

PRODUCTION, PURIFICATION, QUANTITATION AND
KINETICS OF STAPHYLOCOAGULASE

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ABSTRACT

PRODUCTION, PURIFICATION, QUANTITATION, AND KINETICS OF STAPHYLOCOAGULASE

by Fred J. Stutzenberger

Extracellular coagulase, produced by Staphylococcus aureus Strain 70 in a low-protein casein acid hydrolysate medium, was purified in a series of steps involving isoelectric precipitation, ion exchange, ammonium sulfate fractionation, and gel filtration. At least 50% of the original coagulase activity was retained during a 45-fold increase in specific activity to yield a finally purified product having 336,000 reciprocal titer units per mg of protein. This preparation produced a single precipitin band in analysis by immunodiffusion and was free of lipase, phosphatase, DNase, and hemolysins. Thermal inactivation of coagulase suggested the presence of two isoenzymatic forms which persisted in definite proportions (70% and 30%) throughout the purification process. In the finally purified coagulase preparations, the half-life of the more stable component was calculated to be 66 min at 60 C, while that of the labile form was 4.7 min at that temperature.

The inherent inadequacy of the titer method initiated a search for a more quantitative means of measuring coagulase

activity. To fulfill this need, a precise nephelometric method was developed which proved to be a sensitive technique under optimal conditions of pH (7.1-7.3), ionic strength (0.07-0.10), and substrate concentration (2.2 μM fibrinogen). Non-inhibitory human plasma (2% v/v) was included in the substrate as a source of the coagulase-reacting factor (CRF). The observed clotting activity was directly proportional to the coagulase concentration under these optimal conditions.

This nephelometric method was later extended to measure and study inhibition of coagulase by bovine antiserum. The technique was well suited to the quantitation of such coagulase inhibitors. In addition, heated antiserum (56 C, 30 min) not only lost much of its ability to inhibit coagulase, but also exhibited a different type of inhibition. Heated antiserum appeared to act as a competitive inhibitor, while unheated antiserum acted in a non-competitive manner. A mechanism was postulated to explain these differences.

Kinetic analyses, based on the nephelometric assay, indicated that the coagulase reaction followed classical Michaelis-Menton kinetics. The K_m for fibrinogen in the reaction was calculated to be 6.35×10^{-7} M. There appeared to be no interaction between fibrinogen-binding sites, or between those binding CRF on the coagulase molecule. Furthermore, the binding of CRF had no apparent effect on the binding of fibrinogen on the coagulase molecule, nor

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did the binding of fibrinogen have any effect on the binding of CRF. Variation of the K_m value for CRF in proportion to the concentration of coagulase eliminated the possibility that it was a substrate and suggested its role as an essential activator which bound stoichiometrically to the coagulase molecule.

PRODUCTION, PURIFICATION, QUANTITATION
AND KINETICS OF STAPHYLOCOAGULASE

By

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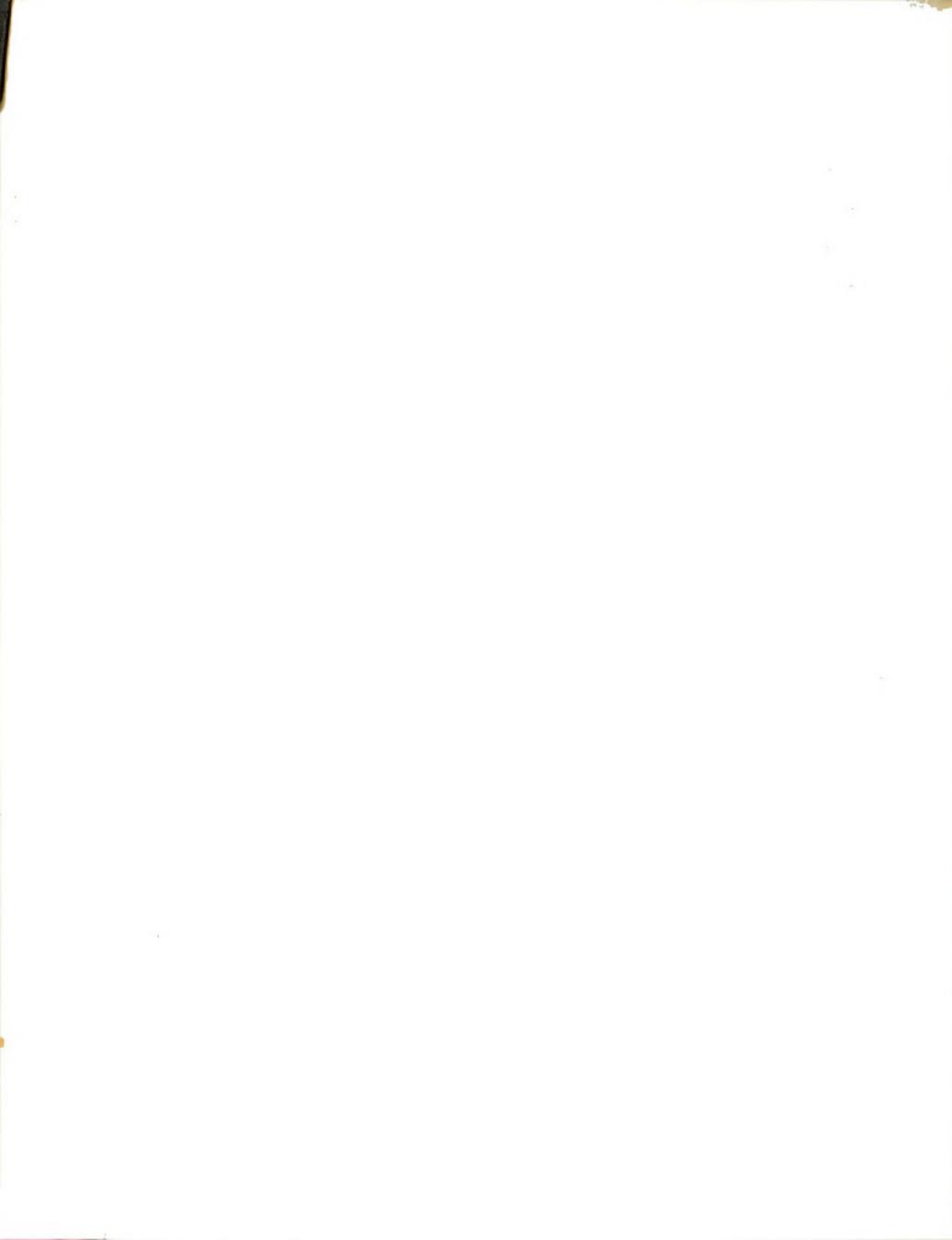


INTRODUCTION

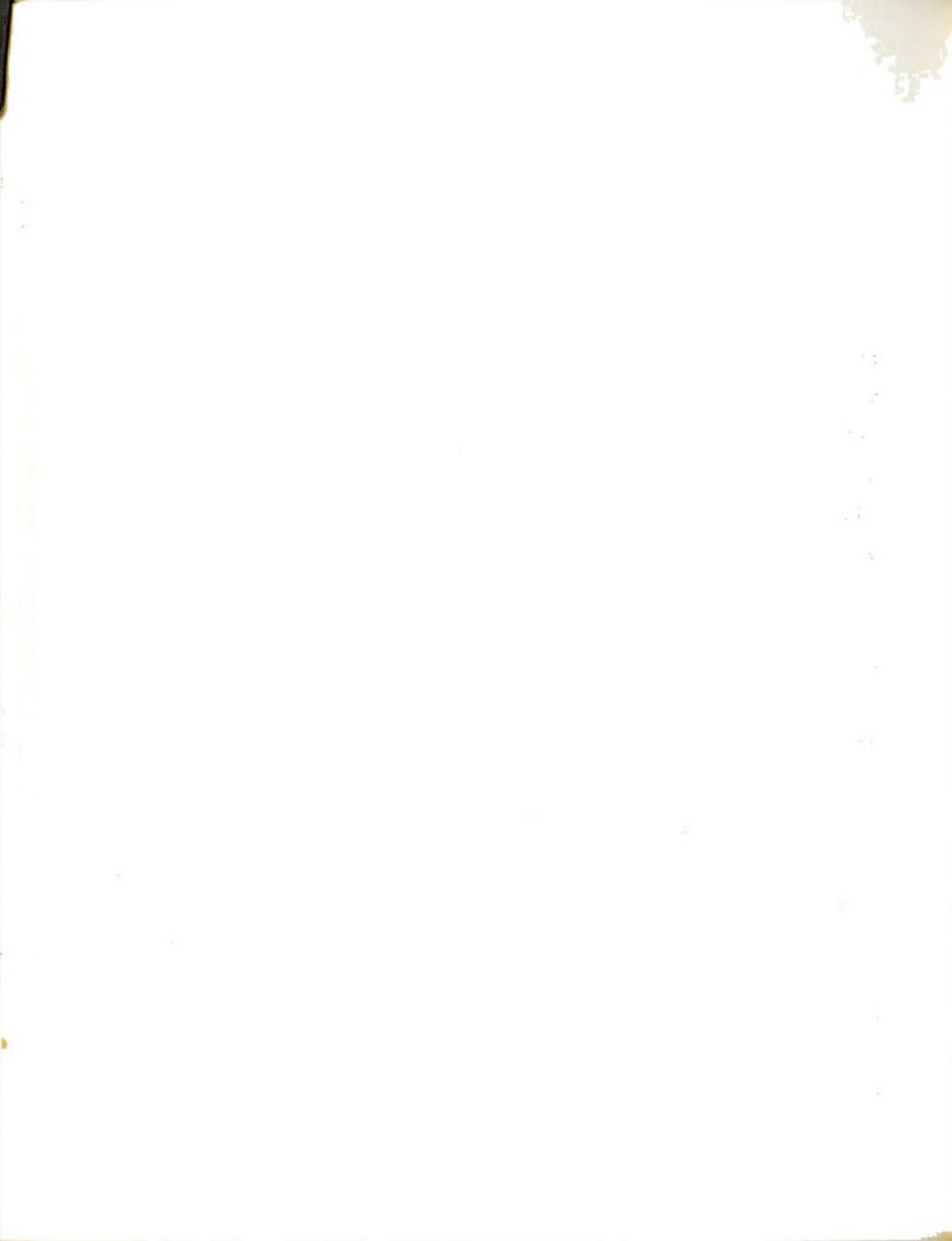
The close relationship between coagulase production and virulence in Staphylococcus aureus (Elek, 1959) has initiated many studies on the nature of the clotting enzyme and its role in staphylococcal infections. Progress in these areas has been hampered by the lack of a precise method for the determination of coagulase activity. Therefore, investigators have been unable to determine the mechanism by which coagulase converts soluble fibrinogen into a fibrin clot.

One aspect of the reaction which has been especially obscure is the relationship of the coagulase-reacting factor (CRF) to coagulase and the fibrinogen substrate. Several plasma protein fragments have been linked with CRF activity (Tager, 1956), and its identity as a prothrombin derivative has been suggested. Although CRF has been implicated as an enzyme (Haughton and Duthie, 1959), as an accelerator (Murray and Gohdes, 1959), and as an essential activator (Tager and Drummond, 1965), the nature of its role in the clotting reaction has not been elucidated.

The purpose of the investigation reported herein was to: a) arrive at a simplified procedure for the production and purification of staphylococcal free coagulase; b) to



develop a precise and quantitative method for the measurement of coagulase activity; c) to employ this quantitative assay as a basis for a series of kinetic studies on the coagulase reaction, with particular stress on the role of CRF.



REVIEW OF LITERATURE

Coagulase Purification

Coagulase, the fibrinogen-clotting factor produced by Staphylococcus aureus, has often been associated with virulence of the staphylococci (Boake, 1956; Ekstedt and Yotis, 1960; Tager and Drummond, 1965). Therefore, many investigators have attempted the purification of the clotting enzyme. However, in this review only the more significant purification procedures will be described.

Tager (1948) was one of the earliest workers to report significant purification of staphylococcal coagulase. Cultures of S. aureus 104 were grown at 37 C for 4-6 days in Brain Heart Infusion. The cells were removed by centrifugation and the supernatant fluid was then acidified at 4 C to pH 3.8 with 4 N HCl to precipitate extracellular coagulase. After 12-24 hrs, this precipitate was collected, washed at pH 3.8 with phosphate buffer, and redissolved at pH 8.2. A second cycle of precipitation was accomplished by acidification to pH 6.5, followed by addition of three volumes of 95% ethanol at -5C. This precipitate was washed, redissolved at pH 8.2, and saturated to 12% with ammonium sulfate to precipitate extraneous protein. After several cycles of this ethanol and ammonium sulfate fractionation, the specific

activity of the purified coagulase had increased 300-400 fold to a final specific activity of 691,666 titer units per mg nitrogen. The finally purified coagulase was found to be a thermolabile, non-dialyzable protein which appeared to be most stable at pH 4 to 7.

Another group of workers (Walker et al., 1948) found that coagulase in its crude state was very heat stable. Cell-free filtrates of S. aureus cultures grown at 37C in beef heart tryptic digest broth were autoclaved at 120 C to denature and precipitate contaminating proteins. The coagulase was then precipitated from the supernatant fluid by acidification to pH 4.0 with HCl. After several hrs at 0 C, the precipitate was washed twice at pH 4.0 with cold sodium acetate buffer and finally with cold distilled water. The redissolved precipitate was adjusted to pH 7.5, the insoluble material was discarded, and the purified coagulase was stored in the lyophilized state. The maximum specific activity was 9000 titer units per mg of protein.

Duthie and Lorenz (1952) employed cadmium sulfate in the purification of coagulase. Cultures of S. aureus Strain Newman were cultivated in nutrient broth at 37 C. After nine hrs stationary growth, followed by three hrs shaking, the cells were removed by mixing with Filter-Cel and subsequent filtration. This cell-free broth was cooled to 4 C, 0.5% cadmium sulfate was added, and the pH was adjusted to 5.8. The resulting precipitate was dissolved in 1 N HCl at pH 2.0. Cadmium was removed by dialysis at this pH and the

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purified coagulase was lyophilized. The finally purified product had a specific activity of 6000 minimal clotting doses per mg of protein.

This procedure was later extended and modified by Duthie and Haughton (1958). A casein hydrolysate medium was employed for culture growth and production of extracellular coagulase. The use of this medium eliminated much of the contaminating protein that was present in such a complex medium as Brain Heart Infusion. However, these workers obtained only poor coagulase production when the inocula were grown in the simplified medium. Therefore, Brain Heart Infusion or Ox Heart Digest was used for cultivation of the inocula, and much of the advantage of the low-protein casein hydrolysate medium was lost.

Murray and Gohdes (1960) also used cadmium sulfate fractionation in their purification procedure. Tryptose broth cultures of S. aureus were cultivated at 37 C for 72 hrs. The cells were removed and 33% (w/v) ammonium sulfate was added to the supernatant fluid. After 24 hrs at 4 C, the precipitate was collected and redissolved in 3.2% (w/v) sodium citrate solution at pH 5.0. Contaminating protein was then removed at 4 C with two volumes of 0.01 M cadmium sulfate at the same pH. The supernatant fluid was adjusted to pH 6.8 and saturated to 30% with ammonium sulfate at 4 C. The resulting precipitate was redissolved in a minimal amount of distilled water and exhaustively dialyzed. The final purification step was passage through a calcium

phosphate hydroxyapatite column at pH 6.8 and elution with increasing concentrations of phosphate buffer. This preparation appeared homogeneous on moving boundary electrophoresis and ultracentrifugation.

Coagulase was partially purified by Blobel et al. (1960) by initially employing acid precipitation and ethanol fractionation. Staphylococci were cultivated at 37 C in Heart Infusion shake cultures for 5 days. After removal of the cells, the free coagulase was precipitated from the cold supernatant fluid at pH 3.8 by addition of 4 N HCl. This precipitate was redissolved at pH 7.4, 70% ethanol was added at -20 C, and the resulting precipitate was dissolved in distilled water. After two cycles of this alcohol precipitation, the purified coagulase was dialyzed against distilled water and lyophilized. Further purification was later achieved by starch block electrophoresis at pH 8.4. After migration for 16 hrs at 5 C, the block was segmented, the active fractions eluted, concentrated, and adsorbed onto a DEAE-Cellulose column at pH 7.5. Increasing concentrations of NaCl were used to elute the purified coagulase, and the active fractions were lyophilized.

Innis and San Clemente (1962) were unable to separate phosphatase from coagulase. Elution from DEAE-Cellulose columns with 0.01 M tris buffer resulted in fractions which possessed both coagulase and phosphatase activity. At this time, it appeared that perhaps one molecular species possessed both activities.

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A year later, Zolli and San Clemente (1963) reported an improved procedure for coagulase purification, based on the technique of Cohn et al. (1946). This technique, originally employed in the fractionation of blood plasma components, consisted of variation of ionic strength, pH, temperature, and protein and alcohol concentrations. Three cycles of dialysis in ethanol-water mixtures under controlled conditions, followed by molecular sieving through Sephadex G-200, yielded a coagulase preparation which appeared homogeneous electrophoretically and immunochemically. Twelve percent of the original activity was retained during a 3,793 fold increase in purity, resulting in a specific activity of 220,000 titer units per mg of protein.

Free coagulase from a culture filtrate of S. aureus 213 was highly purified by Hitokoto (1965). Initial precipitation and concentration of coagulase was accomplished by addition of cold trichloroacetic acid. The redissolved precipitate was purified by ion exchange on a column of Dowex-1 resin, followed by passage through a DEAE-Sephadex A-50 column. The purity of the final fractions was increased 280-fold compared to the crude filtrate. Coagulase purified by this technique was composed of lysine, aspartate, threonine, serine, glutamine, glycine, alanine, valine, and methionine, but no carbohydrate, lipid, or phosphate was detected. The finally purified preparation was found to be homogeneous in immunochemical, electrophoretic, and ultracentrifugal studies.

Quantitation of Coagulase

At least 45 modifications of coagulase assay methods have been reported in the literature (Tager and Drummond, 1965). Most of these assay methods are based on either the two-fold serial dilution titer or the determination of plasma clotting time.

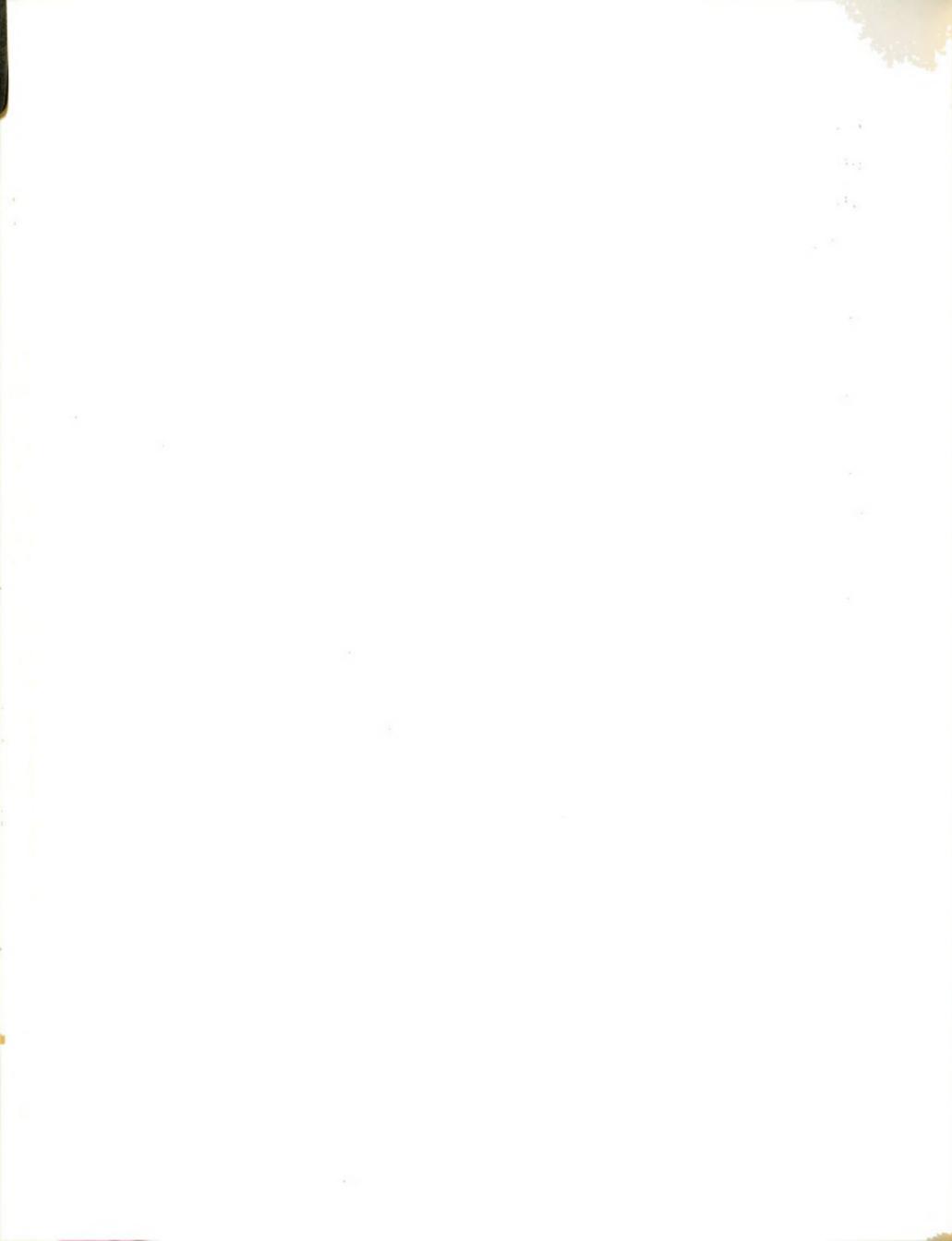
Tager and Hales (1948) employed the two-fold serial dilution titer method for measuring coagulase production by staphylococci of clinical importance. Beginning with a 1:10 dilution of the original preparation, serial two-fold dilutions were performed in total volumes of 0.5 ml; a diluent of 2% peptone-saline containing 1:5000 thimerosal was uniformly employed. After dilution, 0.5 ml of human citrated plasma was added to each tube, followed by incubation at 37 C. Readings were recorded during the first hr and at 24 hrs. The titer was recorded as the highest dilution causing a detectable clot within 24 hrs. A known coagulase preparation was included for titration in order to insure validity of the test conditions.

Plasma clotting time was the basis for the assay of Duthie and Lorenz (1952), later modified by Duthie and Haughton (1958). Fresh human plasma was diluted 1:5 in saline and added in 0.2 ml amounts to test tubes in a 37 C water bath. Immediately before assay, one drop of a 1% suspension of Filter-Cel was added to each tube. A coagulase preparation (0.2 ml) was added to a tube and viewed by indirect light against a black background while being tapped

with one finger. The end point was taken as that amount of time required for a rapid agglutination of the Filter-Cel particles. A curve was constructed which related the concentration of coagulase to the clotting time in seconds. One coagulase unit was defined as the activity causing a clot in 20 seconds under the standardized assay conditions.

Maidanova (1964) reported a turbidimetric method in which the clotting of fibrinogen by coagulase and thrombin was followed by the increase in light absorbance at 413 m μ . Coagulase and thrombin were prepared in stock solutions of 0.5% Tween 80 to a final concentration of 0.05 g enzyme per ml. The total reaction mixture contained three ml of 4% fibrinogen in 0.04 M barbiturate, human plasma as the source of CRF, and 0.1 ml of the stock enzyme preparation. A sharp increase in turbidity was recorded between 15 sec and 5 min incubation. Maximum coagulase activity was attained at 27-33 C with a pH of 6.9-7.0 and a fibrinogen concentration of 0.20 to 0.26%.

Methods for the determination of coagulase inhibition by antiserum have usually been based upon a reduction of the coagulase titer or an increase in the minimal clotting time. Lominski and Roberts (1946) inactivated human antiserum samples at 56 C for 30 min prior to assay. Varying concentrations of antiserum in saline were then incubated with a cell-free filtrate of a coagulase-positive culture grown in 10% rabbit plasma. At least 1.5 to 2 hrs at 37 C were required for maximum inhibition of coagulase activity. This



coagulase-antiserum mixture was then added to equal volumes of fresh, non-inhibitory, citrated human plasma. The reduction in coagulase titer as compared to the uninhibited controls was recorded at intervals up to 24 hrs.

The anticoagulase test of Duthie and Lorenz (1952) specified that the antiserum was not heat inactivated, but rather just incubated with heparin to destroy thrombin. The antiserum was then progressively diluted from 1:2 to 1:256 in 0.1 ml volumes of saline at pH 7.0. Equal volumes of a coagulase preparation containing 20 minimal clotting doses per ml were added to each tube and incubated for 30 min at room temperature. A 0.1% suspension of fibrinogen with 5% rabbit plasma was added in 0.2 ml quantities to each tube and incubated for one hr at 37 C. One drop of 1% Super-cel suspension was then added to each tube, and the tubes were incubated for 30 min at 4 C. The reciprocal of antiserum dilution just sufficient to allow minimal clotting was taken as the titer and expressed in anticoagulase units.

Coagulase Mode of Action

Although staphylococcal coagulase has been studied in a qualitative and semi-quantitative manner for many years, the exact mode of action by which it brings about the clotting of fibrinogen remains obscure (Tager and Drummond, 1965). On the other hand, brilliant advances have been made in elucidating the enzymatic events which result in the clotting of fibrinogen by thrombin. It would be advantageous

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therefore to review these advances as a basis for future study on coagulase activity.

Seegers et al. (1945) prepared purified prothrombin, the precursor of thrombin, by initially concentrating the active material in an isoelectric precipitation with 1% acetic acid. The precipitate was redissolved, and prothrombin was adsorbed onto $Mg(OH)_2$. After elution under two atmospheres of CO_2 , the preparation was fractionated with ammonium sulfate, and finally purified by another cycle of isoelectric precipitation. The purified prothrombin contained 14.5% nitrogen, 10% tyrosine, and 4.3% carbohydrate with a small amount of glucosamine. Prothrombin was later characterized as an α_2 -globulin with an isoelectric point of pH 4.2 and a molecular weight of 63,000 (Lamy and Waugh, 1953).

Prothrombin is converted to active thrombin by a number of thromboplastic substances released from cells during injury. Calcium ions are required in this activation and the system may be readily inhibited by the addition of chelating agents such as citrate or oxalate (West and Todd, 1961).

In the presence of thrombin, fibrinogen is rapidly converted to an insoluble fibrin clot under suitable conditions of pH and ionic strength. This conversion is now known to involve three major steps (Laskowski et al., 1956): (1) fibrinogen is acted upon by thrombin and is converted to activated fibrin monomers; (2) these monomers then

polymerize to form soluble fibrin intermediates; and (3) soluble fibrin monomers then interact further to form the insoluble fibrin clot.

Laki and Mommaerts (1945) were the first workers to demonstrate that thrombin was not involved in the two polymerization phases of the fibrinogen-fibrin transformation. At pH 5.1, only the activation phase occurred, resulting in an accumulation of fibrin monomers. However, upon neutralization, clot formation occurred at a rate proportional to the pre-clotting time at low pH. Later, in 1949, Laki concluded that the actual mode of action of thrombin was the alteration of the fibrinogen molecule by removal of acidic groups. Polymerization could then occur spontaneously, independently of thrombin.

Through the coordinated work of Bailey and his associates (1951), it was discovered that during the primary stages of the clotting reaction, fibrinogen lost N-terminal glutamic acid residues. Subsequently, Lorand (1951) observed that non-protein nitrogen was cleaved off during the action of thrombin upon fibrinogen, and suggested that it was a peptide in nature, more specifically, a "fibrinopeptide". His observations were concurrently confirmed by Laki (1951) who found ninhydrin-positive material in the deproteinated supernatant fluids after iodinated fibrinogen had been clotted by thrombin. The existence of not one but two fibrinopeptides was demonstrated by Bettelheim and Bailey (1952). These two peptides, which they designated A and B,

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were dialyzable, non-precipitable by trichloroacetic acid, and contained most of the common amino acids. Both peptides contained aspartic and glutamic acids, glycine, threonine, valine, leucine, phenylalanine, proline, and arginine. Fibrinopeptide A contained serine, but no alanine or tyrosine, while fibrinopeptide B contained both alanine and tyrosine, but no serine. Although both peptides were rather acidic due to their high content of aspartic and glutamic acids, fibrinopeptide A was more strongly anionic and contained all of the detectable N-terminal glutamyl residues. The B fibrinopeptide contained some ϵ -amino groups, but no N-terminal residues capable of reacting with fluorodinitrobenzene.

Lorand and Middlebrook (1952) determined the N-terminal amino acid residues of both fibrinogen and fibrin by employing the dinitrophenyl method. In the fibrinogen molecule, two chains ended in tyrosine and one in glutamic acid. The same unit weight of fibrin possessed two N-terminal residues of tyrosine and four of glycine. It appeared that the action of thrombin resulted in the cleavage of glycy-peptide bonds within the fibrinogen molecule, resulting in the activated fibrin monomer and free peptides containing the N-terminal glutamyl residues.

Thrombin, already defined in terms of its proteolytic activity, was also shown to hydrolyze synthetic arginine ester substrates as well as fibrinogen. Sherry and Troll (1954) found that alpha-N-tosyl arginine methyl ester (TAME)

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was readily cleaved by thrombin to the corresponding acid and alcohol. Thrombin activity could be measured in this manner, much the same as that of the proteolytic enzymes trypsin and chymotrypsin. Kinetics of the reaction were initially of zero order, the pH optimum in phosphate buffer was 7.6, and the K_m was calculated to be 4.3×10^{-3} M. Elmore and Curragh (1963) studied the kinetics of the reaction in greater detail and concluded that thrombin hydrolyzed TAME in a three-step sequence similar to that proposed for trypsin: (1) formation of a Michaelis-Menton complex; (2) acylation of the enzymic catalytic site with the liberation of one product; (3) deacylation of the enzyme to yield the second product and the free enzyme.

In recent years, research on the hydrolytic activity of thrombin has been extended to include many other synthetic substrates. Sherry et al. (1965) found that thrombin rapidly hydrolyzed various substituted lysine esters as well as arginine esters. The highest maximal activity was obtained with carbobenzoxylysine methyl ester, but benzoylarginine methyl ester possessed the greatest affinity for the enzyme. Kezdy et al. (1965) demonstrated the hydrolytic activity of thrombin on N-benzyloxycarbonyl-L-tyrosine-p-dinitrophenyl ester; the K_m for this synthetic substrate was 7.15×10^{-6} M.

Electrochemical analysis of fibrinogen clotting has provided additional information, not only about the thrombin-fibrinogen relationship, but also concerning the second and third phases of the clotting process. Mihalyi (1954a)

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titrated fibrinogen and fibrin dissolved in unbuffered 3.3 M urea. From pH 5.5 (the lower limit for the clotting phases) to pH 6.8, fibrinogen and fibrin titrated identically. However, during the thrombin-catalyzed reaction at pH 8.5, the formation of fibrin caused a decrease of 0.2 pH units. During electrophoresis in a Tiselius apparatus, fibrin had a lower mobility above its isoelectric point, and a higher mobility below its isoelectric point when compared to fibrinogen.

In further studies, (Mihalyi, 1954b), the fibrinogen clotting process was accompanied by a marked pH change over the entire range of 5.6 to 9.8. Below an unbuffered pH of 8.0, acidification was apparent, while above that pH, an initial acidification occurred, followed again by an increase in pH. Calculations revealed that there was a dissociation of approximately one equivalent of protons per 10^5 g of fibrinogen during the clotting reaction. This phenomenon was attributed to the hydrogen bonding of the activated fibrin monomers, brought about by the donation of protons by amino or tyrosine groups to proton-accepting imidazole groups.

