PURIFICATION AND CHARACTERIZATION
OF STAPHYLOCOAGULASE

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C. L. Sanclemente
Major professor

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ABSTRACT

PURIFICATION AND CHARACTERIZATION

OF STAPHYLOCOAGULASE

by Zeno Zolli, Jr.

The antigenic relationship between coagulases from some twenty phage propagating strains of the International-Blair series of staphylococci was studied. The coagulases were prepared by acid and ethanol precipitation according to methods of Tager (1948) and recent modifications of Blobel et al. (1960). Inconclusive results from these materials stimulated a research program to develop procedures for the isolation and purification of coagulase. Progress in this quest was gauged by a series of serological and chemical characterizations.

Inhibition tests using early preparations with a purification factor of 21 to 79X showed that maximal anticoagulase activities occurred in homologous antigen-antisera systems. Although there was a low degree of cross-inhibition among the strains, no consistent correlation was observed corresponding to phage group. This fact suggested possible

strain specificity of coagulase. Further elucidation of this phenomenon using gel diffusion patterns proved difficult to interpret because of the high number of precipitin bands.

Several approaches were investigated for the increased purification of coagulase from Staphylococcus aureus 70. In the first, coagulase was separated by concentration in the syneretic fluid from the clot complex. This method resulted in a partially purified preparation with a 300-fold purification factor. Another method yielding a partially purified preparation (approximately 300-fold) employed only gel filtration (Sephadex G-200) which permitted separation of proteins by differences in molecular weight. The final procedure achieved extreme purification of this clotting material by using three cycles of dialysis in ethanol-water mixtures under controlled conditions according to modifications of methods of Cohn et al. (1946) and Pillemer et al. (1948) followed by molecular sieving through a column of Sephadex G-200. By manipulation of five variables (pH, ionic strength, temperature, protein and ethanol concentration), the final preparation showed an

approximate 3700-fold increase in activity per mg protein. The final procedure for the separation and isolation of coagulase, evolved from a series of detailed experiments, was composed of four steps as follows:

(a) The first fraction (Cg-I-P) was precipitated from six 200 ml aliquots of cell-free broth filtrate by dialysis against a sodium acetate buffer (pH 3.8 and ionic strength 0.1) containing 10% ethanol (v/v) at -4C. Each precipitate was then redissolved in 50 ml of 0.1 N sodium acetate solution.

(b) Secondly, each solution of Cg-I-P was dialyzed against a sodium acetate buffer (pH 5.2 and ionic strength 0.05) containing 10% ethanol (v/v) at -4C, and the resulting precipitates (Cg-II-P) were each redissolved in 5 ml of 0.1 N sodium acetate solution.

(c) The solution of Cg-II-P was then dialyzed against a phosphate buffer (pH 6.1 and ionic strength 0.1) containing 10% ethanol (v/v) at -4C; in this step, the supernatant fluid (Cg-III-S) was retained while the precipitate was discarded.

(d) In the final step, the solution of fraction Cg-III-S was concentrated (10X) prior to molecular sieving

through a column of Sephadex G-200. The final active fraction was designated as Cg-IV-C.

The successfully isolated coagulase was serologically and chemically characterized. Using gel diffusion techniques, the use of the fourth fraction as antigen against anti-fraction III serum produced one precipitin band. A more critical aspect of this test employing the fourth fraction against anti-fraction I serum produced a second but weak band. Additional confirmation of purity was evidenced by the appearance of a single peak using cellulose acetate paper electrophoresis. Progressive elimination of carbohydrate, deoxyribonuclease, lipase and phosphatase was observed through the four stages of purification. Temperature stability studies showed that increasing purity corresponded to decreasing heat stability.

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INTRODUCTION

The exact role of coagulase in the pathogenic process of Staphylococcus aureus has been a matter of conjecture for over half a century. The means by which this enzyme-like material may enhance virulence is unknown. Although other cellular constituents have been incriminated as factors of pathogenicity, coagulase is demonstrated most consistently and readily among the virulent strains of this organism.

The development of procedures for the purification of coagulase has made possible more critical studies of its antigenicity and mode of action. Barber and Wildy (1958) and Blobel et al. (1960) showed a relationship between the bacteriophage groups of staphylococcal strains and the antigenic specificity of free coagulase produced by corresponding strains. Significant findings along these lines could conceivably lead to simpler diagnostic procedures for staphylococcal strain identification.

This report is concerned with a study of the possible relationship between phage type and antigenic specificity of coagulase. The inconclusive results obtained with partially purified coagulase indicated the need for a preparation of greater purity than the one employed.

Methods were developed to separate and purify coagulase to a high degree. Antigenic and chemical properties of the purified coagulase were determined.

REVIEW OF LITERATURE

Coagulase and Pathogenicity

Since the discovery by Loeb (1903) that S. aureus possesses the ability to clot goose plasma, persistent attempts have been made to determine the exact relationship of this clotting material to the virulence of S. aureus and the role it plays in pathogenesis. The earliest observation that a correlation may exist was reported by Much (1908). Among the reports which followed and agreed with this view were those of Daranyi (1926), Cruickshank (1937), Smith et al. (1947) and Blair (1958).

Other factors which have been reported to be associated with virulence were phosphatase (Rangam and Katdare, 1954), urease (Fusillo and Jaffurs, 1955), mannitol fermentation (Schaub and Merrit, 1960) and alpha hemolysin (Brown, 1960). Jeffries (1961) attempted to correlate coagulase with several activities which included deoxyribonuclease, mannite fermentation, pigment production, reduction of phenyltetrazolium, and growth on tellurite glycine agar. Some 494 strains of staphylococci isolated from routine specimens in the laboratory of the Detroit Receiving Hospital were used in the study. Deoxyribonuclease, mannite

fermentation or pigment production correlated to a high degree with coagulase production and could be used as preliminary tests for potential pathogenicity among the staphylococci. Among the organisms tested, deoxyribonuclease activity correlated with coagulase production to the highest degree.

Citations for other tests for the pathogenicity of S. aureus include those of Blair (1939, 1958) and Angyal (1961) who both reported data supporting the reliability of coagulase production, and suggested its use as sole criterion.

Antigenicity of Staphylocoagulase

The correlation of coagulase production to the virulence of S. aureus led to investigations of the possibility that antibodies specific for coagulase could confer protection for S. aureus.

Initial attempts to elicit antibodies specific for coagulase by culture supernatant fluid proved fruitless (Gross, 1931; Walston, 1935; Smith and Hale, 1944). However, Lominski and Roberts (1946) showed that coagulase inhibition by human sera exhibited "inhibitory substances

which have antibody characteristics, but cannot yet be accepted as such." It was not until Rammelkamp (1948) noted an increase in anticoagulase titers of acute and convalescent phase sera of patients infected with staphylococci that an intensive study of this phenomenon was instigated. Subsequent studies by Rammelkamp et al. (1950) showed anticoagulase titers in monkeys injected with cell-free coagulase preparations. Tager (1948) developed a method for the partial purification of coagulase and paved the way for additional studies involving the experimental production of antibodies to staphylocoagulase (Tager and Hales, 1948). These experiments showed that coagulase was antigenic in rabbits. Exposure to a partially purified preparation necessitated intensive and prolonged injection regimes as well as combination with alpha-lysin.

Aluminum phosphate as an adjuvant with purified coagulase was used by Duthie and Lorenz (1952). A suspension of washed aluminum phosphate (6 mg/ml) adsorbed up to 3.5 mg coagulase per mg of aluminum phosphate from a 1% coagulase preparation at pH 4.8. Intramuscular or subcutaneous inoculations of rabbits with this material consistently yielded higher anticoagulase levels than coagulase alone or in conjunction with alpha lysin.

Barber and Wildy (1958) combined potassium aluminum sulfate with coagulase at pH 6.8 for four or more weekly injections in rabbits.

Blobel et al. (1960) obtained antibodies from rabbits against alcohol precipitated coagulase. Potassium aluminum sulfate was used as an adjuvant in the manner described by Barber and Wildy (1958). Six intramuscular injections were administered at weekly intervals and consisted of a total dosage of 12 mg of lyophilized preparation per animal. An adjuvant was used which consisted of mineral oil and an emulsifying agent (mannide monooleate). The electrophoretically purified preparation of coagulase was dissolved in physiological saline solution (pH 7.0) and mixed with the oil and emulsifying agent in a ratio of 10:9:1 respectively. The presence of either adjuvant doubled the anti-coagulase titer (1:180).

Serological Specificity of Coagulase

Rammelkamp et al. (1950) observed that coagulase may be serologically distinct. Using antisera produced against coagulases derived from four different strains of staphylococci, Duthie (1952) and Duthie and Lorenz (1952) found four

antigenically distinct types of coagulases and suggested the existence of others. In spite of a low degree of cross inhibition, the majority of coagulases were inhibited by only a single antiserum.

A suggestion by Smith (1954) was followed with an attempt by Barber and Wildy (1958) to determine whether a correlation does in fact exist between coagulase antigenic specificity and bacteriophage type. They chose strains which were representative of three main bacteriophage groups (strain M, phage type 52/80 - group I; strain W120, phage types 3B/3C/55 - group II; strain Newman, phage types 7/47/53/54/73/75/77 - group III). Antisera were prepared by injection of alum precipitated partially purified coagulase into young adult rabbits. A close relationship between bacteriophage groups of staphylococcal strains and the antigenic specificity of free coagulase was found from corresponding strains.

Blobel and Berman (1960) reported experiments designed to obtain data concerning the serologic specificity of coagulase. By using rabbit anticoagulase sera against alcohol precipitated coagulases representing phage groups I, II, III, IV and Misc., each coagulase was neutralized

to a greater degree by its homologous antiserum. This indicated a relationship between bacteriophage group and antigenic specificity, but a low level of cross neutralization did occur among all pairs. The difference in neutralization titers did not appear to be sufficiently distinct to justify the use of coagulase cross inhibition tests to classify staphylococci for epidemiological investigations. Using a limited number of organisms, similar results were obtained by Zen-Yoji et al. (1961). More conclusive results may have been obtained by using an extremely purified coagulase preparation.

Purification of Coagulase

The determination of the role of coagulase in the pathogenesis of staphylococci spurred early attempts to purify this material. Walston (1935) and Fisher (1936) were among the first to use alcohol to precipitate crude coagulase from broth cultures of S. aureus. Lominski (1944) separated supernatant fluid from 12 hr broth cultures of S. aureus and found that coagulase passed through Chamberland L3 filters. Similarly, Smith and Hale (1944) found coagulase to be readily filterable through gradacol

membranes of suitable porosity. In addition, they reported coagulase thermostable and particulate, or associated with uniform particles. Rammelkamp et al. (1950) obtained coagulase preparations for serological investigations by growth of S. aureus in albumin enriched broth for 3 days at 37C.

Gerheim et al. (1947) prepared crude coagulase extracts by alcohol precipitation. The cells were removed from the broth of 48 hr cultures of S. aureus by filtration or centrifugation. Ethanol (95%) was added to the cell-free filtrate in a 3:1 ratio respectively at 0C. The yield from 850 ml of broth culture filtrate was approximately 1.5 g of a "greyish-brown powder" which was completely soluble as a 1% solution in citrate-borate buffer (pH 7.55). No inactivation of the "powder" occurred when stored at 5C for 8 months. Solutions remained stable for 10 days at room temperature.

Tager (1948) was one of the earliest workers to purify coagulase. An inoculum was prepared by growing S. aureus #104 in brain heart infusion for 3-6 hr at 37C. A 10-15% inoculum of this culture was transferred to a 1 liter flask containing 175 ml of the same broth. Then flasks were incubated for 4-6 days at 37C. The cells were removed from

15-20 liters of broth culture with a Sharples super-centrifuge. The cell-free supernatant fluid was acidified to pH 3.8 - 4.0 by the addition of 4 N HCl. This mixture was allowed to stand at 4C for 12-24 hr and the precipitate collected. This precipitate was then washed several times with phosphate buffer solution (pH 3.8 - 4.0), resuspended in M/15 phosphate buffer solution (pH 8.2) and the insoluble residues discarded. Reprecipitation was accomplished by reacidification to pH 6.5 followed by the addition of 3 vol ethanol (95%) and storage at -5C. The precipitate was washed, dissolved in phosphate buffer solution (pH 8.2) and insoluble debris removed. Ammonium sulfate (8-12% saturation) was added and the resulting precipitate discarded. Ethanol and ammonium sulfate fractionation were repeated several times. Results showed a 300-400 fold increase in purification and indicated that (a) coagulase was most stable at pH 4.5 - 7.0; (b) crude coagulase was thermostable and resisted autoclaving at 120C but that the partially purified material was more heat labile; (c) purified coagulase was non-dialyzable; and (d) coagulase was protein in nature. Drummond and Tager (1959) showed that coagulase preparations possessed 0.5%

carbohydrate plus esterase activity. When 20 mg samples were used, they obtained a coagulase titer of 1:112,640 in 18 hr.

