BIOCHEMICAL STUDIES ON STAPHYLOCOAGULASE WITH EMPHASIS UPON AN ASSOCIATED PHOSPHATASE ACTIVITY

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
William E. Inniss
1961

This is to certify that the

thesis entitled

Biochemical Studies on Staphylocoagulase with
Emphasis upon an Associated Phosphatase Activity

presented by

William E. Inniss

has been accepted towards fulfillment
of the requirements for

Ph.D. degree in Department of
Microbiology &
Public Health

C. L. San Clemente
Major professor

Date 20 July 1961

O-169

BIOCHEMIC
EMPHASIS UP

It has b

exists between

staphylococci.

to determine

A known

aureus, phage

heart infusio

preparation w

activity as

using para-n

In ord

of studies w

anion excha

paper elect

of enzyme s

activities

(1) whethe

accounted

active gro

(3) or wh

possessed

The

ABSTRACT

BIOCHEMICAL STUDIES ON STAPHYLOCOAGULASE WITH EMPHASIS UPON AN ASSOCIATED PHOSPHATASE ACTIVITY

by William E. Inniss

It has been previously suggested that a correlation exists between the coagulase and phosphatase activity of staphylococci. The present investigation was carried out to determine if this relationship was a functional one.

A known high coagulase producer, Staphylococcus aureus, phage propagating strain 70, was grown in brain heart infusion and the coagulase purified. The coagulase preparation was found to possess a concomitant phosphatase activity as measured spectrophotometrically at 400 mμ using para-nitrophenylphosphate as the substrate.

In order to clarify this dual phenomenon, a series of studies were conducted such as: thermal inactivation, anion exchange chromatography, starch, starch gel, and paper electrophoresis, effect of inhibitors, saturation of enzyme sites, and parallel measurement of specific activities. These approaches were intended to determine (1) whether the same active group on a common protein accounted for both activities; (2) whether different active groups were associated on the same protein entity; (3) or whether two proteins, intimately associated, each possessed a different active group.

Thermal inactivation experimentation, carried out on

the purified co
decrease in bot
subjected to a
gradient eluti
0.25 M NaCl.
of the two act
were associate
when subjecte
phoresis unde
4.2 to 10), e
after migrat
may have bee
conducted w
It was not
complete a
of overlap

Inhi
fluoride,
different
each of
activiti
supposi
which t
an exc
(100-f
coagu

the purified coagulase preparation showed a parallel decrease in both activities. The preparation was subjected to anion exchange chromatography employing gradient elution of DEAE-cellulose at pH 7.5 and 8.6 with 1.25 M NaCl. The occurence of coincident maximum peaks of the two activities suggested that both functional groups were associated on the same protein. The two activities, when subjected to starch, starch gel, and paper electrophoresis under various conditions of buffers and pH (pH 4.2 to 10), exhibited similar maximum peaks of activity after migration. An extremely small degree of separation may have been obtained when electrophoresis was conducted with a discontinuous buffer system. However, it was not possible to recover either activity in the complete absence of the other because of the large amount of overlapping of the two activities.

Inhibitory compounds such as EDTA, iodoacetate, fluoride, azide and para-chloromercuribenzoate exerted different rather than similar degrees of inhibition on each of the two activities indicating that the two activities do not operate by the same mechanism. This supposition was verified by subsequent experiments in which the phosphatase was completely saturated by an excess of para-nitrophenylphosphate substrate (100-fold K_s value). Under these conditions the coagulase activity remained intact.

William E. Inniss

Parallel increases were found in the total activity (on the basis of protein) for both the coagulase and phosphatase after each purification step. It is therefore hypothesized that the coagulase and phosphatase activities represent two different functional groups associated with the same or an extremely similar protein entity.

BIOCHEMICAL STUDIES ON STAPHYLOCOAGULASE WITH EMPHASIS UPON AN ASSOCIATED PHOSPHATASE ACTIVITY

By

William E.dgar Inniss

A THESIS

Submitted to
Michigan State University
in partial fulfillment of the requirements
for the degree of

DOCTOR OF PHILOSOPHY

Department of Microbiology and Public Health

1961

The auth

to Dr. C. L.

and Public Hea

advice.

The nume

by Dr. R. K.

Department of

. L. Fairle

greatly appr

[illegible]277
5/24/62

ACKNOWLEDGEMENTS

The author wishes to express his sincere appreciation to Dr. C. L. San Clemente of the Department of Microbiology and Public Health for his interest, guidance and technical advice.

The numerous helpful suggestions generously given by Dr. R. N. Costilow and Dr. H. L. Sadoff of the Department of Microbiology and Public Health and Dr. J. L. Fairley of the Department of Biochemistry were also greatly appreciated.

TABLE OF CONTENTS

[illegible]

1. Effec o
   coagu as
   the p ri

2. Compa ris
   coag las

LIST OF TABLES

GURE

LIST OF FIGURES

The conti
problems cause
a large amount
research to be
studies have
relatively un
organisms bec
between certa
being coagula
such substanc
arginase hav
capacity of
role which
a staphyloc

Coag
in the vir
accepted a
of potenti
which sta
determina
substance
could cl
involvem
aspect
previous
other a

INTRODUCTION

The continuing emergence of complex therapeutic problems caused by Staphylococcus aureus has stimulated a large amount of both clinical and physiological research to be conducted on this bacterium. Various studies have indicated that the staphylococci occupy a relatively unique position among pathogenic microorganisms because of the supposed correlation existing between certain cellular substances, the most prominent being coagulase, and virulence. In addition to coagulase, such substances as phosphatase, hyaluronidase, and arginase have been associated with the pathogenic capacity of staphylococcal strains. However, the exact role which these activities play in the establishment of a staphylococcal infection has yet to be elucidated.

Coagulase has been assumed to be largely involved in the virulence of staphylococci and is generally accepted as the best single criterion for determination of potential pathogenicity. However, the mechanism by which staphylocoagulase functions is not known. The determination of the biochemical activities of this substance as well as its physical properties potentially could clarify its mode of action and even, perhaps, its involvement in staphylococcal disease. An additional aspect would be to establish whether the correlations, previously indicated to exist between coagulase and other activities, were of a functional nature. To

accomplish this end, the present investigation was undertaken.

REVIEW OF LITERATURE

Staphylococci and Coagulase

One of the earliest descriptions of the ability of *Staphylococcus aureus* to produce coagulase was reported by Loeb (1903) who demonstrated the *in vitro* clotting of goose plasma. Using citrated human plasma Much (1908) found that pathogenic staphylococci exhibited the coagulation phenomenon. Gonzenbach and Uemura (1916) observed the coagulation reaction in oxalated rabbit, goat and sheep plasma, as well as human plasma, with the rabbit plasma being the most coagulable. Later in 1926, Darányi, observing staphylococci from human and animal sources, recognized a correlation between the pathogenicity of these microorganisms and their ability to clot plasma. He advocated that the clotting of citrated rabbit plasma was the best criterion in the separation of pathogenic from nonpathogenic staphylococci. Also finding that citrated plasma was the most suitable for determination of coagulase activity, Gross (1931) similarly concluded that coagulase production was indeed characteristic of pathogenic staphylococci. He also found that the potency of culture filtrates of virulent staphylococci paralleled the activity of the unfiltered cultures. Later authors such as Walston (1935), Fisher (1936) and Cruikshank (1937) confirmed the coagulase activity of culture filtrates.

The production of coagulase and hemolysin by *S.*

aureus as criteria for virulence was examined by Chapman and his coworkers (1934). They found that out of 690 strains, 88% of these strains coagulated oxalated plasma whereas only 51.7% were hemolytic. They also showed that only 11.9% of 1,852 strains of *Staphylococcus albus* were coagulase-positive. The relatively lesser power of the *S. albus* group to coagulate plasma as compared to *S. aureus* was also reported by other workers (Burnet, 1930; Fisher, 1936). Finding that nonhemolytic pathogenic strains of *S. aureus* coagulated plasma and that, regardless of pigment formation, strains which possessed coagulase were usually pathogenic, caused Chapman *et al*. (1934) to conclude that coagulase positivity is a valuable asset in conjunction with hemolysin production in the determination of a staphylococcal pathogen. Fisher (1936) also showed that coagulase-positive strains were nearly always hemolytic whereas coagulase-negative strains were most often nonhemolytic.

Stephan (1935) reported that out of 45 staphylococcal isolates from pus, 37 (82%) were coagulase positive, whereas this was true only in 46% of the 45 isolates from blood, urine and sputum. On the basis of his investigations and on the work of other authors, Cruikshank (1937) suggested that the coagulating ability of *S. aureus* represented the most convenient, reliable *in vitro* method for the determination of the pathogenicity of a given staphylococcal strain whether

it be of hum
such as Chap
Devenish and
Keogh (1940)
have confirm

Howeve
Christie, N
toxin produ
agreed more
did coagula

Howe
staphyloc
accepted
as Blair
reactions
sometime
are suff
Conseque
the use
in vitr

V
positi
method
fundam
animal
supern
Coagu

it be of human or animal origin. Numerous authors such as Chapman *et al*. (1938), Gillespie *et al*. (1939), Devenish and Miles (1939), Blair (1939), Christie and Keogh (1940), Fairbrother (1940) and Smith *et al*. (1947) have confirmed and supported this criterion.

However, there have been a few dissenting reports. Christie, North, and Parkin (1946) suggested that alpha toxin production by pathogenic strains of *S. aureus* agreed more closely with mouse pathogenicity tests than did coagulase production.

However, at the present time, the ability of staphylococci to produce coagulase is generally accepted for the recognition of pathogenic strains and as Blair (1939) stated even though various *in vitro* reactions such as hemolysis and pigment formation sometimes parallel the coagulase reaction, the results are sufficiently varied to make them inadequate. Consequently Blair (1939, 1958) completely endorsed the use of the coagulase test as a sufficient single *in vitro* index of the pathogenicity of staphylococci.

Various methods for the detection of coagulase-positive staphylococci have been proposed. The tube method approach, introduced by Loeb (1903), is fundamentally the inoculation of susceptible human or animal plasma with staphylococcal cells or their supernatant fluids followed by incubation at 37 C. Coagulation of the plasma indicates the presence of

coagulase. The slide method, originated by Much (1908) and also described by Cadness-Graves and his coworkers (1943), consists of the mixing of a staphylococcal suspension with plasma on a glass slide, resulting in nearly immediate clumping of the microorganisms. Because of its ease and rapidity, Williams and Harper (1946) found this method to be advantageous for screening purposes. A plate method (Penfold, 1944; Reid and Jackson, 1945) consisting of the cultivation of the organisms on a solid nutrient medium containing plasma has never become popular (Elek, 1959). Even though this method possesses the advantage of allowing simultaneous cultivation and coagulase determination, it has been found that some coagulase-positive strains fail to produce turbidity while some coagulase-negative strains do. Also, several products of staphylococci have been found to produce opacity on agar containing human plasma, one of them according to Christie and North (1941) and Fulton (1943) is beta toxin. Woods and Parkin (1946) comparing the tube test with the plate method, also found the latter to be inaccurate.

Gillespie and Alder (1952) and Alder _et_ _al_. (1953) reported a modified procedure using egg-yolk medium for the measurement of coagulase positivity. They found that the majority of coagulase-positive _S_. _aureus_ strains isolated from human hosts produced opacity on such a medium.

magni

effec

plasm

and

sugg

the

fou

th

st

Fisk (1940) and Chapman _et_ _al_. (1941) subjected the coagulase tube test to careful scrutiny and evaluated the effects of concentration, source and age of citrated plasma, the number of organisms used, the culture medium, and the temperature of incubation. Both authors suggested that the plasma be diluted with normal saline, the former advocating a 10-fold dilution while the latter found a 4-fold dilution was best. Chapman also showed that plasma, dried _in_ _vacuo_ on blotting paper, has a storage life of many months and may easily be redissolved when required, thus being extremely useful in a laboratory where only a few tests are made over a relatively long period of time. Harper and Conway (1948) have indicated various sources of error which might contribute to an erroneous coagulase determination and pointed out the need for adequately controlled conditions.

The demonstration by Cadness-Graves _et_ _al_. (1943) that a strong correlation existed between the coagulase tube test and the slide method led to the common assumption that both tests were a measurement of the same staphylococcal product, namely coagulase. However, the finding by Linsell and Gorrill (1951) that the subjection of human plasma to 56 C inactivates the tube coagulase activity while not imparing that of the slide test drew attention to the possibility that the two tests were not based on the same mechanism. Duthie

(1954) presented evidence disclosing that in contrast with free coagulase measured by the tube test, the majority of staphylococci also possess the property of adsorbing the fibrinogen of certain species on to the surface of the bacterial cell, thus causing a flocculation or clumping as evidenced in the slide test. This factor was termed bound coagulase or clumping factor and was stated to be independent of free coagulase (Duthie, 1954, 1955). The clumping factor reacted with fibrinogen plus an activator. Jacherts (1956) challenged the concept of the existence of two different coagulases. However, Duthie and Haughton (1958) showed that Jacherts' conclusions were based on misinterpretation of results and that the latter author had not successfully liberated the original bound coagulase as described by Duthie (1955).

Elek (1959) suggested that from the practical point of view the differences between the tube and slide tests for coagulase are of consequence only in a quantitative sense, rather than a qualitative one.

Mode of Action of Staphylocoagulase in the Coagulation of Plasma

Much (1908) was one of the first to describe the coagulation phenomenon in some detail. He found that strains of S. aureus, besides coagulating plasma, also could coagulate certain exudates containing fibrinogen, although at a slower rate. Purified fibrinogen, however, was not clotted by staphylococci but was readily clotted by the addition of thrombin. Any suggestion, however, of a thrombin-like activity was eliminated by the inactivity of antithrombin compounds such as hirudin and heparin (Walston, 1935; Fisher, 1936).

Gratia (1920) reported that staphylococci possessed the ability to clot fibrinogen, chemically isolated from plasma, upon the addition of a solution from which thrombin and fibrinogen had been extracted.

Other authors such as Walston (1935), Fisher (1936) and Cadness-Graves et al. (1943) have found that purified fibrinogen can be coagulated by staphylococcal coagulase although sometimes more slowly and incompletely. However, in all the above investigations, the possibility exists that another substance, or substances, could readily have been added during the procedures, particularly since in two cases, the fibrinogen was obtained by the simple precipitation of plasma with a saturated solution of NaCl.

Evidence reported by Smith and Hale (1944) tended to resolve the conflicting ideas proposed by previous workers. They successfully demonstrated that in order for staphylocoagulase to react with fibrinogen purified by repeated precipitation, a secondary factor is required for activation. This factor, which they termed "activator", was reported to be present in human and rabbit plasma but not in mouse, fowl or guinea-pig plasma.

The latter plasma was able to be coagulated at 20 C instead of 37 C. However, the addition of a human or rabbit testicular extract to any of the normally inactive plasmas rendered them completely active to coagulase. These authors explained that in those cases where human plasma was noncoagulable by coagulase, a deficiency in activator was evidenced.

