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Partial purification and properties of plasmonigen fractionated from horse serum and activated via staphylokinase

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PARTIAL PURIFICATION AND PROPERTIES OF PLASMINOGEN FRACTIONATED FROM HORSE SERUM AND ACTIVATED VIA STAPHYLOKINASE

by

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TOPICAL OUTLINE

I INTRODUCTION AND HISTORICAL REVIEW

The fibrinolytic property of blood was first noted by Dastre(7) in 1893 when he described the liquefaction of a fibrin clot after it stood in serum for 18 hours. Hedin (17) observed that the euglobulin fraction of plasma possessed the blood's proteolytic property. The addition of $CHCl_3$ to serum was demonstrated by Delezenne and Pozerski (1903), to activate a proteolytic enzyme, and Tagnon (9) demonstrated that this chloroform-activated enzyme was similarly present in the acid-insoluble euglobulin fraction of blood. Tillet and Garner (37) found that bacteria-free filtrates of cultures of beta-hemolytic streptococci contained a water soluble substance which could not be dialyzed and which aided rapid lysis of plasma or fibrin clots. This fibrinolytic property was found by Milstone (24) to depend on the presence of a "lytic factor" (i.e., plasminogen) in the euglobulin fraction of plasma. Plasminogen has been shown to be deficient in the plasmas of the bovine (12,15) and rabbit (39) species and it has been demonstrated that streptococcal fibrinolysis will occur only in the presence of this plasma component (24). Remmert and Cohen (30) found that plasminogen has a pronounced tendency to precipitate with other proteins, and this results in its distribution into the various blood fractions. Consequently, bovine fibrinogen and bovine thrombin are used in determining plasminogen activities to prevent incorrect fibrinolytic assays due to plasminogen contamination of a fibrinogen preparation. Christensen (5) reported that

in the presence of the active enzyme (i.e., plasmin), both the plasminogen and plasmin are destroyed. Remmert and Cohen (30) and others, therefore, did not attempt to purify the active serum protease, as they suspected it would prove unstable. Kaulla (22) however, reported that "acid plasmin", capable of liquefying a clot from human plasma in a few hours, is stable in the powdered form for months, but that its solutions rapidly lose their activity at room temperature. Plasminogen activity was reported to be completely destroyed by heating for 3 minutes in 80°C. water bath(33).

Kaplan (21), Christensen and MacLeod (35) and Ratnoff (27) found that the same plasma fraction contains the "lytic factor" and the chloroform-activated proteolytic enzyme, affording strong evidence that the fibrinolytic property of streptococcal filtrates was identical with the chloroform-activated enzyme. Kaplan (20) suggests that the kinase reaction may be regarded as analogous to the transformation of trypsinogen to trypsin via enterokinase. Another hypothesis for explaining the mechanism of activation is the dissociation of a compound analagous to the trypsin:trypsin-inhibitor complex in a strong acid medium. In fact, Kaulla (22) suggests that in his "acid-plasmin fraction, precipitated from an euglobulin solution at pH=1½ 1.2 via ice-cold acetone, "the inhibitory compound has a relatively low molecular weight and probably remains in solution during the precipitation with acetone." Loomis, George and Ryder (23) found that plasmin "is an euglobulin-water insoluble, saline soluble non-dialyzable protein enzyme. Its

point of minimum solubility is near pH=5.5." These same authors also observed that thrombin is immune to the proteolytic action of plasmin, and that while plasmin destroys fibinogen and fibrin it does not clot fibrinogen. It was also found (32) that plasmin is able to digest prothrombin.

