THE INTERRELATIONSHIPS OF SOME DIFFUSIBLE PRODUCTS OF STAPHYLOCOCCUS AUREUS

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ABSTRACT

THE INTERRELATIONSHIPS OF SOME DIFFUSIBLE PRODUCTS OF STAPHYLOCOCCUS AUREUS

Evidence is presented which confirms the accepted view that an approximate parallelism exists between α-hemolysin, dermonecrotic and lethal activities of culture filtrates of Staphylococcus aureus. A loose quantitative relationship is shown to exist between coagulase, α-hemolysin and lethal activities of thirty-seven strains of Staphylococcus when measured by serial dilution, but not between coagulase and dermonecrotic factor. The results of coagulase and α and δ-hemolysin measurements obtained on plates are observed to conflict with those obtained by serial dilution. Relatively small numbers of the strains of S. aureus employed in this investigation produce β or δ-hemolysins. Evidence is presented which confirms the existence of an un-neutralizable hemolysin in some strains of coagulase-negative Staphylococcus.

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TABLE OF CONTENTS

PAGE

LIST OF TABLES

LIST OF FIGURES

INTRODUCTION AND HISTORICAL REVIEW

INTRODUCTION

Staphylococcus aureus is known to produce a variety of extracellular substances. Those which have attracted the most attention are coagulase, the hemolysins, leucocidin, fibrinolysin, hyaluronidase, enterotoxin and dermonecrotic and lethal factors.

Most of these diffusible substances are distinct from each other, but in the case of the α-hemolysin, dermonecrotic and lethal factors, there is some question as to whether these substances represent the same entity.

All of these products except the enterotoxin have generally a qualitative association with the pathogenicity of S. aureus. It has also been established that there is an approximate quantitative relationship between the α-hemolysin, the dermonecrotic and lethal factors. Coagulase is the substance most highly correlated with pathogenicity, but the amount produced in relation to that of the hemolysins, dermonecrotic and lethal factors is not well established.

The purpose of this study has been to enquire into quantitative relationships existing between the coagulase, α and δ-hemolysins, dermonecrotic and lethal factors produced by S. aureus.

HISTORICAL REVIEW

I. COAGULASE

Loeb (1903) was the first to report that cultures of S. aureus would coagulate animal plasma. While his observations were not confined to S. aureus, he nevertheless observed that the coagulating power of the staphylococci was greater for goose plasma than that of any other bacterial species tested. Much (1908) observed that staphylococci could clot plasma to which citrate, hirudin or certain other substances had been added. He believed this property to be peculiar to staphylococci, and then only to certain strains.

Walston (1935) noticed that different strains clotted the plasmas of different animals with varying degrees of ease: for example, one strain clotted human more readily than rabbit plasma and did not clot dog plasma at all; while another strain clotted dog plasma more easily than rabbit plasma, and rabbit more easily than human plasma. Other observations made by Walston were that the clotting principle is dialyzable through cellophane after alcoholic precipitation but not before, and is distinct from hemolysin. The clotting principle was also found to be precipitable by acetic acid and ammonium sulphate, in addition to alcohol. It was found to be filtrable through Berkefeld but not Seitz filters. Pure fibrinogen could also be clotted by a culture filtrate. Fisher (1936) found that bacteria-free culture filtrates of some strains could clot human and rabbit plasma or fibrinogen. He was able to concentrate the active material by precipitation with alcohol. Filtration by both Berkefeld and Seitz filters was observed to retain most of the coagulase activity, and in some cases the clotting activity could not be separated from the cells. This phenomenon did not suggest to him that he might be working with two coagulases (Duthie, 1954).

Smith and Hale (1944) showed that coagulase is particulate, filtrable through Gradocol membranes of pore size 0.5 μ, and is thermostable. Human plasma was generally clotted by coagulase, but guinea-pig, fowl and mouse plasmas as a rule could not be clotted.

Tager (1948b) effected a three hundred-fold purification of coagulase by precipitation at an acid pH with acetate buffer followed by alcohol precipitation and separation of the impurities with ammonium sulphate. Unlike the crude substance, he found the purified product to be thermolabile. The purified product gave negative Molisch and Bial tests, and trypsin and chymotrypsin were effective in destroying its activity, which suggested that it might be protein in nature. Tager also observed the reaction between coagulase and plasma to proceed stoichiometrically, but claimed that under certain circumstances this fact could still fit the picture of coagulase as an enzyme.

Rammelkamp, Hezebicks and Dingle (1950), using cell-free preparations of coagulases, injected monkeys with the coagulases of three selected strains. On the basis of titration of monkey antisera with these coagulases, they were able to detect three types of coagulase, designated types I, II, and III. It was found that types I and II are antigenically distinct, and type III is related to types I and II. Duthie (1952) detected four antigenically distinct types of coagulase in culture supernatants which he labelled A, B, C, and D. Types A, B and C were isolated from three well-known strains of Staphylococcus, and the fourth was found to occur in freshly-isolated human strains. He observed that the majority of human sera neutralize types A and D while animal strains produce coagulase inhibited by antisera B and C. The relationship of Duthie's four coagulases to the three types of Rammelkamp is not known.

Duthie and Lorenz (1952) were able to find anticoagulase in the sera of normal individuals and those suffering from staphylococcal infections. They observed in addition that the injection of coagulase adsorbed on aluminum phosphate produced similar antibody in the sera of rabbits.

Duthie (1954) produced evidence for two forms of coagulase. One was found to be bound to the cell wall and is responsible for agglutination in the slide test.

It is said to act on a susceptible fibrinogen, causing the staphylococci to clump. The other, free coagulase, is said to act on a prothrombin-like substance to give a thrombin-like product, and is responsible for the clotting of plasma in the tube test for coagulase. He found these two forms of coagulase to be antigenically distinct.

Duthie and Houghton (1958) obtained a highly purified preparation of free coagulase by adsorption of the crude product to cadmium sulphate followed by fractionation with ammonium sulphate. The molecular weight of the purified substance was found by sedimentation methods to be about forty-four thousand. The material was toxic to rabbits only when injected intravenously, apparently causing intravascular thrombosis. He observed in addition that lyophilization caused a rapid loss of coagulase activity. Kato and Omori (1959) successfully extracted bound coagulase from cells which were positive for bound and negative for free coagulase, by grinding and by phenol extraction. They found their extract to absorb clumping-inhibiting antibody (antibody to bound coagulase) in immune sera and to adsorb fibrinogen. The fibrinogen-adsorbing substance obtained by the grinding method was different to that obtained by phenol extraction in that the former was non-dialyzable, while the latter was dialyzable. Thus it appeared that bound coagulase is of a dual nature. Blobel et al (1960) purified free coagulase by acid precipitation followed by two cycles of ethanol fractionation. Further purification was achieved by starch column electrophoresis and anion-exchange chromatography. The injection of the electrophoretically-purified enzyme into rabbits stimulated the formation of coagulase-inhibiting antibodies.

Coagulase Reacting Factor[1]

The variation in the behaviour of animal plasmas of different species and of different members of the same species led to the idea that the action of

coagulase might require an accessory factor.

Smith and Hale (1944) found testicular extract to be a suitable source of the accessory factor. They observed that coagulase is the precursor of a thrombin-like substance, and that the production of this thrombin-like substance depends upon the presence in plasma of accessory factor in varying amounts. A deficiency of this coagulase accessory factor would thus explain the non-coagulability of some plasmas, since these plasmas are rendered coagulable if the accessory factor is added.

Duthie and Lorenz (1952) also observed that the plasmas of certain species vary in their content of activator. They found that the activator resembles prothrombin closely in physical properties such as thermolability, behaviour during "salting-out" and removal by adsorbents. Both appeared partly lost in blood clotting and dicoumarol poisoning. They also confirmed the observation of Tager (1948a) that prothrombin is apparently removed from plasma by repeated Seitz filtration, but activator is not. Tager (1956) was able to localize CRF activity in globulin fractions III-1, III-2 and IV-1 by means of controlled ethanol precipitation. Fraction I (fibrinogen) contained no CRF activity after it was further purified, which might explain why free coagulase does not act on purified fibrinogen. Tager has further outlined a number of similarities between prothrombin and CRF: similar inactivation by heat, parallel consumption during physiologic clotting, disappearance in rabbits treated with dicoumarol and phenylindanedione, equal restoration by the anticoagulant effect of vitamin K_1, and acceleration of activity by crystalline trypsin. There are also distinct differences between CRF and prothrombin: physiologic clotting, but not clotting by coagulase, is inhibited by heparin and by citrate or oxalate ions; the plasma of certain animals is deficient in CRF; clotting by coagulase is not affected by

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[1] Also referred to as accessory factor, activator or CRF.

soybean trypsin inhibitor; and the two factors are differently distributed in the various plasma fractions of Cohn.

Murray and Gohdes (1959) have observed, contrary to a number of investigators, that purified coagulase and fibrinogen will clot slowly in the absence of CRF. They did find, however, that the addition of plasma (containing CRF) greatly accelerated the reaction. They proposed the following scheme:

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

Unfortunately, in much of the work, investigators have not made clear whether they were working with free or bound coagulase, and it has been left to the reader to determine which types of coagulase were being studied.

The Relation of Coagulase to Pathogenicity

Loeb (1903) stressed the relation of coagulase to pathogenicity. Cruickshank (1937) investigated coagulase production in strains of S. aureus and observed that coagulase was the substance most highly correlated with pathogenicity to rabbits. He was unable to demonstrate antibody to coagulase in normal or infected individuals and took this fact to mean that coagulase was non-antigenic.

Smith et al (1947), aware of the non-coagulability of guinea-pig plasma in the presence of coagulase, were able to select a few strains of Staphylococcus the coagulase of which did react with guinea-pig plasma. They were able to show that these strains were more virulent for guinea-pigs than strains which produced coagulase not reacting with their plasma. They also discovered that staphylococci were more virulent for mice if they were suspended in a coagulable plasma before injection.

Much has been made of the interesting phenomenon that coagulase-positive staphylococci grow readily in fresh human serum whereas coagulase-negative strains

do not. Wlodarczak and Jeljaszewicz (1959) found this fact to be true, but partially-purified coagulase added to the serum containing coagulase-negative strains did not allow growth. If culture filtrate from coagulase-positive strains was added, the growth of coagulase-negative strains was stimulated, which suggested a non-specific effect. Yotis and Ekstedt (1959), using conventional manometric techniques, showed that coagulase-positive strains of staphylococci respired in human serum more actively than coagulase-negative strains. They observed that the addition of purified coagulase stimulated the respiration of both coagulase-positive and negative strains in human serum, the negative strains being stimulated about twice as much as the positive strains. The use of a method such as manometry might explain the discrepancy of these results with those of Wlodarczak and Jeljaszewicz.

Alami and Kelly (1960), studying the effect of coagulases on virulence of staphylococci for mice, found that mucin enhanced the virulence of free coagulase-positive, bound coagulase-negative strains but not that of others when injected intraperitoneally. Neither free nor bound coagulase appeared to be a critical factor in virulence for mice. Kapral and Li (1960) investigated the virulence and coagulases of S. aureus using a parent strain that was free and bound coagulase-positive. Ultraviolet irradiation of this strain produced three mutants; one having free but not bound coagulase; another having bound but not free coagulase; and a third which produced both types. These mutants were injected into rabbits. The mutants having free but no bound coagulase, and bound but no free coagulase were no more virulent than the parent strain. The mutant having both free and bound coagulase was, unlike the parent strain, avirulent. Kapral and Li have interpreted these results to mean that the possession of either free or bound coagulase is not essential for virulence of S. aureus.

Yotis and Ekstedt (1960) isolated a water-soluble globulin fraction from normal human serum which was shown to have a direct lethal and lytic action upon S. aureus. The antibacterial activity of the fraction could be reversed by treatment with coagulase.