Gerheim et al. (1947) and Kaplan and Spink (1948) confirmed the necessity of the activator for the clotting reaction with coagulase to occur, although some dispute existed as to the nature of this activator for it was confusingly similar to prothrombin. Tager (1948a) attempted to localize the activator material in human plasma which he termed the "coagulase-reacting factor" (C.R.F.). He reported maximal recovery of C.R.F. in the globulin fractions obtained by saturation with 33% and 50% ammonium sulfate. The same result was obtained by precipitation of plasma with acid at pH 5.3. It was also demonstrated by means of ethanol fractionation that C.R.F. is not fibrinogen or albumin. Tager also reported that neither free lipoids nor trace ions had any significant effect on the clotting mechanism. Kaplan and Spink (1948) reported that the accessory factor or C.R.F. occurred in alcoholic fractions and was largely associated with alpha and beta globulins. On the other hand, Gerheim et al. (1947) reported that the C.R.F. was contained in certain plasma fractions especially those with albumin present.

In searching for the identity of the C.R.F. and its relationship to prothrombin, Tager summarized the various prevalent ideas as to the C.R.F.'s role in the clotting mechanism (Tager 1954, 1956a). Later the same author (Tager, 1956b) reported that the C.R.F. of human plasma had been successfully concentrated and purified, as well as having been largely freed of prothrombin by Seitz filtration, absorption on hyflo-amphogel columns, controlled elution with phosphate buffer, and ammonium sulfate fractionation. When the purified C.R.F. was compared to a highly purified prothrombin preparation of Seegers (Seegers and Alkjaersig, 1953) by electrophoresis and ultracentrifugation, significant differences were observed. Both substances caused the coagulation of plasma. Consequently, Tager hypothesized that C.R.F. activity resides in some group or groups of the prothrombin molecule not involved in prothrombin function and devoid of prothrombin activity. Thus, the complete prothrombin entity concomitantly possessed both C.R.F. and prothrombin activity but only certain components of the complete unit were required for C.R.F. activity. As indicated by Tager, this hypothesis provided a suitable explanation for the sometime parallelism of C.R.F. amd prothrombin activities as observed on intramuscular administration of dicumarol and phenylindanediol (Tager, 1953) or the sometime complete divergence of the two activities that occurred following Seitz filtration.

More recently, Murray and Gohdes (1959a) have reported that purified coagulase reacting with two types of purified fibrinogen caused the formation of fibrin strands as viewed via electron microscopy. This reaction, however, required 280 minutes whereas the supplementation of plasma produced fibrin formation in only 2 minutes. Consequently, these authors suggested that the C.R.F. of plasma acts as an enzyme activator and thus is not necessary for the coagulation process, although it increases the rate of the reaction considerably. It can be readily seen, therefore, that at the present time the exact mechanism of staphylocoagulase activity is still undetermined.

Physical and Chemical Properties of Coagulase

Gonzenbach and Uemura (1916) first accomplished the separation of coagulase from staphylococcal cultures by centrifugation. Gross (1931) accomplished separation via filtration as did Walston (1935), Fisher (1936) and Cruikshank (1937). Walston (1935) pointed out that Berkefeld and Seitz filters contained calcium which might be released during filtration sufficiently to cause the clotting of plasma and thus lead to erroneous conclusions. Employing gradocol membranes, Smith and Hale (1944) demonstrated that coagulase may be successfully filtered through filter pore diameters of 0.5 µ whereas coagulase activity is decreased by filtration through the membranes with pore diameters of 0.31 μ and completely negated with those possessing a 0.11 μ pore diameter. Lominski and Milne (1947) indicated Seitz filtration may be successfully accomplished if the pH of the culture to be filtered is adjusted to pH 6.7 or less.

One of the more distinguishing physical properties of coagulase is its heat stability in both cultures and their filtrates (Walston, 1935; Fisher, 1936). The relative thermostability of coagulase, originally noted by Gonzenbach and Uemura (1916), was studied by Gross (1931). This author found no heat inactivation of coagulase-containing filtrates from 56 C to 90 C regardless of the amount of filtrate employed, whereas thermal inactivation even at 100 C occurred only when the amount of filtrate was less that 50% of the total volume in the reaction tube. Fisher (1936) reported that a marked variation existed in the heat resistance of the coagulating substance with the staphylococcal strains under consideration. He found that out of 11 strains studied, the coagulating activity of 2 of them was abolished at a temperature of 60 C for 30 minutes; 2 others at 60 C for 60 minutes; 3 others at 80 C for 60 minutes, and the remaining 4 possessed some activity even after subjection to 100 C for 30 minutes. Cell-free filtrates of the heat stable strains exhibited similar thermal resistance. Walker _et_ _al_. (1948) reported that their coagulase preparation retained its activity even after autoclaving at 120 C for 20 minutes. However Smith and Hale (1944) found that subjection to the same amount of heat caused inactivation within 10 minutes. Variations in the concentration and pH of the active solutions may have contributed to this apparent conflict in experimental results.

On the other hand, purified coagulase, when compared to crude preparations, has been shown to be relatively thermolabile (Walston, 1935). Tager (1948b) demonstrated that heating at 56 C for 30 minutes caused 75% inactivation; 65 C for 30 minutes, 93.8%; 100 C for 15 minutes, 99.22%; and 100 C for 30 minutes, 99.61%.

In examining other properties Tager (1948b) showed purified staphylocoagulase to be unstable in 0.85% saline, being inactivated approximately 63% within 24 hours. However, over the same period of time no inactivation occurred when 2% peptone-saline was employed. The same author observed that lyophilized preparations of coagulase may be successfully stored for 6 months or more in a desiccator at 4 C or even in solution at -15 to -20 C.

The effect of a number of chemical substances on coagulase has been investigated by various workers. Walker, Derow and Schaffer (1948) reported that propylene glycol, sodium azide, and streptomycin

inhibited partially purified staphylocoagulase whereas no inhibition occurred with penicillin, Zephiran, Tyrothricin, Bacitracin, Gramicidin, Tyrocidin, sulfathiazole, sulfadiazine and hydrazine sulfate. However, Miale (1949) found penicillin to be quite inhibitory towards coagulase. This discrepancy, of course, may be due to variations in the media employed and the method and degree of purification.

Tager (1948) found that sodium thioglycollate (0.05% in saline) at pH 6.8 exerted no effect on coagulase while, on the other hand, cysteine retarded the deterioration of coagulase in saline. Ascorbic acid exhibited a strong inhibitory effect as did the oxidizing agent, superoxol. Cysteine was found to block the deleterious effect of the former compound. Crystal violet in a concentration of 1:100,000 and high concentrations of phosphate and borate ions have been reported to be inhibitory (Miale, 1949). Crude preparations of trypsin and pepsin (Walker et al., 1947) as well as crystalline preparations of the same proteolytic enzymes (Tager, 1948b) have been found to inactivate coagulase.

Relationship of Coagulase to other Biological Activities of Coagulase-positive Staphylococci

Various biological properties of staphylococci have been investigated over the years in an attempt to differentiate the pathogenic organisms from the nonpathogenic ones. Since coagulase was early established as the index of pathogenicity, then the finding of another reliable criterion of pathogenicity might shed light on the mechanism by which coagulase functioned.

The differentiation of pathogenic and nonpathogenic staphylococci by mannitol fermentation has been attempted by numerous workers. Hine (1922) found, as did Jullianelle (1937), that the fermentation of mannitol with acid production was indicative of pathogenic staphylococci. Hallman (1937) confirmed this observation in 90.9% of 487 strains. He also found agreement between the crystal-violet reaction of Chapman and Berens (1935) and the coagulase test in 88% of the strains tested. Similarly, Flaum (1938) reported a correlation between coagulation of rabbit plasma and mannitol fermentation, and to a lesser degree, pigment production. Chapman _et al_. (1938) attempted to determine the significance of of various biological tests for pathogenicity and found that the coagulase test was the most reliable single index whereas tests with crystal-violet agar, brom-thymol-blue agar, mannitol fermentation and pigment production were useful only as supplementary tests.

Differentiation of pathogenic and nonpathogenic types by different reactions in litmus milk was originally attempted by Dudgeon (1908) who observed no significant correlation between the two types. Confirmation of this lack of correlation was made by other workers such as Winslow et al. (1920), Minett (1936) and Schlam and Woods (1953).

In 1951 Barber et al. (1951) tested 160 coagulase-positive and 75 coagulase-negative strains of *S. aureus* for their ability to release phenolphthalein sulfate and phenolphthalein glucuronide. They reported that all 160 coagulase-positive strains liberated phenolphthalein from phenolphthalein phosphate whereas only 1 coagulase-negative organism did so. Neither type of staphylococci exhibited any significant activity on the other two substrates. Barber and Kuper (1951) also found that when 100 clinical staphylococcal isolates were incubated overnight on nutrient agar plus 0.01% phenolphthalein phosphate, after exposure to ammonia, 42 exhibited phosphatase-positivity while 58 did not. Colonies from the 58 negative plates proved to be coagulase-negative. When 160 representative colonies were picked from the 42 positive plates, all but one of these were coagulase-positive. Chiarolanza (1955) reported a strict relationship between coagulating power and phosphatase activity. Lovell (1958) confirmed this finding and also devised a solid medium employing disodium p-nitrophenylphosphate as the substrate since he had experimentally showed its superiority over other phosphatase substrates.

On the other hand, Gupta and Chakravati (1954) found a doubtful relationship between coagulase and phosphatase-positivity as did Malik and Singh (1960).

The latter authors reported that in addition to strains of *S. aureus*, some strains of *S. albus* and *S. citreus* also exhibited phosphatase activity. The possibility exists that this discrepancy in results may revolve around the fact that the latter two articles were concerned primarily with staphylococci of animal origin.

A relationship has also been suggested to exist between staphylocoagulase and arginase or an arginase-like enzyme. Fusillo and Jaffurs (1955) found that 23 of 36 organisms grown on arginine-yeast medium produced plasma clotting. They considered that arginine and arginase play an important role in the metabolic processes which form substances that activate the formation of fibrin. Another possible relationship to staphylococcal pathogenicity was reported by Hills (1940) who postulated the possible function of an arginine dihydrolase, which is distinguished from arginase by the fact that it catalyzes the formation of ammonia, carbon dioxide and glutamine from arginine, rather than ornithine and urea as does arginase. By following the growth of *S. aureus* on a special arginine medium by chromatography, Lominski *et al.* (1952) reported that during incubation a citrulline spot occurred which increased in intensity with a corresponding decrease in the amount of arginine present. The presence of an active arginine, ornithine, citrulline cycle was thus indicated. Therefore, as Fusillo and Jaffurs (1955) stated, these studies suggested the possibility that coagulase might be related to an arginase or a similar type enzyme.

Recently, Drummond and Tager (1959a, 1959b) have reported that purified preparations of staphylocoagulase possessed esterase activity upon tributyrin in addition to the normal coagulating activity. However, they (Drummond and Tager, 1959b) later achieved the physical separation of the coagulase and tributyrinase activities.

Isolation, Concentration and Purification of Coagulase

Early realization that an understanding of the mechanism by which staphylocoagulase functions in the plasma clotting mechanism could explain the relationship between coagulase and pathogenicity stimulated attempts on the concentration and purification of staphylocoagulase itself. One of these first attempts was carried out by Walston (1935). He precipitated coagulase from the filtrate of a broth culture of a coagulase-positive strain with 10 volumes of 96% alcohol. He also attempted precipitation with acetic acid and half-saturation with ammonium sulfate but found that alcoholic precipitation yielded a much more highly active coagulase. Also, the precipitate obtained by half-saturation with ammonium sulfate could not be completely dissolved in water. Fisher (1936) showed that the active principle could be isolated from cell-free filtrates by precipitation with 3 or 3½ volumes of 95% alcohol.

According to Smith and Hale (1944) the thermostability of coagulase-containing filtrates allowed them to concentrate the filtrates by distillation at 60 to 70 C under reduced pressure. By this method they obtained a 10-fold concentration together with some purification. They also suggested that partial purification could be achieved by alcoholic precipitation and dialysis.

Walker _et_ _al_. (1948) obtained his coagulase preparations by repeated precipitation with acid at pH 4. He initially autoclaved the coagulase-positive cultures for 20 minutes to remove interfering proteins, and after centrifugation, the supernatant fluid was adjusted to pH 4 with HCl. The resulting precipitate was centrifuged in the cold. After washing with buffers of the same pH value but of progressively decreasing ionic strengths, the acid precipitation was repeated. The products obtained varied between 25 and 60 mg of coagulase containing from 4 to 10 mg of nitrogen.

Tager (1948b) reported a procedure by which he obtained highly purified coagulase preparations. Fifteen to 20-liter lots of crude coagulase in brain heart infusion broth were cleared of cells by a Sharples centrifuge. The supernatant fluid was slowly acidified to pH 3.8 to 4.0 with 4 N HCl. After storage in the cold, the acid precipitate was collected by centrifugation, washed, redissolved in buffer, and then precipitated with three volumes of 95% alcohol. This alcohol treatment was repeated with intermittent precipitation of impurities with low concentrations of ammonium sulfate. The purified preparation was lyophilized and stored in a desiccator. It possessed high biological activity, clotting plasma in 3 or 4 seconds, and was found to be protein in nature.

Purification of free coagulase from staphylococcal cultures in digest broth was described by Duthie and Lorenz (1952). They demonstrated that coagulase was readily precipitated by cadmium salts which could be removed by dialysis at a low pH. This procedure yielded a coagulase which was stable in solution at pH 2. Jacherts (1956) subjected this type of preparation to further purification by column chromatography, employing aluminum hydroxide, and paper electrophoresis. Duthie and Haughton (1958) improved their purification method by obtaining a starting material which possessed a greater degree of purity than that usually obtained in culture supernatant fluids. They took advantage of the fact first demonstrated by Duthie (1954b) that if one volume of a mature shaken culture of S. aureus in digest broth is inoculated into 9 volumes of temperature-equilibrated broth and is then shaken at 37 C, free coagulase is maximally released within 120 minutes. In the present work, Duthie and Haughton (1958) employed a casein-hydrolysate medium and found a maximal ratio of coagulase to extracellular protein within 80 minutes.

Murray and Gohdes (1959b) purified coagulase principally by precipitation with ammonium sulfate (33% weight/volume) and cadmium sulfate, followed by chromatography on a calcium phosphate column using stepwise elution with phosphate buffer. Recently, Blobel *et al*. (1960) reported successful purification based on acid precipitation of the original culture supernatant fluid followed by ethanol fractionation and electrophoresis.

MATERIALS AND METHODS

Organisms and Culture Media

In this study, 3 phage propagating strains of *Staphylococcus aureus* of the International-Blair series (Blair and Carr, 1953) were employed, namely 3B, 7, and 70. These strains were experimentally determined to be representatives of staphylococci which produce a relative low, medium and high amount of coagulase respectively. Strain 70 was the one which was purified and used for the subsequent studies in which purified preparations were employed. Stock cultures were maintained on brain heart infusion agar[1] slants and stored at 4 C.

In all experiments in which growth of *S. aureus* was required, an incubation temperature of 37 C was employed. Similarly brain heart infusion broth[2] was constantly used as the liquid growth medium.

