulase serving as the substrate and the activator as the enzyme. According to this idea coagulase is enzymatically degraded by coagulase activator and the active clotting agent produced is either the enzyme-substrate complex or a degradation product of coagulase. However, as the authors point out, it is possible that coagulase may be destroyed by some factor in plasma other than the activator. Plasmin was excluded as being such a factor because coagulase destruction occurred in the presence of soybean inhibitor, which inhibits plasmin activity. On the other hand, the same workers reported that the synthetic amino acids ester, Nα-toluene-p-sulphonyl-l-argine methyl ester (TAME) which is hydrolyzed by plasmin and thrombin (Sherry and Troll, 1954) is also hydrolyzed by activated coagulase but not by unactivated coagulase. Furthermore, clotting by both thrombin and activated coagulase were inhibited by low concentrations of TAME which suggests that both act on fibrinogen in a similar manner.

Coagulase Production

Although coagulase may regularly be demonstrated in staphylococcal cultures of simple digest as well as infusion media, the conditions required for optimal coagulase production are largely unknown. Staphylococci will grow in relatively simple, protein-free media, but attempts to demonstrate the presence of coagulase in such chemically defined

media have been unsuccessful (Lominski et al., 1950; Szeto and Halick, 1958). Fisher (1936a) noted the enhancement of coagulase production by the addition of whole blood or plasma to various media. The addition of serum or egg yolk to media has also been found to stimulate coagulase production (Davies, 1951). There is evidence that the enhancing effect of the addition of serum to media is due to albumin (Duthie and Lorenz, 1952; Duthie, 1954b; Barber and Wildy, 1958). Lominski et al. (1953) emphasized the possibility that the presence of additional protein in the medium may protect against a coagulase-destroying factor. Lack and Wailing (1954) suggested that albumin may not actually increase coagulase production, but rather it may protect fibrinogen and fibrin against lysis by plasmin. Szeto and Halick (1958) found that cultivation of staphylococci in a dialyzate of heart infusion broth resulted in high titers of coagulase and made its concentration convenient.

Variation in the coagulase yield of different strains of staphylococci is common even when they are grown in the same batch of medium or in a chemically defined medium, and even within populations of a single strain, variants can be found which differ in respect to degree of coagulase production (Smith et al., 1952). High yields of coagulase were associated with R variants and low yields with the S type of growth and the changes in coagulase production observed in the case of some staphylococcal strains were related to a changing proportion of R and S variants within the strain. It was noted, however, that the variants were not absolutely stable; that there was a greater tendency for the R form to give rise to the S form than subsequent variation in the opposite direction. For the selection of high coagulase producing variants, which

incidentally are thought to occur rarely in nature Duthie and Lorenz (1952), Boake (1956), Barber and Wildy (1958) used fibrinogen-plasma-agar plates. Colonies of the "fast-clotting" variants, as indicated by the size of the zone of opacity around the colonies, were often small and rough in appearance. However, a direct relationship between the size of the opaque zone and production of coagulase in broth could not be demonstrated. Several methods have been used by various workers for titration of coagulase production in liquid culture media. Basically these methods measure either the time necessary for the development of a clot or the highest dilution of the coagulase preparation which causes clot formation (Tager and Hales, 1947; Tager, 1948b; Duthie, 1954a,b; Duthie and Haughton, 1958; Szeto and Halik, 1958).

Purification and Characterization of Coagulase

Tager (1956a) obtained a 300-fold purified product of coagulase from cell-free supernatants of cultures grown in brain-heart infusion broth to which a mixture of trace ions had been added. The purification was effected by precipitation of the active principle with acetate buffer, purification by repeated treatment of the precipitate with ethanol in the cold at controlled pH, and separation of impurities from the final product by use of low concentrations of ammonium sulfate. The purified product was not dialyzable through Visking membranes. Its nitrogen content was 14 to 16 per cent; it was inactivated rapidly by crystalline trypsin, chemotrypsin and plasmin. In contrast to the relative thermostability of crude coagulase, it was heat labile. Chromatography of hydrolyzed coagulase indicated the presence of aspartic acid, hydroxyproline or tyrosin, histadine and tryptophan or valine and, thus,

gave further evidence of its protein nature. Tager reported that his purified product was not completely homogeneous on electrophoresis or ultracentrifugation.

Duthie and Haughton (1958) concentrated coagulase from filtrates of casein-hydrolysate medium cultures by cadmium precipitation. Electrophoretic studies showed their product to have an iso-electric point around pH 5.3, and ultracentrifugation of the preparation indicated it had a minimum molecular weight of approximately 44,000. The purified product of Tager as well as that of Duthie and Haughton was highly active biologically: intravenous injection into rabbits caused immediate death; a concentration of 75 uug/ml clotted human plasma in 24 hours and the clotting time was inversely proportional to the concentration of the coagulase.

The filterability of coagulase is controversial. In the hands of certain workers Seitz filtration removed all coagulase activity; whereas others did not find this to be so (Waltson, 1935; Fisher, 1936a; Lominski, 1944). Conflicting results were also obtained when Berkfeld and Chamberland filters were used (Waltson, 1935; Miale, 1949a). Growth of the staphylococci in a medium containing 10 to 25 per cent human plasma considerably increases the filtrability of coagulase or of what may be the resulting active, plasma clotting principle (Lominski, 1944). Coagulase filtrability apparently is affected by the pH of the culture; coagulase produced in broths without plasma was filterable through Seitz pads when the pH was adjusted to 6.7 or less (Lominski and Milne, 1947). Elek (1959) suggested that the filterable product produced in the presence of 10 to 25 per cent plasma was, in fact, not coagulase, but the coagulase-

thrombin complex or the activated coagulase of Haughton and Duthie (1959a) which is readily filterable and which acts directly on fibrinogen.

Recently, Drummond and Tager (1959a,b), associated a second biological activity with coagulase: an esterase activity which has a marked specificity for tributyrin. However, their results suggest that the esterase and the plasma clotting activities of coagulase reside in separate molecular entities, as indicated by studies of the differential effects of heat, trypsin, antiserum fractions, and certain enzyme inhibitors. Also, a physical separation of tributyrinase and the clotting principle was achieved by cellulose column zone electrophoresis.

The antigenicity of coagulase is well established (Tager and Hales, 1948b; Rammelkamp et al., 1949). The existence of antigenically distinct coagulases was first indicated by the studies of Rammelkamp and coworkers (1950) who recognized three antigenic types of coagulase produced by strains of staphylococci isolated from human sources. Duthie (1952) added a fourth antigenic type of coagulase which was produced by a strain of bovine origin. Barber and Wildy (1958) correlated antigenic types of coagulase and bacteriophage groups. These workers also confirmed previous reports of the common occurrence of coagulase neutralizing antibodies in human sera and of the observation that an individual may have antibodies to one type of coagulase but not to another (Rammelkamp et al., 1950; Duthie, 1952).

Coagulase-Reacting Factor

The identity of the coagulase-reacting factor (CRF) and its relation to the known components of the physiological blood clotting mechanism has remained an enigma since it was first proposed by Smith and

Hale in 1944. Many observers believe the key to the problem lies in the relation of the CRF to prothrombin (Smith and Hale, 1944; Tager and Hales, 1948a; Duthie and Lorenz, 1952; Tager, 1956a,b; Haughton and Duthie, 1959a,b). Studies of human prothrombin preparations indicate CRF activity and suggest that prothrombin and CRF may be identical.

Maximum CRF activity has been associated with globulin fractions prepared according to the Mellanby technique or by precipitation with ammonium sulfate at concentrations between 33 and 50 per cent saturation. By ethanol precipitation following the method of Cohn, the CRF activity appears to reside in globulin fractions III-1, III-2, and IV-1. Albumin (Fraction V) contains no CRF activity nor does purified fibrinogen (Fraction I). The CRF activity of crude albumin and fibrinogen preparations are believed to be due to impurities since these two plasma fractions after further purification or crystallization are completely devoid of CRF activity (Tager, 1948a; Miale, 1949a; Tager, 1956a).

The physico-chemical behavior of CRF supports the view that it is protein in nature. Lipoid extracted plasma was found to retain its original CRF activity (Tager, 1948a). Smith and Hale (1944) reported the factor to be heat labile. However, Tager and Hales (1948a) found that slight CRF activity persisted after heating it to 70 C for 30 minutes; and seemingly boiling is required to inactivate it completely (Tager and Hales, 1948a; Kaplan and Spink, 1948; Miale, 1949a). Coagulase reacting factor is destroyed by pepsin as well as by dilute sodium nitrite (Kaplan and Spink, 1948).

Coagulase reacting factor is present in high titers in human and rabbit plasma. Rabbit lung and kidney extracts have only 1/10 the CRF

potency of the plasma, and the CRF content of other tissues appears to be about 1 per cent that of plasma. Extracts of rabbit brain and of human platelets show little CRF activity. Only slight variation has been observed in the amount of CRF in different samples of human plasma (Tager and Hales, 1948c).

Clumping Factor

Much (1908) observed that the addition of a suspension of coagulase-positive staphylococci to a drop of citrated human plasma results in an immediate clumping of the organisms. He also noted clumping of coagulase-positive staphylococci in fibrinogen solution although this solution was not coagulated by the staphylococci. Birch-Hirschfeld (1934) noticed that when coagulase-positive staphylococci were emulsified in a drop of plasma on a slide, rapid clumping of the organisms occurred; that clumping of staphylococci in plasma agreed closely with their plasma clotting property. The plasma of man, horse, calf, and guinea pig were active for staphylococcal clumping regardless of whether oxalate, fluoride, citrate or heparin was used as the anticoagulant. Birch-Hirschfeld demonstrated that crude fibrinogen was responsible for the clumping phenomena, and she thought that clumping in plasma and coagulation of plasma by staphylococci may not be due to the same mechanism. She concluded that in the slide test an agglutination of staphylococci was caused by prothrombin and that in the tube test clotting of fibrinogen was effected.

The significance of the clumping of staphylococci in plasma as an index of coagulase production and, therefore, of potential pathogenicity was soon recognized (Christie, 1940). Cadness-Graves *et al.* (1943) made a large scale comparison of the tube coagulase test and the slide

agglutination test and found a good correlation between them. Of 442 strains recovered from human clinical specimens, all but two gave a positive reaction in both tests. It was commonly assumed that both tests depended on the activity of a single substance, coagulase (Berger, 1943). Linsell and Gorrill (1951) threw some doubt on this assumption when they found that plasma treated by heat or thiomersalate (merthiolate) continued to give a positive slide agglutination test although it failed to clot with the same staphylococci.

Duthie (1954a) presented further evidence that the tests were expressions of different factors and that the "two forms of staphylococcal coagulase" were antigenically distinct. Diluted (1:10) human or rabbit plasma, heated at 56 C for four minutes, no longer clotted with coagulase or with thrombin, whereas it clumped the cocci, though more slowly and less strongly than did unheated plasma. Plasma adjusted to pH 2 and then neutralized behaved in the same way as heated plasma. Duthie designated the factor responsible for clumping of the organisms in plasma as "bound coagulase" or, preferably, "clumping factor" in contrast to the "free coagulase" which causes the clotting of citrated plasma. He concluded that the clumping factor (a) is bound to the cell wall and is released only on cell disintegration or autolysis; (b) acts directly on the fibrinogen of certain animals to cause it to precipitate and thus clump the cells. Free coagulase, he stated, is liberated into the medium during growth; it acts on prothrombin, or some closely related substance, and converts it to a thrombin-like product which will clot any fibrinogen. In his comparative study of the plasma of different animal species (man, sheep, guinea pig, cow, horse, mouse and rabbit) as a source of

activator (CRF) for free coagulase and of fibrinogen which was sensitive to the clumping factor, only 3 of the 8 plasmas (human, horse and rabbit) were good sources of CRF and were clotted readily by free coagulase, whereas all but guinea pig and sheep fibrinogen reacted with the clumping factor.

The agglutination of hemolytic streptococci in sera and plasma from patients suffering from acute bacterial infections (scarlatina, erysipelas, typhoid fever, and pneumococcal pneumonia) has been reported (Weaver, 1904; Tillett and Abernethy, 1932; Tillett and Garner, 1934). The reaction appeared to be caused by a protein which resembled fibrinogen rather than by specific antibodies. When such strains of streptococci were incubated at 37 C in high dilutions of plasma or fibrinogen and then allowed to settle in the cold, they were agglutinated (Tillett and Garner, 1934). Duthie (1954a) suggested that streptococci produce a substance similar to the clumping factor of staphylococci, and that the serum substance involved is a partially denatured fibrinogen similar to that obtained by acid or heat treatment of plasma. Duthie (1955) investigated the action of fibrinogen on certain pathogenic cocci (staphylococci, streptococci and pneumococci), and reported that streptococci of group A, C, and G, but not of other groups, adsorb fibrinogen as do staphylococci but that the streptococci do not proceed to the second or clumping stage as do the staphylococci.

Strong evidence that coagulase and clumping factor are, indeed, separate entities is supplied by recognition of rare strains of staphylococci which possess either coagulase or clumping factor activity, but not both of these properties (Cadness-Graves et al., 1943; Needham

et al., 1945; Williams and Harper, 1946; Duthie, 1954a; Klemperer and Haughton, 1957; Jenkins and Metzger, 1959). However, comparative studies showed that, in general, coagulase-positive staphylococci also clumped in plasma, and thus supported the earlier recommendation of the slide test for the presumptive recognition of potentially pathogenic staphylococci. Rare strains which lack either coagulase or the clumping factor have been isolated from nasal swabs and body surfaces, and they have been considered to be of doubtful pathogenicity. Duthie (1954a, 1955) claimed that strains of coagulase-positive staphylococci which failed to give a positive clumping reaction usually clumped in plasma or fibrinogen, though never in high titer, after several subcultures. He assumed that either the plasma clotting tube test or the slide clumping test may serve as a satisfactory guide to the pathogenicity of staphylococci which are recovered from lesions.

Partial purification of the clumping factor by precipitation with cadium followed by dialysis in acid was achieved by Duthie (1954a). Study of this preparation revealed that clumping factor resists boiling but is destroyed by autoclaving and proteolytic digestion. Like free coagulase, it is more stable in the acid than in the alkaline range. In the same report, Duthie noted that strains of staphylococci may differ in degree of clumping ability as measured by the dilution of fibrinogen or plasma in which the clumping reaction occurred. Steward and Kelly (1959) observed variation of clumping factor activity with reduction or loss of this activity, at different rates in individual strains, after storage of cultures and cell suspensions.

Antibody to the clumping factor has been demonstrated in commercial

antitoxin, and also in antiserum produced by immunization of rabbits with clumping factor preparations of autolyzed cultures that have been partially purified and adsorbed on aluminum phosphate. Antisera which was produced by the intravenous or intracardiac injection of heat-killed staphylococci into rabbits or guinea pigs contained antibodies against the clumping factor. Clumping factor antibody apparently inhibits the absorption of the fibrinogen to the surface of the cocci and thus their clumping in plasma or fibrinogen, but does not inhibit free coagulase activity. Conversely, free coagulase antibody failed to neutralize clumping factor activity. Although antibodies to free coagulase seem to be specific for a given antigenic type of coagulase, there is no evidence for multiplicity of antigenic types of clumping factor (Duthie, 1954a, 1955). Though the presence of specific clumping factor antibody in normal human sera has not been fully investigated (Duthie, 1954a), the use of fibrinogen solution instead of plasma for the demonstration of clumping factor by the slide test has been advocated (Berger, 1943; Duthie, 1954a; Spencer, 1954) to avoid false positive reactions due to staphylococcal agglutinins which may be present in plasma (Hodgson, 1943; Jensen, 1958).

Methods for Detection of Coagulase and Clumping Factor

The plasma clotting tube test is used routinely and the slide agglutination test less commonly for the detection of potentially pathogenic staphylococci. The merits of both tests and the conditions necessary for reliable results in each have been discussed in detail by Elek (1959).

For the demonstration and estimation of the degree of clumping

factor activity, Duthie (1954a) substituted a tube method for the so-called slide agglutination test.

The plasma agar plate method, in which a high concentration (10 to 20 per cent) of plasma is incorporated into the agar culture medium, has been used with some success for the demonstration of coagulase activity (Penfold, 1944; Reid and Jackson, 1945; Williams and Harper, 1946; Clapper and Wood, 1954; Lack and Wailing, 1954). In this method, the action of coagulase results in a zone of precipitation of fibrin around the staphylococcal colony. Some workers have similarly used fibrinogen agar plates which contained small amounts of plasma or serum (Duthie and Lorenz, 1952; Boake, 1956; Klemperer and Haughton, 1957). False negative results were encountered in the case of highly fibrinolytic strains of coagulase-positive staphylococci which digested the precipitated fibrin and thus cleared the zone around the colonies (Duthie and Lorenz, 1952; Lack and Wailing, 1954). The incorporation of traces of soybean trypsin inhibitor into the agar medium was shown to obviate this difficulty in the plate method (Lack and Wailing, 1954; Lack, 1957).

Griffith and Ostander (1959) described a simple and inexpensive capillary tube method for the determination of the coagulase reaction. After 18 hour incubation, the results of the capillary tube test were in full agreement with the standard tube test.

Finkelstein and Sulkin (1958) reported on the colonial characteristics of coagulase-positive and coagulase-negative staphylococci in serum-soft agar. All strains produced elongated, feathery colonies in a basal medium containing 0.15 per cent agar. On addition of 1 per cent normal or immune plasma or serum, the coagulase-positive staphylococci

formed compact, round colonies, whereas the colonies of coagulase-negative strains were elongated and essentially like those grown in the basal soft agar medium. The authors explained this observation on the basis of antigen-antibody reaction.

Virulence of Staphylococci

Role of Coagulase

Despite general acceptance of a positive coagulase test as the best indication of a potentially pathogenic staphylococcus, little is known about the exact role and significance of coagulase as a virulence factor (Rammelkamp and Lebovitz, 1956). It is generally held that coagulase, in some way, facilitates the *in vivo* establishment of staphylococci either by rendering them more resistant to phagocytosis (Hale and Smith, 1945; Smith *et al.*, 1947; Rogers and Tompsett, 1952; Selbie and Simon, 1952; Lack and Wailing, 1954; Tompsett, 1954, 1956; Jackson *et al.*, 1955) or by protecting them against the bactericidal activity of serum (Spink and Vivino, 1942; Ekstedt and Nungester, 1955; Ekstedt, 1956).

In vitro effect of coagulase on the antistaphylococcal action of serum. Coagulase-positive staphylococci grow luxuriantly in normal human serum which inhibits growth of coagulase-negative staphylococci. Pillemer *et al.* (1954) noted the resistance of coagulase-positive staphylococci to the properdin system. It was postulated that free coagulase, or a substance associated with it, may be a factor destroying the bactericidal properties of serum. The antistaphylococcal serum factor was shown to be heat labile, and not specific in the immunological sense (Ekstedt, 1956).

Recently, Wlodarczak and Jeljaszewics (1959) demonstrated that coagulase-negative staphylococci are not always destroyed by serum. They found that, in most instances, the number of bacteria which survived 24 hours incubation in serum was about the same as before incubation. The same workers reported that fresh human serum had full bactericidal action, while those stored in frozen state were bacteriostatic, and those stored at 4 C for seven days were neither bacteriostatic or bactericidal. These workers confirmed previous observations (Ekstedt, 1956; Ekstedt and Yotis, 1959) that the addition, to serum, of a cell-free Seitz culture filtrate containing a high concentration of coagulase will establish the growth of coagulase-negative staphylococci in the serum, but they also found that preparation of purified coagulase did not promote the growth of coagulase-negative staphylococci in human serum. It has been shown that the addition of partially purified coagulase to normal serum stimulated the respiration of both coagulase-positive and coagulase-negative staphylococci; however, the resulting increase in the respiratory rate of the coagulase-negative staphylococci was twice as great as that of the coagulase-positive organisms (Ekstedt and Yotis, 1959; Yotis and Ekstedt, 1959). Evidence does not justify the idea that coagulase per se is the factor which impairs the antistaphylococcal activity of human serum. The identity of this factor has yet to be determined (Wlodarczak and Jeljaszewics, 1959).

In vitro effect of coagulase on phagocytosis. Depression of phagocytosis by staphylococcal products was reported by Pike (1934) who suspected the presence of a leucocidin in the culture filtrates. The ability of coagulase to inhibit phagocytosis was advanced by Hale and Smith

(1945) as an explanation for the pathogenicity of coagulase-positive staphylococci. Previously, the same workers (Smith and Hale, 1944) had shown that the correlation between coagulase production and pathogenicity held only when the plasma of the animal concerned was clotted by coagulase. Thus, they postulated, the inhibition of phagocytic activity may be an important, if not the factor which determines the host range of staphylococci with the susceptible animal species comprising those whose plasma is clotted by coagulase. Their studies of phagocytosis of staphylococci in defibrinated and citrated human plasma revealed less effective phagocytosis of coagulase-positive than coagulase-negative strains. Their observations of stained smears suggested that coagulase-positive staphylococci resist phagocytosis due to the presence of a protective fibrin coat around these organisms. Furthermore, phagocytosis of coagulase-positive staphylococci was inhibited when the organisms were first treated with plasma, whereas serum was without this effect.

Rogers and Tompsett (1952) using a suspension of human polymorphonuclear leukocytes in plasma, observed an initial rapid fall in the number of culturable coagulase-positive and coagulase-negative staphylococci, but, thereafter, they noted a significant difference in the rate of the two types of organisms. Whereas the number of coagulase-negative staphylococci continued steadily to decline, the number of viable coagulase-positive organisms, after an initial 4 to 8 hour decline, rapidly increased and multiplication soon assumed a logarithmic form. The latter course of events was associated with a steady fall in the total leukocyte count. Prior removal of fibrin from the system did not influence the sequence for either type of organism. It was evident that coagulase-

positive staphylococci survived phagocytosis by human polymorphonuclear leukocytes, whereas coagulase-negative staphylococci did not. The same results were obtained when Tompsett (1954) studied the fate of staphylococci within mononuclear phagocytes obtained from rabbits. Electron microscopic studies of the interaction between staphylococci and human neutrophils further confirmed the ability of coagulase-positive staphylococci to survive within the phagocytes (Goodman and Moore, 1956; Goodman et al., 1956). Thus, as in the case of Brucella (Shaffer et al., 1953), tubercle bacilli (Mackaness, 1952) and Neisseria (Thayer et al., 1957), coagulase-positive staphylococci may find, within the host cell, a shelter from unfavorable body climates (Rogers, 1959). Braude and Feltes (1953) indicated however that the initial fall in the number of culturable organisms may be inherent in the experimental method. The authors suggested that the intracellular and extracellular clumping of the cocci would lower the count of culturable organisms.

Recently, Kapral and Shayegani (1959) used a tissue culture procedure which permitted quantitative evaluation of the intracellular survival of staphylococci. They reported that normal human and rabbit leukocytes did not permit intracellular multiplication, but only survival of virulent staphylococci; that, within human and rabbit leukocytes, coagulase-negative staphylococci were destroyed. On the other hand, rat monocytes destroyed both coagulase-positive and coagulase-negative staphylococci. The experiments of Kapral and Shayegani were performed in the presence of streptomycin in order to control extracellular multiplication of the organisms as well as in its absence.

Cohn and Morse (1959) investigated the fate of staphylococci in

suspensions of rabbit polymorphonuclear leukocytes by a method which simultaneously evaluated both phagocytosis, i.e., ingestion, and intracellular inactivation of the bacteria. Coagulase-positive strains of S. aureus were not efficiently ingested in the presence of normal rabbit serum, whereas coagulase-negative staphylococci were rapidly engulfed and inactivated. Immune serum which was prepared against a coagulase-positive strain and which contained thermostable opsonins for this strain, enhanced the ingestion of the homologous organism as well as of three heterologous strains of S. aureus. Once the staphylococci were ingested by the phagocyte, prompt intracellular killing of the organisms occurred. A comparison between polymorphonuclear leukocytes obtained from normal and immune animals revealed no difference in their ability either to ingest or to kill coagulase-positive staphylococci. The fact that staphylococcal antibodies are common in human sera may explain previous reports that virulent and avirulent strains of staphylococci are ingested with equal avidity by phagocytes.