II. THE HEMOLYSINS

The hemolytic action of Staphylococcus on various species of animal erythrocytes was well-known before 1900. The disaster at Bundaberg, Australia, in 1928 in which a number of children died who had received diphtheria toxin-antitoxin contaminated with S. aureus, stimulated fresh research into its extracellular products. Since that time, several specific diffusible products have been discovered which are able to lake animal erythrocytes to varying degree. Some of these hemolysins have been shown in addition to be lethal or dermonecrotic or both.

The α-Hemolysin[2]

Before the multiplicity of the hemolysins of S. aureus was discovered, the activity of culture filtrates against rabbit erythrocytes was noted. Today this activity is attributed to the α-hemolysin.

Arrhenius (1907) found that the α-hemolysin exhibited paradoxical behaviour toward heat. He stated that heating α-hemolysin to 60°C. for thirty minutes inactivates it, but that heating it at 100°C. for ten minutes restores half its activity. Landsteiner and von Rauchenbichler (1909) suggested that the heat anomaly was due to the action of an extraneous factor. They obtained evidence that inactivating products unite with the hemolysin at 65°C., but that higher temperatures dissolve the union, thus re-activating the hemolysin. Tager (1941), by means of acetone precipitation, isolated the inactivating products, and his

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[2]Hemolysin and toxin are used interchangeably when referring to S. aureus.

observations supported the earlier explanation of Landsteiner and von Rauchenbichler. He found that the α-hemolysin itself was relatively heat-stable.

Pillemer and Robbins (1949) have reviewed the work of Wittler and Pillemer (1948) in which staphylococcal α-toxin has been precipitated from the parent filtrate at pH 4.0 in 15 per cent methanol at a temperature of -5°C. It was re-precipitated at pH 4.3 and 0°C., and the toxin was extracted from this precipitate with 0.15 M sodium acetate buffer at pH 5.0. Impurities were removed in a final step at pH 6.2 in 10 per cent methanol at a temperature of -5°C. The purified product contained over 2000 Lf per mg. nitrogen, and was dermonecrotic, lethal and hemolytic. Jackson and Little (1957), using an α-hemolysin preparation free of detectable quantities of β and δ-hemolysins, found its hemolytic activity to be maximal at a pH of 6.8-7.0. A linear relationship was observed to hold between hemolysin concentration and extent of hemolysis, within 25-75 per cent limits of the latter. They also found the α-hemolysin to be partially inhibited by glucose and citrate. Robinson et al (1960a) separated α-hemolysin from other components of a culture filtrate by treatment of the filtrate with ethanol at -20°C., calcium phosphate gel and ammonium sulphate. They subjected this crude hemolysin to carboxymethylcellulose column chromatography and observed the α-hemolysin so obtained to be lethal, hemolytic, dermonecrotic and proteolytic. Upon additional purification of the preparation by zone electrophoresis, a minor non-hemolytic dermonecrotic component was isolated (1960b). Three out of six strains produced this component, and it was suggested that such a component could explain the lack of parallelism between α-hemolysin and dermonecrotic titres noted by some investigators.

The question of whether the hemolytic, dermonecrotic and lethal effects of the α-toxin are due to the same molecular entity has stimulated a great deal of research. The monistic view of α-toxin has gained almost universal

acceptance since the careful analysis of Burnet (1931b), who found that the optimal flocculating ratio and the neutral point of the toxin were not identical, but were close enough to be considered within the range of experimental error.

In the work cited in this thesis, the hemolytic, dermonecrotic and lethal properties of the α -toxin were never separable by the means employed.

The β -Hemolysin

Glenny and Stevens (1935) found evidence for a new hemolysin which they designated "β". They observed that for a particular α -hemolysin the same amount of antiserum was required to neutralize both the hemolytic and dermonecrotic effects. For another unknown toxin, it was found that ten times as much antiserum was required to neutralize the hemolytic effect as was required for the dermonecrotic activity. The hemolytic effect of this second, unknown toxin was greatly intensified by incubation at 37°C. followed by a further period at room temperature or 4°C. This "hot-cold" β -hemolysin was observed to be lethal for rabbits but not for mice, unlike its α -counterpart. It produced an erythematous flush rather than full necrosis. Bryce and Rountree (1936) found that the β -hemolysin was produced largely by strains of animal origin. They prepared β-hemolysin free of α -hemolysin by noting that the pure preparation withstood heating at 60°C. for fifteen minutes whereas α -hemolysin was inactivated at this temperature. The β -hemolysin was antigenic and could be toxoided. The toxicity of the β -hemolysin observed by Glenny and Stevens was confirmed. The work of Bryce and Rountree indicated that the erythrocytes of sheep and oxen were susceptible to the action of the β -hemolysin, while those of the ferret, rabbit, rat, guinea-pig and koala were quite resistant. The hemolysin proved to be only feebly lytic toward human erythrocytes. In their hands the α and β -hemolysins were also antigenically distinct.

Christie and North (1941) found that β-hemolysin could be produced under anaerobic conditions.

Jackson and Mayman (1958c) have found that the crude lysin loses much of its effect upon dialysis, but that its activity is restored upon the addition of Mg^{++} and Mn^{++} ions. Lytic activity appeared to be maximal at neutral reactions and decreased sharply below pH 6.8. The time-hemolysis curve was found by them to be sigmoid.

According to Elek (1959) there is reason to believe that the β-toxin exhibits the same paradoxical behaviour toward heat as the α-toxin.

The δ-Hemolysin

Williams and Harper (1947) discovered a new hemolysin which they labelled "δ". They found that on sheep-blood agar plates containing α-or $\alpha\beta$-antiserum, a type of hemolysis was produced which was different from that due to α- or β- hemolysins. The δ-hemolysin was found to have a wider hemolytic spectrum than α- and β- hemolysins, being lytic towards the erythrocytes of rabbit, sheep, man, monkey, horse, rat, mouse, and guinea-pig. It also differed from the α and β-hemolysins in that it could not be produced in a fluid medium. Williams and Harper were able to separate the δ from the α-hemolysin by ethanol and ether precipitation. Marks and Vaughan (1950) confirmed the existence of the δ-hemolysin. They found it to be dermonecrotic, and said that it could be recognized by its lysis of human and horse cells at 37°C. It was observed to act synergistically with β-toxin on human and sheep erythrocytes, and was noted to be soluble in ethanol but not in ether or acetone. Jackson and Little (1958a) studied the δ-hemolysin and found that hemolysis of human erythrocytes by δ-hemolysin was unaffected by the presence of α-hemolysin. A number of proteins, particularly serum were, however, found to be inhibitory

to its action. Glucose, citrate and Mg^{++} ions were observed not to have any effect on hemolysis due to the hemolysin. Hemoglobin and erythrocyte ghosts were inhibitory. They found a linear relationship to exist between degree of hemolysis of human erythrocytes and hemolysin concentration within 30-65 per cent limits of hemolysis.

Jackson and Little (1958b) partially purified the δ-hemolysin by precipitating it from the crude preparation under controlled conditions of ethanol concentration, temperature and pH, followed by extraction of the δ-hemolysin from the precipitate with 75 per cent ethanol in saline-phosphate buffer at pH 6.8. Crude δ-hemolysin was seen to consist of two fractions; one stable to heat at 60°C., and the other rapidly inactivated under these conditions. α and δ-hemolysins could be separated by heating a crude preparation at 60°C. for fifteen minutes, or by filtering through ultrafine sintered glass, the δ-hemolysin being adsorbed.

Other Hemolysins

Various other staphylococcal hemolysins have been postulated from time to time. The separate existence of the α, β and δ-hemolysins has been confirmed by numerous investigators, but considerable doubt exists as to the nature of other hemolysins which have been described.

Morgan and Graydon (1936) presented evidence that the α-hemolysin really consisted of two distinct hemolysins which they designated "α_1", and "α_2". They observed different end-points when two α-toxins containing no β-toxin were neutralized. All culture filtrates were found to contain the α_1 and about two-thirds of the filtrates contained small amounts of α_2. Both α_1 and α_2-hemolysins appeared to be antigenic, and the antitoxins for each toxin were partially separable.

Smith and Price (1938) postulated a γ-hemolysin. They found the γ-hemolysin to be less stable than α and β-hemolysins; it was completely destroyed by heating for thirty minutes at 55°C. They were able to produce a preparation of γ-hemolysin which contained no α-hemolysin and only small amounts of β-hemolysin. They found its lytic spectrum to be wide, the sensitivity of the following animal erythrocytes increasing in the order: horse, rat, ox, guinea-pig, sheep, man and rabbit. The γ-hemolysin was observed to be very slightly dermonecrotic for guinea-pigs and rabbits, was found to be toxic to rabbits but not guinea-pigs and mice, was antigenic, and could be toxoided. Furthermore, they found that antisera selected for a high γ/α ratio also had a high α_2/α ratio, which led them to suggest that the α_2-hemolysin of Morgan and Graydon might be identical with their γ-hemolysin.

Elek and Levy (1950) made a study of the distribution of hemolysins of S. aureus on rabbit and sheep-blood agar plates. They confirmed evidence for the three distinct hemolysins; α, β and δ[3]. The possible combinations would be α, β, δ, $\alpha\beta$, $\alpha\delta$, $\beta\delta$, and $\alpha\beta\delta$, all of which were observed by them. The recognition of the combinations was based on qualitative effects and quantitative neutralization, plus a little imagination. The experiences of Elek and Levy with their plate method inclined them to the view that the α_2, γ and δ-hemolysins are identical. Marks (1951) showed that the δ-hemolysin is identical with the α_2-hemolysin. δ-hemolysin was shown to react in the same manner with concentrated (ammonium sulphate-precipitated) and native antitoxins as did the α_2-hemolysin. As mentioned before, Morgan and Graydon found that most culture filtrates contained a small amount of α_2, and this obscured the end-point of hemolytic titrations by producing a tail of minimal lysis. The α_2-hemolysin also acted on rabbit and sheep cells, and

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[3] Vide supra.

was slightly antigenic. All these findings are in accord with those for δ-hemolysin. It is also noteworthy that the lytic spectrum of γ-hemolysin is identical with that of δ-hemolysin.

The separate existence of the α, β and δ-hemolysins is now well established, but the existence of other hemolysins is controversial.

Mode of Action of the Hemolysins

Whether α-toxin and erythrocytes unite stoichiometrically or whether they exhibit an enzyme-substrate relationship has been a question of some depth. Forsman (1934) regarded staphylococcal hemolysis as an enzyme effect, since he found that fixation of the hemolysin by erythrocytes is not essential for lysis to occur; sensitive red cells do not bind the lysin. Fixation when it occurred appeared to be irregular and easily reversible. Using constant amounts of rabbit erythrocytes, Levine (1938) plotted the disappearance of the hemolysin from mixtures of varying hemolysin concentrations and found that he obtained a parabola of the Freundlich adsorption isotherm type.

The "hot-cold" effect of the β-hemolysin has been attributed to the presence of various electrolytes. Bigger (1933) believed interfering substances resulting from the use of unsuitable media, prolonged incubation, or too alkaline reaction were possibly the cause of the "hot-cold" effect. Other than these suggestions, there is a dearth of information on the mode of action of the β-hemolysin.

Nothing is known of the mode of action of δ-hemolysin.

The Relation of the Hemolysins to Pathogenicity

Schwabacher et al (1945) have stated that the property most closely associated with pathogenicity is the α-toxin. Christie et al (1946) produced evidence that, of the various tests employed, the production of α-hemolysin agreed most closely with mouse pathogenicity tests. Whether or not this fact is applicable to human

beings is not known with certainty. Marks (1952) claimed that the production of α-toxin is a more accurate and convenient criterion of pathogenicity of staphylococci than is the coagulase test. Lack and Wailling (1954) have not been able to confirm the contention that α-toxin is more closely associated with pathogenicity than is coagulase. They found only 82 per cent of 435 strains to produce α-toxin, and suggested that pathogenicity of S. aureus is correlated with a broad spectrum of toxins rather than a single entity.