Assay Methods

Coagulase

Coagulase activity was determined by a slight modification of the method of Tager and Hales (1947). In most cases, 1 ml of coagulase-containing solution was added to 1 ml of a 2% peptone-saline solution (2 gm of peptone in 100 ml of 0.85% saline) containing

[1,2] Difco Laboratories, Inc., Detroit, Michigan

merthiolate at a concentration of 1:5000. After suitable dilutions were made, 1 ml of a 1:5 dilution of standardized human plasma in saline was added to each tube, thoroughly mixed, and incubated at 37 C. The final tube in which a clot was visible after 24 hr was taken as the endpoint.

Phosphatase

Procedure I. Phosphatase activity was determined by a modification of the method of Barnes and Morris (1957). The stock substrate solution employed was p-nitrophenylphosphoric acid, disodium salt[1] at a concentration of 0.005 gm/ml and was stored at 4 C until needed. Usually a 1-ml sample was placed in a standardized cuvette (12 mm by 100 mm) containing 1.4 ml of tris buffer, pH 7.2. To this was added 0.6 ml of stock substrate solution. After incubation in a water bath at 37 C for 30 min, 3 ml of 0.01 N NaOH (pH 12) was added to stop the reaction and to completely develop the color of the liberated p-nitrophenol. Optical density of the alkaline solution was determined using a Bausch and Lomb Spectronic 20 spectrophotometer at a wavelength of 400 mμ. A control tube consisting of buffer and substrate was treated similarly. By comparison of the intensity of the color produced to that of a standard curve of p-nitrophenol, the activity

[1] Aldrich Chemical Co., Milwaukee, Wisconsin

of the enzyme in terms of µM p-nitrophenol liberated was obtained.

Procedure II. For the measurement of the amount of inorganic phosphorous released, a modification of Allen's method (1940) was employed. To a 2-ml sample was added 8 ml of 10% trichloroacetic acid and the precipitate thus formed was millipore filtered. To the protein-free filtrate was added 2 ml of 10 N H_2SO_4, 2 ml of monomethyl-para-aminophenol sulfate (Pictol), 1 ml of ammonium molybdate, and 10 ml of distilled water to bring the total volume to 25 ml. Incubation at room temperature for 15 min was followed by spectrophotometric measurement at 625 mµ. The amount of phosphorous released was then determined via a previously devised standard curve.

Protein

Protein was determined by the Folin-Ciocalteu reagent (Lowry et al., 1951). One ml of 1 N NaOH was added to a 1-ml sample, mixed well and incubated at room temperature for 10 min. To this was added 1.5 ml distilled water and 5 ml of Reagent A (1 ml of 2.7% sodium potassium tartrate .4 H_2O and 1 ml of 1% copper sulphate .5 H_2O added to 100 ml of 2% sodium bicarbonate). After standing at room temperature for 15 min, 0.5 ml of Reagent B (commercial Folin-Ciocalteu reagent) was added, mixed immediately, and stored at room temperature for 25 min. Optical density readings were made at 660 mµ and protein content determined from a standard curve for bovine albumin, fraction V.[1]

Isolation and Purification of Coagulase

Six tubes, each containing 10 ml of broth, were inoculated from a brain heart infusion agar slant culture. After incubation for 18 hr, 5 ml of each of the above tubes were correspondingly inoculated into six 500-ml Erlenmeyer flasks, each containing 100 ml of broth, and incubated for 7 hr. This logarithmic-phase growth was in turn used to inoculate a total of 6.6 liters of broth medium using 6-liter Florence flasks each containing 3.3 liters. The organisms were incubated for 12 hr on a rotary shaker (150 cycle/min). The organisms were removed using a Servall continuous flow, superspeed centrifuge[2] at a flow rate of 100 ml/min. The coagulase in the supernatant fluid was purified principally by the methods developed by Tager (1948b) and more recently modified by Blobel et al. (1960). Since some procedures and reagents were further changed or modified, the actual process employed will be briefly outlined. The supernatant fluid was slowly adjusted to pH 3.8 by 4 N HCl producing a precipitate of coagulase. This precipitation process was continued at 4 C for 20 hr. The acid precipitate was then collected by

---

[1] Nutritional Biochemicals Corporation, Cleveland, Ohio

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

continuous flow superspeed centrifugation, dissolved in 50 ml of distilled water and adjusted to pH 7.2 with 0.066 M disodium phosphate buffer. After thorough mixing in the cold, the insoluble residues were removed by a centrifugal force of 12,100 x G for 20 minutes. The cooled supernatant fluid was then precipitated by adding 95% ethanol kept at -20 C to a final ethanol concentration of 70% (v/v). The recommendations by Cohn and his coworkers (1946) for alcoholic precipitations were observed by maintaining the environment at a temperature not higher than -5 C. Also, the precooled ethanol was added very slowly through a syringe needle with constant stirring. This precipitation was allowed to continue for 20 hr after which time the resulting precipitate was collected by centrifugation at 1,935 x G at -20 C. The alcoholic supernatant fluid was discarded, and the precipitate was resuspended in 50 ml of distilled water and adjusted to pH 7.2 with 0.066 M potassium dihydrogen phosphate buffer. After thorough mixing, the insoluble residues were again removed by centrifugation and the supernatant fluid subjected to another complete cycle of ethanol precipitation. This final clear solution was concentrated about 4-fold by dialysis against polyvinylpyrrolidine[1] for 24 hr at 4 C. The resulting coagulase preparation was then lyophilized and stored at -20 C.

---

[1] Oxford Laboratories, Redwood City, California

Anion Exchange Chromatography

N,N-diethylaminoethylcellulose (DEAE-cellulose) was thoroughly washed with 0.01 M 2-amino-2-hydroxymethyl-1, 3-propanediol (tris) buffer at pH 7.5 for 16 hr. This slurry was poured into a glass column (30 cm in length and 1 cm in diameter), and allowed to settle, maintaining a head of buffer to prevent drying out of the adsorbent. The adsorption column was then packed to a height of 18 cm by air pressure, washed well with buffer at 4 C to obtain pH and temperature equilibrium, and set up over a fraction collector[1]. Thirty mg of lyophilized purified coagulase, which had been dissolved in 1 ml of 0.01 M tris buffer pH 7.5 and dialyzed against the same buffer for 24 hr at 4 C, was introduced into the column. The coagulase was then subjected to gradient elution with the reservoir containing 1.25 M NaCl. The flow rate was such that 5 ml fractions were collected every 10 minutes. Elution was continued at 4 C for approximately 15 hr and a total of 76 fractions was obtained. Each fraction was assayed for coagulase and phosphatase activity as well as protein. The same general procedure was followed when buffers of other pH values were used.

Electrophoresis

Starch block electrophoresis

The following general procedure was followed for

---

[1] Rinco Instrument Co., Greenville, Illinois

the complete series of starch block electrophoretic experiments. In certain individual experiments various buffers of different pH values were employed, which will be indicated in the appropriate sections. In order to remove any protein impurities, insoluble potato starch was washed three times with 500-ml amounts of 0.02 N KOH. This treatment was followed by similar washings with three 500-ml amounts of distilled water and finally with the appropriate buffer. The starch was then molded into a block, 24 cm by 2 cm, by means of a plastic template. By careful blotting with highly absorbent paper, excess buffer was removed without disturbing the surface of the settled starch granules. Twelve mg of the lyophilized coagulase preparation, which had been dissolved in 0.3 ml of appropriate buffer and dialyzed for 24 hr at 4 C against this same buffer, were applied to the block by excising a 2 mm segment of starch and replacing it with a slurry of coagulase and starch granules. The starch block was placed in a leveled horizontal position between two plastic electrolytic containers enclosed within a plastic box prior to equilibration to 4 C. Heavy filter paper strips were used as the electrolytic contacts. The material was then subjected to 200 v for 16 hr with an average current of 2 ma. At the end of the electrophoretic run the starch block was carefully segmented at 1-cm intervals, placed in separate tubes and resuspended. Upon settling of the starch granules

the contents of each tube were assayed for coagulase and phosphatase activity. Protein content was also determined in certain instances.

Starch gel electrophoresis:

The starch gel used for this type of electrophoresis was prepared according to the procedure of Smithies (1955). Hydrolyzed starch[1] (6.24 gm) was dissolved in 50 ml of 0.01 M tris buffer at the desired pH values. The gel was heated through its semi-solid state to a viscous liquid, quickly degassed by negative pressure, and poured onto a plastic template, 24 cm in length by 2 cm in width. After setting of the gel, a 2-mm segment was removed and replaced by a mixture of coagulase (12 mg/0.3 ml buffer) and starch granules. White petrolatum was melted, cooled, and layered over the surface of the gel to prevent evaporation. Strips of filter paper served as contacts between the starch gel block and the buffer in the plastic electrolytic vessels. The coagulase preparation was subjected to 200 v resulting in a current of 2 ma. One-cm segments were removed, placed in 5 ml of distilled water, and homogenized with a glass pestle. After settling of the homogenized gel, sometimes aided by low speed centrifugation, each of the supernatant fluids were assayed for coagulase and phosphatase activity.

[1] Connaught Research Laboratories, Toronto, Ontario

Paper electrophoresis

The horizontal open strip method of paper electrophoresis was conducted at 4 C. Whatman 3 MM paper strips, 47 mm wide and 32 cm in length were employed. The paper strip was dampened with 0.01 M tris buffer at a given pH, stretched taut between the electrolytic vessels and equilibrated to 4 C. By means of a micropipette, 25×10^{-4} ml of coagulase (12 mg/0.3 ml) in buffer was applied in a straight line across one end of the paper strip. After subjection to 200 v and a current of 1 ma for 16 hr, the strip was cut into 2 equal portions lengthwise and at 1-cm intervals crossways. Consequently, 2 equal portions of the paper strip for each centimeter distance in the migration path were obtained. One of these was eluted with 1 ml of distilled water and assayed for coagulase while the other corresponding portion was similarly eluted but tested for its phosphatase activity.

RESULTS

Coagulase Production in a Biphasic and Nonbiphasic Growth Medium

We have previously found in our laboratory that brain heart infusion broth is a suitable medium to yield a maximum amount of staphylococcal growth. In order to obtain initially as large an amount of coagulase as possible, the value of a biphasic growth system over that of a nonbiphasic system was investigated. Tyrrell *et al.* (1957) reported that yields up to 6×10^{11} viable organisms per ml were obtained with a biphasic system consisting of a layer of solid nutrient medium overlaid with nutrient broth. With this system an increase in the concentration of cells of 2- to 30-fold was obtained without sacrificing total yield. In our studies, three strains of *S. aureus* (3B, 7, 70) were used. Logarithmic phase growth from each of these three strains was spectrophotometrically standardized to 0.05 OD and used as inoculum. The biphasic system consisted of 100 ml of broth over a layer of 50 ml of solidified brain heart infusion agar in a 500-ml flask. The nonbiphasic system consisted of 50 ml of broth alone. After inoculation the flasks were incubated on a rotary shaker (150 cycles/min). At various intervals of time, samples were removed and growth determined by means of optical density at 650 mμ. The coagulase activity of the supernatant fluid of the same growth sample was also determined. The biphasic system began to show superior growth over the nonbiphasic system within 8 hours and after 24 hours the difference in growth was quite pronounced (Fig. 1). For example the increase in growth of strain 70 grown biphasically compared to strain 70 grown nonbiphasically was 4.5-fold as measured by viable cell counts. These results agree with the fundamental hypothesis that the biphasic system by continued elution and adsorption of growth-enhancing substances and inhibitory cell products will result in the production of a greater concentration of cells. However, the amount of coagulase produced by each strain grown in either type of growth system was quite similar (Fig. 2). It was also found that the maximum production of coagulase was followed by a decrease in coagulase activity. In the case of strain 3B, it was more pronounced than with strain 70. It was also observed in both growth systems that maximum coagulase production was obtained prior to maximum growth of cells. A similar observation was indicated by Duthie and Haughton (1958) for their growth system. It was, therefore, concluded that brain heart infusion broth supplies a satisfactory medium for the production and isolation of a maximum amount of coagulase.

Studies on the Possible Possession of Arginase Activity by Coagulase

It has previously been suggested that a correlation exists between the coagulase and arginase

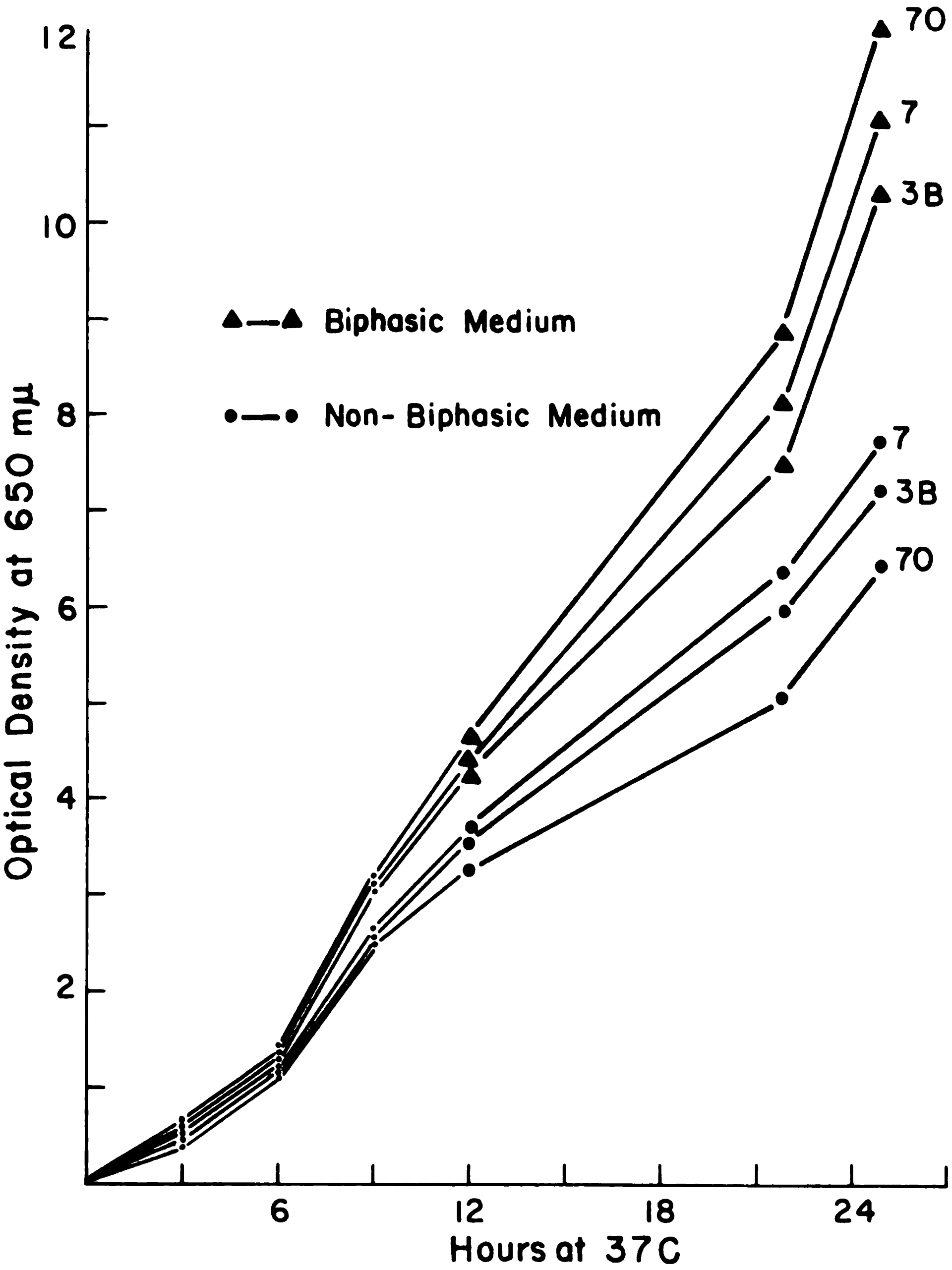

Fig. 1. Comparison of growth of selected strains (3B, 7, 70) of <u>Staphylococcus aureus</u> in biphasic and nonbiphasic media.