Boake (1956) modified Tager's (1948) technique by eliminating the addition of ammonium sulfate and relied only on alcohol and pH adjustments for purification. From 10 liters of culture supernatant fluid, 2 g of concentrated, partially purified coagulase were recovered.

Taking advantage of the thermostability of crude coagulase preparations, Walker et al. (1948) cultivated S. aureus in beef heart tryptic digest broth and autoclaved the culture filtrates for 20 min at 120C in order to remove certain interfering proteins by coagulation. Then the supernatant fluid was acidified to pH 4.0 with HCl, cooled to OC and the precipitate was allowed to settle overnight. The precipitate was washed three times with 0.1 original volume of filtrate as follows: (1) cold sodium acetate buffer solution at pH 4.0 and ionic strength 0.1 (2) cold sodium acetate buffer solution (pH 4.0, ionic strength 0.01) (3) cold distilled water. Finally, the precipitate was adjusted to pH 7.5 and the insoluble debris discarded. This final product was stored in vacuum dried form. Yields

varied between 25 mg and 60 mg per 100 ml of original supernatant fluid.

Duthie and Lorenz (1952) used cadmium sulfate to partially purify coagulase. Two liter flasks containing 400 ml nutrient broth were seeded with S. aureus strain Newman (NTCT 8178) and incubated at 37C for 9 hr without shaking. An additional 3 hr of cultivation was conducted with shaking. It was shown that shaken cultures contained four to five times more coagulase activity than non-shaken cultures did. Bacteria were removed from the broth culture by mixing with Filter-Cel and passage through filter paper. After cooling to 4C, cadmium sulfate was added to a final concentration of 0.5% (w/v) and the pH adjusted to 5.8. The precipitate, which resulted from overnight storage at 4C, was dissolved in 1 N HCl solution and adjusted to pH 2.0. Dialysis against tap water removed the cadmium at this pH and the material was stored in lyophilized form. The resulting preparation contained 6000 MCD (minimal clotting doses) per mg. Using cultures grown in 200 ml quantities of peptone yeast extract media and incubated in shallow layers in Roux bottles for 1 to 3 days at 37C, Barber and Wildy (1958) obtained coagulase preparations which were

then purified with cadmium sulfate according to methods of Duthie and Lorenz (1952).

Duthie and Haughton (1958) used casein hydrolysate as a growth medium and continuous shaking to obtain optimal coagulase production. After removal of the cells from the broth by filtration through Buchner funnels containing Hyflo Super-Cel, cadmium sulfate was added to the culture free fluid and allowed to stand for 16 hr at 4C. A water slurry was then made of the precipitate and HCl was added to a pH of 2.0. This solution was dialyzed for 48 hr and ammonium sulfate added at a concentration of 67% saturation. Reprecipitation with ammonium sulfate was performed.

Murray and Gohdes (1959) also used cadmium sulfate to purify coagulase. Cultures of S. aureus were grown in tryptose broth for 72 hr at 37C. After removal of the cells, ammonium sulfate was added to the supernatant fluid (33% w/v) and allowed to stand at 4C for 24 hr. The precipitate was harvested by centrifugation, dispersed in sodium citrate solution (3.2% w/v) and adjusted to pH 5.0. Cadmium sulfate was added and the precipitate discarded. Coagulase was then precipitated from the supernatant fluid

by the addition of ammonium sulfate (33% w/v) and subsequently solubilized with a 3.2% solution of sodium citrate. Further purification was accomplished by stepwise elutions from a cadmium sulfate chromatographic column with increasing concentrations of phosphate buffer.

Using S. aureus grown in heart infusion for 5 days on a shaker at 37C, Blobel et al. (1960) partially purified coagulase by acid and ethanol precipitation. Addition of ammonium sulfate proved useless since even at 5% saturation considerable amounts of contaminating proteins were precipitated. Additional purification was accomplished by starch block electrophoresis (pH 8.4, 200 v, 20-30 ma) for 16 hr. Migration of active coagulase was toward the anode. Although an overall purification factor of 387.5 was achieved and the final preparation contained high activity per unit nitrogen, electropherograms revealed a heterogeneous mixture. The use of anion exchange chromatography produced a slight increase in purification; and, recovery of active coagulase was less than 50%. Using high voltage column electrophoresis, a contaminating egg yolk factor was separated from purified coagulase preparations by Blobel et al. (1961).

Using methods similar to those of Blobel et al. (1960), Inniss and San Clemente (1961, 1962) were unable to separate phosphatase from coagulase activity. Results obtained by anion exchange chromatographic studies, in which gradient elutions were made from DEAE-cellulose columns with 0.01 M tris buffer solution, showed that maximal coagulase and phosphatase activity occurred in the same fraction. Subsequent experiments using starch block electrophoresis (pH 8.6, 200 v, 2 ma) gave similar results. A small degree of separation by electrophoresis did occur when a discontinuous buffering system was used.

According to Cohn et al. (1946, 1950), the separation of components from blood plasma by fractional precipitation has the advantage that the material which remains insoluble is protected from various chemical and enzymatic changes which may rapidly occur in solution. By use of variations in ionic strength, pH, temperature, protein and alcohol concentration, they were able to separate several proteins successfully from plasma. Using this same technique, Pillemer et al. (1948) crystallized tetanal toxin. Their procedures involved the use of methanol, since toxin denaturation by ethanol was encountered.

Initial attempts to utilize these procedures to purify coagulase were made by Tager and Lodge (1951). Cell-free culture filtrate was acidified to pH 5.2 with 5 M acetic acid solution and cold methanol was added to a final concentration of 20%. Maintenance of the temperature below 1C for 18 hr resulted in a precipitate which was separated and then redissolved in 0.15 M sodium acetate solution (pH 7.4). This step was followed by adjustment to pH 5.4 and addition of cold methyl alcohol (final concentration of 17%). Finally, a third precipitation was carried out at pH 5.3 with 0.075 M sodium acetate, 20% methyl alcohol and -5C. An increase of approximately 125-fold in purification and 50% recovery of coagulase was found.

Further attempts to utilize alcohol precipitation for purification of coagulase were made by Blobel (1959). After precipitating coagulase from cell-free broth filtrate at pH 3.8, the material was suspended in M/15 phosphate buffer solution (pH 7.4) and methanol added to final concentrations of 25%, 50% and 75%. Temperatures were maintained at below -10C for 16 hr. No precipitation occurred at alcohol concentrations of less than 25% under these conditions. When methanol was used in final

concentrations of 50%, approximately 85% of coagulase activity was lost. The best yield with only 28% loss of total coagulase activity was found at alcohol concentrations of 75%. Similarly designed experiments indicated that optimal coagulase precipitation occurred when the final concentration of ethanol was 70%.

MATERIALS AND METHODS

Cultures and Their Maintenance

Twenty phage propagating strains of S. aureus of the International-Blair series (Blair and Carr, 1953 and 1960) were used in these studies. These organisms were designated as follows: Group I - phage types 52A/79, 80; Group II - phage types 3A, 3B, 3C, 55; Group III - phage types 187, 53, 83(VA4), 73, 6, 77, 71, 47, 54, 75, 7, 70; Group IV - phage type 42D; Group Miscellaneous - phage type 81. Stock cultures were maintained on brain heart infusion agar[1] slants at 4C and were transferred approximately every two months. To eliminate strain variation with respect to loss of coagulase production, periodic transfers were made onto brain heart infusion agar plates containing 10% human plasma. Typical colonies around which the largest zone of fibrin occurred were chosen for restocking.

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[1]Difco Laboratories, Inc., Detroit, Michigan.

Concentration and Purification of Coagulase

Several methods were used to concentrate and purify coagulase. In initial investigations, coagulase was partially purified according to methods of Tager (1947) and recent modifications of Blobel et al. (1960). Experimental results showed the need for a preparation of increased purity. Thus, several other techniques were developed in an attempt to purify this clotting material to a high degree.

Partial purification by acid and ethanol precipitation according to methods of Tager (1948) and Blobel et al. (1960)

Twenty strains of S. aureus representing phage groups I, II, III, IV and Miscellaneous were used as sources of coagulase for partial purification. Three hundred ml of culture in log phase growth were added to 6 liter Florence flasks which contained 3 liters of brain heart infusion. These flasks were uncubated for 12 hr at 37C on a rotary shaker (ca 150 cycles/min). The cells were removed by use of a Servall continuous-flow superspeed centrifuge[1] (Model

[1] Ivan Sorvall, Inc., Norwalk, Connecticut.

KSB-1) at a flow rate of 50 ml/min. Using 4 N HCl, the supernatant fluid was acidified to pH 3.8 and allowed to stand at 4C for 18 hr. The precipitate was collected, 50 ml distilled water added and the solution adjusted to pH 7.2 with 0.066 M disodium phosphate buffer. After stirring, the insoluble residues were removed by centrifugation at 12,000 x g for 20 min. To the filtrate, ethanol (95%) was added slowly to a final concentration of 70% (v/v). Precipitation was allowed to occurr at -20C for 18 hr. The precipitate was removed at -20C, 50 ml distilled water and enough of 0.066 M potassium dihydrogen phosphate buffer solution were added to adjust the pH to 7.4. Three cycles of ethanol precipitation were repeated before concentration of the product by dialysis overnight against polyvinylpyrrolidone[1] and subsequent lyophilization.

Partial purification by separation from clot complex

Elek (1959) suggested that the conversion of fibrinogen to fibrin by coagulase may involve the formation of a complex with substances in the clot. This observation prompted a series of investigations to determine whether a

[1]Oxford Laboratories, Redwood City, California.

complex did indeed occur and whether this system would yield purified coagulase.

Partial purification by gel filtration (Sephadex alone)

The use of particular dextran gels for separation of amino acids, peptides and proteins has been described by Porath (1960). These methods using Sephadex[1] G-200 were adapted and modified to the purification of coagulase.

Extreme purification using ethanol-water mixtures under controlled conditions, and Sephadex

The separation of extremely purified coagulase from cell-free supernatant fluid was accomplished in four steps, three of which employed modifications of procedures of Cohn et al. (1946) and Pillemer et al. (1948). The fourth step utilized gel filtration (Sephadex G-200) according to modifications of methods of Porath (1960).

[1]Pharmacia Fine Chemicals, Uppsala, Sweden.

Characterization of the Various Fractions

Specialized procedures

Electrophoretic analysis. With partially purified material, starch block electrophoresis was used as an attempt to separate coagulase from phosphatase activity. Procedures according to Blobel et al. (1960) and modified by Inniss (1961) were used. Approximately 500 g of insoluble potato starch were washed 2-3 times with 1 liter amounts of 0.02 N potassium hydroxide solution followed by washing with distilled water and then the desired buffer. To mold the starch block, a thick slurry was prepared and added to a plastic template (2 x 24 cm). After hardening, a cross-wise well was excised from the starch block and the sample in the desired concentration was placed in the well. The plastic template was subsequently arranged in the Universal apparatus[1] in a manner similar to that used for cellulose acetate paper electrophoresis. Migration of components was allowed in an electrical field. The material was subjected to 200 v and 2 ma current for 16 hr at 4C.

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[1]Shandon Scientific Co., London, England.

The starch block was segmented at 1 cm intervals and the contents eluted with barbital buffer solution (pH 8.6).

With extremely purified coagulase preparations, small scale electrophoresis using cellulose acetate strips was performed as described by Smith (1960). Oxoid strips (2.5 x 12 cm) were impregnated by floating them on the surface of the desired buffer solution. This technique eliminated opaque spots (entrapped air) which occurred when the strips were rapidly submerged. After the strips were removed from the buffer solution and lightly blotted with filter paper, they were applied across the bridge gap of a Universal apparatus. Using a micropipette and straight edge ruler, in order to obtain narrow zones of origin, 0.01 ml sample was applied to the strip. The time interval varied with the conditions, for example, when barbitone buffer (pH 8.6, ionic strength 0.07) and a current of 0.4 ma/cm were used, optimal separation was achieved in 2 hr. Immediately after removal of the strips from the electrophetic apparatus, they were "fixed" by immersion into 5% trichloracetic acid for 20 min. Protein components were stained by either 0.2% Ponceau S[1] in 3% aqueous trichloracetic acid, or 0.002%

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[1]Allied Chemical Corp., New York, New York.