Plasminogen activation via the streptococcal exotoxin (i.e., streptokinase) occurs almost instantaneously (2,21), [i.e., clot lysis time ≐ 6 minutes (15)], but the same activation reaction employing staphylococcal filtrates is considerably slower, complete lysis occurring in 3 hours (15). Tillet and Garner (38) proved that the streptococcal factor (i.e., streptokinase) does not act as a proteolytic enzyme by itself, but merely serves as a kinase activator for the plasma precursor. Similarly, the staphylococcal factor is not a protease in its own right (15). It appears evident that fibrinolysis via the addition to serum of either chloroform, strepto- or staphylokinase occurs through the activation of the same serum fraction, namely, plasminogen; the difference in clot lysis time in the presence of a particular kinase, probably due either to a difference in the time required for activation or to incomplete activation, may indicate a difference in the activation mechanism for the respective activators.

Goodpasture(16) suggested that rapid fibrinolysis in patients with liver disease was due to a decrease in the concentration of an inhibitor of plasma proteolytic enzyme rather than an excess of the enzyme itself. The addition of normal plasma to patients with atrophic cirrhosis prevented rapid clot lysis. The inhib-

itory activity of plasma was found to decrease upon incubation at 37°C., and the clot lysis time was found to be related to the deterioration of the unstable inhibitor (28). Ratnoff (28) also found that in patients whose clots lysed rapidly, the inhibitory activity of the plasma, serum or albumin reached a minimum more rapidly than normally, but that after fibrinolysis, little or no further decrease in the amount of the inhibitor present occurred; however, the inhibition was not completely destroyed. In this manner the clot lysis time has been correlated with the decrease in inhibitory activity against the activated enzyme. Fibrinolytic activity has been seen in patients with liver diseases, in toxaemia of pregnancy, in shock, in violent death, during surgical operations and in the course of resorption of thrombin (22). The rapid digestion of fibrinogen in vivo may lead to failure of the hemostatic mechanism (35).

Christensen (3,4) and Christensen and MacLeod (5) ascertain that plasmin is not identical with trypsin. They found that plasmin is distinct from trypsin in its pH of optimum activity, its reactions with specific protease inhibitors and in its reactions on a casein substrate; in the latter experiments it was demonstrated that trypsin can continue the hydrolysis of casein after hydrolysis by plasmin is complete. Kaplan (20) reviewed the literature pertaining to a comparison of the properties of plasmin and trypsin; this author demonstrated that the two enzymes were not identical. The evidence for this conclusion was based on the distinct specificities of their kinases; i.e., strepto-or staphylokinase and enterokinase respectively. The respective kinases were found to be highly specific. Also,

the antibodies to plasmin were found ineffective on enterokinase.

Plasminogen cannot be assayed directly, but must first be activated into plasmin. The plasminogen may be activated via several methods. Christensen (3, 4) prepared serum fractions by dilution and acidification to pH 5.3 and by 1/3 saturation with $(NH_4)_2SO_4$ and found them to develop proteolytic activity without the addition of kinase or chloroform after a variable period of aging in the cold, when the inhibitor content is low. Incubation also causes activation (35). Chloroform or ether treatment presumably abolishes the activity of the inhibitor and activation of plasminogen takes place (35), presumably by autocatalysis (3). Strepto- or staphylokinase activation, however, occurs in a matter of minutes (12, 3) even in the presence of plasmin inhibitor (35). Schmitz (31) obtained activation by treatment with trichloroacetic acid. Most significantly, however, Astrup and Permin (1,2) reported that aqueous extracts of certain tissues are able to activate plasminogen into plasmin. These authors found fibrinokinase (the tissue extract) from pig's heart to be inactive on chicken fibrinogen and fibrin. Upon incubating the pig fibrinokinase with ox pro-enzyme, the solution exhibited lytic activity on the chicken fibrin. The specificity and kinase activity of the tissue extract on a blood fibrinolytic enzyme was thus demonstrated. These authors also demonstrated that ox serum contains only one pro-enzyme which is activated by means of fibrinokinase, while human plasma possesses two pro-enzymes, one of which is activated by fibrinokinase and the other by streptokinase. Tagnon and Palade (35) found a fraction obtained by differential centri-

fugation from a suspension of rat lung tissue to be able to activate plasminogen into plasmin. These authors suggest that the activation is a kinase acting on the substrate plasminogen to transform it into plasmin.