The pH curve obtained during the clotting of fibrinogen in an unbuffered system could later be resolved into two first order reactions (Mihalyi and Billick, 1963). The initial acidification was the result of the proteolytic step; the following shift back toward the alkaline range was a consequence of the polymerization phases.

Sturtevant and co-workers (1955) employed thermodynamic analysis in their studies on the two polymerization phases of fibrinogen clotting. Fibrin was dissolved in 1 M NaBr at pH 5.1 to yield the soluble fibrin monomer. When the pH of this preparation was raised from 5.08 to 6.08, there was a large heat absorption due to the instantaneous ionization of histidyl residues. This was followed by a slower first order evolution of heat ($\Delta H = -19$ Kc/mole). No clot formed, indicating that only the intermediate polymerization phase had occurred. Raising the pH to 6.20 or above caused another first order heat evolution ($\Delta H = -44.5$ Kc/mole). These results indicated intermolecular hydrogen bonding with tyrosyl or amino groups being proton donors and histidyl groups being the acceptors. Such donors should lose their protons above pH 10.5, while the acceptors should gain protons below pH 5.5. This postulated range did in fact correspond quite well to the pH range in which the clotting of fibrinogen could occur.

The information gained in the analysis of the thrombin-catalyzed clotting reaction should also aid in the studies on coagulase activity. However, coagulase differs from thrombin in one respect, i.e., its requirement for an accessory factor which Smith and Hale (1944) termed the coagulase-reacting factor (CRF). CRF was purified by Tager (1956); several protein fragments possessing CRF activity were isolated from human plasma by chromatography on aluminum hydroxide gel and fractionation by ammonium sulfate. In a comparison of CRF

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and prothrombin, electrophoresis of purified CRF revealed that the main activity band migrated between the beta and gamma peaks of normal serum, while purified prothrombin appeared between the α_2 and beta peaks. In ultracentrifugal studies, the major CRF component appeared to have a molecular weight in the range of 30,000, while prothrombin was calculated to have a molecular weight of 140,000. In spite of these marked differences in biochemical characteristics, human prothrombin was highly reactive with coagulase and could substitute for CRF in the clotting reaction. Since CRF could not substitute for prothrombin however, it was suggested that CRF was a derivative of the intact prothrombin molecule.

Although coagulase differed from thrombin in that it required CRF for activity, it shared many common characteristics with the latter enzyme. Drummond and Tager (1962) reported that activity of both enzymes was inhibited by diisopropylfluorophosphate, an agent shown by Miller and Van Vunakis (1956) to inhibit a variety of proteases with esterase activity. Coagulase in the presence of CRF could also cleave TAME, the K_m for the reaction being 2.8×10^{-3} M.

More evidence for the similarity between the activities of coagulase and thrombin was reported by Drummond and Tager (1963). Coagulase, acting upon fibrinogen in the presence of CRF, caused the release of characteristic fibrinopeptides which could be separated by chromatography on a Dowex-50x2 column. Amino acid analysis, electrophoretic mobilities,



sedimentation constants, and determination of N-terminal groups all indicated that these peptides were identical to those released during the clotting of fibrinogen by thrombin.



MATERIALS AND METHODS

Cultures and Their Maintenance

Staphylococcus aureus Strain 70 of the International-Blair Series (Blair and Carr, 1960) was employed for the production of coagulase in these studies. Stock cultures were maintained on trypticase soy agar slants at 4 C and transferred once a month.

Production of Coagulase

Media.--A semi-defined casein acid hydrolysate medium was used for culture growth and coagulase production (Table 1). Solutions of casein hydrolysate and mineral salts were sterilized by autoclaving 15 min at 121 C; the glucose and vitamins were sterilized with membrane filters.¹

In preliminary experiments, pH of the medium was varied to determine the optimal range for coagulase production. Those amino acids degraded by acid hydrolysis of casein (tryptophane, tyrosine, cystine, and serine) were added to the medium in 0.1% concentrations as a possible stimulation of coagulase production. In several experiments, energy sources in addition to glucose (1.0% beta-glycerol phosphate or 0.5% ammonium lactate) were also added as supplements.

¹Millipore Filter Corp., Bedford, Mass.

TABLE 1.--Composition of the semi-defined casein acid hydrolysate medium.

Substance	Quantity
Casamino Acids (Difco)	30.00 g
Glucose	1.00 g
K_2HPO_4	7.60 g
KH_2PO_4	2.40 g
Thiamine	10.00 mg
Niacin	10.00 mg
$MgSO_4 \cdot 7H_2O$	10.00 mg
$MnSO_4 \cdot H_2O$	10.00 mg
$FeSO_4 \cdot 7H_2O$	10.00 mg
H_2O	1.00 liter
Trace mineral solution*	1.00 ml

*The trace mineral solution of Tager and Hales (1948) consisted of the following: H_3BO_3 (60.00 mg), $CuSO_4 \cdot 5H_2O$ (40.00 mg), $MoO_3 \cdot H_2O$ (20.00 mg), $ZnSO_4$ (200.00 mg), KI (100.00 mg), and H_2O (1.00 liter).

Cultural conditions.--Two percent lag phase inocula, cultivated in the casein acid hydrolysate medium for 10 to 15 hrs at 37 C, were added to fresh volumes of medium and shaken at 37 C on a rotary shaker at 220 RPM. At various intervals, culture optical density at 625 mu, coagulase production, and extracellular protein concentration were determined. During production of coagulase for purification, culture growth was terminated at the time of maximal specific coagulase activity (about 18 to 20 hrs).

Quantitative Methods

During the development of the purification procedure, coagulase was measured by the titer method of Tager and Hales (1948). Titers were recorded as the highest dilution which caused an organized clot (2+) after 24 hrs at 37C. Protein determinations in the crude, cell-free supernatant fluids were made by the trichloroacetic acid method of Stadtman et al. (1951). Protein concentrations in the dialyzed supernatant fluids and in subsequent purification steps were determined by the method of Lowry et al. (1951). Crystallized bovine Fraction V albumin was used as the protein standard in all determinations.

The presence of contaminating enzymes during purification of coagulase was determined as one criterion to evaluate purity. DNase activity was detected by placing 0.1 ml of each coagulase preparation onto DNase agar.² Phosphatase

²Baltimore Biological Laboratories, Baltimore, Md.

was assayed by the colorimetric method of Barnes and Morris (1957). Hemolysins were detected by placing 0.1 ml of each preparation onto sheep blood and human blood agar plates. Lipase activity was measured by the recently developed instrumental method of San Clemente and Vadehra (1967).

Coagulase Purification

Isoelectric precipitation.--Maximal specific coagulase activity (reciprocal titer units per mg extracellular protein) was reached at a culture optical density of 0.6 to 0.7 at 625 mu. The cells were removed by centrifugation at 4 C and the supernatant fluid was adjusted to 0 C by stirring in an ice bath. Acidification to various pH values was accomplished by dropwise addition of 6 N HCl. That pH was chosen which consistently gave total coagulase precipitation with the minimal amount of protein. This precipitate was then dissolved to one-tenth original volume in 0.01 M phosphate buffer at pH 7.8.

Fractionation by ion exchange.--After two cycles of acid precipitation, the concentrated coagulase preparation was stirred into a cold slurry of activated Diethylaminoethyl-Cellulose³ in 0.01 M phosphate buffer, pH 8.0. After stirring for 4-5 hrs at 4 C, the fluid was filtered from the cellulose and discarded. The adsorbed coagulase was

³Eastman Organic Chemicals Division, Rochester, New York.

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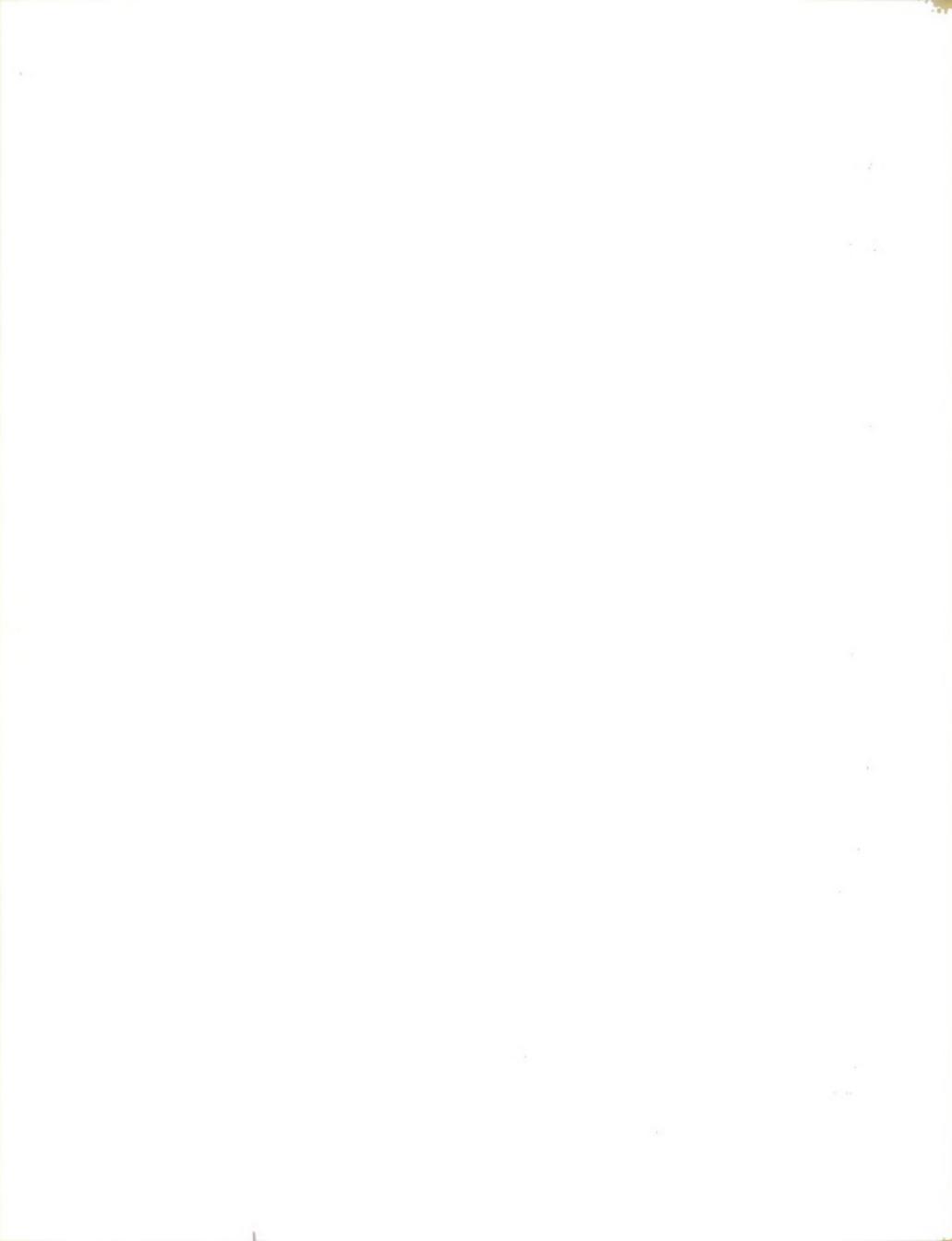
then eluted at pH 7.0 with increasing concentrations of NaCl in 0.01 M phosphate.

Ammonium sulfate fractionation.--The eluted coagulase preparation was acidified to pH 3.0 with 1 N HCl. Saturated ammonium sulfate solution was added dropwise to the preparation during constant stirring at 4 C. Precipitation of coagulase was allowed to occur at 4 C for 5 to 10 hrs. This precipitate was redissolved to 0.01 previous volume in phosphate buffer, pH 7.0.

Gel filtration.--Final purification of coagulase was effected by gel filtration at 6 C through 2.5 x 45 cm columns of Sephadex G-200.⁴ The effluent buffer (pH 7.0) was 0.10 NaCl in 0.01 M phosphate. Ten ml fractions were collected and assayed for coagulase activity and protein.

Immunological characterization of purified coagulase.--Anticoagulase serum was prepared by subcutaneously injecting three New Zealand white rabbits with crude coagulase preparations (first acid precipitate) in Freund's Incomplete Adjuvant (Difco). All rabbits were injected each week with approximately 0.2 mg of crude coagulase for six consecutive weeks, followed by a final injection about one month later. The rabbits were bled from the ear vein ten days after the final injection; their sera were preserved with 0.01% thimerosal and frozen.

⁴Pharmacia Fine Chemicals, Inc., Piscataway, New Market, New Jersey.



The homogeneity of the purified coagulase was tested against rabbit antiserum in immunodiffusion employing the methods of Ouchterlony (1958). Zones of precipitation were stained by the triple stain method of Crowle (1961).

Coagulase thermal denaturation studies.--Two ml aliquots of coagulase in various stages of purification were heated at 60 C in 0.01 M phosphate buffer, pH 7.0. Residual coagulase activity was measured at intervals and the denaturation curves were analyzed mathematically as outlined by Ray and Koshland (1960).

Development of the Nephelometric Coagulase Assay

Early in these studies, it became apparent that the serial dilution titer method was inadequate for accurately measuring coagulase activity. On the other hand, the increase in light scattering which accompanied the clotting of fibrinogen seemed to offer a precise means of measuring reaction rates. Therefore, the development of a nephelometric method for the precise quantitation of coagulase activity was undertaken.

Substrate system in the nephelometric assay.--A fresh stock solution of 0.3% (w/v) Bovine Fraction I citrated fibrinogen⁵ was prepared daily in 0.01 M phosphate buffer at pH 7.2. To determine optimal fibrinogen concentrations

⁵Sigma Chemical Co., St. Louis, Mo.

for the assay in initial experiments, amounts were varied as follows: 12.5, 25.0, 50, 100, 150, 200, 250, 300, 350, 400, 450, and 500 mg per 100 ml. Higher concentrations of fibrinogen could be attained only by increasing the ionic strength of the solution. All substrate preparations were clarified with membrane filters.⁶

Two percent non-inhibitory human plasma, clarified by filtration or centrifugation, was added to each concentration of fibrinogen to provide the coagulase-reacting factor (CRF). To determine the optimal concentration of CRF, the amounts of plasma were varied as follows: 0.0, 0.1, 0.2, 0.5, 1.0, 1.5, and 2.0%. Effect of ionic strength upon the clotting rate was studied over a range of 0.025 to 0.20; the pH was also varied from 6.2 to 9.2. Spontaneous precipitation of the fibrinogen below pH 6.2 precluded any studies at lower values.

Assay procedure.--The assay instrument was a Lumetron Model 402E colorimeter⁷ with a nephelometric attachment; the galvanometer was replaced by a Sargent Model SR strip chart recorder.⁸ A Kahn antigen tube, cut to fit the instrument, served as the cuvette. The nephelometer was calibrated with a BaSO₄ suspension as described by Kunkel et al. (1948).

⁶Millipore Filter Corp., Bedford, Mass.

⁷Photovolt Corp., New York, N. Y.

⁸E. H. Sargent and Co., Detroit, Mich.

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A stock $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$ solution was prepared in a concentration of 1.15 g per 100 ml distilled water. Three ml of this solution were diluted to 100 ml with 0.2N H_2SO_4 . The resulting turbidity of the BaSO_4 suspension was assigned an arbitrary value of 20 nephelometric units. This suspension was serially diluted with 0.2N H_2SO_4 to prepare standards for a calibration curve in which nephelometric units were plotted against recorder response in the 1.25 mv range.

Coagulase activity was quantitated in the following manner: one ml of the coagulase preparation to be assayed was thoroughly mixed with two ml of the fibrinogen-plasma substrate in the same cuvette used in calibration of the instrument. The increase in light scattering which accompanied the clotting reaction was automatically recorded and the rate of the reaction was determined from the slope. A coagulase unit was defined as an increase of 0.01 nephelometric units per min.

The clotting rates of similar reaction mixtures were determined in some exploratory experiments using the Brice-Phoenix light scattering photometer.⁹ The instrument employed in these studies was equipped with a laser as a light source emitting wavelengths of 632.8 mu. The increase in scattered light was measured at an angle of 90 degrees from the incident light path. These increases were recorded

⁹Phoenix Precision Instrument Co., Philadelphia, Pa.

on a Honeywell strip chart recorder,¹⁰ and the reaction rates determined.

Unavailability of temperature control for either the Lumetron or the Brice-Phoenix instrument precluded any studies involving effects of temperature. All data reported in these studies were obtained at 22 C.

Development of the Nephelometric Anticoagulase Assay

A procedure for the reproducible assay of coagulase activity presented the opportunity to extend this method into the measurement of coagulase-inhibiting serum factors. Our interest in bovine staphylococcal mastitis prompted us to employ antiserum produced in dairy cows during the course of these studies.

Production of bovine anticoagulase serum.--Two cows were injected subcutaneously with coagulase alone, while two others received coagulase mixed with a staphylococcal purified antigen (SPA) prepared by the method of Fisher et al. (1963). All cows were injected three times with the cellular products in Freund's Incomplete Adjuvant (Difco), the first two injections a week apart, and a third about two months later. An aqueous injection was administered on the eighteenth week. The total amount of SPA given to each of one pair was six and 14 mg respectively; total coagulase received by both pairs of cows was four and eight mg. The

¹⁰Honeywell Corp., Minneapolis, Minn.

two cows which developed the highest anticoagulase titers were given two more injections. Blood samples were collected, the sera were pooled, preserved with 0.01% thimerosal, and frozen.

Nephelometric anticoagulase assay procedure.--Suitable dilutions of antiserum were incubated with a constant amount of purified coagulase at pH 7.3 in phosphate-buffered physiological saline solution. One ml of this incubation mixture was added to two ml of the previously described coagulase substrate. Anticoagulase activity was measured nephelometrically as the difference between the clotting rates of the coagulase-anticoagulase mixtures and the uninhibited controls. An anticoagulase unit was defined as the neutralization equivalent of one coagulase unit.

Pre-assay dialysis of anticoagulase serum.--To eliminate possible effects of ionic strength upon the coagulase reaction, some serum samples were exhaustively dialyzed against the same phosphate-buffered saline solution used as diluent in adjusting the concentration of antiserum. Their capacity to inhibit coagulase was then compared to that of identical undialyzed samples.

Effect of time upon coagulase-antiserum reactions.--The minimal time required for the completion of the coagulase-antiserum reaction prior to assay was determined at both 4 C and 22 C.

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Heat inactivation of anticoagulase serum.--Samples of antiserum heated at 56 C were removed at regular intervals to determine the time required for inactivation of heat labile coagulase inhibitors. One percent complement (Difco) was added to several heated and unheated samples to determine a possible effect upon anticoagulase activity.

Anticoagulase extraction.--The crude gamma globulin fraction of some pooled antisera was prepared by the ammonium sulfate technique of Campbell et al. (1963). The relative anticoagulase activity of this preparation, the supernatant serum fluid, and the reconstituted mixture of the two fractions was tested after dialysis.

Effect of coagulase and anticoagulase concentrations.--Varying dilutions of antiserum were incubated with a constant amount of coagulase to relate coagulase inhibition to the quantity of antiserum added. Conversely, coagulase preparations of varying activities were incubated with a constant amount of antiserum to determine the measurable range of this nephelometric method.

Survey of coagulase inhibitors in sera of dairy cows.--Sera obtained from 16 randomly selected dairy cows of the Michigan State University dairy herds were tested for the presence of heat stable (56 C, 30 min) and heat labile coagulase inhibitors.

Effect of Fibrinogen Concentration
upon Coagulase Inhibition

Graphical treatment of data.--The effect of fibrinogen concentration upon coagulase inhibition by heated and unheated antisera was initially analyzed by the graphical method of Cinader and Lafferty (1963). Data obtained in later experiments were plotted by the method of Lineweaver and Burke (1934). The slopes and intercepts of these plots were calculated by the least squares method for unweighted data.

Experimental procedure.--The molecular weight of the fibrinogen used in these studies was taken to be 3.40×10^5 (Katz et al., 1952). In the presence of excess coagulase, 62.4% of the fibrinogen protein was found to be clottable. These values were used in converting dry weight of fibrinogen to moles per liter of reaction mixture.

Both heated (56 C, 30 min) and unheated antisera were used in these studies. Mixtures of coagulase and antiserum were assayed in fibrinogen concentrations ranging from 0.365 to 2.86 μM . In several experiments, coagulase was incubated at 4 C or 22 C with varying concentrations of fibrinogen, but without plasma-CRF so as to prevent clotting. Antiserum was later added and the reduction in coagulase activity was measured in the usual manner.



Kinetic Studies on Coagulase Activity

In spite of the apparent impurity of the reaction mixture due to the addition of whole plasma (2%) as a source of CRF, we initiated a kinetic analysis of the coagulase reaction to gain a better insight into the mode of action.

Stability of coagulase during the clotting process.--

There has been some dispute as to whether coagulase is an enzyme or a substrate (Tager and Drummond, 1965). If coagulase was indeed a substrate, then it should seem reasonable that it would be consumed in the clotting process. An experiment was devised to ascertain whether coagulase was a substrate or a catalyst. A highly active coagulase preparation was mixed with an equal volume of substrate and the rate of clotting was nephelometrically determined. The clotted fibrinogen was then removed by centrifugation and the supernatant fluid was added to an equal volume of fresh fibrinogen solution containing no plasma-CRF. The rate of that clotting reaction was determined and the clot was again removed. This cycle was repeated several times until coagulase activity was greatly reduced due to the dilution factor. The decrease in observed coagulase activity was plotted and compared to that calculated on the basis of dilution.

Application of Michaelis-Menton kinetics.--

A simple graphical analysis may be used to test the validity of the Michaelis-Menton equation in its application to the kinetics

of any enzymatic system (Kistiakowsky and Rosenberg, 1952). This method consists of plotting $\log_{10} S(V_m - V)/V$ against \log_{10} of the substrate concentration. The quantity $S(V_m - V)/V$ should be independent of substrate concentration, since by rearrangement of the Michaelis-Menton equation, it can be shown to equal K_m , and therefore the slope of such a plot should equal zero. Such an analysis was employed to determine the validity of the Michaelis-Menton equation in its application to the kinetics of the coagulase reaction.

Michaelis-Menton constant for the coagulase reaction.--

Fibrinogen concentrations in the substrate were varied from 0.091 to 3.65 μ M, while plasma-CRF concentration (2%) was held constant. Coagulase reaction rates were recorded for these fibrinogen concentrations and plotted by the method of Hanes (1932) in order to determine the K_m for fibrinogen in the reaction. This method was used because it has been shown to be statistically superior to the Lineweaver-Burke method for the determination of K_m and V_m (Dowd and Riggs, 1965).

Interaction of catalytic sites on the coagulase molecule.--Hill (1913), while studying the oxygenation of hemoglobin, observed that the oxygen-binding sites on the hemoglobin molecule exerted influence upon one another. Oxygenation of one site increased the affinity of other sites for oxygen molecules. Such an effect resulted in a sigmoid curve when hemoglobin oxygenation was plotted against the partial pressure of oxygen. A graphical analysis

was developed to detect interaction of catalytic sites on enzyme molecules. This method consisted of plotting the \log_{10} of $V/(V_m - V)$ against \log_{10} of the substrate concentration to obtain a positive slope with some value N . A similar method of graphic analysis may be developed from the Michaelis-Menton equation: the \log_{10} of $(V_m/V) - 1$ is plotted against \log_{10} of the substrate concentration, resulting in a negative slope. The slopes of either of these plots may be taken as an indication of the degree of interaction between catalytic sites on an enzyme molecule.

This kinetic approach was employed in the study of coagulase, treating both fibrinogen and CRF as substrate in the clotting reaction. Fibrinogen concentrations were varied while CRF concentration was held constant; conversely, CRF concentrations were varied at constant fibrinogen concentration. The slopes for both plots were calculated by the least squares method for unweighted data.

The coagulase reaction as a two-substrate system.--

The method of Florini and Vestling (1957) was employed in the analysis of the coagulase reaction as a two-substrate system. Coagulase reaction rates were determined for several concentrations of fibrinogen at a constant concentration of CRF; the concentration of CRF was then changed and the procedure was repeated to provide several such lines in a Lineweaver-Burke plot. Similar plots were constructed in which CRF was considered to be the substrate while fibrinogen



concentration was held constant for each curve. A series of maximum velocities was then obtained from each plot. These values were then incorporated into a third graph. Maximum velocities at infinite fibrinogen concentration for all concentrations of CRF were plotted against the reciprocal of the CRF concentration. Likewise, maximum velocities at infinite CRF concentration were plotted against the reciprocal of fibrinogen concentration. The intersection of these lines at the ordinate was designated as V_f , the final maximum velocity when both fibrinogen and CRF concentrations were non-limiting.

Identification of CRF as an activator or a substrate.--

An experiment was designed to determine whether CRF acted as an activator or a substrate in the coagulase reaction. The basis of this experiment was as follows: if CRF was a substrate, its apparent K_m value would be independent of the enzyme concentration, since by definition its value is a constant. On the other hand, if CRF was an activator which combined with the enzyme and yet was not consumed in the reaction, the apparent K_m would not remain constant, but rather would reflect the concentration of the enzyme.

The effect of CRF concentration upon the rate of the coagulase reaction was observed at several coagulase concentrations. A Hanes plot was used in the analysis of these data, and the apparent K_m value for CRF was determined at each coagulase concentration.

RESULTS

Coagulase Production

Media.--In the semi-defined casein acid hydrolysate medium, maximal coagulase production was obtained at a pH of 7.2 to 7.6, with a glucose concentration of 0.05 to 0.2%. Higher glucose concentrations stimulated rate of growth, but resulted in decreased coagulase production. Supplementation of the medium with 0.1% tryptophane, tyrosine, cystine, and serine did not enhance coagulase production, nor did the addition of 1.0% beta-glycerol phosphate or 0.5% ammonium lactate have any significant effect. However, the casein hydrolysate concentration of the medium markedly influenced coagulase production (Fig. 1). The maximal production of coagulase increased logarithmically with increase in casein hydrolysate, and the greatest production was attained at a 3.0% concentration.

Cultural conditions.--Rapidly shaken cultures (220 RPM) produced consistently higher coagulase titers than stationary cultures or those shaken slowly (130 RPM). The culture volume/flask volume ratio also greatly affected coagulase production (Fig. 2). Coagulase production was best effected by using small amounts of medium shaken rapidly in large flasks.



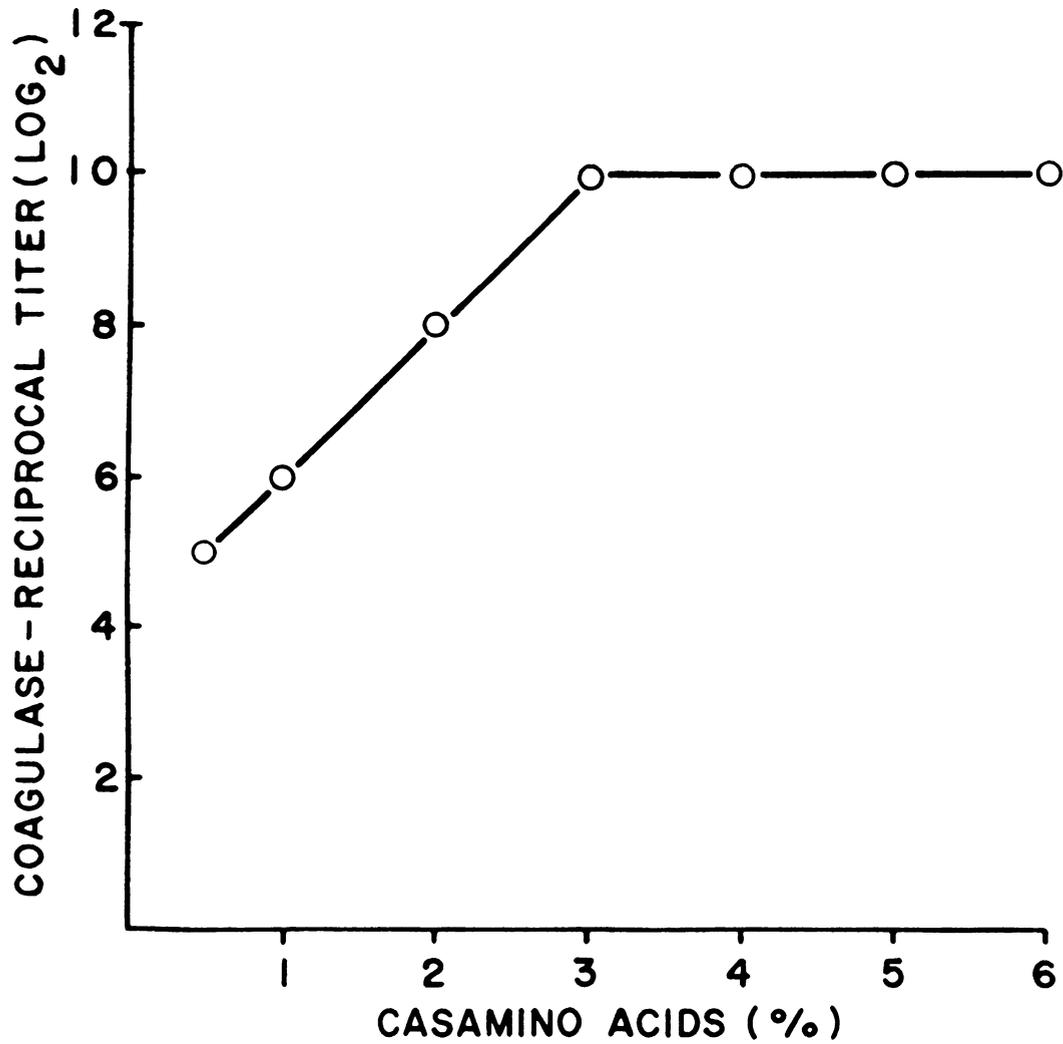


Fig. 1.--Influence of casein hydrolysate concentration on the maximal yield of extracellular coagulase. Cultures were shaken (220 RPM) at 37 C, pH 7.2, with a culture volume to flask volume ratio of 1:10.