Nigrosin[1] in 2% acetic acid solution. Staining was more rapid with Ponceau S and gave satisfactory results in 10-20 min. With Nigrosin, overnight immersion of the strips was necessary. After staining, the strips were transferred to a washing solution (5% aqueous acetic acid) until a colorless background appeared. The strips were dried by placing them between two pieces of paper towel and pressing them with glass. An instrument consisting of a double beam recording and integrating reflectance densitometer (Chromoscan[2]) was used to determine the relative concentration of electrical components on the strip.

Production of anticoagulase. In our earlier studies employing acid and ethanol precipitated coagulase preparations, either mature Dutch Belt rabbits (2-4 lb) or New Zealand White rabbits (4-6 lb) were used for production of anticoagulase serum. Freund[3] adjuvant (1:1) was used as an antibody enhancing agent in all coagulase solutions. For proper emulsification, the addition of adjuvant to sample

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[1]Allied Chemical Corp., New York, New York.

[2]Joyce, Loebl and Co., Gateshead-on-Tyne, England.

[3]Difco Laboratories, Detroit, Michigan.

was done in small increments while carefully drawing and withdrawing the mixture with a one ml pipette; this technique resulted in a stable emulsion which did not separate upon standing overnight at 4C. The rabbits were inoculated subcutaneously by multiple injections in the subscapular region at weekly intervals for a period of 5 weeks. For this particular route of administration, 5 mg of coagulase mixture was injected each week for a total of 25 mg. Ten days after the last injection, the rabbits were carefully restrained on a board and blood was removed by cardiac puncture using a 10 ml syringe with attached 2 in. needle (20 gauge).

With extremely purified coagulase (3700X) preparations, only mature Dutch Belt rabbits (2-4 lb) were used. An antibody enhancing agent (Freund adjuvant) was used as previously described. However, this material was injected according to techniques of Leskowitz and Waksman (1960) via the foot pad route instead of the subscapular region. In this case, the total inoculation consisting of 2 mg of extremely purified antigen contained in 0.8 ml of a 0.1 N sodium acetate solution and Freund adjuvant mixture (1:1) was given in 0.2 ml quantities per foot of each rabbit.

The rabbits were then bled approximately 10 days after this initial injection. Obviously, since this route required only one injection, some 5 weeks were saved over the subscapular route. Adequate quantities of blood (50 ml) were obtained from the marginal ear vein while keeping the appendage warm with a special, electrically heated test tube.

Serological techniques. The coagulase inhibition test was used to determine the highest dilution of anti-serum which completely inhibited the clotting activity of coagulase. To a series of tubes containing a minimal amount of coagulase sufficient in 0.2 ml to yield a 4+ clot there was added 0.2 ml of serially diluted serum. The tubes were incubated in a water bath at 37C for 30 minutes. Then, all tubes received 0.5 ml of human plasma (diluted 1:5 in 0.85% NaCl solution), mixed well, and incubated at 37C for three more hours. In some instances, reinforcement of this system with rabbit serum was followed by an increase in coagulase activity. This enhancement was caused by an adequate level of coagulase reacting factor (CRF). To eliminate the masking of coagulase antibodies, normal serum was used in

control tubes. In place of human plasma as the source of fibrinogen, an alternate method involved the use of 1.5% (w/v) human fibrinogen plus 1% normal rabbit serum.

In our earlier studies, gel diffusion evaluation of coagulase preparations was made according to modifications of methods of Ouchterlony (1958). A good quality agar was dissolved in water at a concentration of 1%. Following the addition of calcium chloride and removal of the precipitates by filtration through glass wool, the agar was cut into small cubes and washed for several days in running tap water prior to final washing in distilled water. The agar was melted and to it was added sodium chloride (0.85%). Antibiotic glass petri dishes (90 mm diameter) were used because their flat surfaces were ideal for layering a thin film of this agar. Perfectly flat bottoms are essential for the maintenance of constant agar depth across the plate. Before addition of the agar, six strips of filter paper (Whatman 1) measuring approximately 1.5 x 0.5 in. were folded over both ends of the lip of the bottom plate so that part of the strip extended along the bottom and toward the center of the plate and part folded upward and out over the lip. A stainless steel wire bent to form a

circle was placed inside of the bottom plate to hold the strips in place. After sterilization of the petri plates in a hot air oven (300F) for 3 hr, 30 ml of sterile agar were poured into each plate and allowed to cool slowly. Before use, the plates were stored at 4C overnight. A central well and six circumferential wells were cut so that distances between each well were equal (10 mm). The antigen was placed in the central well and the serum in the peripheral wells, precipitation zones were observed after 5 days incubation at room temperature.

A gel diffusion method (Murty, 1960) was used with the extremely purified coagulase preparations. Difco ionagar at a final concentration of 1% was added to phosphate buffered physiological saline solution (pH 7.4). Ten ml quantities of this agar were layered on standard glass lantern slides (3 1/4 x 4 in.) and allowed to harden at 4C for 2 hr. After cutting wells to the desired pattern, antigen and serum samples were added. The glass slides were stored at room temperature under humid conditions for 3-5 days. With this system, the development time of precipitin zones was reduced from 5-6 days to 48 hr. After immersing the slides into 0.85% NaCl solution for 4 hr, they were washed

in distilled water overnight and the agar dried to a thin film by use of a warm air heater. The zones of precipitation were stained according to methods described by Crowle (1961). The slides containing the dried agar film were immersed into stain solution (thiazine red R, 10%; amidoswarz 10B, 0.1%; light green SF, 0.1%; acetic acid, 2%; mercuric chloride, 0.1%; to a desired volume with distilled water) for 5 minutes. The slides were then washed in 5% acetic acid solution to remove excess non-specific stain.

Assay methods

Coagulase. Coagulase activity was assayed by methods similar to those of Tager and Hales (1948). To the first of a series of non-etched, scrupulously clean test tubes (13 x 100 mm) containing 1 ml 0.85% NaCl solution, 1 ml of coagulase solution was added and serially diluted two-fold. Each tube then received 1 ml diluted human plasma. The plasma was prepared by adding 4 parts of 0.15 M NaCl solution and enough thimerosal to give a final concentration of 1:5000. After thorough mixing, the tubes were incubated in a 37C bath and clot formation was observed at 3 and 24 hr. Reciprocals of the highest dilution of coagulase

solution in which any visible clot occurred were recorded as "coagulase reciprocal titers." Importance of early initial readings was essential to detect those samples containing fibrin lysing substances with consequent dissolution of the clot.

Phosphatase. Modifications of methods of Pelczar et al. (1956), Barnes and Morris (1957) and Inniss and San Clemente (1962) were used to determine quantitatively any residual phosphatase activity in representative samples from successive fractions of increasingly purified coagulase. A 0.4% solution of disodium p-nitrophenylphosphate was used as the stock substrate. P-nitrophenol standard was prepared so that 1 ml contained a concentration of 0.2 μM. Acid phosphatase was measured in citrate buffer (pH 5.6) while alkaline phosphatase was determined in glycine buffer (pH 10.4). Tris buffer (pH 7.2) was also used.

To 1.4 ml of the desired buffer solution contained in a test tube (20 x 150 mm), 1 ml of sample and 0.6 ml stock substrate solution were added. After incubation in a 37C bath for 30 min, 1 ml of 1 N NaOH solution was added to each tube to stop the reaction and to develop the color of the liberated p-nitrophenol. Optical density was

determined with a Bausch and Lomb Spectronic 20 spectrophotometer using a filter with a wavelength of 425 mμ. Enzyme activity was determined from a calibrated curve in which optical density was plotted against μM of p-nitrophenol.

Total protein. Quantitative determination of total protein was made with techniques established by Lowry et al. (1951). Bovine serum albumin[1] was prepared so that values on the standard curve ranged from 7.8 to 500 μg per test tube. One ml samples were added to tubes containing 1 ml of 1 N NaOH solution and allowed to stand at room temperature for 10 min. To each tube there were added 1.5 ml triple distilled water and 5 ml reagent A (1 ml of 2% copper sulfate $5H_2O$ and 1 ml of 2.7% sodium potassium tartrate $4H_2O$ added to 98 ml of 2% sodium carbonate). After standing at room temperature for 15 min, each tube received 0.5 ml Folin-Ciocalteu reagent, carefully mixed, and was incubated an additional 35 min at the same temperature. A wavelength of 660 mμ on a Bausch and Lomb Spectronic 20 spectrophotometer was used to determine the

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

optical density. Total protein per ml of sample was determined from a calibrated chart currently prepared.

Nitrogen. The micro-Kjeldahl method was used to determine protein nitrogen as described by Kabat and Mayer (1961). To a 10 ml micro-Kjeldahl flask containing 1 ml of sample there was added 1 ml of a sulfuric acid solution. The sulfuric acid solution was prepared by adding 0.2 g copper sulfate to 20 ml of concentrated H_2SO_4. Approximately 0.25 g of potassium sulfate and some spherical glass beads were added to each digestion flask and allowed to boil. Digestion was carried out by boiling on a gas heated apparatus[1] until all particles disappeared and the solution became clear. Boiling was continued for an additional one-half hour. Distillations were made with a micro-Kjeldahl distillation apparatus. The ammonia was distilled into a boric acid-methyl red indicator solution. This indicator solution was prepared by the addition of 2 ml methyl red solution (saturated methyl red in 50% ethyl alcohol) to 100 ml saturated boric acid solution, and adding 100 ml distilled water. The distillate was titrated with N/70 HCl

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[1]Precision Scientific Co., Chicago, Illinois.

and the endpoint recorded when the sample agreed in color intensity with that of the distilled blank.

Phosphorus. The Fiske and Subbarow method (1925) was used for the colorimetric determination of phosphorus. A series of test tubes receiving 1 ml sample and 4 ml of 10% trichloracetic acid were allowed to set at room temperature for 10 min. After centrifugation and decantation, the filtrate received 0.5 ml molybdate reagent (2.5% ammonium molybdate in 3 N sulfuric acid), 0.5 ml aminonaphtholsulfonic acid powder in 195 ml of 15% sodium bisulfite and 5 ml of 20% sodium sulfite), and 2 ml distilled water. The tubes were thoroughly mixed and stored in the dark for 10 min. The optical density was measured at a wavelength of 660 mμ using a Bausch and Lomb Spectronic 20 spectrophotometer. Phosphorus was determined from a calibrated curve prepared with known amounts of standard phosphate solution (1 ml = 0.004 mg P).

Miscellaneous tests

Estimation of carbohydrate. The Molisch test according to the method of Gunsalus (1959) was used for the qualitative detection of carbohydrate. One-half ml of solution

was placed into a small test tube. Two drops of 5% alcoholic a-naphthol solution were added and the contents thoroughly mixed. One ml of concentrated sulfuric acid was added slowly down the side of the tube forming a layer beneath the aqueous phase. A pink band at the interface indicated a positive carbohydrate reaction.

Estimation of lipase. According to Tietz et al. (1959), lipase activity was satisfactorily estimated when olive oil was used as a substrate. The oil substrate was an emulsion of 93 ml of a solution containing 0.2 g sodium benzoate and 7 g gum arabic and 93 ml olive oil. Three ml of this substrate were added to a test tube containing 1 ml sample, 2.5 ml water and 1 ml buffer (0.02 M tris, pH 8.0). The tube was mixed thoroughly before incubating in a 37C bath for 14 hr. After adding 3 ml of ethanol (95%), the entire contents were transferred to a 50 ml beaker. A control tube was similarly treated. The contents of both beakers were then titrated electrometrically with N/20 NaOH to pH 10. By calculating the difference between the control and sample value, the units of lipase activity were recorded as the number of ml of N/20 NaOH used to neutralize the liberated free fatty acids.

Estimation of deoxyribonuclease. DNase test agar[1] was used to detect deoxyribonuclease activity of each fraction obtained during the process of coagulase purification. A thin layer of turbid agar was placed into the base of a sterile pertri dish. Shallow wells similar to those used for the gel diffusion technique were then cut into the agar at desired intervals. Several drops of sample were added to each well and diffusion allowed to occur at room temperature for 6-8 hr. After flooding the plates with N/1 HCl, positive reactions were recognized by clear zones around the well.

Effect of pH. The effect of pH was studied for the various fractions of purified coagulase. Addition of appropriate buffer to the coagulase reaction system resulted in the desired pH value. Coagulase activity was measured using a two-fold serial dilution technique as previously described but in this case we adjusted the pH of the system to 5.0, 6.0, 6.8, 7.0, 7.2, 8.0 and 9.0. A citrate buffer was used to obtain values of pH 5.0 and 6.0. Phosphate buffer was used for ranges between pH 6.8 and 7.2, while tris buffer was used for pH values of 8.0 and 9.0.

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[1]Difco Laboratories, Detroit, Michigan.