Plasminogen has been isolated and partially purified by a variety of procedures. Low temperature ethanol-water mixtures (6) and alting out via $(NH_4)_2SO_4$ and Na_2SO_4 (35), though resulting in the dispersal of plasminogen into several fractions (30) have been employed. The Remmert and Cohen (30) procedures and others utilize dialysis followed by isoelectric precipitation at several ionic strengths and adsorption and elution from kaolin. Remmert and Cohen (30) assert purification of plasminogen by a factor of 136 to 165 compared to the original human serum. Schmitz (31) has employed trichloroacetic adid in fractionation procedures as well as adsorption on Ca-fibrin. Remmert and Cohen (30) present an analysis of several of these procedures.

Shinowara (33) and Milstone (24) reviewed several of the methods employed in assaying plasminogen activity. Among those listed are the following: (a) the liquefaction of a fibrin clot (24); (b) the hydrolysis of casein as measured by either an assay of the acid soluble tyrosine and/or nitrogen produced or, an assay of the precipitatable protein performed turbidimetrically before and after digestion (26, 27, 28); (c) the alteration of fibrinogen as measured by the increased clotting time with thrombin (12) (i.e., "fibrinogenolysis"); (d) the hydrolysis of hemoglobin and also (e) an assay of the change in gelatin viscosity as determined via a decrease in efflux time. Remmert

and Cohen (30) tested the activities of plasmin samples against casein and fibrin clots. These authors obtained considerable caseinolysis and found that the two plasminogen preparations employed, though differing in purity by a factor of 17.6, exhibited the same ratio of activities toward the casein and fibrin. They suggest that human serum contains only one proteolytic enzyme activated by streptokinase. Shinowara (33), however, suggests that "the liquefaction of a fibrin clot, the alteration of fibrinogen as measured by clotting time with thrombin, the change in gelatin viscosity, and the increase in acid soluble nitrogen are not representative of an identical enzyme system." Remmert and Cohen (30) found that the plasma of human serum does not hydrolyze hippuramide.

Walker, Derow and Schaffer (40) have reviewed the literature pertaining to the coagulative aiding property of filtrates of staphylococci. In an earlier paper (41) these same authors titrated coagulase activity by a serial dilution procedure in which 0.5 ml of each dilution was mixed with an equal volume of fresh, sterile human plasma. Staphylocoagulation is produced by a bacterial product, prostaphylocoagulase, plus a plasma cofactor other than prothrombin (15). The term staphylokinase is reserved for the second factor which acts like streptokinase (15). Gengou (14) found staphylocoagulase extremely resistant to thermal inactivation. Walker, Schaffer and Derow (41) confirmed the complete destruction of coagulase activity by trypsin or pepsin, and also verified its stability when heated. (120°C. does not abolish the coagulase activity). However, Gerheim, Ferguson

et al (15) demonstrated the separability of the coagulative and lytic aiding properties associated with staphylococci by virtue of the fact that incubation at 75°C. for 2 hours resulted in a complete loss of coagulase activity while the lysis time of the preparation was little altered. These authors proposed the term "staphylocoagulase" for the active coagulant and "prostaphylocoagulase" for the bacterial product.

From a teleological point of view, two hypotheses have recently been offered to explain the presence of a potentially active fibrinolytic enzyme in plasma. Nolf (25) suggested that proteolysis is an essential step in the clotting of blood and the observation that pancreatic trypsin clotted oxalated plasma (8) seemed to support his theory. However, the proteolytic enzyme prepared from plasma which was deficient in the known clot-promoting substances did not possess such thromboplastic activity (29). It is therefore apparent that the clot-promoting property is not related to the presence of the plasminogen or plasmin, but is rather a function of the clot-promoting substances fractionated with it from the plasma.

The second hypothesis deals with the physiological activation of plasminogen in the organism and is discussed by Tagnon, Davidson and Taylor (36) and by Ratnoff, Hartman and Conley (29). Tagnon and Palade (35) suggest that the tissue activator may provide the mechanism by which blood clots formed in tissues are eventually resorbed. Some tissues (i.e., liver tissue) are deficient in the activator (35).