Elek and Levy (1950) found three hemolysins to be associated with coagulase positive strains. These were the α, β and δ-hemolysins. The occurrence of α, β or δ-hemolysins alone was not absolutely correlated with coagulase production, but α and δ-hemolysins together occurred most frequently in cultures derived from human sources. α, β and δ-hemolysins occurring together were found most often in cultures derived from animal sources. They found that out of two hundred strains examined from human sources, 96 per cent produced α-hemolysin, 11 per cent produced β-hemolysin, and 97 per cent produced δ-hemolysin. None was found to produce no hemolysin when measured on plates. It is, however, noteworthy that since coagulase production is in complete agreement with pathogenicity as established by the majority of investigators, coagulase-negative strains generally produce none of the three hemolysins associated with coagulase-positive strains. Elek and Levy pointed out in addition that their observation of a hemolysin produced by 95 per cent of coagulase-negative staphylococci invalidates the assumption that a "hemolytic Staphylococcus" is pathogenic. Marks (1952) was, however, unable to find any evidence of this hemolysin produced by coagulase-negative staphylococci.

Brown (1960) suggested that although coagulase production may well be necessary for the pathogenicity of a strain, the degree of virulence can be most conveniently estimated by its α-hemolysin production.

By means of a sampling device attached to the side of the animals, Gladstone and Glencross (1960) made the interesting observation that high yields of α -hemolysin were produced by four coagulase-positive strains growing for twenty-four hours *in vivo* in mice, rabbits, guinea-pigs and rats. The same cultures growing *in vitro* under optimal conditions produced no α-hemolysin. Coagulase-negative strains which were non-hemolytic *in vitro* were also non-hemolytic *in vivo*. In view of the results, it was pointed out that tests for toxigenicity on cultures grown *in vitro* may not always be valid.

III. THE QUANTITATIVE RELATIONSHIPS OF COAGULASE AND THE HEMOLYSINS OF *S. AUREUS*

In a study of ten strains, Dolman (1932) found an approximate parallelism between the initial dermonecrotic and hemolytic titres of different samples of toxin. Panton and Valentine (1932) reported that the α-hemolysin, dermonecrotic and lethal factors were closely correlated in a series of twenty-two strains derived from a variety of lesions. Neither Dolman nor Panton and Valentine ascertained the degree of coagulase production in relation to that of α-toxin.

Tager and Hales (1947), in a study of 230 strains derived from various human lesions, reported no significant correlation between quantities of coagulase and α, β and δ -hemolysins produced. The β and δ -hemolysins were titrated to hemolytic end-points using sheep and human erythrocytes respectively, and the Lh dose of the α -hemolysin was determined with a standard antiserum. Coagulase was assayed in the presence of added trace elements in serial dilutions of whole culture which had been treated with merthiolate, and clotting was assessed after twenty-four hours. On the other hand, Marks (1952), working with one hundred strains derived from a community of Welsh coal miners, found that α-

hemolysin and coagulase were quantitatively correlated. The α-hemolysin was measured in terms of the diameter of the zone of hemolysis occurring on sheep-blood agar. Coagulase was titrated using live cultures which were serially diluted for the test, and clotting activity noted after four hours. No statistical analysis of Marks' results was undertaken, however.

Differences in method may well be responsible for the discrepancies between the results of Tager and Hales and those of Marks. Tager and Hales inhibited growth of the staphylococci by the addition of merthiolate in their titrations of coagulase, and read the end-point after twenty-four hours. Marks made no attempt to inhibit growth and assessed clotting activity after four hours. Twenty-four-hour readings could allow fibrinolysis of the clot by staphylokinase to alter the titre. The action of merthiolate on the plasma-coagulase-CRF system is not known. In addition to merthiolate, Tager and Hales added a solution of trace elements which was supposed to enhance coagulase production, but they gave no evidence to support this assumption. Another difference worth noting is that Marks used sheep erythrocytes contained in a "diagnostic" medium for the detection of α-hemolysin, whereas Tager and Hales used rabbit erythrocytes and a standard anti-toxin to calculate the Lh dose of the α-hemolysin. No doubt the calculation of the Lh, test dose or hemolytic end-point of the α-hemolysin is a more accurate measurement than the estimation in millimetres of the diameter of a zone of hemolysis on a blood agar medium. It is also necessary to point out that Tager and Hales tested more than twice the number of strains employed by Marks.

Using different methods, then, one group has found no correlation between coagulase and α, β and δ-hemolysins, and another has found some correlation between coagulase and α-hemolysin.

MATERIALS AND METHODS

MATERIALS and METHODS

Source of Strains of Staphylococcus

Forty-four strains have been used in this study, thirty-three of which are coagulase-positive and eleven of which are coagulase-negative. Twenty-six of the strains are recent isolates from the clinical bacteriology laboratory of the Winnipeg General Hospital. The remaining eighteen are old laboratory strains, the origin of which is obscure. All strains fitted a description of Staphylococcus given in Bergey's Manual (1957)[1].

All strains were subcultured on nutrient agar slants once monthly, stored at 4°C., and were periodically checked for purity.

Qualitative Coagulase Measurement

Slide test. The slide test was performed according to the method of Cadness-Graves et al (1943). Emulsions of the organisms grown overnight on nutrient agar were made in each of two drops of saline on a clean glass slide. Bacto Coagulase Plasma[2] was added to one drop and mixed for a few seconds, and saline was likewise added to the other drop, which serves as a control, and mixed. Clumping of cells occurring in the presence of plasma within 15-20 seconds indicated a positive reaction.

Tube test. An emulsion of a twenty-four-hour agar culture was made in three drops of Brain Heart Infusion broth[3] in a tube measuring 1.0 x 8.5 cm., to which was then added 0.5 ml. Bacto Coagulase Plasma. The tube was then immersed in a water-bath at 37°C. A positive test was indicated by the presence of a firm clot at the end of a three-hour period.

Quantitative Coagulase Measurement

Tube methods with whole cultures. A loopful of growth from a twenty-four-

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[1]See Appendix A. [2]Difco Laboratories, Detroit, Mich. [3]Ibid.

hour agar slant culture was inoculated into 25 ml. Brain Heart Infusion broth and incubated for twenty-four hours at 37°C. 2.5 ml. of the broth culture was placed in a Klett calibrated test tube, and saline added up to the 5 ml. mark. After thorough mixing, the tube was placed in a Klett Colorimeter and brought to a standard optical density of 0.252, which corresponded to 7.2×10^8 cells per ml. Cell counts corresponding to an optical density of 0.252 were obtained using a Petroff-Hausser bacteria counter[4]. Serial whole log dilutions of the standarized culture were made in tubes measuring 1.0 x 8.5 cm., containing 0.9 ml. saline. An equal volume of freshly-drawn pooled plasma from four rabbits was added to the culture dilutions and the tubes were incubated in a water-bath at 37°C. for three hours. The end-point was indicated by the highest culture dilution which showed a trace of clotting[5].

Tube methods with culture filtrates. Brain Heart Infusion broth cultures were prepared and standarized as described in the preceding section. 5 ml. of culture was then filtered through a Millipore membrane filter[6] of pore size 0.45u. Serial whole log dilutions of the culture filtrate were incubated and the end-point assessed as described above for whole culture.

Plate method. Plate tests for coagulase activity were carried out using the medium of Blobel et al (1960). A solution of Heart Infusion Broth[7] containing 3 per cent agar was diluted with an equal volume of 3 per cent bovine fibrinogen[8] in 0.85 per cent saline at 45°C. The fibrinogen solution was sterilized previously by Seitz filtration. 2 per cent rabbit serum was added as cofactor for coagulase

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[4]Arthur H. Thomas Co., Philadelphia, Pa.

[5]Slight coagulation or fibrin filaments.

[6]Millipore Filter Corp., Bedford, Mass.

[7]Difco Laboratories, Detroit, Mich.

[8]Nutritional Biochemicals Corp., Cleveland, Ohio.

activity and the whole poured into Petri dishes. Plates of the medium were stab-inoculated eight times from a twenty-four-hour broth culture and incubated at 37°. for twenty-four hours followed by a further period of twenty-four hours at room temperature. Fibrin precipitation around the four most regular colonies was measured in terms of the diameter of the zone of precipitation minus the diameter of the colony (see figure). Each diameter in mm. was measured in two directions at right angles to one another and the mean of the two measurements taken. The mean value of the four most regular colonies measured in this way was taken as an expression of coagulase activity.

Toxin Production

Staphylococcal toxin was prepared according to the method of Dolman and Wilson (1940)[9]. The plates of medium were seeded over the surface from a five-hour broth culture, and incubated at 37°C. under 10 per cent carbon dioxide and 90 per cent oxygen for seventy-two hours. A smear of the incubated medium was then made and Gram-stained. The whole of the medium was passed through a Buchner funnel containing coarse filter paper. Each 100 ml. of medium yielded 50-60 ml. of crude toxin which was then centrifuged to free it of residual agar particles, and the supernatant fluid sterilized by passage through a Seitz filter. Seitz filtrates were immediately tested for sterility and stored at 4°C. until required.

Experimental results have been compiled in connection with the effect of filtration on the toxin[10].

Preparation of Antitoxin in Rabbits

Antitoxin against the L-16 strain of S. aureus (possessing α, β and δ-hemolysins) was prepared in two rabbits according to the method of Pillet et al

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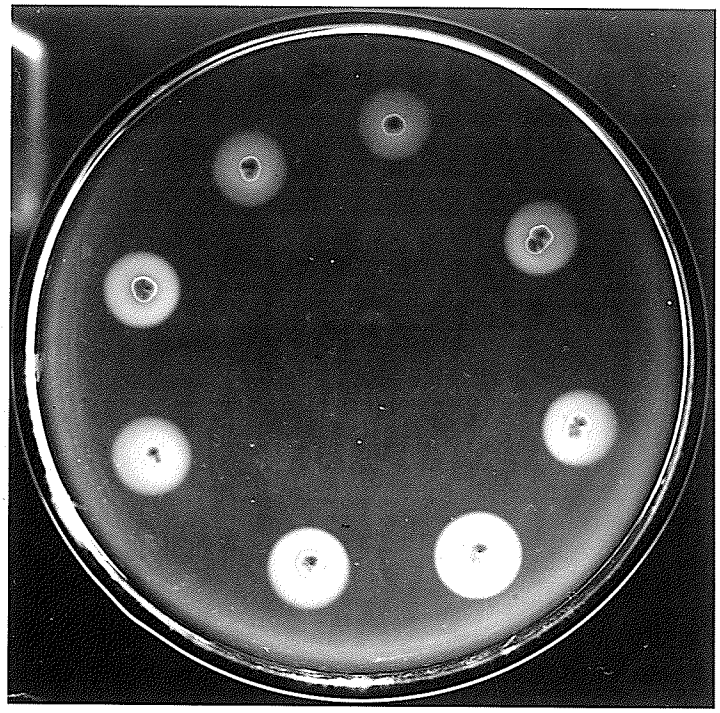
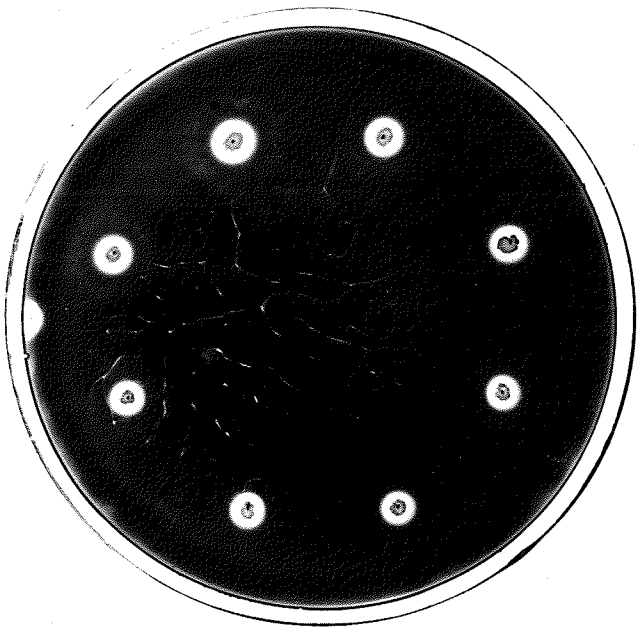
[9]See Appendix B. [10]See Results.