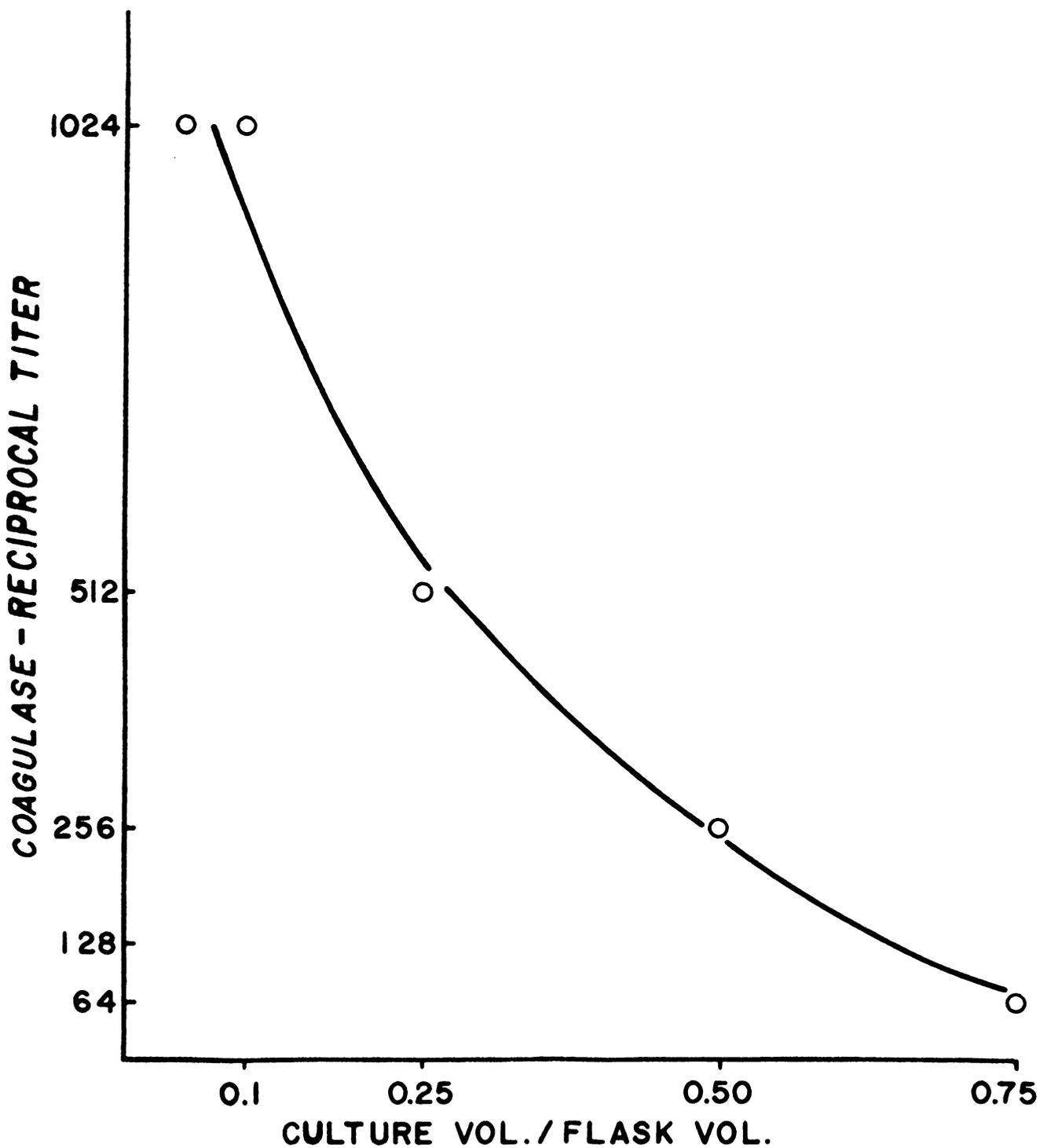


Fig. 2.--Effect of the culture volume to flask volume ratio upon the production of extracellular coagulase in the 3% casein hydrolysate medium. Cultures were shaken (220 RPM) at 37 C, pH 7.2.



Figure 3 depicts culture growth, coagulase production, and liberation of extracellular protein under conditions of constant optimal pH (7.2), agitation (220 RPM), casein hydrolysate concentration (3%), and a culture volume to flask volume ratio of 1:10. Liberation of coagulase into the medium closely followed the rate of growth and the maximal production was reached at 18 to 24 hrs.

Purification of Coagulase

During the purification of coagulase, the enzyme was found to be rather stable under acid conditions, but very labile at alkaline pH values, especially when purified. Therefore, acid precipitation was employed for primary concentration and purification.

Acid precipitation.--As indicated in Figure 4, 100% of the active extracellular coagulase in the crude cell-free medium could be precipitated over a pH range of 2 to 4, while maximal protein was precipitated at pH 2.5 to 3.0. A pH of 3.5 to 3.8 was used for both cycles of acid precipitation because this range consistently rendered the supernatant fluid free of coagulase activity. A ten-fold concentration and a two- or three-fold increase in purity were attained in this step.

Ion exchange.--The redissolved precipitate was subjected to ion exchange on DEAE-Cellulose. Figure 5 illustrates the stepwise elution pattern of coagulase and protein

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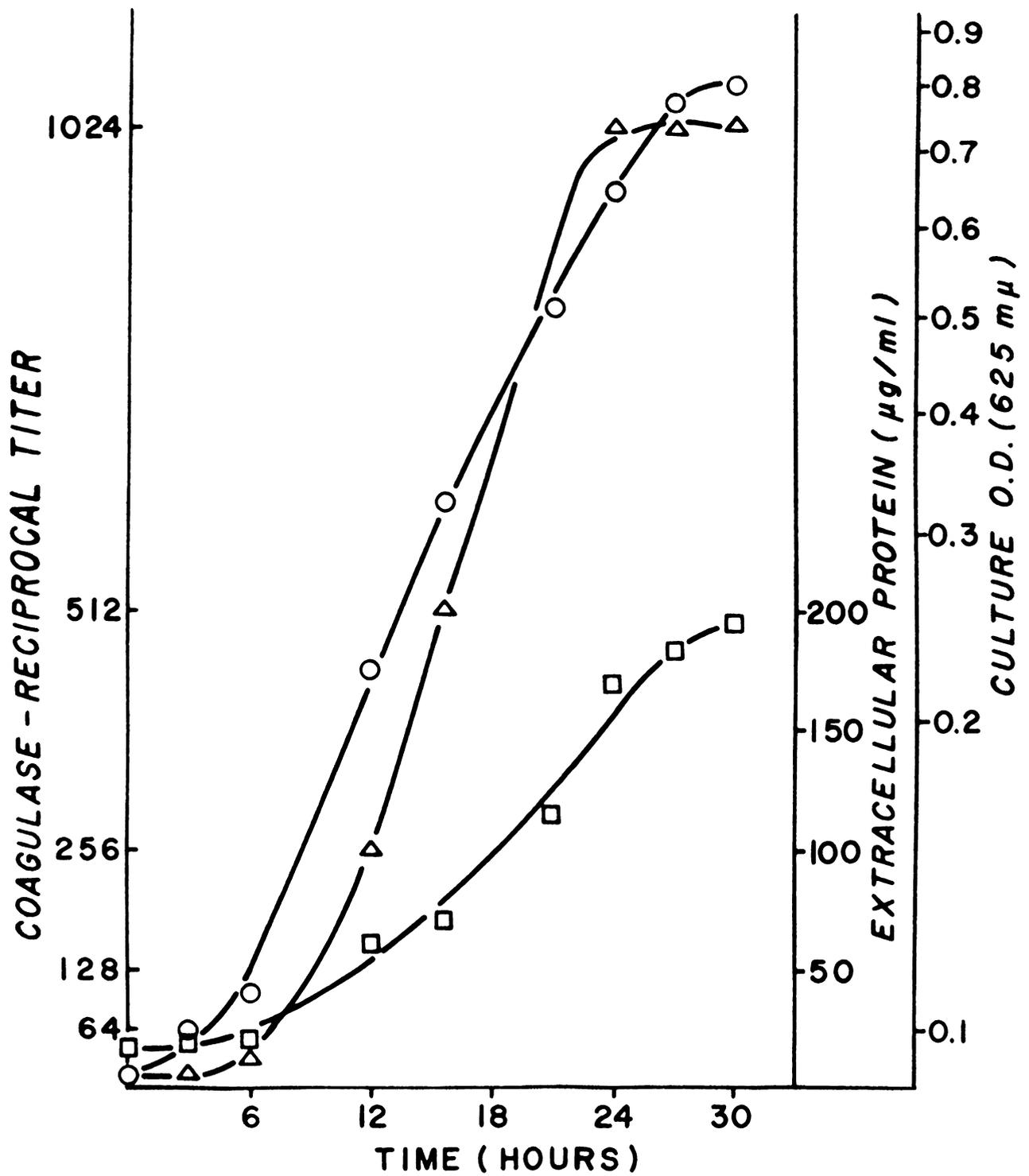
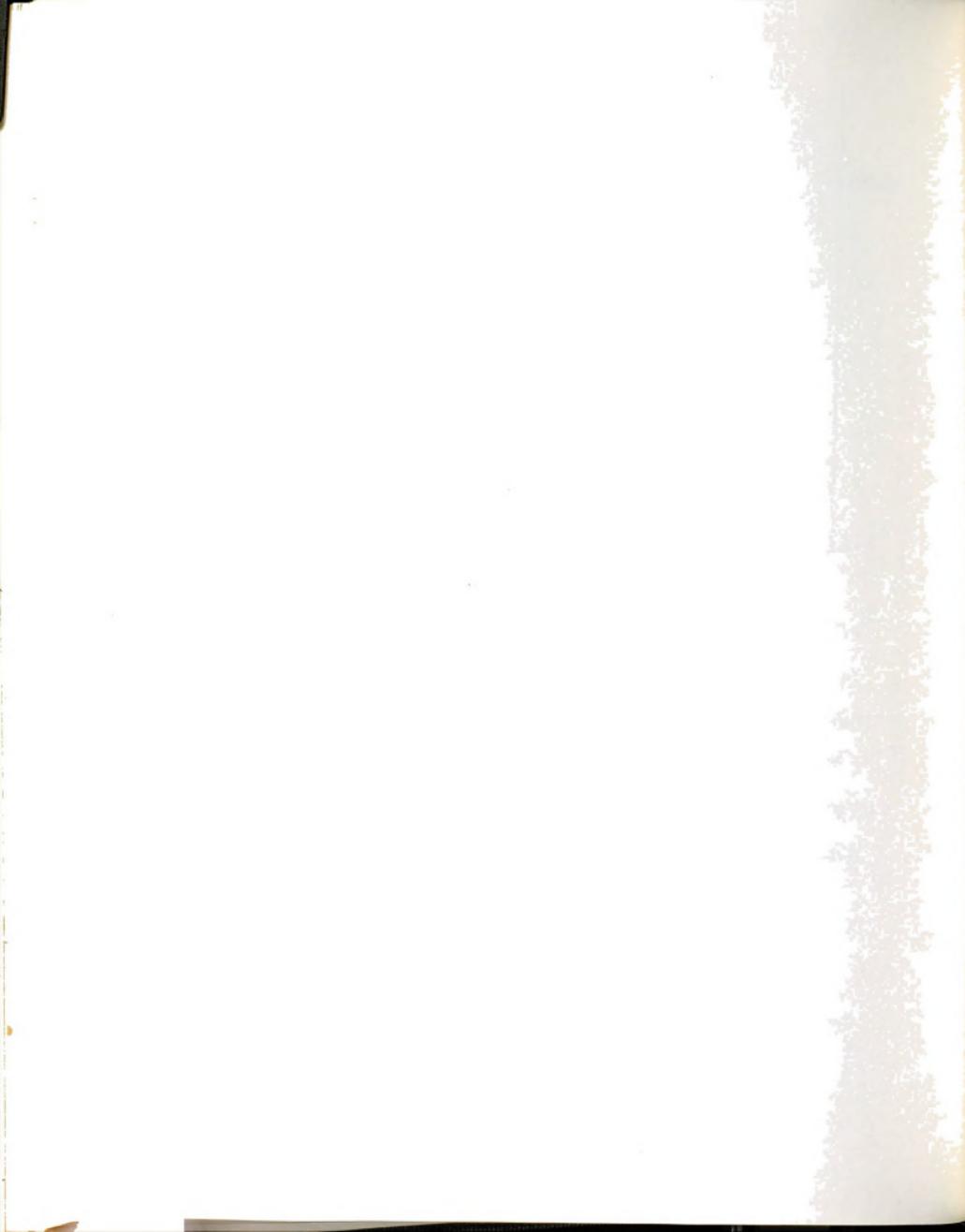


Fig. 3.--Increase in culture and optical density (-○-), production of extracellular coagulase (-Δ-), and liberation of cell-free protein (-□-) under optimal conditions in the casein hydrolysate medium.



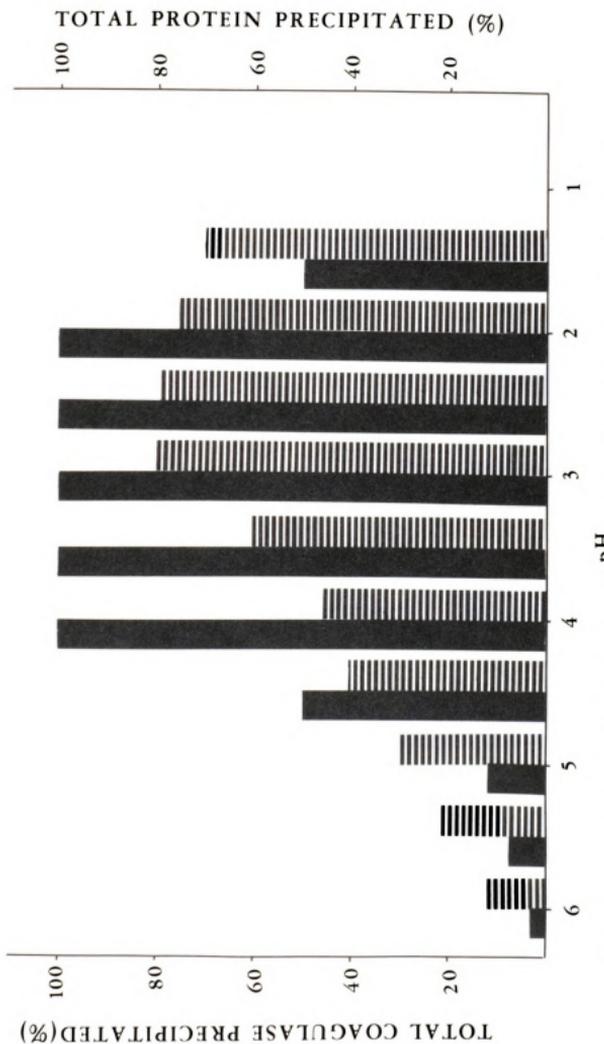


Fig. 4.--Acid precipitation of coagulase (solid bars) and protein (striated bars) at various pH values in the cell-free supernatant fluids.

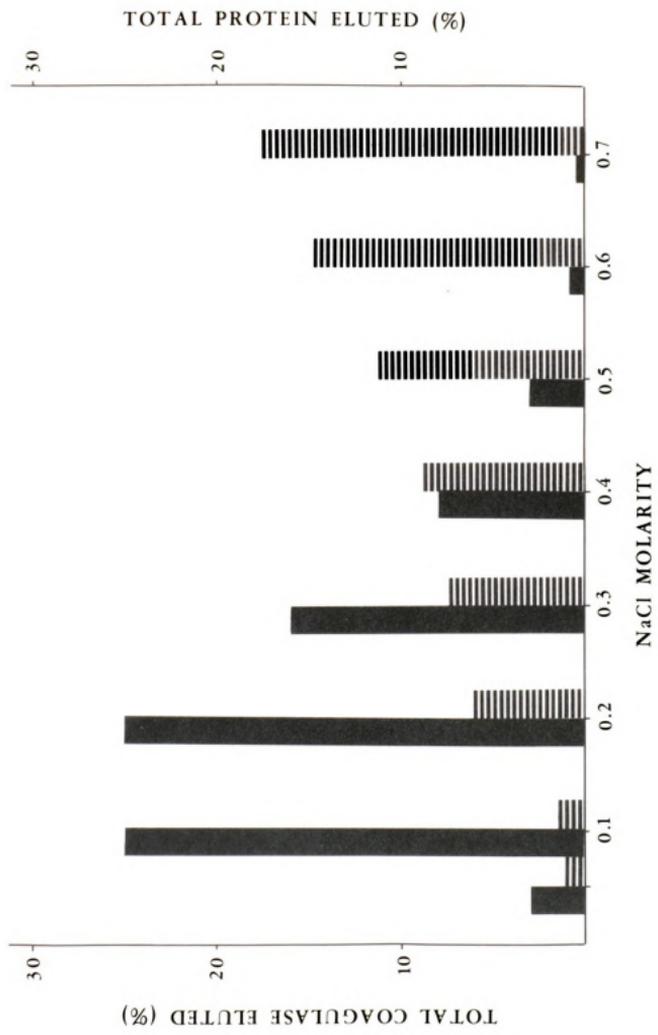


Fig. 5.--Stepwise elution pattern of coagulase (solid bars) and protein (striated bars) after adsorption onto DEAE-Cellulose. The eluant was NaCl in 0.01 M phosphate (pH 7.0).

adsorbed to the cellulose when treated with increasing concentrations of NaCl at pH 7.0. When 0.15 M NaCl in 0.01 M phosphate was used as eluant, 50% of the original coagulase activity could be recovered with a total increase in purity of 12 to 15 fold.

Ammonium sulfate fractionation.--Acidification of the eluted material caused no visible precipitation. However, 50% saturation with ammonium sulfate at pH 3.0 allowed precipitation of all the active coagulase. Further concentration was achieved together with a doubling in specific activity over the preceeding step.

Gel filtration.--Figure 6 illustrates the typical pattern which was obtained when the redissolved precipitate was passed through a column of Sephadex G-200. The activity peak corresponded with the protein peak, indicating a relatively homogeneous molecular species. All activity of the previous step was retained within the experimental error of the coagulase titer assay method.

Evaluation and summary of the purification procedure.--One criterion of purity for an enzyme preparation is the absence of contaminating enzymes. Table 2 outlines the progressive elimination of some contaminating enzymes from the coagulase preparation. No DNase, lipase, hemolysins, or phosphatase was detected in the finally purified fractions.

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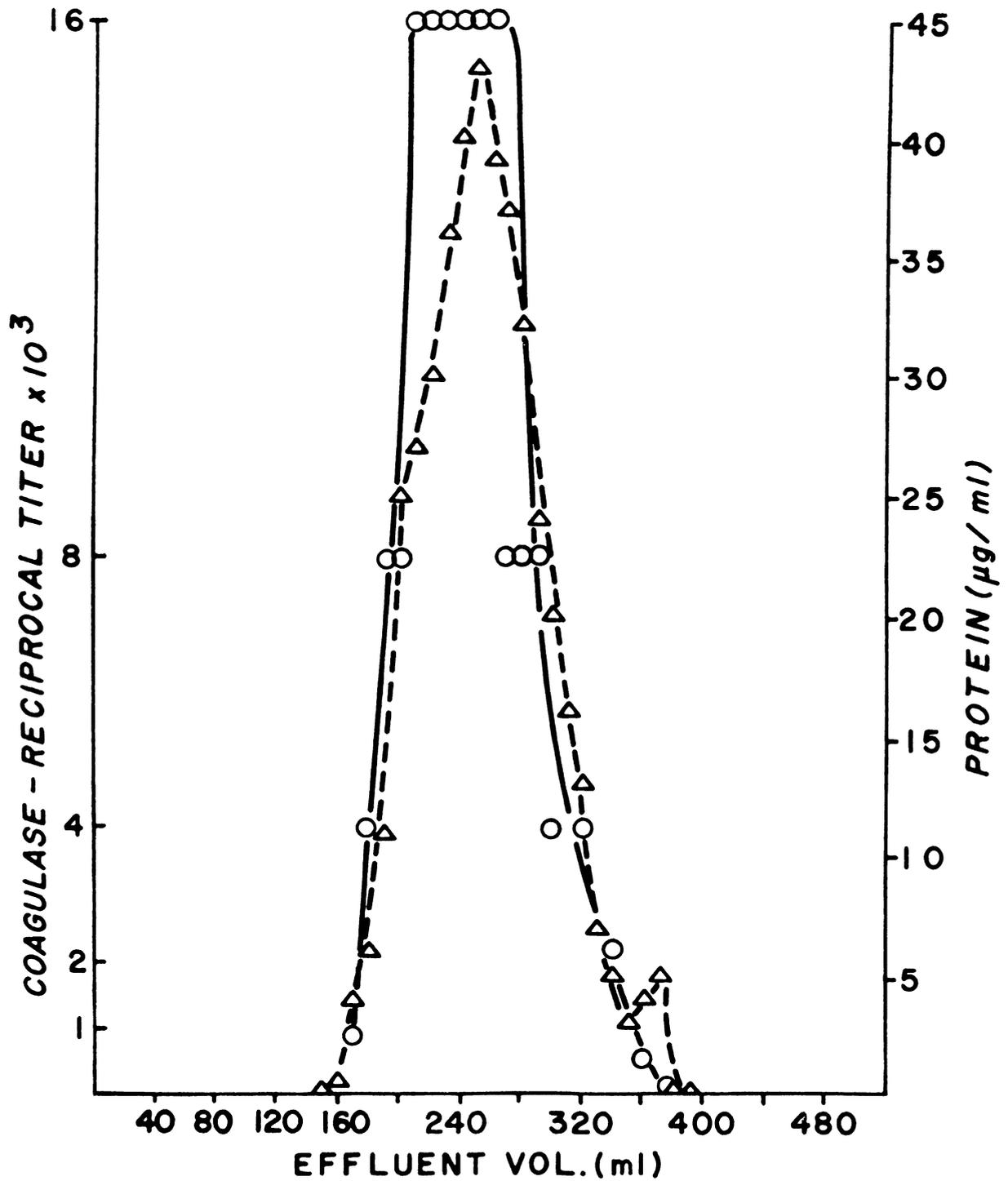


Fig. 6.--Correspondence of coagulase activity (-O-) and protein (-Δ-) during gel filtration through Sephadex G-200.

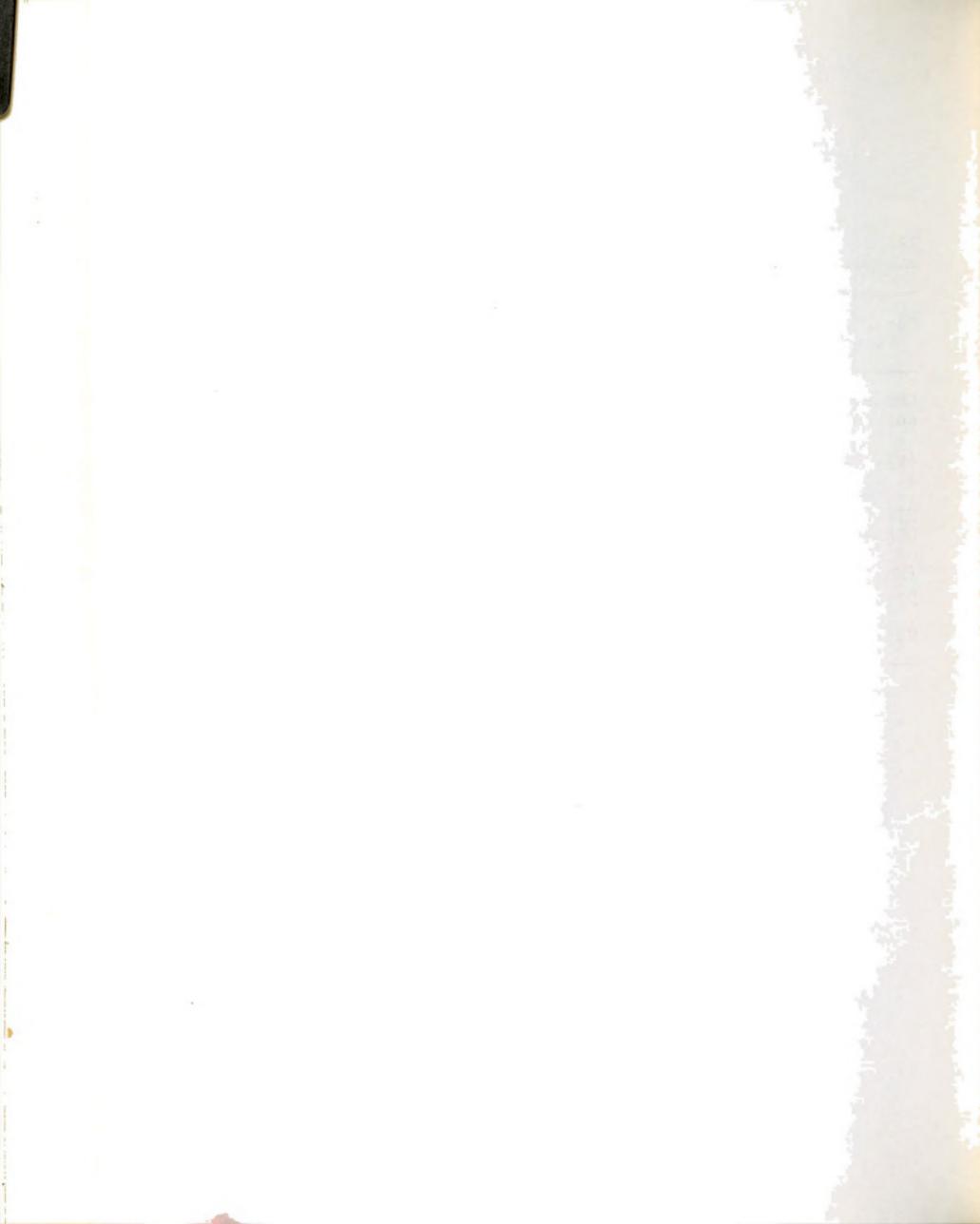


TABLE 2.--Progressive elimination of several contaminating enzymes during the purification of coagulase.

Purification stage	Contaminating enzymes present			
	DNase	Lipase	Hemolysins	Phosphatase
Crude, cell-free supernatant fluid	+	+	-	+
Acid precipitate at pH 3.5-3.8	+	+	-	+
Eluate from DEAE-Cellulose	-	+	-	+
(NH ₄) ₂ SO ₄ precipitate	-	+	-	<u>+</u>
Gel filtration	-	-	-	-



A summary of the purification procedure and the evaluation of its efficiency is given in Table 3. Specific coagulase activity (reciprocal titer units per mg of protein) was increased approximately 45-fold. Recovery of the original enzyme activity was maintained at 50% or above, within the limit of accuracy of the coagulase titer method.

Immunological characterization of purified coagulase.--

When finally purified coagulase preparations were tested against rabbit antiserum in immunodiffusion studies, single precipitin bands were generally observed after staining (Fig. 7). Purified coagulase preparations appeared to be free of contaminating antigens.

Thermal inactivation of coagulase.--Thermal stability of coagulase preparations in various stages of purification was tested at 60 C. Crude, partially purified, and finally purified coagulase preparations appeared to be degraded in two first order reactions, one phase proceeding at a much more rapid rate than the other (Fig. 8). Ray and Koshland (1960) reported a mathematical treatment of these biphasic denaturation curves. In an enzyme preparation, two isoenzymatic forms, X and Y, may exist in certain proportions. Form X may be more heat stable than form Y. If each form is independently inactivated in a first order reaction, the fractions of each of these residues remaining at any time, T, can be calculated from the following equations:

TABLE 3.--Summary and evaluation of the coagulase purification procedure.

Purification stage	Total activity* x 10 ⁶	Recovery %	Total protein mg	Specific activity x 10 ⁵	Purification factor
Cell-free supernatant	2.56	-	350.00	0.0748	-
First acid precipitate at pH 3.4-3.8	2.56	100	170.00	0.1540	2.1
Second acid precipitate at pH 3.5-3.8	2.56	100	120.60	0.2170	2.9
Eluate from DEAE Cellulose with 0.15 M NaCl	1.28	50	12.50	1.0240	13.7
(NH ₄) ₂ SO ₄ (50%) precipitate at pH 3.0	1.28	50	7.15	1.7900	23.7
Gel filtration	1.44	56	4.29	3.3600	44.9

*Expressed as coagulase reciprocal titer.

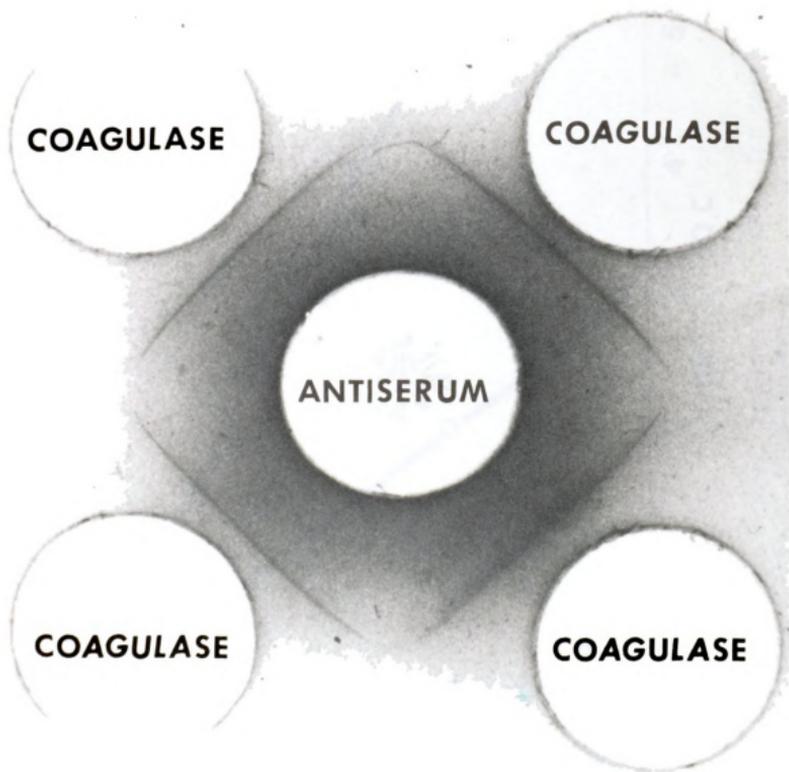


Fig. 7.--Agar gel immunodiffusion pattern of highly purified coagulase (quadruplicate samples) and homologous rabbit antiserum prepared against crude coagulase (first acid precipitate).

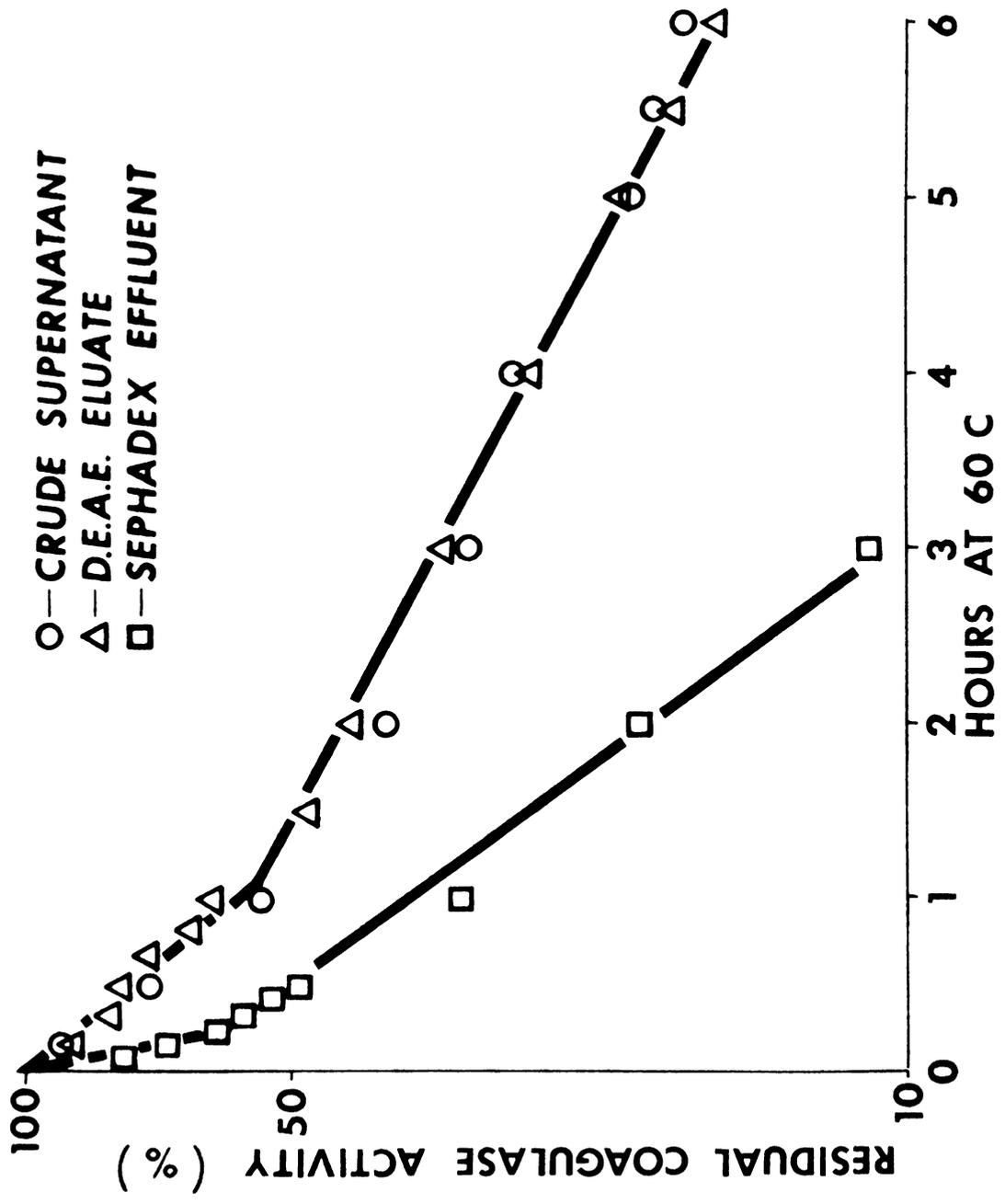


Fig. 8.--Biphasic thermal inactivation curves for coagulase in three progressive stages of purification.

$$X/X_0 = e^{-k_x T} \quad (1)$$

$$Y/Y_0 = e^{-k_y T} \quad (2)$$

where k_x and k_y are the inactivation constants for the respective forms.