Nomenclature (27, 5, 10, 23, 30)

The fibrinolytic enzyme of plasma or serum has been called

plasma protease, tryptase, trypsin, fibrinolysin, plasmin or proteinase. The term "plasmin" will be used throughout. The precursor ot this enzyme has been variously termed plasminogen, tryptogen or profibrinolysin; "plasminogen" will be used here. The activating principle obtained from beta-hemolytic streptococci and originally called fibrinolysin will be termed "streptokinase"; similarly, the staphylococcal activator will be designated "staphylokinase". The precursor, when activated by either chloroform, strepto- or staphylokinase, or via spontaneous action will be designated as "plasmin" as the active principle in each instance is thought to be identical. The term "enzyme" as used in this paper will mean the crude, partially fractionated enzyme preparation, and not a single purified substance. Antifibrinolysin or antiplasmin is the naturally occurring plasma or serum fraction that inhibits the activity of plasmin.

Assays

Unit of fibrinolytic activity: Since there is no set unit for plasmin activity (23), one was defined here as follows: one unit of fibrinolytic activity is the highest dilution of the staphylococcal preparation which will completely lyse a fibrin clot (prepared via .2 ml of a bovine fibrinogen, Fraction I, solution containing .44 gms/100ml q. s. 1.5ml to which 2 drops of a bovine thrombine solution are added) at 37°C. in 3 hours in a .1M phosphate - .9% NaCl buffer, pH = 7.4.

Unit of caseinolytic activity: Caseinolytic activity was expressed as mg of tyrosine liberated after a one hour incubation period at 37°C. due to the plasmin activity in a 0.7 % casein solution. The plasminogen was initially completely activ-

ated by the preliminary incubation of the plasminogen with its kinase at 37°C. for 1 1/2 to 2 hours.

II Statement of the Problem

The principal objects of this study are to verify methods for the purification of plasminogen via fractionation from horse serum and to determine plasmin activity on casein and fibrin substrates in order to ascertain the relationship between the two activities. A comparison between a procedure yielding the inactive proteolytic precursor [i.e., Remmert and Cohen (30)], and the active "acid-plasmin" [Kaulla (22)] is performed. The kinase employed in all assays was prepared from a culture of staphylococcus aureus.

III Materials and their Preparation

A. Partial purification of plasminogen

A 500 ml sample of partially hemolyzed, normal horse serum, prepared 6/19/50 by the Antitoxin and Vaccine Laboratory, Boston, Mass., was divided into three portions as follows:

1: 10-cc for the preliminary tests; i.e., to determine the initial concentration and activity of the plasminogen in the unfractionated horse serum;

2: 220 cc for the isolation and partial purification of the fibrinolytic precursor, plasminogen, via the procedure of Remmert and Cohen (30), and finally

3: a 220 cc aliquot for the preparation of "acid-plasmin" as suggested by Kaulla (22) and said to be the active principle isolated from its serum inhibitors. The procedures numbered 1 and 2 above will be found outlined in figures 1a and 1b respectively.

At every point in either case where the discarding of a supernatant fraction is designated, the solution or mixture, as the case may be, was placed in the refrigerator for 48 hours. At the end of this period, which was to allow for complete precipitation, the pH was rechecked and readjusted if necessary, and the precipitate obtained upon centrifugation combined with the fraction obtained 48 hours earlier from the same mother liquor.

All preparations of plasminogen and its bacterial exotoxin activator, staphylokinase, were kept in the refrigerator when not in use, and all tests were prepared and conducted at room temperature except where otherwise stated.

fig. 1a Partial Purification of Plasminogen as slightly modified from Remmert and Cohen (30).

place two 110-ml portions of normal horse serum in each of two dialyzing bags; dialyze against cold running water for 24-hrs. and then against six changes of distilled water in the refrigerator for 24-hrs.