In vivo action of coagulase. Whether or not the in vitro plasma clotting activity of coagulase also occurs in vivo is controversial. Fisher (1936a) found that intravenous injection of large doses of culture filtrates of coagulase-positive staphylococci into rabbits failed to produce evidence, either during the life of the animal or at postmortem, of intravascular clotting. He concluded that coagulase action is primarily a test tube phenomenon. However, more recently, the intravenous injection of small amounts of purified coagulase, free of alpha hemolysin, has been found to be lethal for rabbits (Tager, 1956a; Duthie and Haughton, 1958). Smith and Johnstone (1956, 1958) claimed that coagulase

can exercise its clotting activity in vivo. The intravenous injection into rabbits of a potent preparation of purified coagulase produced fibrinogen depletion and intravascular clotting with multiple fibrin deposits occluding capillaries and small vessels especially in the lungs and to some extent in liver and kidneys. On the other hand, mice survived the intravenous injection of the same coagulase preparations and no changes were observed in sacrificed mice.

Evidence for coagulase as a virulence factor in experimental staphylococcal infections. Studies of staphylococcal virulence in experimental animals have recently been reviewed by Elek (1959). A variety of animal species, including the rabbit, mouse, guinea pig, rat, hamster, adult birds as well as the developing chick embryo have been used. The virulence of staphylococci for various animal species has been related to the susceptibility of the animal plasma to clotting by coagulase (Hale and Smith, 1945). Animals, such as guinea pigs and mice whose plasma is generally not clotted by coagulase are relatively refractory to staphylococcal infection. However, infection has been produced experimentally in an occasional guinea pig whose plasma was clotted by certain strains of staphylococci (Smith et al., 1947). Although enormous intravenous doses of coagulase-positive staphylococci (more than 200 million viable organisms) were required to kill mice, virulence of these organisms was enhanced if they were suspended in a coagulable menstruum before intravenous or intraperitoneal injection into mice. Likewise, the production of abscesses in the guinea pig was made possible by subcutaneous injection of staphylococci at a site previously prepared by introduction of a coagulable plasma from another animal species. These results suggested that

coagulase plays a major role in the initiation and development of the staphylococcal lesion, probably by laying down a fibrin barrier around the organisms, after which other factors, such as toxins, come into action.

Boake (1956) reported on the protective effects of anti-coagulase in experimental staphylococcal infections. Rabbits actively immunized with coagulase were more resistant to intravenous challenge with coagulase-positive, but not with coagulase-negative staphylococci. Boake injected mice intraperitoneally with coagulase-positive staphylococci suspended in a clotting system (fibrinogen and CRF). Serum from rabbits immunized with coagulase preparation diminished the virulence of these organisms when it was allowed to come in contact with them for 30 minutes before the addition of the clotting system. Similar results were obtained, under the same conditions, by using human serum which contained coagulase inhibiting antibodies. This diminution of virulence was not effected by alpha antitoxin or by staphylococcal agglutinating serum. Boake concluded that the decrease in virulence observed in mice was due to neutralization of coagulase and that the active immunity observed in rabbits was probably associated with the in vivo inhibition of coagulase activity.

More direct evidence of the enhancing effect of coagulase on staphylococcal virulence was reported by Ekstedt and Yotis (1959). When coagulase-negative staphylococci were treated with purified coagulase and a standard dose (1 to 5 million organisms) was injected intracerebrally into mice, as high as 72 per cent mortality resulted instead of the usual 1 per cent mortality produced by the untreated staphylococcal

suspensions.

The influence of route of injection on mouse susceptibility to staphylococcal infections is not clear. A good correlation was shown between the severity of the disease in the patient and the pathogenic effect of staphylococci on mice when injected intravenously (Smith et al., 1947) or intraperitoneally (Fisher and Thompson, 1956). The studies of Christie et al. (1946) and Lack and Wailing (1954) suggested the relative resistance of mice to staphylococci injected via the intraperitoneal route. Fisher and Thompson (1956) indicated that if very large inocula are used for the intraperitoneal injection of mice, death of the animals may result when potentially virulent staphylococci are used. Gorrill (1951) remarked that mice are extremely resistant to staphylococci injected intraperitoneally or intranasally, but are susceptible to the intravenous injection of coagulase-positive staphylococci. In contrast, Dutton (1955) observed that staphylococci were less lethal for mice by the intravenous route than by the intraperitoneal or subcutaneous routes.

Mice have been found susceptible to the intravenous injection of coagulase-positive staphylococci. The intravenous injection of 0.1 ml overnight broth culture which contained 400 million cells of coagulase-positive staphylococci proved lethal for mice, whereas similar doses of coagulase-negative strains were ineffective (Gorrill, 1951). Eleven out of the 12 coagulase-positive strains tested by Gorrill clotted mouse plasma regularly, while the other strain clotted 2 out of 8 different mouse plasmas (Gorrill, 1951).

Smith and Dubos (1956a) injected mice intravenously with graded doses, 0.03 to 0.6 ml, of 18 hour broth cultures of staphylococcal strains

that failed to clot mouse plasma. Whatever the dose injected, coagulase-negative and weakly coagulase-positive strains caused no death in mice. In contrast, all coagulase-positive staphylococci caused a certain percentage of deaths, depending on the strength of coagulase production, when the injected dose was 0.1 ml of cultures. Strong coagulase producers were lethal in various percentages when 0.03 to 0.1 ml doses were injected. Although virulence appeared to be correlated with the production of coagulase, it did not seem however to depend upon the activity of this substance to interfere with the bactericidal mechanism of the mouse organs during the early phase of infection. Virulence was manifested by kidney abscess formation from few cocci that escaped initial destruction.

Subsequently, Gray et al. (1957) made a systemic investigation similar to that of Smith and Dubos (1956a) to further characterize experimental staphylococcal infection of mice by histopathological, hematological, and quantitative bacteriological studies. When introduced by the intravenous route, an inoculum of 0.2 ml of coagulase-positive staphylococcal broth culture containing 100 million cells was effective and a 15 per cent mortality in 120 infected animals was recorded. The majority of these deaths occurred between the 7th and 14th days after infection. In general, the results of Gray and his group were in agreement with those of Smith (1956) and Smith and Dubos (1956a). There was an initial high bacterial count in the various organs followed by a steady decline after which there was a slight rise at the 9th to 14th day, except for the kidneys in which there was an actual increase in the number of organisms recovered. This increase reached its maximum between the 5th

and the 7th day after which there was a levelling off. Multiple abscess formation in the kidneys was most extensive 10 days post infection. The histopathological picture revealed the development of suppurative nephritis, pyelonephritis and urocystitis.

The development of abscesses in the mouse kidney, following intravenous inoculation of coagulase-positive staphylococci was investigated further by Gorrill (1958). The studies of Gorrill included a comparison of the death rates and the rates of renal infection; the latter was found to be a more sensitive indicator of infection. Graded doses of 0.5 ml, which contained 2.7×10^5 to 2.1×10^7 of saline suspended cocci, were injected intravenously. A roughly linear relationship was obtained between the number of kidneys infected or the number of mice killed and the logarithm of the dose injected. This led the author to the use of K.I.D. 50 dose, which is the number of cocci which on intravenous injection into mice will infect half the kidneys. Thus, it was possible for him to relate the number of cells deposited in the kidney to the frequency of renal infection. Following the intravenous injection, the organisms were distributed throughout the body with the vast majority in the reticuloendothelial tissues. It was thought that few cocci are deposited in the kidneys, possibly purely mechanically trapped in vessels or by being trapped in vessels which were closed by physiological changes. The number of organisms lodged in the kidneys depended on the size of the dose; with larger doses, some would divide and establish themselves before the cellular defenses of the body are alerted. Gorrill also suggested that abscess formation in the kidneys leads to the death due to disturbed renal function and toxemia. Infection may however be overcome gradually

and the damage is repaired with fibrous tissue. These findings are essentially in agreement with those of other investigators (Smith et al., 1947; Smith and Dubos, 1956a; Gray et al., 1957; Rogers, 1959).

Other changes observed in mice infected with staphylococci via the intravenous route included marked neutrophilia (Gray et al., 1957) as well as dehydration and loss of weight (Smith and Dubos, 1956a; Gray et al., 1957; Sellers and LeMaistree, 1960). Lesions were occasionally observed in organs other than the kidney, especially in the lungs and spleen (Smith, 1956; Smith and Dubos, 1956a; Gray et al., 1957; Sellers and LeMaistree, 1960). In some mice there were also myocardial abscesses and areas of acute interstitial pneumonia (Gray et al., 1957).

The intraperitoneal route of injection for the study of virulence of staphylococci for mice has been used by several investigators. On the average, high numbers of staphylococci were required to produce lethal effects in mice (Christie et al., 1946; Howard, 1954; Lack and Wailing, 1954; Fisher and Thompson, 1956; Hunt and Moses, 1958; Myers, 1959). The suspension of staphylococci in mucin was shown to enhance their virulence for mice by the intraperitoneal route (Schneierson and Amsterdam, 1956; Amsterdam and Schneierson, 1957; Hunt and Moses, 1958; Fisher, 1959).

Hunt and Moses (1958) reported on the production of acute infection of mice by the intraperitoneal injection of a variant of the Smith staphylococcus strain. The differentiation and isolation of this variant was accomplished by the application of the soft agar technique of Finkelstein and Sulkin (1958). While the parent strain formed compact colonies in plasma-soft agar, the variant showed elongated, diffuse colonial growth. Both strains, however, produced coagulase as determined by the tube test.

The LD_{50} of the variant strain was approximately 4 x 10^6 when a broth culture was injected, but when the organisms were suspended in 5 per cent hog gastric mucin, the LD_{50} was approximately 580 cells per mouse. In contrast, studies of ordinary strains of coagulase-positive staphylococci, including the parent Smith strain, resulted in an LD_{50} of 1 x 10^6 viable cells in mucin, although these organisms did not consistently cause death when 1 x 10^9 viable cells were injected without mucin. The response of mice to infection included prompt leukocytosis and phagocytosis of the staphylococci regardless of whether the compact or the diffuse colony type was injected. Organisms of the compact type were viable but multiplied only slightly within the phagocytes. In contrast, the diffuse type showed a marked proliferation within the leukocytes 8 to 12 hours after the injection and the length of the intracellular proliferation period depended on the size of the challenging dose. At the end of the 8 to 12 hour period, there was abrupt appearance of overwhelming number of extracellular organisms and the mice died within the next 20 to 40 minutes. Intracellular growth and high concentration of delta hemolysin within the leukocytes were postulated to account for the sudden extracellular appearance of the diffuse type staphylococci. Fisher and Thompson (1956) indicated earlier the rapid lethal effect for mice of high challenging doses of staphylococci, and a toxic, heat-labile substance, which was inseparable from actively growing bacteria, was incriminated. The histopathological studies of Fisher and Thompson revealed production of pleural effusion with positive fluid cultures and visible pulmonary congestion or hemorrhage in the mice infected with virulent strains.

Significance of the Clumping Factor in the Virulence of Staphylococci

Johanovsky (1957b) has been the only one to investigate the significance of the clumping factor in the virulence of staphylococci. He injected mice intraperitoneally with strains of coagulase-positive staphylococci possessing the clumping factor and others lacking it, and found that the amount of the lethal dose depended on the formation of alpha toxin and the presence of the clumping factor. The author suggested that the clumping factor rather than coagulase may contribute to the resistance of pathogenic staphylococci to phagocytosis and to their survival in leukocytes.

Physiological Properties and Metabolic Products Associated with Coagulase and/or Pathogenicity of Staphylococci

It is well known that there is a high degree of correlation between the ability of staphylococci to cause disease in man and experimental animals and certain in vitro properties of the organism other than coagulase activity. Staphylococci which produce coagulase regularly possess a number of these properties which have been associated with staphylococcal virulence (Blair, 1958; Elek, 1959; Sonnenwirth, 1959).

Toxins

Since early in the century, it has been known that when certain staphylococci are grown under suitable conditions, they give rise to a toxic filtrate which has a variety of effects. Such a toxic filtrate causes acute and fatal toxemia when injected intravenously into rabbits or mice. When injected intradermally into the skin of rabbits or guinea

pigs, it causes necrosis. It is hemolytic, especially for rabbit and sheep red blood cells, and it has a destructive effect on leukocytes (Parker, 1924; Wilson and Miles, 1955; Blair, 1958; Elek, 1959). Although opinions differ, it appears that most investigators still accept the monistic view proposed by Burnet (1929), and which considers the various effects of the toxic filtrate as different manifestations of the activity of a single substance (Blair, 1958). This view is strengthened by several observations such as that of Pillemer and Robbins (1949) who found that the dermonecrotic, lethal, and hemolytic activities are associated with a purified toxin. Since the activity of alpha hemolysin can be most conveniently measured in vitro, it gradually became the basis for detecting and measuring staphylococcal toxin and antitoxin (Elek, 1959).

Kleiger and Blair (1940) pointed out the close similarity between the response of experimental animals to intravenous injection of toxic filtrates and the clinical symptoms of acute staphylococcal toxemia in children from whom highly toxigenic staphylococci were isolated. The same workers (Kleiger and Blair, 1943) suggested that the toxin plays a considerable role in the pathogenesis of acute human staphylococcal toxemia since adequate therapeutic doses of antitoxin often improved the clinical picture. On the other hand, the production of intense necrosis following the intradermal injection of potent toxic filtrates in rabbits and the common observation that many, if not all, strains from cutaneous lesions are toxigenic (Marks, 1952), led to the assumption that the exotoxin may be important in the initiation of infection in intact skin (Blair, 1958). Elek (1956), however, indicated that multiplication of cocci is necessary to the establishment of purulent lesions in the skin

of human volunteers, whereas injection of sterile toxic filtrates failed to produce such lesions.

Some strains of coagulase-positive staphylococci produce under favorable conditions an enterotoxin which is distinct from other staphylococcal products and is capable of giving rise to acute food poisoning in man (Dack et al., 1930; Dack, 1937, 1956). Studies on enterotoxin properties are confusing and incomplete chiefly because of lack of suitable assay method (Wilson and Miles, 1955; Blair, 1958).

Highly purified enterotoxin has been characterized by Bergdoll (1956) as a water soluble, antigenic protein with a high percentage of lysine. It has a molecular weight of 15,000 to 25,000 and an isolecteric point near pH 8.5. However, it is resistant to trypsin. Earlier, Davison and Dack (1939) reported that boiling for 30 minutes causes little reduction in the potency of the enterotoxin, but 60 minutes boiling leads to an appreciable loss, and autoclaving results in almost complete destruction.

The mode of action of enterotoxin is not known (Dack, 1956; Elek, 1959). It has been suggested that the site of emetic action may be localized in the peripheral sensory structures, the probable principal pathways being the vagi (Bayliss, 1940), or the enterotoxin may act directly on the gastric mucosa (Palmer, 1951). Symptomatology of staphylococcal food poisoning strongly suggests involvement of the nervous system (Holtman and Peterson, 1952). These workers used a nerve-muscle preparation of the frog to prove that a direct neurotoxic action takes place. When the sciatic nerve was treated directly with staphylococcal filtrates prepared by the method of Dolman et al. (1936) and heat treated to destroy

alpha and beta toxins, the kymographic effect was found to resemble the action of tetanus toxin.

Hemolysins

The main contender with coagulase for primary role as a virulence factor is alpha hemolysin, an extracellular product which lyses rabbit, and to a lesser degree sheep, but not human red blood cells. Alpha hemolysin is commonly associated with the lethal and necrotizing effects of toxic staphylococcal culture filtrates (Elek and Levy, 1950b; Blair, 1958; Brown and Scherer, 1958; Johanovsky, 1958a). Several investigators claimed that there is a closer correlation between alpha hemolysin production and pathogenicity than between coagulase and pathogenicity, and some advocated the use of alpha hemolysin rather than coagulase production as the in vitro criterion for the potential pathogenicity of staphylococci (Christie et al., 1946; Marks, 1952; Selbie and Simon, 1952; Fisher and Thompson, 1956). Others reported, however, that a varying proportion of staphylococcal strains from human lesions produce no alpha hemolysin (Lack and Wailing, 1954; Jackson et al., 1955; Lack, 1956; Jessen et al., 1959a).

Close correlation of alpha hemolysin with coagulase production was demonstrated by Chapman et al. (1934). With improved methods for the detection of alpha hemolysin, between 93 and 96 per cent of coagulase-positive strains were found to produce alpha lysin (Williams and Harper, 1946, 1947; Elek and Levy, 1950b). Elek and Levy (1950b) suggested that the term "hemolytic staphylococci" be discarded as meaningless because a high proportion of coagulase-negative staphylococci produce nonspecific hemolysis of rabbit and sheep cells. Blair (1958) commented that alpha

hemolysin per se seems to be of little importance in the pathogenesis of staphylococcal infections in man because of its effect on human erythrocytes is negligible and some pathogenic strains do not produce it.

A second, serologically distinct hemolysin, beta hemolysin, was first demonstrated by Glenny and Stevens (1935). Beta hemolysin lyses sheep erythrocytes only, and its activity is enhanced when refrigeration follows a 37 C incubation period or by "hot-cold lysis." It has no effect on rabbit or human erythrocytes. It is produced chiefly by strains of animal origin and is uncommon in human strains (Cowan, 1938; Christie and Keogh, 1940; Christie et al., 1946; Williams and Harper, 1946, 1947; Elek and Levy, 1950b). Hinton and Orr (1957), however, indicated that hospital staphylococcal strains are characterized by the common ability to produce beta hemolysin besides their tendency to show a remarkable incidence of antibiotic-resistance and they belong in great majority to phage group III (Blair, 1956).

Williams and Harper (1947) reported the presence of a third staphylococcal lysin, delta hemolysin, which was not neutralized by either alpha or beta antitoxins. It produces a narrow, well-defined zone of hemolysis around staphylococcal colonies growing on agar plates which contain sheep, rabbit, human, or mouse blood. The delta hemolysin is produced by over 90 per cent of the strains isolated from human staphylococcal infections, and in these strains it is usually associated with alpha hemolysin. However, it occurs as the only hemolysin in about 4 per cent of human strains (Elek and Levy, 1950b, 1954; Marks and Vaughan, 1950).

Lack and Wailing (1954) doubted if beta and delta hemolysins are

virulence factors of staphylococci. Johanovsky (1957a) investigated the significance of delta hemolysin and noted that neutralization of this lysin determined the outcome of staphylococcal infection in experimental animals. Jackson and Little (1956, 1957) suggested that the delta lysin may be identical with leucocidin. Hunt and Moses (1958) indicated that the concentration of delta hemolysin in mouse peritoneal leukocytes may be the cause of the abrupt appearance of extracellular staphylococci following phagocytosis and the subsequent rapid death of the animal.

The characteristics and relationships of alpha, beta, and delta hemolysins have been described by Elek and Levy (1950b). The superiority of the plate over the tube method for the detection of individual hemolysins, due to their differential diffusion rates, was advocated by the same workers. This view is strengthened by the observation that alpha and beta hemolysins are mutually antagonistic, whereas beta and delta hemolysins have a synergistic effect. Differentiation of the three hemolysins can best be achieved by a combination of the appearance of hemolysis on rabbit, sheep, and human blood agar plates with specific antibody neutralization of the hemolysins (Elek and Levy, 1950b, 1954; Elek, 1959).

Leucocidin

Pathogenic staphylococci are capable of producing one or more leukocyte destroying substances. Gladstone and van Heynigen (1957) gave evidence of at least three staphylococcal leucocidins. In addition to the Neisser-Wechsberg (Neisser and Wechsberg, 1900; Panton and Valentine 1932) leucocidin, which acts on rabbit leukocytes only and which is merely another effect of alpha hemolysin, the authors differentiated two

other factors. One of these was specific for human and rabbit white cells, while the other, "leucolysin," acted on the leukocytes of all animal species tested except sheep white cells. They reported that the first of these two factors is produced by many coagulase-positive staphylococci, but not by any coagulase-negative strains, whereas leucolysin is produced by a large number of coagulase-positive staphylococci and by some coagulase-negative strains.

Definite information concerning the exact role of leucocidin in the virulence of staphylococci is scarce. It is only recently that a partial purification of leucocidin has been accomplished (Woodin, 1959). The importance of determining leucocidin activity in evaluating the pathogenicity of staphylococci was emphasized by Vancurik and Svejcar (1960), who found a high degree of correlation between leucocidin production and pathogenicity of the organism.

Enzymes

Staphylokinase. The exhibition of fibrinolytic activity by staphylococci was encountered by early workers. Much (1908) noted that clotting of human blood by staphylococci was followed by dissolution of the clot. Fibrinolytic activity is restricted essentially to coagulase-positive strains of human origin (Gerheim, 1948). Lack and Wailing (1954) reported that, with few exceptions, staphylococci which produce beta hemolysin and are chiefly of animal origin fail to produce fibrinolysin. Fisher (1936b) found that approximately 81 per cent of coagulase-positive strains dissolved human plasma- or fibrinogen-clots, whereas, others reported this figure as 70 per cent (Lack and Wailing, 1954; Lack, 1956). Fisher (1936b) and Neter (1937) suggested that the potential

danger of a staphylococcus is greater if it is capable of dissolving the fibrin barrier and thus spreading the infection. Small emboli may be liberated by fibrinolysin from an affected thrombus, to lodge in the body and establish metastasis (Fisher, 1936b; Cruikshank, 1937). There is some evidence that streptokinase, by its interaction with plasminogen, contributes to the pathogenicity of streptococci for mice; that combinations of certain concentrations of streptokinase and plasminogen increase the pathogenicity of fibrinolysin negative staphylococcal strain for mice to a greater extent than did either substance alone (Krasner and Young, 1959). However, little information is available concerning the possible role of fibrinolysin in the pathogenesis of staphylococcal infection.

Fisher (1936b) found the active principle, which was responsible for the lysis of the fibrin clot, was present in precipitates obtained by treating staphylococcal culture filtrates with alcohol. Lack (1948a) showed that staphylococcal fibrinolysin was similar to streptococcal fibrinolysin and suggested that the active principle is not a true fibrinolysin but, rather, a kinase which activates plasminogen. Soybean trypsin inhibitor which inhibits plasmin also inhibited staphylokinase activity and this observation was used to distinguish the fibrinolysin obtained by kinase activation of plasmin from the effect of proteases (Lack and Wailing, 1954; Lack, 1956, 1957; Hinton and Orr, 1957).

Hyaluronidase. The majority of pathogenic staphylococci produce hyaluronidase, an enzymatic "spreading factor" which attacks the hyaluronic acid constituent of the intracellular ground substances of many body tissues (Schwabacher et al., 1945; Blair, 1958; Lack, 1956). Schwabacher et al. (1945) reported that 93.6 per cent of coagulase-

positive staphylococci produced the enzyme, while none of the coagulase-negative strains produced it. Myers (1959) described an ammonium molybdate chemical (AMC) test as an indirect measurement of the amount of hyaluronidase produced by staphylococci. Close association between an "invasive" type of staphylococcal infection and a positive AMC reaction of the infecting strain, led the author to speculate on staphylococcal hyaluronidase as an important virulence factor. The exact role of hyaluronidase in the pathogenicity of staphylococci is, however, not established and is a matter of conjecture. Lack (1956) suggested that it may serve to remove a potential inhibitor of the organism by depolymerizing hyaluronic acid in connective tissue. Rogers (1956) indicated that certain extracellular enzymes, including hyaluronidase, are formed at or near the surface of cell and are extruded in the form of a capsule. Certain negatively charged synthetic polymers, "macroanions," were found to inhibit the production of the enzymes. It was suggested that a similar effect may be exerted in the body by natural macroanions such as chondritin sulfate, hyaluronic acid, heparin or nucleic acids. There is some evidence that staphylococcal hyaluronidase may contribute to the severity of infections in which staphylococci are mixed with certain other bacteria or viruses (Lack, 1948b; Blair, 1958; Elek, 1959). Elek (1959) concluded that the presence of the enzyme cannot be correlated with the invasiveness of individual strains because it is produced by the great majority of coagulase-positive staphylococci; if hyaluronidase does play any role at all, it is likely to be effective only in the initial stages of infection since the earliest inflammatory response of the host suppresses it.