To calculate k for each component of the biphasic inactivation curve, the slower of the two phases (phase X in this case) is extrapolated back to zero time, and the fraction of the total activity it represents is obtained. The inactivation constant for the slow phase is then calculated using formula (1). Then, the contribution which the X form makes to the total activity, A , at any time during the inactivation process, is subtracted from the total activity remaining at that time. The remainder is the contribution of the Y component to the total activity at that time. A second curve is plotted from these calculated data, and the inactivation constant, k_y , is obtained from formula (2).

Thermal denaturation of coagulase at 60 C indicated no difference in the heat stability of the crude cell-free supernatant fluids and the DEAE-Cellulose eluates. Therefore, their inactivation curves were treated as one. The finally purified coagulase was much more heat labile. In spite of this difference, all preparations had the characteristic biphasic curves with the more heat stable phase extrapolating back to 70% of the total activity. All preparations appeared to contain the same proportion of

two thermal isozymes; a heat stable component accounted for 70% of the total activity and a more heat labile form contributed the remaining 30%. The inactivation constants and half-life values for these two forms are given in Table 4.

TABLE 4.--Inactivation constants and half-life values at 60 C for coagulase in varying stages of purification.

Purification stage	Inactivation constant (hr ⁻¹)	Half-life (min)
Crude or partially purified:		
Form X	0.236	174
Form Y	1.808	26
Finally purified coagulase:		
Form X	0.637	66
Form Y	8.700	4.7

Development of the Nephelometric
Coagulase Assay

Instrument standardization.--The BaSO₄ suspension used in standardization of the nephelometer was diluted to give nephelometric units equivalent to 2.5, 1.25, and 0.625. The recorder response to each value was then determined. A linear relationship existed between nephelometric units and recorder response. This relationship was used in the conversion of recorder response to nephelometric units.

Effect of fibrinogen concentration upon the reaction rate.--Fibrinogen concentrations of the stock substrate preparations were varied from 12.5 to 500 mg per 100 ml while 2% plasma-CRF was held constant. One ml of the highly active coagulase preparation was mixed with two ml of each fibrinogen concentration and the reaction rate determined (Fig. 9). Maximal activity was attained with 300 mg fibrinogen per 100 ml of substrate (2.2 μ M). Higher concentrations resulted in no increase of the clotting rate.

Effect of CRF concentration upon the reaction rate.--The coagulase reaction rate was dependent upon the percentage of plasma-CRF added to the substrate. Maximal activity occurred in excess of 1.5% plasma (Fig. 10).

pH dependence of the reaction.--It was found that pH had a marked influence upon the rate of the coagulase reaction (Fig. 11). A change of one pH unit from the optimal value resulted in approximately 50% decrease in the clotting rate. The optimal pH for this system appeared to be in the range of 7.1 to 7.3.

Influence of ionic strength upon the coagulase reaction.--Although fibrinogen is usually prepared in a physiological saline solution (ionic strength, 0.15), the optimal ionic strength for the coagulase reaction appeared to be in the range of 0.07 to 0.10. These data (Fig. 12) obtained with the Lumetron nephelometer were verified in



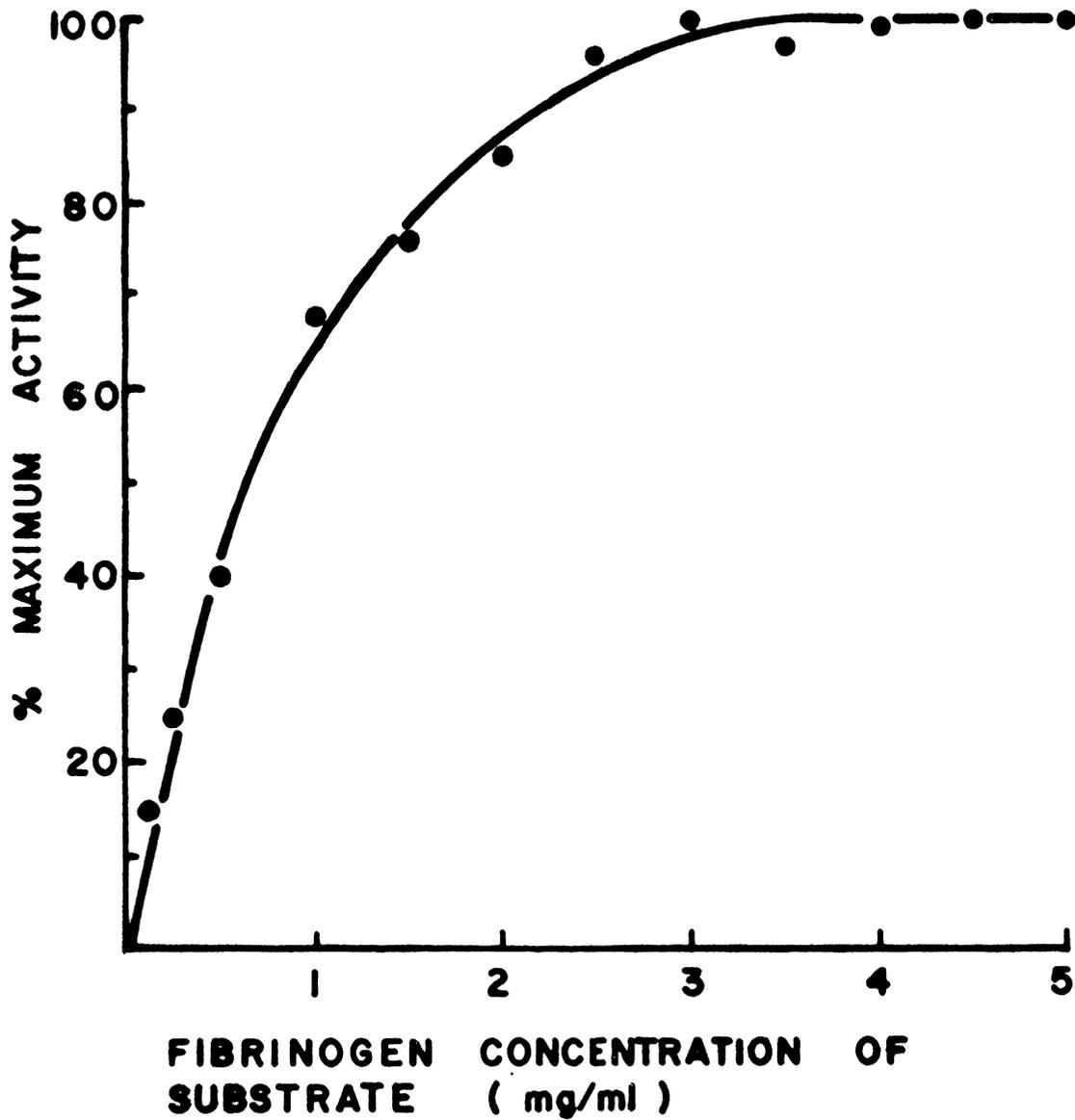


Fig. 9.--Influence of fibrinogen concentration on the clotting reaction rates under conditions of optimal plasma-CRF, pH, and ionic strength, using a constant amount of coagulase (titer of 1/2048 per ml).



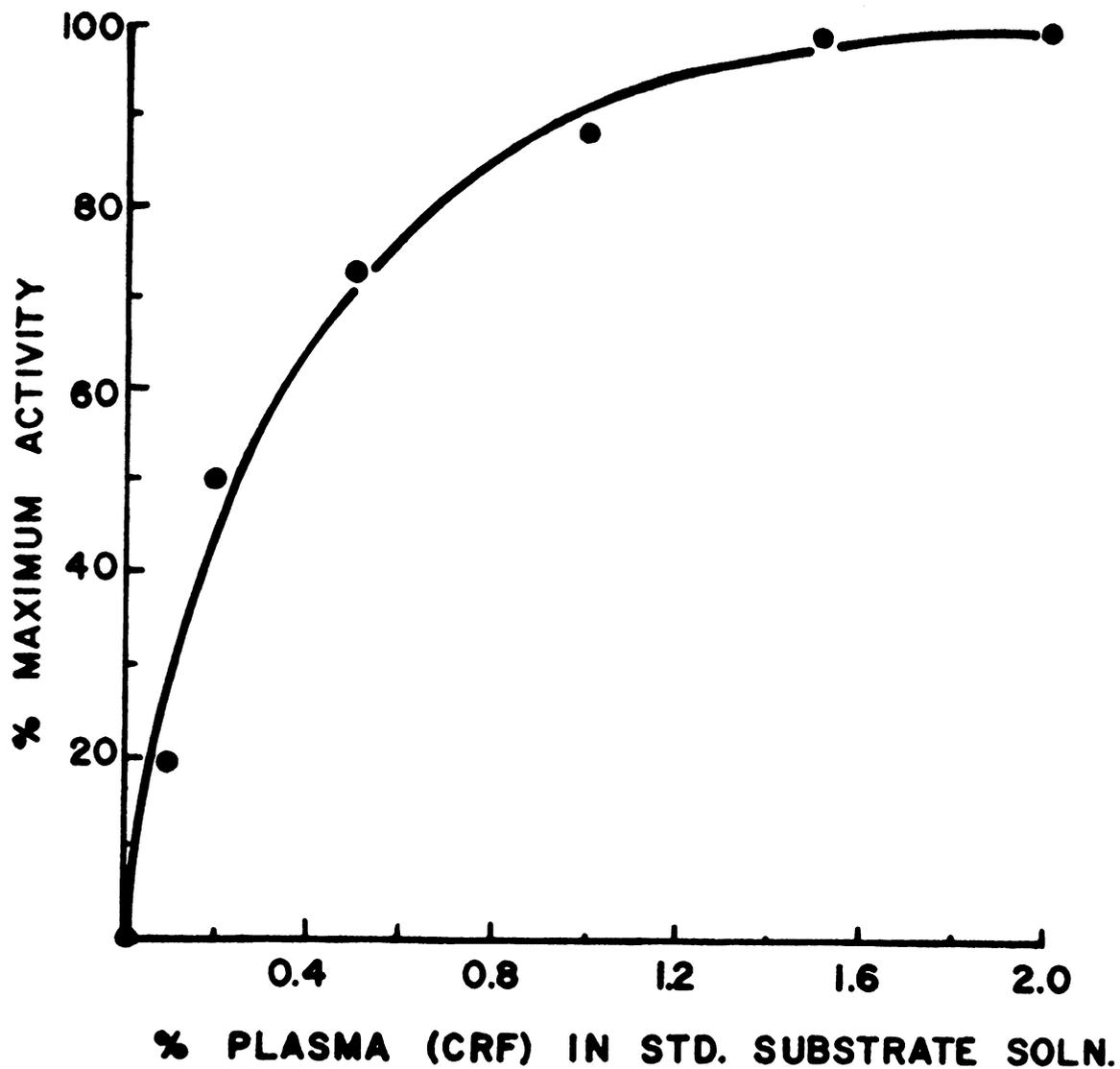


Fig. 10.--Effect of CRF (fresh plasma) concentration on the clotting reaction rates under conditions of optimal fibrinogen concentration, pH, and ionic strength, using a constant amount of coagulase (titer of 1/2048 per ml).

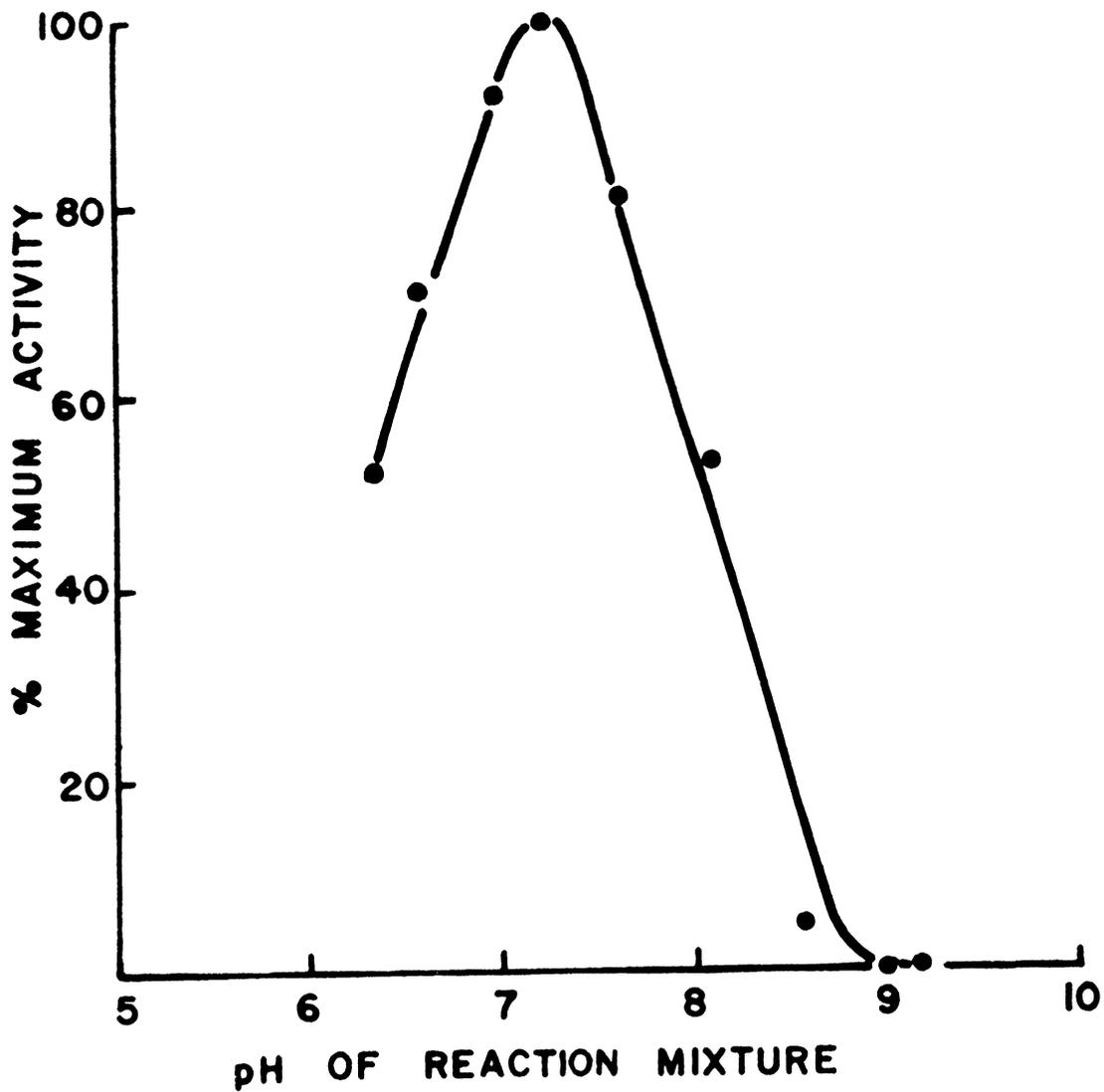


Fig. 11.--Effect of pH on the rate of the clotting reaction under optimal conditions of ionic strength and fibrinogen and plasma-CRF concentrations, using a constant amount of coagulase (titer of 1/2048 per ml). Spontaneous precipitation of fibrinogen occurred at pH values below 6.2.



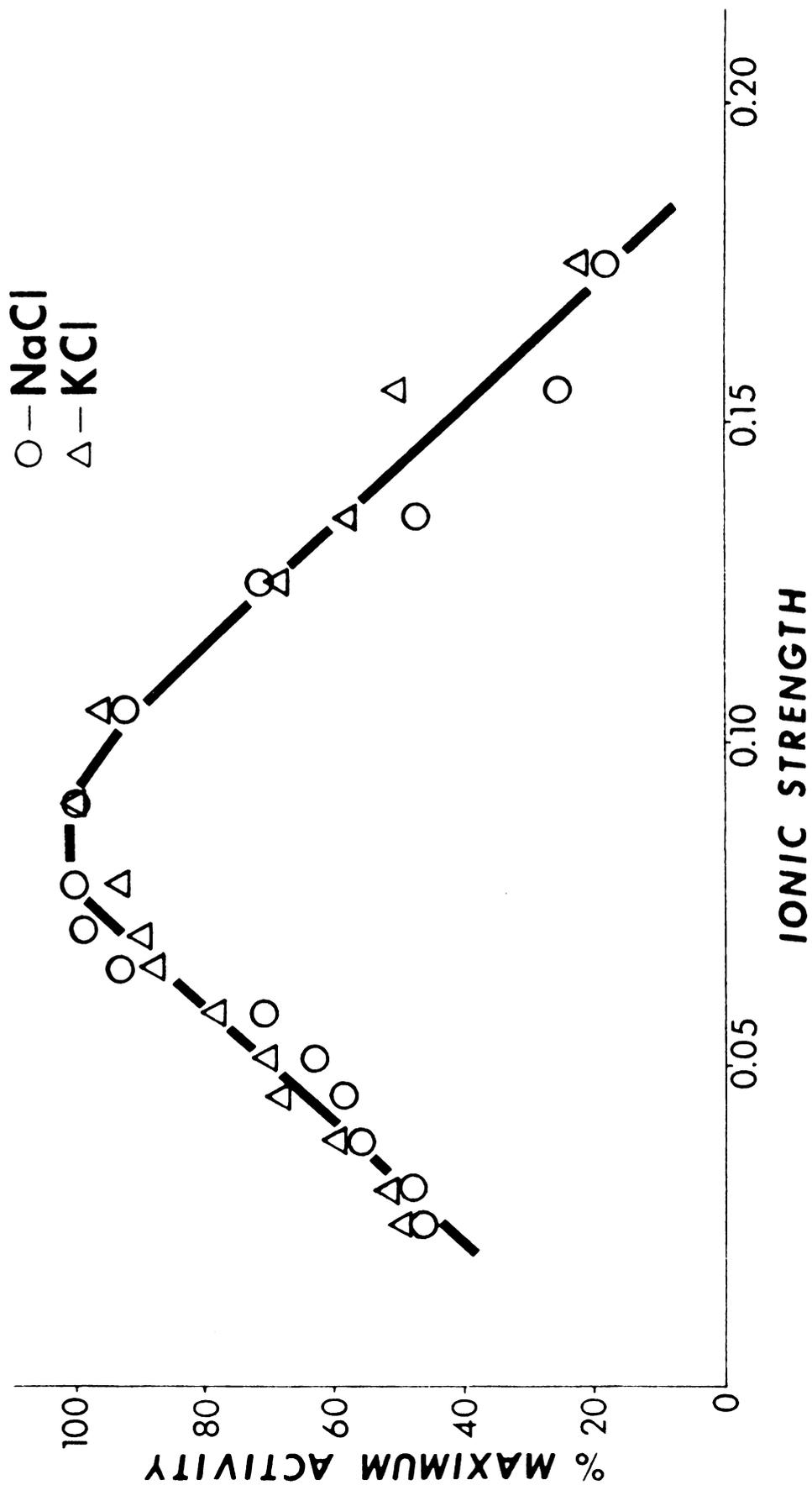
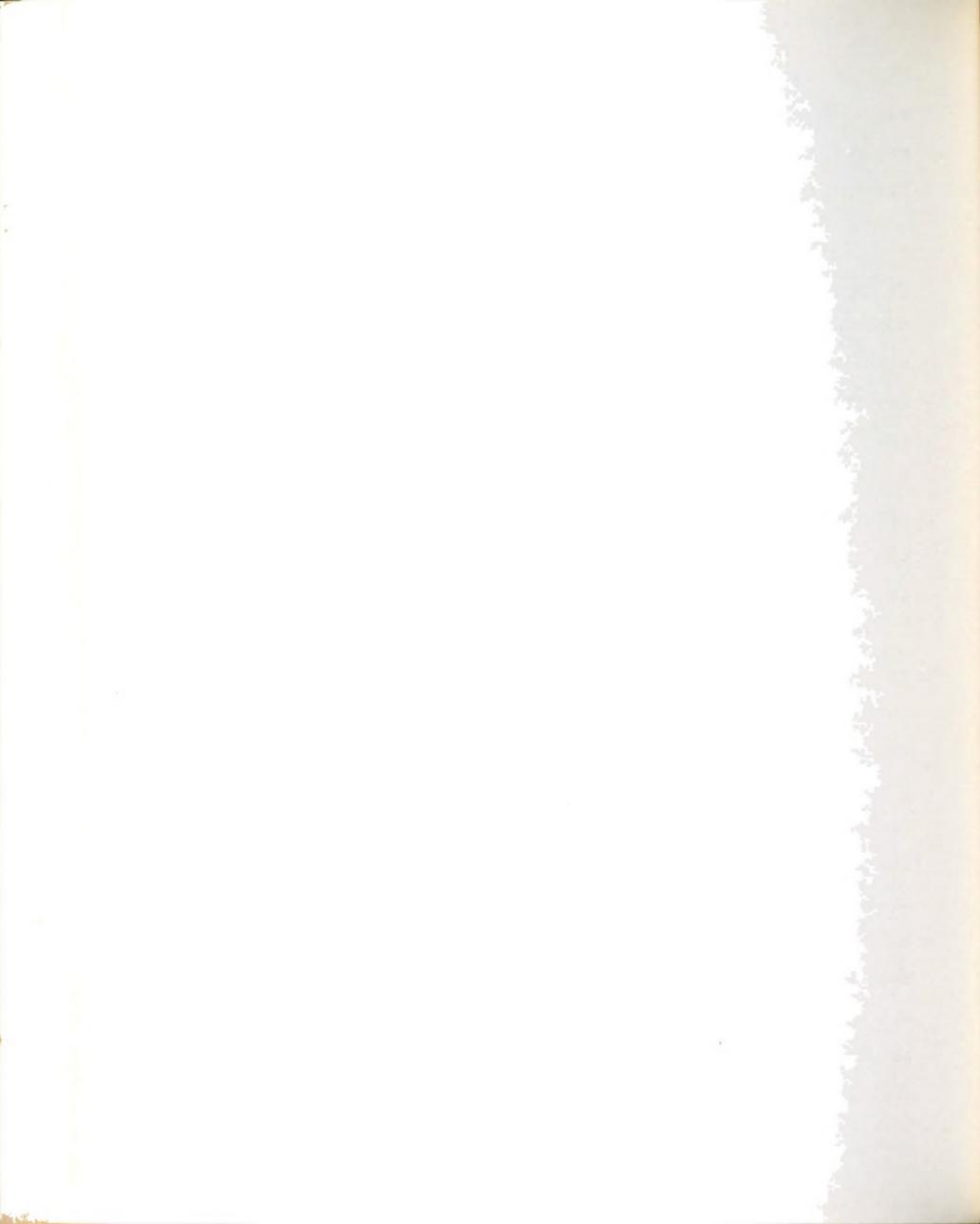


Fig. 12.--Effect of ionic strength on the rate of the coagulase reaction. All reaction mixtures contained a phosphate concentration of 0.01 M. Increased ionic strength was attained by addition of NaCl or KCl.



visually observed test tube experiments by plotting the reciprocal of the minimal clotting time (minutes required for the formation of a 4+ clot) against ionic strength.

Effect of coagulase concentration upon the reaction rate.--A linear relationship existed between the coagulase concentration and the observed clotting rate at room temperature when followed nephelometrically under the following conditions: fibrinogen concentration, 2.2 μM , CRF concentration, 2.0% (v/v), pH 7.2, and ionic strength, 0.07 to 0.10. This proportional relationship between coagulase concentration and recorded rate (Fig. 13) was the basis for the precise quantitation of coagulase activity. A coagulase unit was defined as that amount of activity which caused an increase of 0.01 nephelometric units per min under the specified conditions. The relationship which existed between the nephelometric unit and the reciprocal titer unit is illustrated in Fig. 14.

The Brice-Phoenix light scattering photometer could also be readily utilized in the assay procedure. Rates of increase in light scattering by the clotting mixtures, measured in preliminary tests simply as recorder units per min, were plotted against coagulase concentration (Fig. 15). Because of the greater sensitivity of this instrument, the minimal amount of coagulase which could be accurately measured was about one-half that required with the Lumetron nephelometer. Also, with the more sensitive instrument, an

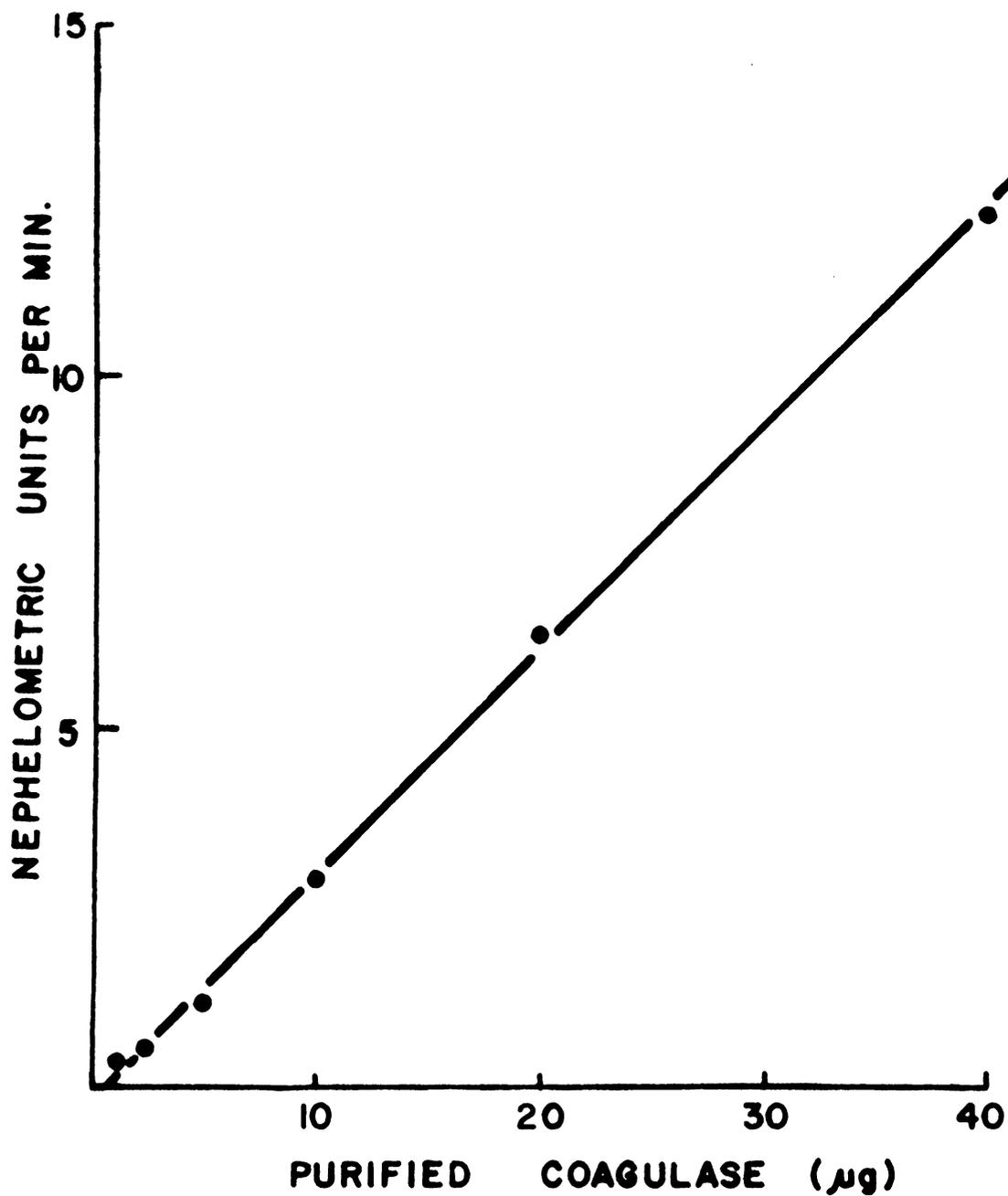


Fig. 13.--Standard curve of the linear relationship between a wide range of coagulase concentrations and their corresponding reaction rates using the Lumetron nephelometer.