↓

adjust the dialysate (i.e., serum dialyzed to low ionic strength) to pH = 5.3 with dil. AcOH; allow to stand in refrigerator overnight and centrifuge at 5,000 r.p.m., 35°F → 40°F for 20 → 30 min.

ppt.: dissolve in minimum vol. 0.16 M NaCl, pH = 7.0 → 7.4; dilute to 0.08 M NaCl, adjust to pH = 5.3 with dil. AcOH and centrifuge at 5,000 r.p.m., 35°F → 40°F for 20 → 30 min.

Supernatant: discard

ppt.: dissolve in $PO_4^{\equiv}$ - saline buffer, pH = 7.4; centrifuge at 5,000 r.p.m, 35 → 40°F for 20 → 30 min. to remove insoluble proteins.

Supernatant: place in cold room at -4°C to -6°C for 24 hrs.; adjust to pH = 5.3 if necessary, centrifuge as above and combine with above ppt.

ppt.: discard

Supernatant: dialyze against 0.3 M NaCl for 24-hrs. in the refrig.; adjust dialysate to pH = 5.0 with dil. AcOH and add 500-mg. of dry kaolin (previously washed with distilled water and thoroughly dried and powdered). Stir 1½ hrs. via mechanical stirrer at -4°C to -6°C, allow to freeze, then thaw in refrig. and centrifuge at 5,000 r.p.m., 35 → 40°F for 20 → 30 min.

ppt.: suspend in minimum vol. (20 → 25 mls used) .1M $PO_4^{\equiv}$ - .9% NaCl, pH = 5.4; stir 15 min. and centrifuge as above

Supernatant: discard

ppt.: suspend in minimum vol. .1M $PO_4^{\equiv}$ - .9% saline buffer, pH = 5.4; stir 15 min. and centrifuge as above

Supernatant: discard

ppt.: suspend in minimum vol. (20 → 25 mls used) .1M $PO_4^{\equiv}$ - .9% NaCl buffer, pH = 7.40; stir 3 hrs. at -4°C → -6°C and allow to freeze; thaw in refrig. overnight; centrifuge as above.

ppt.: discard kaolin ppt.

Supernatant: eluate is the stock plasminogen preparation; store in refrigerator when not in use.

fig. 1b Partial Purification of Plasminogen via method of Kaulla (22) (slightly modified).

220-mls normal horse serum + 220 mls distilled water and bubble in CO_2 ↑ till solution is saturated; allow to stand overnight in refrig. for complete precipitation of the euglobulin fraction; centrifuge at 5,000 r.p.m., 35→40°F for 20→30 min.

Supernatant: discard

ppt. (euglobulin): dissolve in minimum vol. of .1N AcOH (total vol. = 25 mls) and adjust to pH = 1→1.2 with 2N HCl; add 4 vol. ice-cold acetone [this acid precipitation of the euglobulin enzyme brings about activation of the plasminogen, as the low molecular weight inhibitor remains in the acetone layer (32)]; centrifuge as above.

Supernatant: discard

ppt.: redissolve in minimum vol. (70 mls used) of .1N AcOH, add N NaOH to pH = 5.0 and centrifuge as above.

Supernatant: add 4 vol ice-cold acetone (270 mls added) and allow to stand in refrig. overnight; centrifuge at 5,000 r.p.m., 35→40°F for 10→15 min.

ppt.: save this ppt. and test its activity by dissolving same in minimum vol. (20 mls used) .1M $PO_4^{\equiv}$ - .9% NaCl buffer, pH = 7.4, just before testing.

Supernatant: discard

ppt.: extract with cold acetate buffer (½ hr. at −4°C → −6°C via the mechanical stirrer) pH = 4.92, corresponding to ½ the original vol. of serum (i.e., 110 ml); allow to freeze; thaw in refrigerator; dialyze the buffer for 24 hrs. against several changes of cold distilled water previously adjusted to pH = 1.2.