Phosphatase. Phosphatase production by coagulase-positive

staphylococci and its detection by the use of phenolphthalein phosphate agar were described by Barber and coworkers (Barber et al., 1951; Barber and Kuper, 1951). They reported that after 24 hours incubation coagulase-positive staphylococci regularly gave a positive phosphatase test, whereas coagulase-negative strains were always negative. The authors, and subsequently White and Pickett (1953), recommended the use of the phosphatase test for the rapid screening of coagulase-positive staphylococci. A quantitative test for phosphatase activity was reported by Barnes and Morris (1957). Shaw et al. (1951) and Elek (1959) observed that a low percentage of coagulase-positive staphylococci were phosphatase negative and some coagulase-negative strains gave a positive phosphatase test. The combination of coagulase, mannitol and phosphatase tests was recommended by Capocaccia (1956) as a more reliable index of pathogenicity. When positive results were obtained, there was a strong indication of the organism's potential virulence.

Deoxyribonuclease. Catlin (1956) demonstrated the synthesis of deoxyribonuclease in staphylococci. A rapid method for detecting activity on nucleic acids of microorganisms grown on a semi-synthetic medium was reported by Jeffries et al. (1957). Subsequent reports (Di Salvo, 1958; Fusillo and Weiss, 1959) described methods for the demonstration and the qualitative estimation of staphylococcal deoxyribonuclease (DNase). Weckman and Catlin (1957) reported that high DNase activity is a property of all coagulase-positive staphylococci, whereas all coagulase-negative strains have significantly lower DNase activity. Burns and Holtman (1959) agreed that the ability to split deoxyribonucleic acid appears to be a common characteristic among potentially

pathogenic strains; that it is lacking in strains of doubtful virulence, since there is a good correlation between DNase activity and other properties of staphylococci (coagulase-positive, pigmentation and mannitol fermentation) which are usually associated with virulence.

Egg-yolk reaction. Gillespie and Alder (1952) and Alder et al. (1953) reported on a stable property (Andreoni, 1958), the egg-yolk (EY) reaction, which is demonstrable by living cultures of S. aureus and by ammonium sulfate precipitates of staphylococcal culture filtrates. It was observed that most strains of coagulase-positive staphylococci produced an opacity when grown in egg-yolk broth, and a slower, less distinct, though definite reaction on egg-yolk agar medium in the form of zones of opacity around the colonies. Coagulase-negative strains did not have this effect. The evidence suggested that the reaction may be caused by a lipase which is active against certain triglycerides rather than by the general lipolytic activity exhibited by nearly all coagulase-positive and many coagulase-negative staphylococci (Christie and Graydon, 1941; Williams and Harper, 1947). This assumption was subsequently supported by Jessen et al. (1959a) who also found almost complete correlation between the EY-reaction and the presence of Tween-splitting lipase. Parker (1958) indicated that a perfect correlation existed between the EY-reaction and the serum opacity which is produced by staphylococci and which is not due to coagulase. Graber et al. (1958) related the EY-reaction to phage typability of staphylococci. Recently, Drummond and Tager (1959a,b) observed the production of turbidity when a purified preparation of coagulase was incubated with egg yolk. Their analysis of this phenomenon resulted in the description of a second biological

activity for coagulase, that of an esterase, which has a marked specificity for tributyrin.

Lipase. The production of a diffusible staphylococcal lipase, which can be demonstrated on solid agar medium, was reported by Davies (1954). It is formed independently of coagulase, and is widely distributed among staphylococci. There is, apparently, no correlation between lipase production and the source of the organism. However, more coagulase-positive than coagulase-negative strains were found to form the enzyme. Davies (1954) found that lipase activity is present in high dilutions in culture filtrates; that the enzyme is antigenic. Elek (1959) commented that there is no evidence at the present that lipase plays any part in the pathogenicity of staphylococci.

Urease. The production of urease by coagulase-negative staphylococci and its absence in coagulase-positive strains was claimed by Fusillo and Jaffurs (1955). Though the correlation was not complete, the authors proposed the use of the urease test for differentiating pathogenic from non-pathogenic staphylococci. Fahlberg and Marston (1959) reported that urease production by staphylococci is inconsistent and Elek (1959) regarded this property to be of no taxonomic value.

Pigment Production and Mannitol Fermentation

The production of pigment and mannitol fermentation have for long been associated with potentially pathogenic staphylococci (Julianelle, 1937; Chapman et al., 1938; Chapman, 1940). In general, it was accepted that the pigmented, coagulase-positive staphylococci fermented mannitol, whereas the white, coagulase-negative staphylococci failed to do so. The correlation of these two physiological properties and a few others, such

as salt tolerance, with coagulase production led to the development and recommendation of a variety of selective media for the recognition of potentially pathogenic staphylococci (Chapman, 1945, 1946; Esber and Faulconer, 1959). Recently, Schaub and Merritt (1960) described a broth medium containing plasma and mannitol for the simultaneous detection of mannitol fermentation and coagulase production by S. aureus. However, discrepancies between mannitol fermentation and coagulase production have been reported (Evans, 1947). Evans pointed out that more complete correlation existed between anaerobic than aerobic fermentation of mannitol and coagulase production. Furthermore, non-pigmented variants which are capable of coagulase production have been encountered (Barber, 1955; Boniece et al., 1957). The white variants obtained by Barber (1955) resembled the pigmented parent strains in the elaboration of coagulase and in certain other characters, but not in their ability to clump in plasma. Rosenblum and Jackson (1957) concluded that a high degree of correlation existed between pigmentation, bound coagulase (clumping factor) and sensitivity to typing phages in staphylococci freshly isolated from routine clinical specimens.

Susceptibility to Antibiotics

Ingram (1951) found no correlation between certain physiological properties of staphylococci, such as pigmentation, beta hemolysin and coagulase production, and their susceptibility to penicillin. Nor did Amsterdam and Schneierson (1957) observe any distinct relationship between penicillin susceptibility and virulence of coagulase-positive strains of S. aureus. The same workers found no change in the virulence of originally penicillin sensitive strains following their artificial

induction of resistance to the antibiotic.

Subsequent to the observation of the differential susceptibility of staphylococcal strains to neomycin (Greer and Menard, 1957-1958), mannitol-neomycin agar medium was described (Greer and Britt, 1959; Greer and Menard, 1959). It was found that coagulase-negative staphylococci as well as certain other pathogenic bacteria were inhibited by concentrations of neomycin which had no effect on coagulase-positive staphylococci. The use of mannitol-neomycin agar was recommended by these workers as a selective media for the potentially pathogenic staphylococci, especially from skin and nasal specimens.

Phage Typing and Serological Typing

Serological typing, as developed by various investigators (Cowan, 1938, 1939; Christie and Keogh, 1940; Hobbs, 1948; Brodie, 1957; Lofkvist, 1957; Oeding, 1952, 1957; Pettison and Mathews, 1957; Stern and Elek, 1957; Oeding and Sompolinsky, 1958; Oeding and Williams, 1958) did not prove applicable for routine use in the recognition of potentially pathogenic staphylococci. No particular serotype of S. aureus has been associated with virulence. Phage-typing on the other hand, has been widely and successfully employed in epidemiological studies (Blair, 1956) since its development (Fisk, 1942a,b). Only coagulase-positive staphylococci are regularly lysed by the specific staphylococcal bacteriophages. Borchardt (1953) and Levy et al. (1953) could demonstrate no significant association between diffusible antigens, hemolysins, fibrinolysin, and pigment production with the phage groups of staphylococci isolated from human sources. Likewise, Fahlberg and Marston (1959) were unable to establish any direct relationship between certain groups of phage-typable,

coagulase-positive staphylococci and the production of alpha, beta or delta hemolysins. Several unsuccessful attempts have been made to relate staphylococcal serotypes and phage types (Oeding and Sompolinsky, 1958). Barber and Wildy (1958), however, related the antigenic specificity of coagulase to the bacteriophage group of the staphylococcus.

Relationship of *in vitro* Staphylococcal Antigen-Antibody Reactions to Virulence

Elek and Levy (1950a) used a double diffusion gradient method to estimate the number of diffusible antigens of staphylococci obtained from various sources. The mean number of precipitation lines of antigen-antibody reactions was greatest with strains isolated from human lesions. All coagulase-positive strains from lesions produced antigens and this suggested to the authors that these diffusible substances are prerequisites of virulence. Animal strains, and on the average strains from nasal carriers, showed fewer antigens, but the majority of the latter group resembled those from lesions. Nasal strains deficient in antigens were also poor in hemolysins production. Howard (1954) found the number of antigen-antibody flocculation lines is directly related to the mouse virulence of different staphylococcal strains when the mice are inoculated by the intramuscular method of Selbie and Simon (1952). The number of lines varied from eight in the most virulent to zero in the avirulent strains. Similar results were reported by Anderson (1956). In their study of the factors which may contribute to pathogenicity, Lack and Wailing (1954) found that greater pathogenicity was associated with a broad spectrum of toxins rather than with greater production of any one toxin. Hinton and Orr (1957) investigated the distribution of toxins in

coagulase-positive staphylococci isolated from patients and carriers. They found that strains from furuncles and carbuncles produce more diffusible antigens, alpha and delta hemolysin, as well as staphylokinase than did strains isolated from wound infections. However, all of these strains were more active in these respects than those strains isolated from throats of the same patients. The authors considered the production of alpha and beta hemolysins and staphylokinase as characteristic of the more virulent strains. Recently, Thomas and Penikett (1959) were unsuccessful in their attempt to grade hospital coagulase-positive staphylococci on the basis of identifiable properties and number of diffusible antigens.

Host Factors and Pathogenesis of Staphylococcal Disease

It is evident, from the wide distribution of staphylococci in the environment and on body surfaces, that man is, ordinarily, relatively resistant to staphylococcal infections. The studies of Elek (1956) demonstrated that, regardless of the source or toxigenicity of the organism, a minimal infecting dose of 2 to 7 million staphylococci was necessary to produce a purulent lesion in the skin of human volunteers. The effect of subinfectious doses was not enhanced by plasma, toxin, starch, or mucin. The infective capacity of the staphylococci was, however, enhanced 500 to 10,000 fold by the presence of foreign bodies such as sutures (Elek, 1956; Elek and Conen, 1957).

Susceptibility to infection by staphylococci appears to be influenced by certain consititutional factors and to be markedly altered by nutritional disturbances and certain debilitating diseases (Elek, 1959).

Smith and Dubos (1956b) found that when mice were completely deprived of food for 36 to 48 hours immediately before infection, their susceptibility to staphylococcal infection was increased. This effect was enhanced by allowing the animal to drink 5 per cent glucose solution instead of water or saline during the fasting period. Mice fed inadequate diet for several weeks appeared to be just as resistant to staphylococcal infections as did mice which gained weight rapidly on unrestricted, complete diet. However, when mice were maintained on a diet containing non-lethal but weight retarding amounts of dinitrophenol or thyroid extracts for one or two weeks before infection, Smith and Dubos (1956c) found that most of them died within 12 days after infection with staphylococci, and much more rapidly than mice fed a normal diet. Deaths occurred even when the organism injected was ordinarily a non-virulent staphylococcus, unable to cause fatal disease in mice fed a normal diet.

Prigal and Dubos (1956) reported that when mice, which had previously been sensitized to bovine serum, were injected intravenously with staphylococci, many more organisms were recovered from the kidneys, liver and lungs of these animals than from the same organs of similarly infected, non-sensitized mice. Johanovsky (1958b) reported that a state of increased susceptibility to staphylococcal infection together with the delayed hypersensitivity to staphylococcal antigens can be transferred to normal rabbit recipient by means of living cells in spleen homogenate. A non-lethal substance, probably nucleoprotein, which is present in extracts of pathogenic staphylococcal cells and which is unrelated to the extracellular products found in culture filtrates, may be the toxic or allergenic factor (Dworetzky et al., 1956).

Purpose of the Present Investigation

From the preceding survey it is obvious that several properties of staphylococci are associated with their ability to produce coagulase and directly or indirectly with their virulence. Several classifications were proposed in an attempt to differentiate pathogenic from non-pathogenic staphylococci (Shaw *et al.*, 1951; Cowan and Shaw, 1954; Thatcher and Simon, 1957); however, the production of coagulase appears to be the most widely accepted criterion for the detection of potentially pathogenic staphylococci (Blair, 1958; Elek, 1959). In the seventh edition of Bergy's Manual of Determinitive Bacteriology (Breed *et al.*, 1957) staphylococci are divided into two species on the basis of two main properties: coagulase production and mannitol fermentation. Accordingly, if both of these tests are positive, the organism is assigned the name *Staphylococcus aureus*; if both are negative, the organism is named *Staphylococcus epidermidis*.

The chief purposes of the present investigation were:

1. To modify and evaluate the use of a soft agar medium for the simultaneous demonstration of staphylococcal coagulase and clumping factor activities.
2. To compare the mouse virulence of ordinary strains of staphylococci with that of rare strains which exhibit either coagulase or clumping factor activity but not both of these properties.
3. To investigate the effect of the route of injection on the mouse virulence and infectivity of these staphylococcal strains.
4. To determine if other *in vitro* properties of the selected strains of staphylococci could be correlated with their virulence and infectivity for mice.

5. To observe the effect of *in vivo* cultivation on the properties of the test organisms, with particular reference to coagulase and clumping factor activities.

CHAPTER II

MATERIALS AND METHODS

Staphylococcal Strains

More than 350 strains of staphylococci were examined by a variety of methods for their coagulase and clumping factor properties. The great majority of the strains tested were isolated from hospital patients and personnel; a few were laboratory stock cultures. Two additional strains, the Smith strain (S) and its mouse virulent variant (Sv), were kindly supplied by Dr. George A. Hunt of Bristol Laboratories.

All strains were maintained on trypticase soy agar (Baltimore Biological Laboratories) slants at 4 to 6 C and were transferred at monthly intervals. Periodic observations were made for homogeneity of the cultures by plating them on blood trypticase soy agar. Similarly, single colony isolates, which were typical of each strain, were maintained and checked at regular intervals for stability of coagulase and clumping factor activities. The single colony isolates were used in the virulence studies and in determination of properties usually associated with the pathogenicity of staphylococci. Strains selected for such studies possessed both coagulase and clumping factor, coagulase or clumping factor, or neither of these properties.

Strains Positive for Coagulase and Clumping Factor

Four strains selected for study were positive for both coagulase and clumping activity. These were the Smith parent (S), Villafor (V), Hutton (H) and Steward 33 (S 33) strains. The Smith strain was originally isolated from a human case of osteomyelitis in 1930 by Dubos and later was briefly described by Smith and Dubos (1956a). The Villafor strain was isolated in 1959 from the blood of a terminal leukemia patient. The Hutton strain is a recent isolate from a throat culture of a healthy carrier. The Steward 33 strain originated in an infected leg wound; it was held in the frozen state (-28 C) for more than two years before the present study began.

Strains Positive for Coagulase and Negative for Clumping Factor

Four of the 13 strains selected for study, the Smith mouse virulent variant (Sv), K 6, K 93 and K 93m, were unusual in that they possessed coagulase but no clumping factor activity. Isolation of the mouse virulent variant of the Smith strain was reported by Hunt and Moses (1958). The unusual character of two strains, obtained from clinical specimens taken from patients at local hospitals, were noted in this laboratory. Strain K 6 was isolated from a nasopharyngeal swab. K 93 originated in a mixed culture from an ulcer on the foot of a patient. K 93m is a mucoid variant of the K 93 strain; it was recovered from organs of mice which were sacrificed five weeks after intravenous injection with the K 93 strain. Each organism of this strain appears to be surrounded by an envelop of mucoid material which is less discreet than the usual bacterial capsule.

Strains Negative for Coagulase and Positive for Clumping Factor

Staphylococci which do not produce coagulase but which exhibit clumping factor activity are probably even more rare than those which are negative for clumping factor and positive for coagulase. Three such rare strains, recognized in this laboratory, Hv 1, S 35 and S 35s, were included in the study. The Hv 1 strain first appeared as a coagulase-negative variant in a soft agar culture of the coagulase-positive, clumping factor-positive H strain. Strain S 35 was coagulase-negative, but clumping factor-positive, and its growth was noticeably rough when it was first isolated from a bronchoscopy specimen. The strain designated S 35s is a smooth variant of the S 35 strain. This smooth variant was recovered from the organs of mice injected with the parent strain. It differs from the parent organism not only in its smooth type growth, but also in the formation of larger compact colonies in fibrinogen containing soft agar.

Strains Negative for Coagulase and Clumping Factor

Two strains selected for further study were Hv 2 and S 33v, *in vitro* variants of the H strain and S 33 strain respectively, which were distinguished from the parent organisms in soft agar cultures by their lack of both coagulase and clumping factor activity.

Demonstration of Coagulase and Clumping Factor

Conventional Methods

The production of coagulase was demonstrated by the routine plasma clotting test. Rehydrated compounded plasma (Difco) was inoculated with

12 to 16 hour broth cultures of the organisms and incubated at 37 C. The tubes were observed for clotting at the end of 1, 3 and 24 hours incubation.

Clumping factor activity was determined simultaneously by the slide and tube agglutination methods (Duthie, 1954a). In each procedure, an equal volume of a saline suspension of the organism was mixed with (a) undiluted fresh human pooled plasma, (b) rehydrated compounded plasma (Difco) and (c) a saline solution of 0.4 per cent bovine fibrinogen (Armour fraction I), a common concentration of fibrinogen in normal human plasma (Miller, 1957). Slide tests were considered to be positive if visible clumping of the organisms occurred within 15 to 30 seconds. In cases of delayed or no clumping in the slide test, a loop of growth from a 16 to 18 hour trypticase soy agar slant culture was emulsified directly in a drop of undiluted plasma and in the fibrinogen-saline; the slides were observed for the same time interval. In the tube agglutination test, readings were made after mixtures of the cell suspension and plasma or the cell suspension and fibrinogen solution were shaken vigorously on a Kahn shaker for five minutes.

The Soft Agar Method

Media devised for simultaneous detection of coagulase and clumping factor. During the course of these studies a new soft agar medium was developed to demonstrate both coagulase and clumping factor in a single system (Alami and Kelly, 1959). Preliminary observations were made on (a) the nature of the growth of approximately 350 strains of staphylococci in a basal soft agar medium and (b) the effect on the growth of the organisms on the addition of varying amounts of sterile plasma, serum,

fibrinogen or albumin or combinations of these substances to the basal medium. For each strain, results of the usual plasma clotting coagulase test and slide agglutination in plasma and in fibrinogen were compared with the type of colonies observed in the various soft agar media. The method which proved to be most satisfactory as to correlation of results required (a) a basal soft agar medium made by adding 0.15 per cent agar to trypticase soy broth (BBL), (b) fibrinogen soft agar which incorporates 0.4 per cent bovine fibrinogen (Armour fraction I) in the basal medium, and (c) fibrinogen-plasma-albumin soft agar which has a final concentration of 1 per cent pooled human plasma, 0.4 per cent bovine fibrinogen, and 0.3 per cent bovine albumin (Armour fraction V), added to the basal medium.

For convenience the basal medium was prepared in two different agar concentrations: a 0.15 per cent agar and a 0.3 per cent agar medium. The former was dispensed in 10 ml amounts and the latter in 5 ml amounts to 1.5 x 12 cm screw capped tubes before autoclaving. To make 0.4 per cent fibrinogen soft agar, 0.8 per cent solution of fibrinogen in trypticase soy broth was sterilized by positive pressure Seitz filtration and added in 5 ml amounts to tubes containing 5 ml melted, cooled, 0.3 per cent agar basal medium. In the third type of medium, either rabbit or human plasma proved to be satisfactory source of the necessary coagulase reacting factor (CRF). The practice of pooling several lots of human plasma routinely employed here was a precaution against the presence of a high concentration of staphylococcal antibodies in any one plasma. A solution of 3 per cent albumin in trypticase soy broth was sterilized by positive pressure Seitz filtration before it was incorporated at 0.3 per

cent final concentration in the plasma-fibrinogen soft agar.

Inoculation of soft agar media. An inoculum which resulted in an appropriate number of colonies, i.e., 20 to 50 per tube of soft agar medium was obtained as follows. For each strain to be tested, a tube containing 5 ml trypticase soy broth was inoculated from an overnight trypticase soy agar slant culture. After 4 to 6 hours incubation at 37 C, the young broth culture was diluted serially using a 4 mm loop to introduce the organisms successively to (1) 10 ml sterile saline containing 0.05 per cent peptone, (2) 3 ml of the same sterile peptone-saline and (3) 10 ml of the soft agar medium. In this manner each culture to be tested for coagulase and clumping factor activity was inoculated to a tube of fibrinogen-plasma-albumin soft agar as well as to tubes of the two control media: the basal soft agar and the fibrinogen soft agar.

When a primary plate culture of the staphylococci recovered from infected mice was to be tested, a sufficient amount of the growth was taken from the confluent area to make a turbid suspension in 2 ml of sterile trypticase soy broth. A loop of this suspension could then be substituted for the young broth culture to make the serial dilutions described above.

All soft agar cultures were examined after 12 to 16 and 24 hours incubation at 37 C.

Titration of Coagulase

Because filtration of broth cultures of coagulase-positive staphylococci removed much, if not all, of the coagulase activity, titration of coagulase was next attempted with supernatants of centrifuged broth

cultures. This method, although fairly satisfactory, proved cumbersome. The method finally followed was the one advocated by Lominski (1944) who discovered that the incorporation of serum into the culture medium enables coagulase to pass through Seitz filters.

Five ml amounts of sterile brain heart infusion (Difco) which contained 10 per cent of pooled human serum were inoculated from stock agar slant cultures of the organisms. After eight hours incubation at 37 C, 0.05 ml of the young serum-broth culture was transferred to a tube of 10 ml sterile serum-broth and this tube was incubated at 37 C for 18 hours. The culture was then centrifuged for 20 minutes at 2500 rpm. The supernatant was passed through a Boerner type Seitz filter. Two-fold serial dilutions of the sterile filtrate were made in 1 x 9.5 cm tubes containing 0.5 ml of sterile trypticase soy broth. Next, 0.5 ml of the same clotting system used in the soft agar method, i.e., a sterile mixture of 1 per cent pooled human plasma, 0.4 per cent fibrinogen and 0.3 per cent albumin in trypticase soy broth, was added to each tube. The tubes, with appropriate controls, were incubated at 37 C and were examined for clot formation at five minute intervals during the first hour, at 15 minute intervals for the next four hours, every hour for the following five hours and, finally, after 24 hours incubation. The amount of coagulase in the culture filtrate was graded on the basis of the highest dilution of the filtrate which caused clot formation and the time required to form the first clot in the dilutions of each filtrate.

Titration of Clumping Factor

The organisms were tested for clumping factor activity in decreasing concentrations (0.4, 0.2, 0.1, 0.05, 0.02 and 0.002 per cent) of

bovine fibrinogen (Armour fraction I) by the slide and the tube agglutination methods, as well as for their ability to form compact colonies in the basal soft agar medium containing similar decreasing concentrations of fibrinogen. Equally turbid saline suspensions of each strain were prepared for the slide and tube agglutination tests from 18 hour trypticase soy agar slant cultures. Fibrinogen agar was inoculated as described above. Included in the slide and agglutination tests were saline controls which ruled out auto-agglutination and other controls which gave the expected results with known clumping factor positive and negative strains (Cadness-Graves et al., 1943; Duthie, 1954a; Steward and Kelly, 1959). A basal soft agar medium culture was included as a control for the fibrinogen-soft agar test. The amount of clumping factor present was judged in the slide and tube agglutination procedures by the highest dilution of fibrinogen in which clumping of the organisms occurred; in the case of the soft agar method, it was indicated by the highest dilution of fibrinogen which supported the formation of compact colonies.

Further Characterization of Staphylococcal Strains

In addition to coagulase and clumping factor, the organisms were also examined for other in vitro properties which have been associated with typical S. aureus strains, coagulase production and staphylococcal virulence (Blair, 1939, 1958; Evans, 1948; Elek and Levy, 1950a; Shaw et al., 1951; Cowan and Shaw, 1954; Hinton and Orr, 1957; Thatcher and Simon, 1957; Jessen et al., 1959a,b).