Effect of inhibitors. Each of the increasingly purified coagulase fractions was exposed to trypsin, ethylenediaminetetraacetate (EDTA) and sodium fluoride to determine their effect on the clotting activity of coagulase. To 2 ml of each coagulase fraction was added 1 ml of a highly active (1:250) trypsin preparation[1] to a final concentration of 40 μg/ml. Final concentrations of 0.025 M were used with sodium fluoride and EDTA. After incubation of these mixtures in a 37C bath for 1 hr, coagulase activities were measured using a two-fold serial dilution system as previously described.

Temperature stability. Two ml aliquots of each coagulase sample in 0.1 N sodium acetate were placed into separate test tubes and incubated at 56C, 37C and 25C. Each of the fractions was standardized to obtain approximately equivalent initial coagulase titers. Samples were tested for coagulase at various intervals over a 2 day period.

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[1] Difco Laboratories, Detroit, Michigan.

RESULTS

Evaluation of Various Cultural Conditions for Optimal Production of Coagulase

During the development of procedures to purify coagulase frustration was caused by continually limited yields. Initial enhancement of coagulase activity in the culture filtrate was attempted to overcome this problem. Recently, Solomon (1962) developed a synthetic growth medium for S. aureus which was not as effective as brain heart infusion in the production of coagulase. In addition to confirming the observations of Tyrrell et al. (1957) that a biphasic growth medium enhanced bacterial growth 2-30 times over a nonbiphasic system, Inniss (1961) also found no significant change in coagulase production by S. aureus 70. A series of investigations was thus conducted to determine whether temperatures of incubation, increased aeration, or addition of various additives to the medium would affect coagulase production.

Effect of incubation temperature

When incubation using brain heart infusion was carried out on a rotary shaker at various temperatures, maximal

coagulase was produced at 12 hr and 37C (FIG. 1). A slight delay and reduction in peak activity was noted at 25C. No coagulase appeared at the lowest temperature.

Fukui et al. (1960) reported that initial storage of Pasteurella pestis at 5C for 24 hr followed by incubation at 37C for 6 hr increased the elaboration of certain antigens. No significant increase was noted in viable or total cell count over that which occurred during normal incubation at 37C. Experiments were conducted to determine whether a similar system would be beneficial for coagulase production by staphylococci. Primary incubation at 4C was carried out for 24, 48, 72 and 96 hr followed by incubation at 37C for 2, 8, 12 and 24 hr. Neither increase in viable count nor evidence of coagulase activity were observed for periods up to 96 hr at 4C. Additional incubation of these cultures at 37C showed no further increase in coagulase activity over those which were not initially incubated in the cold.

Effect of shaking

Duthie and Haughton (1958) showed that shake cultures gave higher and more rapid coagulase titers than did non-shake cultures. To substantiate these studies coagulase activity of shake versus non-shake cultures were compared

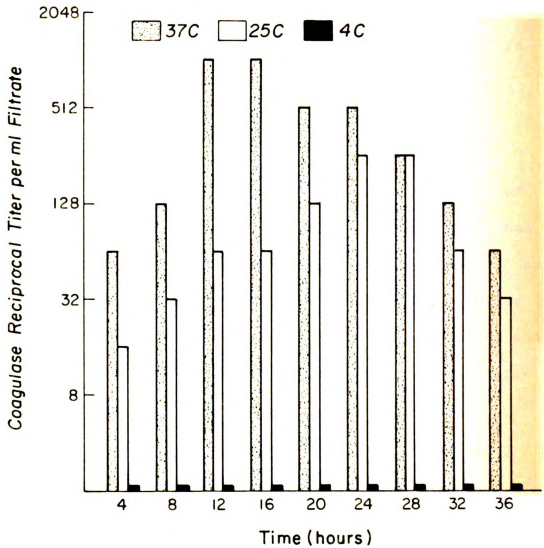


FIG. 1. Effect of temperature on coagulase production by Staphylococcus aureus 70 in brain heart infusion.

over a 5 day incubation period. When S. aureus 70 was incubated on a rotary shaker at 37C, maximal titers were obtained at 12 hr. However, within 48 hr continued shaking resulted in a sharp decrease in coagulase and little or no activity remaining at 72 hr. In contrast, highest coagulase activity under non-shake conditions occurred in 4 days followed by a slight decrease the next day (TABLE 1).

Effect of addition of trace minerals to the medium

Tager and Hales (1948) suggested that the addition of trace elements to brain heart infusion increased coagulase production. The composition of this mineral "cocktail," as Tager called it, is indicated on TABLE 1. When coagulase titers were compared between brain heart infusion alone and brain heart infusion plus 1 ml of the mineral solution per liter of medium, no enhancement of activity occurred (TABLE 1).

Concentration and Purification of Coagulase

Partial purification by acid and ethanol precipitation

The early attempts to purify coagulase included methods originally developed by Tager (1948) and more recently

TABLE 1. Effect of shaking and the addition of trace minerals to brain heart infusion upon the production of coagulase by Staphylococcus aureus 70

Time (Hr)	Coagulase Reciprocal Titers			
	Shake		Non-shake	
	BHI	BHI+Minerals*	BHI	BHI+Minerals*
12	1024	1024	128	128
18	512	256	128	128
24	512	256	512	512
48	16	16	1024	1024
72	0	0	2048	2048
96	0	0	4096	4096
120	0	0	2048	2048

*Composition of trace mineral solution (g/liter). One ml of this solution was added to a liter of brain heart infusion.

$MnSO_4 \cdot H_2O$	-	0.03
Boric acid	-	0.06
$CuSO_4 \cdot 5H_2O$	-	0.04
Molybdic acid	-	0.02
$FeCl_3 \cdot 6H_2O$	-	0.25
$ZnSO_4$	-	0.20
KI	-	0.10

TABLE 2. Purification factors of coagulases prepared by acid and ethanol precipitation

Phage Group	Phage Type	Purification Factor	Phage Group	Phage Type	Purification Factor
I	52A/79	26.6	III	83(VA4)	21.6
	80	66.2		73	65.0
II	3A	33.3		6	42.4
	3B	58.3		77	42.4
	3C	58.3		71	50.3
	55	35.0		47	26.6
III	7	42.0		54	50.2
	70	56.0		75	56.0
	187	42.6	IV	42D	79.0
	53	28.7	Misc.	81	36.6

modified by Blobel et al. (1960). Twenty strains of S. aureus were used representing phage groups I, II, III, IV and Miscellaneous. The inadequate degree of purification of each coagulase is indicated in TABLE 2. The purification factor was determined by dividing the coagulase reciprocal titer per mg protein of the purified material by the coagulase reciprocal titer per mg protein of the original culture filtrate. Although the purification factors tend to vary among the different strains of staphylococci, an average of these results (46.8) agrees closely with a factor of 48.4 obtained by Blobel et al. (1960).

Partial purification by separation from clot complex

Since Elek (1959) suggested that the conversion of fibrinogen to fibrin by coagulase may involve the formation of a complex with substances in the clot, we devised a series of experiments to determine whether this system would yield purified coagulase. We added enough partially purified coagulase to plasma to obtain a 2+ clot (approximately 25% of the total volume) after incubation at 37C for 10 min. The clot was removed by low speed centrifugation and washed several times with 0.85% NaCl solution. After

carefully disrupting the clot and removing the fibrin debris by centrifugation, the syneretic fluid was tested for protein, phosphatase and coagulase activity. Results (TABLE 3) indicate an 8-fold increase in coagulase purification. This purification factor was similar to that obtained by Blobel et al. (1960) who used starch block electrophoresis for further purification of the acid and ethanol precipitated coagulase preparation.

Furthermore, to determine the extent to which available coagulase would become trapped within the clot, trials were conducted in which 5 mg of partially purified material were added to 4.9 ml of physiological saline solution. To this mixture was then added 0.1 ml normal rabbit serum (source of coagulase reacting factor) and purified human fibrinogen. By adding the aforementioned three substances at various stages and removal of the clot when formed, it appeared that the clot consisted of fibrin, coagulase and coagulase reacting factor (TABLE 4).

Partial purification by gel filtration (Sephadex alone)

Separation of coagulase from undesirable protein was attempted using Sephadex G-200 chromatography. After 1300

TABLE 3. Separation of coagulase from phosphatase by concentration in the syneretic fluid from the clot complex

Fraction	Reciprocal of Coagulase Titer per Mg Protein	Coagulase Purification Factor	Residual Phosphatase Activity[a]
I[b]	5,120	-	-
II[c]	194	0.04	0.30
III[d]	40,960	8.00	0.02

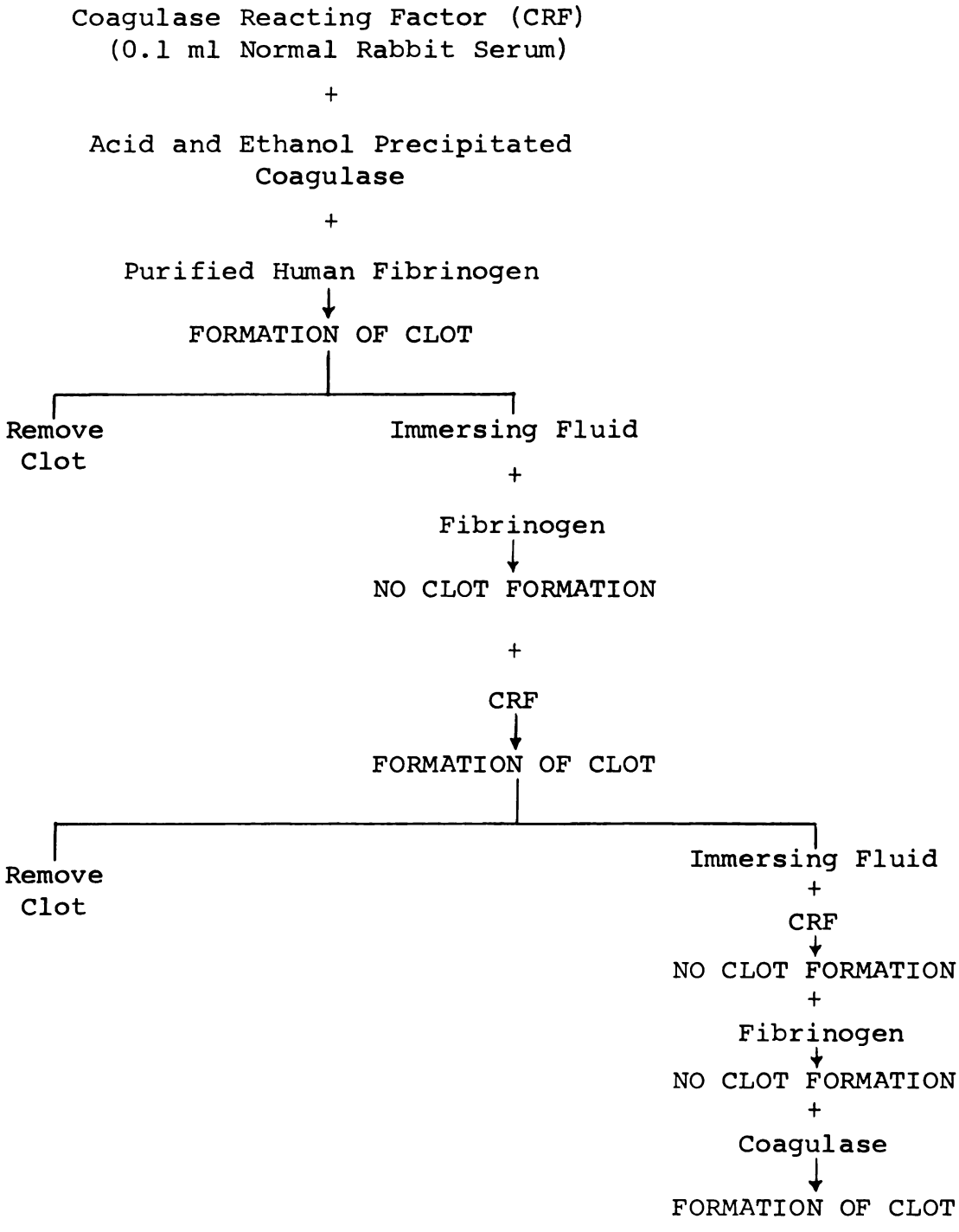
[a] μM p-nitrophenol liberated per ml.

[b] Partially purified coagulase from Staphylococcus aureus 70.

[c] Free fluid in which clot was immersed.

[d] Recovered syneretic fluid.