↓

dialysate: adjust to pH = 6→6.2 with 1N NaOH and place in refrig. for 72 hrs. for complete precipitation. Centrifuge for 20→30 min. as above.

Supernatant: discard

ppt.: dissolve in 1/10 vol. $PO_4^{\equiv}$ - saline buffer, pH = 7.4 (i.e., 22 mls of buffer) and store in the refrigerator when not in use.

B Partial Purification of Staphylokinase via Method of Blaustein (pre-doctoral work)

Plasminogen in blood plasma, serum or in partially purified fractions cannot be assayed directly but must first be activated into plasmin. Streptokinase, kindly prepared by Dr. Weinstein of the Haynes Memorial Hospital, Boston, Mass., was used in the preliminary activation tests, as complete activity via this kinase is practically instantaneous and complete lysis occurs within a six minute incubation period (19). However, this particular preparation proved inactive (also, it was highly insoluble), and consequently the slower acting staphylokinase was prepared to serve as activator in all the experiments. The staphylokinase is slower acting than the streptococcus exotoxin, but it does produce complete clot lysis in three hours (19).

A stock culture of Staphylococcus aureus was obtained through the courtesy of E. Blaustein (pre-doctoral candidate at the Boston University Graduate School of Bacteriology), and the kinase was partially purified via the procedure he employed in his pre-doctoral studies(unpublished); the culture media consisted of 15 gms. casamino acids and 5 gms. yeast extract in one liter of distilled water and the mixture adjusted to pH = 7.8. The method of preparation is outlined in fig. 2 below. The final fraction, brown in color, was dissolved in .1M $PO_4^{\equiv}$ - .9% NaCl buffer, pH=7.4, 1/10 the volume of the culture filtrate. It was not wholly soluble, but it did produce a fairly uniform mixture when thoroughly shaken before use, and it proved quite active on a sample of human plasma known to contain plasminogen.

fig. 2 Partial Purification of Staphylokinase via Method of Blaustein (predoctoral work)

* Culture media used consisted of 15 gms casamino acids and 5 gms yeast extract in one-liter of distilled water, and the entire mixture adjusted to pH = 7.8 on a Beckman pH meter.

inoculate test tube containing several mls of the above medium from a slant culture of S. aureus and incubate for 18→24 hrs. at 37°C.

↓ 18→24 hrs.

pipett 1 ml of above culture into each of six-250 ml Erlenmeyer flasks containing 100 mls of the culture media; incubate the flasks for 48 hrs. at 37°C.

↓ 48 hrs.

centrifuge all the cultures at 5,000 r.p.m., 35→40°F for 20→30 min.

ppt.: discard

supernatant: filter via Berkfeld filter, using sufficient suction to allow a drop by drop collection and thus avoid bacterial contamination of the filtrate

↓

filtrate: adjust to pH = 4.0 via 0.1 N NaOH; place in deep freeze till nearly frozen, and then add 2 volumes cold absolute ethanol and place in refrigerator overnight for maximum precipitation; centrifuge alcoholic precipitate at 5,000 r.p.m., 35→40°F for 20→30 min.

supernatant: discard

ppt.: resuspend in absolute ethanol, stir thoroughly and centrifuge; discard supernatant and wash several more times with absolute ethanol

washings: discard

ppt.: place in vacuum dessicator overnight; grind up the dry powder and suspend in volume of $PO_4^{\equiv}$-saline buffer, pH = 7.40, 1/10 that of culture filtrate (i.e., 56 mls used). Store in refrigerator when not in use. This stock preparation will be used for plasminogen activation in all the tests conducted.

C Preparation of Substrates

1. Casein

a. Preparation

Unhomogenized milk was centrifuged, and the fatty layer was discarded. The remaining fraction was adjusted to pH=4.7 with dil. H Cl and the casein precipitate allowed to settle. After decantation of the supernantant, the precipitate was dissolved several times in water to which dil. NaOH was added and reprecipitated by the addition of dil. HCl. The casein precipitate was next filtered, washed several times with water and then suspended and stirred in 95% ethyl alcohol. This mixture was again filtered, and the casein pressed between filter papers to remove the alcohol and water. The casein was next suspended in ethyl ether and stirred and heated over a water bath. The ether-casein mixture was again filtered and allowed to dry in air.