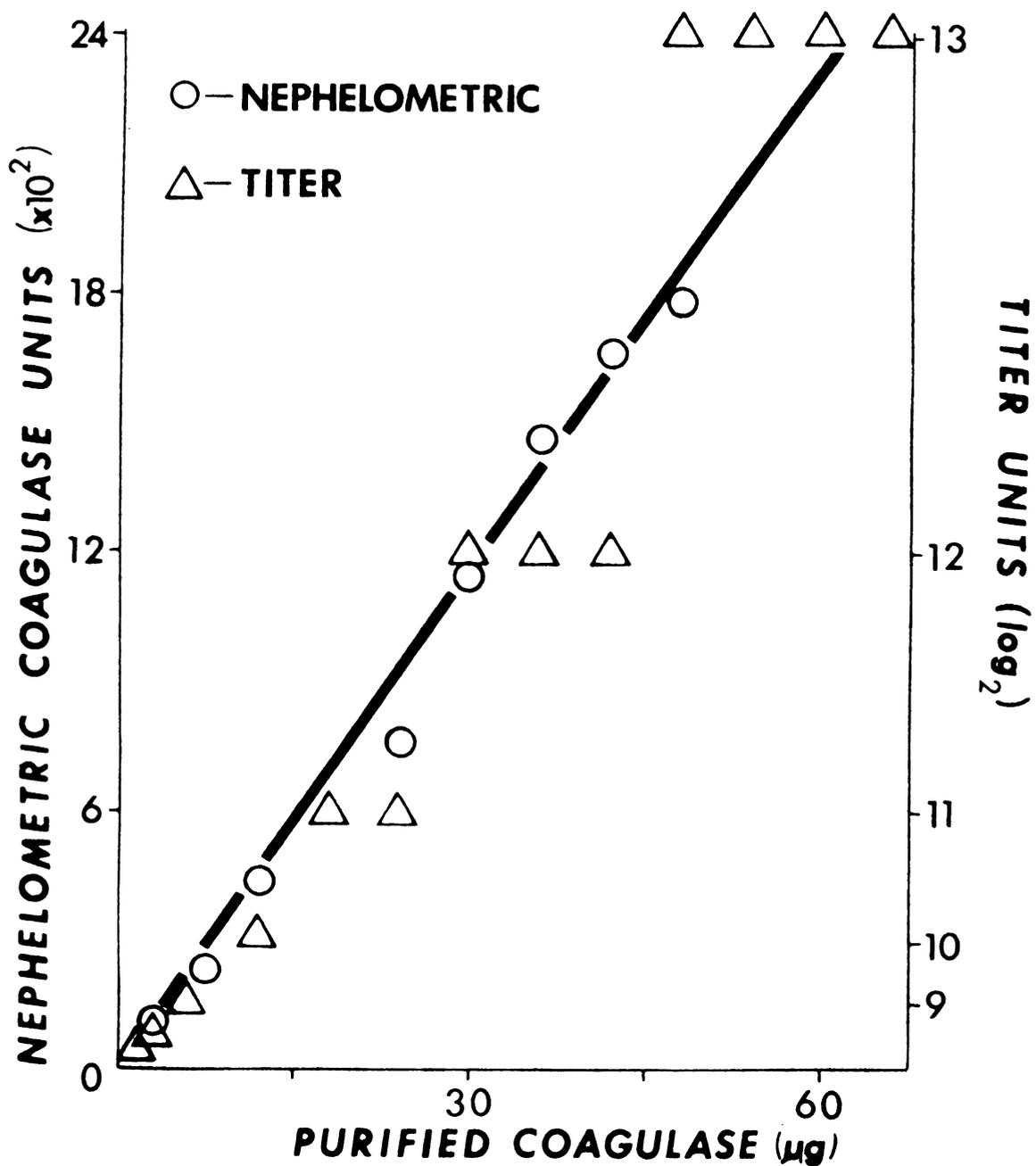


Fig. 14.--Relationship between the nephelometrically determined coagulase units and the reciprocal titer units of the previously-employed two-fold dilution titer method.



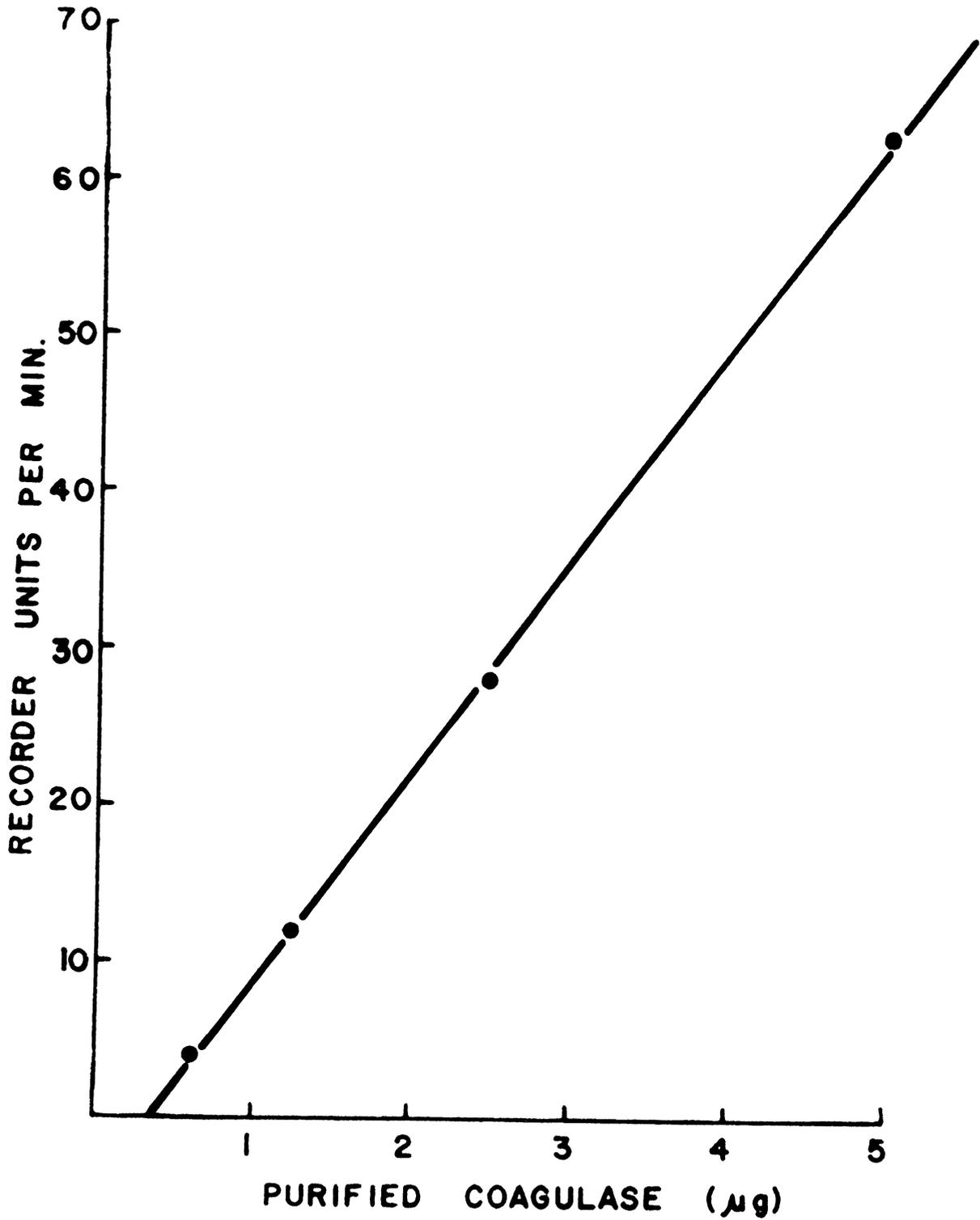
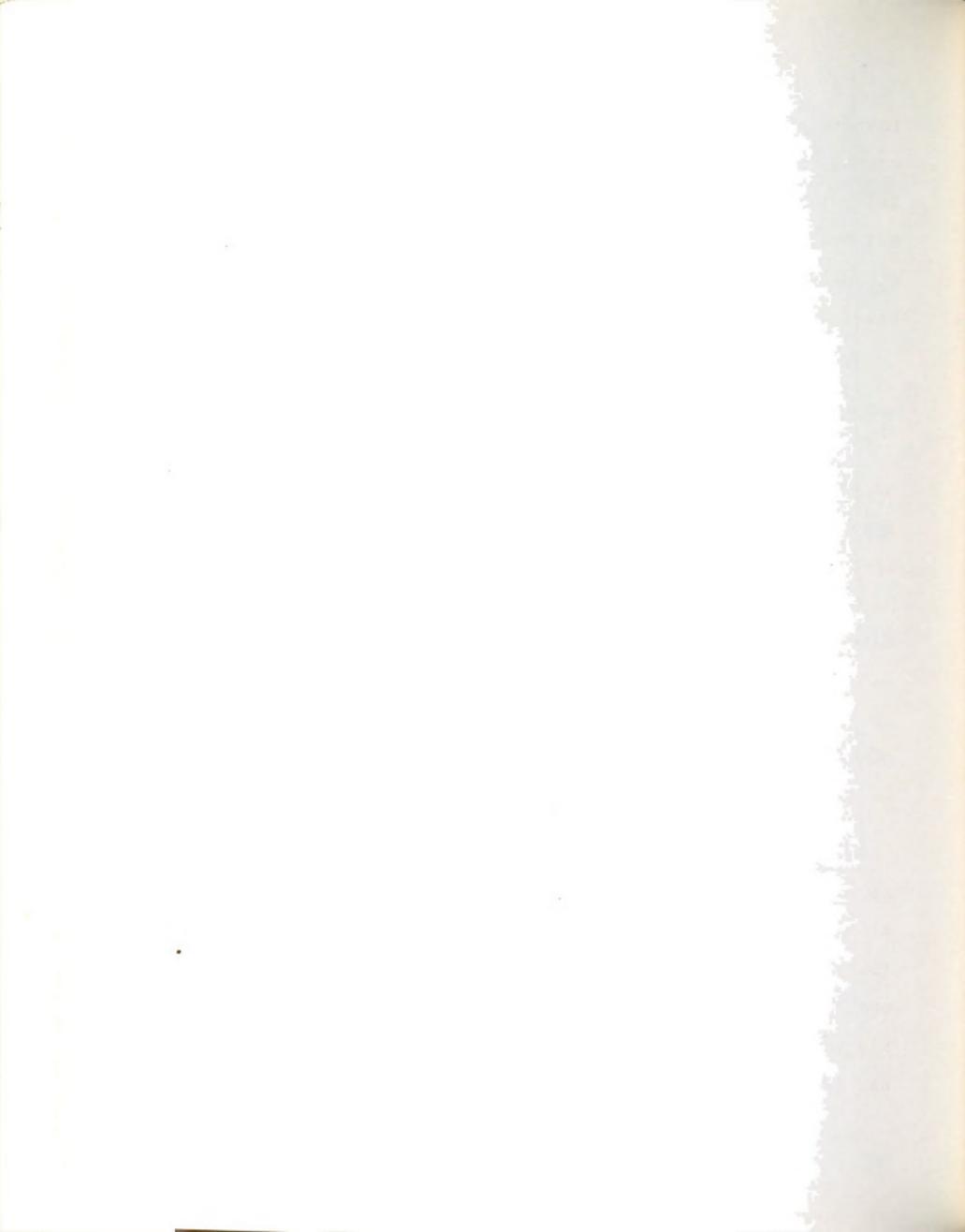


Fig. 15.--Linear correspondence between coagulase concentration and recorder response using the Brice-Phoenix light scattering photometer.



inverse linear relationship (Fig. 16) existed between the coagulase concentration and the lag period (time required for initiation of a detectable increase in light scattering after mixing of coagulase and substrate). With very active coagulase preparations however, this lag period was quite brief and therefore difficult to measure accurately.

Development of the Nephelometric
Anticoagulase Assay Method

Ionic strength.--The coagulase reaction possessed a marked sensitivity to variations in ionic strength of the reaction mixture. In the nephelometric anticoagulase assay, some coagulase inhibition by antiserum could have been caused merely by a change in ionic strength which was uncompensated in the final reaction mixture. This possibility was eliminated by exhaustive dialysis of serum samples against phosphate-buffered physiological saline solutions (ionic strength, 0.15); no significant change in coagulase inhibition could be detected in the dialyzed samples.

Time required for complete coagulase-antiserum reaction.--Incubation of the coagulase preparation with inhibitory antiserum for 15 min at 22 C or one hr at 4 C was sufficient to obtain maximal inhibition (Fig. 17). No visible precipitation occurred in incubation mixtures of coagulase and antiserum; centrifugation at 4×10^4 G for one hr at 4 C had no effect upon the degree of inhibition.

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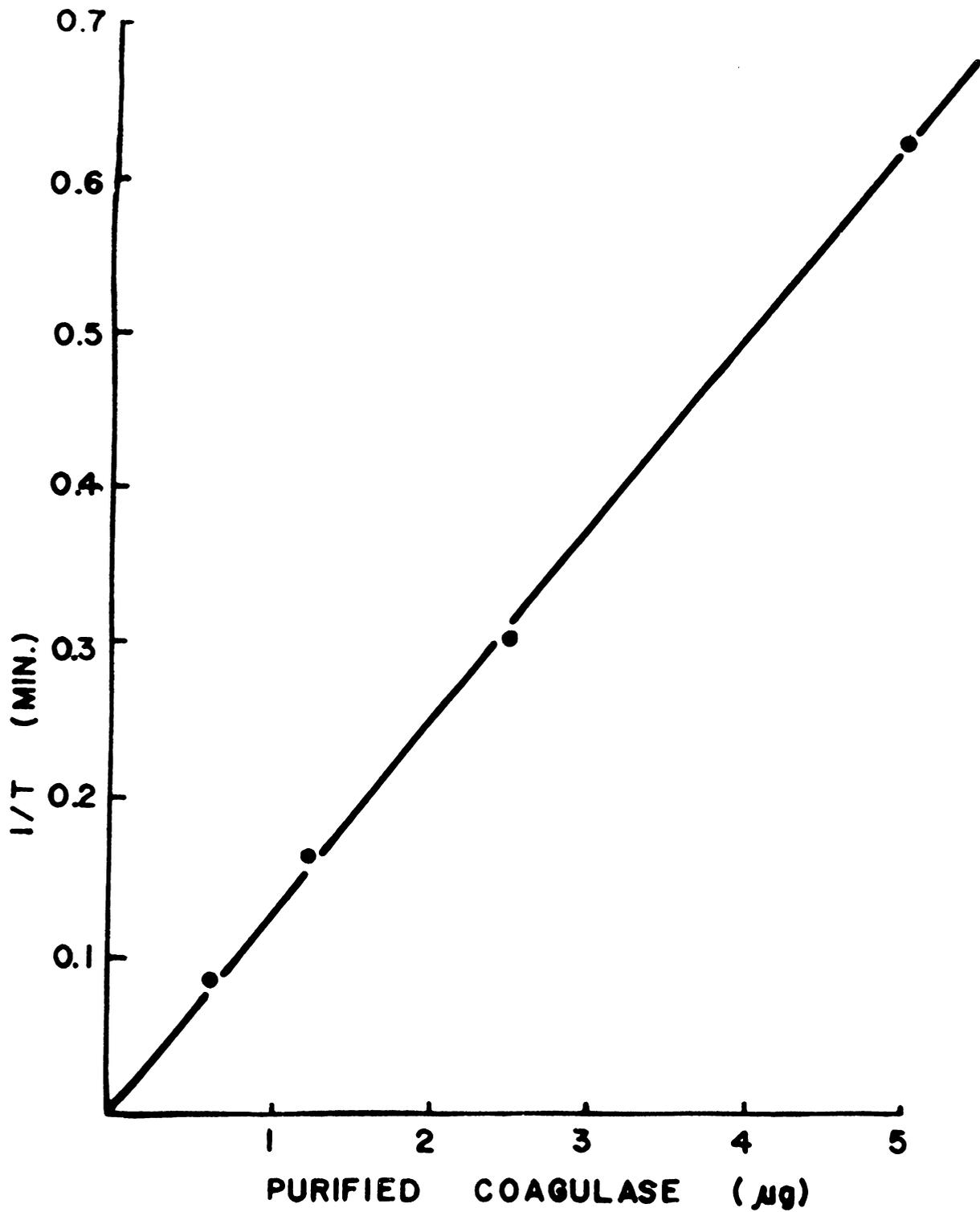
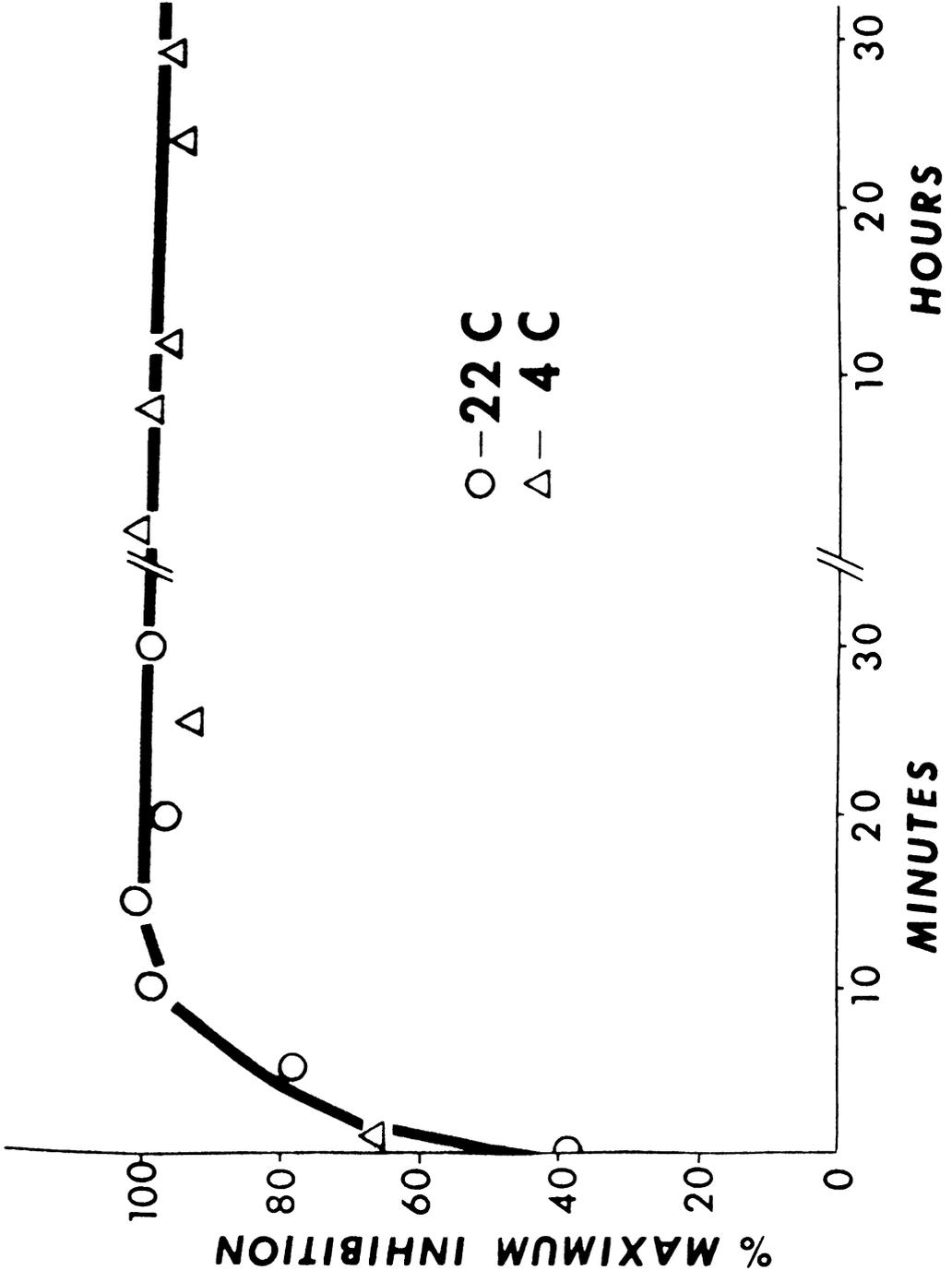


Fig. 16.--Inverse relationship between coagulate concentration and time required for the initiation of recorder response using the Brice-Phoenix light scattering photometer.







Heat inactivation of anticoagulase serum.--Heating of the pooled antiserum for 10 min at 56 C reduced coagulase inhibition 50-55% (Fig. 18). Further heating up to 40 min had no significant effect. Addition of complement did not restore the inhibitory activity of heated antiserum, nor did complement alone have any inhibitory effect upon the coagulase reaction.

Anticoagulase standard curves.--A linear relationship existed between the concentration of antiserum, either heated or unheated, and the corresponding reduction in coagulase activity (Fig. 19). However, below residual coagulase activity of 150-200 units, the curvature of the lines increased rapidly and became asymptotic to the abscissa. Residual coagulase activity (approximately 75 units) persisted even in the presence of excess antiserum. Therefore, concentrations of the antiserum were always adjusted to allow residual coagulase activity in excess of 200 units.

Effect of variable coagulase-anticoagulase proportions.--The data in Figure 20 support the validity of the anticoagulase assay with various coagulase concentrations. Addition of a given amount of antiserum resulted in the same reduction of coagulase activity over a thousand unit range. The measurable range of uninhibited coagulase activity was 0-1800 units. Activity much in excess of that range was difficult to measure accurately as the recorded



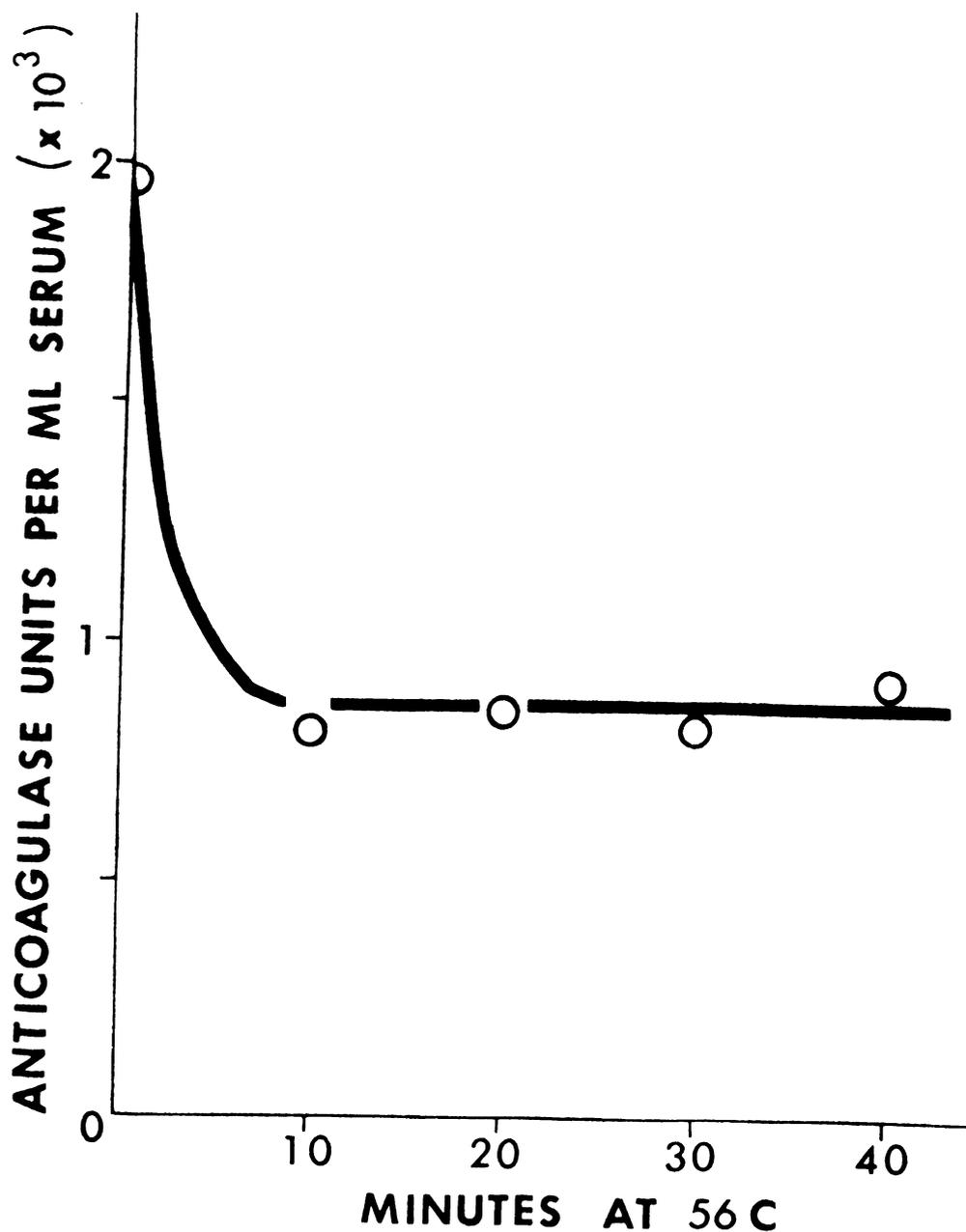


Fig. 18.--Reduction in the coagulase-neutralizing ability of bovine antiserum during thermal inactivation at 56 C prior to incubation with coagulase for subsequent nephelometric assay.



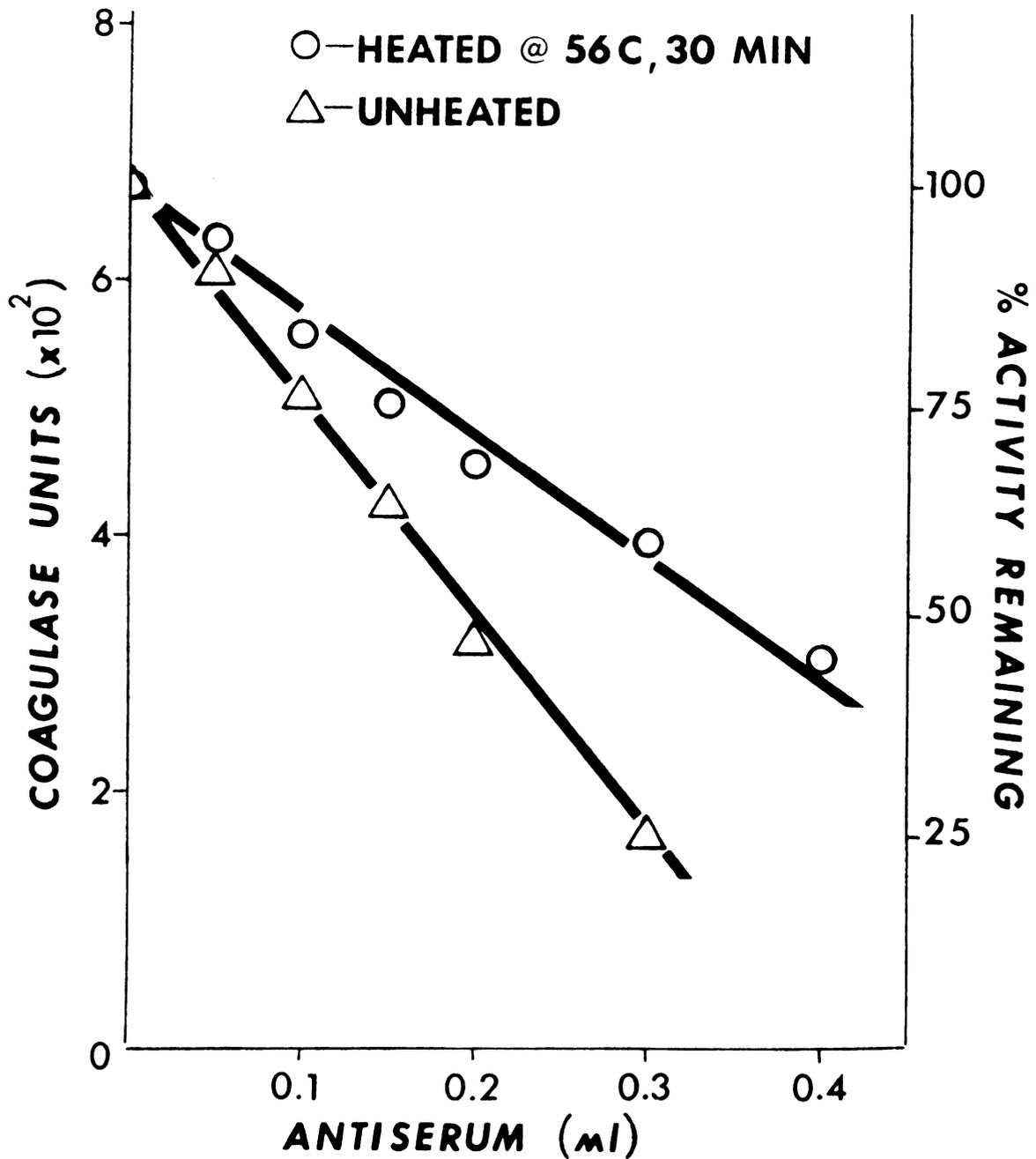


Fig. 19.--Linear relationships between coagulase neutralization and the respective amounts of heated or unheated bovine antiserum added per ml of coagulase.



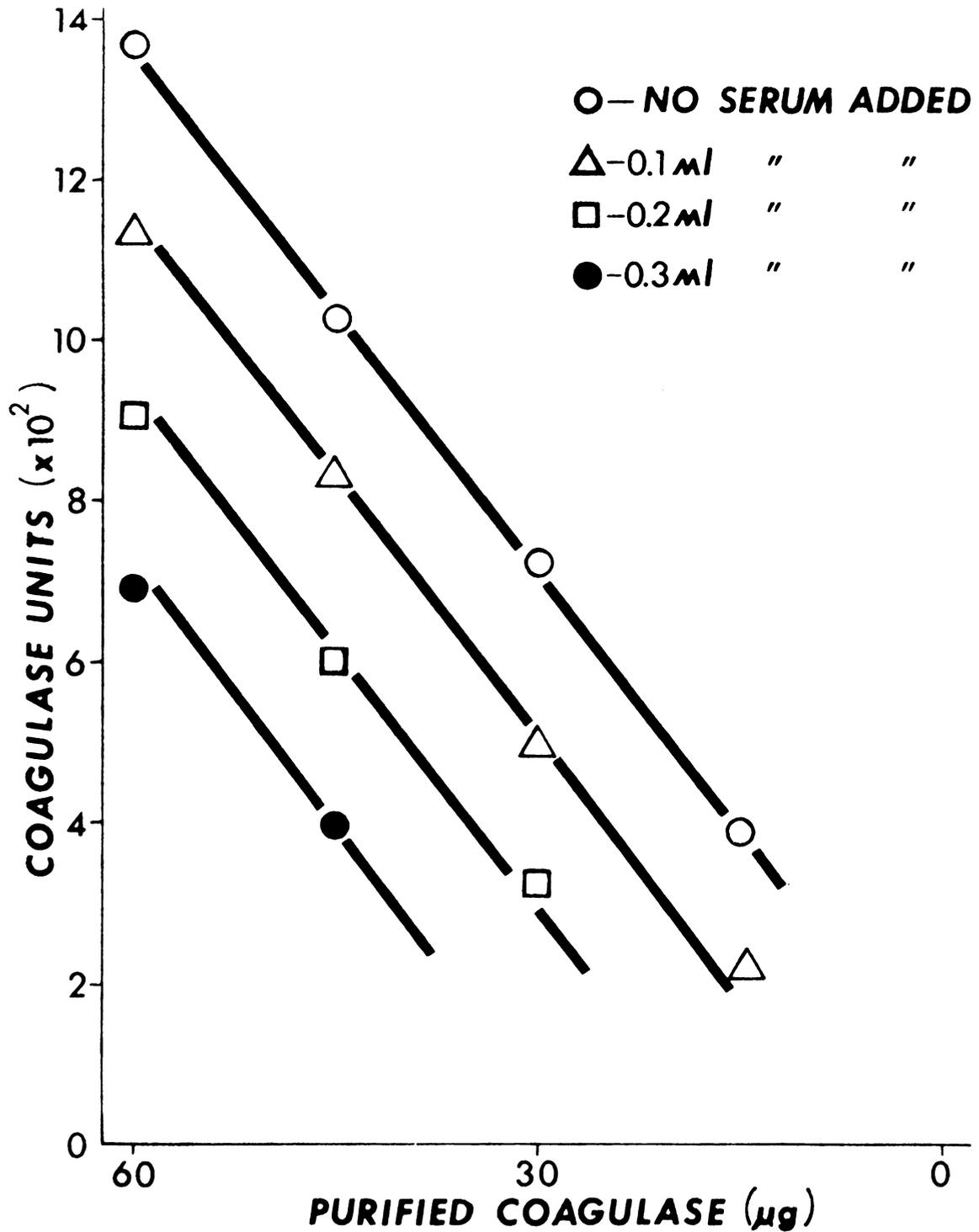


Fig. 20.--Standard curves obtained by incubating constant amounts of antiserum with varying amounts of coagulase over a range of 200-1400 coagulase units.



rate of change approached infinity. Standard deviation of the assay method, based on the observed rates of 12 identical samples, was calculated to be $\pm 3.9\%$.