TABLE 4. Experimental proof for the retention of coagulase in the clotting complex



ml of cooled cell-free supernatant fluid were adjusted to pH 3.8 with 4 N HCl, the resulting precipitate was removed and dissolved in 50 ml of 0.1 N sodium acetate. Concentration to 5 ml was accomplished by evaporation at 4 C through dialysis tubing. Application of 2 ml (20 mg/ml) of this material to a column containing Sephadex G-200 and subsequent collection of 5 ml aliquots resulted in the removal of about 75% of the inert protein present in the original sample (FIG. 2).

Extreme purification using ethanol-water mixtures under controlled conditions, and Sephadex

Preliminary experiments. Modifications of methods of Cohn et al. (1946) and Pillemer et al. (1948) were used to purify coagulase. These techniques involving rigid control of pH, ionic strength, temperature, protein and alcohol concentration have been successfully used by the above workers for the separation of various components of blood plasma as well as purification of several toxins. It was deemed feasable to use a similar approach for the isolation and purification of coagulase. The following preliminary experiments were devised to arrive at conditions for the optimal recovery of coagulase:

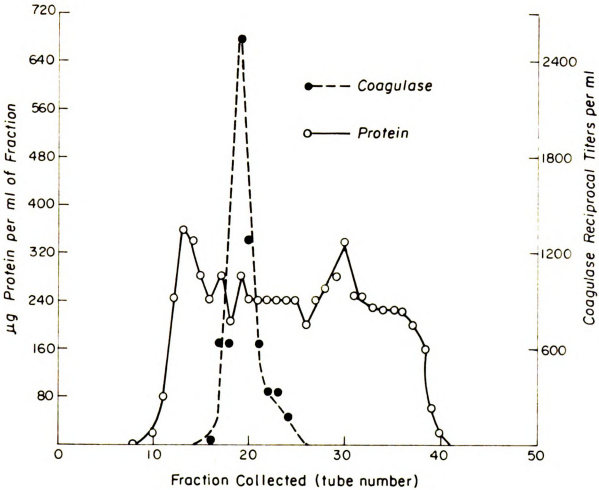


FIG. 2. Separation by gel filtration (Sephadex G-200) of a coagulase preparation obtained by precipitation of a cell-free filtrate at pH 3.8.

1. Fraction Cg-I-P. As a starting point, it seemed desirable to obtain maximal coagulase by precipitating it from the cell-free broth filtrate. Since Tager (1948) found that most of the active material precipitated at pH 3.8, we repeated this value. In addition, we used an acetate buffer at a constant ionic strength of 0.1 and various concentrations of ethanol between 0 and 60% (v/v). A temperature of -4C was used throughout these trials except where noted. TABLE 5 indicates that optimal activity was found in the precipitate that appeared at an ethanol concentration of 10% (v/v).

Fixing the pH at 3.8, the ethanol concentration at 10% (v/v) and the ionic strength of the acetate buffer at various values from 0.01 to 0.1, optimal coagulase activity was found in the precipitate obtained at 0.1 ionic strength (TABLE 6).

Furthermore, when ionic strength was held constant at 0.1 and two different ethanol concentrations were used for a series of pH values, some paired, ideal precipitating conditions prevailed at pH 3.8 (TABLE 7).

2. Fraction Cg-II-P. To obtain this fraction, satisfactory results were obtained by first redissolving fraction

Cg-I-P in 0.1 N sodium acetate followed by reprecipitation at favorable conditions. Ionic strength and pH of acetate buffer and ethanol concentrations were individually varied to determine optimal conditions. Maximal activity was obtained at pH 5.2, ionic strength 0.05, ethanol concentration 10% (v/v) and protein concentration of 3.1 mg/ml (TABLE 8).

3. Fraction Cg-III-S. In the preparation of the third fraction, it was considered appropriate to remove inert protein by precipitation and keeping the active coagulase in solution. Anticipating the need for higher pH values, a change was made to a phosphate buffer system. TABLE 9 represents the set of conditions for the solubility of coagulase fraction Cg-III-S in ethanol-water mixtures varying in pH but at a fixed temperature, protein concentration and ionic strength of the phosphate buffer.

Summary of final procedure. As a result of the foregoing series of detailed experiments, we arrived at exact conditions for the separation of coagulase to a high degree of purity. TABLE 10 represents a summary of the purification factors and per cent recovery of successive fractions showing an apparent similar degree of purification (3,793X) for both fractions Cg-III-S and Cg-IV-C. Although these

results might indicate that the Sephadex step was not essential, one must remember that the immunological tests are far more critical and sensitive than the protein or coagulase determinations. Subsequent studies revealed that the third fraction contained three immunological components which were reduced to one precipitin band by the additional use of Sephadex to remove residual but interfering protein. The following protocol, therefore, represents a summary of the final procedure for the isolation of coagulase in ethanol-water mixtures under carefully controlled conditions followed by molecular sieving through a column of Sephadex G-200.

1. Culture conditions. Brain heart infusion cultures (10 ml) in logarithmic phase of growth were used as inocula for 100 ml of the same infusion contained in a group of 1 liter Erlenmeyer flasks. Following incubation on a rotary shaker (ca 150 cycles/min) at 37C for 7 hr, a final inoculation with the contents of one Erlenmeyer flask was made into a 6 liter Florence flask containing 1200 ml broth. Additional incubation was then allowed for either 12 hr on a shaker or 96 hr without shaking. The organisms were then

TABLE 5. Precipitation of coagulase fraction Cg-I-P using various concentrations of ethanol at fixed conditions of temperature,[a] protein concentration,[b] pH and ionic strength of acetate buffer

pH	Ionic Strength	% Ethanol (95%) (v/v)	Coagulase Activity[c]
3.8	0.1	0	1,000
		10	5,300
		20	2,000
		40	900
		60	400

[a]Temperature of bath was -4C.

[b]Protein concentration of 12 mg/ml.

[c]Coagulase reciprocal titer per mg protein in precipitate.

TABLE 6. Precipitation of coagulase fraction Cg-I-P using an acetate buffer of various ionic strengths at fixed conditions of temperature,[a] protein concentration,[b] pH and ethanol

pH	Ionic Strength	% Ethanol (95%) (v/v)	Coagulase Activity[c]
3.8	0.01	10	1,200
	0.02		1,200
	0.04		1,100
	0.06		1,000
	0.08		3,600
	0.10		5,500

[a]Temperature of bath was -4C.

[b]Protein concentration of 12 mg/ml.

[c]Coagulase reciprocal titer per mg protein in precipitate.

TABLE 7. Precipitation of coagulase fraction Cg-I-P at two strengths of ethanol using different pH values of acetate buffer at fixed conditions of temperature,[a] protein concentration[b] and ionic strength

pH	Ionic Strength	% Ethanol (95%) (v/v)	Coagulase Activity[c]
3.4	0.1	10	3,600
3.6		10	3,300
3.6		20	3,200
3.8		10	5,300
3.8		20	2,000
4.0		10	4,400
4.0		20	1,000
4.2		10	3,300
4.6		10	3,300
5.0		10	2,400
5.4		10	No ppt
5.8		10	No ppt
6.2		10	No ppt
7.0		10	No ppt
8.0		10	No ppt

[a]Temperature of bath was -4C.

[b]Protein concentration of 12 mg/ml.

[c]Coagulase reciprocal titer per mg protein in precipitate.

TABLE 8. A set of optimal conditions for the precipitation of coagulase fraction Cg-II-P in ethanol-water mixtures at constant temperature[a] and protein concentration[b] but at various pH values and ionic strengths of acetate

pH	Ionic Strength	% Ethanol (95%) (v/v)	Coagulase Activity[c]
5.2	0.01	10	10,700
	0.01	20	4,200
	0.05	10	99,300
	0.05	20	17,400
5.4	0.01	10	46,800
	0.01	20	26,400
	0.03	10	11,700
	0.03	20	15,200
	0.05	10	49,600
	0.05	20	37,000
	0.06	10	26,000
	0.06	20	45,000
5.6	0.01	10	24,000
	0.01	20	41,900
	0.05	10	25,600
	0.05	20	21,500

[a] Temperature of bath was -4C.

[b] Protein concentration of 3.1 mg/ml.

[c] Coagulase reciprocal titer per mg protein in precipitate.

TABLE 9. The set of conditions for the solubility of coagulase fraction Cg-III-S in ethanol-water mixtures varying in pH but at a fixed temperature,[a] protein concentration[b] and ionic strength of phosphate buffer

pH	Ionic Strength	% Ethanol (95%) (v/v)	Coagulase Activity[c]
5.5	0.1	10	1,400
		20	900
6.1		10	218,000
		20	600
6.5		10	20,000
		20	4,000
7.1		10	20,000
		20	10,000
7.5		10	19,000
		20	8,000
7.9		10	19,000
		20	11,000

[a]Temperature of bath was -4C.

[b]Protein concentration of 0.7 mg/ml.

[c]Coagulase reciprocal titer per mg protein in the supernatant fluid.

removed by use of a Servall continuous-flow superspeed centrifuge at a flow rate of 50 ml/min.

2. Separation of fraction Cg-I-P. Two liters of acetate buffer solution (pH 3.8, ionic strength 0.1, 10% ethanol v/v) were placed into each of six heavy walled cylindrical glass jars (15 x 20 cm) and transferred to a cold bath (-4C). Since a suitable water bath was not available for studies with temperatures below the freezing range, and since similar sized commercial laboratory coolers were economically prohibitive, a unique innovation using a "wet-type" Coca-Cola cooler merits mention here. By manipulation of the thermostat and addition of alcohol to the water bath, a constant temperature below zero centigrade was obtained. After the temperature of the buffer solution was equilibrated with that of the surrounding medium, 200 ml aliquots of cell-free culture filtrate, transferred to each of 6 bags prepared from cellulose dialyzing tubing, were immersed into each of the six jars containing buffer solution. With constant stirring, dialysis was allowed to proceed for 12 hr. At this time, a freshly prepared 2 liter batch of buffer solution was added to each jar replacing that already present and dialysis was allowed to occur for

an additional 12 hr. The precipitate accumulating in the dialyzing bag was removed by use of a refrigerated centrifuge (International PR-1, 2000 rpm/60 min at -4C) and rehydrated to 50 ml with 0.1 N sodium acetate solution. After thorough mixing, insoluble residues were removed by centrifugation at 2000 rpm for 30 min and discarded.

3. Separation of fraction Cg-II-P. For this step, 2-liter quantities of acetate buffer solution (pH 5.2, ionic strength 0.05, 10% ethanol v/v) were placed into jars and treated in the manner described in the preceding step. Each of the 50 ml aliquots obtained as fraction Cg-I-P was placed into six dialyzing bags and immersed individually into six jars containing buffer solution. Following dialysis for 24 hr, the precipitate was removed by centrifugation in a Servall superspeed centrifuge (15,000 x g) for 10 min and dissolved in 5 ml 0.1 N sodium acetate solution.

4. Separation of fraction Cg-III-S. In contrast to recovery of the active coagulase in the precipitate as done in fractions Cg-I-P and Cg-II-P, conditions were arranged so that coagulase for this fraction was retained in solution and inactive material was precipitated. Since six 5 ml samples were obtained from the preceding step, only one jar

containing 2 liters of phosphate buffer (pH 6.1, ionic strength 0.1, 10% ethanol v/v) was necessary. Again, after equilibration of the buffer to -4C, each of the samples was placed into individual dialysis bags and immersed into the buffer solution and stirred. Dialyzing time, temperature and centrifugation speed were similar to those of the preceding step. However, the precipitate was now discarded. The supernatant fluid was recovered since it contained maximal coagulase activity per unit protein.

5. Separation of fraction Cg-IV-C. Five grams of dry Sephadex powder (G-200) were added to 250 ml of 0.15 M sodium chloride solution and allowed to swell. Henceforth, all operations were carried out at 4C. After transfer of this mixture to a glass chromatographic tube (2.5 x 45 cm), enough material was allowed to settle and pack overnight to achieve a height of 35 cm. To prevent the loss of the salt solution and subsequent dehydration of the gel, a rubber tube was clamped to the outlet of the column. A disc of filter paper whose diameter measured slightly less than that of the bore of the column tube was carefully placed on the surface of the bed. Above the disc 20 ml of 0.1 N sodium acetate (pH 7.2) were carefully pipetted and an

equal volume was allowed to drain from the column. Next, 2 ml of fraction Cg-III-S (concentrated to approximately 1 mg per ml of protein) were dissolved in the same buffer solution and added to the column in the same manner. To avoid disturbance of the surface of the gel column and subsequent skewing of the components to be separated, the filter disc was absolutely essential. After the sample was started through the column, approximately 20 ml of eluant was added again. This was followed by the application of a continuous, regulated flow (20 ml/hr) of eluant from a reservoir which fed into the top of the column by capillary tubing through a rubber stopper. Mechanical collection of 5 ml samples was accomplished by one of two available instruments (Preparative Fraction Collector,[1] Model No. 85000; Rotary Fraction Collector,[2] Model No. 1205A). To reduce the possibility of bacterial contamination of the polysaccharide gel, merthiolate solution (1:20,000) was allowed to flow through the column between sample runs. FIG. 3 shows the concomitant protein and coagulase peaks by molecular sieving of Cg-III-S through a column of Sephadex G-200.