FIBRIN PRECIPITATION AROUND COLONIES



HEMOLYSIS AROUND COLONIES

FIGURE

PLATE MEASUREMENTS OF COAGULASE AND HEMOLYSIN ACTIVITY.

(1959). A culture filtrate of the L-16 strain was toxoided with 0.5 per cent formalin by allowing it to stand at 37°C. for fifteen days (Burnet, 1929).

Each of two rabbits was bled and then given 0.2 ml. of the toxoid subcutaneously on the first day. After ten days the rabbits were injected subcutaneously with a further 1.0 ml. of toxoid. Ten days after the second injection, both rabbits were bled and the sera collected and stored at 4°C. until required. A control rabbit received the same course of injections of toxin medium only.

Preparation of Erythrocyte Suspensions

Rabbit erythrocytes were obtained by bleeding four adult animals directly from the heart. The blood was pooled and immediately added to an equal volume of Alsever's solution[11] and stored at 4°C. until required for hemolysin titrations.

Human erythrocytes were obtained by venipuncture from four volunteers, pooled, and stored in the same manner.

Sheep erythrocytes collected in Alsever's colution were obtained from the Colorado Serum Company of Denver, Colorado.

Erythrocytes stored in Alsever's solution were centrifuged and washed in 0.85 per cent saline and resuspended to a final concentration of 2 per cent. Cells were used the same day as prepared.

Quantitative Hemolysin Measurement

Tube method. Culture filtrates of staphylococcal toxin [12] were assayed for hemolysin activity against human and rabbit erythrocytes. Serial two-fold dilutions were made in 1 ml. volumes of saline in tubes measuring 1.0 x 8.5 cm. An equal volume of 2 per cent erythrocyte suspension was added to the tubes so as to give a final concentration of 1 per cent. The tubes were then agitated

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[11] See Appendix B, [12] Vide Supra.

and placed in a water-bath at 37°C. for one hour. After incubation, all tubes were centrifuged at one thousand r.p.m. for five minutes.

The amount of hemolysin present was expressed in terms of Burnet's 50 per cent end-point (1931a) by comparison with a hemolytic standard.[13] The titration was stored for a further twelve-hour period at 4°C. and a reading once again taken.

Plate method. Ten ml. of a 50 per cent suspension of washed erythrocytes was added to 200 ml. of Dolman-Wilson medium containing 1.5 per cent agar held in a water-bath at 50°C. Approximately 15 ml. was poured into each Petri dish. Rabbit, sheep and human blood agar plates prepared in this manner were used to detect α, β and δ -hemolysins respectively.

Each plate was stab-inoculated eight times from a twelve-hour broth culture of the strain being tested, using a straight inoculating needle. All plates were incubated in air at 37°C. for twenty-four hours followed by an equal period at room temperature. The sheep cells used for the detection of β -hemolysin were allowed to stand for an additional twenty-four-hour period at 4°C., since it was found that incubation at room temperature did not allow proper development of the "hot-cold" effect (Glenny and Stevens, 1935).

Hemolysin activity was measured in the same way as coagulase on plates, except that a zone of hemolysis was observed rather than one of precipitation.

Dermonecrotic Factor

The skin-necrotizing property of staphylococcal culture filtrates was assayed in adult rabbits. The back of the rabbit was closely shaved, and the skin divided into sections with a marking pencil. Each rabbit was injected subcutaneously with duplicate 0.1 ml. quantities of the filtrate. Up to six filtrates, including a medium control, could be injected into the back of one

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[13] See Appendix C.

rabbit. Two rabbits were used for every assay. The dermonecrotic factor was measured in terms of the diameter in centimetres of the area of necrosis appearing after five days.

Lethal Factor

The lethal activity of culture filtrates was assayed in mice of the CFW strain. Whole log dilutions, i.e., undiluted to 10^{-3}, of the filtrates were made. 1.0 ml. quantities of each dilution were injected intra-peritoneally into a group of three mice, and only deaths occurring within a twenty-four-hour period were accepted as evidence of the lethal activity of the filtrate.

The LD_{50} of each toxin was calculated by the method of Reed and Muench (1938).

EXPERIMENTAL RESULTS

EXPERIMENTAL RESULTS

Filtrability and Stability of α-Hemolysin

To render fluids which contain α-hemolysin bacteria-free necessitated the use of a filtration procedure. The Seitz filter is one of the simplest types to use for this work, but its asbestos filter pad is noted for its adsorptive capacity. Before employing the Seitz filter for experimental work, it was decided that a comparison of its adsorptive power should be made with that of a Millipore HA membrane filter, [1] the adsorption by which is claimed to be negligible.

Twenty ml. of a single batch of centrifuged culture fluid, prepared from strain Wood-46, was passed through each of two Seitz EK filters and also through two Millipore filters, the degree of vacuum being the same in each case. Filtration by Seitz was found to proceed more rapidly than by Millipore.

Alpha-hemolysin assays were carried out on the filtrates with the results shown in Table I. It is evident that there is no greater adsorption of the α-hemolysin to the Seitz pad than to the Millipore filter, and the former, because it is easier to handle than the latter, was employed in all further work.

Once it had been decided to employ Seitz EK filtration in the experimental work, it was necessary to know at what point adsorption of the toxin became minimal as it passed through the pad, so that the highest possible yields could be obtained. In an experiment designed to determine this point, toxin was passed through a Seitz filter and an aliquot was taken after 5 ml. of filtrate had been collected. Further aliquots were taken after 10 and 20 ml. quantities had passed through.

Table II shows that the α-hemolysin titre of all three aliquots was the same. Consequently, the quantity of toxin which passed through the filter was of

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[1] See Materials & Methods.

TABLE I

COMPARISON OF SEITZ AND MEMBRANE FILTRATION OF α-LYSIN

Filter	α -lysin titres	mean titre
Seitz EK	320	480
	640	
Millipore	640	480
	320	

TABLE II

EFFECT OF SEITZ FILTRATION ON α-LYSIN TITRE

Aliquot*	α-lysin titre
5 ml.	640
10 ml.	640
20 ml.	640

*Taken after passage through Seitz filter.

no importance as long as it was greater than 5 ml. No extra adsorption was thus detected when larger amounts were filtered. The α-hemolysin titre of unfiltered culture was not determined.

It was often necessary to store sterile culture filtrates for several days before testing, and the stability of the toxin over a period of time had to be determined. Two strains were selected the culture filtrates of which had initial α-hemolysin titres of 2560 and 640 respectively. After seven days' storage at 4°C., the α-hemolysin titre of the two strains was again measured. As shown in Table III, no changes in titre were recorded which lay outside the range of experimental error, and it was decided that the toxin could be safely stored under sterile conditions for up to seven days at 4°C. without detectable denaturation.

During the course of the experimental work, it was further observed that the toxin was stable if stored at 4°C. for up to three months.

Age of Erythrocytes

In hemolysin assays, 2 per cent erythrocyte suspensions were always used on the same day they were prepared from the Alsever stock suspension, but it was not always possible to use the erythrocytes as soon as they were collected from animals or human volunteers. An experiment was performed which was designed to test the effect of age of the Alsever's stock suspension on a titration of standard toxin. A toxin less than one week old, the titre of which was known (it has been shown that the hemolytic properties of the toxin are stable for one week at 4°C.), was assayed for α and δ-hemolysins using freshly-drawn erythrocytes and erythrocytes which had been stored in Alsever's solution for six days. Reference to Table IV shows that storage in Alsever's solution for up to six days does not influence the sensitivity of the erythrocytes to α or δ-hemo-

TABLE III

STABILITY OF α-LYSIN STORED AT 4°C.

Strain	α-LYSIN TITRE	
	Initial	Day 7
103	2560	5120
104	640	640

TABLE IV

EFFECT OF AGE OF ERYTHROCYTES ON α AND δ -LYSIN TITRES OF CULTURE FILTRATES

Strain	Erythrocyte species	AGE OF ERYTHROCYTES 0 Days	6 Days
103	Rabbit	2560	5120
	Human	< 20	< 20
104	Rabbit	640	640
	Human	< 20	< 20

lysin. In the case of strain 103, the difference between the initial and final titres was considered to be within the limits of experimental error.

Coagulase Assay in Whole Cultures and Culture Filtrates

Because of the discovery by Duthie (1954) of two forms of coagulase, one bound to the cell wall and the other occurring free in the medium, it was felt that some attempt should be made to define more precisely the nature of the coagulase being assayed in this study.

Sterile Brain Heart Infusion broth culture filtrates of twenty-four strains were tested for free coagulase activity as described under Methods, but none was observed in any case. Fahlberg and Marston (1960) claimed that the addition of 0.5 per cent lithium chloride to nutrient media would inhibit bound but not free coagulase in their titrations. Their claim could not be substantiated in this laboratory, as the addition of 0.5 per cent lithium chloride was found to inhibit neither free nor bound coagulase.

Tager and Hales (1947) employed a solution of trace elements (boric acid, manganese chloride, copper sulphate, molybdic acid, ferric chloride, zinc sulphate and potassium iodide) presumably to enhance coagulase production. The effect of such a solution on coagulase production in culture filtrates and whole cultures was assessed in this laboratory, but no enhancement was observed in either case.

The present method of coagulase titration by serial dilution of whole cultures was employed in view of the lack of success with the methods just outlined.

Quantitative Interrelationships of Coagulase, α and δ -Hemolysins, Dermonecrotic and Lethal Factors

A total of forty-four strains of Staphylococcus was acquired during the course of this investigation. The physiological properties and coagulase activity

of all strains were tested at the time they were received. Thirty-five strains were initially coagulase-positive. A culture filtrate was prepared from each strain by methods described earlier, and the α and δ-hemolysins and lethal factor measured by serial dilution. The dermonecrotic factor was measured by the area of necrosis on a rabbit's back; an area less than 1 cm. being assigned +, between 1-2 cm., ++, and greater than 2 cm., +++. The α and δ-hemolysins, dermonecrotic and lethal factors of the strains were all assayed within a few days of each other, but since the capacity of the carbon dioxide incubator was small, toxins could be produced only for six or seven strains at a time.

Coagulase activity was measured by serial dilution of whole cultures grown in Brain Heart Infusion broth for twenty-four hours at 37°C., as described under Methods. The interval between coagulase and the hemolysin, dermonecrotic and lethal factor assays was generally a month.

The results of this survey of quantitative interrelationships of the hemolysins, dermonecrotic and lethal factors and coagulase measured by serial dilution methods are presented in Tables V and VI. The coagulase-negative strains are dealt with in a later section.