Fractionated anticoagulase preparations.--The gamma globulin fraction, precipitated by 33% saturation with ammonium sulfate, contained 35-45% of the anticoagulase activity of whole antiserum. This fraction was stable at 56 C for 30 min. After dialysis, the supernatant fluid contained an additional 5-15% of the original inhibitory activity. Combination of the two fractions resulted in only 60-70% of the original anticoagulase activity. Complement had no effect upon its restoration.

Survey of coagulase inhibitors in sera of dairy cows.--Data obtained in a survey of sera from 16 dairy cows (Table 5) indicated that the heat labile coagulase inhibitor accounted for most of the coagulase neutralization in the majority of those sera tested. Sera from 15 cows lost 50% or more of their neutralizing ability after heating at 56 C for 30 min.

Modification of Coagulase Inhibition by
Heat Inactivation of Antiserum

Heating of bovine anticoagulase serum decreased its inhibitory ability from 50 to 55%. Besides this quantitative difference, heating of the antiserum changed the nature of the coagulase inhibition (Fig. 21). With unheated antiserum, increasing the fibrinogen concentration of the

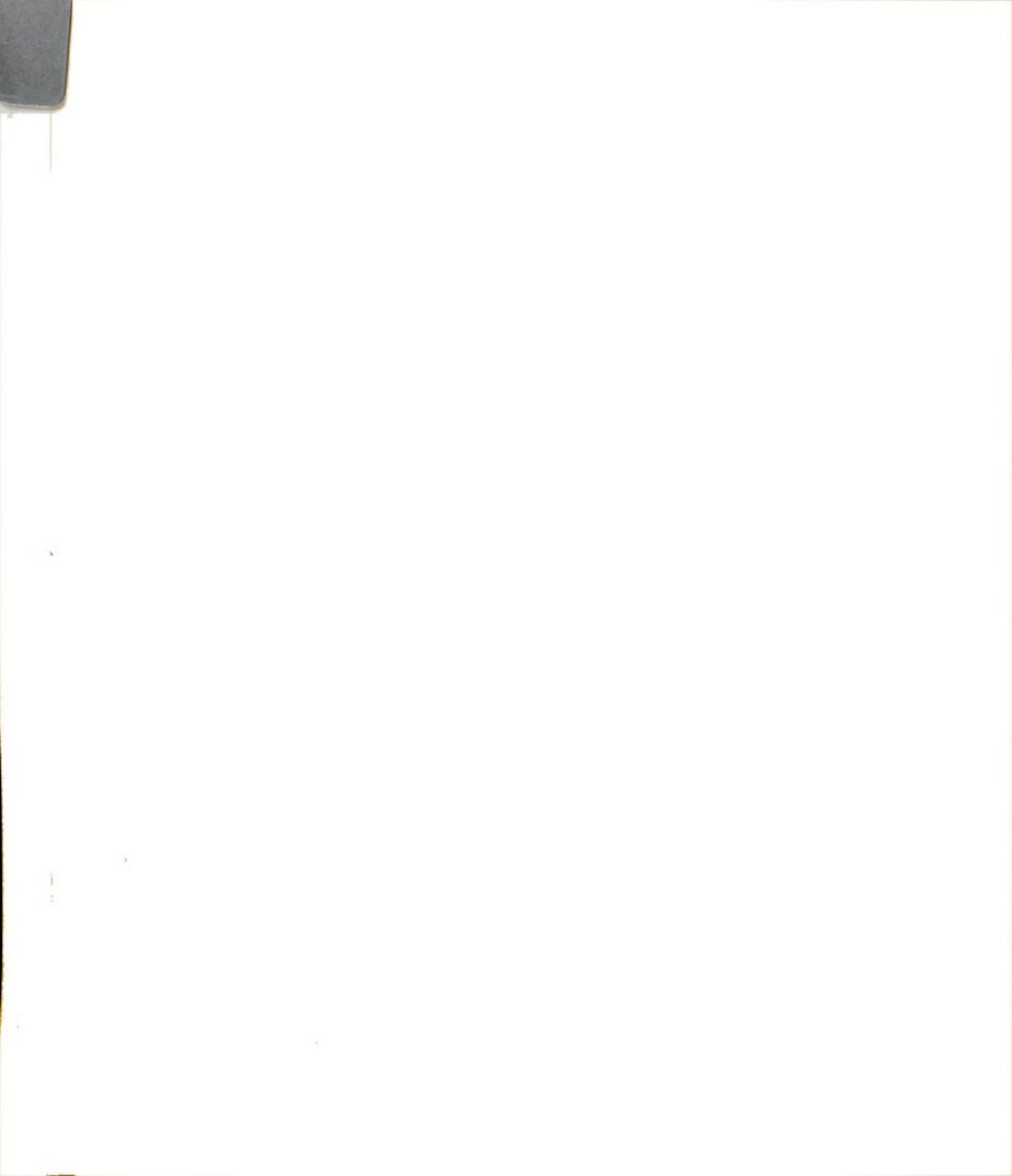


TABLE 5.--Effect of heating (56 C, 30 min) upon the coagulase-neutralizing ability of sera from dairy cows.

Cow number	*Anticoagulase units per ml serum	
	Heated serum	Unheated serum
1	940	1875
2	281	3470
3	0	1004
4	0	140
5	140	3130
6	275	740
7	10	2873
8	140	1475
9	275	1540
10	40	1270
11	285	1940
12	0	1535
13	140	675
14	190	140
15	0	675
16	0	215

*An anticoagulase unit was defined as the neutralization equivalent of one coagulase unit.



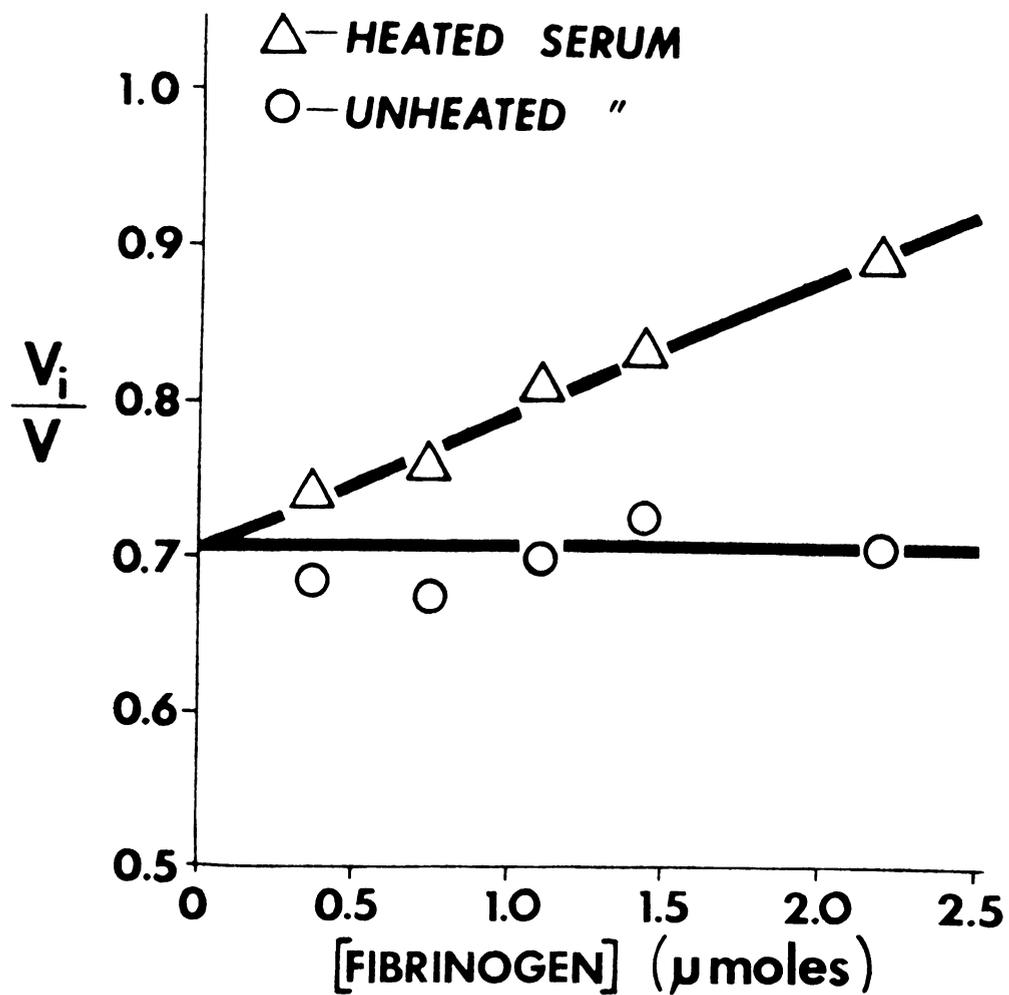


Fig. 21.--Effect of fibrinogen substrate concentration (micromoles per liter of reaction mixture) on coagulase inhibition by heated and unheated bovine antiserum. This effect has been expressed as the ratio of inhibited activity (V_i) to uninhibited activity (V).



reaction mixture had no appreciable effect upon the ratio of inhibited coagulase activity (V_i) to uninhibited activity (V). This indicated that a constant amount of coagulase remained inactivated regardless of the substrate concentration. However, when coagulase was inhibited by heat antiserum, increasing substrate concentrations caused a corresponding increase in the V_i/V ratio, indicating a rapid reversal of inhibition during the assay period.

In later experiments, the effects of substrate concentration upon coagulase inhibition were analyzed by Lineweaver-Burke plots. Figure 22 presents the results of a typical experiment in which coagulase was inhibited by two concentrations of unheated antiserum. Intercepts on the ordinate were 0.56 for the uninhibited reactions, 0.74 for the reactions inhibited by 0.05 ml antiserum, and 1.10 for the reactions inhibited by 0.10 ml antiserum. There was no significant change in the K_m value, which was calculated to be 6.45×10^{-7} M for fibrinogen. These data indicated that unheated antiserum inhibited coagulase in a non-competitive fashion.

Data obtained in the same experiment using heated antiserum (Fig. 23) indicated a modification of this inhibition. Intercepts on the ordinate were 0.52 for the reactions inhibited by 0.05 ml antiserum, and 0.62 for reactions inhibited by 0.10 ml antiserum. The respective K_m values were 10.0 and 12.9×10^{-7} M. Heated antiserum appeared to act as a competitive inhibitor, as its inhibition

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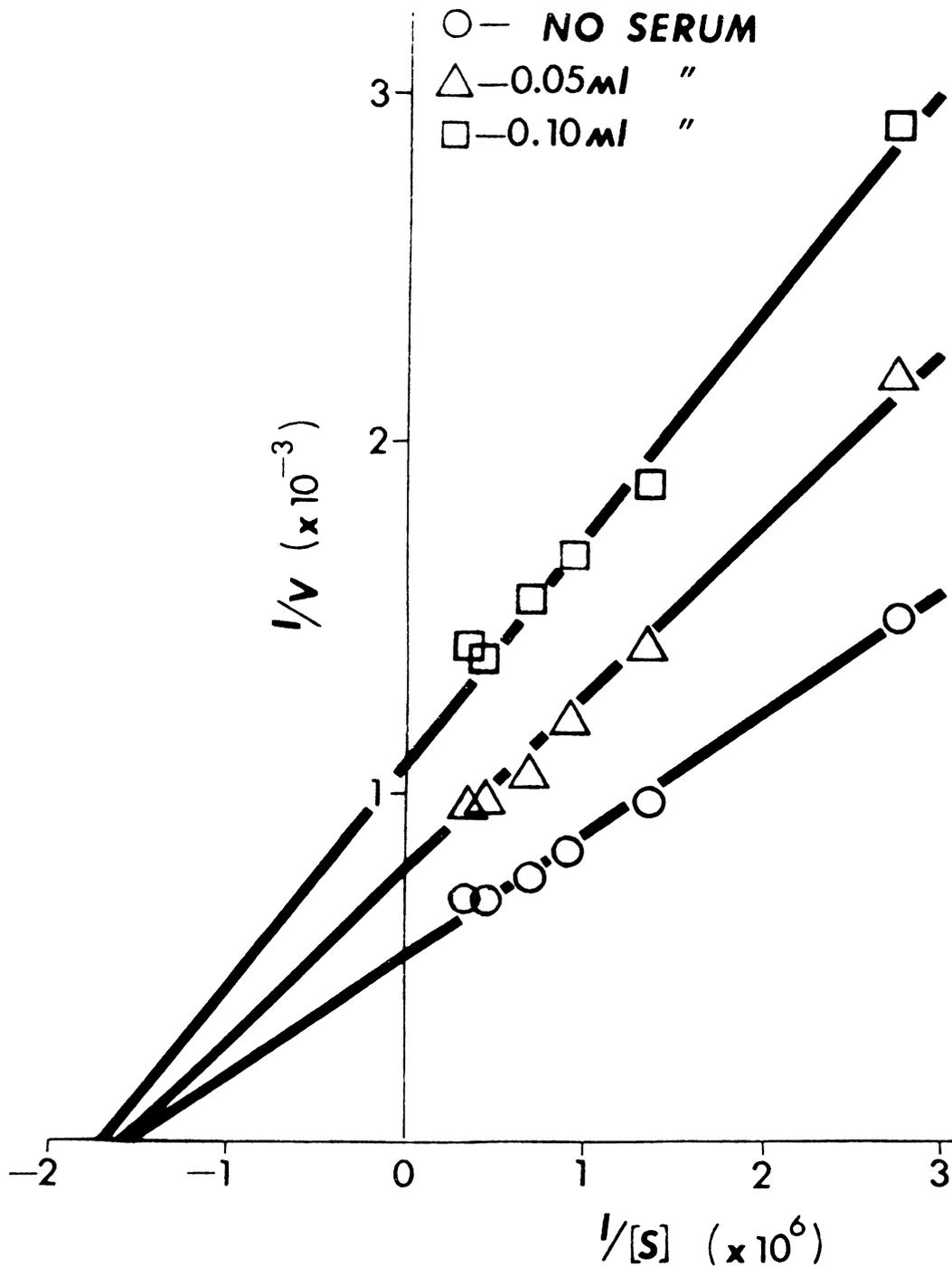


Fig. 22.--Lineweaver-Burke plot of the relationship of clotting velocity to fibrinogen substrate concentration for uninhibited coagulase, and coagulase inhibited by two concentrations of unheated antiserum.



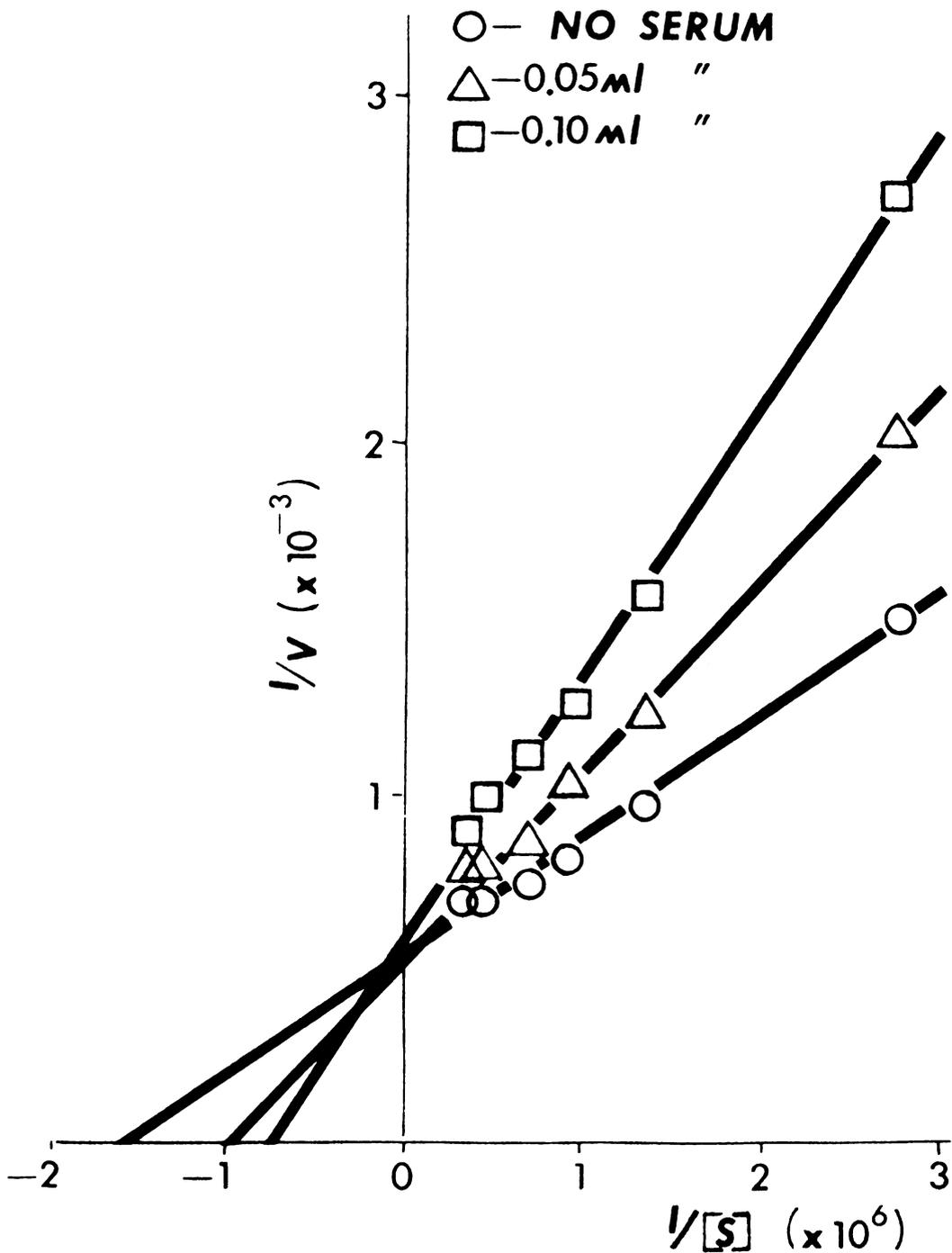


Fig. 23.--Lineweaver-Burke plot of the relationship of clotting velocity to fibrinogen substrate concentration for uninhibited coagulase, and coagulase inhibited by two concentrations of heated antiserum.



could be reversed as the concentration of fibrinogen approached infinity.

In several experiments, coagulase was also pre-incubated at 4 C or 22 C with varying concentrations of fibrinogen lacking plasma-CRF. No clotting was observed even after 30 min. Heat and unheated antisera were then added and the anticoagulase assay was carried out in the usual manner. Pre-incubation of coagulase with fibrinogen prior to the addition of heated or unheated antisera had no significant effect upon coagulase inhibition.

Kinetics of the Coagulase Reaction

The catalytic nature of coagulase.--A series of successive clotting reactions, each being initiated by the addition of fresh fibrinogen (lacking CRF), was employed to eliminate the possibility that coagulase was consumed as a substrate. The results of such a series of experiments are given in Figure 24. After removal of the clotted fibrinogen by centrifugation of the original reaction mixture, addition of an equal volume of fresh fibrinogen diluted the initial coagulase and CRF concentrations to one-half. As a consequence, the clotting rate of the second reaction should have been reduced 50%. However, the observed activity of the second reaction was 70-80% of the first. When subsequent reactions were initiated in the same manner, measurement of the clotting rates produced a



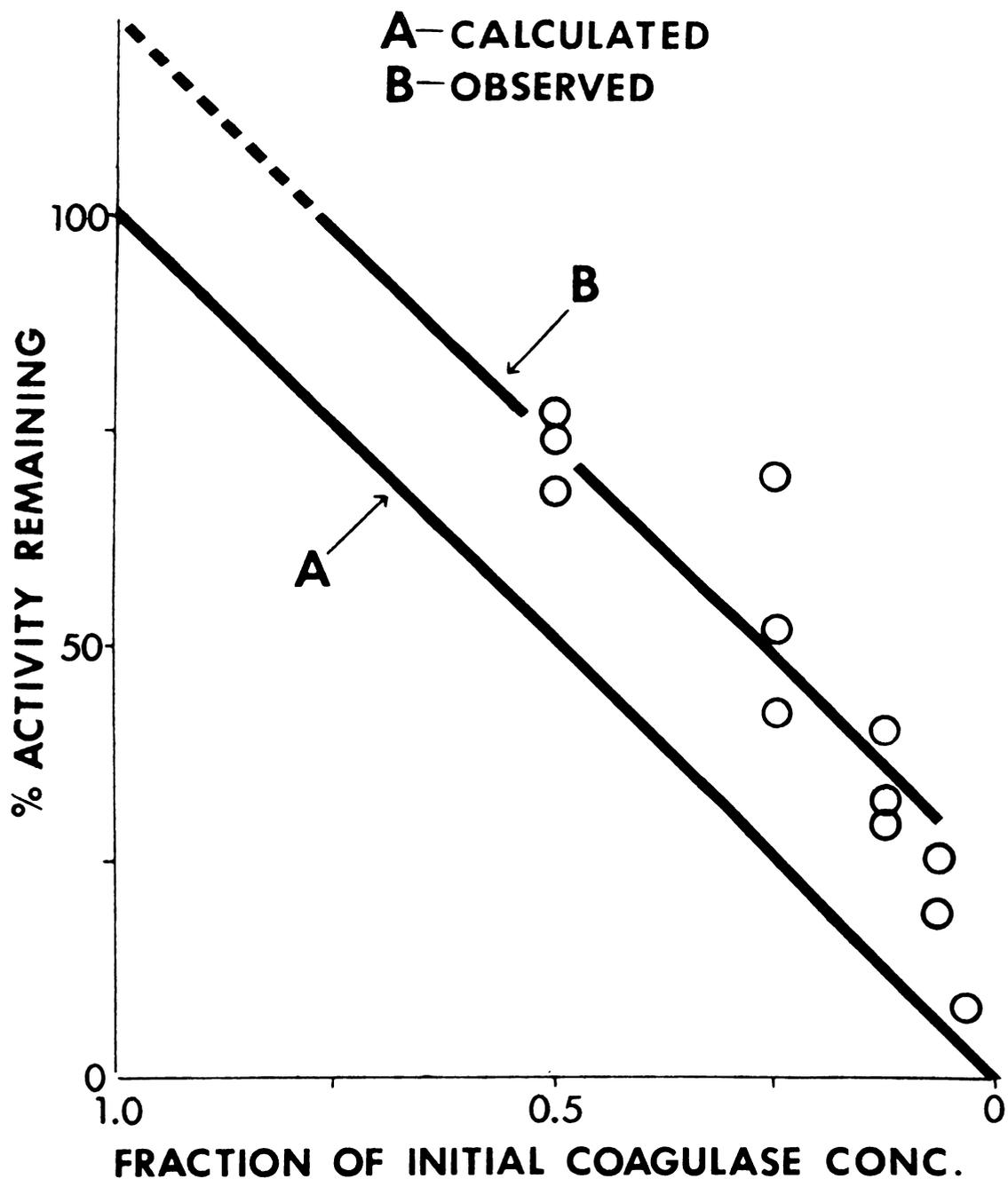


Fig. 24.--Experiment designed to demonstrate the catalytic nature of coagulase in the clotting reaction. This graph represents the results of a series of two-fold dilutions of the original reaction mixture (coagulase concentration 1.0) by equal volumes of fresh fibrinogen lacking plasma-CRF. Each subsequent addition of fresh fibrinogen was made only after the fibrin clot of the previous reaction was compressed and removed by centrifugation.



plot that was approximately parallel to the calculated line, but always in excess of the expected activity.

Michaelis-Menton equation in coagulase kinetics.--

Before application of the Michaelis-Menton equation to the study of coagulase kinetics, it was necessary to determine whether the equation fitted the observed characteristics of the reaction. Figure 25 illustrates the independence of $S(V_m - V)/V$ in regard to both fibrinogen and CRF concentrations. This independence satisfied the criterion suggested by Kistiakowsky and Rosenberg (1953) for testing the validity of the application of the Michaelis-Menton equation.

Determination of the Michaelis-Menton constant.--The

Michaelis-Menton constant (K_m) for the fibrinogen substrate in the coagulase reaction was calculated in a Hanes plot (Fig. 26). This graphical method produced a value of 6.35×10^{-7} M, which correlated closely with values obtained in Lineweaver-Burke plots during characterization of coagulase inhibition by bovine antiserum.

Interaction of catalytic sites on the coagulase mole-

cule.--The lack of knowledge concerning the relationship of CRF and fibrinogen to coagulase prompted a study of interaction between catalytic sites on the coagulase molecule. To determine interactions between fibrinogen-binding sites, a Hill plot (Fig. 27) was constructed to relate the quantity $(V_m/V) - 1$ to the concentration of fibrinogen substrate (S).



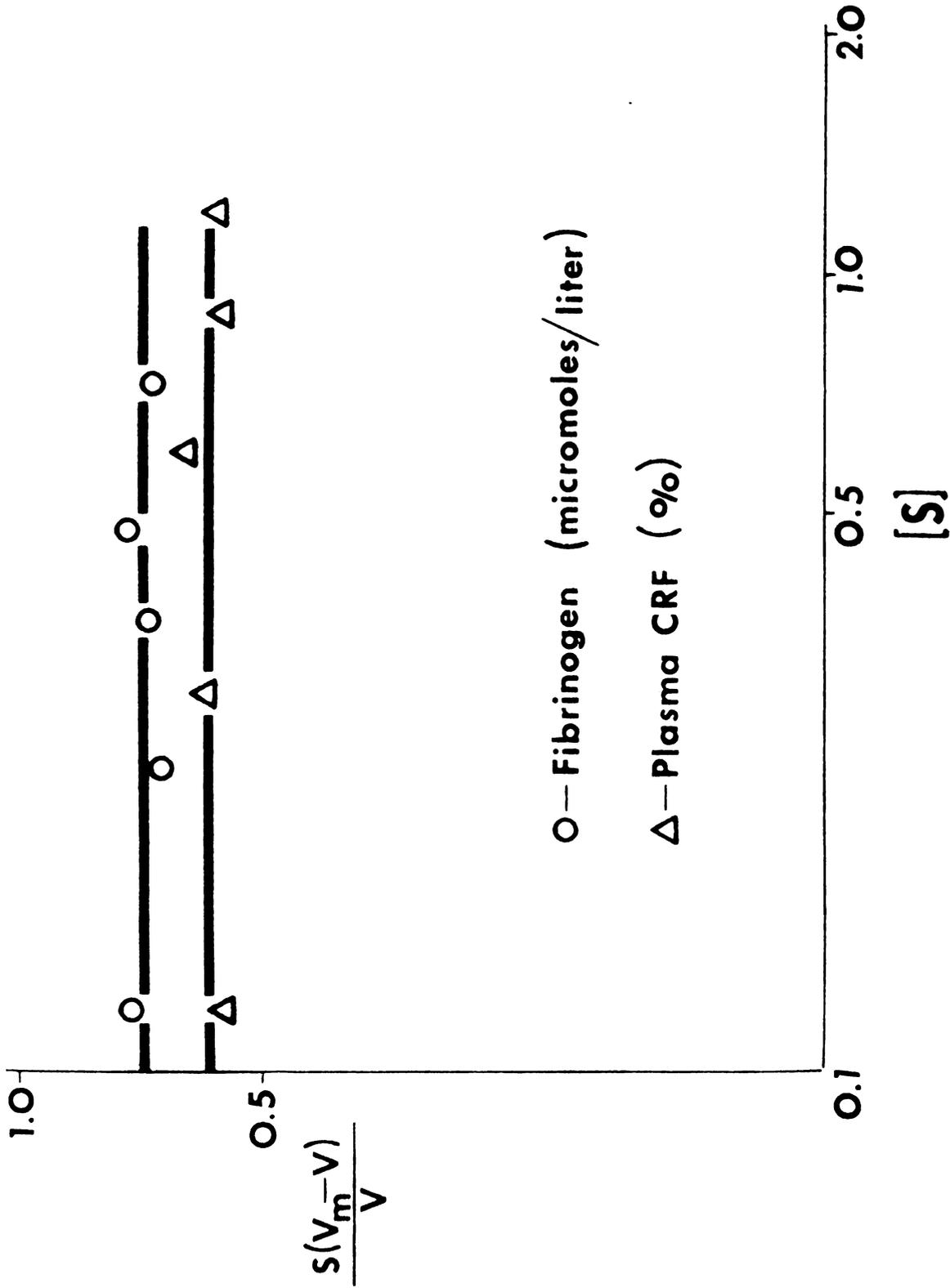


Fig. 25.--Test to insure the validity of the application of Michaelis-Menton kinetics to the study of the coagulase reaction. Each point is the average of three determinations.



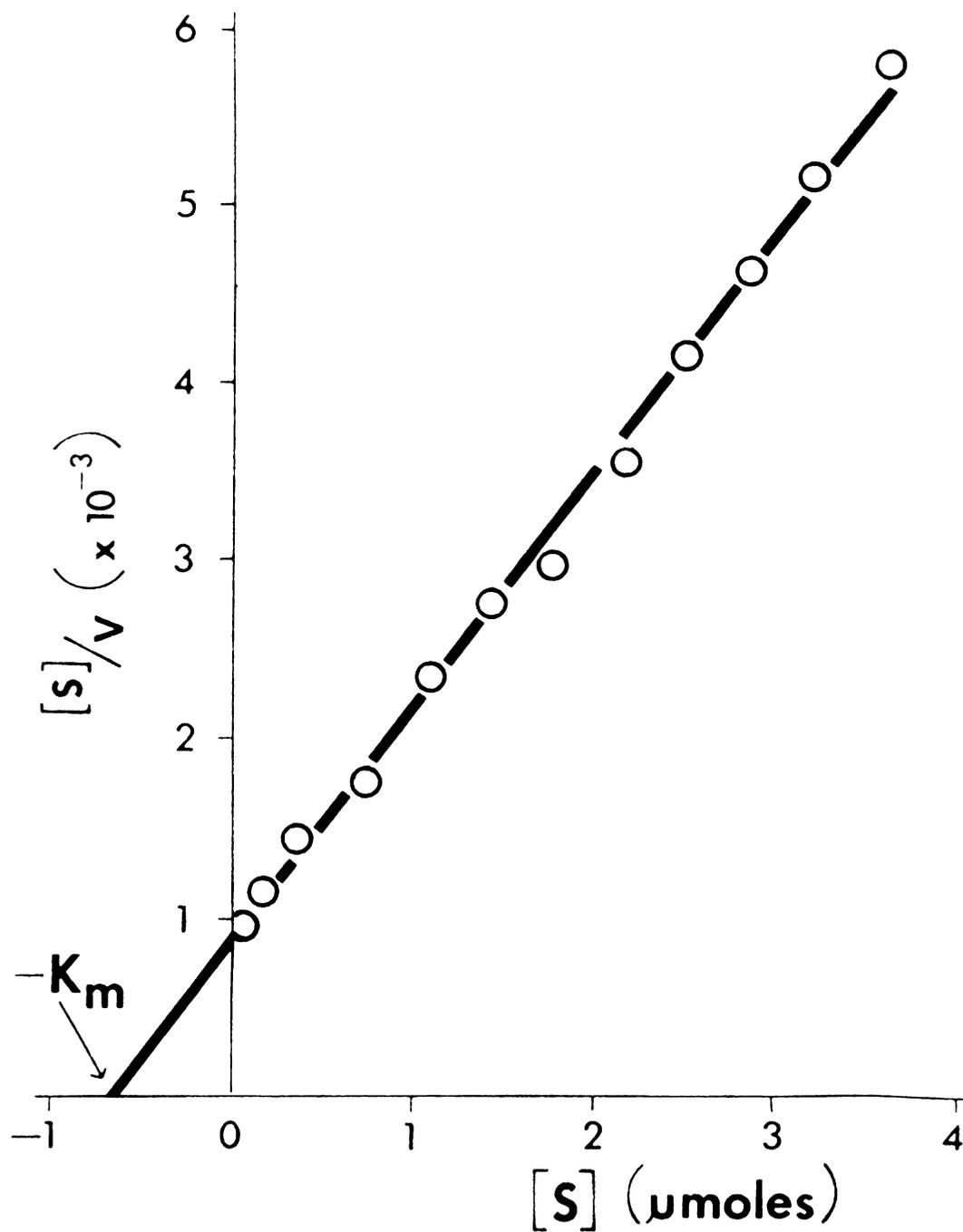


Fig. 26.--Determination of the Michaelis-Menton constant (K_m) for fibrinogen in the coagulase reaction. ^mFibrinogen substrate concentration is expressed as micromoles per liter of reaction mixture.