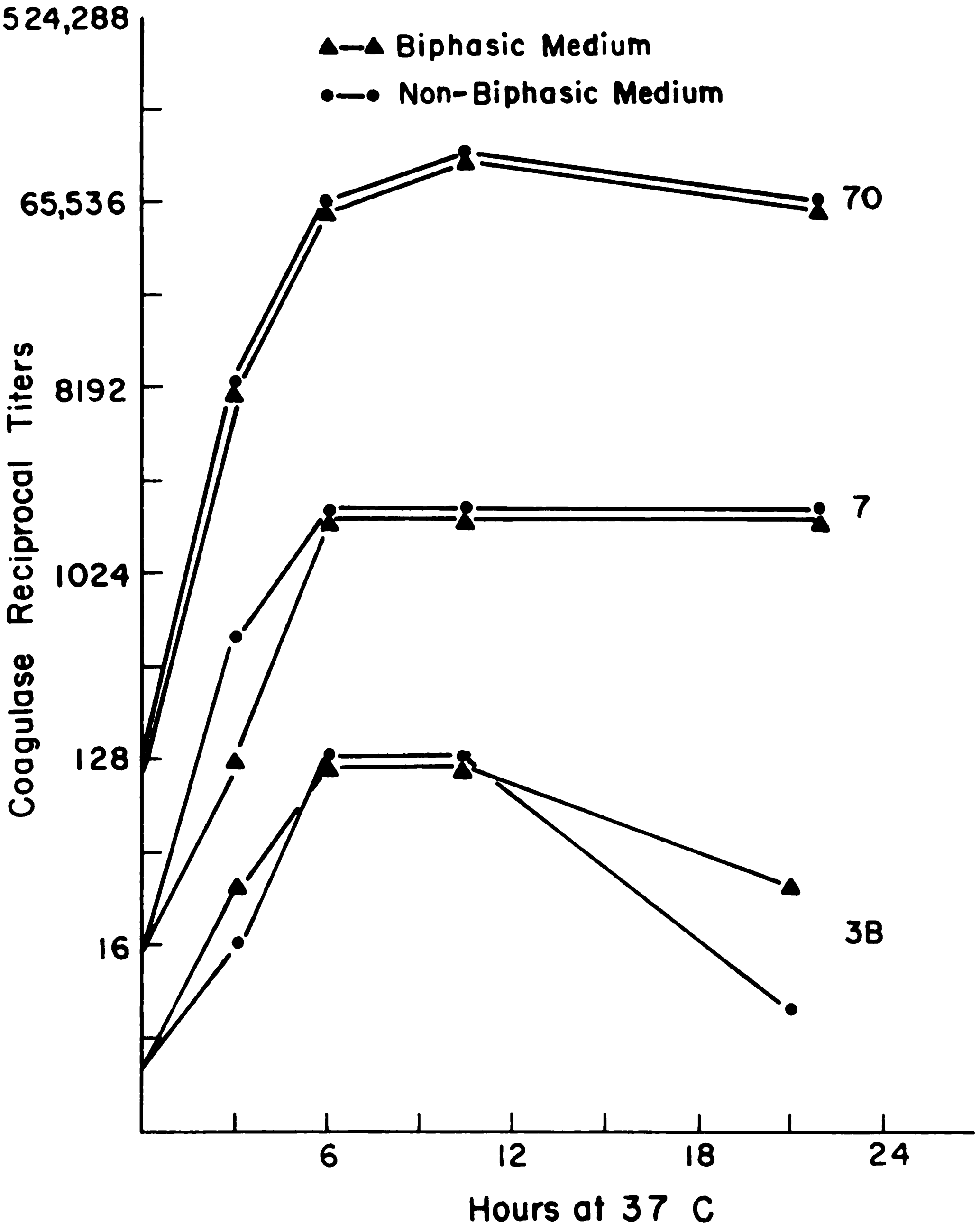

Fig. 2. Comparison of coagulase production by selected strains (3B, 7, 70) of *Staphylococcus aureus* in biphasic and nonbiphasic media.

activities of *S. aureus* (Fusillo and Jaffurs, 1955). If a correlation did exist, it might be due to (1) a chance relationship between two biochemically unrelated phenomena, or (2) a biochemical relationship functionwise. To resolve the basis of this correlation, standard manometric procedure (Umbreit *et al.*, 1957) was used. The main compartments of one set of exogenous Warburg flasks each contained 1 ml of 0.05 M phosphate buffer (pH 4.8) and 1 ml of purified coagulase solution in the same buffer (0.5 mg/ml). The side-arms each contained 1 ml of arginine (0.005 M). Another set of exogenous flasks contained, in addition to the above, 0.2 ml of 20% KOH in the center well to absorb any CO_2 present. After equilibration for 15 min at 37 C, the stopcocks were closed and measurements begun. No significant O_2 uptake or evolution of gas occurred.

Since most arginases are activated by Mn^{++} and generally have an optimum pH of 9-10, another series of manometric experiments were conducted employing 0.05 M phosphate buffer of pH 9 and 0.001 M Mn^{++}, as well as enzyme preparation and substrate. Similar studies were also carried out in an atmosphere of 5% CO_2 and 95% N_2. Where necessary, double side-armed flasks were employed with one of the side-arms containing 1 ml of 0.1 N HCl which was tipped into the main compartment at the end of the manometric run in order to release any CO_2 adsorbed. However, no significant O_2 uptake or gas evolution was observed in any of the reaction vessels. Employing Nessler's reagent, the reaction mixtures from these manometric studies were found to possess no NH_3. They were also found to contain no urea as determined spectrophotometrically at 475 mµ using Hycel 2, 3-butanedione reagent[1].

The contents of representative exogenous flasks were treated by paper chromatography. Samples of 0.05 ml were spotted on Whatman #1 paper and water-saturated phenol was used as solvent. After 12 hr, the chromatogram was developed by spraying with a 0.1% alcoholic solution of ninhydrin followed by heating at approximately 80 C. Only one spot was observed which, by comparison to a control, was identified as arginine, the original substrate.

Consequently, even though the coagulase preparation was provided with a specific cofactor and optimum pH for arginase enzyme, no indication either manometrically or chromatographically was found to suggest that purified staphylocoagulase possessed an arginase or an arginase-like activity.

Possession of Phosphatase Activity by Coagulase

The investigations of various workers have indicated that a correlation exists between the coagulase and

---

[1] Scientific Products, Evanston, Illinois

phosphatase activity of staphylococci. Preliminary studies showed that the purified coagulase preparation from S. aureus phage propagating strain 70, possessed a concomitant phosphatase activity as measured spectrophotometrically at 400 mμ using p-nitrophenylphosphate as the substrate. Since the actual mechanism by which staphylocoagulase clots citrated plasma is poorly understood, the finding of a significant phosphatase activity in a purified preparation of coagulase was of considerable interest. In order to clarify this dual phenomenon a series of investigations consisting of various approaches was conducted. These approaches were intended to determine (1) whether the same active group on a common protein accounted for both activities; (2) whether different active groups were associated with the same protein entity; or (3) whether two proteins, intimately associated, each possessed a different active group.

The pH-Dependence of the Coagulase and Phosphatase Reactions

The pH-dependence of enzymic reactions is usually a critical function. In these studies the staphylocoagulase and staphylophosphatase reactions were performed at different pH values within the range of pH 5.1 to 8.8. Since no one buffer can be employed over such a pH range, two different buffer systems were used, namely, citrate and tris. It is well known that certain ionic constituents of various buffers activate or inhibit enzymatic systems, sometimes skewing the pH-dependence curve. Consequently, the use of phosphate buffer was avoided since phosphate ions have been reported to inhibit acid phosphatase (Roche, 1950). Citrate buffer was used within the limits of pH 5.1 to 6.7, whereas tris buffer was used for the pH range of 7.2 to 8.8. The concentration of coagulase preparation in the reaction tubes was 0.5 mg per tube. As seen in Fig. 3, the coagulase and phosphatase reactions have the same optimum pH (pH 7.2). However, the coagulase was more sensitive to pH values on both the acid and alkaline side of pH 7.2. The fact that both reactions occurred optimally at the same pH might not be of possible significance. The basis for this conclusion was that the two reactions under study were involved with two different substrates whereas it is well known that even one enzyme might exhibit different pH optima on different substrates.

When the effect of pH on the rate of an enzymatic reaction is studied, one of the major factors to consider is the stability of the enzyme. In these experiments, the influence of pH on the stability of the enzymes was not differentiated from the pH-dependence of the actual catalytic reactions. Therefore, the results presented in Fig. 3 represent the total effect of the various factors which contribute to pH-dependence curves such

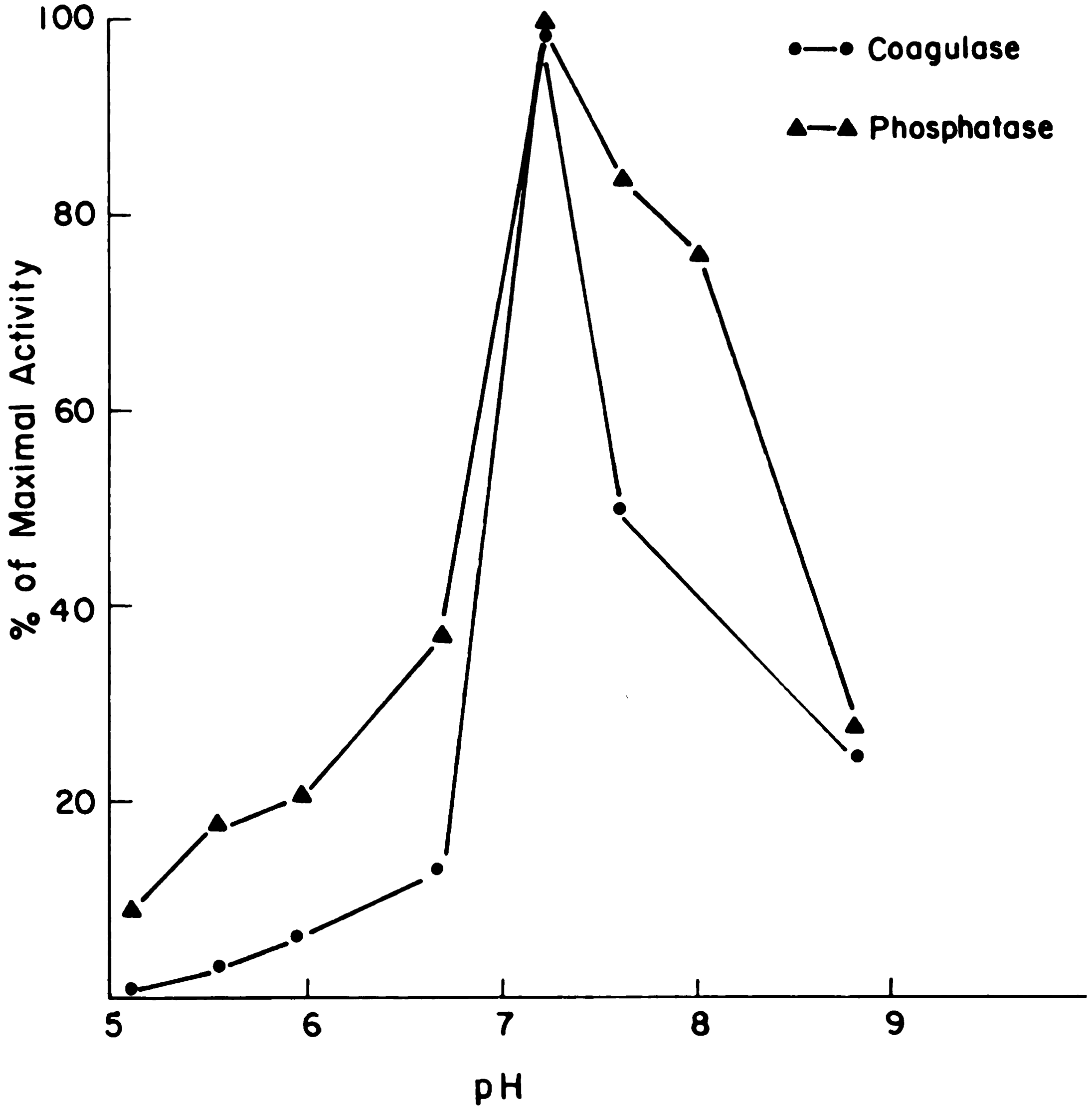

Fig. 3. The effect of pH upon coagulase and phosphatase activity.

as the degree of dissociation of enzyme protein and substrate, and enzyme stability. The elucidation of these pH optima as they existed under standard test conditions determined the pH at which future phosphatase and coagulase assays were conducted when maximum activity was desired.

Thermal Inactivation Studies

Thermal inactivation studies were carried out on the phosphatase and plasma clotting activities of purified coagulase in order to differentiate the two substances by their susceptibilities to heat. Samples of coagulase preparation (0.5 mg/ml) were placed in thermoregulated water baths at 37 C and 56 C. After ½ hr, 2½ hr, 5¼ hr, and 9 hr, samples were removed, immediately cooled and assayed for coagulase and phosphatase activity. The effects of these temperatures on the two activities were then compared (Fig. 4). It is apparent that the curves for the two activities appear quite similar with parallel decreases in both. After heating at 37 C for 4 hr, the residual activity of the coagulase was 85% and that of the phosphatase, 83,2%. Similarly, the preparation after heating at 56 C for 5¼ hr retained coagulase and phosphatase activities of 12.5% and 7.4% respectively. This similarity in thermal inactivation suggested that the same protein entity was involved with both activities.