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[1]California Corp. for Biochem. Res., Los Angeles, Calif.

[2]Research Specialties Co., Richmond, California.

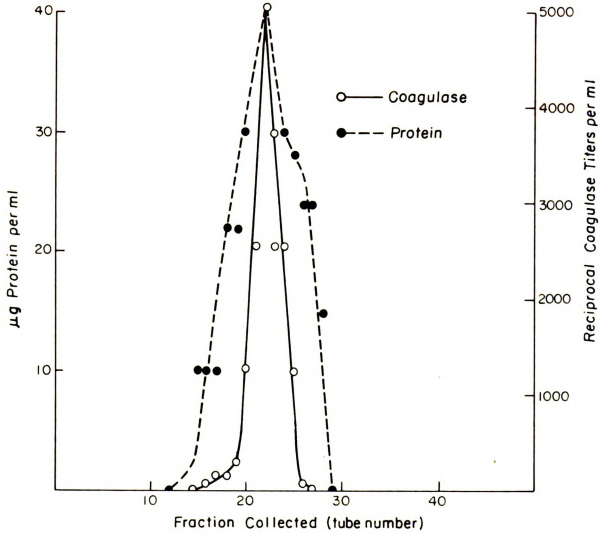

FIG. 3. Preparation of extremely purified coagulase, Cg-IV-C, by molecular sieving of Cg-III-S through a column of Sephadex G-200. The eluant was 0.1 N sodium acetate (pH 7.2).

TABLE 10. Summary of the purification factors and per cent recovery of successive fractions of coagulase

Fraction	Coagulase Activity[a]	Purification Factor	% Recovery
Filtrate	58	-	-
Cg-I-P	2,600	45	61
Cg-II-P	92,000	1,586	24
Cg-III-S	220,000	3,793	12
Cg-IV-C	220,000	3,793	12

[a]Coagulase reciprocal titer per mg protein (average value of at least 5 separate trials).

Characterization of the Various Fractions

Results of specialized procedures

Electrophoretic analysis. Evaluation of protein homogeneity was obtained by use of paper electrophoresis using cellulose acetate strips. Duplicate strips were prepared to detect the coagulase component. One strip was stained while the other was cut into small squares (2 x 2 mm) and immersed into 0.5 ml of 0.1 N sodium acetate solution. After elution of the component, the pieces of oxoid strip were removed with tweezers. To this solution was then added 0.5 ml of 0.85% NaCl solution followed by measurement of coagulase activity with a two-fold serial dilution system as previously described. For comparative purposes, electrophoretic patterns of coagulase fractions Cg-I-P and Cg-IV-C are indicated in FIG. 4. The extremely purified preparation (Cg-IV-C) showed only one peak while the relatively crude fraction (Cg-I-P) showed multiple components.

Serological studies. Suggestions by Barber and Wildy (1958) indicated a possible antigenic relationship between

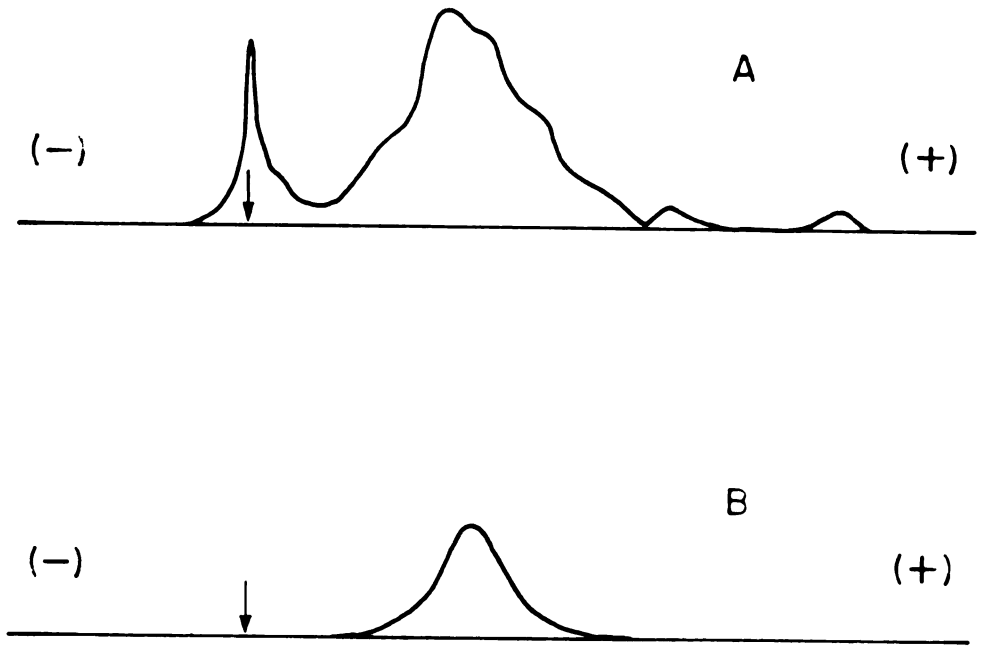

FIG. 4. Patterns of the first fraction (A) of coagulase, Cg-I-P, as contrasted with the final fraction (B) of coagulase, Cg-IV-C, indicating electrophoretic homogeneity on cellulose acetate strips using barbital buffer at pH 8.6 and ionic strength 0.07.

coagulases within major phage groups of S. aureus and prompted an extensive series of studies to confirm or deny these results. For these early studies, we isolated coagulase from each strain of the International-Blair series of staphylococci by acid and alcohol precipitation according to modifications of techniques of Tager (1948). Rabbits were used for serological studies, the material being injected subcutaneously along with Freund adjuvant. The sera from two rabbits used for each sample were pooled and employed in the coagulase inhibition and agar diffusion tests to determine antigenic specificity. These experiments were arranged to detect any possible cross-inhibition as well as cross-precipitation reactions. Results (TABLE 11) indicate that highest coagulase inhibition occurred in the homologous antigen-antiserum system. Although low cross-inhibition titers occurred in heterologous combinations, no definite relationships could be noted corresponding to phage groups. Therefore, these results may indicate that coagulases are strain specific. Using agar diffusion techniques, further confirmation of this phenomenon proved exceptionally difficult to interpret because of the appearance of a high number of precipitin zones.

TABLE 11. Cross-neutralization of constant amounts (depending upon purity of preparation) of the coagulases from staphylococcal strains of the International-Blair groups by their respective antisera

Antisera	Strains Acting as Sources of Coagulase Preparations																		
	52A/79	80	3A	3B	3C	55	187	53	VA4	73	6	77	71	47	54	75	7	42D	81
I 52A/79	40*	10	0	5	5	5	0	5	5	0	5	5	5	0	0	5	0	5	5
80	10	80	5	0	5	5	5	5	5	10	10	0	5	5	5	0	5	0	5
II 3A	5	10	80	5	5	5	10	5	5	10	5	5	0	10	0	5	10	5	5
3B	10	5	5	160	40	20	5	5	10	20	10	5	5	10	5	5	10	10	20
3C	0	10	5	20	40	10	5	0	0	5	0	0	10	0	10	5	0	5	5
55	5	0	10	5	5	40	0	5	5	10	5	0	10	5	0	5	5	5	10
III 187	0	0	5	0	5	0	40	5	5	0	10	0	0	5	5	5	0	5	5
53	5	10	0	5	0	5	5	80	10	10	5	5	10	5	10	10	0	0	5
VA4	5	10	0	10	5	5	5	5	40	5	10	20	10	20	10	10	20	10	5
73	0	20	0	5	5	10	10	5	10	20	20	10	10	5	10	10	5	5	10
6	10	20	10	10	10	20	10	10	5	20	160	10	10	20	20	10	10	10	20
77	5	5	5	0	0	0	0	5	10	5	20	80	20	5	0	5	10	0	10
71	5	10	0	0	5	5	5	5	5	10	10	10	40	10	5	5	5	0	5
47	10	5	5	5	0	0	5	10	10	5	10	10	10	40	20	10	5	0	10
54	5	5	10	5	5	0	0	10	5	10	10	5	10	10	40	5	0	0	5
75	5	0	5	5	5	5	0	5	5	5	5	10	10	5	10	40	0	0	5
7	0	5	0	5	5	5	5	0	5	10	10	20	10	0	10	0	40	10	0
IV 42D	10	10	5	10	5	10	5	5	5	20	20	5	0	0	5	10	5	80	10
Misc 81	5	5	10	20	10	5	5	10	5	10	40	10	5	5	5	20	10	5	80

*Figures represent reciprocals of the highest dilution which just neutralized a constant predetermined amount of each coagulase. The predetermined amount is that which just causes a 4+ clot with appropriate plasma.

Because of the obvious complexity of the coagulase material isolated by the Tager (1948) method and subsequent difficulties in interpretation when precipitin reactions were conducted, new techniques were needed to separate coagulase from inert protein. Using only S. aureus 70, coagulase fractions were obtained by acid and alcohol precipitation under controlled conditions, and Sephadex. Again, rabbits were used for anticoagulase production; however, this time the material was injected via the foot pad route with Freund adjuvant. In contrast to anticoagulase titers being produced in 6-8 weeks with multiple subcutaneous inoculations, high titers were obtained in 2 weeks with peak titers occurring at 4 weeks. In this case, the agar diffusion method was used as one of several criteria for the determination of protein homogeneity. By injection of coagulase fractions Cg-I-P (relatively crude) and Cg-III-S (relatively pure) into rabbits and testing the antisera against each of the increasingly pure antigenic fractions, it was hoped that a progressively decreasing number of precipitin zones would occur and that our extremely purified material (Cg-IV-C) would show only one precipitin band. Furthermore, the reaction in the gel

diffusion system between antiserum prepared against crude material and the antigen composed of extremely purified coagulase posed a rather severe test of homogeneity. A series of precipitin patterns were derived to include extreme variation in reactant concentrations. Results (FIG. 5) showed only one precipitin band between our extremely purified antigen (Cg-IV-C) and the antiserum against a relatively pure fraction (Cg-III-S). The other three fractions (Cg-I-P, Cg-II-P, Cg-III-S) exhibited multiple zones of precipitation against this same antiserum. When Cg-IV-C was tested against anti-Cg-I-P rabbit serum, the appearance of a second weak component indicated the presence of only a trace impurity.

Results of assays

Phosphatase. The presence of phosphatase activity in purified preparations of coagulase has recently been a matter of debate. Inniss and San Clemente (1961, 1962) suggested that coagulase and phosphatase activities represented two different functional groups associated with the same protein entity. By use of starch block electrophoresis and anion exchange chromatography, they were unable to

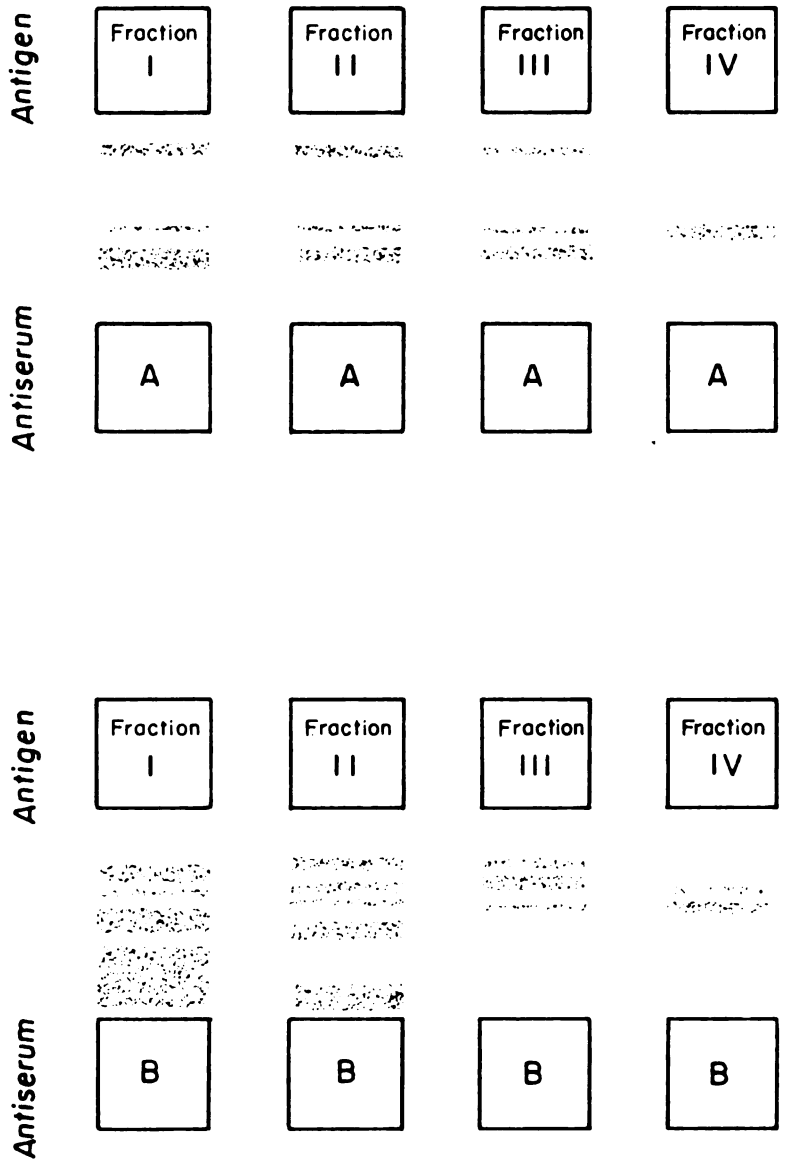

FIG. 5. Precipitation bands of the gel diffusion technique indicating the progressive purity of each of the four fractions of coagulase. Wells A (top) contain antisera prepared in rabbits against the third fraction Cg-III-S. Wells B (bottom) contain antisera prepared in rabbits against the first fraction of coagulase, Cg-I-P.

separate these two activities. In our studies, similar results were obtained when fraction Cg-I-P was applied to starch block electrophoresis. Later, Drummond and Tager (1961) indicated that acid phosphatase is an enzyme separate and distinct from the coagulating principal and demonstrated separability by results obtained on cellulose column zone electrophoresis. Their data showed that although a distinct decrease in activity of either of the components was affected, no complete separation was obtained.