The results as shown in Table V suggest that both the dermonecrotic and lethal activities of the filtrate vary directly with the α-hemolysin content. The relationship of the lethal factor, however, appears to be somewhat more close than that between dermonecrotic factor and α-hemolysin. For example, of eleven strains having an α-hemolysin titre of less than 640, six have a dermonecrotic activity of $0-^{+}$, four have an activity of ++, and one has an activity of +++. Of eighteen strains having an α-hemolysin titre of 640-2560, two have an activity of $0-^{+}$, eleven have $^{++}$ and five have an activity of $^{+++}$.

The closer relationship between LD_{50} and α-hemolysin titres becomes clear when it is noted that of eleven strains having an α-hemolysin titre of less

TABLE V

QUANTITATIVE RELATIONSHIPS OF α-LYSIN, DERMONECROTIC AND LETHAL FACTORS MEASURED IN CULTURE FILTRATES OF THIRTY-SEVEN STRAINS OF S. AUREUS

Dermonecrotic activity	α-lysin titre			
	< 640	640-2560	> 2560	
0 - +	6	2	0	8
++	4	11	4	19
+++	1	5	4	10
	11	18	8	37
LD_{50}/ml.				
< 3.2	10	1	0	11
3.2-5.6	1	17	4	22
> 5.6	0	0	4	4
	11	18	8	37

than 640, ten have an LD_{50} titre of less than 3.2, one has an LD_{50} titre in the range 3.2-5.6, and none have an LD_{50} titre greater than 5.6. Of eighteen strains having an α-hemolysin titre of 640-2560, seventeen have an LD_{50} titre in the range 3.2-5.6, and none have an LD_{50} titre greater than 5.6. Of the eight strains possessing an α-hemolysin titre of greater than 2560, however, all have a dermonecrotic activity of at least $^{++}$, and all have an LD_{50} of at least 3.2.

The coefficient of correlation for α-hemolysin and LD_{50} is 0.8812 (see Table IX, line four), and that for α-hemolysin and dermonecrotic factor is 0.6129 (see Table IX, line five). Both coefficients are significant at less than the 0.1 per cent level ($p < 0.001$). The value for α-hemolysin and dermonecrotic factor is lower, however, indicating less correlation, though still highly significant.

Coagulase titres have been ranked from lowest to highest values in Table VI, in which a complete breakdown of their relationship to α and δ-hemolysins, dermonecrotic and lethal factors is presented.

In the comparison of coagulase and α-hemolysin, low α-hemolysin titres appear generally to be associated with low coagulase titres, and high with high, but there are outstanding exceptions; for example, strain 203, with coagulase titre of less than 20 and α-hemolysin titre of 640; strain 201, with a coagulase titre of 200 and α-hemolysin titre of 5120; and strain 210 with a coagulase titre greater than 2×10^7 and α-hemolysin titre of 80.

The relation of dermonecrotic factor to coagulase is obscure upon visual inspection. There are six strains with a dermonecrotic activity of $^{+++}$ associated with high coagulase titres, and four which have a dermonecrotic activity of $^{+++}$ associated with low coagulase titres. Of eighteen strains having a dermonecrotic activity of $^{++}$, nine have high and nine have low coagulase activity.

A comparison of coagulase and LD_{50} titres in Table VI shows that more LD_{50} titres of less than one are located in the upper half of the table corresponding to low coagulase titres. It will be noted, however, that the first and last strains in the table have an LD_{50} titre of less than one.

The coefficient of correlation for coagulase and α-hemolysin is 0.3996 (Table IX, line one). The coefficient for coagulase and LD_{50} is 0.3925, and that for coagulase and dermonecrotic factor if 0.1795 (lines 2-3, Table IX). The first two values are not highly significant ($p < 0.02 > 0.01$) while the last is not significant ($p > 0.05$).

Reference to Table VI shows that δ-hemolysin titres appear to bear no relation to those of coagulase, α-hemolysin, dermonecrotic and lethal factors. For example, Table VI shows that strain 101 has a coagulase titre of 200, a δ-hemolysin titre of less than 20, and an α-hemolysin titre of 640; strain 211 has coagulase titre of 2000, a δ-hemolysin titre of 160, and an α-hemolysin titre of 1280; and the O'Hara strain has a coagulase titre of 2×10^5, a δ-hemolysin titre of 160, and an α-hemolysin titre of 80.

Measurements of Coagulase and α, β and δ-Hemolysins by the Plate Method

In view of the discrepancies observed by some investigators (Elek and Levy, 1954) and suggested by the work of others (Marks, 1952; Tager and Hales, 1947) in comparing tube and plate methods for the assay of coagulase and hemolysins, it was decided to measure these activities of the strains by a plate method in addition to the tube method previously described. In these experiments, β-hemolysin activity as well as α and δ-hemolysin and coagulase activities was measured. Assays of all coagulase-positive strains together with the Smith strain and 203 strain were made simultaneously (in a single day) some three months after the tube experiments had been completed.

TABLE VI

QUANTITATIVE RELATIONSHIPS OF COAGULASE, α AND δ-LYSINS, DERMONECROTIC AND LETHAL FACTORS OF S. AUREUS

Strain	Coagulase titre	α-Lysin titre	δ-lysin titre	Dermo-necrotic factor*	LD_{50}/ml.
Smith	< 20	< 20	40	0	< 1
203	< 20	640	< 20	+	3.2
100	200	40	< 20	++	< 1
101	200	640	< 20	+++	1.8
102	200	40	< 20	++	< 1
201	200	5120	20	++	31.6
3A	2000	160	< 20	++	< 1
L-16	2000	160	< 20	+++	< 1
Newman	2000	160	40	++	< 1
191	2000	160	< 20	+	< 1
211	2000	1280	160	+++	3.2
3099	2000	640	< 20	++	3.2
3038	2000	2560	< 20	++	3.2
103	2×10^4	2560	< 20	+++	5.6
13908	2×10^4	2560	< 20	+++	3.2
13889	2×10^4	640	20	++	3.2
Wood-46	2×10^4	1280	< 20	+++	3.2
206	2×10^4	5120	20	++	31.6
3053	2×10^4	1280	< 20	++	3.2
2364	2×10^4	640	< 20	+	3.2
MAM	2×10^4	1280	< 20	++	3.2
3023	2×10^4	2560	< 20	++	3.2

TABLE VI (Continued)

Strain	Coagulase titre	α-lysin titre	δ-lysin titre	Dermo-necrotic factor*	LD_{50}/ml.
104	2×10^5	640	20	++	3.2
13819	2×10^5	640	≤ 20	++	3.2
13896	2×10^5	20	≤ 20	+	≤ 1
53	2×10^5	1280	≤ 20	++	3.2
O'Hara	2×10^5	80	160	0	≤ 1
Giorgio	2×10^5	>10240	20	++	31.6
3251	2×10^5	320	≤ 20	+	3.2
105	2×10^6	5120	≤ 20	+++	31.6
106	2×10^6	5120	≤ 20	+++	3.2
107	2×10^6	1280	≤ 20	++	3.2
109	2×10^6	5120	≤ 20	+++	3.2
13853	2×10^6	640	≤ 20	++	3.2
212	2×10^7	5120	20	++	3.2
108	$>2 \times 10^7$	>5120	20	+++	5.6
210	$>2 \times 10^7$	80	≤ 20	0	≤ 1

* 1 cm. +
1-2 cm. ++
2 cm. +++

Coagulase activity was assessed on plates by the size of the zone of fibrin-precipitate which developed around a colony twenty-four hours after stab-inoculation of a fibrinogen agar plate. Alpha and δ-hemolysins were measured by the size of the zones of hemolysis which occurred twenty-four hours after stab-inoculation of rabbit and human blood agar plates respectively.

Beta hemolysin activity was detected on sheep-blood agar plates which were stab-inoculated and incubated at 37°C. for twenty-four hours followed by equal periods at room temperature and at 4°C. The activity of this hemolysin was measured by the increase in diameter of the zone of hemolysis after incubation at 4°C. Tube measurements of β-hemolysin were not made.

An examination of Table VII in which zones of fibrin precipitation which indicate coagulase activity have been ranked from smallest to greatest, shows at first inspection that there may be some correlation between coagulase and α-hemolysin activity, since most strains with no detectable hemolysin activity are found in the upper half of the table corresponding to low coagulase activity. Closer inspection of the table, however, indicates that there is a large amount of variation. For example, seven strains have zones of hemolysis ranging from one to two millimetres in the upper half corresponding to low coagulase activity; and in the lower half, corresponding to high coagulase activity, twelve also have diameters of zones of hemolysis in this same range. Such an observation is not consistent with the existence of a positive degree of correlation between coagulase and α-hemolysin.

It is to be noted that only seven of the thirty-seven strains produce measurable quantities of δ-hemolysin on human blood agar plates. There is no apparent relationship between δ-hemolysin and coagulase activities, but large zones of hemolysis due to α-hemolysin (greater than 2.5 mm.) are generally associated with δ-hemolysin activity, as Table VII shows, but again not all

TABLE VII

QUANTITATIVE RELATIONSHIP OF COAGULASE, α , β AND δ -LYSINS MEASURED ON PLATES*

Strain	Fibrin precipitation (mm.)	α-lysin (mm.)	δ-lysin (mm.)	β-lysin (mm.)
Newman	0	0	0	0
203	0	1.5	0	0
Smith	0	0	0	6.5
103	1.5	3.5	2.0	0
211	1.5	0	0	5.0
13889	1.5	0	0	0
3A	2.5	0	0	0
Wood-46	3.5	0	0	6.0
109	4.0	2.0	0	0
13819	4.0	1.5	1.0	0
O'Hara	4.0	0	0	9.0
101	4.5	3.5	1.5	0
102	5.0	1.0	0	0
212	5.5	3.5	1.0	1.0
3038	5.5	2.5	0	0
Giorgio	5.5	2.0	0	0
106	6.0	2.5	1.5	0
L-16	6.0	1.5	0	0
201	6.0	1.5	0	0
3099	6.0	2.0	0	0
3053	6.0	1.5	0	0
MAM	6.0	2.5	0	0
100	6.5	0	0	0

TABLE VII (Continued)

Strain	Fibrin precipitation (mm.)	α -lysin (mm.)	δ-lysin (mm.)	β-lysin (mm.)
13896	6.5	2.0	0	0
53	6.5	1.5	0	0
191	6.5	0	0	0
2364	6.5	1.0	0	0
105	7.0	3.0	2.0	0
108	7.0	2.0	0	9.5
13853	7.0	2.0	0	3.5
13908	7.0	1.5	0	0
210	7.0	1.0	0	2.0
3023	7.0	1.0	0	0
3251	7.0	0	0	0
206	7.5	3.5	2.5	0
107	8.0	2.0	0	0
104	8.5	1.0	0	0

*Diameter of zone of hemolysis or fibrin minus diameter of colony.

strains which produce a zone of greater than 2.5 mm. also produce δ-hemolysin; for example, strains MAM and 3038.

Reference again to Table IX (line six) shows that the coefficient of correlation for α-hemolysin and coagulase activities measured on plates is 0.1736, which is not significant ($p > 0.05$). It will be noticed that this coefficient is not the same as that for α-hemolysin and coagulase measured by serial dilution which has a value of 0.3996 ($p < 0.02 > 0.01$).

Only eight of the thirty-seven strains investigated produce detectable β-hemolysin as shown in Table VII.. No particular relationship of β-hemolysin to coagulase, α or δ-hemolysin is evident. Beta hemolysin varies independently of these properties. It is noteworthy that the Smith strain, which is coagulase-negative, produces no α or δ (on plates only) but does produce β-hemolysin when measured on plates. According to Parker (1960), the Smith strain is of exceptional virulence, but, as has been shown earlier (Table X) its culture filtrate is not lethal.