Pigmentation

Pigment production was observed in plate cultures of milk agar which were incubated at 37 C for 18 to 24 hours and then held at room temperature for at least one week (Fujita and Yoshioka, 1923, 1938; Christie and Keogh, 1940; Anderson, 1956). Milk agar was prepared by adding autoclaved fresh skim milk to an equal volume of sterile, melted trypticase soy agar (3.0 per cent agar) medium.

Catalase

The test for catalase was performed by suspending a loop of the growth from an 18 to 24 hour trypticase soy agar culture in 3 per cent hydrogen peroxide. Bubbling indicated a positive test.

Mannitol Fermentation

Aerobic phenol red mannitol agar (Difco) as well as aerobic and anaerobic phenol red mannitol broth (Difco) cultures of each strain were incubated at 37 C for 48 hours. A positive fermentation test was evidenced by change of the indicator color from red to yellow. Plate cultures of Staphylococcus Medium No. 110 (BBL) were also streaked with each strain. After aerobic incubation at 30 C for 48 hours, phenol red indicator was applied at the site of confluent growth and change in color of the indicator was noted.

Hemolysins

A young (10 hour) trypticase soy agar culture of each strain was inoculated to produce a band of growth on plates of human, rabbit and sheep 5 per cent blood agar. In each instance the blood agar was prepared by adding a saline suspension of washed erythrocytes to trypticase

soy agar. All plates were examined after 24 hours incubation at 37 C. The sheep blood agar plates were incubated an additional 24 hours in the cold (4 C) in order to demonstrate the hot-cold lytic effect of beta hemolysin. Incubation of the human and rabbit blood agar plates was continued at 37 C for another 24 hours before a second reading was made. The occurrence and appearance of hemolytic zones were noted and interpreted as to the presence of alpha, beta and delta hemolysins (Williams and Harper, 1947; Elek and Levy, 1954; Howard, 1954; Lack and Wailing, 1954; Blair, 1958; Elek, 1959). Similarly mouse blood agar plates were inoculated, incubated and examined.

Phosphatase

The method followed to detect phosphatase was essentially the one described by Barber and Kuper (1951). A 0.01 M solution of the substrate, phenolphthalein diphosphoric acid (Sigma Chemical Co.), was prepared in phosphate buffered saline and was adjusted to pH 7.4. This stock solution was passed through a Seitz filter and stored in the refrigerator when not in use. The phenolphthalein diphosphoric acid agar plates were prepared by adding, aseptically, one part of the stock solution to 49 parts of melted, cooled trypticase soy agar. The cultures were streaked in the usual manner or by band streaks which allowed the testing of several strains on each plate. After 16 to 18 hours incubation at 37 C, the agar surface of each plate was held over an open bottle of aqueous ammonia. If the organisms produced sufficient phosphatase to liberate phenolphthalein, exposure to ammonia caused the growth to become bright pink, whereas the growth of phosphatase-negative organisms remained unchanged in color.

Urease

Urea broth (Difco) was inoculated from an 18 to 24 hour trypticase soy agar culture. The tubes were examined after 12, 24, 48 and 72 hours incubation at 37 C. A positive reaction was indicated by the production of red color in the culture medium.

Deoxyribonuclease

Deoxyribonuclease (DNase) activity was determined according to the method of Di Salvo (1958). The medium used was trypticase soy agar which contained 2 mg of DNA (Nutritional Biochemical Corporation) per ml and 0.8 mg of anhydrous calcium chloride per ml. Six to 8 strains were band streaked on each plate of the autoclaved medium and the cultures were incubated for no longer than 36 hours at 37 C. The DNase activity was demonstrated by flooding the plates with 6 N HCl, which was found more satisfactory than the 1 N HCl recommended by Di Salvo. A positive reaction was indicated by a clear zone surrounding the inoculated area and the width of this zone was interpreted as an expression of the amount of extracellular DNase produced (Jeffries et al., 1957).

Staphylokinase

The method used to detect staphylokinase activity has been recommended by a number of workers (Christie and Wilson, 1941; Lack and Wailing, 1954; Lack, 1956, 1957; Elek, 1956; Hinton and Orr, 1957). The fibrin agar plates were prepared by adding pooled human blood bank plasma, in a final concentration of 15 per cent, to melted trypticase soy agar which had been cooled to 56 C and then holding the medium in a 56 C water bath for 20 minutes before pouring into plates. Fibrin agar plates

containing crystalline soy bean trypsin inhibitor (Nutritional Biochemicals Corporation) in a final concentration of 1:10,000 were also prepared in the same manner. Six to 8 strains were spot inoculated to a plate of fibrin agar and to a plate of the soy bean trypsin inhibitor-fibrin agar. Plates were incubated at 37 C. Fibrinolytic activity was evidenced by the appearance of a clear zone around the growth after 24 to 48 hours incubation. Soy bean trypsin inhibitor prevents lysis of fibrin by staphylokinase-activated plasmin but does not affect the fibrinolytic activity of bacterial protease (Lack and Wailing, 1954; Lack, 1957). Thus, since staphylokinase producing strains induce clearing only in the absence of soy bean trypsin inhibitor, it was possible to distinguish fibrinolysis caused by staphylokinase activation of plasmin from that caused by proteases.

Egg-Yolk Reaction

Each strain was studied for the changes it produced in an egg-yolk medium. Egg-yolk agar was prepared according to the directions given in the Manual of Microbiological Methods (Conn, 1957). Fresh egg yolk was collected aseptically and was diluted with an equal volume of sterile normal saline. Ten ml of the yolk suspension was added to each 100 ml of melted, cooled trypticase soy agar and the medium was poured into sterile plates. The plates were spot inoculated from young (10 hour) trypticase soy agar slant cultures and, after 24 and 48 hours incubation at 37 C, were examined for a zone of opacity and/or clearing around each spot of growth. The formation of a zone of opacity indicated the positive egg-yolk (EY) reaction of Gillespie and Alder (1952) or, possibly, the tributyrinase activity of coagulase described by Drummond and Tager

(1959a,b); a clearing zone, such as that observed by Graber (1958), may represent general lipolytic or lipoproteolytic activity.

Lipase

Demonstration of extracellular lipase was accomplished by the method of Davies (1954). Nile blue sulfate-fat agar was prepared by adding a hot, distilled water suspension of neutral hog fat (lard) in a final concentration of 0.3 per cent to trypticase soy agar which contained 0.1 per cent nile blue sulfate. The plates were spot inoculated with each of the staphylococcal strains, incubated at 37 C and examined daily for three days. Positive results were indicated by the appearance of blue droplets on and around the spot of growth due to the liberation of fatty acids.

Phage Typing

The staphylococci were phage typed by the method of Blair and Carr (1953, 1960) using the set of 25 staphylococcal phages originally supplied by Dr. J. E. Blair. A young (4 to 6 hours) trypticase soy broth culture was swabbed over the entire surface of the trypticase soy agar in a cross hatched petri plate. A drop of the routine test dilution of each of the high titered phage filtration was applied to the appropriate square on the plate. Plates were examined for lysis after 18 to 24 hours incubation at 30 C.

Susceptibility to Antibiotics and Other Chemotherapeutic Agents

A blood agar plate was inoculated heavily and uniformly with a 10 hour trypticase soy broth culture of each strain of staphylococci. Sensidiscs (BBL) of the following agents, using high and low concentrations in

most instances, were placed on the surface of the plates: penicillin, dihydrostreptomycin, chloramphenicol, tetracycline, chlortetracycline, erythromycin, neomycin, kanamycin, Albamycin(R), vancomycin, triacetyloleandomycin, polymyxin B, bacitracin, Spontin(R), triple sulfa, Gantricin(R), and Furadantin(R). Plates were examined for zones of growth inhibition after 24 hours incubation at 37 C.

Capsule Stain

The mucoid (K 93m) strain of staphylococci was stained by both the Hiss and Anthony capsule staining methods as described in the Manual of Microbiological Methods (Conn, 1957). More satisfactory results were obtained when the same methods were modified by gently heating the preparations for five minutes while the slides were flooded with the basic dye. Several non-mucoid strains of staphylococci, including K 93, K 6 and S, were subjected to the same capsule stains.

Virulence Studies

Animals

Equal numbers of male and female albino mice, aged 6 to 8 weeks and weighing from 20 to 30 grams, purchased from the National Laboratory Animal Company, Creve Cour, Mo., were used throughout the study. The animals were fed Rockland mouse diet.

Quantitative Estimate of Viable Numbers of Staphylococci

Viable counts of broth cultures and phosphate buffered saline suspensions of staphylococci were estimated by making ten-fold serial

dilutions in sterile, phosphate-buffered saline and inoculating trypticase soy agar plates in triplicates with 1.0, 0.5, and 0.1 ml amounts of each of two or three suitable dilutions. After 48 hours incubation at 37 C, colony counts were made of those plate cultures which developed 20 to 300 colonies and from this data the viable unit counts were determined.

Additional counts were made by inoculating tubes of soft agar, (in each instance the basal soft agar, fibrinogen soft agar and fibrinogen-plasma-albumin soft agar) with measured amounts of suitable dilutions. The soft agar cultures also served to demonstrate the homogeneity of the population in the culture with respect to coagulase and clumping factor activity.

Preparation of Inoculum

Staphylococci were grown in trypticase soy broth (BBL) for 8 to 10 hours at 37 C and 0.5 ml of this culture was transferred to 125 ml of the same medium contained in 250 ml centrifuge bottles. These cultures were incubated at 37 C for 20 to 24 hours. The number of bottles inoculated differed with the strain and was determined by previous results of viable counts obtained with similar preparations of each strain. The cultures were centrifuged for 40 minutes at 2000 rpm. The sediment in each bottle was suspended in 25 ml of phosphate-buffered saline and the suspensions of cells from the same strain were pooled. The pool of cells was recentrifuged and the process of washing the cells with phosphate-buffered saline was repeated twice. Finally, the cells were suspended in the appropriate amount of phosphate-buffered saline to obtain a challenge dose of high count in a volume of 0.3 ml. A preliminary estimate of the desired concentration of cells was determined, for each strain, by trial inoculations of mice.

Determinations of LD_{50} and ID_{50}

For each of the 13 strains of staphylococci, five suitable four-fold dilutions were prepared in phosphate-buffered saline from the washed cell suspension described above. A 0.3 ml dose of each of the five dilutions was injected intravenously into each of six mice (three males and three females) and intraperitoneally into another group of six mice. The viable number of bacteria in each dose was determined as previously described immediately after the injection procedure. As controls, groups of six mice were injected intraperitoneally and intravenously with 0.3 ml amounts of (1) phosphate-buffered saline, (2) sterile trypticase soy broth, (3) a sterile supernatant of a trypticase soy broth culture of each strain and (4) a heat killed (75 C for 45 minutes) cell buffered saline suspension of the highest challenge dose of each strain.

The mice were observed for an experimental period of 14 days (Smith and Dubos, 1956a; Gray et al., 1957; Gorrill, 1958) and the time of death was recorded. On death, the mice were autopsied and cultures were made of organs and body fluids as described below. Mice surviving the experimental period were chloroformed and examined immediately for infection both grossly and microscopically. The ID_{50} was based on recovery of culturable staphylococci from dead and sacrificed mice. The Reed-Muench method (1938) was followed to estimate the LD_{50} and ID_{50} of each staphylococcal strain.

Recovery of Staphylococci from Infected Mice

Peritoneal fluid, heart's blood and, if present, pus from abscess were streaked on blood trypticase soy agar plates. The kidneys, liver, spleen and lungs were removed aseptically and impression inoculations

were made by pressing the cut surfaces of the organs to the surface of blood agar plates. At least one whole kidney from each animal was ground with 2 ml of phosphate-buffered saline in a small sterile mortar. Occasionally, as a check on the impression inoculation method, homogenates of other organs were also prepared. A 4 mm loop of the organ homogenate was streaked on a blood agar plate. After incubation, plate cultures were examined for purity, colonial morphology and the relative numbers of colonies which developed from similar inocula (Braude et al., 1959). The same amount of the homogenate, suitably diluted, served as an inoculum for soft agar cultures which revealed the coagulase and clumping factor activities of the culture population.

Tissue Sections

Sample specimens of organs from normal and infected mice were fixed in 10 per cent formalin. The tissues were imbedded in paraffin, sectioned at 4 mm and stained by the Harris hematoxylin-eosin method. These sections were prepared by Miss Lucy Pitts and they were examined by members of the Department of Pathology.

Studies of in vivo Strain Stability

The in vivo stability of the 13 staphylococcal strains, with special reference to coagulase production and clumping factor, was studied by injecting mice with at least two sublethal but infective doses of the organisms. For each dose a group of six mice were injected intravenously and another similar group intraperitoneally. At two week intervals for a period of six weeks, two mice from each group were sacrificed and the organisms were recovered as previously described. Colonial morphology

was observed on blood agar plates and the homogeneity of the mouse passed strains as to coagulase and clumping factor activity was tested by the soft agar culture method. In like manner, these characteristics were examined in organisms recovered from mice which survived the 14 day period of the second LD_{50} and ID_{50} determinations.

CHAPTER III

RESULTS

The Soft Agar Technique for the Simultaneous Detection of Staphylococcal Coagulase and Clumping Factor

Of the 320 strains reported (Table 1), 240 were positive for coagulase and 80 were negative according to the routine plasma clotting test. Results of the slide and tube agglutination tests with 0.4 per cent fibrinogen, rehydrated plasma, and human plasma agreed. All morphologically typical strains of staphylococci tested produced an elongated, feathery type of colonial growth in basal soft agar (0.15 per cent agar) medium. On the addition of either 1 per cent plasma or 0.4 per cent fibrinogen to basal soft agar medium, the great majority of coagulase-positive staphylococci formed compact colonies, whereas the colonies of coagulase-negative strains were the same elongated, diffuse type like observed in the basal soft agar medium. Preliminary experiments indicated that an agar concentration of 0.15 per cent is optimal and minor increase or decrease in its concentration affected the characteristic colonial morphology in basal soft agar, fibrinogen soft agar and in plasma soft agar. With rare exceptions, all strains which formed compact colonies in 1 per cent plasma soft agar also produced the same type colonies in 0.4 per cent fibrinogen soft agar after 14 to 24 hours incubation at 37 C. Conversely, strains which failed to develop compact colonies in fibrinogen

TABLE 1

CLUMPING REACTION AND COMPACT COLONY FORMATION BY COAGULASE-POSITIVE AND COAGULASE-NEGATIVE STAPHYLOCOCCI

Total Number	Slide and Tube Agglutination			Compact Colonies		
	0.4% Fibrinogen	Rehydrated Plasma (Difco)	Undiluted Human Plasma	0.4% Fibrinogen Soft Agar	1% Plasma Soft Agar	Control: Basal Soft Agar
240 Coagulase-	208 +	208 +	208 +	217 +	225 +	239 -
Positive Strains	4 -	4 -	4 -	3 -	3 -	1 +
	28 ±[a]	28 ±	28 ±	8 ±		
				12 +/-[b]	12 +/-	
80 Coagulase-	3 +	2 +	2 +	3 +	2 +	80 -
Negative Strains	65 -	66 -	66 -	77 -	78 -	
	12 ±	12 ±	12 ±			

[a]Doubtful results: rough strains in agglutinations; compact, slightly elongated base in soft agar.

[b]x/-, mixtures of compact and diffuse colonies in soft agar.

soft agar were negative for this feature in plasma soft agar.

Among the 240 coagulase-positive strains, four strains gave negative slide and tube agglutination tests, and three of these (Sv, K 6 and K 93) formed the diffuse type colony in fibrinogen soft agar and plasma soft agar (Table 1). The fourth strain (K 93m) is a mucoid variant of one of these three strains. Within the first 24 hours incubation, it produced large, spherical colonies in fibrinogen soft agar and plasma soft agar as well as in the control basal soft agar medium. However, these colonies exhibited slight diffusion in all soft agar media after an incubation period of more than 24 hours.

Twenty eight of the 240 coagulase-positive strains were autoagglutinating and gave equivocal results in the slide and tube agglutination tests. Twelve of these strains proved to be mixtures of diffuse and compact colonies in plasma soft agar and in fibrinogen soft agar cultures, i.e., mixtures of clumping factor negative and clumping factor positive organisms, respectively. On subsequent isolation and purification of these mixed cultures, the diffuse type colonies were found to be negative for coagulase activity, whereas the compact type colonies were coagulase-positive. Eight of the 28 strains produced compact colonies in plasma soft agar, but in 0.4 per cent fibrinogen soft agar they formed an intermediate type colony which was compact and spherical except for slight elongation of its under surface.

Of the 80 coagulase-negative strains reported in Table 1, 12 strains were doubtful for clumping factor by the slide and tube agglutination tests, but these strains produced diffuse colonies in fibrinogen soft agar and plasma soft agar. Two other strains were positive in all tests

for the clumping factor. One strain clumped only in 0.4 per cent or more fibrinogen, regardless of the test method and thus proved to be notably deficient in clumping factor activity compared to other strains.

When strains were compared in soft agar which contained varied concentrations of plasma, serum or fibrinogen, the great majority formed compact colonies in 0.4 per cent fibrinogen soft agar, in 0.1 per cent plasma soft agar, and in 1 per cent serum soft agar (Table 2). In the case of a very few strains, such as the one described above, a higher concentration, at least 0.4 per cent, of fibrinogen was required for compact colony formation.

For expression of coagulase activity in soft agar, a combination of plasma (or serum) and fibrinogen was required in the medium. Well defined, opaque, coagulation zones were observed around colonies of coagulase-positive strains growing in 1 per cent plasma-0.4 per cent fibrinogen soft agar (Figure 1). Substitution of the same concentration of normal rabbit serum or plasma for human serum or plasma in these experiments yielded similar results. The addition of 0.3 per cent albumin to the system intensified the coagulation zone reaction and supplied a highly satisfactory medium for demonstrating both clumping factor and coagulase activity, provided a 0.4 per cent fibrinogen soft agar medium was also inoculated as a control. An additional control culture of basal soft agar medium proved necessary when testing the mucoid strain. The soft agar method revealed a few staphylococcal strains which were positive for one of these properties exclusively and several strains which were mixed populations with regard to clumping factor or coagulase activity (Table 1; Figure 2).

TABLE 2

EFFECTS OF DIFFERENT CONCENTRATIONS OF PLASMA, SERUM AND FIBRINOGEN ON FORMATION OF STAPHYLOCOCCAL COMPACT COLONIES

Strain	Clumping in Plasma or Fibrinogen[a]	Compact Colonies in Soft Agar Containing								
		Plasma (%)			Serum (%)			Fibrinogen (%)		
		1.0	0.1	0.01	1.0	0.1	0.01	0.4	0.04	0.004
K, V, 5, 22, S 33, 96	+	+	+	-	+	$\pm$[b]	-	+	+	$\pm$
75, 79	+	+	+	-	+	-	-	+	+	$\pm$
2, 28	-	-	-	-	-	-	-	-	-	-

[a]Slide and tube agglutinations.

[b]Intermediate colony type; round, compact colony with slightly elongated base after 14 hours incubation at 37 C.

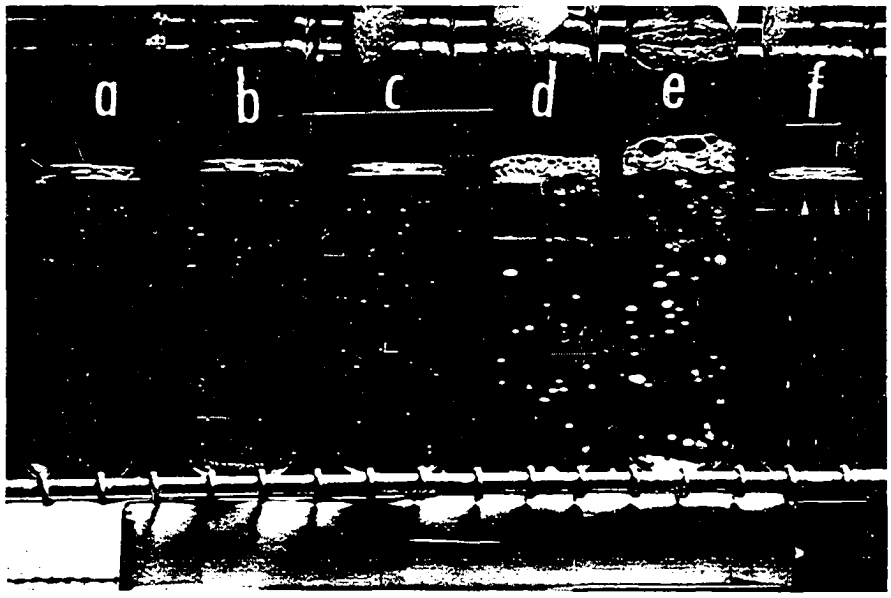

Fig. 1.—A staphylococcal strain which is positive for coagulase and clumping factor after 14 hours incubation at 37 C in soft agar medium containing: (a) 1 per cent plasma; (b) 5 per cent plasma; (c) 0.4 per cent fibrinogen; (d) 1 per cent plasma and 0.4 per cent fibrinogen; (e) 1 per cent plasma, 0.4 per cent fibrinogen, and 0.3 per cent albumin; (f) basal medium only (control). Note compact colonies in all except control culture; coagulation zones in b, d and e.

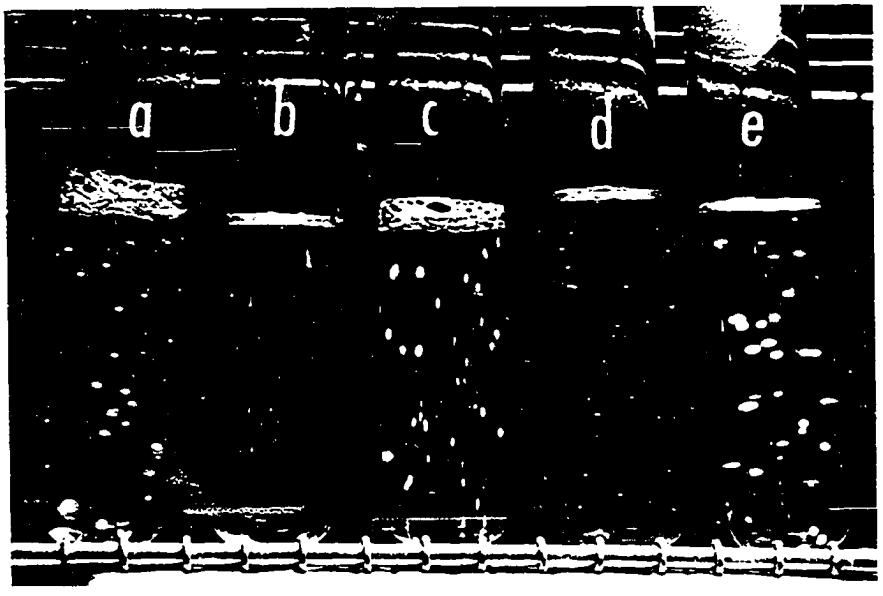

Fig. 2.—Growth of five different strains of staphylococci in plasma-fibrinogen-albumin soft agar: (a) positive for both coagulase and clumping factor; (b) negative for both coagulase and clumping factor; (c) positive for coagulase only; (d) positive for clumping factor only; (e) mixed population with respect to coagulase and clumping factor.

Less distinct coagulation zones were demonstrable in plasma soft agar containing higher concentrations (5 to 10 per cent) of pooled human plasma. The characteristic appearance, in plasma-fibrinogen-albumin soft agar, of strains which are positive for coagulase and negative for the clumping factor appeared to be extremely sensitive to minor changes in the agar concentration. Typically, however, this group of staphylococci form a "parachute" shaped colony in plasma-fibrinogen-albumin soft agar when the routinely used agar concentration of 0.15 per cent is accurately measured (Figure 3).