In our studies, a hint that coagulase and phosphatase may be separable appeared in the results of the clot technique. However, the complete separation of these two entities was not realized until we resorted to gel filtration. In fact, two methods have been established in our laboratories for this purpose and are described as follows:

1. Gel filtration. When S. aureus 70 was grown in brain heart infusion and the resulting cell-free supernatant fluid adjusted to pH 3.8 with 4 N HCl, the resulting precipitate was redissolved in 0.1 N sodium acetate and passed through a Sephadex G-200 column. Since Inniss and San Clemente (1961, 1962) found that optimal phosphatase

activity occurred at pH 7.2 while a pH of 5.6 was used by Drummond and Tager (1961), we, therefore, used both buffer systems in our phosphatase assay procedures. Distinct and separate coagulase and phosphatase peaks were obtained (FIG. 6). The different phosphatase titers at both pH values suggested a family of phosphatases varying with staphylococcal strain.

2. Multiple dialysis under controlled conditions. TABLE 12 represents phosphatase activity of each of the successive fractions of purified coagulase. Again, phosphatase activity was assayed at pH 5.6 and 7.2 Results indicate that phosphatase was coprecipitated with coagulase in the first two fractions (Cg-I-P and Cg-II-P). In the third step, maximal coagulase activity remained in solution (Cg-III-S) while the majority of phosphatase was removed with the precipitate. Subsequent passage of fraction Cg-III-S through Sephadex G-200 completely eliminated any remaining traces of phosphatase activity.

Nitrogen and phosphorus. We determined the nitrogen and phosphorus content in each of the fractions (TABLE 13). Because of the limited amount of sample, both elements were calculated on the basis of weight of protein instead of the

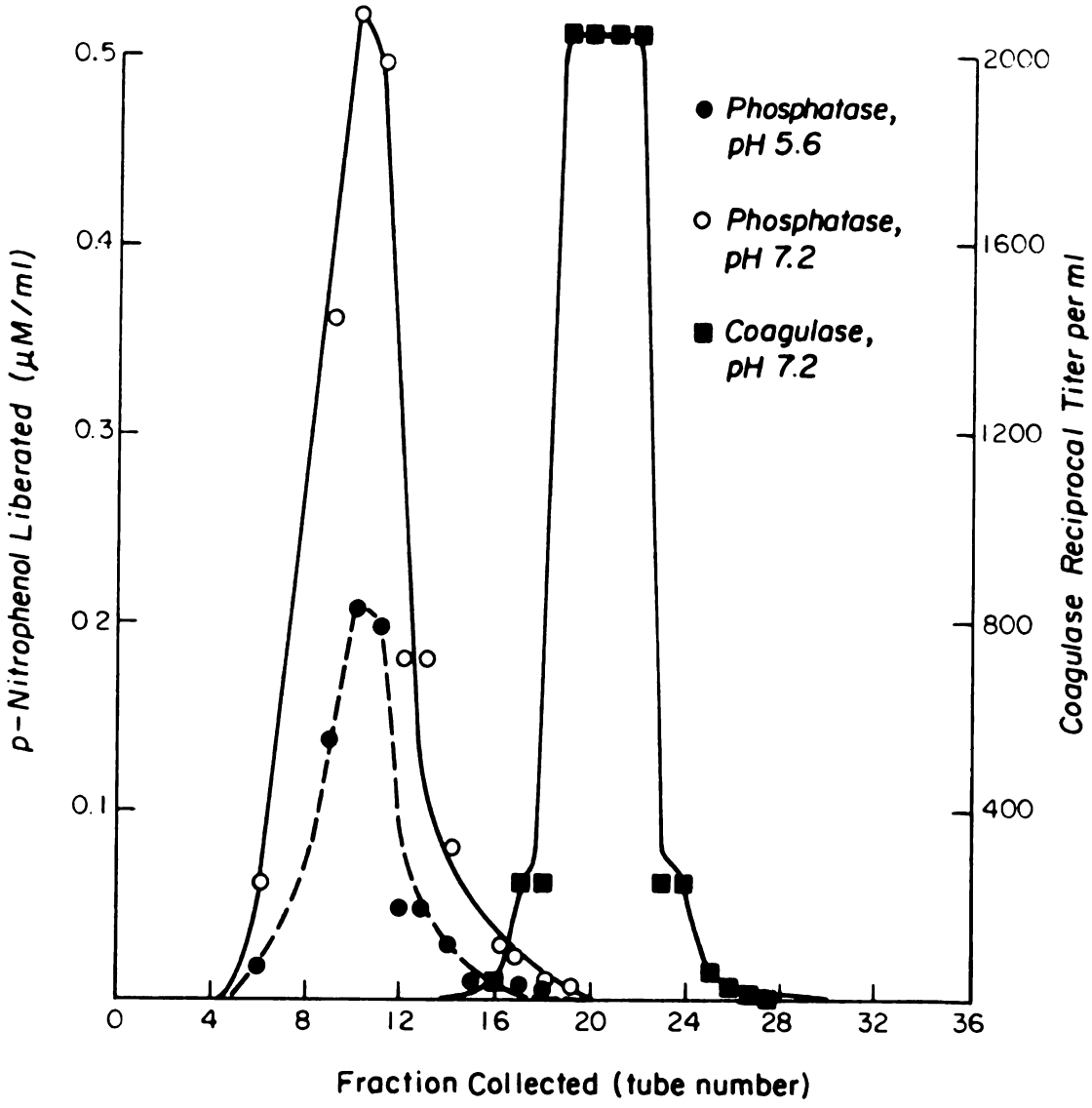

FIG. 6. Separation of phosphatase from coagulase by molecular sieving the first fraction (20 mg protein per ml of sample) of coagulase (Cg-I-P) through a column of Sephadex G-200. The eluant was 0.1 N sodium acetate (pH 7.2). Phosphatase activity was measured at both pH 5.6 and 7.2.

TABLE 12. Amounts of residual phosphatase and its eventual elimination during the purification of coagulase as measured in the precipitates and corresponding supernatant fluids of successive fractions obtained in three cycles of ethanol-water mixtures under controlled conditions followed by passage through Sephadex G-200. Proof of separability required measurement of phosphatase at two pH values

Purification Step	Fraction	Phosphatase Activity[a]		Coagulase Activity[b]
		pH 5.6	pH 7.2	
I	Cg-I-P	0.05	0.13	2,600
	Cg-I-S	0.02	0.02	0
II	Cg-II-P	1.31	3.92	92,000
	Cg-II-S	0.84	2.60	1,960
III	Cg-III-P	1.31	3.94	1,200
	Cg-III-S	0.02	0.06	220,000
IV	Cg-IV-C	0.00	0.00	220,000

[a] μM p-nitrophenol liberated per mg protein.

[b] Coagulase reciprocal titer per mg protein.

usual dry weight basis. Micro-Kjeldahl analysis showed gradual increase in nitrogen content from 13 to a maximum of 15% which corresponded with increasing purity of coagulase. A phosphorus-protein relationship was difficult to interpret after the second precipitate because a phosphate buffer was used in the next fractionation.

Results of miscellaneous tests

Estimation of carbohydrate. Determination for the presence of carbohydrate was made with the Molisch test. It was found that carbohydrate was not precipitated in the very first fraction, Cg-I-P (TABLE 14).

Estimation of lipase. This enzyme is widely distributed among the staphylococci. Little information is available concerning the properties of staphylococcal lipase. Preliminary investigations indicated that this enzyme existed in the cell-free culture filtrate of S. aureus 70. Lipase was found in fraction Cg-I-P (precipitate) and Cg-I-S (supernatant fluid). During the second step of the fractionation process all of the activity was found in the supernatant fluid (Cg-II-S) while the precipitate (Cg-II-P) was free of

this material. The absence of lipase was confirmed in all subsequent fractions derived from Cg-II-P (TABLE 14).

Estimation of deoxyribonuclease. Estimation of this enzyme showed that it persisted in both the precipitates and supernatant fluids of the first three fractions (TABLE 14). It was not until the separating power of Sephadex G-200 was used that deoxyribonuclease was definitely separated from coagulase.

Effect of pH. It can be observed (FIG. 7) that a pH range of 6.8 to 7.2 showed optimal coagulase activity for fraction Cg-IV-C. A relatively sharp decline in activity was noted on both sides of this range. All other fractions were tested in the same manner and optimal coagulase activity occurred in the same pH range regardless of the degree of purity.

Effect of inhibitors. An endopeptidase, trypsin, was found by Tager (1948) to inhibit coagulase activity. In addition, Elek (1959) reported that ethylenediaminetetra-acetate (EDTA) and sodium fluoride had no effect on the clotting capacity of coagulase. To substantiate these

results, we tested these same three compounds against each of the successively purer coagulase fractions. It was noted (TABLE 15) that trypsin caused about a 98.5% loss of activity to each fraction while sodium fluoride and EDTA exerted no detrimental effects under those conditions.

Temperature stability. The effect of three temperatures on coagulase activity of all of the fractions was studied (FIG. 8). At all temperatures tested, the degree of destruction was greatest for the purest fractions. Within each temperature range, a decrease in coagulase activity corresponded with an increase in purity.

TABLE 13. Phosphorus and nitrogen determination of each successive fraction of coagulase obtained from three cycles of ethanol-water mixtures under controlled conditions followed by molecular sieving through Sephadex G-200

	Per Cent on Protein Basis	
Fraction	Phosphorus	Nitrogen
Cg-I-P	0.5	13.0
Cg-II-P	1.5	12.9
Cg-III-S	-*	14.0
Cg-IV-C	-	15.0

*The use of phosphate buffer in the third fraction precluded P determination.