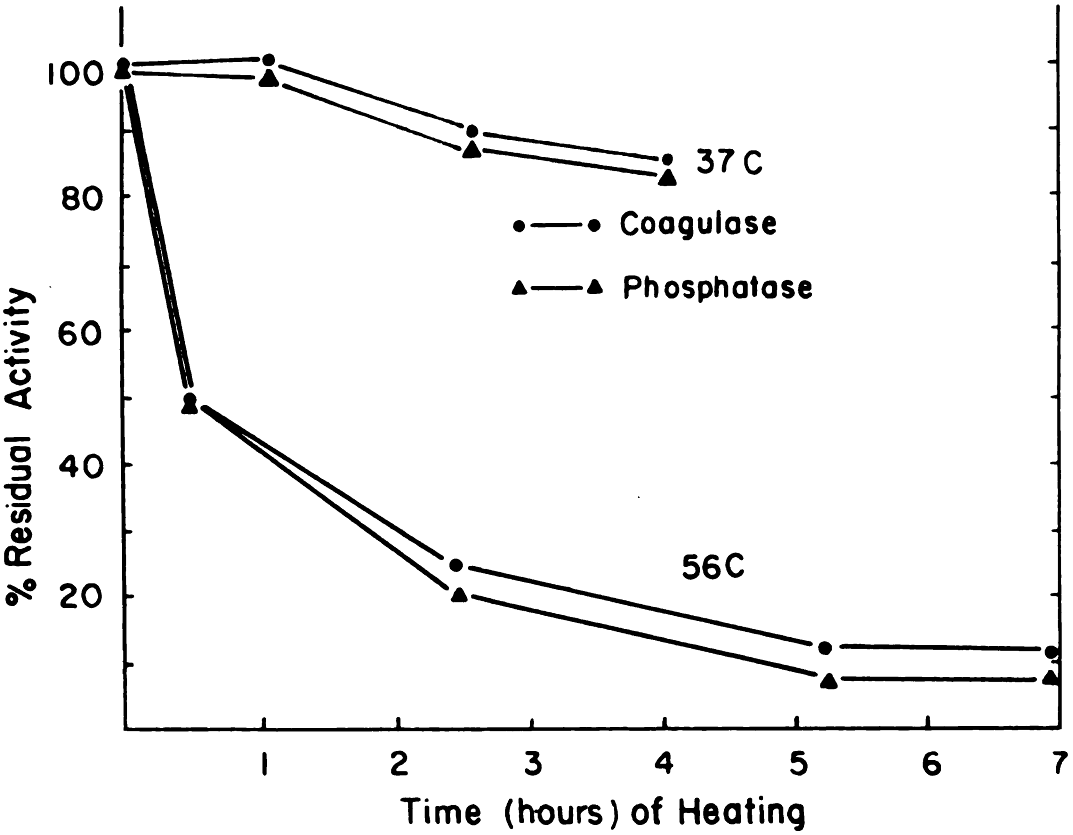

Fig. 4. Comparison of the degree of thermal inactivation of coagulase and phosphatase at 37 C and 56 C.

Anion Exchange Chromatography

Anion exchange chromatography was employed in an attempt to determine whether or not the coagulase and phosphatase activities were physically separable. A DEAE-cellulose column was prepared using 0.01 M tris buffer. Lyophilized coagulase preparation (30 mg/ml) was applied to the column, gradiently eluted with sodium chloride and the effluent collected in fractions each of which was assayed for coagulase and phosphatase activity as well as protein. Fig. 5 indicates the results obtained at pH 7.5 with the eluate collected in 5-ml fractions at the constant rate of 5 ml/10 min. A total of 76 fractions were collected. The two activities occurred maximally in the same fraction. A single protein peak emerged simultaneously with the two activities and the maximum peaks of activity for both the coagulase and phosphatase expressed in terms of specific activity (units of enzyme activity per µg of protein) also coincided.

When chromatography was carried out in the same manner but at pH 8.6, similar results were obtained (Fig. 6). The two activities again showed similar maximum peaks of activity with remarkably sharp resolution. The obvious decrease in the activities of the fractions collected at pH 8.6 as compared to pH 7.5 was due to the lesser stability of both substances at the higher pH and the one-third decrease in concentration

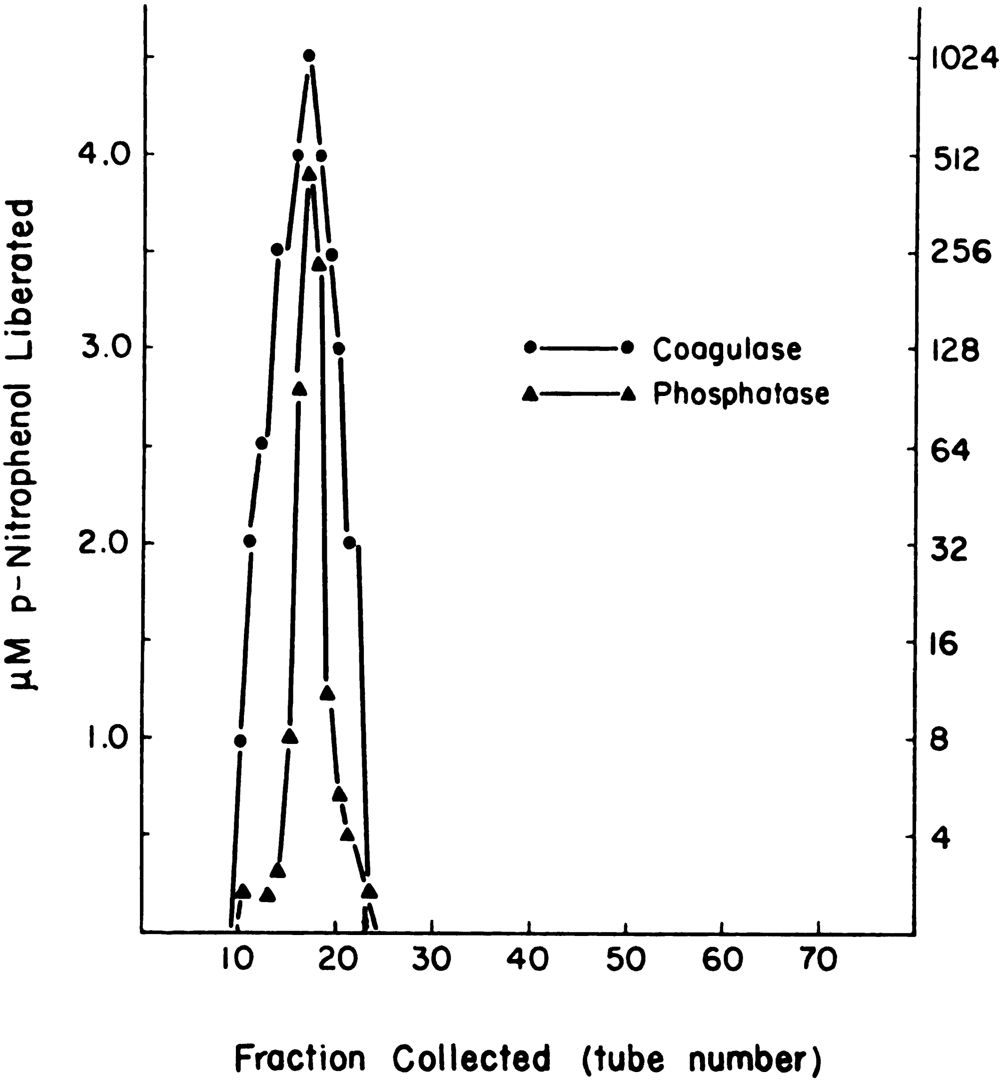

Fig. 5. Inseparability of the coagulase and phosphatase activities by anion exchange chromatography employing DEAE-cellulose and 0.01 M tris buffer at pH 7.5.

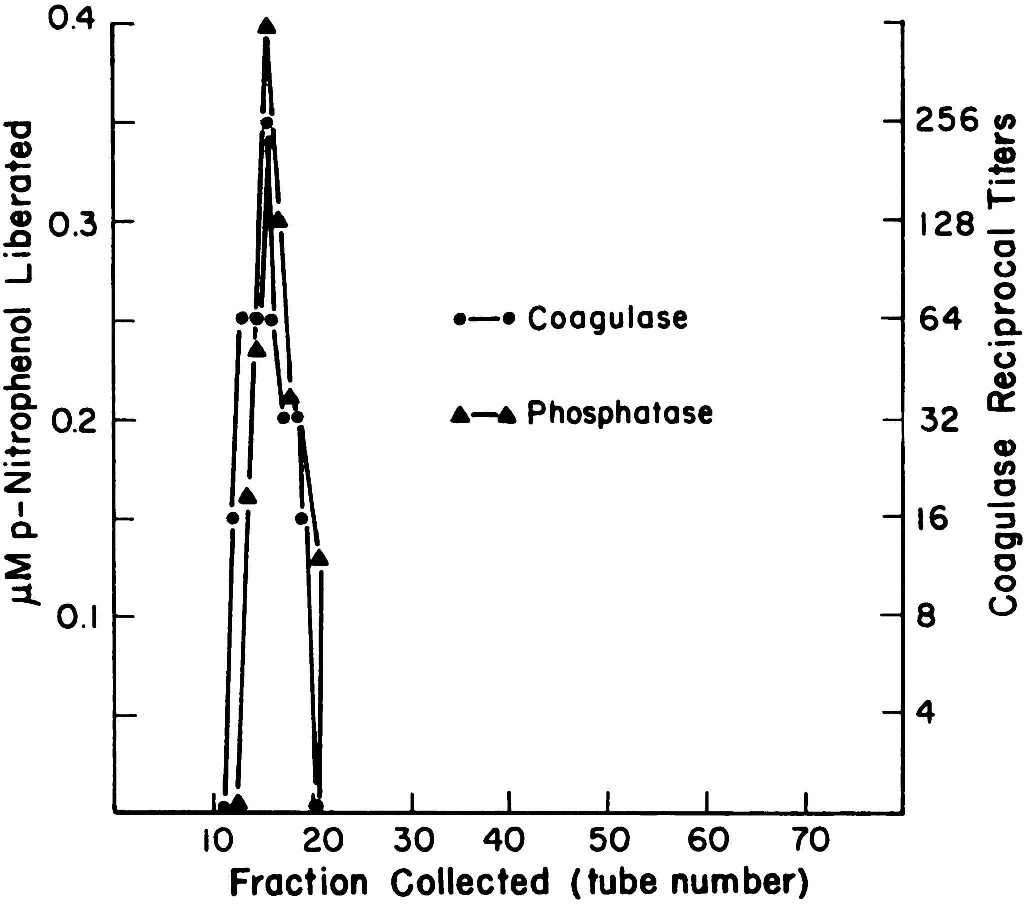

Fig. 6. Inseparability of the coagulase and phosphatase activities by anion exchange chromatography employing DEAE-cellulose and 0.01 M tris buffer at pH 8.6.

of coagulase preparation originally applied to the column.

Electrophoretic Studies

A complete series of electrophoretic studies was carried out on the purified coagulase preparation to determine if the coagulase and phosphatase were separable. If one found that the two activities showed similar maximum peaks of activity after migration, then one could conclude that the two activities were electrophoretically indistinguishable. The results of a typical experiment employing insoluble starch as the stabilizing medium and 0.01 M tris buffer at pH 8.6 are shown in Fig. 7. At the end of the electrophoretic run the block was segmented, resuspended, and upon settling of the starch granules, coagulase and phosphatase determinations were made. It is evident that both the coagulase and phosphatase possessed similar maximum peaks of activity after migration. The electrophoregram thus indicates that the two activities are associated with the same protein under the given experimental conditions. Fig. 8 indicates that similar results were obtained when electrophoresis was carried out using the same buffer at pH 7.5. The preparation moved the same distance but in the opposite direction, showing that the isoelectric point was between pH 8.6 and pH 7.5.

Other electrophoretic studies involving different buffer systems, pH values, and stabilizing media were

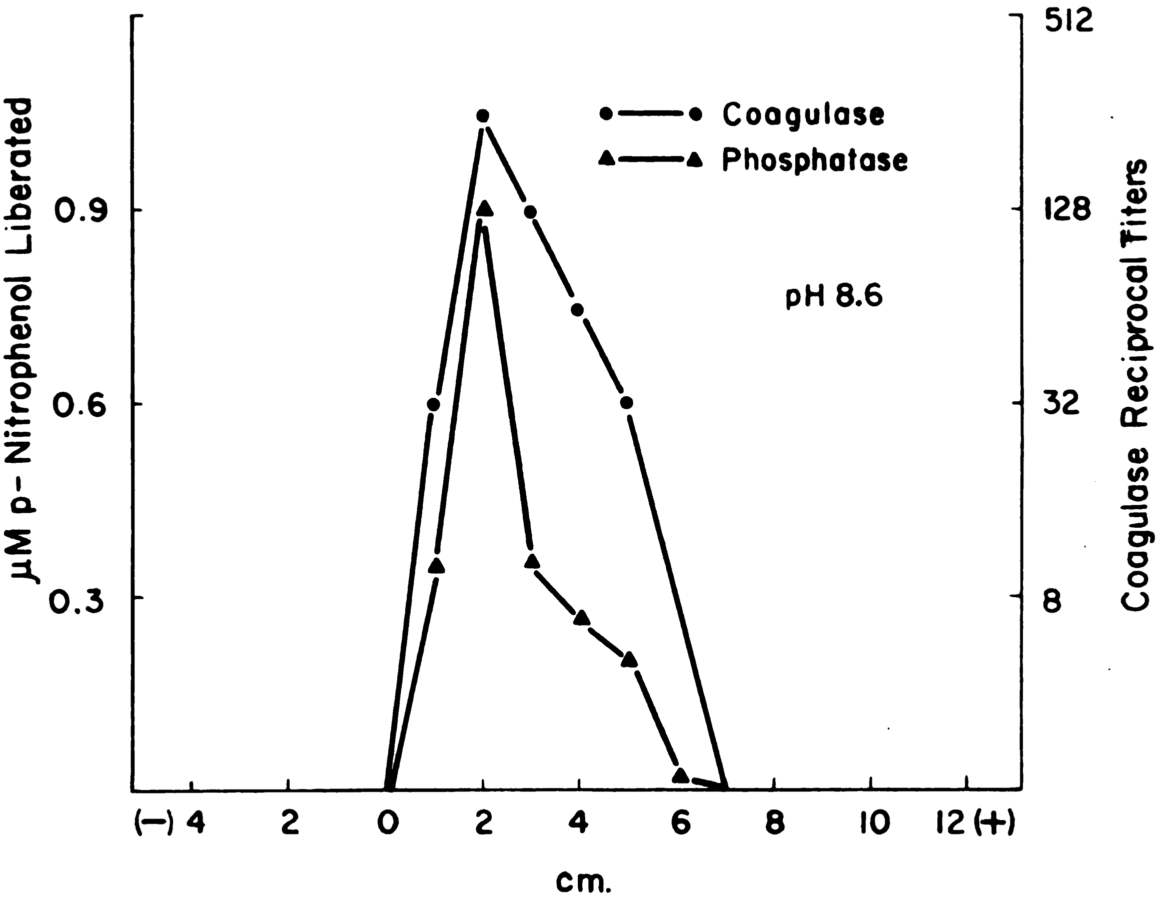

Fig. 7. Inseparability of the coagulase and phosphatase activities by starch block electrophoresis employing 0.01 M tris buffer at pH 8.6.