Table 3 summarized the typical results which correlate clumping factor and coagulase activity with the production, in soft agar media, of the compact colonies and coagulation zones, respectively. Staphylococci of the Sv strain produce compact colonies in plasma-fibrinogen-albumin soft agar which show evidence of slight elongation after 24 hours incubation. However, typical diffuse colonies were obtained when the organism was cultured in plasma-fibrinogen-albumin soft agar which contained lowered concentrations of plasma (0.1 per cent) and fibrinogen (0.2 per cent), as well as 1 per cent plasma soft agar and 0.4 per cent fibrinogen soft agar. The parent (S) strain appears to be unstable; when subcultured, isolated compact colonies gave rise regularly to a mixture of predominantly compact colonies and a small, variable percentage of diffuse colonies in plasma soft agar and in fibrinogen soft agar. However, by removal of individual colonies from mixed soft agar cultures with a capillary pipette, it was possible to separate colonial types and to obtain pure substrains of diffuse or compact colonies in fibrinogen soft agar and in plasma soft agar.

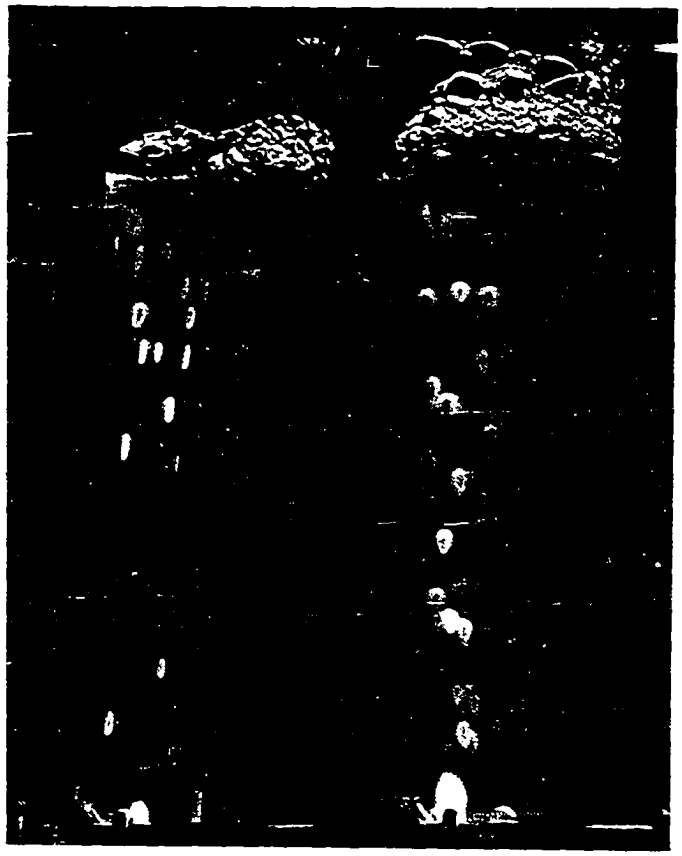

Fig. 3.—Typical appearance of colonies of coagulase-positive and clumping factor-negative staphylococci in plasma-fibrinogen-albumin soft agar.

TABLE 3

COMPARISON OF DEMONSTRATION OF COAGULASE AND CLUMPING FACTOR ACTIVITY OF REPRESENTATIVE STAPHYLOCOCCAL STRAINS BY CONVENTIONAL METHODS AND BY THE SOFT AGAR TECHNIQUE

Strain	Coagulase (Routine Test)	Clumping Factor (Slide Agglutination)[a]	Basal Soft Agar Medium Contain									
			1% Plasma		5% Plasma		0.4% Fibrinogen		1% Plasma Plus 0.4% Fibrinogen		Control	
			C[b]	CF[c]	C	CF	C	CF	C	CF	C	CF
H, K, V, 5, 79, 96	+	+	-	+	+	+	-	+	+	+	-	-
22, S 33, 75	+	+	-	+	±[d]	+	-	+	+	+	-	-
2, 28, 64, S 33v	-	-	-	-	-	-	-	-	-	-	-	-
Hv 2	-	±	-	-	-	-	-	-	-	-	-	-
K 6, K 93	+	-	-	-	+	-	-	-	+	-	-	-
K 93m	+	-	-	+	+	+	-	+	+	+	-	+[e]
Sv	+	-	-	-	+	±	-	-	+	+	-	-
Hv 1, S 35s	-	+	-	+	-	+	-	+	-	+	-	-
S 35	-	+[f]	-	-	-	+	-	+	-	+	-	-
18, 32, 95	+	+	Mixtures									
			-	+	±	+	-	+	+	+	-	-
			-	-	-	-	-	-	-	-		
S	+	+	Mixtures									
			-	+	+	+	-	+	+	+	-	-
			-	-	-	±	-	-				

[a]Results in 0.4 per cent fibrinogen and in rehydrated plasma.

[b]C, Coagulation zone around colonies.

[c]CF, Compact colonies.

[d]Narrow or indistinct coagulation zones.

[e]Unusually mucoid strain; in all soft agar media, forms large, round colonies which diffuse slightly after 24 hours incubation at 37 C.

[f]Positive in 0.4 per cent fibrinogen, but not in rehydrated plasma or lower dilutions of fibrinogen.

Virulence of Staphylococci for Mice

Physiological Properties and Metabolic Products of Selected Staphylococcal Strains

Results of tests for the *in vitro* properties commonly associated with *Staphylococcus aureus* are reported for the 13 strains selected for virulence studies (Table 4). Table 5 summarizes the results in relation to the four groups of staphylococci as divided according to their coagulase and clumping factor properties.

Regardless of coagulase activity, the clumping factor-positive strains were pigmented, whereas clumping factor-negative strains included both pigmented and non-pigmented ones. All the coagulase-positive strains fermented mannitol. Two of the 3 coagulase-negative, clumping factor-positive strains fermented mannitol, while both of the coagulase-negative, clumping factor-negative strains failed to do so. All of the 13 strains were catalase positive.

The hemolysin pattern of each of the 13 strains was determined by comparing the characteristic hemolysis produced on the human, rabbit and sheep blood agar plates following the recommendations of Elek and Levy (1950b, 1954). *Delta* hemolysin was produced by 12 of the 13 strains; it was the sole hemolysin in 9 strains, 4 of which were coagulase-positive, clumping factor-negative staphylococci. Only 2 of the 13 strains, Hv 2 and S 35, produced *alpha* hemolysin, which produced a wide zone of complete hemolysis on all of the blood agar media tested, as compared to narrow zone of complete hemolysis caused by *delta* hemolysin. *Beta* hemolysin was produced by 3 of the 13 strains. It occurred as the sole hemolysin in S 33 strain which produced a characteristic narrow zone of

TABLE 4

IN VITRO PROPERTIES OF STAPHYLOCOCCAL STRAINS SELECTED FOR VIRULENCE STUDIES

Strain	Coagulase	Clumping Factor	Pigment[a]	Catalase	Mannitol Fermentation	Hemolysis				Hemolysins Pattern	Staphylo-kinase	DNase	Phosphatase	Urease	Lipase	Egg-Yolk (EY) Reaction	Phage Pattern
						Human	Rabbit	Sheep	Mouse								
S	+	+	Y	+	+	+	+	+	+	delta	+	+	+	-	+	-	29
H	+	+	O	+	+	+	+	+	+	delta	+	+	+	-	+	+	52/80/81
V	+	+	O	+	+	+	+	+	+	delta	+	+	+	-	+	+	52/80/81
S 33	+	+	C	+	+	+	-	+	-	beta	+	+	+	-	+	+	NT[b]
Sv	+	-	Y	+	+	+	+	+	+	delta	+	+	+	-	+	-	NT
K 6	+	-	W	+	+	+	+	+	+	delta	+	+	+	-	+	+	NT
K 93	+	-	W	+	+	+	+	+	+	delta	+	+	+	-	-	-	NT
K 93m	+	-	W	+	+	+	+	+	+	delta	+	+	+	-	+	+	NT
Hv 1	-	+	O	+	+	+	+	+	+	delta	+	+	+	-	+	+	52/80/81
S 35	-	+	C	+	-	+	+	+	+	alpha, delta	-	-	-	+	+	+	NT
S 35s	-	+	O	+	+	+	+	+	+	delta	+	+	+	-	+	+	52/80/81
Hv 2	-	-	O	+	-	+	+	+	+	alpha, beta, delta	-	+	-	+	-	-	NT
S 33v	-	-	C	+	-	+	+	+	+	beta, delta	-	+	+	+	+	+	NT

[a] O, orange; Y, yellow; C, cream or buff; W, white.

[b] NT, non-typable.

TABLE 5

SUMMARY OF *IN VITRO* PROPERTIES OF SELECTED STAPHYLOCOCCAL STRAINS AS RELATED TO THEIR COAGULASE AND CLUMPING FACTOR ACTIVITY

Property Tested	Coagulase-Positive		Coagulase-Negative	
	Clumping Factor Positive	Clumping Factor Negative	Clumping Factor Positive	Clumping Factor Negative
Catalase	4/4[a]	4/4	3/3	4/4
Pigment	4/4	1/4	3/3	2/2
Mannitol Fermentation	4/4	4/4	2/3	0/2
Hemolysin				
alpha	0/4	0/4	1/3	1/3
beta	1/4	0/4	0/3	2/2
delta	3/4	4/4	3/3	2/2
Hemolysis of mouse blood	3/4	4/4	3/3	2/2
Staphylokinase	4/4	4/4	2/3	0/2
DNase	4/4	4/4	2/3	2/2
Phosphatase	4/4	4/4	2/3	1/2
Urease	0/4	0/4	1/3	2/2
Lipase	4/4	4/4	3/3	1/2
Egg-Yolk Reaction	3/4	3/4	3/3	1/2
Phage Typable	3/4	0/4	2/3	0/2

[a]Ratio: Number of strains with positive reaction/total number of strains tested in the particular coagulase-clumping factor group.

partial hemolysis on human and sheep, but no hemolysis on rabbit blood agar plates. However, the hot-cold phenomenon of beta hemolysin was not operative in this case; there was no distinct change on reincubation of the sheep blood agar plate at 4 C following its incubation at 37 C. Hemolysis on mouse blood agar plates was tested since the mouse was chosen as an experimental animal in the present virulence studies. Twelve of 13 strains showed zones of complete hemolysis, whereas S 33 strain failed to cause any degree of hemolysis on mouse blood agar plates.

With the exception of the two coagulase-negative, clumping factor-negative strains (Hv 2 and S 33v) and the coagulase-negative, clumping factor-positive S 35 strain, staphylokinase was produced by all strains. Twelve strains, but not the S 35 strain, produced deoxyribonuclease (DNase) in varied amounts as estimated by the size of clearing zone which resulted on the DNA containing trypticase soy agar medium. Relatively high DNase activity was observed in the case of the S 33 strain; otherwise, comparable amounts were produced by the other DNase positive strains. Only 2 of the 13 strains, S 35 and Hv 2, failed to liberate phosphatase. Urease was not produced by any of the coagulase-positive strains. Two strains (Hv 1 and S 35s) of the clumping factor-positive, coagulase-negative group did not produce urease, whereas S 35 strain and both of the coagulase and clumping factor-negative strains produced the enzyme. Lipase was produced by 12 of the 13 strains, but not by the Hv 2 strain. In contrast, only two of the other strains (S and Sv) gave a negative egg-yolk reaction.

None of the four coagulase-positive, clumping factor-negative strains were phage-typable, nor were the two coagulase-negative, clumping

factor-negative strains. Five of the 7 clumping factor-positive strains were phage typable, and 4 of them had a 52/80/81 phage pattern.

Some of the variant strains, which were differentiated on the basis of morphological changes or by their coagulase and/or clumping activity, retained other properties in common with their parent strains. The Smith virulent (Sv) strains which was differentiated from its parent, the Smith (S) strain, by its loss of clumping factor activity, had similar properties as those of its parent except for being phage non-typable. A complete correlation existed between the properties of K 93m variant strain and its parent, the K 93 strain, from which it differed by its unusually mucoid character. There was complete correlation between the properties of Hv 1 variant strain and its parent H strain from which it was differentiated by the loss of coagulase activity. There was no such complete correlation between the H strain and its other variant, the Hv 2 strain, which lost both coagulase and clumping factor activities. Compared to its parent strain, the Hv 2 variant strain lost the ability to ferment mannitol, to produce staphylokinase, phosphatase, and lipase, to give a positive egg-yolk reaction, and to be lysed by phages. Furthermore, it differed from its parent H strain by positive urease activity and the production of alpha and beta hemolysins in addition to delta hemolysin. The S 33v variant strain, which lost coagulase and clumping factor activities, retained some, but not all, of the parent strain (S 33) properties (Table 4). In addition to stronger clumping factor activity and diffuse growth in broth cultures, the smooth variant S 35s was different from the S 35 strain in that it fermented mannitol, produced a deeper, orange pigment, and was positive for staphylokinase as well as

for deoxyribonuclease. Furthermore, compared to its parent strain, the S 35s variant was phage typable, and produced no urease or alpha hemolysin.

The 13 strains of staphylococci were susceptible to the majority of the chemotherapeutic agents tested (Table 6). Eight of the 17 agents were effective against all of the strains, whereas occasional resistant strains were encountered with the other agents. Four (H, Hv 1, S 35s, and S 33v) of the 13 strains were resistant to penicillin. Of all strains, the H strain showed the greatest resistance; it was resistant to 6 out of the 17 chemotherapeutic agents, and to the lower concentrations of two other agents. All but two strains (S 35 and S 33v) were resistant to polymyxin B, and 9 of the 13 strains were resistant to triple sulfa and Gantricin. Some of the variant strains (Sv, K 93m and Hv 1) showed the same susceptibility to the chemotherapeutic agents tested as did their respective parent strains (S, K 93 and H). Other variants (S 33v, S 35s, and Hv 2), however, did not have the same susceptibilities as the parent strains.

Titration of coagulase in supernatants of centrifuged broth cultures proved to be unsatisfactory because of the chance that viable staphylococci might be present in the supernatants. The addition of 1:1000 and 1:2000 merthiolate inactivated most, if not all, of coagulase activity. Coagulase activity of culture supernatants was lost after Seitz filtration. Titration of coagulase production was possible only when serum-culture sterile filtrates were used. Different degrees of coagulase activity by the various strains were indicated by coagulase titration results (Table 7). The higher the coagulase titer, the less time

TABLE 6

IN VITRO SUSCEPTIBILITY OF STAPHYLOCOCCI TO ANTIBIOTICS AND OTHER CHEMOTHERAPEUTIC AGENTS[a]

Strain	Penicillin		Dihydrostreptomycin		Chloramphenicol		Tetracycline		Chlorotetracycline	Erythromycin		Neomycin		Kanamycin		Albamycin(R)	Vancomycin	Triacetyloleandomycin	Polymyxin B	Bacitracin	Spontin(R)	Triple Sulfa		Gantricin(R)	Furadantin(R)
	2 u	10 u	10 mcg	50 mcg	10 mcg	50 mcg	10 mcg	50 mcg	30 mcg	2 mcg	15 mcg	5 mcg	30 mcg	10 mcg	30 mcg	30 mcg	30 mcg	15 mcg	30 mcg	10 u	30 mcg	0.25 mg	1 mg	0.25 mg	100 mcg
S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S
H	R	R	R	R	S	S	R	S	R	R	S	S	S	S	S	S	S	S	R	S	S	R	R	R	S
Y	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S
S 33	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	R	R	R	S
Sv	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	S	S	S	S
K 6	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	R	R	R	S
K 93	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	R	R	R	S
K 93m	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	R	R	R	S
Hv 1	R	R	R	R	S	S	R	S	R	S	S	S	S	S	S	S	S	S	R	S	S	R	R	R	S
S 35	S	S	R	S	R	R	R	S	R	S	S	S	S	S	S	S	S	S	S	S	S	R	R	R	S
S 35s	R	R	R	R	S	S	R	R	R	R	S	S	S	S	S	S	S	S	R	S	S	R	R	R	S
Hv 2	S	S	S	S	R	R	S	S	S	S	S	S	S	S	S	S	S	S	R	S	S	R	R	R	S
S 33v	R	R	S	S	S	S	R	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S	S

[a]S, susceptible; R, resistant.

TABLE 7

COAGULASE TITRATION OF SERUM-CULTURE FILTRATES OF SELECTED STAPHYLOCOCCAL STRAINS

Strain	Clumping Factor	Coagulase Titer	Coagulation Time (Minutes)											
			Serum-Culture Sterile Filtrate Dilution											
			1:1	1:2	1:4	1:8	1:16	1:32	1:64	1:128	1:256	1:512	1:1024	1:2048
S	+	1:1024	15	30	75	105	120	150	180	240	300	420	540	–[a]
H	+	1:64	45	90	120	240	300	480	1440	–	–	–	–	–
V	+	1:256	35	90	120	150	300	360	540	1440	1440	–	–	–
S 33	+	1:128	20	45	90	120	150	300	480	600	–	–	–	–
Sv	–	1:256	15	60	90	120	180	210	300	360	1440	–	–	–
K 6	–	1:8	60	120	240	480	–	–	–	–	–	–	–	–
K 93	–	1:8	60	120	240	480	–	–	–	–	–	–	–	–
K 93m	–	1:8	90	420	600	1440	–	–	–	–	–	–	–	–
Hv 1	+	0	–	–	–	–	–	–	–	–	–	–	–	–
S 35	+	0	–	–	–	–	–	–	–	–	–	–	–	–
S 35s	+	0	–	–	–	–	–	–	–	–	–	–	–	–
Hv 2	–	0	–	–	–	–	–	–	–	–	–	–	–	–
S 33v	–	0	–	–	–	–	–	–	–	–	–	–	–	–

[a]No coagulation after 24 hours incubation.

was required for dilutions of the serum-culture sterile filtrates to clot a coagulation system of 1 per cent plasma-0.4 per cent fibrinogen-0.3 per cent albumin, the same system that was selected for use in the soft agar technique. Furthermore, the clots formed by strains of a high coagulase titer were regularly more firm than those formed by low coagulase titer strains.

Loss of clumping factor activity by the Sv variant strain was accompanied by lowered coagulase titer (1:256) in comparison to 1:1024 coagulase titer of its parent S strain. Strain K 93 and its variant strain (K 93m) as well as K 6 strain, all of which were coagulase-positive, clumping factor-negative, showed a relatively low coagulase titer of 1:8. The three other strains (H, S 33 and V) which were positive for clumping factor, had coagulase titers of 1:64; 1:128 and 1:256, respectively.

Less variation was observed in the clumping factor activity of the various strains than in their coagulase production (Table 8). All but one of the clumping factor-positive strains were clumped in 0.02 per cent fibrinogen solution. The exceptional strain, S 35s, required a minimal fibrinogen concentration of 0.4 per cent in order to exhibit positive clumping factor activity. This strain also differed from all others by growing in the bottom of broth culture tubes with almost a clear supernatant. An in vivo smooth variant, S 35s, which produced diffuse growth in broth cultures, emerged in organs of mice infected with the S 35 parent strain. The variant showed a stronger clumping factor activity; it clumped in 0.02 per cent fibrinogen. Equivocal results were obtained in the slide and tube agglutination test of the self agglutinating Hv 2

TABLE 8

CLUMPING FACTOR TITRATION OF SELECTED STAPHYLOCOCCAL STRAINS

Strain	Coagulase	Clumping Factor Titer[a]	Per Cent Fibrinogen																									
			Slide Agglutination								Tube Agglutination								Compact Colonies in Soft Agar							
			0.4	0.2	0.1	0.04	0.02	0.004	0.002	Control	0.4	0.2	0.1	0.04	0.02	0.004	0.002	Control	0.4	0.2	0.1	0.04	0.02	0.004	0.002	Control
S	+	0.02	+	+	+	±[b]	±	-	-	-	+	+	+	+	±	-	-	-	+	+	+	+	+	±	-	-
H	+	0.02	+	+	+	+	±	-	-	-	+	+	+	+	±	-	-	-	+	+	+	+	+	±	-	-
V	+	0.02	+	+	+	±	±	±	-	-	+	+	+	+	±	-	-	-	+	+	+	+	+	±	±	-
S 33	+	0.02	+	+	+	+	±	-	-	-	+	+	+	+	±	-	-	-	+	+	+	+	+	±	±	-
Sv	+	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
K 6	+	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
K 93	+	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
K 93m	+	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
Hv 1	-	0.02	+	+	+	+	±	-	-	-	+	+	+	+	±	-	-	-	+	+	+	+	+	±	±	-
S 35	-	0.4	+	±	±	-	-	-	-	-	+	±	±	±	-	-	-	-	+	±	±	±	±	-	-	-
S 35s	-	0.02	+	+	+	+	±	-	-	-	+	+	+	+	±	-	-	-	+	+	+	+	+	±	-	-
Hv 2	-	0	R[c]	R	R	R	R	R	R	R	R	R	R	R	R	R	R	R	-	-	-	-	-	-	-	-
S 33v	-	0	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a]Highest dilution of fibrinogen in which clumping of the organisms occurred or the one which supported formation of compact colonies in fibrinogen soft agar cultures.

[b]± Indicates doubtful results.

[c]R Indicates autoagglutination.

strain. However, it formed diffuse colonies in soft agar, and on this basis it lacked clumping factor activity.

When tested in soft agar media, all strains gave results comparable to those obtained in the conventional coagulase and clumping factor tests, with the exception of the autoagglutination property of Hv 2 strain mentioned above. The substitution of pooled mouse plasma for pooled human plasma as the substrate in coagulase and clumping factor yielded interesting results. When tested by the slide agglutination and soft agar technique, all of the 13 strains gave the same clumping factor results in mouse plasma as they did in human plasma or in bovine fibrinogen. Two of the 8 coagulase-positive strains (S and Sv) caused weak clotting of undiluted and diluted (1:2 and 1:4) mouse plasma in the conventional coagulase test. Strains S and Sv were also the only staphylococci tested which clotted a 0.4 per cent fibrinogen-1.5 per cent mouse plasma mixture. Here, however, clotting was slow; both the S and Sv strains required four hours to give a positive coagulase test with mouse plasma as the source of the coagulase reacting factor as compared to 30 minutes clotting time when human plasma was used in the same way. In 0.4 per cent fibrinogen-1.5 per cent mouse plasma soft agar, narrow coagulation zones were formed around the colonies of the S and Sv strains. The addition of 0.3 per cent albumin to the system did not affect the appearance of their coagulation zones (Table 9).

Results of the LD_{50} Determination

A comparison of the virulence of the selected strains was made by determining the LD_{50} of each strain, with the LD_{50} being defined as the minimum number of staphylococcal units required to cause the death of

TABLE 9

COMPARISON OF STAPHYLOCOCCAL COAGULASE AND CLUMPING FACTOR REACTIONS IN MOUSE PLASMA AND HUMAN PLASMA

Strain	Coagulation (Tube Test)				Clumping Factor (Slide Agglutination)		Colonies in Soft Agar Medium Containing									
	Human Plasma	Mouse Plasma	0.4% Fibrinogen Plus 1.5% Mouse Plasma	0.4% Fibrinogen (Control)	0.4% Fibrinogen	Mouse Plasma	0.4% Fibrinogen		1.5% Mouse Plasma		Fibrinogen Plus 1.5% Mouse Plasma		0.4% Fibrinogen, 1.5% Mouse Plasma Plus 0.3 Albumin		Basal Soft Agar (Control)	
							C[a]	CF[b]	C	CF	C	CF	C	CF	C	CF
S	+	±[c]	+	-	+	+	-	+	-	+	±	+	±	+	-	-
H	+	-	-	-	+	+	-	+	-	+	-	+	-	+	-	-
V	+	-	-	-	+	+	-	+	-	+	-	+	-	+	-	-
S 33	+	-	-	-	+	+	-	+	-	+	-	+	-	+	-	-
Sv	+	±	+	-	-	-	-	-	-	-	±	-	±	-	-	-
K 6	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
K 93	+	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
K 93m	+	-	-	-	-	-	-	+[d]	-	+[d]	-	+[d]	-	+[d]	-	+[d]
Hv 1	-	-	-	-	-	-	-	+	-	+	-	+	-	+	-	-
S 35	-	-	-	-	+	±	-	+	-	±	-	+	-	+	-	-
S 35s	-	-	-	-	+	+	-	+	-	+	-	+	-	+	-	-
Hv 2	-	-	-	-	±	±	-	-	-	-	-	-	-	-	-	-
S 33v	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-

[a]Coagulase as indicated by coagulation zones around colonies.