Comparison of Plate and Tube Methods of Measuring α and δ-Hemolysins and Coagulase

In Table VIII, strains are shown which produce both α and δ-hemolysins by either the serial dilution or plate methods. It has already been mentioned that there is some correlation between tube and plate measurements of coagulase and α-hemolysin, but reference to the table points up a number of discrepancies: The Newman strain produces coagulase by the tube method but not on fibrinogen agar; strains 211, Newman, 13889, and O'Hara produce α-hemolysin when their culture filtrate is measured but not on rabbit-blood agar. Strains 201, 211, Newman, 13889, O'Hara, 104, Giorgio and 108 produce δ-hemolysin when culture filtrate is measured but not on human-blood agar; and strains 101, 103, 13819, 106 and 105

TABLE VIII

STRAINS OF S. AUREUS PRODUCING BOTH α AND δ -LYSINS BY EITHER SERIAL DILUTION OR PLATE METHODS

Strain	Serial Dilution Coagulase	α-lysin	δ-lysin	Fibrin	Plate Zone (mm.)* α-lysin	δ-lysin
101	200	640	< 20	4.5	3.5	1.5
201	200	5120	20	6.0	1.5	0
211	2000	1280	160	1.5	0	0
Newman	2000	160	40	0	0	0
13889	2×10^4	640	20	1.5	0	0
206	2×10^4	5120	20	7.5	3.5	2.5
103	2×10^4	2560	< 20	1.5	3.5	2.0
13819	2×10^5	640	< 20	4.0	1.5	1.0
O'Hara	2×10^5	80	160	4.0	0	0
104	2×10^5	640	20	8.5	1.0	0
Giorgio	2×10^5	> 10240	20	5.5	2.0	0
106	2×10^6	5120	< 20	6.0	2.5	1.5
105	2×10^6	5120	< 20	7.0	3.0	2.0
212	2×10^7	5120	20	5.5	3.5	1.0
108	> 2×10^7	> 5120	20	7.0	2.0	0

*Diameter of zone of hemolysis or fibrin minus diameter of colony.

TABLE IX

COEFFICIENTS OF CORRELATION (r_s) BASED ON THIRTY-SEVEN STRAINS OF S. AUREUS*

1.	Serial dilution	Coagulase-α-lysin	r_s = 0.3996	Significant at 2% level.
2.	" "	Coagulase-LD_{50}	0.3925	Significant at 2% level.
3.	" "	Coagulase-Dermonecrotic factor	0.1795	Not significant.
4.	" "	α-lysin-LD_{50}	0.8812	Significant at $<$0.1% level.
5.	" "	α-lysin-Dermonecrotic factor	0.6129	Significant at $<$0.1% level.
6.	Plate measurement	Coagulase-α-lysin	0.1736	Not significant
7.	Plate vs. tube method	Coagulase-Coagulase	0.3351	Significant at 5% level.
8.	" " "	α-lysin-α-lysin	0.6626	Significant at $<$0.1% level.

*Calculated by the method of Spearman (1904).

do not produce detectable δ-hemolysin in culture filtrates, but do so on human-blood agar plates. Thus, coagulase and hemolysin activities of strains measured on plates in many cases do not correlate with these properties measured by serial dilution of culture filtrate or whole culture.

Table IX shows that there is some correlation between coagulase activity measured on plates and in tubes, and between α-hemolysin activity measured on plates and in tubes. Lines seven and eight show the coefficients to be 0.3351 ($p < 0.05 > 0.02$) and 0.6626 ($p < 0.001$) respectively. The latter is highly significant but the former is not.

Coagulase-Negative Strains

Culture filtrates of nine coagulase-negative strains were assayed for α- and δ-hemolysins, dermonecrotic and lethal factors in the same manner as described earlier for coagulase-positive strains. Reference to Table X shows that only the Smith strain produces δ-hemolysin, and strain 203 is the only one which produces α-hemolysin, dermonecrotic and lethal factors. None of the culture filtrates of any of the other strains is hemolytic and none, including Smith, is dermonecrotic or lethal.

Stability of Toxin and Coagulase Production

Titres of α and δ-hemolysins, dermonecrotic and lethal factors in culture filtrates of any one strain were measured at the same time. As mentioned previously, there was generally a month's delay before coagulase activity was assayed. Because of this delay and because of the possibility, suggested by the results of Table VI, that there may have been changes in the capacity of a strain to produce coagulase or hemolysin in the interval, nine strains were selected at random and tested again for coagulase and α-hemolysin activity at the conclusion of experimental work.

TABLE X

α AND δ -HEMOLYSIN, DERMONECROTIC AND LETHAL TITRES OF CULTURE FILTRATES OF COAGULASE-NEGATIVE STRAINS OF STAPHYLOCOCCUS

Strain	α-lysin	δ-lysin	Dermonecrotic factor	LD_{50}/ml.
Smith	<20	40	0	<1
200	<20	<20	0	<1
202	<20	<20	0	<1
203	640	<20	+	3.2
204	<20	<20	0	<1
205	<20	<20	0	<1
207	<20	<20	0	<1
208	<20	<20	0	<1
209	<20	<20	0	<1

Culture filtrates of these selected strains were prepared and assays of α and δ-hemolysins carried out by serial dilution as described earlier. Dermonecrotic and lethal titres were not measured again because of the demonstrated parallelism between the α-hemolysin and these properties. Coagulase assays were carried out simultaneously on overnight Brain Heart Infusion broth cultures by serial dilution.

Table XI shows that two of nine strains suffered changes in α-hemolysin titre. The initial α-hemolysin titre of strain 211 was 1280, and when tested three months later it had fallen to 160. The δ-hemolysin titre also changed, falling from 160 to less than 20. Strain 13889 also exhibited a change of α-hemolysin titre, the initial being 640 and the final 160. Only one-tube differences (within the limits of experimental error) were observed between all other initial and final titres of α and δ-hemolysins. There was no change in the coagulase activity of either of these two strains.

Three of nine strains suffered a change in coagulase activity. Extreme changes occurred in strain 210, where the initial titre was greater than 2×10^7, and three months later it had fallen to 2×10^3. Strains 105 and 108 showed some change, but it was not as marked as that occurring in strain 210. Again there was no significant change in the capacity of these three to produce α and δ-hemolysin. Thus, of nine strains tested, five showed a significant change in their capacity to produce either coagulase or α-hemolysin over a three-month period.

Qualitative Changes in Coagulase Production

Ten coagulase-negative strains of Staphylococcus were originally acquired for this investigation from the clinical laboratory of the Winnipeg General Hospital, as mentioned earlier. At the time of acquisition, all ten were coag-

TABLE XI

VARIABILITY OF HEMOLYSIN AND COAGULASE ACTIVITY OVER A PERIOD OF THREE MONTHS

Strain	α-lysin titre		δ-lysin titre		Coagulase titre	
	Initial	3 months	Initial	3 months	Initial	3 months
3A	160	80	<20	<20	2×10^3	2×10^2
211	1280	160	160	<20	2×10^3	2×10^4
191	160	160	<20	<20	2×10^3	2×10^4
13889	640	160	20	<20	2×10^4	2×10^4
210	80	40	<20	<20	$>2 \times 10^7$	2×10^3
105	5120	5120	20	20	2×10^6	2×10^8
108	>5120	>10240	20	40	$>2 \times 10^7$	2×10^6
Wood-46	640	1280	<20	<20	2×10^4	2×10^5
13896	20	40	<20	<20	2×10^5	2×10^5

ulase-negative by both slide and tube tests. One additional strain, Smith, was acquired several months later and it has been described in the literature as coagulase-positive (Hunt and Moses, 1958). It was, however, coagulase-negative in this laboratory. Independent tests in another laboratory (Parker, 1960) confirmed this fact.

Of the ten strains (not including Smith), strains 201 and 206 had become coagulase-positive after about two months and two subcultures. These strains were included in the analysis of coagulase, hemolysin, dermonecrotic and lethal activity carried out on strains in Table VI. Four others, strains 200, 204, 205, and 208 (see Table XII) became coagulase-positive about five months and five subcultures later.

Table XII shows the present qualitative hemolytic properties on human, rabbit and sheep-blood agar of the strains originally coagulase-negative and of the six which have become coagulase-positive. These tests were made about four months after the original culture filtrates were tested and found negative. While a comparison of results obtained on blood agar with those obtained by the assay of culture filtrates may not be valid (see Table X for comparison), it is interesting to note that all ten strains (200-209) produce some hemolysin on human, rabbit or sheep-blood agar.

All strains in Table XII except Smith were tested with standard $\alpha\delta$-antitoxin[2] on human, rabbit and sheep-blood agar plates. Hemolysis caused on rabbit-blood agar by the coagulase-negative strains 202 and 203 was inhibited by the antitoxin. Hemolysis on human, rabbit and sheep-blood agar caused by the coagulase-negative strains 207 and 209 was not inhibited by the antitoxin.

The hemolysis of all strains in Table XII which have become coagulase-positive was inhibited by the antitoxin on the two types of blood agar. The

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[2]Wellcome Research Laboratories, Beckenham, Kent. Potency: 20 α units/ml.

TABLE XII

HEMOLYSIN ACTIVITY OF STRAINS ORIGINALLY COAGULASE-NEGATIVE

Strain	Coagulase tube test	Hemolysis on Blood Agar		
		Human	Rabbit	Sheep
Smith	-	-	-	+
200	+	-	±	+(I)
201	+	-	+(I)	+(I)
202	-	±	+(I)*	-
203	-	±	+(I)	±
204	+	+(no I)	+(I)	+(I)
205	+	+(no I)	+(I)	+(I)
206	+	+(no I)	+(I)	+(I)
207	-	+(no I)**	+(no I)	+(no I)
208	+	-	±(I)	±(I)
209	-	+(no I)	+(no I)	+(no I)

* (I) Inhibition of hemolysis by antitoxin.

** (no I) No inhibition.

ability of a culture filtrate of strain Smith to lyse human erythrocytes was inhibited by the antitoxin, but no tests were done on blood agar.

Antitoxin Inhibition of Hemolytic Properties of Coagulase-Positive Strains

As an aid to further characterization of strains of S. aureus, all hemolytic culture filtrates of these coagulase-positive strains were tested with either a standard antitoxin or with an antitoxin prepared in this laboratory in two rabbits.[3] The pooled antitoxin titre of the two rabbit sera was 1280 Burnet Antihemolytic Units per ml.[4]

All human and rabbit-cell hemolysins of coagulase-positive strains were found to be neutralizable by antitoxin.

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[3] See Methods.

[4] One Burnet Antihemolytic Unit of antitoxin is that amount which will neutralize one Burnet Unit of toxin.

DISCUSSION

DISCUSSION

It has been shown that α-hemolysin, dermonecrotic and lethal activities of culture filtrates vary directly with one another. This parallelism is one of the main reasons why the monistic view of α-toxin is widely accepted today. In the present work, while dermonecrotic and lethal activities were both highly correlated with that of α-hemolysin, the coefficient of correlation for dermonecrotic factor and α-hemolysin was much lower than the coefficient for LD_{50} and α-hemolysin. There are at least two possibilities which might explain this discrepancy; the first is that the method of measuring dermonecrotic activity is more crude than that of LD_{50} and hemolysin measurement by serial dilution; secondly, the existence, as first shown by Robinson et al (1960b), of a minor non-hemolytic dermonecrotic component which is produced by some but not all strains of S. aureus.

Some quantitative correlation has been found between coagulase, α-hemolysin and LD_{50} titres, but not between coagulase and dermonecrotic factor. The coefficient of correlation for coagulase and α-hemolysin titres is significant, but not highly so. This fact is also true of the coefficient for coagulase and LD_{50} titres. The relationship of coagulase to dermonecrotic factor is not significant, or even nearly so. Again, the existence of a minor non-hemolytic dermonecrotic component or the crudeness of measurement might explain this lack of correlation.