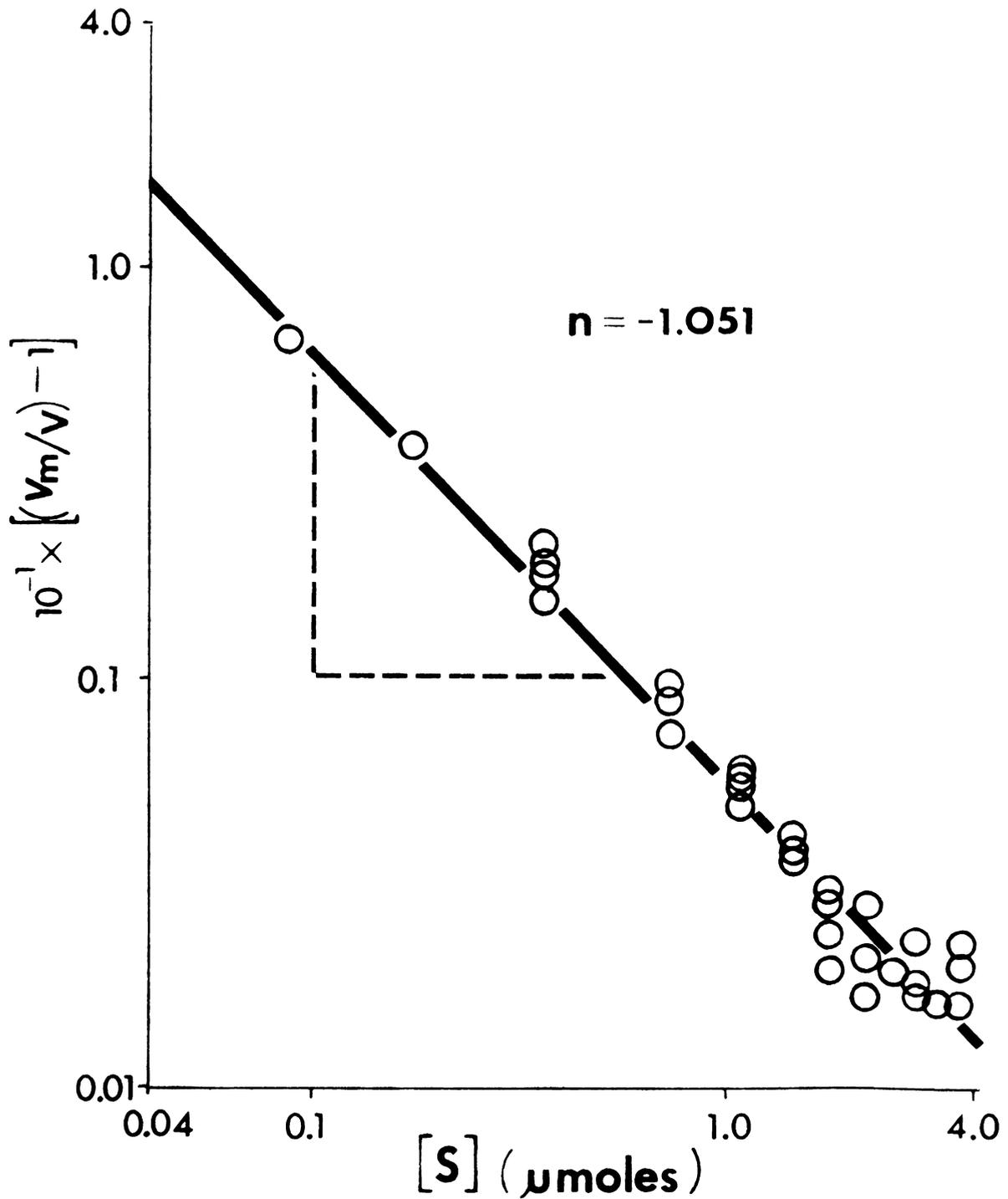


Fig. 27.--Hill plot to determine possible interaction between fibrinogen-binding sites on the coagulase molecule. Fibrinogen substrate concentrations are expressed as micromoles per liter of reaction mixture.



The Hill equation plotted in this manner resulted in a negative slope with a value of -1.051 . The similar plot, $V/(V_m - V)$ vs. CRF concentration, was used to determine any interaction between CRF-binding sites (Fig. 28). A positive slope of 0.983 was obtained in this case. Slopes for both fibrinogen and CRF were very close to unity and indicated no significant interaction between catalytic sites for either on the coagulase molecule.

The coagulase reaction as a two-substrate system.--

The requirements of the coagulase reaction indicated that it was a system involving either two substrates or a substrate and an activator. Figures 29 and 30 are plotted according to Florini and Vestling (1957). The ordinate at the point of intersection for the lines in each graph is important; it is an indication of the effect that the presence of one bound substrate or activator has upon the binding of the second substrate or activator to the enzyme. Coordinates at the point of intersection for all lines are independent of all substrate concentrations and so can be expected to fall at a single point in each double reciprocal plot. If the points of intersection on the ordinates of both graphs equal zero, the K_m values for either substrate (or activator) are not influenced by the concentration of the other. These conditions fit the data for the coagulase reaction; the binding of either fibrinogen or CRF has no influence upon the binding of the other.

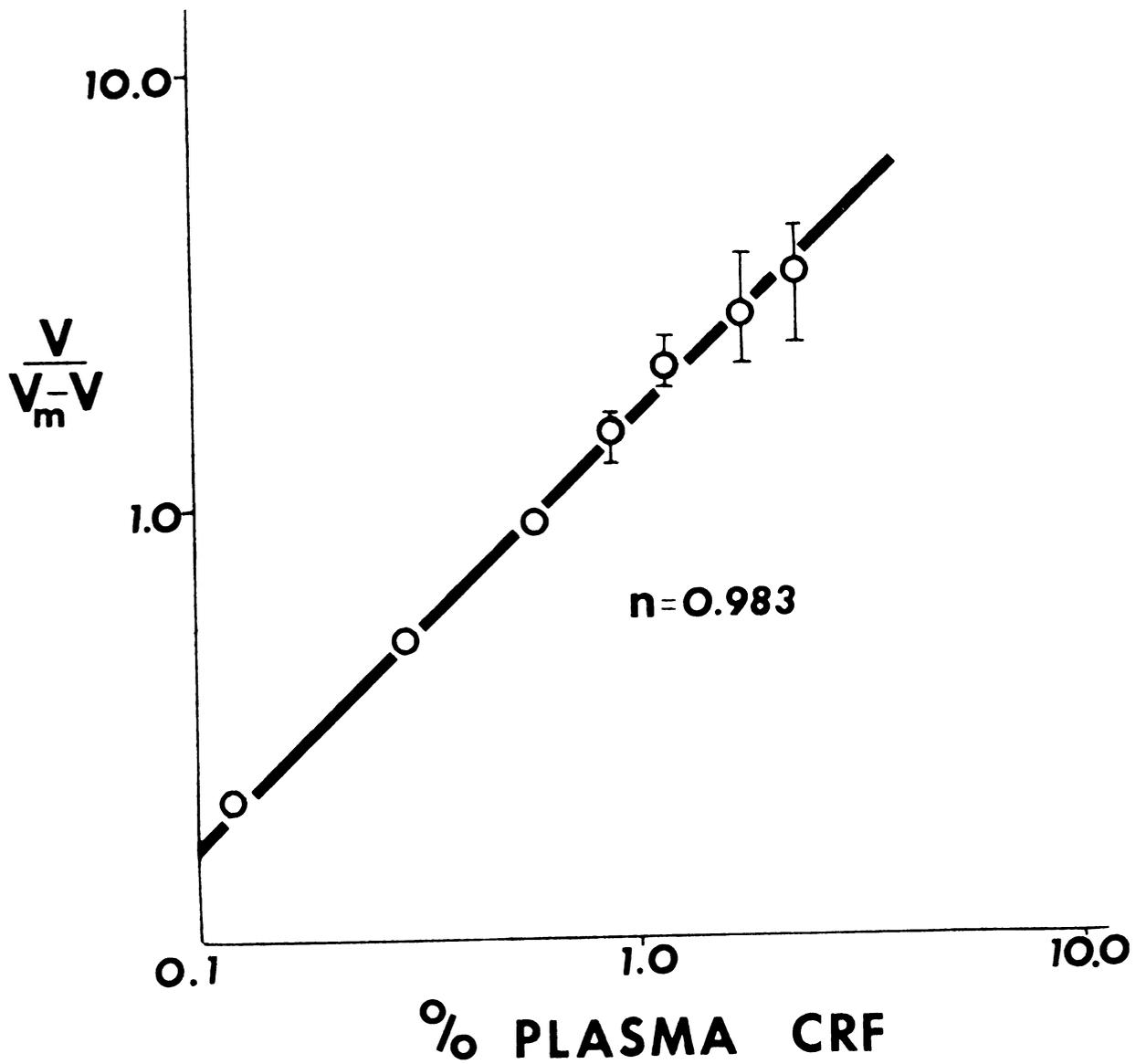


Fig. 28.--Hill plot to determine possible interaction between CRF-binding sites on the coagulase molecule. The points are the averages of five determinations.



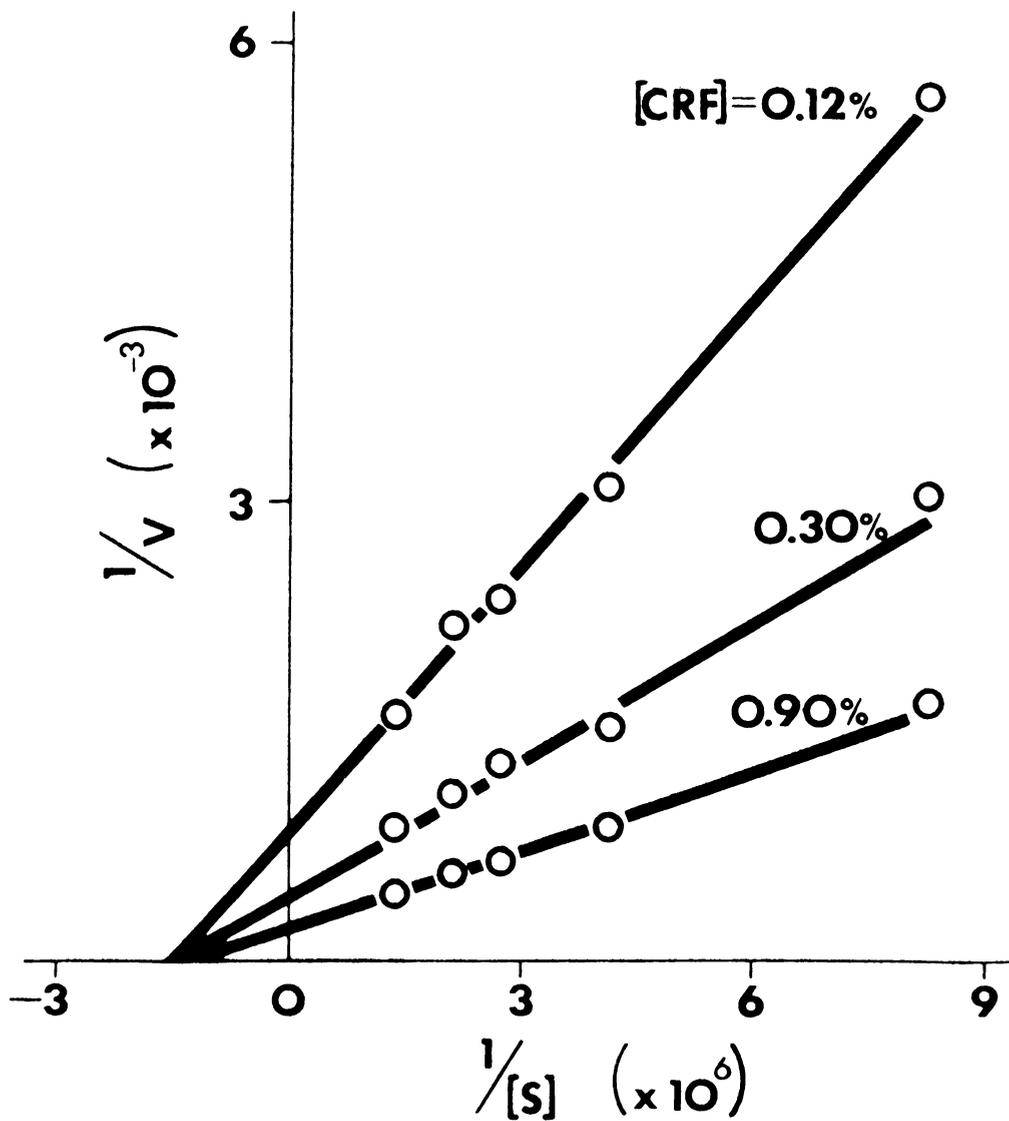


Fig. 29.--Lineweaver-Burke plot of clotting velocity versus fibrinogen substrate concentration at three limiting concentrations of plasma-CRF. The K_m for fibrinogen was 7.14×10^{-7} M.



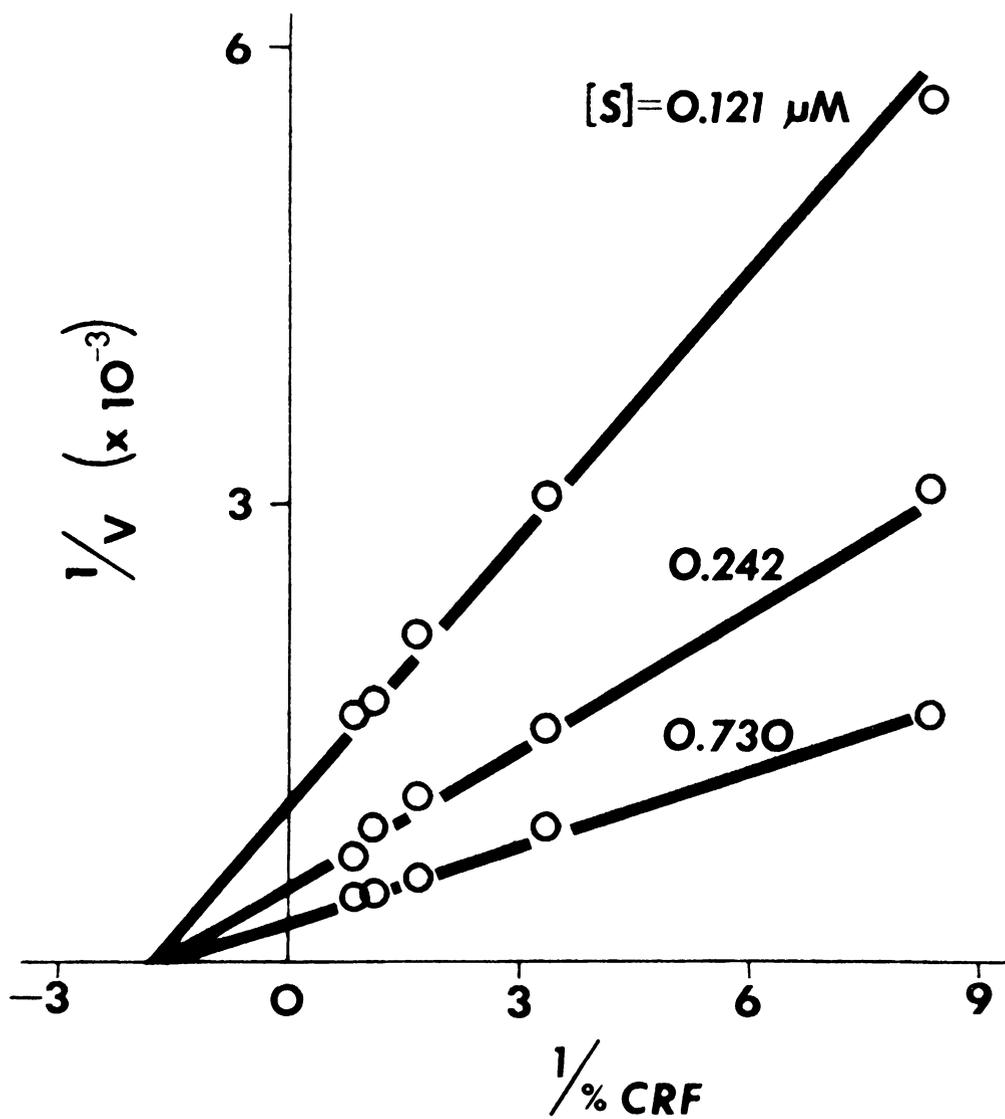


Fig. 30.--Lineweaver-Burke plot of clotting velocity versus plasma-CRF concentration at three limiting concentrations of fibrinogen substrate.



Maximal velocity values obtained from such graphs were incorporated into a third graph (Fig. 31) to calculate the final maximal velocity (V_f), and more importantly, to check the validity of the analysis. Maximal velocities for five limiting concentrations of fibrinogen at infinite CRF concentration were plotted against the concentration of fibrinogen. Likewise, maximal velocities for five limiting concentrations of CRF at infinite fibrinogen concentration were plotted against the concentration of CRF on the same double reciprocal graph. Lines for these two plots should theoretically intersect the ordinate at the same point at infinite concentrations of both components if the application of Michaelis-Menton kinetics is valid and if there is no substrate inhibition. The analysis of the coagulase reaction conformed to the expected characteristics of a two-substrate system, or a substrate-activator system, in which the binding of one component to the enzyme has no effect upon the binding of the other.

Role of CRF in the clotting reaction.--Having arrived at this point in the analysis of the coagulase reaction, the important question was the identity of CRF as a substrate or as an activator. A simple kinetic approach was employed to make this distinction. If CRF was a substrate, the K_m should not be influenced by variation in the coagulase concentration. However, if it was an activator which combined with coagulase without being consumed, the

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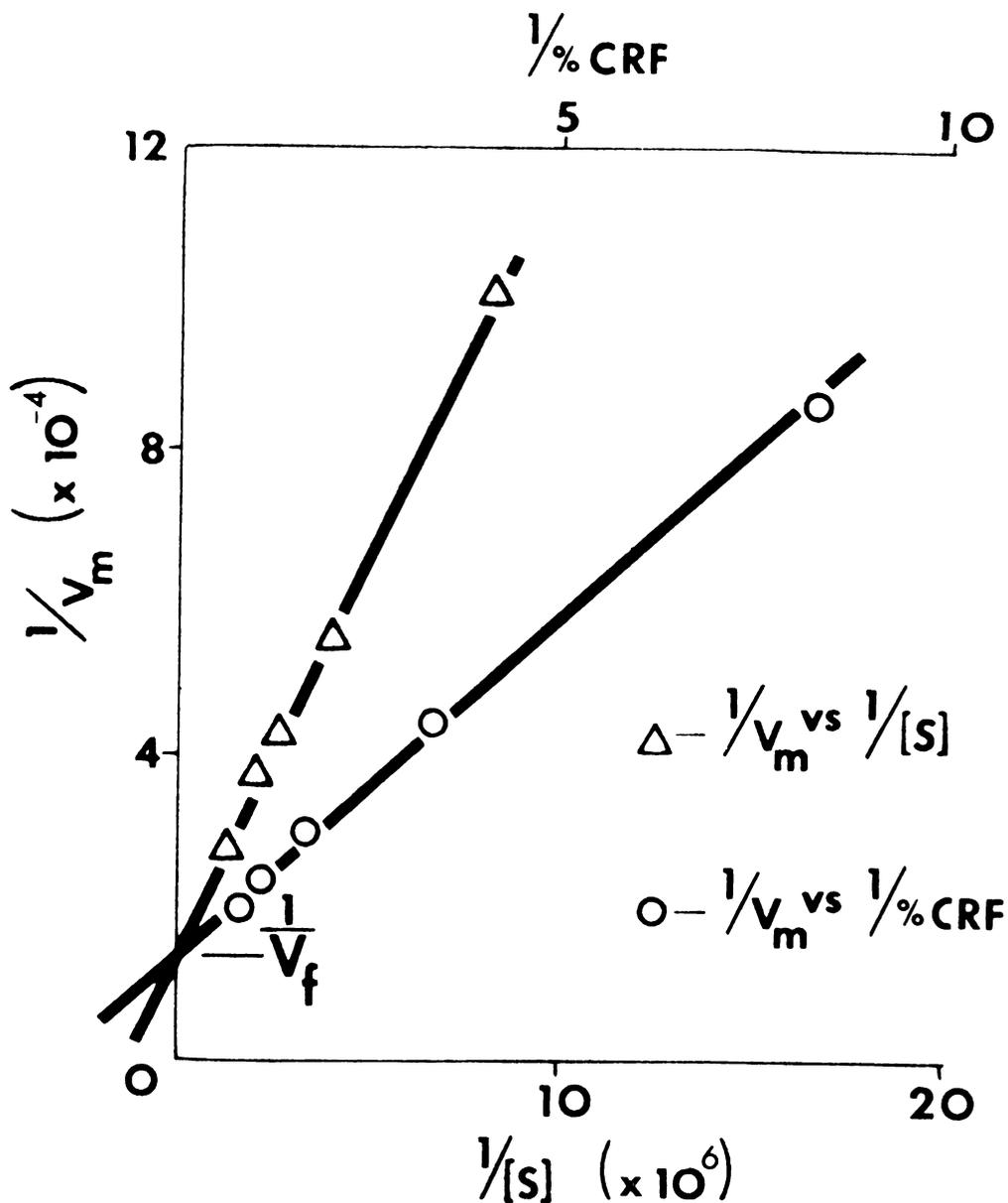
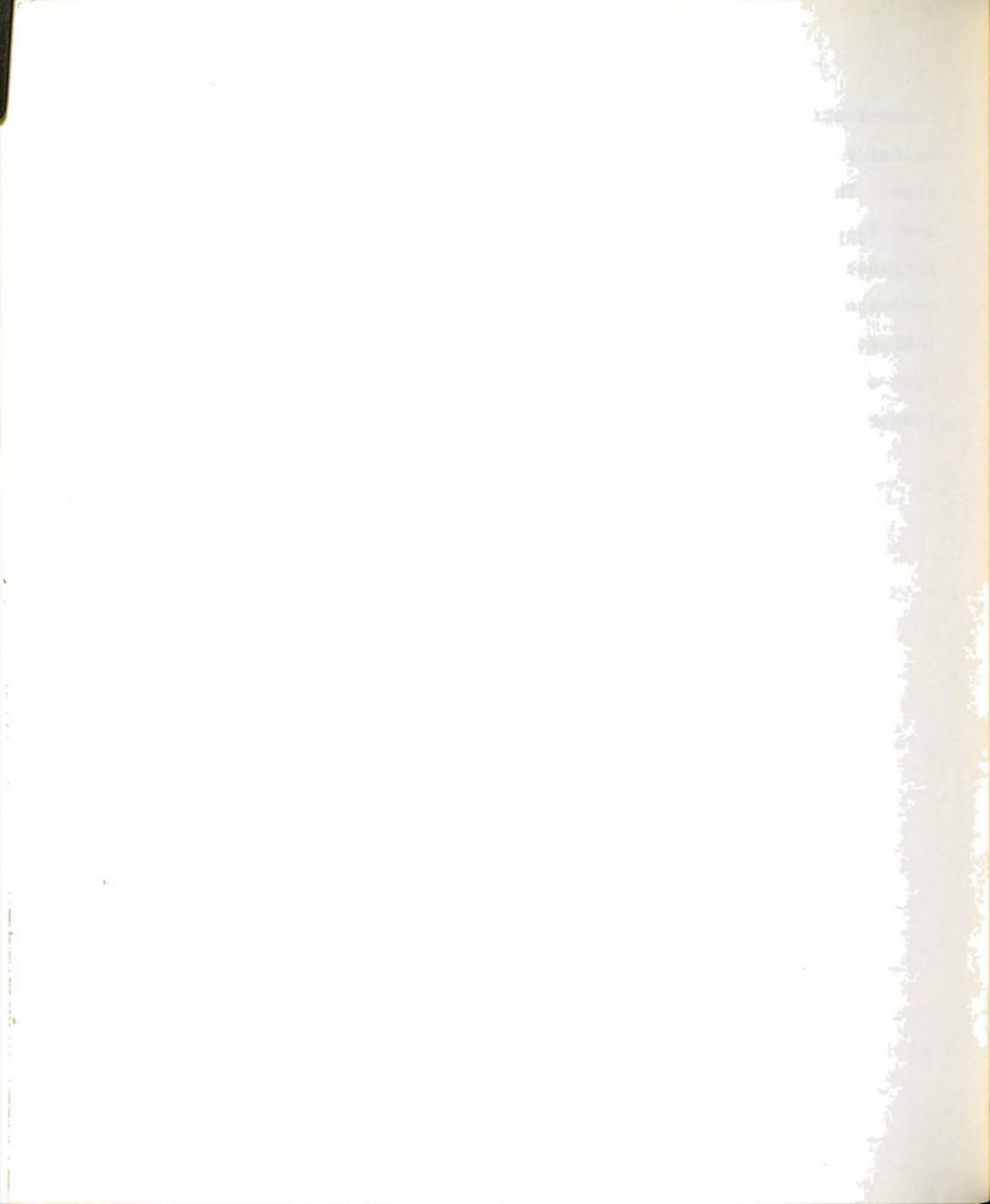


Fig. 31.--Plot of maximal velocities obtained with limiting concentrations of plasma-CRF (%) or fibrinogen substrate (moles per liter of reaction mixture). Both lines intersect the ordinate at a value of $1/V_f$. V_f was calculated to be 6,896 coagulase activity units, the maximal velocity at infinite concentrations of both fibrinogen substrate and plasma-CRF.



concentration necessary to support one-half the maximal velocity would be proportional to the coagulase concentration. The effect of variation in coagulase concentration upon K_{CRF} was determined in a Hanes plot (Fig. 32). An increase in the coagulase concentration resulted in a corresponding increase of the K_{CRF} value. Such behavior indicated that CRF was not a substrate, but rather some type of activator which combined with coagulase in definite proportions.



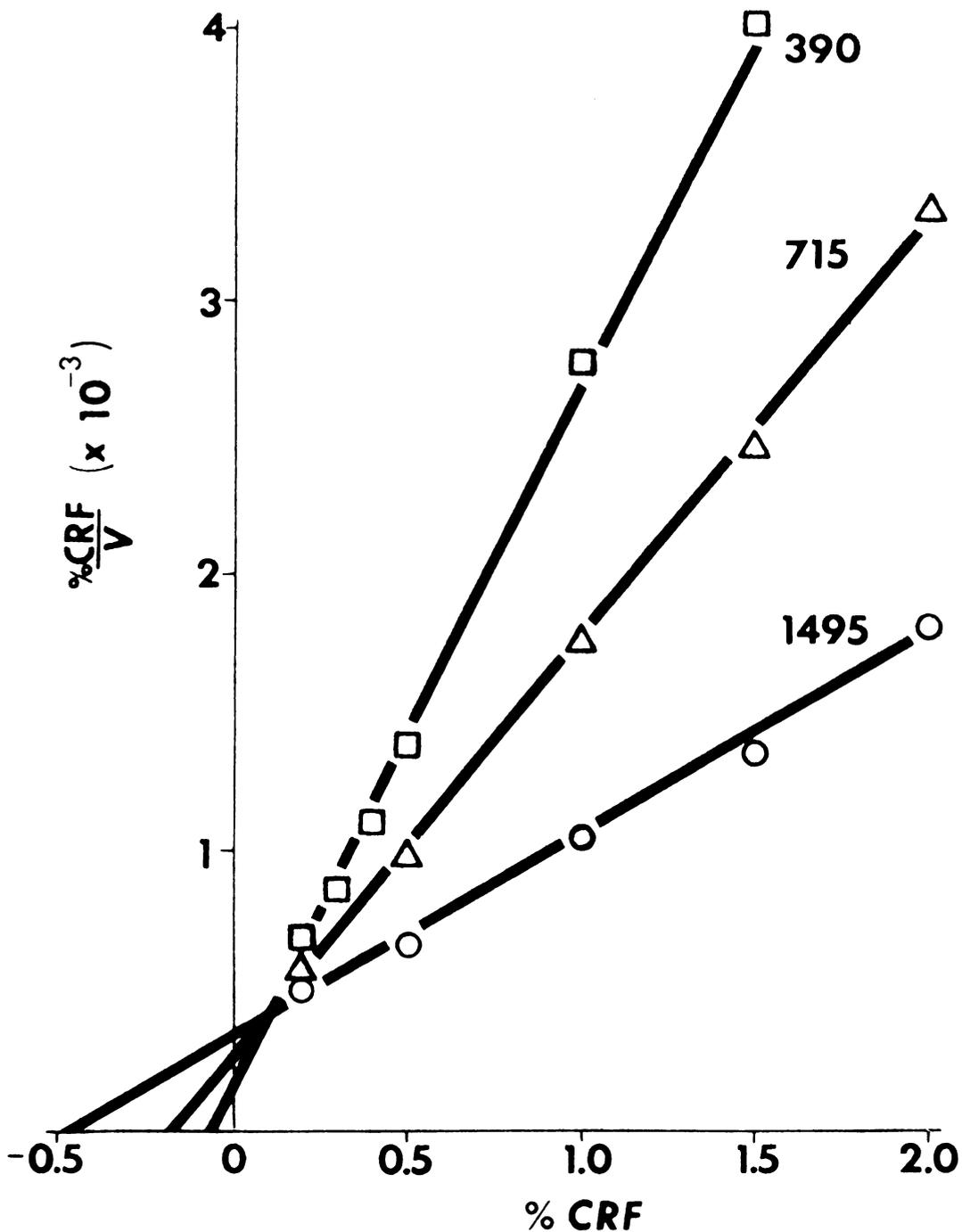


Fig. 32.--Hanes plot demonstrating the variation of K_{CRF} in proportion to the coagulase concentration. The three coagulase concentrations used in this experiment had maximal velocities of 390, 715, and 1495 activity units as shown in the graph.



DISCUSSION

The results obtained in these studies have indicated that a complex, high-protein medium such as Brain Heart Infusion is not necessary for satisfactory coagulase production. A casein acid hydrolysate medium supported comparable yields and offered the advantage of a greatly decreased protein concentration. This characteristic is important in enzyme purification, as the initial specific activity (activity units per mg protein) determines to some extent the difficulty of the purification process. Duthie and Haughton (1958) acknowledged the advantages of a low-protein casein hydrolysate medium for coagulase production. However, they were forced to cultivate their inocula in Ox Heart Digest Broth in order to obtain high yields of the clotting enzyme, and therefore lost much of the advantage of the simple medium. The purification procedure was simplified in the present studies because a casein acid hydrolysate medium could be used exclusively.