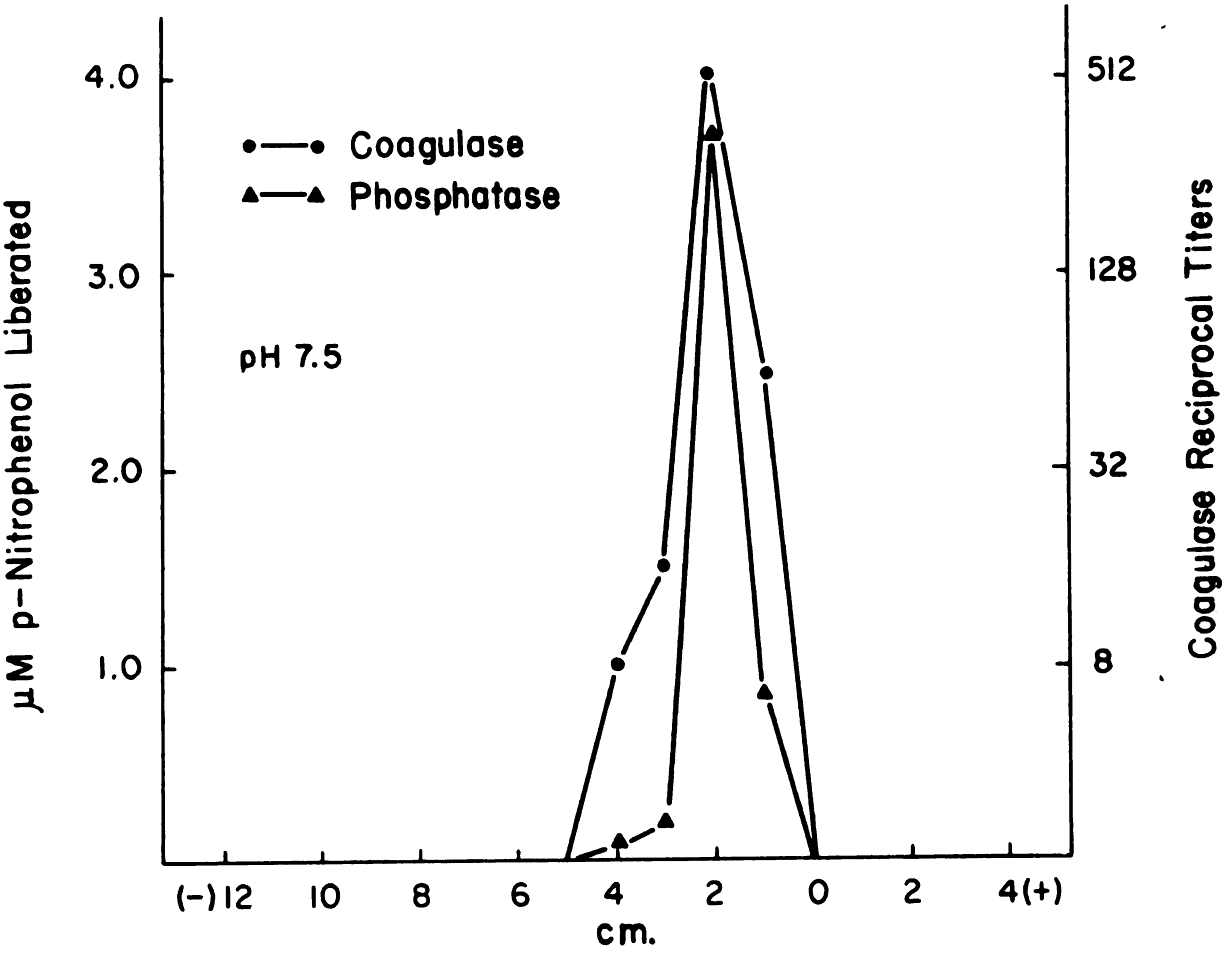

Fig. 8. Inseparability of the coagulase and phosphatase activities by starch block electrophoresis employing 0.01 M tris buffer at pH 7.5.

conducted. Electrophoresis was carried out using barbital buffer as well as tris and at pH values from 4.2 to 10. The stabilizing media employed included paper and starch gel. A total of 20 electrophoretic determinations were made with the same results being obtained, thus suggesting that the same protein was involved with both activities.

The coagulase preparation was also subjected to electrophoresis using a discontinuous buffer system (Goldberg, 1959). The same procedure described for starch block electrophoresis was employed except that the starch block buffer was 0.1 M tris (pH 8.0) whereas the well buffer was 0.05 M barbital buffer (pH 6.8). Electrophoresis was carried out at 50 v for 20 hr with an average current of 4 ma. Under these experimental conditions a small degree of separation of the two activities seemed to occur (Fig. 9). However, it was not possible to recover appreciable amounts of either activity in the complete absence of the other, because of the large amount of overlapping of the two activities. If a true separation of the two activities was obtained, then the fact that it was of such a small degree after treatment with a very critical electrophoretic system reemphasizes the probable similarity of the two activities.

Studies on the Effects of Various Inhibitors on the Coagulase and Phosphatase Activities

The effect of certain enzyme inhibitors upon the

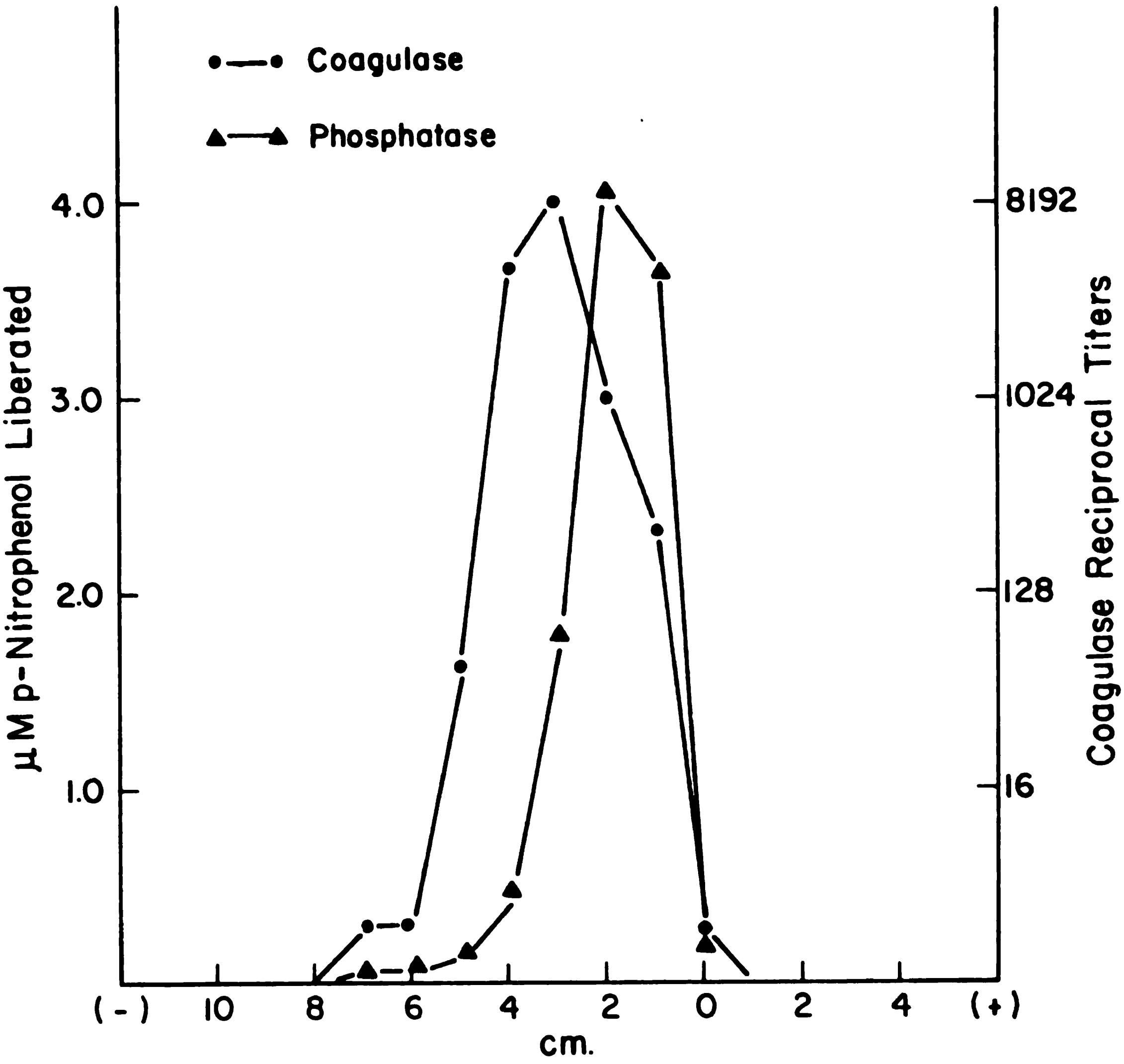

Fig. 9. Attempt at separation of the coagulase and phosphatase activities by starch block electrophoresis employing a discontinuous buffer system; strip buffer composed of 0.1 M tris at pH 8.0 and well buffer, 0.05 M barbital at pH 6.8.

two activities of the purified coagulase preparation was investigated. If coagulase and phosphatase were the same catalytic substance, then the degree of inhibition exerted by these compounds on each activity would be the same. On the other hand, if the preparation possessed two different catalytic groups, then different degrees of inhibition could occur. The inhibitors employed were ethylenediaminetetraacetate (EDTA), sodium fluoride, sodium azide, sodium iodoacetate, and p-chloromercuribenzoate (Table 1). A 2.5-ml sample of coagulase solution (0.5 mg/ml) was incubated with an equal volume of each inhibitor. After incubation at 37 C for 120 min, samples were removed and coagulase and phosphatase determinations made. The controls consisted of coagulase at the appropriate concentrations in the absence of inhibitors.

It was apparent that the degree of inhibition caused by each of the compounds was different, suggesting that the two activities were not caused by the same functional group. It also seemed that sulfhydryl groups were involved, particularly with respect to the phosphatase since it was inhibited 97% by the p-chloromercuribenzoate. The inhibition by iodoacetate further confirmed this interpretation. Sodium fluoride, a metal inactivator, and EDTA, a chelating agent, were especially inhibitory to the phosphatase.

However, disregarding these other facts of interest, the main conclusion to be derived is that the two

TABLE 1. Effect of various inhibitors upon the coagulase and phosphatase activities of the purified preparation

Inhibitor	Final Conc. (M)	Per Cent Residual Activity Coagulase	Per Cent Residual Activity Phosphatase
EDTA*	2.5×10^{-2}	50	2
Iodoacetate	2.5×10^{-2}	50	76
Fluoride	5.0×10^{-1}	50	26
Azide	5.0×10^{-1}	50	60
p-Chloromercuribenzoate	2.5×10^{-2}	50	3

*Ethylenediaminetetraacetate

activities do not function by the same mechanism.

Saturation of Phosphatase Activity with Excess Substrate

The supposition that the coagulase and phosphatase activities are not caused by the same functional group was verified by the failure to inhibit coagulase activity when the phosphatase was saturated with excess substrate. This experiment was accomplished in the following manner: into 5 cuvettes there was placed p-nitophenylphosphate so that the cuvettes contained a final concentration of $1.9\text{x}10^{-2}$ M, $9.5\text{x}10^{-3}$ M, $4.75\text{x}10^{-3}$ M, $2.38\text{x}10^{-3}$ M, $1.19\text{x}10^{-3}$ M respectively. To each of these cuvettes was added 2.4 ml of purified coagulase (0.5 mg/ml). Controls consisted of the appropriate concentration of substrate to which 2.4 ml of distilled water was added instead of enzyme. The reaction tubes and controls were incubated in a water bath at 37 C. At various periods of time, the phosphatase activity of the cuvettes was determined spectrophotometrically at 400 mμ, using the appropriate controls as blanks. Consequently the velocities of the phosphatase reaction at different concentrations of substrate were obtained (Fig. 10).

From these data the values for $\frac{S}{v}$ (S=substrate concentration, v=velocity of reaction) at the various concentrations of p-nitrophenylphosphate substrate were calculated. As cited by Wilson (1950) the typical Michaelis-Menton equation of $K_s = \frac{V.S}{v}$ -S may be

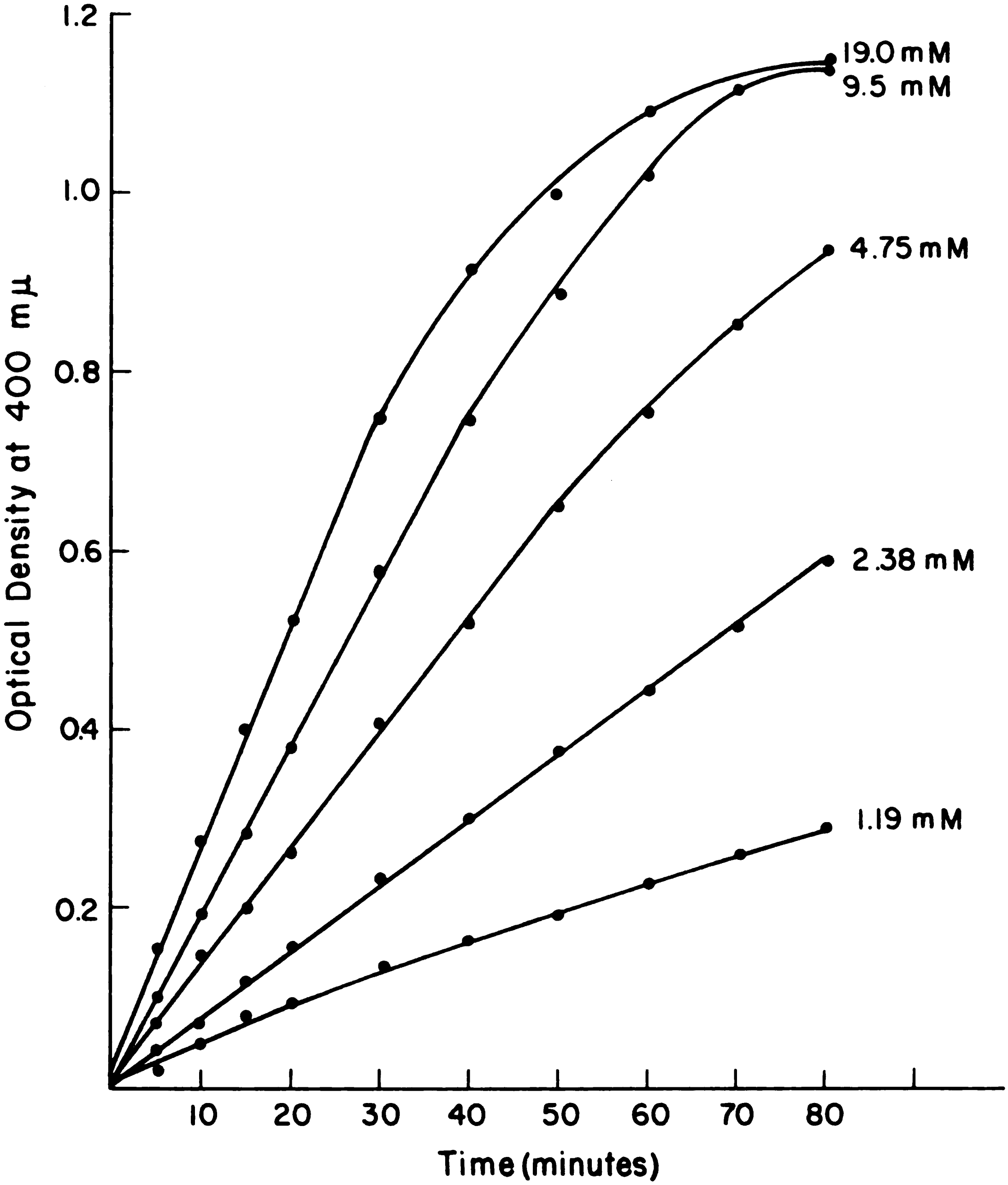

Fig. 10. The velocities of the phosphatase reaction at various concentrations of substrate (p-nitrophenylphosphate) at 37 C.

rearranged to the linear equation, $\frac{S}{v} = \frac{1}{V} \cdot S + \frac{K_s}{V}$. A Lineweaver-Burk plot was made (Fig. 11); that is, $\frac{S}{v}$ was plotted as the ordinate and S as the abscissa. The slope of the resulting straight line is $\frac{1}{V}$ and the ordinate intercept, $\frac{K_s}{V}$. Thus the value for the K_s of the phosphatase reaction was calculated to be 1.599×10^{-3} M.