There are one or two other factors which are possibly implicated in the lack of agreement, in a number of cases, between coagulase, α-hemolysin, dermonecrotic and LD_{50} titres. It was shown that both the coagulase and α-hemolysin titres of five of nine strains tested again at the end of experimental work varied significantly. Since coagulase activity was not assayed at the same time as the hemolysin, dermonecrotic and lethal activity, it has to be admitted

that such variation could lead to the demonstration of a false relationship between these properties.

The other important factor, which might explain the discrepancy between the results of Tager and Hales (1947), those of Marks (1952) and the author is the method used to obtain the results. As shown in this laboratory, there is no significant correlation between coagulase and α-hemolysin activity measured on plates, and this fact is in contradiction to the demonstration of a correlation between coagulase and α-hemolysin titres as measured by serial dilution. Elek (1959) has stated that the use of plasma or fibrinogen agar for the detection of coagulase may often yield equivocal results, as there are other staphylococcal products than coagulase which produce a turbidity in plasma or fibrinogen agar. In general, the author would place more reliance on data obtained by direct titration rather than by measurements of the size of zones of activity on agar plates. The measurement of diameters of zones of hemolysis or fibrin precipitation is a less accurate method of expressing results than the use of an Lh, test dose or hemolytic end-point in a tube titration. It appears that differences in the composition of media, in methods of incubation and titration might well account for some of the disagreement in results observed between tube and plate methods.

One unusual finding has been the relatively small number of strains which were found to produce δ-hemolysin. Elek and Levy (1950) have observed that 97 per cent of a total of 359 strains produce δ-hemolysin alone or in combination with other hemolysins, when measured on blood agar. The present investigation, employing a much smaller number of strains and a somewhat different method than Elek and Levy, has found that only sixteen (about 45 per cent) strains out of thirty-seven tested produce δ-hemolysin on blood agar. When culture filtrates were tested, only eleven (about 30 per cent) produced δ-hemolysin, and moreover

only to low titre. A likely reason for the low production of δ-hemolysin is that the cultural conditions employed in this laboratory were not optimal for its production. On the one hand, it is claimed that δ-hemolysin is not produced in fluid cultures; whether or not the 0.3 per cent agar employed in Dolman-Wilson medium in this investigation is sufficient for optimal production of δ-hemolysin, is not known. On the other hand, Jackson et al (1958a) have found that hemolysis of human erythrocytes by δ-hemolysin is minimal at pH 7, and since the erythrocyte-hemolysin mixtures of the present work were not buffered, it is just possible that δ-hemolysin was present to higher titre in a larger number of cultures but was not detected.

Elek and Levy (1950) found 88 per cent of fifty-nine coagulase-positive animal strains and 11 per cent of two hundred coagulase-positive human strains to produce β-hemolysin. In the present investigation, 20 per cent of coagulase-positive strains derived from human sources were found to produce β-hemolysin (see Table VII). β-hemolysin is produced chiefly by strains derived from animal rather than human sources.

An interesting finding has been the number of coagulase-negative strains which have become coagulase-positive. In this investigation, six of ten strains which were originally coagulase-negative (excluding Smith) became positive after 2-5 subcultures. Explanations accounting for this phenomenon might be: (1) mutation, and (2) contamination by a coagulase-positive culture when subculturing. One would expect coagulase-positive strains to lose this property much more commonly than he would expect coagulase-negative strains to become positive. A possible way to avoid the situation arising from explanation (1) would be to plate out all cultures periodically and pick from a single clone each time. Also it is felt that contamination is not likely to have played a role, because of the careful technique employed.

No examples involving coagulase-positive cells which have become coagulase-negative were encountered. However, as mentioned before, the Smith strain, which has been described as coagulase-positive in the literature, was coagulase-negative when tested in this laboratory and in an adjacent laboratory.

Elek and Levy (1950) have discussed the occurrence in 95 per cent of their coagulase-negative strains of a hemolysin which was not inhibited by standard staphylococcal antitoxin. They called this hemolysin "ε". Two of the coagulase-negative strains examined in this laboratory produced a hemolysin which was not inhibited by Wellcome standard antitoxin. Reference to Table XII indicates that strains 207 and 209 produced hemolysis on rabbit, sheep and human blood agar plates, which was not inhibited in the presence of a filter strip soaked in antitoxin. The hemolytic properties of the coagulase-negative strain 203 on rabbit blood agar and in culture filtrates tested with rabbit erythrocytes, were inhibited by antitoxin. Hemolysis by strain 202 on rabbit blood agar was also inhibited. A culture filtrate of the Smith strain was hemolytic to human cells, but the activity could be neutralized by antitoxin.

The existence of the ε-hemolysin of coagulase-negative strains has been questioned by some investigators, particularly Marks (1952). In the present work, however, the author suggests that un-neutralizable hemolysis produced on rabbit, sheep and human blood agar by strains 207 and 209 is due to such a hemolysin. It is also suggested that coagulase-negative strains which produce α, β or δ-hemolysins occur more frequently than reported.

Because of the observed frequency of the change from the coagulase-negative to positive state, and because of the observation of α, β and δ-hemolysins in coagulase-negative strains, a question is raised as to the use of coagulase production as a taxonomic criterion within the genus Staphylococcus (Bergey's Manual, 1957).

SUMMARY

Alpha hemolysin of culture filtrates of S. aureus has been shown to be stable for a period of seven days if kept at 4°C.

The accepted view that there is a quantitative correlation between the α-hemolysin, dermonecrotic and lethal factors is confirmed.

Evidence is presented which shows some correlation between coagulase and α-hemolysin measured by serial dilution methods, and between coagulase and lethal factor, but not between coagulase and dermonecrotic factor. The δ-hemolysin of culture filtrates also bears no relationship to these properties.

No relationship has been observed between coagulase and α-hemolysin activity measured on plates, which opposes the observed correlation between coagulase and α-hemolysin activities measured by serial dilution. Some correlation, however, appears to exist between the degree of α-hemolysin production on plates and the ability of the strains being measured to produce δ-hemolysin. β-hemolysin activity appears to vary independently of coagulase, α and δ-hemolysin, dermonecrotic and lethal activities.

Confirmation of the existence in two strains of coagulase-negative Staphylococcus of a hemolysin un-neutralizable by $\alpha\,\delta$-antitoxin is presented.

The question is raised as to the validity of coagulase production as a taxonomic criterion within the genus Staphylococcus.

BIBLIOGRAPHY

BIBLIOGRAPHY

Alami, S.Y., and Kelly, F.C. (1960). Influence of coagulases and route of injection on staphylococcal virulence in mice. Proc. Soc. Exper. Biol. Med. 105, 589.

Arrhenius, S.A. (1907). Immunochemistry: the application of the principles of physical chemistry to the study of biological antibodies. Macmillan Co., New York.

Bigger, J.W. (1933). The production of staphylococcal hemolysin with observations on its mode of action. J. Path. Bact. 36, 87-114.

Blobel, H., Berman, D.T., and Simon, J. (1960). Purification of staphylococcal coagulase. J. Bact. 79, 807-815.

Breed, R.S., Murray, E.G.D., and Smith, N.R. (eds.) (1957). Bergey's manual of determinative bacteriology. Williams and Wilkins Co., Baltimore.

Brown, J. (1960). Some properties of staphylococci related to pathogenicity. J. Path. Bact. 79, 257-262.

Bryce, L.M., and Rountree, P.M. (1936). The production of β-toxin by staphylococci. J. Path. Bact. 43, 173-189.

Burnet, F.M. (1929). The exotoxins of Staphylococcus pyogenes aureus. J. Path. Bact. 32, 717-734.

Burnet, F.M. (1931a). The interactions of Staphylococcus toxin, antitoxin, and anatoxin. J. Path. Bact. 34, 471-492.

Burnet, F.M. (1931b). The flocculation reaction with staphylococcal toxin. J. Path. Bact. 34, 759-769.

Cadness-Graves, B., Williams, R., Harper, G.J., and Miles, A.A. (1943). Slide-test for coagulase-positive staphylococci. Lancet 1, 446.

Christie, R., and North, E.A. (1941). Observations on staphylococcal beta toxin and its production by strains isolated from staphylococcal lesions in man. Aust. J. exp. Biol. Med. Sci. 19, 323-327.

Christie, R., North, E.A., and Parkin, B.J. (1946). Criteria of pathogenicity in staphylococci. Aust. J. exp. Biol. Med. Sci. 24, 73-78.

Cruickshank, R. (1937). Staphylocoagulase. J. Path. Bact. 45, 295-303.

Dolman, C.E. (1932). Pathogenic and antigenic properties of Staphylococcus toxin. Canad. publ. Hlth. J. 23, 125-132.

Dolman, C.E., and Wilson, R.J. (1940). The kitten test for Staphylococcus enterotoxin. Canad. publ. Hlth. J. 31, 68-71.

BIBLIOGRAPHY (Continued)

Duthie, E.S. (1952). Variation in the antigenic composition of staphylococcal coagulase. J. gen. Microbiol. 7, 320-326.

Duthie, E.S. (1954). Evidence for two forms of staphylococcal coagulase. J. gen. Microbiol. 10, 427-436.

Duthie, E.S., and Lorenz, L.L. (1952). Staphylococcal coagulase: mode of action and antigenicity. J. gen. Microbiol. 6, 95-107.

Duthie, E.S., and Houghton, G. (1958). Purification of free staphylococcal coagulase. Biochem. J. (Lond.) 70, 125-134.

Elek, S.D. (1959). Staphylococcus pyogenes and its relation to disease. Livingstone Ltd., Edinburgh.

Elek, S.D., and Levy, E. (1950). Distribution of hemolysins in pathogenic and non-pathogenic staphylococci. J. Path. Bact. 62, 541-554.

Elek, S.D., and Levy, E. (1954). The nature of discrepancies between hemolysins in culture filtrates and plate hemolysin patterns of staphylococci. J. Path. Bact. 68, 31-40.

Fahlberg, W.J., and Marston, J. (1960). Coagulase production by Staphylococcus aureus.-I. Factors influencing coagulase production. J. Inf. Diseases 106, 111-115.

Fisher, A.M. (1936). The plasma-coagulating properties of staphylococci. Bull. Johns Hopk. Hosp. 59, 393-414.

Forssman, J. (1934). Staphylolysinstudien-II. Acta Path. Microbiol. Scand. 11, 214-226.

Gladstone, G.P., and Glencross, E.J.G. (1960) Growth and toxin production of staphylococci in cellophane sacs in vivo. Brit. J. exp. Path. 41, 313-333.

Glenny, A.T., and Stevens, M.F. (1935). Staphylococcus toxins and antitoxins. J. Path. Bact. 40, 201-210.

Hunt, G.A., and Moses, A.J. (1958). Acute infection of mice with Smith strain of Staphylococcus aureus. Science 128, 1574-1575.

Jackson, A.W., and Little, R.M. (1957). Staphylococcal toxins.-I. Factors affecting the hemolytic activity of alpha toxin. Canad. J. Microbiol. 3, 47-54.

Jackson, A.W., and Little, R.M. (1958a). Staphylococcal toxins.-II. Factors affecting hemolysis by δ-lysin. Canad. J. Microbiol. 4, 435-444.

Jackson, A.W., and Little, R.M. (1958b). Staphylococcal toxins.-III. Partial purification and some properties of δ-lysin. Canad. J. Microbiol. 4, 453-462.

BIBLIOGRAPHY (Continued)

Jackson, A.W., and Mayman, D. (1958c). Staphylococcal toxins.-IV. Factors affecting hemolysis by β-lysin. Canad. J. Microbiol. 4, 477-486.

Kapral, F.A., and Li, I.W. (1960). Virulence and coagulases of Staphylococcus aureus. Proc. Soc. Exper. Biol. Med. 104, 151-153.