[b]Clumping factor as indicated by compact colonies.

[c]Weak or doubtful results.

[d]Mucoid strain K 93m forms spherical colonies in all soft agar media.

50 per cent of the mice within 14 days after either intravenous or intraperitoneal injection. Each challenge dose consisted of washed staphylococci suspended in 0.3 ml of phosphate-buffered saline. The experimental period of 14 days was found satisfactory since, as shown in preliminary trials, only rarely did death of an animal occur after this time.

No deaths occurred among mice which were injected intravenously or intraperitoneally with 0.3 ml amounts of (1) phosphate-buffered saline, (2) sterile trypticase soy broth, (3) a sterile supernatant of a trypticase soy broth culture of each strain and (4) a heat killed (75 C for 45 minutes) cell buffered saline suspension of the highest challenge dose of each strain.

The averages of two LD_{50} determinations of each of the 13 strains are recorded in Table 10. In this table, these strains are divided into four groups on the basis of their coagulase and clumping factor activities: Group I, positive for coagulase and clumping factor; Group II, positive for coagulase and negative for clumping factor; Group III, coagulase-negative and clumping factor-positive; Group IV, negative for both coagulase and clumping factor.

Influence of the route of injection on LD_{50}. The results of LD_{50} determinations of strains and the average LD_{50} for each group of staphylococci (Table 10) indicate there is an interaction between their virulence for mice and the route of injection. The coagulase-positive, clumping factor-positive staphylococci of Group I were somewhat more virulent for mice by the intravenous route than when they were injected intraperitoneally, with average LD_{50} values of 97.5×10^7 and 212.8×10^7, respectively. In contrast, Group II organisms of the coagulase-positive,

TABLE 10

LD_{50} OF STAPHYLOCOCCI INOCULATED INTRAVENOUSLY AND INTRAPERITONEALLY INTO MICE

Group	Strain	Titers		LD_{50} ($X10^7$)[a]		Group Average LD_{50} ($X10^7$)	
		Coagulase	Clumping Factor	IV	IP	IV	IP
I	S	1:1024	0.02	45.0	114.9	97.5	212.8
	H	1:64	0.02	64.2	279.4		
	V	1:256	0.02	27.4	31.9		
	S 33	1:128	0.02	254.8	425.0		
II	Sv	1:256	0	4.0	1.1	128.6	25.4
	K 6	1:8	0	183.5	20.8		
	K 93	1:8	0	167.0	18.1		
	K 93m	1:8	0	160.1	61.5		
III	Hv 1	0	0.02	97.2	358.0	109.1	284.2
	S 35	0	0.4	145.0	156.3		
	S 35s	0	0.02	85.1	284.2		
IV	Hv 2	0	0	940.0	315.0	1087.9	382.5
	S 33v	0	0	1235.8	450.0		

[a]The figures presented are arithmetical means of two LD_{50} determination values of each of the strains.

clumping factor-negative strains were more virulent intraperitoneally (average LD_{50}, 25.4 x 10^7) than by intravenous inoculation (LD_{50}, 128.6 x 10^7). Indeed, when introduced by the intraperitoneal route, Group II was the most virulent of all the groups; Group I was next most virulent and Group IV with an average intraperitoneal LD_{50} of 382.5 x 10^7 was least virulent of all. However, although the coagulase-negative, clumping factor-negative staphylococci of Group IV were least virulent by either route of injection, they were more virulent when injected intraperitoneally than intravenously (average LD_{50}, 1087.9 x 10^7). These observations, illustrated in Figure 4, were shown to be statistically significant when the over all mean LD_{50} values of strains within each of the four groups, with the random elimination of S 35 strain of Group III, were compared by the Duncan multiple range test (Li, 1957). The analysis of variance (Snedecor, 1956) showed that the route by group interaction is statistically significant at the 1 per cent level (Table 11). The F value of the analysis signifies that such an interaction can be attributed to chance less than 1 time in a 100.

With two exceptions, staphylococci of Group I appear to be more virulent intravenously than those of other groups. The LD_{50} of the first exceptional strain (S 33) was 254.8 x 10^7, which is higher than the mean LD_{50} value (45.5 x 10^7) obtained for the other three strains of Group I, and than the LD_{50} value of all staphylococci in Groups II and III. The variation in LD_{50} value of S 33 may be due to its peculiar predilection for skeletal muscle rather than for the tissues of more vital organs such as the kidney, which was the usual site of lesions and persisting infection. The second exception is the Sv strain of Group II; its

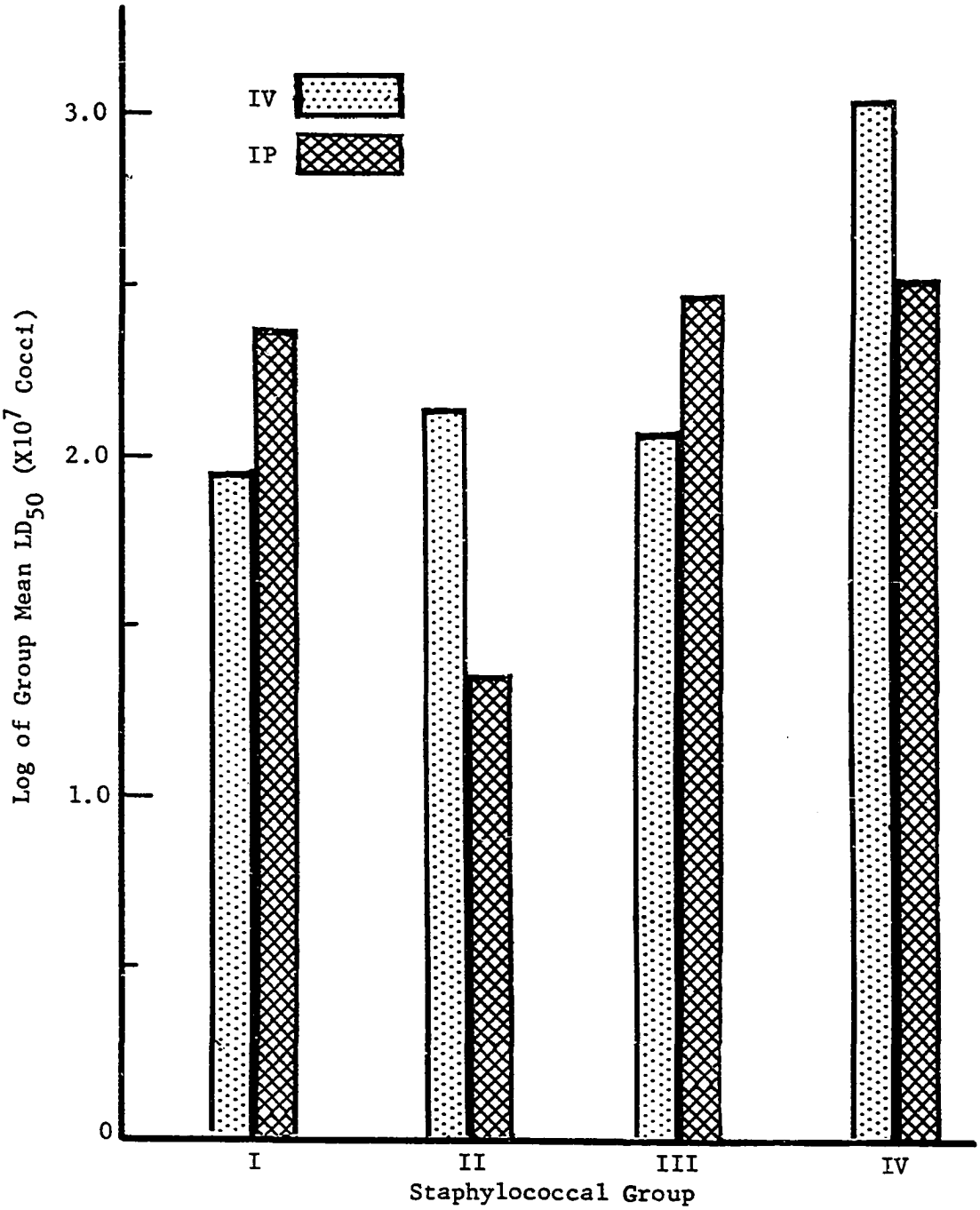

Fig. 4.—Interaction of virulence (LD_{50}) of groups of staphylococci and the route of injection. Group I: coagulase-positive, clumping factor-positive; Group II: coagulase-positive only; Group III: clumping factor-positive only; Group IV: negative for both factors.

TABLE 11

ANALYSIS OF VARIANCE OF THE INTERACTION AMONG STAPHYLOCOCCAL GROUPS BY VIRULENCE FOR MICE AND THE ROUTE OF INJECTION

Source of Variation	Degrees of Freedom	Sums of Squares	Mean Squares
Total	47	254,210.5	
Among groups	3	103,930.0	34,643.3
Between routes	1	1,354.7	1,354.7
Strain in group	8	104,395.7	13,049.5
Route by group interaction	3	32,125.4	10,708.5[a]
Replicates; strain by route in group	24	7,119.5	296.6
Strain by routes in group (error)	8	5,285.2	660.6

[a] $F_{(318)} = \frac{10,788.5}{660.6} = 16.21$, $P < 0.01$; P is the probability which indicates that such an F value can be attributed to chance alone.

intravenous LD_{50} value of 4.0×10^7 was smaller than any of the other strains tested including the Group I organisms.

On the other hand, with one exception, staphylococci of Group II were more virulent by the intraperitoneal route than any organisms of the other groups. The exceptional strain (K 93m) had an LD_{50} value of 61.5×10^7; it was relatively less virulent than the V strain of Group I, which had an LD_{50} value of 31.9×10^7. The most virulent strain of Group II by the intraperitoneal route was the Sv strain, which had an LD_{50} of 1.1×10^7. K 93 and K 6 strains had LD_{50} values of 18.1×10^7 respectively, which were much lower than those obtained for staphylococci of the other groups (Table 10).

The coagulase-negative, clumping factor-positive staphylococci of Group III were, on the average, more virulent intravenously and intraperitoneally than the coagulase-negative, clumping factor-negative strains of Group IV. Nevertheless, staphylococci of Group III were more virulent intravenously than when injected intraperitoneally, with mean LD_{50} values of 109.1×10^7 and 284.2×10^7, respectively. The LD_{50} values obtained by intravenous and intraperitoneal injection of strain S 35, however, were closely similar.

Results in Table 10 suggest that coagulase-positive staphylococci, i.e., Groups I and II, were, on the average, more virulent intraperitoneally than the coagulase-negative strains of Groups III and IV. On the other hand, clumping factor-positive organisms of Groups I and III were more virulent intravenously than clumping factor-negative strains of Group IV and to a lesser extent than organisms of Group II.

When variant strains are compared with their respective parent

strains, relative variation in their virulence may be noted (Table 10). The Sv strain was more virulent intravenously (LD_{50}, 4.0×10^7) and intraperitoneally (LD_{50}, 1.1×10^7) than its parent, the S strain, which showed an LD_{50} of 45.0×10^7 when injected intravenously and of 114.9×10^7 when injected intraperitoneally. The Sv strain was originally differentiated from its parent strain by loss of clumping factor activity. Further study also showed that it was phage non-typable and that it possessed a lower coagulase titer than its parent strain (Tables 4 and 7).

The Hv 1 strain, which differed from its parent H strain, only by loss of coagulase activity, was relatively less virulent, than the parent strain both intravenously and intraperitoneally. In contrast, the second variant of the H strain, the Hv 2 strain, which lost both coagulase and clumping factor activities and differed from its parent in other aspects (Tables 4 and 6), was much less virulent intravenously and less so intraperitoneally than its parent. The same situation was obtained when the S 33v variant strain, which is coagulase-negative, clumping factor-negative, was compared with its coagulase-positive, clumping factor-positive parent, the S 33 strain (Table 10).

The mucoid K 93m variant strain had an intravenous LD_{50} value of 160.1×10^7, which was comparable to that of its parent K 93 strain (167.0×10^7), whereas intraperitoneally, its LD_{50} (61.5×10^7) was significantly higher than that of the parent strain (18.1×10^7).

A more complicated situation arises when the virulence of the S 33s variant strains is compared with that of its parent strain (S 35) from which it differed in several aspects besides possessing stronger clumping

factor activity (Tables 4, 6 and 8). The variant S 35s strain was relatively more virulent intravenously, but not intraperitoneally (Table 10).

Variation in virulence among the staphylococci tested is further evidenced by the death rate and the speed with which the different strains killed the mice (Table 12). Depending on the strain and the dose of staphylococci, most of the animal deaths occurred during the first week after inoculation.

As indicated in Table 12, relatively large numbers of organisms (8.0×10^9 or more) of the coagulase-negative, clumping factor-negative strains were required to cause death of the animals when injected intravenously or intraperitoneally. In contrast, the more virulent strains, which were characterized by lower LD_{50} values, killed all of the mice with lower challenge doses, i.e., 4.0×10^9 or less. Furthermore, depending on the route of injection, virulent strains, e.g., Sv, V, K 93, K 6 and S strains, killed a varied percentage of the animals with doses (2.5×10^8 or less) which were completely non-lethal in the case of the less virulent strains. Thus, while there was a gradual decrease in the death rate with decreased doses of the virulent strains, a sharp drop in the killing level was associated with the less virulent strains. This gradual decrease in the death rate of mice injected with the virulent strains was also accompanied by prolonged death time (Table 12).

Results of ID_{50} Determinations

Determination of the ID_{50} was made on the basis of organisms recovered from infected mice which survived for 14 days after their injection intravenously or intraperitoneally with four-fold serially diluted doses of washed, phosphate-buffered saline suspended staphylococci.

TABLE 12

DEATH RATE AND TIME OF DEATH OF MICE INJECTED WITH STAPHYLOCOCCI BY THE INTRAVENOUS AND INTRAPERITONEAL ROUTES[a]

Strain	Titers		Average Number of Staphylococcal Units Injected[b]					
	Coagulase	Clumping Factor	8.0×10^9		4.0×10^9[c]		1.0×10^9	
			IV	IP	IV	IP	IV	IP
S	1:1024	0.02			$6/6^{5}$	$6/6^{7}$	$6/6^{1D}$	$6/6^{14}$
H	1:64	0.02	$6/6^{5}$	$6/6^{12}$	$6/6^{5}$	$4/6^{2D}$	$4/6^{4D}$	$1/6^{5D}$
V	1:256	0.02			$6/6^{7}$	$6/6^{5}$	$5/6^{2D}$	$5/6^{14}$
S 33	1:128	0.02	$6/6^{3}$	$3/6^{2D}$			$1/6^{5D}$	0/6
Sv	1:256	0			$6/6^{10}$	$6/6^{5}$	$6/6^{3D}$	$6/6^{5}$
K 6	1:8	0			$6/6^{4D}$	$6/6^{5}$	$1/6^{3D}$	$5/6^{10}$
K 93	1:8	0			$6/6^{18}$	$6/6^{5}$	$1/6^{3D}$	$6/6^{18}$
K 93m	1:8	0			$6/6^{18}$	$6/6^{14}$	$1/6^{2D}$	$3/6^{14}$
Hv 1	0	0.02	$6/6^{3}$	$6/6^{12}$			$2/6^{6D}$	$1/6^{1D}$
S 35	0	0.4	$6/6^{7}$	$6/6^{14}$			$3/6^{14}$	$2/6^{2D}$
S 35s	0	0.02	$6/6^{2}$	$6/6^{10}$			$3/6^{18}$	$1/6^{1D}$
Hv 2	0	0	$1/6^{6}$	$6/6^{14}$			0/6	$3/6^{10}$
S 33v	0	0	0/6	$6/6^{14}$			0/6	0/6

[a]Expressed ratio: number died/number injected in a given time. Time of death (figure recorded at upper right of ratio) is expressed in hours if death occurred within 24 hours, or in days (D) if death occurred after 24 hours.

[b]Figures derived from averages of viable number of staphylococcal units injected.

[c]Where blanks occur, challenge dose was not within 15 per cent of the stated average number of viable staphylococcal units injected.

TABLE 12—Continued

Average Number of Staphylococcal Units Injected[b]							
2.5×10^8		6.2×10^7		1.6×10^7		4.0×10^6	
IV	IP	IV	IP	IV	IP	IV	IP
$3/6^{3D}$	$1/6^{14}$	0/6	0/6	→			
$1/6^{6D}$	0/6	0/6	→				
$3/6^{5D}$	$2/6^{2D}$	$1/6^{6D}$	0/6	0/6	→		
0/6	→						
$5/6^{8D}$	$6/6^{7}$	$3/6^{8D}$	$5/6^{2D}$	$1/6^{13D}$	$4/6^{3D}$	0/6	0/6
0/6	$4/6^{14}$	0/6	0/6	→			
0/6	$3/6^{18}$	0/6	$2/6^{7D}$	0/6	0/6	→	
0/6	0/6	→					
0/6	0/6	→					
0/6	0/6	→					
$1/6^{4D}$	0/6	0/6	→				
0/6	0/6	→					
→							

The ID_{50} dose can then be defined as that number of cocci which caused infection and persisted for a period of 14 days or longer in 50 per cent of intravenously or intraperitoneally injected mice. Arithmetical means of two ID_{50} determinations for each of the 13 staphylococcal strains are recorded in Table 13.

Twelve of the 13 staphylococcal strains were relatively more effective in establishing infection when injected intravenously than intraperitoneally. The one exception, strain S 33 of Group I, was more effective by the intraperitoneal route. In other words, on the average, the four groups of staphylococci had relatively lower ID_{50} values when injected intravenously than intraperitoneally (Table 13; Figure 5). However, the ID_{50} values of intravenously and intraperitoneally injected coagulase-positive staphylococci of Groups I and II were notably lower than those of similarly injected staphylococci of the coagulase-negative groups. When injected intraperitoneally, the ID_{50} values for Group I and Group II were comparable, whereas intravenously, the mean ID_{50} (9.8×10^7) of Group I was relatively higher than that of Group II (5.2×10^7).

Among the coagulase-positive, clumping factor-positive staphylococci of Group I, the S 33 strain was the least effective in establishing infection regardless of the route of injection. Comparable ID_{50} results were obtained with the other (S, H and V) strains of Group I when they were injected intraperitoneally, but when introduced intravenously the V strain was the most effective of these three strains in establishing infection (ID_{50}, 0.6×10^7) whereas the H strain was the least effective (ID_{50}, 6.0×10^7).

TABLE 13

ID_{50} OF STAPHYLOCOCCI INOCULATED INTRAVENOUSLY AND INTRAPERITONEALLY INTO MICE

Group	Strain	Titers		ID_{50} $(X10^7)$[a]		Group Average ID_{50} $(X10^7)$	
		Coagulase	Clumping Factor	IV	IP	IV	IP
I	S	1:1024	0.02	1.3	13.6	9.8	16.5
	H	1:64	0.02	6.0	15.8		
	V	1:256	0.02	0.6	13.3		
	S 33	1:128	0.02	31.3	23.2		
II	Sv	1:256	0	0.3	0.7	5.2	18.0
	K 6	1:8	0	7.5	14.0		
	K 93	1:8	0	7.0	10.4		
	K 93m	1:8	0	5.9	47.0		
III	Hv 1	0	0.02	4.4	40.8	40.7	73.5
	S 35	0	0.4	114.7	156.3		
	S 35s	0	0.02	2.9	23.5		
IV	Hv 2	0	0	32.0	55.0	44.2	252.5
	S 33v	0	0	56.3	450.0		

[a]The figures presented are arithmetical means of two ID_{50} determination values of each of the strains.

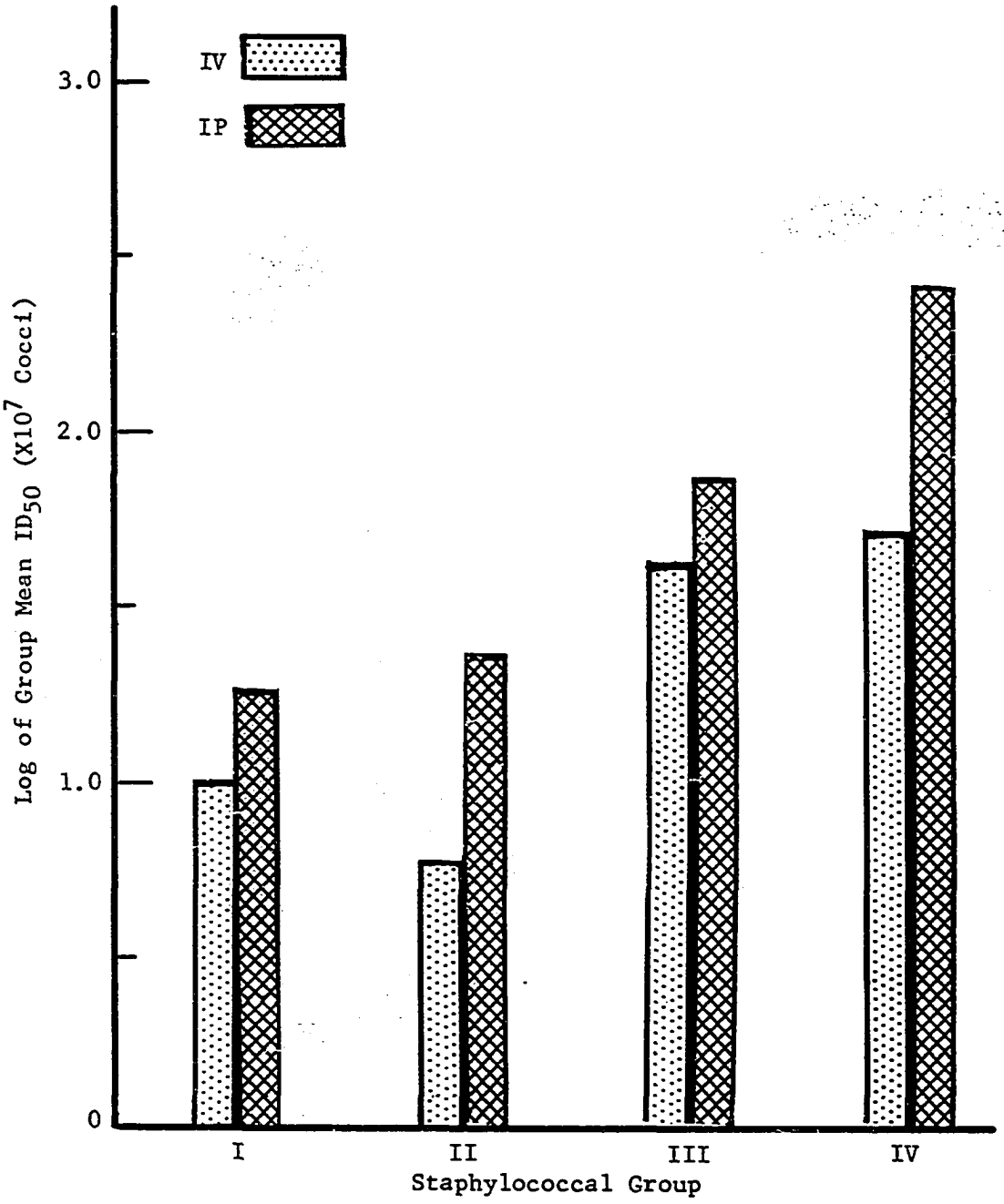

Fig. 5.—Interaction of infectivity (ID_{50}) of staphylococcal groups and the routes of injection. Group I: coagulase-positive, clumping factor-positive; Group II: coagulase-positive only; Group III: clumping factor-positive only; Group IV: negative for both factors.

Of the 13 strains, the strain with highest infectivity, whether by the intravenous or intraperitoneal route, was the Sv strain of Group II. Its ID_{50} values were 0.3×10^7 intravenously and 0.7×10^7 intraperitoneally. The ID_{50} values obtained for K 6 and K 93 strains of Group II when injected intravenously as well as intraperitoneally were similar. The K 6 strain gave ID_{50} values of 7.5×10^7 and 14.0×10^7 when injected intravenously and intraperitoneally, respectively, as compared to an intravenous ID_{50} of 7.0×10^7 and an intraperitoneal ID_{50} of 10.4×10^7 for the K 93 strain. The mucoid variant, K 93m, was notably more successful in establishing infection intravenously (ID_{50}, 5.9×10^7) than intraperitoneally (ID_{50}, 47.0×10^7).