TABLE 14. The progressive elimination of carbohydrate, lipase, deoxyribonuclease and phosphatase as measured in the precipitates and corresponding supernatant fluids of successive fractions obtained from three cycles of ethanol-water mixtures under controlled conditions followed by molecular sieving through Sephadex G-200

Fraction	Qualitative Analysis			
	Carbohydrate	Lipase	DNA-ase	Phosphatase
Cg-I-P	-	+	+	+
Cg-I-S	+	+	+	+
Cg-II-P	-	-	+	+
Cg-II-S	-	+	+	+
Cg-III-P	-	-	+	+
Cg-III-S	-	-	+	+
Cg-IV-C	-	-	-	-

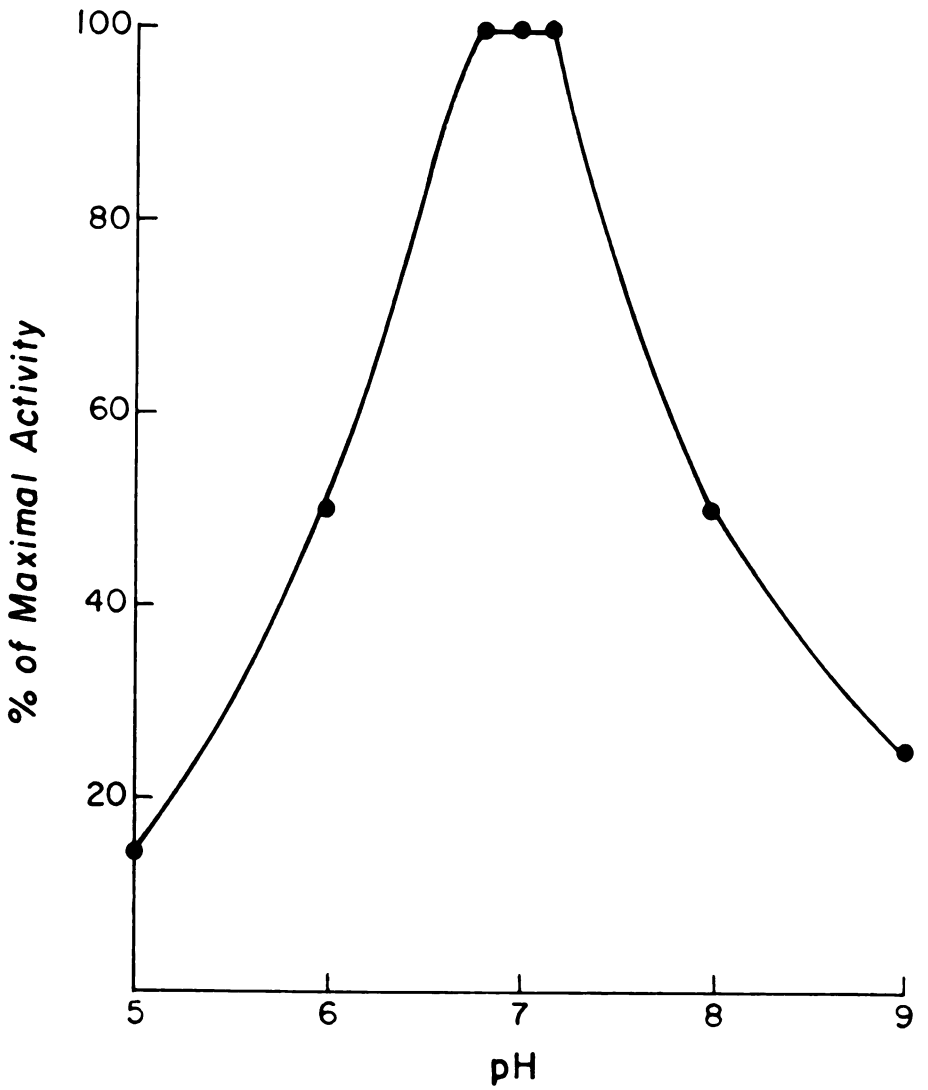

FIG. 7. Effect of pH on the clotting activity of extremely purified coagulase fraction Cg-IV-C.

TABLE 15. Effect of trypsin, sodium fluoride and EDTA on the activity of successive fractions of increasingly purified coagulase

Fraction	Per Cent Residual Coagulase Activity		
	Trypsin[a]	Fluoride[b]	EDTA[c]
Cg-I-P	1.5	100	100
Cg-II-P	1.0	100	100
Cg-III-S	1.5	100	100
Cg-IV-C	1.5	100	100

[a]Final concentration (40 mcg/ml).

[b]Final concentration (0.025 M).

[c]Final concentration (0.025 M).

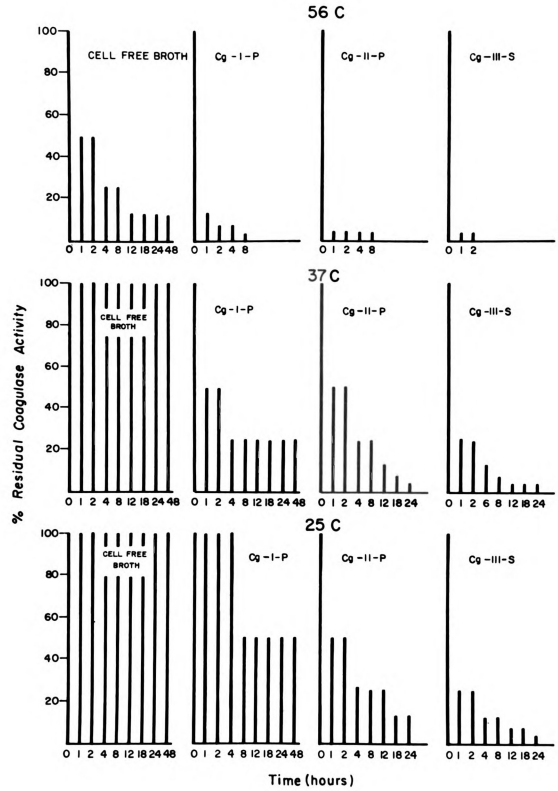

FIG. 8. Effect of temperature at various intervals upon the stability of successive fractions of purified coagulase.

DISCUSSION

The initial objective of these studies was to determine whether coagulases isolated from each of the phage propagating strains of the International-Blair series of S. aureus were antigenically distinct or related. To evaluate this phenomenon, purified preparations made according to modifications of methods of Tager (1948) were used in a series of studies. When this material was injected into rabbits to elicit coagulase antibody, the highest anticoagulase activities occurred in homologous antigen-antisera systems. Although these results suggested possible strain antigenic specificity of coagulase, there was a low degree of cross-inhibition between strains. Differentiation of antigenic specificity using gel diffusion patterns proved difficult to interpret because of the appearance of multiple precipitin bands. We needed a pure antigenic entity of coagulase. If coagulase could be purified to the degree whereby preferably one or even two immunological components were observed, then interpretation of antigenic specificity data would be facilitated.

Therefore, the forgeoing inconclusive results instigated a search for procedures to purify coagulase to a high

degree. Several approaches were investigated using only coagulase from S. aureus 70. The first attempt consisted of a "clot technique" whereby fluid entrapped within the fibrin network of a coagulase-plasma system was recovered and tested for clotting ability. An approximate 300-fold increase in activity per mg protein over the original activity in the cell-free filtrate was noted in this system. Although this procedure appeared somewhat unsophisticated, the purification factor was almost equivalent to that obtained by Blobel et al. (1960) who used starch block electrophoresis. The manner in which coagulase is retained within the fibrin network remains unknown. Our results indicate that after the clot has occurred, most of the coagulase is present in the fibrin-entrapped fluid. Further development of this procedure was discontinued because we anticipated inadequate yields.

Another method of purification employed gel filtration alone. This method permitted a separation of proteins by differences in molecular weight. For these procedures we used an insoluble substance made of cross-linked polysaccharide (dextran) known as Sephadex. When this material was hydrated and placed within a chromatographic column,

each gel grain acted as a tiny dialyzing bag. With these preliminary trials, a purification factor was obtained which was similar to the preceding method. Extensive development of this technique was also discouraged since Sephadex G-200 was not readily available commercially.

We finally settled on a procedure using acid and alcohol precipitation under controlled conditions, as well as Sephadex. By manipulation of five variables (pH, ionic strength, temperature, protein and alcohol concentration), we removed most of the undesirable components associated with coagulase. An additional fractionation using gel filtration separated this clotting material to an extremely high degree. Final preparations showed an approximate 3700-fold increase in activity per mg protein. Previous methods by Blobel et al. (1960) showed a final purification factor of only 387.5 while Tager (1948) obtained products which were approximately 300 times purer than the original material. It was therefore concluded that our method gave an additional 10-fold increase in purity over that achieved by previous investigators.

Since material of high specific activity was now available, we conducted immuno-diffusion studies to determine

serological distinction. It was most desirable to obtain only one or two precipitin bands in the coagulase-anticoagulase system. Kabat and Mayer (1961) stated that gel diffusion methods are among the most powerful tools which the immunochemist has available for establishing the presence of mixtures of antigens and antibodies; however, these procedures also possess certain limitations. For instance, the quantity of antibody may be insufficient to produce discernible precipitin bands. Also, the presence of non-precipitating antibody may be responsible for similar results. On the other hand, the presence of two bands does not always indicate a mixture of antigenic or antibody components since an excess of antigen may diffuse beyond the original zone of specific precipitate to form a broad or second band. Keeping these pitfalls in mind, we devised a system using extensive variations in reactant concentrations. To increase the critical aspects of this immunological test, we prepared antisera against crude as well as purified coagulase preparations. When a relatively pure fraction (Cg-III-S) was injected into rabbits and the antiserum tested against each of the successively purified fractions of coagulase in a gel diffusion system, a single

zone of precipitation appeared with the extremely purified fraction (Cg-IV-C). When a relatively crude coagulase preparation (Cg-I-P) containing substantial amounts of contaminating protein was injected into rabbits and the antisera tested against each of the fractions in a similar gel diffusion system, there appeared a second but weak zone of precipitation against our extremely purified preparation (Cg-IV-C). It was concluded from these immuno-diffusion studies that this material was suitable for future studies on antigenic specificity.

Besides immuno-diffusion, other studies were made to determine the homogeneity of this enzyme-like material. Electrophoretic analysis by Tager (1948) revealed that his preparations were heterogeneous. When our final fraction (Cg-IV-C) was analyzed by paper electrophoresis using cellulose acetate strips, we obtained a single peak.

Another test of homogeneity was obtained chromatographically. The third fraction (Cg-III-S) was passed through a column of Sephadex G-200. Concomitant total protein and coagulase peaks were observed.

As a further criterion for the homogeneity of our coagulase preparation, we assayed for specific enzymes associated

with coagulase and known to be elaborated by S. aureus. Although lipase, deoxyribonuclease and phosphatase were found in initial fractions, they were not present in the final product. Carbohydrate was removed during the first fractionation.

The controversy concerning the separability of phosphatase and coagulase (Inniss and San Clemente, 1961, 1962; Drummond and Tager, 1961) had been unsettled up to this time. In our system using S. aureus 70 we found a pH of 7.2 to be optimal for phosphatase activity. Drummond and Tager (1961) used the methods of Barnes and Morris (1957) in which pH 5.6 was recommended. By using both pH 5.6 and 7.2, we obtained consistently higher activity at the latter pH. The apparent diverse conclusions of these two groups of workers may be reconciled by realizing that Drummond and Tager were justified in assuming separability from the relatively low values of phosphatase which they obtained at pH 5.6. On the other hand, Inniss and San Clemente justified their conclusion of inseparability by the relatively high value of accompanying phosphatase measured at pH 7.2. However, final proof of separability required a different technique which was supplied by molecular sieving through Sephadex G-200.

Several other miscellaneous tests were conducted to determine their effect on the various fractions. Thermal stability studies indicated that an increase in purity was associated with a decrease in heat stability. When the effect of pH was tested, optimal coagulase activity occurred in a range of pH 6.8 - 7.2 for all fractions regardless of purity. EDTA and fluoride exerted no deleterious effect on the clotting capacity of each fraction while trypsin almost destroyed it completely.

In conclusion, a brief summary of the projected areas of research that are suggested from the previous studies are as follows: (a) a detailed study of the antigenic specificity using extremely purified coagulase preparations; (b) isolation and purification of phosphatase and lipase using modifications of the procedures to purify coagulase to a high degree; (c) studies on the mode of action of coagulase; and (d) the incorporation of this highly purified clotting material in a vaccine system against S. aureus.

SUMMARY

Although preliminary attempts to determine antigenic specificity of coagulase were inconclusive, there was a suggestion of strain specificity. More critical evaluation of this phenomenon using agar diffusion techniques necessitated highly purified coagulase preparations. We, therefore, affected the isolation and purification of coagulase prepared from S. aureus 70 by modifications of the Cohn et al. (1946) and Pillemer et al. (1948) systems to fractionate proteins in ethanol-water mixtures of controlled pH, ionic strength, temperature and protein concentration. Additionally, final purification was accomplished by gel filtration (Sephadex). An extremely purified preparation, about 3700-fold, was obtained by three cycles of dialysis and one using Sephadex G-200. This factor represented an approximate 10-fold increase in purity over that found by previous investigators. Containing 15% nitrogen, the isolated coagulase gave one peak with cellulose acetate paper electrophoresis and one band on the agar diffusion test; it was easily inactivated by trypsin; it gave a negative Molisch test; and it was free of detectable lipase, phosphatase, and deoxyribonuclease activities. Thermal

inactivation studies showed that an increase in purity corresponded with a decrease in heat stability. In addition, a pH range of 6.8 - 7.2 was found to be optimal for coagulase activity regardless of the degree of purity.

The debate over the separability of phosphatase and coagulase activities appears to be finally settled. A distinct separation of these two components was accomplished using Sephadex G-200.

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