Most reports on the production of extracellular coagulase (Tager, 1948; Duthie and Haughton, 1958; Blobel, 1959) have emphasized the requirement for a large surface to volume ratio, although Hayashi (1960) reported maximal yields under anaerobic conditions. Satisfactory production of coagulase in our casein acid hydrolysate medium



required rapid shaking and a large surface to volume ratio. It is unknown whether this dependence is based on oxygen requirements, or is merely the result of liberating more coagulase from the cell walls by agitation.

Since production and purification of coagulase have often been reported, the primary purpose of these studies was not just the mechanics of another purification procedure. However, in order to obtain sufficient quantities of the purified enzyme for the development of a precise assay for coagulase activity and a kinetic analysis of the clotting reaction, a simplified purification procedure was necessary as a means to an end. The procedure reported here allowed a high percentage of recovery (50%) of an immunologically homogeneous coagulase preparation.

The presence of two thermal isozymes, one more heat stable than the other, appeared to exist in definite proportions (70% to 30%) throughout the course of the purification procedure. Although such isoenzymatic states seem to be not unusual (Kaplan, 1962; Staples et al., 1965), Coleman and Eley (1963) have pointed out that caution must be maintained in assuming that differences in thermal stability are a reflection of conditions in their native state. It could be possible that during heat inactivation of a homogeneous enzyme preparation, the formation of a second, less active enzyme may occur, both species inactivating independently. Resolution of this problem might be accomplished by physical isolation of the postulated



thermal isozymes by a highly sensitive technique such as discontinuous electrophoresis.

During the course of our research on coagulase purification, it became apparent that the routine titer method was inadequate for all but qualitative or semi-quantitative purposes. This inadequacy stimulated research towards a more precise method of quantitating coagulase activity. Because light scattering techniques had been widely used in the study of polymers (Kratohvil, 1964), this approach seemed well suited for the study of fibrinogen polymerization. A precise nephelometric assay for coagulase activity was developed upon this basis.

In determining the parameters which exerted an effect upon the rate of the fibrinogen clotting reaction, consideration of fibrinogen and CRF concentrations, pH, and ionic strength was found to be important. Maximal coagulase activity occurred at a fibrinogen concentration of 2.2 μM or greater; the K_m was calculated to be 6.35×10^{-7} M. The optimal pH was 7.2, and an ionic strength of 0.07 to 0.10 was most favorable. These results agree rather well with the observations of Shinowara (1966) in studies on thrombin activity. Maximum thrombin activity was attained at fibrinogen concentrations of 2.85 to 7.39 μM , and the K_m was calculated to be 4.38×10^{-7} M. The pH and ionic strength optima for the thrombin-catalyzed reaction were 7.2 and 0.092 respectively. Comparison of these results obtained with coagulase and thrombin,

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together with the findings of Drummond and Tager (1963), support the view that the mode of action might be identical or at least quite similar.

In any coagulase or thrombin assay based on the clotting of fibrinogen per se, it should be emphasized that the enzyme is responsible for only the activation of the fibrinogen molecules. The formation of the intermediate fibrin polymers and their subsequent polymerization into the insoluble fibrin clot occur independently of enzymatic activity. Since it is the end product of this reaction sequence (i. e. the fibrin clot) which is measured, the question arises as to whether the physico-chemical requirements of the observed reaction, such as pH, ionic strength, and temperature, reflect the nature of the enzymatic or the polymerization reactions. Thrombin activity has been shown to proceed at pH 5.1 in M NaBr, conditions which completely inhibit the polymerization reaction (Sturtevant et al., 1955). Under such conditions, any technique measuring clot formation would not accurately estimate enzyme activity. The validity of an enzyme assay method based on the detection of clot formation would depend on the activation of fibrinogen being the limiting step in the chain of events leading to the fibrin clot. This appears to hold true in the nephelometric coagulase assay, for the observed activity is directly proportional to coagulase concentration.

The precision and reproducibility of this nephelometric coagulase assay warranted extension of its use into



the studies of coagulase inhibition by bovine antiserum. Under optimal conditions, the nephelometric assay proved to be suitable in this regard. However, linear relationships between antiserum concentration and coagulase inhibition could only be obtained when the residual coagulase activity exceeded 200 units. Below this value, the lines became curved and asymptotic to the abscissa at 50-75 coagulase units; this small amount of residual activity persisted even in the presence of excess antiserum.

This failure of excess antiserum to reduce coagulase activity to zero is not at all unusual in view of previous reports (Samuels, 1963; Roberts, 1966). In fact, most enzyme-antibody systems do exhibit a constant amount of residual enzyme activity no matter how much antiserum is added (Cinader, 1963). This phenomenon may probably be best explained by the theory that antiserum contains both inhibiting and non-inhibiting antibody (Cinader and Lafferty, 1963). The residual level of enzyme activity is due to the presence of combining non-inhibiting antibody which interferes with attachment of inhibiting antibody to the enzyme.

Biphasic heat inactivation curves and antiserum fractionation suggested the presence of both antibody and a heat labile coagulase inhibitor in bovine antiserum. In an attempt to establish the presence of both heat stable and heat labile coagulase inhibitors in bovine sera, a survey of randomly selected dairy cows was undertaken. Heating of the sera greatly reduced (and in some cases completely



abolished) coagulase inhibition in 15 of the 16 samples tested. Such results obtained from cows not previously injected with coagulase introduced the possibility that the heat labile factor was a normal serum constituent, in contrast to antibody which reached significant proportions only in immunized cows. The four distinct alpha globulins of human serum which inhibit the proteolytic enzymes of Aspergillus oryzae (Bergkvist, 1963) and the thrombin and plasmin inhibitors in human plasma (Rimon et al., 1966) appear to be such factors, and could be closely related to the heat labile coagulase inhibitor in bovine serum.

An interesting characteristic of bovine antiserum was discovered in this connection. Unheated antiserum inhibited coagulase in a non-competitive manner, and remained unaffected by the concentration of fibrinogen substrate in the reaction mixture. In contrast, antiserum heated at 56 C for 30 min not only exerted a reduced capacity to inhibit the coagulase reaction; the residual inhibitory activity appeared to be competitive, and could be reversed by increasing the concentration of fibrinogen substrate in the reaction mixture during assay.

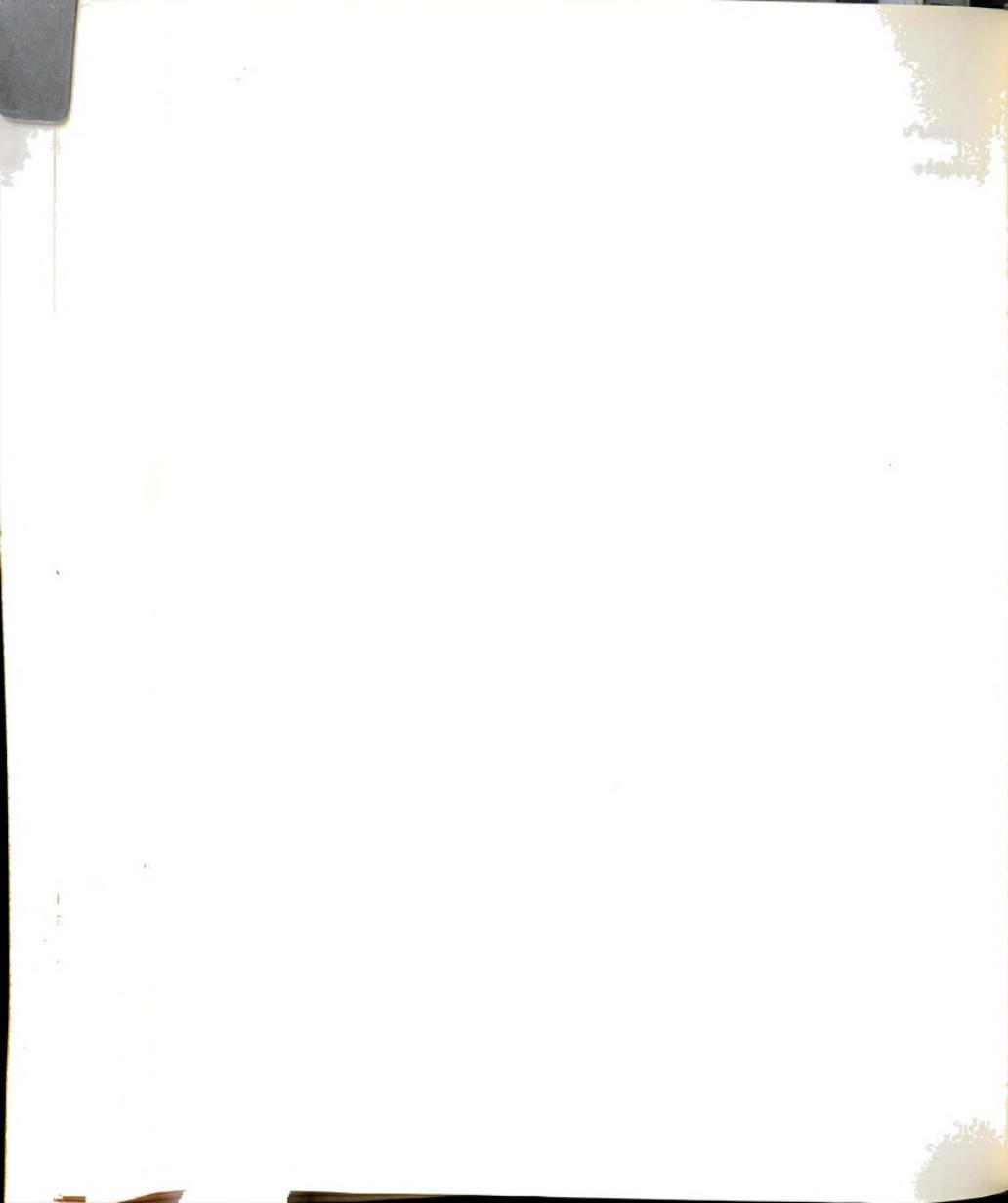
Some reports on enzyme-antibody reactions (Ullmann and Feigelson, 1963; Roberts, 1966) have indicated that antibody inhibition of enzymatic activity is of a non-competitive type, while others (Sevag et al., 1954; Samuels, 1963) have demonstrated that certain systems are competitive. However, a new phenomenon is introduced by the

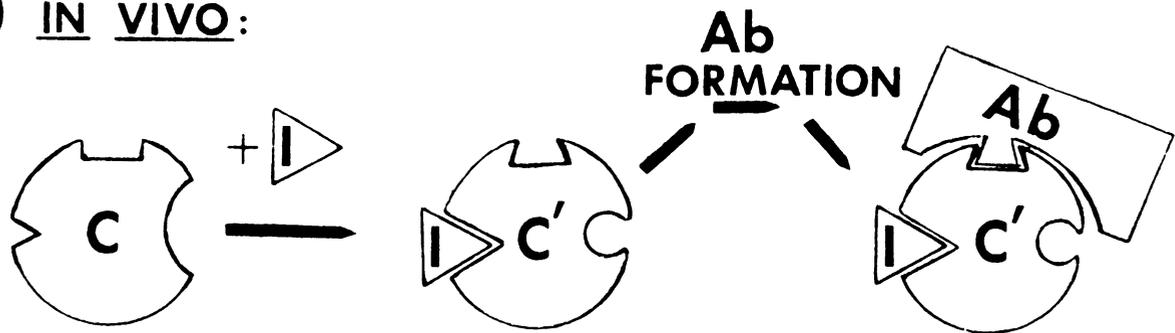
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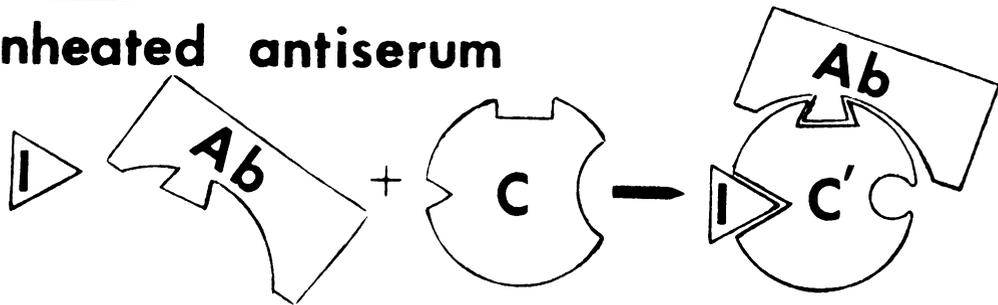
destruction of the heat labile coagulase inhibitor and the subsequent modification of coagulase inhibition from a non-competitive to a competitive type. The observed characteristics of this system might be best explained as a direct or indirect cooperative interaction of the heat labile and heat stable coagulase inhibitors.

A mechanism for such an interaction is proposed in Figure 33. When purified coagulase (designated at C) is injected into cows, the heat labile inhibitor (I), which appears to be a normal serum constituent, rapidly combines with the coagulase, inhibits the enzyme, and converts it to a modified configuration (C'). These modified coagulase molecules then go on during the course of immunization to induce formation of antibody specific not for the normal coagulase molecule, C, but rather for the C' configuration. When unheated bovine antiserum is mixed in vitro with purified coagulase prior to assay, the heat labile inhibitor converts the coagulase molecules to the configuration for which the antibody is specific (C'). A stable inhibitor-coagulase-antibody complex is formed which is unaffected by the presence of substrate. In the case of heated antiserum, however, the heat labile coagulase inhibitor is destroyed and is therefore unable to convert C to C'. The antibody, being specific for the C' coagulase configuration, can manage only a weak attachment to the C structure, making it vulnerable to displacement by the fibrinogen substrate (F).



1) IN VIVO:2) IN VITRO ASSAY:

a) unheated antiserum



b) heated antiserum

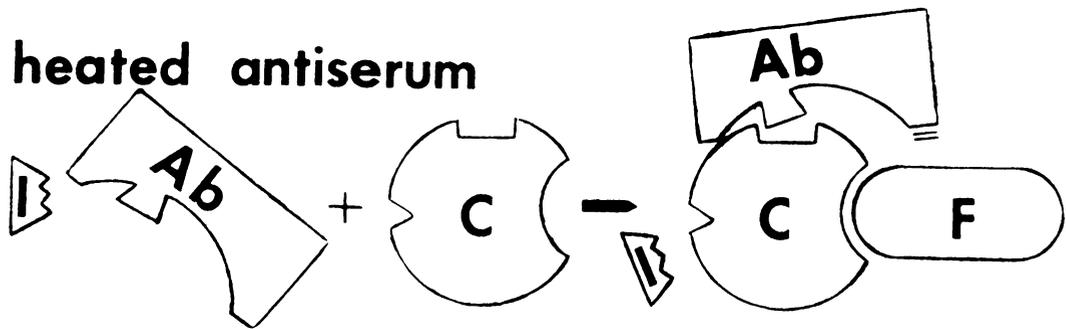


Fig. 33.--Mechanism proposed to explain the modification of coagulase inhibition by heat inactivation of antiserum.



Such a mechanism is compatible with the characteristics of the bovine antikoagulase system and might be also of value in other enzyme-antibody systems involving participation by non-antibody inhibitors. This proposal shares some common features with the hypothesis of Najjar and Fisher (1955) that during the later stages of prolonged immunization, antibodies are formed which are adapted not only to the antigen, but the complex of antigen and earlier antibody. Such a binding might stabilize the antibody-enzyme complex, perhaps to the extent that the presence of specific substrate would have no effect upon inhibition. In the absence of one component, however, the binding would be weaker, and therefore more susceptible to dissociation by substrate.

During kinetic studies on the coagulase reaction, it was necessary to initially determine whether coagulase was a catalyst or a substrate. Haughton and Duthie (1959) proposed that CRF was the enzyme and that coagulase was a substrate, basing their conclusion upon the loss of coagulase activity after 30 to 70 hr incubation with CRF. During this same period, no loss of CRF activity was detected. The specificity of this coagulase degradation has been questioned by Tager and Drummond (1965); these latter workers failed to detect loss in activity of either coagulase or CRF over an 18 hr incubation period. Ultracentrifugal studies of coagulase-CRF incubation mixtures did not



disclose any new molecular species which would indicate a combination of the two proteins.

The experiment in our studies designed to determine the catalytic nature of coagulase consisted of initiating successive clotting reactions by the repeated addition of fresh fibrinogen substrate to an initial reaction mixture of coagulase and CRF. If either coagulase or CRF were substrates, they could be consumed during this series of reactions. This was not found to be the case however; in fact, residual activity of the second reaction exceeded that which was expected on the basis of the dilution by addition of fresh fibrinogen. This observed activity, somewhat greater than expected, might have been caused by the persistence of activated fibrin monomers in the reaction mixture after removal of the clot. Presence of such intermediates would probably have an accelerating effect on subsequent reactions. In spite of this unexpected effect, a plot of successive reaction rates was approximately parallel to the calculated line. This would indicate that neither coagulase nor CRF was consumed in the series of clotting reactions.

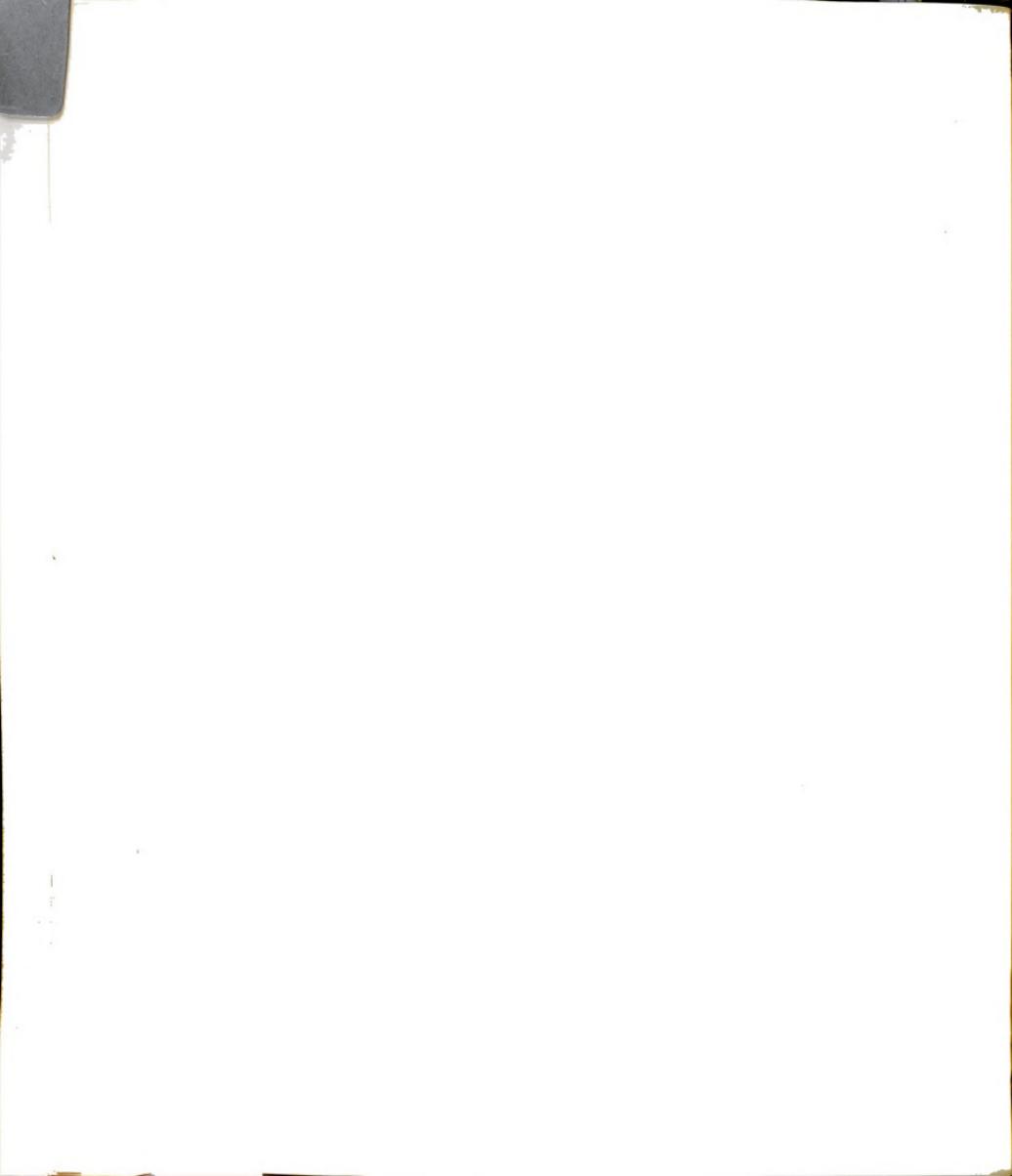
The lack of knowledge concerning the relationship of CRF and even fibrinogen to the coagulase molecule made it necessary to determine some of the fundamental kinetic properties of the enzyme. One approach concerned interaction between catalytic sites on the coagulase molecule. The basis for this determination was the method originally



suggested by Hill (1913); a plot was made of the relationship of the $\log_{10} V/(V_m - V)$ to \log_{10} of the substrate concentration, resulting in a slope with some value N . The significance of N was explained by Atkinson *et al.* (1965) as a function of the number of interacting substrate-binding sites per enzyme molecule and of the degree of interaction between those sites. When the interactions are strong, the slope will equal N as the number of interacting sites. The slope will decrease to a value of 1.0, regardless of the number of sites, as the interaction between catalytic sites is weakened. A slope with a value of 1.0 corresponds to complete independence of sites, and this value should remain constant at all substrate concentrations.

In this analysis, slopes calculated for both fibrinogen and CRF were very close to 1.0, indicating negligible interaction. Unfortunately, this analysis does not provide the actual number of catalytic sites on the coagulase molecule. However, since coagulase is a rather small enzyme (mol. wgt. of 44,000 as calculated by Duthie and Haughton, 1958), it would hardly seem possible that it could accommodate the simultaneous binding of more than one fibrinogen molecule (mol. wgt. of 340,000) and one CRF molecule (mol. wgt. in the range of 30,000).

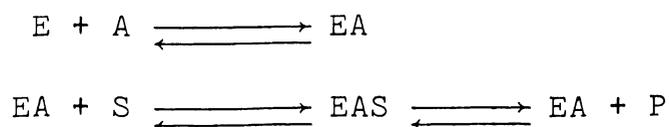
The role of CRF in the coagulase reaction is still unclear. Therefore, a series of experiments were initiated to define its function from a kinetic standpoint. Florini and Vestling (1957) reported a graphical method for the



analysis of a two-substrate system. Application of this analysis to the coagulase reaction led to the conclusion that a two-substrate system was involved in which the binding of either substrate was completely independent of the other. However, the acceptance of CRF as a substrate was incongruous with the report of Tager and Drummond (1965) that no change in CRF was noticed after prolonged incubation with coagulase. Also, CRF did not appear to be consumed in the series of clotting reactions designed in our own studies to demonstrate the catalytic nature of coagulase.

If CRF was indeed a substrate, the apparent K_m value should have remained constant regardless of coagulase concentration. Experiments were initiated in which the K_m for CRF was determined at different coagulase concentrations. As was expected, the K_m for CRF did not remain constant, but varied proportionally to the concentration of coagulase. Although these findings seemed to eliminate CRF as a substrate, positive evidence for its role as an activator was still lacking.

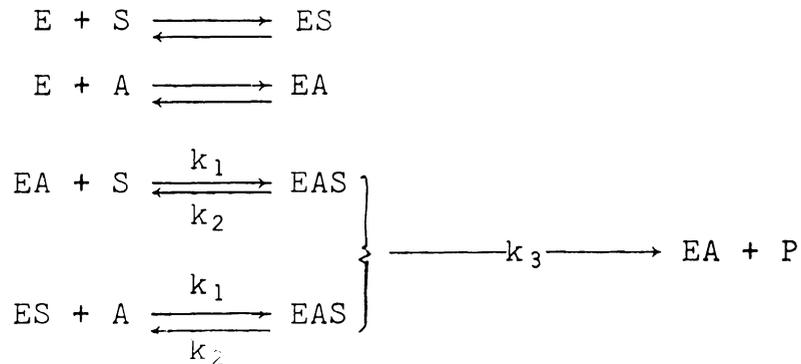
Suggestions on the treatment of enzyme kinetic data by Frieden (1964) provided several possibilities concerning the role of CRF as an activator. There are two limiting cases for which there is no enzyme activity except in the presence of the activator. In the first case, the reaction may be described as:





where E is the enzyme, A is the activator, S is the substrate, and P is the product. In this system, the ES complex can only form in the presence of A, and therefore, increasing concentrations of A always decrease the K_m for substrate until saturation by A is reached. This possibility was eliminated in regard to the coagulase reaction because the K_m for fibrinogen was not affected by CRF concentration.

In the second limiting case, the reactions may be outlined as :



The ES complex cannot break down to E + P in the absence of A. The reaction velocity is zero in the absence of A, increases, and eventually becomes constant at high concentrations of A. The K_m for the substrate in this case may be increased, decreased, or unchanged by increasing levels of A, depending on the relative values of k_2 and k_3 . In the coagulase reaction, the K_m for fibrinogen is unaffected by the concentration of CRF. If the K_m is considered to be equal to $(k_2+k_3)/k_1$, it would then seem likely that k_3 is much smaller than k_2 and k_1 , even in the presence of CRF.



Therefore, the dissociation of the EAS complex to yield EA + P would be the limiting step as is assumed in the classical Michaelis-Menton hypothesis. In view of the circumstantial evidence, it seems quite possible that CRF may accept the role of an activator outlined in the second limiting case postulated by Frieden. Physical and chemical characterization of highly purified CRF should help in the evaluation of this mechanism.



SUMMARY

The production and purification of staphylocoagulase in a semi-defined casein acid hydrolysate medium were investigated. Optimal yields of extracellular coagulase were obtained in small, rapidly shaken volumes of a medium containing 3.0% casein acid hydrolysate supplemented with glucose, vitamins, and mineral salts. Purification of coagulase was accomplished by a procedure consisting of two cycles of acid precipitation, followed by ion exchange on DEAE-Cellulose, fractionation with ammonium sulfate, and molecular sieving through Sephadex G-200. The finally purified enzyme was immunologically homogeneous and has a specific activity of 336,000 reciprocal titer units per mg of protein.

Thermal inactivation of coagulase at 60 C proceeded in two first order reactions, one phase more rapid than the other, and indicated the presence of two thermal isozymes. This phenomenon was observed in both crude and highly purified coagulase preparations, and so the inactivation constants and half-lives of both isozymes were calculated for several stages of purification.

The requirement for a coagulase assay more quantitative than the titer method was fulfilled by the development of a new nephelometric technique. This nephelometric coagulase

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assay allowed rapid and reproducible quantitation of coagulase activity by measuring the clotting of a substrate containing 2.2 μ M fibrinogen and 2.0% human plasma-CRF at pH 7.2 and an ionic strength of 0.07-0.10.

The nephelometric method also allowed accurate measurement of coagulase inhibition by bovine antiserum. During kinetic studies in this regard, unheated antiserum acted as a non-competitive inhibitor, while heated antiserum (56 C, 30 min) behaved as a competitive inhibitor and could be displaced by the fibrinogen substrate. Heat inactivation and ammonium sulfate fractionation of antiserum disclosed the presence of a heat stable antibody and a heat labile coagulase inhibitor.

The stability of both coagulase and CRF during the clotting reaction indicated that neither component was a substrate. Kinetic investigation revealed that the coagulase reaction could be treated by Michaelis-Menton enzyme kinetics, and the K_m for fibrinogen was calculated to be 6.35×10^{-7} M. No significant interaction between fibrinogen-binding sites and CRF-binding sites could be detected. The presence of CRF did not affect the K_m for fibrinogen, and the K_{CRF} varied in proportion to coagulase concentration. Such behavior suggested that it was an activator essential for the dissociation of the coagulase-fibrinogen complex into free coagulase and activated fibrin monomer.



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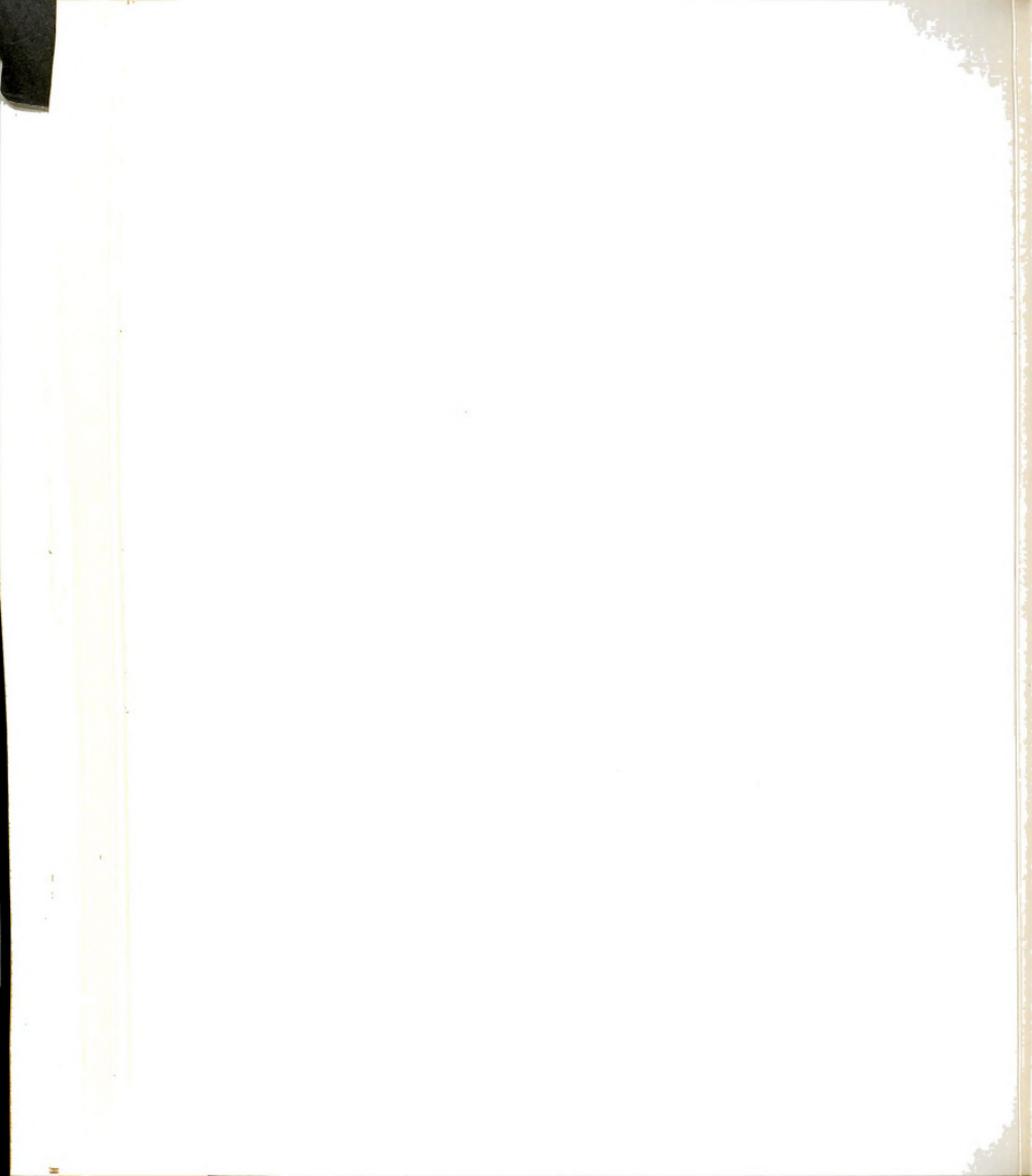


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