In order to saturate the phosphatase of the purified coagulase preparation, 2.4 ml of the preparation (0.5 mg/ml) was placed in a cuvette containing 100 times the K_s value of phosphatase substrate. A control consisting of the same concentration of substrate without enzyme was also prepared. The cuvettes were incubated at 37 C and the reaction allowed to proceed to completion as determined spectrophotometrically and by measurement of the release of inorganic phosphorous. The coagulase activity of the coagulase preparation was then determined and found to be completely intact.

The fact that the phosphatase was completely saturated even in the presence of plasma substrate was verified by the complete lack of liberated inorganic phosphorous during the coagulation of the plasma by the saturated preparation. Phosphorous was released from plasma, however, when the phosphatase was not saturated with p-nitrophenylphosphate. It may be concluded therefore, that the two activities of the purified preparation do not represent the same functional groups.

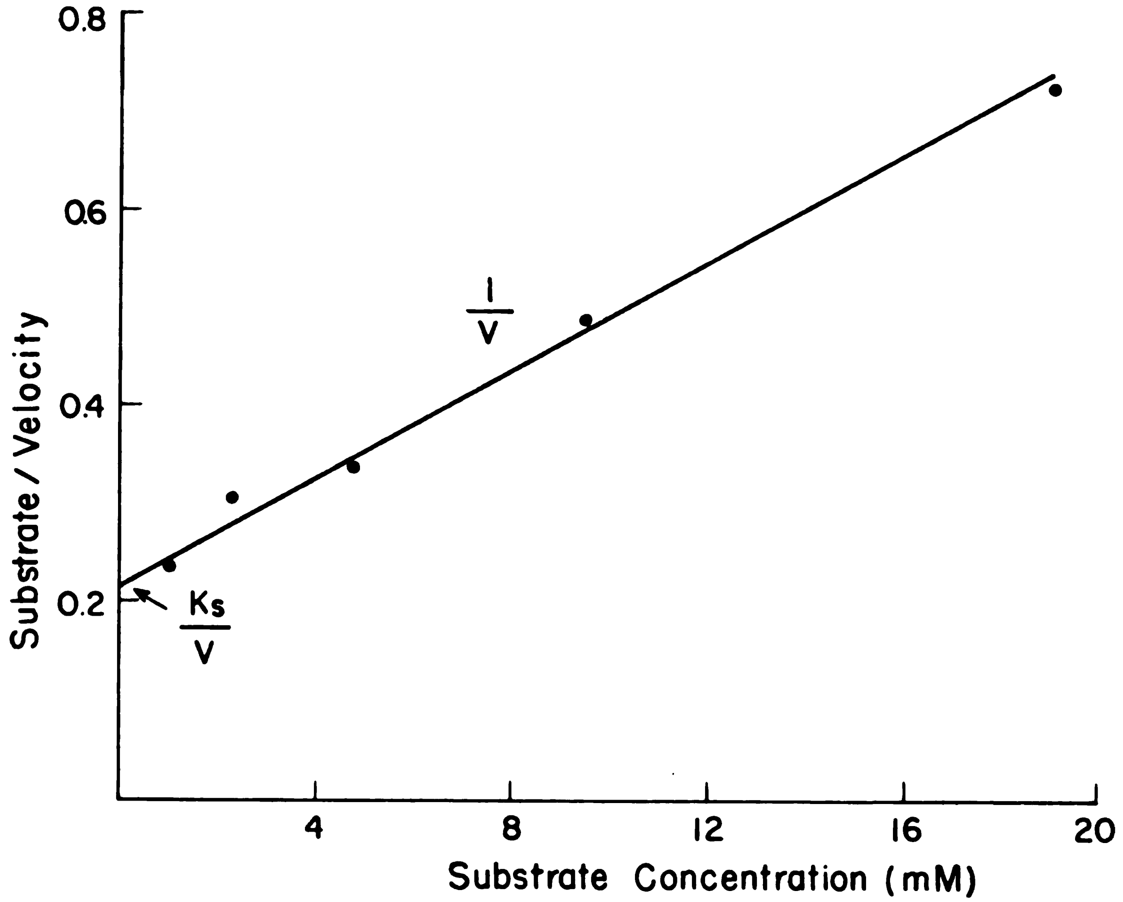

Fig. 11. Lineweaver-Burk plot of phosphatase (p-nitrophenylphosphate substrate) for the purpose of calculating the Michaelis constant.

Increases in Specific Activity of Coagulase and Phosphatase during Purification

Another approach was carried out to determine if the two activities were associated with the same protein. If two different catalytic groups resided on the same protein entity, then during the process of purification, one should find parallel increases in activity on the basis of protein. Consequently, coagulase was isolated from *S. aureus* strain 70 and purified as previously described. After each stage of purification, the material was assayed for its coagulase and phosphatase activity and protein content. The results obtained during the progressive purification steps of the coagulase and phosphatase are seen in Table 2. Examination of the specific activity of both activities revealed a parallel increase after each purification step. The evidence again suggested that the coagulase and phosphatase were associated with the same protein entity.

Studies on the Possible Possession of Lecithinase Activity by Coagulase

Drummond and Tager (1959a) reported Tager's earlier observation that when incubated with egg yolk, purified staphylocoagulase produced a turbidity resembling that formed by the toxin (lecithinase) of *Clostridium perfringens*. Chu (1947) showed that *Bacillus cereus* was capable of causing opacity in egg yolk medium due to its lecithinase. Staphylococci also produce this opacity (Gillespie and Alder, 1952). Since

TABLE 2. Comparison of specific activities of coagulase and phosphatase during purification.

Purification Step+	Specific Activity		Fold Increases in Activity	
	Coagulase*	Phosphatase** (x 10^{-3})	Coagulase	Phosphatase
A	0.70	0.85		
B	3.23	3.38	4.6	4.0
C	3.00	3.18	2.5	2.9
D	7.87	9.80	2.7	3.1
E	8.09	10.0		
F	15.33	18.0	1.8	1.8
G	36.10	32.0	2.3	1.8

+A = Initial broth supernatant fluid; B = Acid ppt, pH 3.8; C = Redissolved acid ppt from which insoluble residues removed by centrifugation; D = 1st ethanol ppt; E = 1st ethanol ppt, redissolved, and insoluble residues removed by centrifugation; F = 2nd ethanol ppt; G = Lyophilized, dialized residue.

*Reciprocal titer/µg protein

**µM p-nitrophenol liberated/µg protein

purified coagulase preparations were found, as reported in this thesis and elsewhere (Inniss and San Clemente, 1961), to possess a concomitant phosphatase activity, it seemed reasonable that the action on egg yolk might be due to this phosphatase activity. Consequently, studies were conducted to determine the possible lecithinase activity of the coagulase preparation. The lecithinase of Cl. welchii and B. cereus split lecithin into acid-soluble phosphorylcholine and the rate of increase of acid-soluble phosphorous served as a convenient measure of the enzyme activity. In the present investigation lecithinase was measured by determination of the increase of acid-soluble phosphorous. The method employed was essentially the same as that used by Macfarlane and Knight (1941) and Chu (1949). The reaction mixtures consisted of 1 ml of purified coagulase (0.5 mg/ml), 1.4 ml tris buffer pH 7.2 and 1.6 ml of a 3% lecithin emulsion. Controls consisted of coagulase without lecithin substrate and substrate alone. After incubation at 37 C for 30 min, the reaction was terminated by the addition of 20% trichloroacetic acid which also flocculated the unused lecithin. The contents of the tubes were then filtered through millipore filters and the total phosphorous in the filtrates was determined (Umbreit et al., 1957). No increase in total phosphate was found over that of the controls. The coagulase-phosphatase preparation, therefore, possessed no lecithinase activity.

DISCUSSION

One of the initial requirements for these investigative attempts at elucidation of the biochemical activities of staphylocoagulase was the production, isolation and purification of the clotting activity. The first step in the production of a coagulase preparation was the selection of a strain of S. aureus which possessed the ability to produce a large quantity of coagulase. A member of the International-Blair series of phage propagating strains (Blair and Carr, 1953) was considered desirable since considerable data had been previously obtained about these organisms in our laboratory and consequently the possibility was ever present that some fruitful relative information might develop. Preliminary investigations revealed that phage propagating strain 70 was the organism which best fulfilled this criterion.

The optimal conditions for the production of coagulase in relatively large quantities were also investigated. A biphasic growth system (Tyrrell et al., 1958) of brain heart infusion composition gave a greater yield of organisms than that obtained from growth in a nonbiphasic system of brain heart infusion broth. However, the latter system produced the same amount of coagulase and since it possessed the added advantage of ease of preparation, it was employed. Shaken cultures were employed to provide sufficient aeration and to facilitate release of the free coagulase into the growth medium. Under the cultural conditions used it was also observed that coagulase production was approximately proportional to growth up to 12 hours but after that time some loss of activity occurred even though growth continued. Consequently, the coagulase was harvested after 12 hours of growth.

As previously indicated, the isolation and purification procedures employed were essentially those developed by Tager (1948b) and more recently modified by Blobel _et al_. (1960). However, the use of ammonium sulfate as suggested by the former author was avoided since it has been reported to be unsuitable for the removal of impurities from coagulase solutions (Blobel _et al_., 1960).

The actual mechanism by which staphylocoagulase coagulates plasma is not known. Consequently, the finding of phosphatase activity in the purified preparations of coagulase suggested a new concept as to its biochemical mode of action. The possibility existed that a functional relationship existed between the two activities or indeed, perhaps, coagulase functioned in some manner with phosphatase action. However the occurrence of another activity in a protein preparation, which has been purified, presents one with the questions of whether the two activities represent the same or different functional groups on the same protein, or on different proteins.

When studies on the effect of temperature on the coagulase and phosphatase were carried out no differential effects were revealed. Both activities were inactivated to the same extent after the same times of heating. Since thermal inactivation is usually considered to be responsible for the rupture of chemical bondings throughout a protein structure and not any one specific type of bond, the results suggested that the same or a very similar protein was involved with both activities.

To ascertain if the above possibility did, in fact, exist, and that the phosphatase was not simply a separate molecular entity, co-purified with the plasma clotting activity, numerous attempts were made to bring about separation of the two activities. Anion exchange chromatography with DEAE-cellulose proved unsuccessful. At both pH 7.5 and pH 8.6, the coagulase and phosphatase activities emerged simultaneously from the column when subjected to gradient elution with sodium chloride. A series of electrophoretic studies were also conducted to achieve separation. Different buffer systems of various pH values were employed. Insoluble starch, starch gel and paper were used as a stabilizing media. Under the numerous experimental conditions employed, no successful separation of the two activities was obtained. Consequently, the possibility that the coagulase and phosphatase activities were associated with two different proteins was, indeed, reduced, although not entirely eliminated. However, all one can state with certainty in the light of the experimental results obtained, is that the two activities were physically inseparable under the experimental means employed.

Since the indication was that one protein was associated with both the coagulase and phosphatase activities, it remained to be resolved whether these two activities functioned by the same mechanism. The purified preparation was treated with various enzyme inhibitors to determine whether any differential effects were present. Since in no case was the degree of inhibition exerted by these compounds on each activity the same, it was concluded that the same functional groups were not involved.

Further problems of interest are posed by the studies with the enzyme inhibitors. Contrary to the findings of Drummond and Tager (1959b), it appears from the results that both the phosphatase and clotting activities of the coagulase are dependent upon sulfhydryl groups, the latter activity to a lesser extent. Similarly both activities were inhibited by sodium fluoride and EDTA suggesting the involvement of some essential metal(s). However many extensive studies of the effect of inhibitors on the plasma clotting system must be conducted before any firm conclusions may be drawn concerning the mode of coagulation.

Subsequent experiments involving the saturation of the phosphatase activity of the coagulase preparation with excess substrate verified the assumption that the same functional group was not involved with the two activities.

Studies were also carried out to determine the increases in specific activity of the staphylophosphatase and staphylocoagulase during various stages of the purification process. Since these values paralleled each other, the evidence again indicated that the same protein was associated with both activities. However it should be realized that for proteins of a very similar but not identical structure, it is undetermined whether any of the methods utilized would discriminate between two such compounds.

The evidence presented in this thesis represents an attempt to shed some light on the mode of action of staphylocoagulase. The results of our experimentation suggests the hypothesis that the coagulase and phosphatase activities represent two different functional groups associated with the same or a very similar protein entity. However, the author wishes to reemphasize the fact that to prove that this type of phenomenon exists, one must employ data which are usually of a suggestive, rather than conclusive nature. For example, even though numerous electrophoresis and exchange chromatography investigations indicated that the two activities were inseparable, this does not eliminate the possibility that another procedure might yield two separate entities. The absolute resolution of this problem will eventually require precise determination of molecular structure.

SUMMARY

A purified preparation of coagulase from S. aureus, phage propagating strain 70, was found to possess a concomitant phosphatase activity. In order to clarify this dual phenomenon a series of studies was undertaken. Thermal inactivation at 37 C and 56 C showed a parallel decrease in both activities. The preparation was subjected to DEAE-cellulose column chromatography at pH 7.5 and pH 8.6. Coincident maximum peaks of the two activities suggested that both functional groups were associated on the same protein. The two activities, when subjected to starch, starch gel, and paper electrophoresis under differing conditions of buffers and pH (pH 4.2 to 10), exhibited similar maximum peaks of activity after migration. An extremely small degree of separation may have been obtained when electrophoresis was carried out with a discontinuous buffer system. However, it was not possible to recover either activity in the complete absence of the other, because of the large amount of overlapping of the two activities.

EDTA, iodoacetate, fluoride, azide, and p-chloromercuri-benzoate exerted different rather than similar degrees of inhibition on each of the two activities indicating that they do not function by the same mechanism. This supposition was supported by complete saturation of the phosphatase with excess p-nitrophenylphosphate substrate (100-fold K_s value) and the demonstration that under this condition the coagulase was not inhibited.