The coagulase-negative, clumping factor-positive staphylococci of Group III were more infective than Group IV when injected intraperitoneally, but much less so when injected intravenously. Group III included the S 35 strain which was weakly infectious by either route as compared to the two organisms of the same group. The smooth variant (S 35s strain) was more infectious than its rough parent, the S 35 strain, when injected intravenously as well as intraperitoneally. Its intravenous and intraperitoneal ID_{50} values of 2.9×10^7 and 23.5×10^7, respectively, were much lower than those of the parent S 35 strain (Table 13).

Course of Infection and Persistance of Staphylococci in Mice

The course of infection in mice during the 14 day experimental period was followed by post-mortem examination for gross and microscopic lesions, as well as by determination of viable organisms in the organs and exudates, of all mice at the time of their deaths or sacrifice.

Mice injected with sublethal doses of the 13 strains of staphylococci were examined for persistance of the infecting organisms at two week intervals up to six weeks and, in some instances, eight weeks after injection.

Course of infection and persistance of staphylococci following intravenous inoculation. All strains, regardless of their coagulase or clumping factor character, dessiminated and were abundant in the blood, lungs, liver, spleen and kidneys of the mice within two hours after intravenous injection. As would be expected this was most pronounced with high challenging doses. High numbers of viable staphylococci were present in the blood, spleen, liver and lungs at 2 to 3 days post-infection time. Thereafter, a steady decline in the bacterial numbers in these organs occurred and usually ended with complete disappearance of staphylococci by the 10th to 14th day. In rare instances, a small number of organisms persisted for more than two weeks. Such a persistance of the organisms in the lungs, liver, or spleen generally was accompanied by abscess formation in the case of the more virulent strains such as the Sv, V and S.

A different course of events took place when infection was established in the kidneys, a common site for multiplication and persistance of the staphylococci (Table 14). After an initial low level in the numbers of staphylococci in the kidneys, which appeared to last for a few hours, a steady increase in the bacterial population began. The maximal count of viable staphylococci in the kidneys was usually reached by the 3rd to 5th day and was followed by a leveling off of the numbers recovered up to the 14th post-infection day. Subsequently, as indicated

TABLE 14

RELATIVE PERSISTANCE OF STAPHYLOCOCCI IN ORGANS OF MICE INJECTED INTRAVENOUSLY[a]

Group	Strain	Coagulase	Clumping Factor	ID_{50} ($X10^7$)	2 Weeks						4 Weeks						6 Weeks					
					Kidney	Heart's Blood	Spleen	Liver	Lung	Muscle	Kidney	Heart's Blood	Spleen	Liver	Lung	Muscle	Kidney	Heart's Blood	Spleen	Liver	Lung	Muscle
	S	+	+	1.3	4	(1)	(1)	(1)	(1)	(2)	4					(2)	3	(1)				
I	H	+ +	+	6.0	4						3						2					
	V	+ +	+	0.6	4	(1)	(1)	(1)	(1)	(3)	4					(2)	3	(1)	(1)			(2)
	S 33	+	+	31.3	(1)		(1)			3	(1)					3						2
	Sv	+	-	0.3	4	(1)	(1)	(1)	(1)	(3)	4					(3)	3	(1)				(2)
II	K 6	+	-	7.5	3					(2)	3					(2)	2					(1)
	K 93	+	-	7.0	3			(1)		(2)	3					(2)	2	(1)				(1)
	K 93m	+	-	5.9	3			(1)			2						2					
	Hv 1	-	+	4.4	4		(1)				3						2	(1)	(1)			
III	S 35	-	+	114.7	(1)																	
	S 35s	-	+	2.9	4	(1)				(2)	3						(2)					(1)
IV	Hv 2	-	-	32.0	2					(1)	2											
	S 33v	-	-	56.3	2						(1)											

[a]The relative number of organisms is graded from 1 to 4, with 1 indicating a few and 4 an abundant number of organisms recovered. Parentheses indicate organisms were recovered only occasionally.

in Table 14, there was a gradual decline in the number of viable organisms of the more virulent strains (Sv, S and V strains) for as long as the mice were examined, whereas the organisms of the less virulent or avirulent strains (S 35, Hv 2 and S 33v) had disappeared from the kidneys by the fourth week.

When examined one week after challenge, the gross appearance of the infected kidneys was apt to differ with the strain of staphylococcus injected (Figure 6). This was most evident when the infecting staphylococcus was clumping factor-positive or when it was the clumping factor-negative, coagulase-positive Sv strain. However, formation of multiple kidney abscesses (Figure 6a,b) was characteristic of the clumping factor-positive staphylococci of Groups I and III (Table 14). Miscroscopic examination* of such kidneys revealed focal septic infarctions with infiltration of polymorphonuclear leukocytes and cocci throughout the kidney. Many of the glomeruli appeared acutely inflamed. Septic infarction was initiated and was most extensive in the cortex, but the medulla was also involved in cases of severely infected kidneys. Necrosis of the adjacent adrenal glands was not an unusual observation.

In contrast to the pathology produced by the clumping factor-positive staphylococci, grossly visible subcapsular abscesses were rarely observed in the kidneys of mice which died within 14 days after injection of clumping factor-negative strains (Group II and Group IV). Instead, such kidneys were usually partially or entirely pale in appearance (Figure 6d). When occasional, visible kidney abscesses did develop

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*Histopathological examinations were made by members of the Department of Pathology, University of Oklahoma Medical Center.

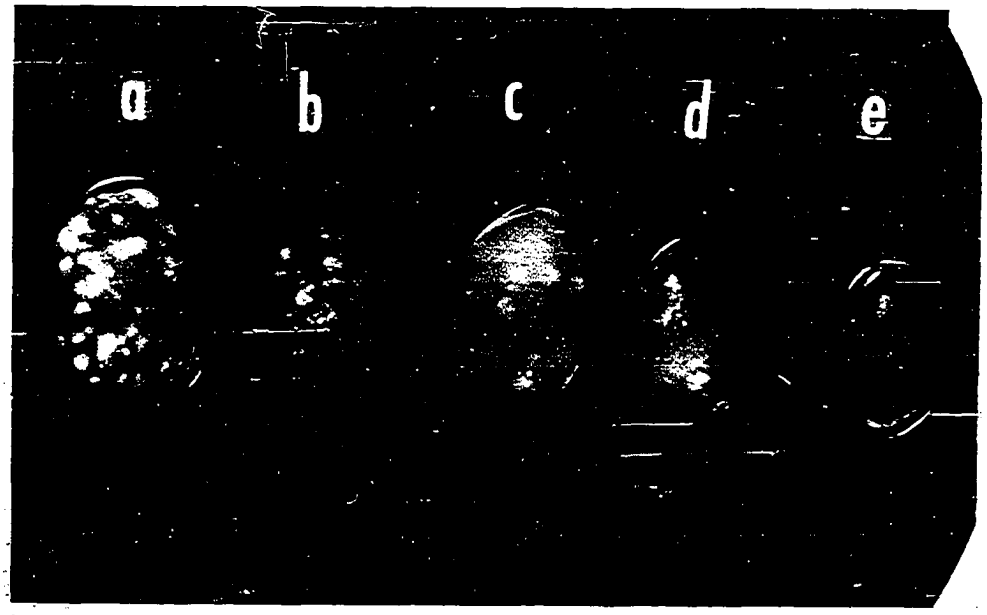

Fig. 6.—Variation in gross appearance of kidneys from mice infected with staphylococci: a and b are characteristic of clumping factor-positive staphylococci infected kidneys, whereas c and d are typical of infections of prolonged and short duration, respectively, with clumping factor-negative staphylococci; e is a normal mouse kidney.

(Figure 6c), it was late in the course of infection, about 4 to 6 weeks after inoculation of the animals. The histopathological picture of these kidneys revealed pyelitis with encapsulated abscesses in the region of the papillae and pus in the pelvis of the kidney.

The degree of reaction in the kidneys then depended on the staphylococcal strain, the size of the challenge dose and the time of death or sacrifice of the animal. It was most extensive with the more virulent strains and, in general, reached a peak at 7 to 10 days after injection. Such kidneys were necrotic when examined 6 or 8 weeks post-infection. Animals with extensive kidney damage were usually obviously ill by the 6th to 8th post-infection week.

Microscopic infection of kidneys, without gross pathological changes other than enlargement, was evident in mice infected with large doses of the less virulent strains (S 35, Hv 2 and S 33v) and in mice infected with lower doses of more virulent strains. In these animals dilation of the tubules of these kidneys was the usual microscopic impression. However, such kidneys were usually sterile by the fourth post-infection week.

Although the kidney was the most common site of infection, other organs were occasionally involved. Abscesses were frequently observed in the skeletal muscles and also in the heart muscle. Liver, spleen and brain abscesses were seen much less frequently and were usually characteristic of strains which were relatively virulent by the intravenous route. Enlargement of the spleen with or without abscess formation was common. Persistance of infection for 14 days in organs other than the kidneys, without grossly visible lesions, usually followed intravenous challenged with large doses of the more virulent staphylococci.

The blood stream of the mice was usually free of the organisms by the second week after injection, but reappearance of a small number of organisms in the blood was not infrequent six weeks after infection with most of the clumping factor-positive staphylococci (Table 14).

Strain S 33 showed a remarkable predilection for skeletal muscle. Formation of abscesses in the muscles of thighs and/or shoulders, rather than in the kidneys, was the usual consequence of infection (Table 14). Miscroscopic examination revealed bone, as well as muscle, involvement and gave an impression of osteomyelitis. Only when high doses of strain S 33 were injected intravenously were organisms recovered from the kidneys and then no gross pathology was observed.

Course of infection and persistance of staphylococci following intraperitoneal inoculation. Large challenge doses of intraperitoneally injected virulent staphylococci, in particular those of Group II, killed mice rapidly, within 2 to 5 hours. Similar and large inocula of the less virulent strains failed to produce any more than transitory signs of illness for the first few hours. Early death of the animals was abrupt and was preceded by marked respiratory distress and convulsions. On post-mortem examination, the peritoneal fluid, blood, liver and spleen of such animals were heavily infected with the organisms, whereas fewer organisms were recovered from the lungs and kidneys.

The events of the first week after intraperitoneal injection were, in general, similar to those observed following the intravenous inoculation. However, fewer organisms were present in the organs of mice injected intraperitoneally than in those injected intravenously with comparable doses of clumping factor-positive staphylococci. On the

other hand, the clumping factor-negative staphylococci, in particular strains of Group II, did not show such a difference.

Recovery of relatively small numbers of staphylococci from the kidneys was a common characteristic of all of the 13 staphylococcal strains throughout the course of intraperitoneal infection as compared to the high numbers recovered after their intravenous inoculation. Persistance of infection with abscess formation in the peritoneal wall and viscera was also observed following the intraperitoneal injection of clumping factor-positive staphylococci (Groups I and III) and of the Sv strain of Group II, but much less frequently than with the other, clumping factor-negative, organisms.

After intraperitoneal injection persisting infection beyond the experimental period of 14 days was localized mainly in the peritoneal and/or in the kidneys (Table 15). The extent of persisting infection varied with the staphylococcus injected, its coagulase and clumping characters and, as expected, with the size of the challenging dose. No organisms, however, were recovered from the peritoneal fluid after 10 days, but abscess formation in the peritoneal wall, spleen and liver ligaments as well as intestinal mesentery were common. Enlargement of the spleen was a striking and common observation when virulent strains were injected intraperitoneally. Miscroscopic examination of such spleens revealed hyperplasia of germinal centers and the conspicuous presence of giant mononuclear leukocytes. On the other hand, following intraperitoneal challenge there was much less kidney damage than when comparable doses of the same strains of staphylococci were injected intravenously.

TABLE 15

RELATIVE PERSISTANCE OF STAPHYLOCOCCI IN ORGANS OF MICE INJECTED INTRAPERITONEALLY[a]

Group	Strain	Coagulase	Clumping Factor	ID_{50} ($X10^7$)	2 Weeks							4 Weeks						6 Weeks					
					Kidney	Heart's Blood	Peritoneum	Spleen	Liver	Lung	Muscle	Kidney	Heart's Blood	Peritoneum	Spleen	Liver	Lung	Muscle	Kidney	Heart's Blood	Peritoneum	Spleen	Liver	Lung	Muscle
I	S	+	+	13.6	3	(1)	4	(1)				3		4					2		4				
	H	+	+	15.8	3		4					3		4					2		3				
	V	+	+	13.3	3	(1)	4	(1)	(1)			3		4					3	(1)	3				
	S 33	+	+	23.2	(1)		4					(1)		3							3				
II	Sv	+	-	0.7	3		3	(1)	(1)			3		3					3		2				
	K 6	+	-	14.0	3		1				(2)	1							1						
	K 93	+	-	10.4	3							2							2						
	K 93m	+	-	47.0	3	(1)	(2)	(1)	(1)	(1)	1	2		(2)					1		(2)				
III	Hv 1	-	+	40.8	3		3					2		2					1		1				
	S 35	-	+	156.3																					
	S 35s	-	+	23.5	3		3					2		3					1		1				
IV	Hv 2	-	-	55.3	2																				
	S 33v	-	-	450.0	2																				

[a]The relative number of organisms is graded from 1 to 4, with 1 indicating a few and 4 an abundant number of organisms recovered. Parentheses indicate organisms were recovered only occasionally.

Moderate numbers of the coagulase-negative, clumping factor-negative organisms of Group IV persisted in the kidneys for two weeks but were almost completely eliminated by the fourth post-infection week. Likewise, organisms of the S 35 strain of Group III were completely eliminated two weeks after their intraperitoneal injection. No viable organisms were ever recovered from purulent material of abscesses produced by the S 35 strain. Organisms of other strains of Group III declined gradually in number from the kidneys and peritoneal abscesses, but they persisted in lowered numbers for six weeks post-infection. A gradual decrease in the numbers of coagulase-positive staphylococci (Groups I and II) in abscesses was also noted, but these organisms persisted in all types of kidney lesions for 6 to 8 weeks.

In vivo Stability of Staphylococci

Staphylococci recovered from infected mice were compared with the organisms injected in order to evaluate the in vivo stability or variation of strains, particularly with respect to coagulase and clumping factor activity.

A morphologically distinct mucoid variant, the K 93m strain described above, was recovered from the kidneys of several mice five weeks after their intravenous injection with the K 93 strain. The mucoid character of this variant was stable in vitro after more than 30 culture passages or as long as examined. Likewise, it appeared to be stable in vivo since organisms of the same general character were isolated from mice in the eighth week of infection. In a few instances, however, the isolates were even more mucoid and, unlike the parent K 93 and the originally isolated K 93m strain, some were weakly pigmented.

A stable, smooth variant, the S 35s strain, was recovered from kidneys of mice which were sacrificed two weeks after intravenous injection with the rough S 35 parent strain. The smooth variant was easily detected in fibrinogen soft agar cultures by its large colony size as compared to that of the parent strain. It had a high clumping factor titer than the original strain (Table 7) and it also differed in certain other characteristics including phage typability (Tables 4 and 6).

Two other variants, Sv and Hv 1, were encountered regularly, although in varied proportions, in soft agar cultures of staphylococci which were recovered from mice infected with the S and H strains, respectively. Both of these variants also occurred as a result of in vitro cultivation of their respective parent strains.

The Sv strain used in the present studies was originally isolated from the Smith (S) strain by Hunt and Moses (1958) who noted that it differed from the parent (S) by formation of diffuse colonies in plasma soft agar. These workers reported that the Smith "mouse virulent" strain (Sv) emerged in high proportion from mice inoculated intraperitoneally with the Smith "mouse avirulent" strain. In the present study, the Sv variant proved to be completely stable in vivo and in vitro. When mice were infected with the S strain, however, the Sv variant emerged in vivo as a small minority (5 to 10 per cent) of the staphylococcal populations recovered from the infected mice.

The Hv 1 variant of the H strain was distinguished by its loss of coagulase activity. It was recovered in various proportions (5 to 20 per cent) from infected organs of all mice injected with the H strain. The Hv 1 variant appeared to retain all of the parental properties other

than coagulase activity and it was stable *in vivo* as well as *in vitro* for as long as examined.

Dwarf colonies, similar to those described by Wise (1956), were commonly observed in cultures made from injected mice, particularly from mice sacrificed two weeks after injection. These dwarf colonies, however, always reverted to regular colony size by the second or third *in vitro* subculture.

Except for the variations noted above, the staphylococcal strains tested exhibited no other detectable changes after residence within the infected mice.

CHAPTER IV

DISCUSSION

Demonstration of Staphylococcal Clumping Factor and Coagulase in Soft Agar

The soft agar method proved to be effective for demonstrating both the clumping factor and coagulase activities. This technique was found to have certain advantages over conventional methods. It overcomes the problem presented in slide and tube agglutination tests by rough strains. It may reveal the heterogeneity of a strain with regard to coagulase and clumping factor as expressed by coagulation zones and colonial compactness, respectively. Thus, the discovery in pure or mixed populations of rare strains which possess either coagulase or clumping factor, but not both of these characters, is readily accomplished by this method. There was no evidence that the coagulation zones which form around the colonies of coagulase-positive staphylococci in plasma-fibrinogen-albumin soft agar failed to develop or disappeared as the result of staphylokinase activity. The soft agar method also proved valuable for simultaneous quantitative estimation of staphylococci recovered from organs and exudates of infected mice and the detection of their coagulase and clumping factor activities without any intermediate cultivation which might affect the *in vivo* character of the organisms. Previous attempts have been made to modify the conventional tests for coagulase and the ability of

staphylococci to agglutinate in plasma or fibrinogen in order to obviate irregular results (Chapman et al., 1941; Miale and Faye, 1948; Elek, 1959; Jenkins and Metzger, 1959). At the research level at least, the soft agar method appears to be more accurate and consistent than the previously described methods for detection of these properties. In addition, the soft agar technique lends itself to titration of the clumping factor activity.

Most coagulase-positive staphylococci produce spherical, compact colonies in 0.04 per cent fibrinogen soft agar, in 0.1 per cent plasma soft agar and in 1 per cent serum soft agar medium. Jensen (1958) reported on the regular occurrence in human serum of an antibody which reacts with a cellular component of staphylococci belonging to serological type I of Cowan. Antibody action cannot be excluded as a contributing factor in the colony compacting mechanism in plasma soft agar or in serum soft agar, but it is not necessarily the sole factor since all strains which are positive for clumping factor also form compact colonies in bovine fibrinogen soft agar. Various investigators (McCarty, 1946; Pike, 1946; Pittman and Davis, 1950; Lankford et al., 1955) have used a soft agar technique successfully in studies of variation. The present soft agar method could undoubtedly be adapted to study of other staphylococcal variations, particularly antigenic variations, as well as to variations in the coagulase and clumping factor activities of staphylococci.

The presence of serum or plasma, which contain coagulase-reacting factor, is essential for the coagulation zone phenomenon in soft agar as it is for the plasma clotting reaction in the routine coagulase test. Finkelstein and Sulkin (1958) noted that halos were visible occasionally

around compact staphylococcal colonies in soft agar medium which contained high concentrations of serum. The combination of 0.3 per cent albumin, 1 per cent plasma, and 0.4 per cent fibrinogen in soft agar provides a medium in which even weak producers of coagulase form remarkably clear coagulation zones. In basal soft agar any concentration of plasma or serum which is adequate for distinct coagulation zone formation is capable of compacting colonies of strains which have the clumping factor. Less distinct coagulation zones were formed by some, but not all, of the coagulase-positive staphylococci tested in 5 to 10 per cent human plasma. The absence of distinct coagulation zones in such high concentrations of plasma in soft agar cultures may be due to the presence of anticoagulase antibodies or, possibly, to digestion of the fibrin by staphylokinase although, as mentioned above, there was no evidence for the latter in 1 per cent plasma containing soft agar cultures.

Narrow and indistinct coagulation zones were formed by two high coagulase producing strains (S and Sv) when 1.5 per cent mouse plasma was substituted for 1 per cent human plasma as a source of coagulase reacting factor in plasma-fibrinogen soft agar. A 0.4 per cent fibrinogen-1.5 per cent mouse plasma (or serum) coagulation system was slowly but firmly clotted by the same two strains and no others when examined by the conventional plasma clotting test. Unusual clotting of mouse plasma by staphylococci was reported by Gorrill (1951). Tager and Hales (1948a) indicated that coagulase-reacting factor (CRF) is present in high titers in human and rabbit plasmas and that there is little variation in the amount of CRF in different samples of human plasma (Tager and Hales, 1948c). Plasmas of other animal species, including the mouse, were less

effective in activating coagulase when used in the unmodified state due to low CRF titer, but additional CRF was released by acid precipitation of these plasmas (Tager and Hales, 1948a).

The formation of compact colonies by coagulase-positive staphylococci in serum- or plasma-containing soft agar has been attributed to the action of specific antibody or to factor(s) resembling antibody in normal rabbit and human plasma and sera (Finkelstein and Sulkin, 1958; Hunt and Moses, 1958). If the findings which contribute to this interpretation are re-examined with respect to the effect of staphylococcal clumping factor on colonial form in fibrinogen soft agar, it appears that antibody action in the usual sense is not the only possible explanation. Undoubtedly, specific antibody is operating at serum dilution of 1:10,000 and more, but the compact "tailed" colonies which develop in soft agar containing 1:1000 rabbit antiserum (Finkelstein and Sulkin, 1958) appear to be the same as the intermediate colony type, compact with elongated base, which were observed in the 0.1 per cent normal serum soft agar and the 0.004 per cent fibrinogen soft agar cultures herein described. Higher concentrations of serum, plasma, or fibrinogen in soft agar media result in the formation of spherical, compact colonies by strains of staphylococci which are positive for clumping factor regardless of their coagulase activity or probably, serological type.

In the demonstration of coagulase and clumping factor in plasma containing soft agar, a fibrinogen soft agar culture is a necessary control. In certain strains, such as the Sv strain (Table 3), colonial diffusion is retarded in plasma-fibrinogen-albumin soft agar, but not in fibrinogen soft agar or plasma soft agar in which no clotting occurs.

The fact that the Sv strain is a stronger coagulase producer than the other clumping factor-negative strains tested (Table 7) may account for inhibition of elongation of its colonies. Only after 24 hours incubation that slight elongation of the Sv colonies is observed. Nevertheless, a typical picture (Figure 4) of the Sv strain may still be seen in 0.1 per cent plasma-0.04 per cent fibrinogen-0.3 per cent albumin soft agar, i.e., with soft agar which contains only 1/10 the usually employed concentrations of plasma and fibrinogen.

In the case of the unusually mucoid K 93m strain (Table 3), an additional basal soft agar medium was required as a control in the soft agar method. This strain formed large, spherical colonies in all soft agar media, including the basal soft agar, during the first 24 hours incubation and only slight diffusion of the colonies occurred after that time. The delay in colonial elongation appears to be due to the elaboration of a substance, probably a mucopolysaccharide, which is extruded in the form of a slime layer around the cocci. Such a layer, not discrete enough to call a true capsule, was seen in preparations stained by special capsule staining methods. Colonies of this mucoid strain were extraordinarily large in soft agar as well as on blood agar plates where their viscid consistency was remarkable. The isolation of mucoid staphylococcal strains was reported by several workers (Elek, 1959), including Reimann (1957) and Wiley (1959).

The use of the soft agar technique is efficient in the discovery of rare staphylococcal strains or variants in mixed populations which possess coagulase or clumping factor but not both of these properties. Hunt and Moses (1958) reported isolation of an exceptionally mouse

virulent strain, the Smith "mouse virulent" (Sv) strain, which forms diffuse colonies in plasma soft agar, as compared to its relatively avirulent parent strain, the Smith (S) strain, which forms compact colonies in plasma soft agar. The present study shows that other rare coagulase-positive staphylococci which also form elongated, diffuse colonies in fibrinogen soft agar (Figure 4) and fail to agglutinate in fibrinogen are also more virulent for mice when inoculated intraperitoneally than any of the coagulase-positive, compact colony forming staphylococci tested.