Kato, Y., and Omori, G. (1959). Extraction of bound coagulase from staphylococcal cells. Biken's J. 2, 321-332.

Lack, C.H., and Wailling, D.G. (1954). A study of 435 strains of Staphylococcus pyogenes with reference to factors which may contribute to pathogenicity. J. Path. Bact. 68, 431-443.

Landsteiner, K., and von Rauchenbichler, R. (1908-09). Ueber das Verhalten des Staphylolysins beim Erwarmen. Z. ImmunForsch. 1, 439-448.

Levine, B.S. (1938). Studies of staphylococcal toxin. The toxin-red-cell reaction. J. Immunol. 35, 131-139.

Loeb, L. (1903). The influence of certain bacteria on the coagulation of the blood. J. Med. Res. 10, 407-419.

Marks, J. (1951). The standardization of staphylococcal α-antitoxin, with special reference to anomalous hemolysins including δ-lysin. J. Hyg. (Lond.) 49, 52-66.

Marks, J. (1952). Recognition of pathogenic staphylococci; with notes on non-specific staphylococcal hemolysin. J. Path. Bact. 64, 175-186.

Marks, J. and Vaughan, A.C.T. (1950). Staphylococcal δ-hemolysin. J. Path. Bact. 62, 597-615.

Morgan, F.G., and Graydon, J.J. (1936). Toxins of the Staphylococcus, with especial reference to the estimation of potency. J. Path. Bact. 43, 385-401.

Much, H. (1908). Uber eine Vorstufe der Fibrinfermentes in Kulteren von Staphylokokkus aureus. Biochem. Z. 14, 143-155.

Murray, M., and Gohdes, P. (1959). Role of coagulase-reacting factor in the activation of fibrinogen. J. Bact. 78, 450.

Panton, P.N., and Valentine, F.C.O. (1932). Staphylococcal toxin. Lancet 1, 506-508.

Parker, S. (1960). Unpublished observations.

Pillemer, L., and Robbins, K.C. (1949). Ann. Rev. Microbiol. 3, 265-288.

BIBLIOGRAPHY (Continued)

Pillet, J., Orta, B., and Perrier, M. (1959). Immunisation du lapin en deux injections par l'anatoxine staphylococcique alpha. Ann. Inst. Pasteur 97, 353-359.

Rammelkamp, C.H., Jr., Hezebicks, M.M., and Dingle, J.H. (1950). Specific coagulases of Staphylococcus aureus. J. exp. Med. 91, 295-307.

Reed, L.J., and Muench, H.A. (1938). A simple method of estimating fifty per cent end-points. Am. J. Hyg. 27, 493-497.

Robinson, J., Thatcher, F.S., and Montford, J. (1960a). Studies with staphylococcal toxins.-V. Possible identification of alpha hemolysin with a proteolytic enzyme. Canad. J. Microbiol. 6, 183-194.

Robinson, J., Thatcher, F.S., and Montford, J. (1960b). Studies with staphylococcal toxins.-VI. Some properties of a non-hemolytic dermonecrotic toxin produced by some strains of Staphylococcus aureus. Canad. J. Microbiol. 6, 195-202.

Schwabacher, H., Cunliffe, A.C., Williams, R.E.O., and Harper, G.J. (1945). Hyaluronidase production by staphylococci. Brit. J. exp. Path. 26, 124-129.

Smith, W., and Hale, J.H. (1944). The nature and mode of action of Staphylococcus coagulase. Brit. J. exp. Path. 25, 101-109.

Smith, W., Hale, J.H., and Smith, M.M. (1947). The role of coagulase in staphylococcal infections. Brit. J. exp. Path. 28, 57-67.

Smith, M.L., and Price, S.A. (1938). Staphylococcus γ-hemolysin. J. Path. Bact. 47, 379-393.

Spearman, C. (1904). Am. J. Psychol. 15, 88.

Tager, M. (1941). Factors modifying the reaction of alpha staphylolysin to heat. Yale J. Biol. Med. 14, 68-88.

Tager, M. (1948a). Studies on the coagulase-reacting factor.-I. The reaction of staphylocoagulase with the components of human plasma. Yale J. Biol. Med. 20, 369-380.

Tager, M. (1948b). Concentration, partial purification, properties, and nature of staphylocoagulase. Yale J. Biol. Med. 20, 487-501.

Tager, M. (1956). Problems in the coagulation of plasma by staphylocoagulase. Ann. N.Y. Acad. Sci. 65, 109-118.

Tager, M., and Hales, H.B. (1947). Quantitative coagulase and toxin production by staphylococci in relation to the clinical source of the organisms. Yale J. Biol. Med. 20, 41-49.

BIBLIOGRAPHY (Continued)

Walston, H.D. (1935). The clotting of plasma through staphylococci and their products. J. Hyg. (Lond.) 35, 549-558.

Williams, R.E.O., and Harper, G.J. (1947). Staphylococcal hemolysins on sheep-blood agar with evidence for a fourth hemolysin. J. Path. Bact. 59, 69-78.

Wlodarczak, S., and Jeljaszewicz, J. (1959). Influence of coagulase on growth of staphylococci in normal human serum. Nature 184, 1514.

Yotis, W.W., and Ekstedt, R.D. (1959). Studies on staphylococci; I. Effect of serum and coagulase on the metabolism of coagulase-positive and negative strains. J. Bact. 78, 567.

Yotis, W.W., and Ekstedt, R.D. (1960). Studies on staphylococci; III. Further studies on purification and mechanism of action of an antibacterial human serum factor. J. Bact. 80, 719-725.

APPENDICES

APPENDIX A

PHYSIOLOGICAL PROPERTIES OF COAGULASE-POSITIVE AND NEGATIVE STRAINS OF STAPHYLOCOCCUS

Strain	Coagulase Slide	Coagulase Tube	Mannitol	Glucose	Nitrite	Gelatin liqu.	10% NaCl	Pigment	Litmus milk	$NH_4H_2PO_4$ utilized
100	+	+	A	A	+	+	g	Gw	A	ng
101	-	+	A	A	+	+	g	Gs	A	ng
102	+	+	A	A	+	+	g	Gw	A	ng
103	+	+	A	A	+	+	g	Gs	AC	ng
104	+	+	A	A	+	+	g	Gw	A	ng
105	+	+	A	A	+	+	g	Gs	AC	ng
106	+	+	A	A	+	+	g	Gw	AC	ng
107	+	+	A	A	+	+	g	Gw	AC	ng
108	+	+	A	A	+	+	g	Gw	AC	ng
109	+	+	A	A	+	+	g	Gw	AC	ng
210	+	+	A	A	+	+	±g	Gw	A	ng
211	+	+	A	A	+	+	±g	-	A	ng
212	+	+	-	A	±	-	ng	-	AC	ng
191	+	+	A	A	+	+	g	Gs	A	ng
Newman	+	+	A	A	+	-	g	-	AC	ng
13819	+	+	A	A	+	-	g	Gs	A	ng
13889	+	+	A	A	+	-	g	-	A	ng
13896	+	+	A	A	+	-	ng	Gs	AC	ng
L-16	+	+	A	A	+	-	g	Gs	AC	ng
Wood-46	+	+	A	A	+	+	g	Gw	AC	ng
13853	+	+	A	A	+	+	g	Gs	AC	ng
13908	+	+	A	A	+	+	g	Gs	AC	ng

APPENDIX A (Continued)

Strain	Coagulase Slide	Coagulase Tube	Manni-tol	Gluc-ose	Nitr-ite	Gelatin liqu.	10% NaCl	Pig-ment	Litmus milk	$NH_4H_2PO_4$ utilized
53	+	+	A	A	+	-	g	Gw	AC	ng
3A	+	+	A	A	+	-	g	Gs	A	ng
Smith	-	-	A	A	-	+	g	-	AC	ng
3099	+	+	A	A	+	-	g	Gs	AC	ng
3053	+	+	A	A	+	-	g	Gw	AC	ng
3038	+	+	A	A	+	-	g	Gw	AC	ng
2364	+	+	A	A	+	-	g	Ys	AC	ng
3023	+	+	A	A	+	+	g	Gw	A	ng
3251	+	+	A	A	+	-	g	Gs	AC	ng
MAM	+	+	A	A	+	-	g	Yw	A	ng
O'Hara	+	+	A	A	+	-	g	Yw	AC	ng
Giorgio	+	+	A	A	+	-	g	Yw	AC	ng
200	-	-	-	A	+	+	g	-	AC	ng
201	-	-	-	A	±	+	g	-	AC	ng
202	-	-	-	A	+	-	g	-	A	ng
203	-	-	-	A	-	+	g	-	AC	ng
204	-	-	-	A	+	+	g	-	AC	ng
205	-	-	-	A	+	+	g	-	AC	ng
206	-	-	-	A	+	+	g	-	AC	ng
207	-	-	a	A	+	-	g	-	A	ng
208	-	-	-	A	+	+	g	-	AC	ng
209	-	-	-	A	+	+	g	-	AC	ng

Symbols employed:

A (acid production)
a " " slight
C (clot)
g (growth)
ng (no growth)

G (golden)
Y (yellow)
w (weakly developed)
s (strongly developed)

APPENDIX B

FORMULAE AND METHODS FOR PREPARATION OF MEDIA AND SOLUTIONS

Dolman-Wilson Medium:

The "semi-synthetic" medium used is prepared as follows:

Difco Proteose Peptone.....20g.

Sodium chloride.....5g.

Dissolve above substances in 500 ml. distilled water.

To this solution, add the following salts in aqueous solution:

Ammonium lactate*.....5g.

Dipotassium hydrogen phosphate..1g.

Potassium dihydrogen phosphate..1g.

Magnesium sulphate.....0.2g.

Calcium chloride.....0.1g.

Bring to boiling, make up to 1 l. volume, adjust to pH 7.4, and autoclave at thirty pounds pressure for fifteen minutes. This medium is the nutrient base, and can be converted into a medium of semi-solid consistency when required for use by adding 0.3 per cent agar, followed by re-sterilization.

One hundred ml. of the medium is poured into 150 x 20 mm. Petri dishes and allowed to set before seeding with culture.

Alsever's Solution:

Alsever's solution is prepared in distilled water as follows:

Glucose.....2.05%

Sodium citrate.....0.80%

Sodium chloride.....0.42%

Citric acid.....0.055%

Autoclave at fifteen pounds pressure for fifteen minutes.

An equal volume of blood is added to one volume of this solution.

*An 80 per cent solution of ammonium lactate was employed, 6.2 ml. of this being added to the medium.

APPENDIX C

PREPARATION OF HEMOLYTIC STANDARD

Erythrocytes are washed with saline, centrifuged and re-suspended in distilled water to obtain a hemolysed cell concentration of 4 per cent. This solution is shaken thoroughly and is added to an equal volume of double-strength saline (1.7 per cent), which results in a final hemolysed cell concentration of 2 per cent in normal saline (0.85 per cent).

A series of eleven tubes measuring 1 x 8.5 cm. is set up, and quantities of hemolysed cells are added, ranging from 0-1.0 ml. by an arithmetic progression of 0.1 ml.

A 2 per cent suspension in normal saline of normal erythrocytes is added to these tubes, in amounts ranging from 1.0 ml.-0, by an arithmetic progression of 0.1 ml. One ml. of normal saline is then added to each of the eleven tubes, which brings the volume per tube up to 2.0 ml., the same as that in the hemolysin titration.

Thus, a hemolytic standard is obtained which consists of a series of eleven tubes, the hemolysis in which varies from 0-100 per cent, by increments of ten.

The standard is best compared with the hemolysin titration by viewing both in an illuminated box with a black background super-imposed on white*.

*Arthur H. Thomas Co., Philadelphia, Pa.