Comparable increases were found in the total activity (on the basis of protein) for both the coagulase and phosphatase after each purification step. It is therefore hypothesized that the coagulase and phosphatase represent two different functional groups associated with the same or an extremely similar protein entity.

BIBLIOGRAPHY

ALDER, V. G., GILLESPIE, W. A., and HERDAN, G. 1953 Production of opacity in egg-yolk broth by staphylococci from various sources. J. Pathol. Bacteriol., 66, 205-210.

ALLEN, R. G. L. 1940 The estimation of phosphorous. Biochem. J., 34, 858-865.

BARBER, M., BROOKSBANK, B. W. L., and KUPER, S. W. A. 1951 Staphylococcal phosphatase, glucuronidase and sulphatase. J. Pathol. Bacteriol., 63, 57-64.

BARBER, M. and KUPER, S. W. A. 1951 Identification of Staphylococcus pyogenes by the phosphatase reaction. J. Pathol. Bacteriol., 63, 65-68.

BARNES, E. H. and MORRIS, J. F. 1957 A quantitative study of the phosphatase activity of Micrococcus pyogenes. J. Bacteriol., 73, 100-104.

BLAIR, J. E. 1939 The pathogenic staphylococci. Bacteriol. Rev., 3, 97-146.

BLAIR, J. E. 1958 Factors determining the pathogenicity of staphylococci. Ann. Rev. Microbiol., 12, 491-506.

BLAIR, J. E. and CARR, M. 1953 The bacteriophage typing of staphylococci. J. Infect. Dis., 93, 1-13.

BLOBEL, H., BERMAN, D. T., and SIMON, J. 1960 Purification of staphylococcal coagulase. J. Bacteriol., 79, 807-815.

BURNET, F. M. 1930 The production of staphylococcal toxin. J. Pathol. Bacteriol., 33 1-16.

CADNESS-GRAVES, B., WILLIAMS, R., HARPER, G. J., MILES, A. A. 1943 Slide-test for coagulase-positive staphylococci. Lancet 1, 736-738.

CHAPMAN, G. H. and BERENS, C. 1935 Crystal violet agar as a differential medium for staphylococci. J. Bacteriol., 29, 437-448.

CHAPMAN, G. H., BERENS, C., NILSON, E. L., and CURCIO, L. G. 1938 The differentiation of pathogenic staphylococci from non-pathogenic types. J. Bacteriol., 35, 311-334.

CHAPMAN, G. H., BERENS, C., PETERS, A., and CURCIO, L. G. 1934 Coagulase and haemolysin tests as measures of the pathogenicity of staphylococci. J. Bacteriol., 28, 343-363.

CHAPMAN, G. H., BERENS, C., and STILES, M. H. 1941 The coagulation of plasma by staphylococci. J. Bacteriol., 41, 431-440.

CHIAROLANZA, V. 1955 Contribution to the study of the pathogenicity of staphylococci. Brit. Abstr. Med. Sci., 2, 95.

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. Exptl. Biol. Med. Sci., 19, 323-327.

CHRISTIE, R. and KEOGH, E. V. 1940 Physiological and serological characteristics of staphylococci of human origin. J. Pathol. Bacteriol., 51, 189-197.

CHRISTIE, R., NORTH, E. A. and PARKIN, B. J. 1946 Criteria of pathogenicity in staphylococci. Aust. J. Exptl. Biol. Med. Sci., 24, 73-78.

CHU, H. P. 1949 The lecithinase of Bacillus cereus and its comparison with Clostridium welchii α-toxin. J. Gen. Microbiol., 3, 255-273.

COHN, E. J., STRONG, L. E., HUGHES, W. L., MULFOR, D. J., ASHWORTH, J. N., MELIN, M., and TAYLOR, H. L. 1946 Preparation and properties of serum and plasma proteins. J. Am. Chem. Soc., 68, 459-475.

CRUICKSHANK, R. 1937 Staphylocoagulase. J. Pathol. Bacteriol., 45, 295-303.

DARÁNYI, J. Von. 1926 Pathogenitat und Einteilung der Staphylokokken. Zentr. Bakteriol., I. Abstr. Orig., 99, 74-79.

DEVENISH, E. A. and MILES, A. A. 1939 Control of Staphylococcus aureus in an operating theatre. Lancet 1, 1088-1094.

DRUMMOND, M. C. and TAGER, M. 1959a Enzymatic activity of staphylocoagulase. I. Characterization of an esterase associated with purified preparations. J. Bacteriol., 78, 407-412.

DRUMMOND, M. C. and TAGER, M. 1959b Enzymatic activity of staphylocoagulase. II. Dissociation of plasma clotting from tributyrinase activity. J. Bacteriol., 78, 413-421.

DUDGEON, L. S. 1908 The differentiation of the staphylococci. J. Pathol. Bacteriol., 12, 242-257.

DUTHIE, E. S. 1954a Evidence for two forms of staphylococcal coagulase. J. Gen. Microbiol., 10, 427-436.

DUTHIE, E. S. 1954b The production of free staphylococcal coagulase. J. Gen. Microbiol., 10, 437-444.

DUTHIE, E. S. 1955 The action of fibrinogen on certain pathogenic cocci. J. Gen. Microbiol., 13, 383-386.

DUTHIE, E. S. and HAUGHTON, G. 1958 Purification of free staphylococcal coagulase. Biochem. J., 70, 125-134.

DUTHIE, E. S. and LORENZ, L. 1952 Staphylococcal coagulase: mode of action and antigenicity. J. Gen. Microbiol., 6, 95-107.

ELEK, S. D. 1959 Staphylococcus pyogenes and its relation to disease. E. and S. Livingstone Ltd., Edinburgh and London.

FAIRBROTHER, R. W. 1940 Coagulase production as a criterion for the classification of the staphylococci. J. Pathol. Bacteriol., 50, 83-88.

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

FISK, A. 1940 The technique of the coagulase test for staphylococci. Brit. J. Exptl. Pathol., 21, 311-314.

FLAUM, A. 1938 Studies on staphylococci and staphylococcal immunity. Acta Pathol. Microbiol. Scand. Suppl., 35, 1-137.

FULTON, F. 1943 Staphylococcal enterotoxin - with special reference to the kitten test. Brit. J. Exptl. Pathol., 24, 65-73.

FUSILLO, M. H. and JAFFURS, W. J. 1955 Relationship of coagulase and urease in Micrococcus pyogenes. J. Bacteriol., 70, 481.

GERHEIM, E. B., FERGUSON, J. H., and TRAVIS, B. L. 1947 Activation of staphylocoagulase. Proc. Soc. Exptl. Biol. Med., 66, 525-527.

GILLESPIE, E. H., DEVENISH, E. A., and COWAN, S. T. 1939 Pathogenic staphylococci: their incidence in the nose and on the skin. Lancet, 2, 870-873.

GILLESPIE, W. A. and ALDER, V. G. 1952 Production of opacity in egg-yolk media by coagulase-positive staphylococci. J. Pathol. Bacteriol., *64*, 187-200.

GOLDBERG, C. A. J. 1959 A discontinuous buffer system for paper electrophoresis of human hemoglobins. Clin. Chem., *5*, 446-451.

GONZENBACH, W. Von and UEMURA, H. 1916 Beitrag zur Gerinnung von Plasma durch Wirkung des *Staphylococcus pyogenes aureus*. Zentr. Bakteriol., I. Abstr. Orig., *78*, 97-103.

GRATIA, A. 1920 Influence du milieu sur la production de la "staphylocoagulase." Compt. Rend. Soc. Biol., *83*, 585-587.

GROSS, H. 1931 Der Plasmagerinnungstoff der Staphylokokken. Zentr. Bakteriol., I. Abst. Orig., *122*, 354-361.

GUPTA, S. P. and CHAKRAVARTI, R. N. 1954 Observations on some biochemical reactions of staphylococci with special reference to coagulase and phosphatase production. Ind. J. Med. Res., *42*, 131-134.

HALLMAN, F. A. 1937 Pathogenic staphylococci in the anterior nares: their incidence and differentiation. Proc. Soc. Exptl. Biol. Med., *36*, 789-794.

HARPER, E. M. and CONWAY, N. S. 1948 Clotting of human citrated plasma by gram-negative organisms. J. Pathol. Bacteriol., *60*, 247-251.

HILLS, G. M. 1940 Ammonia production by pathogenic bacteria. Biochem. J., *34*, 1057-1382.

HINE, T. G. M. 1922 Serological classification of the staphylococci. Lancet, *2*, 1380-1382.

INNISS, W. E. and SAN CLEMENTE, C. L. 1961 Association of staphylocoagulase and staphylophosphatase activities in partially purified preparations. Bacteriol. Proc., 110.

JACHERTS, D. 1956 Experimentelle Untersuchungen über die Identität freier und gebundener Coagulase. Z. Hyg. Infekt-Krankh., *142*, 502-509.

JULLIANELLE, L. A. 1937 Determination of staphylococcal types by fermentation of mannite. Proc. Soc. Exptl. Biol. Med., *36*, 117-119.

KAPLAN, M. H. and SPINK, W. W. 1948 Studies of the staphylocoagulase reaction: nature and properties of a plasma activator and inhibitor. Blood, 3, 573-585.

LINSELL, W. D. and GORRILL, R. H. 1951 The effects of heat and sodium ethyl mercuri-thio-salicylate on the staphylocoagulase test. J. Clin. Pathol., 4, 73-77.

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

LOMINSKI, I. and MILNE, J. A. 1947 Heat resistance and filterability of staphylocoagulase. J. Pathol. Bacteriol., 59, 516-518.

LOMINSKI, I., MORRISON, R. B., and PORTER, I. A. 1952 Formation of citrulline by Staphylococcus aureus in an arginine-containing medium. Biochem. J., 51, xvii.

LOVELL, H. R. 1958 Incorporation of disodium p-nitrophenyl-phosphate in solid medium for the detection of phosphatase production by Micrococcus pyogenes var. aureus. Nature, 181, 995-996.

LOWRY, O. H., ROSEBROUGH, N. J., FARR, A. L., and RANDAL, R. R. 1951 Protein measurement with the Folin phenol reagent. J. Biol. Chem., 193, 265-275.

MACFARLANE, M. G. and KNIGHT, B. C. G. J. 1941 The biochemistry of bacterial toxins. I. The lecithinase activity of Cl. welchii toxins. Biochem. J., 35, 884-902.

MALIK, B. S. and SINGH, C. M. 1960 Studies on staphylococci with particular reference to strains from bovine udder. I. Biochemical activities. Proc. Nat. Acad. Sci. India, 30, 101-108.

MIALE, J. B. 1949 The role of staphylocoagulase in blood coagulation. I. The reaction of staphylocoagulase with coagulase-globulin (C.G.) to form coagulase-thrombin (C.T.). Blood, 4, 1039-1048.

MINETT, F. C. 1936 Staphylococci from animals with particular reference to toxin production. J. Pathol. Bacteriol., 42, 247-263.

MUCH, H. 1908 Ueber eine Vorstufe der Fibrinfermentes in Kulturen von Staphylokokkus aureus. Biochem. Z., 14, 143-155.

MURRAY, M. and GOHDES, P. 1959a Role of coagulase reacting factor in the activation of fibrinogen. J. Bacteriol., **78**, 450-451.

MURRAY, M. and GOHDES, P. 1959b The purification of staphylococcus coagulase. Federation Proc., **18**, 1956.

PENFOLD, J. B. 1944 Coagulase production by staphylococci on a solid medium. J. Pathol. Bacteriol., **56**, 247-250.

REID, J. D. and JACKSON, R. M. 1945 An improved method for determining the coagulase activity of staphylococci by means of plasma agar plate. J. Lab. Clin. Med., **30**, 155-160.

ROCHE, J. 1950 The phosphatases. In *The enzymes*. Sumner, J. B. and Myrback, K., editors. Academic Press, Inc., New York, N.Y.

SCHLAM, O. W. and WOODS, G. M. 1953 *Micrococcus pyogenes* in bovine milk. I. Identification. Am. J. Vet. Res., **14**, 530-533.

SEEGERS, W. H. and ALKJAERSIG, N. 1953 Comparative properties of purified human and bovine prothrombin. Am. J. Physiol., **172**, 731-736.

SMITH, W. and HALE, J. H. 1944 The nature and mode of action of staphylococcus coagulase. Brit. J. Exptl. Pathol., **25**, 101-110.

SMITH, W., HALE, J. H., and SMITH, M. M. 1947 The role of coagulase in staphylococcal infections. Brit. J. Exptl. Pathol., **28**, 57-67.

SMITHIES, O. 1955 Zone electrophoresis of serum in starch gels: group variations in the serum proteins of normal human adults. Biochem. J., **61**, 629-641.

STEPHAN, F. 1935 Der Wert der Plasma-Agglutinationsreaktion zur Differenzierung der Staphylokokken. Z. Hyg., **116**, 550-555.

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**, 487-501.

TAGER, M. 1948b Concentration, partial purification and nature of staphylocoagulase. Yale J. Biol. Med., **20**, 487-501.

TAGER, M. 1953 The comparative action of dicumarol and of phenylindanediol on the coagulase-reacting factor and on prothrombin. Yale J. Biol. Med., 25, 374-382.

TAGER, M. 1954 Staphylococcus coagulase. Bull. N.Y. Acad. Med., 30, 475-477.

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

TAGER, M. 1956b Studies on the nature and the purification of the coagulase-reacting factor and its relation to prothrombin. J. Exptl. Med., 104, 657-686.

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.

TYRRELL, E. A., MacDONALD, R. E., and GERHARDT, P. 1958 Biphasic system for growing bacteria in concentrated culture. J. Bacteriol., 75, 1-4.

UMBREIT, W. W., BURRIS, R. H., and STAUFFER, J. F. 1957 Manometric techniques. Burgess Publishing Co., Minneapolis.

WALKER, B. S., DEROW, M. A., and SCHAFFER, N. K. 1948 The partial purification of staphylocoagulase and the effect of certain presumptive inhibitors upon its plasma-coagulating action. J. Bacteriol., 56, 191-194.

WALKER, B. S., SCHAFFER, N. K., and DEROW, M. A. 1947 Inactivation of staphylocoagulase by trypsin and pepsin. Science, 106, 347.

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

WILLIAMS, R. E. O. and HARPER, G. J. 1946 Determination of coagulase and alpha-hemolysin production by staphylococci. Brit. J. Exptl. Pathol., 27, 72-81.

WILSON, P. W. 1950 Kinetics and mechanisms of enzyme reactions. In Respiratory enzymes. Lardy, H. A., editor. Burgess Publishing Co., Minneapolis.

WINSLOW, C. E. A., ROTHBERG, W., and PARSONS, E. I. 1920 Notes on the classification of the white and orange staphylococci. J. Bacteriol., 5, 145-167.

WOODS, E. F. and PARKIN, B. 1946 The plate test for coagulase production by staphylococci. Aust. J. Exptl. Biol. Med. Sci., 24, 33-35.