Comparative Virulence of Staphylococci for Mice

It is generally believed that mice are more susceptible to bacterial infection by the intraperitoneal route than by the intravenous route of injection. Dutton (1955), in his comparative study of the influence of the route of injection on infections in mice caused by a variety of microorganisms concluded that a lower lethality was obtained when most organisms, including staphylococci, were injected intravenously rather than intraperitoneally. The author suggested that this difference in the susceptibility of mice to the two routes of injection may be a phenomenon of non-specific immunity. Controversial statements have appeared in the literature as to the influence of route of injection on the susceptibility of mice to experimental staphylococcal infection (Elek, 1959). While some investigators have used the intravenous route (Gorrill, 1951, 1958; McCune et al., 1956; Smith, 1956; Smith and Dubos, 1956a; Gray et al., 1957; Smith et al., 1957; Sellers and Le Maistre, 1960), others have inoculated mice with staphylococci by the intraperitoneal route (Christie et al., 1946; Fisher and Thompson, 1956; Hunt and Moses, 1958; Fisher,

1959). The mouse is, at best, relatively resistant to infection with staphylococci and large inocula of these organisms are required to kill mice or even to establish a staphylococcal infection, regardless of the route of injection.

In the present investigation, a definite interaction between the virulence of staphylococci and the route of injection is indicated. This relationship, however, depended on the nature of the staphylococcus injected with respect to its coagulase and clumping factor activities. Among the limited number of staphylococci tested (13 strains), clumping factor-positive staphylococci (Groups I and III) were found to be more virulent and more infective for mice when injected intravenously than by the intraperitoneal route, regardless of coagulase activity (Tables 10 and 11). The coagulase-positive, clumping factor-positive staphylococci of Group I were the most virulent of all when injected by the intravenous route.

On the other hand, the clumping factor-negative staphylococci of Groups II and IV were more virulent for mice when injected intraperitoneally than when the same organisms were introduced intravenously regardless of their coagulase activity (Table 10). Furthermore, the coagulase-positive, clumping factor-negative staphylococci of Group II were more virulent by the intraperitoneal route than any of the other staphylococci tested (Table 10; Figure 4).

With two exceptions (Johanovsky, 1957b; Hunt and Moses, 1958) previous staphylococcal virulence studies have differentiated between coagulase-positive and coagulase-negative staphylococci without regard to clumping factor activity. Several investigators have indicated the

existence of rare staphylococcal strains which possess coagulase or clumping factor activity exclusively (Cadness-Graves et al., 1943; Needham et al., 1945; Williams and Harper, 1946; Duthie, 1954a, 1955; Klemperer and Haughton, 1957; Ness, 1957; Jenkins and Metzger, 1959). However, such strains were generally considered to be of doubtful pathogenicity (Duthie, 1955).

Johanovsky (1957b) was the only one to associate the clumping factor with the virulence of staphylococci. He suggested that the clumping factor rather than coagulase may contribute to the survival of staphylococci inside phagocytes. Although he used staphylococcal strains which were clumping factor-positive and others which were clumping factor-negative, the coagulase activity of both groups was not clearly indicated. Since coagulase-negative, clumping factor-positive strains are rarely encountered, it is suspected, from the number of strains tested, that he most probably utilized strains that were positive for coagulase and clumping factor and others which were negative for both factors, although from his report this is not clear.

Hunt and Moses (1958) reported on the unusual mouse virulence of a variant of the Smith strain of S. aureus by the intraperitoneal route. The Smith parent strain had previously been recognized as being exceptionally virulent for intravenously injected mice (Smith and Dubos, 1956a; Gray et al., 1957). The Smith variant strain, recognized by its diffuse colonial growth in plasma soft agar, was reported to be antigenically distinct from the parent strain. It was found to have an LD_{50} value of 4×10^6, whereas 1×10^9 organisms of other coagulase-positive staphylococci, including the Smith strain, failed to kill mice consistently.

A relatively higher intraperitoneal LD_{50} value (1.1×10^7) of the Smith variant strain was obtained in the present investigation. However, this difference may very well be explained on the basis of experimental variation. Results are influenced by the age and inherent susceptibility of the strain of the mice. The viable count of staphylococci is subject to considerable variation following minor changes in procedure. Furthermore, the volume of the challenge dose was found to influence the estimate of the virulence of staphylococci for mice. A 0.5 ml dose (used by Hunt and Moses, 1958) killed more mice than a 0.3 ml dose (used in the present study) containing the same number of viable cocci.

The virulence of staphylococci for mice appears to be greatly enhanced when the organisms are suspended in hog gastric mucin before their intraperitoneal inoculation (Anderson and Oag, 1939; Schneierson and Amsterdam, 1956). However, the effect of mucin is actually one which decreases the defense mechanisms of the host (Lambert and Richley, 1952; Wilson and Miles, 1955). By the use of mucin, Hunt and Moses (1958) reported exaggerated differences between the LD_{50} values of the "Smith virulent" variant and the "Smith mouse avirulent" parent strain. Thus, the LD_{50} of the Sv strain was lowered to about 580 cells per mouse, whereas that of other coagulase-positive staphylococci, including the parent Smith strain, was at the level of 1×10^6 viable cells.

Between 1956 and 1957 two coagulase-positive, clumping factor-negative strains (K 6 and K 93) as well as two coagulase-negative, clumping factor-positive strains (Hv 1 and S 35) were recognized in this laboratory. During the present study, other investigators* have

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*Personal communications from Dr. George A. Hunt, Bristol Laboratories, Inc. and Dr. Myron W. Fisher, Parke-Davis and Company.

examined the K 6 and K 93 strains for mouse virulence by intraperitoneal injection of mucin suspensions of the organisms. From their results it is evident that, first, these strains, like the Smith "mouse virulent" variant, are unusually virulent for mice by the intraperitoneal route, and, second, compared to the results presented here, mucin greatly exaggerates their virulence.

Fisher and Thompson (1956) found that a toxic substance, which was heat labile and inseparable from actively growing bacteria, was responsible for the rapid killing of mice when they were inoculated intraperitoneally with certain strains of staphylococci. The same workers indicated that formation and absorption of a highly toxic substance in the peritoneal cavity, rather than dessimination of the organisms into the organs or blood stream, was responsible for the death of the animals. Hunt and Moses (1958) noted that the Smith "mouse virulent" variant only grew rapidly within the peritoneal leukocytes, whereas the Smith "mouse avirulent" parent strain and other coagulase-positive staphylococci tested did not, although they remained viable for many hours. Depending upon the challenge dose, Hunt and Moses observed a marked and consistent proliferation of the Smith variant strain within the leukocytes for 8 to 12 hours. At the end of this period, there was an abrupt appearance of overwhelming numbers of extracellular staphylococci and the mice died within the next 20 to 40 minutes. The authors suggested that a leucocidic activity, due to high concentrations of delta hemolysin, may have caused the abrupt death of the animals.

It is reasonable to assume that clumping of the staphylococci may permit the phagocytes to engulf a greater number of organisms than would

be the case with staphylococci which fail to clump in the presence of fibrinogen. This assumption is further supported by the observation that localized infection in the peritoneal cavity and viscera was absent after the intraperitoneal injection of clumping factor-negative organisms, but was usually observed with the clumping factor-positive organisms regardless of their coagulase activity (Table 15).

Following the intraperitoneal injection of clumping factor-negative organisms, irrespective of their coagulase property, fewer organisms may be subject to initial phagocytosis in the peritoneum. A relatively high proportion of the organisms would then dessiminate and multiply in the blood and other organs and thus escape the phagocytes which are subsequently mobilized into the peritoneal cavity (Wilson and Miles, 1955). With the extensive dessimination and multiplication of the organisms combined with high concentrations of toxins including delta hemolysin, the staphylococci would then overcome the animal defense mechanisms. The evidence presented here indicates that this sequence of events is more apt to occur and it requires relatively smaller doses when clumping factor-negative, coagulase-positive staphylococci are injected intraperitoneally than when clumping factor-negative, coagulase-negative organisms are inoculated. The possibility that loss of clumping factor activity may also be associated with a change in the antigenic composition of the cells must also be considered as an explanation of the unusual virulence of these organisms.

In contrast, following the injection of a comparable dose of clumping factor-positive staphylococci, a higher proportion of the organisms are probably subjected to initial phagocytosis in the peritoneum.

Likewise, more efficient and localized phagocytosis of the dessiminated organisms may occur in the blood and other organs. Death time of the animal, if it occurs, would then be prolonged, unless the challenge doses were overwhelmingly large.

Rogers (1956a,b,c, 1959, 1960) reported that 10 to 15 minutes after the intravenous injection of rabbits with staphylococci, the great majority of the organisms are trapped in the liver and spleen and 90 per cent of the organisms circulating in the blood appeared to be phagocytized. With the intravenous inoculation of large doses, the splanchnic tissue no longer traps staphylococci and, as a result, a prolonged bacteremia, lasting for hours to several days, results. Benacerraf et al. (1959) reported that when heat killed, P^{-32} labelled staphylococci were injected intravenously into mice, rapid clearance of the blood occurred. This clearance followed an exponential function of time, and within 10 minutes most of the bacteria were phagocytized in the reticuloendothelial system, principally the liver and spleen.

Considering the above observations and the results of intravenous inoculation of mice reported here, it may be assumed that, due to their dispersion in the blood, clumping factor-negative staphylococci are phagocytized less efficiently by the circulating leukocytes. If this is the case a large portion of the inoculum would then be free to dessiminate into the various organs. Conversely, a relatively larger number of clumping factor-positive staphylococci would be readily available for the circulating phagocytes. This assumption is in line with the idea that intravascular clumping of bacteria is a mechanism contributing to their removal from the blood stream (Rogers, 1960).

Reappearance of an overwhelming number of the coagulase-positive staphylococci, regardless of the clumping factor property, would then occur in the blood stream following their intracellular survival and multiplication. This event may be accompanied with marked transient granulocytopenia due to the active destruction of leukocytes (Gray et al., 1957; Rogers and Melly, 1957). Reappearance in the blood of larger numbers of coagulase-positive, clumping factor-positive organisms than of clumping factor-negative, coagulase-positive staphylococci would be expected if initially more of the former cells were trapped in the circulating phagocytes. Subsequently, earlier death of the animals would occur following intravenous injection with coagulase-positive, clumping factor-positive organisms than with comparable doses of coagulase-positive, clumping factor-negative organisms.

Unless overwhelming doses are injected, destruction and elimination of coagulase-negative organisms appear to follow their intravenous injection. However, clumping factor-positive, coagulase-negative organisms were more efficient in establishing infection than are the coagulase-negative, clumping factor-negative staphylococci.

Several investigators (Tompsett, 1954; Goodman and Moore, 1956; Goodman et al., 1956; Rogers, 1959), have indicated that whereas coagulase-positive staphylococci survive and multiply inside phagocytes with subsequent destruction of the phagocytes, coagulase-negative staphylococci are destroyed by the same phagocytes. It would be of interest to investigate the interaction between phagocytes and staphylococcal clumping factor. In the present investigations, two strains of coagulase-negative, clumping factor-positive survived in mice and were almost as successful in

establishing infection as were the coagulase-positive strains. These staphylococci showed average LD_{50} and ID_{50} values comparable to those of the coagulase-positive strains and higher than those of the coagulase-negative, clumping factor-negative organisms (Tables 10 and 11). It is noteworthy that coagulase-negative staphylococci which possessed strong clumping factor activity (Hv 1 and S 35s) exhibited all of the other in vitro properties which are usually associated with pathogenic staphylococci.

Exceptions to these general observations were encountered. The Smith "mouse virulent" variant (the Sv strain of Group I) like the other coagulase-positive, clumping factor-negative staphylococci, was more virulent when injected intraperitoneally than intravenously, but it was also the most virulent of the 13 strains by the intravenous route. There is no doubt that the Sv strain possesses some factor or factors yet to be recognized which contribute to its unusual virulence by both routes of injection. It may be justifiable, however, to consider that its high coagulase activity, which incidentally was remarkable because it clotted mouse plasma (Table 9), combined with its failure to clump in fibrinogen may contribute to increased dessimination and resistance to phagocytosis. On the other hand, the coagulase-positive, clumping factor-positive V strain of Group I was almost equally virulent whether injected intravenously or intraperitoneally. It was true that it possessed strong coagulase activity, but its coagulase titer was not as high as that of the less virulent S strain of the same group. No significant difference was noted in the other properties of this strain.

The reported differences in the LD_{50} values of the staphylococci

tested are expressed further by the time which was required for comparable doses of each of the staphylococci to kill the mice (Table 12). The more virulent the strain the quicker the mice died. Likewise, the effect of the route of injection was noted here. Mice were killed in a shorter time when injected intraperitoneally with clumping factor-negative staphylococci, which were more virulent by this route, than when injected with the same dose intravenously. The opposite was true for the clumping factor-positive strains, which were more virulent intravenously. This observation is more striking when lower doses of these strains are compared, since death caused by the higher doses may very well be due to the overwhelming numbers of organisms injected.

When injected intravenously into an experimental animal, various strains of staphylococci are reported to have a peculiar and predictable organ tropism (Elek, 1959; Rogers, 1959). Although most staphylococci are trapped initially in the liver and spleen following their intravenous inoculation, they subsequently multiply within the renal tissues (Dyke, 1923; De Navasquez, 1950; Gorrill, 1951; Smith, 1956; Smith and Dubos, 1956a; Gray et al., 1957; Sellers and Le Maistre, 1960). In agreement with previous observations, the present investigation indicates the progressive multiplication and persistence of staphylococci in the kidneys of mice injected intravenously and, to lesser extent, following intraperitoneal injection.

Kidney infection resulted in progressive renal damage and the degree of this damage seemed to determine the course and outcome of infection. It is of interest that different gross and microscopic pathological manifestations were observed in the kidney depending on the coagulase and

clumping factor characters of the strain. The multiple, grossly visible abscesses formed by clumping factor-positive organisms might be explained on the basis of the aggregation of organisms, due to their clumping in plasma. In contrast, clumping factor-negative organisms might cause diffuse rather than localized infection. It has been suggested that coagulase-negative staphylococci readily traverse the renal capillaries of rabbit kidneys, whereas coagulase-positive staphylococci produce emboli in the smaller vessels which lead to abscess formation (Dyke, 1923; De Navasquez, 1950).

Thompson and Dubos (1938) were able to produce osteomyelitis regularly in rabbits by intravenous injection of a staphylococcal strain. One of the 13 staphylococci tested, the coagulase-positive, clumping factor-positive S 33 strain, had a noticeable predilection for the skeletal muscles, with subsequent bone involvement, rather than for the kidney. The involvement of a less vital organ may have resulted in the comparably high LD_{50} values of this strain (Table 10).

Organ selectivity by staphylococci is a puzzling phenomenon and much more understanding of host-parasite relationships is required before any explanation of its mechanisms can be attempted. Nevertheless, susceptibility of the kidney, which is a highly vascularized organ, may be due to the absence of fixed phagocytes such as those present in the liver and spleen. Renal abscesses resulted more frequently and extensively following the intravenous than the intraperitoneal inoculation of comparable doses of the organisms. Smith and Dubos (1956a) and Gorrill (1958) suggested that kidney infection and abscess formation are initiated by a few mechanically trapped organisms. However, the number of organisms

trapped appeared to depend on the size of the dose as well as on the nature of the staphylococci injected. As Gorrill (1958) pointed out, if enough organisms are present, some begin to divide and establish themselves before the cellular defenses of the body are alerted.

Staphylococci injected intravenously were more successful in establishing infection than those injected intraperitoneally. The ID_{50} values of 12 of the 13 staphylococcal strains tested were lower for the intravenous than for the intraperitoneal route of injection.

Several properties have been associated with the ability of staphylococci to produce disease in man and experimental animals (Dubos, 1956; Elek, 1959). Previous speculations emphasize mainly coagulase and alpha hemolysin as virulence factors. Recently, North (1959), by the use of specific alpha antitoxin, was successful in protecting mice against experimental staphylococcal infection. In the present investigation, however, alpha hemolysin was produced by only 2 of the 13 strains (S 35 and Hv 1) and these were among the less virulent strain studied (Tables 4 and 10). The production of delta hemolysin by 12 of the 13 strains would support previous suggestions as to its significance in the virulence of staphylococci (Jackson and Little, 1956, 1957; Johanovsky, 1957b). The S 33 strain, which produced beta hemolysin only and failed to hemolyze mouse blood, was one of the least virulent strains and it had a predilection for the skeletal muscle.

The ability of coagulase to exert its plasma coagulating activity in vivo is a controversial issue. Moreover, mouse plasma is not ordinarily clotted in vitro by coagulase. Nevertheless, the difference in the survival of coagulase-positive and coagulase-negative staphylococci in

the host animal supports the role of coagulase as a virulence factor, as does the usual observation that only coagulase-positive staphylococci are pathogenic for experimental animals. There is also strong evidence for a protective role of coagulase against the bactericidal power of serum as well as against that of phagocytosis.

Although Smith and Dubos (1956a) could arrange strains of staphylococci in a continuous spectrum according to their ability to cause disease in mice, which, in turn, appeared to correlate with coagulase production, they disputed the idea that coagulase was a factor of pathogenesis in this infection. The present study suggests that coagulase-negative as well as coagulase-positive staphylococci are able to resist early phagocytosis and to establish infection in mice. However, results here indicate, that, in general, coagulase-positive staphylococci were more virulent and more infective for mice than coagulase-negative strains regardless of clumping factor activity (Tables 10 and 13; Figures 4 and 5).

The prolonged persistance of coagulase-positive organisms in infected tissues may suggest a protective role for coagulase at the later stages of infection. Coagulase-negative staphylococci were eliminated from infected tissues at a much earlier stage and when they persisted for two weeks post-infection, they were present in relatively low numbers. On the other hand, within each of the two coagulase-positive staphylococcal groups there is some correlation between virulence and coagulase production (Table 10). An exception is encountered when the V and S strains of Group II are compared. The S strain produced coagulase to a high titer (1:1024) and was able to clot mouse plasma <u>in vitro</u>, but it was less virulent intravenously and intraperitoneally than the V strain

which exhibited a 1:256 coagulase titer, and failed to clot mouse plasma. The factors which allow multiplication and persistence of staphylococci in the host tissues are not known, and better understanding of the organisms as well as of the host factors are required before any speculations in this area can be made.

Some support of the significance of coagulase in the pathogenicity of staphylococci may be obtained when the virulence and infectivity of parent strains are compared with those of their variants. The Hv 1 (Group III) and Hv 2 (Group IV) strains had lower intravenous and intraperitoneal LD_{50} and ID_{50} values than their parent, the H strain (Group II), from which they differed by loss of coagulase activity. The coagulase-negative S 33v (Group IV) variant was much less virulent intravenously, but less so when injected intraperitoneally, than its parent, the coagulase-positive S 33 strain. Also, its intravenous and intraperitoneal ID_{50} were much higher than those of the parent strain. As noted above, the coagulase-negative Hv 2 and S 33v variants were more virulent for mice when injected intraperitoneally than intravenously although less so than their respective coagulase-positive strains.

Further study of a larger series of representative strains is necessary before any correlation can be made regarding the possible relationship between the in vitro properties of staphylococci and their virulence for mice. However, there was, in general, better correlation between the in vitro properties, which are often associated with virulence, and clumping factor property than between these properties and coagulase production. This observation is quite evident in the case of pigment production and phage typability (Table 4). It is also apparent that no complete

correlation can be made between the various properties tested and any single character such as coagulase production.

Although S. aureus does not normally possess a capsule, there are references in the literature to the isolation of encapsulated, mucoid staphylococcal strains (Elek, 1959). Lyons (1937) demonstrated a capsule in very young (1 to 3 hour) staphylococcal cultures, and considered the capsule to be responsible for the invasiveness of the organism. Price and Kneeland (1954) obtained an encapsulated variant, which was the same phage type as the parent strain, after passage of the non-capsulated parent strain in embryonated hen's eggs. Wiley (1959) isolated mucoid encapsulated variants of S. aureus, the cells of which exhibited capsular swelling reactions with specific immune serum. He also demonstrated passive protection with hyperimmune rabbit and rooster serum, as well as with human globulin, against infection of embryonated hen's eggs by an encapsulated strain of S. aureus. The same worker (Wiley, 1960) reported that sera of 80 per cent of a group of blood donors contained anti-capsular antibodies although only 4 of the 109 donors examined were nasal carriers of encapsulated staphylococci. The production of what may be a mucopolysaccharide substance by the mucoid K 93m variant of the K 93 strain resulted in an increase in its intraperitoneal LD_{50} and ID_{50} values. It is possible that the formation of a non-protective slime layer would retard the dessimination of the organisms from the peritoneal cavity and subject the organisms to earlier phagocytosis.

There are several implications of these results. They emphasize the dependence of measures of virulence, such as the LD_{50}, on the route of injection. In association with the route of injection, re-evaluation of

the significance of coagulase and clumping factor as factors of staphylococci virulence seems necessary. The present investigation fails to confirm the assumption that rare strains of staphylococci which are positive for either coagulase or clumping factor, but not both of these properties, are of doubtful pathogenicity. Although coagulase may contribute to the persistance of staphylococci in an infected animal, its absence in the clumping factor-positive organisms did not deprive them of their ability to persist in vivo. However, when the staphylococci lacked both coagulase and clumping factor, lowered virulence and infectivity resulted. On the other hand, the coagulase-positive, clumping factor-negative staphylococci tested were notably more virulent than any of the staphylococci tested when injected intraperitoneally into mice. Thus, it seems reasonable to suggest that both coagulase and clumping factor are of significance in the virulence of staphylococci. The clumping factor appears to play its role in the early stages of infection, whereas coagulase may exert its (protective) influence at a latter stage of infection.

CHAPTER V

SUMMARY

A soft agar medium which is efficient in the simultaneous demonstration of the coagulase and clumping factor activities of staphylococci is described. This technique reveals rare strains of staphylococci which are positive for one of these properties exclusively, and it facilitates isolation of such variants from mixed staphylococcal populations.

Thirteen strains representing typical coagulase-positive and coagulase-negative staphylococci, as well as staphylococcal variants with unusual combinations of coagulase and clumping factor activities, were selected for virulence studies. The LD_{50} and ID_{50} values of each strain were determined by injecting challenge doses of washed cells into mice by the intravenous and intraperitoneal routes.

Regardless of their coagulase activity, clumping factor-negative strains of staphylococci were more virulent, as judged by LD_{50} values, when injected intraperitoneally than when introduced by the intravenous route. Conversely, staphylococci which possess the clumping factor, either with or without coagulase, appeared to be more virulent by the intravenous route. The most virulent of all strains tested by the intraperitoneal injection of mice were the coagulase-positive, clumping factor-negative strains.

In the experimental infections, the degree of kidney involvement appeared to determine whether or not the animal survived. A strain which showed remarkable predilection for skeletal muscles, rather than the usual tendency of staphylococci to localize in kidney tissues, was less virulent than other strains with similar coagulase and clumping factor activities.

In general, ID_{50} values indicate that infection was established more readily when the staphylococci were introduced by the intravenous rather than by the intraperitoneal route.

The least virulent of the staphylococci tested were the first to be eliminated. They disappeared from the tissues by the second to the fourth post-infection week, whereas the more virulent staphylococci persisted in the tissues of mice for as long as 6 to 8 weeks. The kidney was the most common site of persisting infection.

During the course of the virulence studies, certain variations in the in vitro properties of the selected strains were observed. Noteworthy among these was a mucoid variant which was recovered from the infected mice. The cells of this variant were enveloped in a demonstrable slime layer if not in a discrete capsule. The mouse virulence of the mucoid strain was less than that of the coagulase-positive, clumping factor-negative parental type.

From the evidence of these studies it is not possible to assign staphylococcal virulence to any particular in vitro property of the organisms. In general, the coagulase-positive strains were more virulent than the coagulase-negative strains. However, in certain instances, coagulase production could not explain the degree of virulence. Strains

with the same or greater coagulase producing ability than others were not necessarily equal or proportionally higher in virulence.

Lack of an undefined cell surface component or activity, known variously as bound coagulase, clumping factor or, in terms of the soft agar technique, colony compacting factor, was a common property of the rare strains of staphylococci which have unusually high virulence for mice when injected by the intraperitoneal route.

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