For use, the purified casein was dissolved in .1M $PO_4^{\equiv}$ .9% NaCl buffer, pH=7.4, and the entire solution adjusted to pH=7.4 with dil. NaOH. The solution was then heated 15 min. in a boiling water bath, filtered and the pH readjusted to 7.4. A micro-Kjeldahl-Nesslerization determination was next performed on one ml of a 1 ml q.s 35 ml and a 1 q. s. 50 dilution of the solution, and the latter was subsequently diluted to obtain approximately a .9 to 1% casein solution. All readings were made at λ = 515.

Stock standard of $(NH_4)_2SO_4$ = .1 N/ml

blank = [1ml distilled water + .5cc digestion mixture + 15cc Nessler's q. s. 50 mls distilled water]

standard = [1 ml containing .1 mg N/ml + .5cc digestion mixture + 15cc Nessler's] q.s. 50 mls ℄ distilled water

casein solutions: { [1 ml of 1 q. s. 35 dilution + .5ml digestion misture + 15ml Nessler's] q. s. 50 ml ℄ distilled water

{ [1 ml of 1 q. s. 50 dilution + .5 ml digestion mixture + 15ml Nessler's] q. s. 50 ml ℄ distilled water

Results

$\lambda = 515$

reading of blank = 100% Transmittance
reading of standard (.1 mg N/ml) = 76.5% T
reading of 1/35 dilution of casein sol = 52.0% T
reading of 1/50 dil. of casein sol. = 68.5% T

$\frac{\text{reading of stand.}}{\text{reading of unknown}} \times$ mg N/ml in stand. $\times$ dil. = mg N present in casein solution

$$\frac{76.5}{52.0} \times .1 \times 35 = 5.3$$

$$\frac{76.5}{68.5} \times .1 \times 35 = \underline{5.5}$$

av. = 5.4 mg N/ml in 185 ml of casein sol.

185 ml × 5.4 mg N/ml = 999 mg N = .999 gms N in 185 ml of casein sol.

no. of gms of casein present = $\frac{1 \times 100}{16.4}$ = 6.1 gms. of casein in 185 mls of solution

(6.1 gms of casein) q. s. 650 ml ⟶ .94% casein

1b. Preparation of tyrosine calibration curve

A tyrosine stock standard in 0.1 N HCl and containing 1 mg/ml was diluted as indicated below (fig. 3) with 0.1 N HCl. A 1ml aliquot of each dilution in a Coleman Jr. Spectrophotometer tube was treated with 6ml clear 12.5% Na_2CO_3 and 1 ml of the prepared Folin-Ciocalteu reagent diluted 1:2 and the resulting solution incubated for 10 to 15 minutes at 37°C. for complete color development. These tubes were read against a

blank set at 100% T containing 1 ml 0.1 N HCl, 6 ml 12.5% Na_2CO_3 and 1 ml of the phenol reagent at λ=650 in the Coleman Jr. Spectrophotometer.

Fig. 3 Preparation of a Tyrosine Calibration Curve

Stock standard = 1 $\frac{\text{mg tyrosine}}{\text{ml}}$

Dilution of stock standard	λ tyrosine/ml	Reading
1:13	71.4	15.0
1:14	66.7	16.0
1:18	52.7	22.5
1:19	50.0	27.7
1:24	40.0	32.5
1:29	33.3	41.0
1:30	32.25	42.5
1:34	27.1	49.0
1:35	27.8	49.5
1:39	25.0	50.0
.5:29.5	16.6	63.0
.5:34.5	14.3	67.5
.5:35.5	13.9	68.5 to 69.0
.5:39.5	12.5	69.5 to 70.0
.5: 49.5	10.0	72.5 to 73.5
.5:99.5	5.0	84.0 to 85.5

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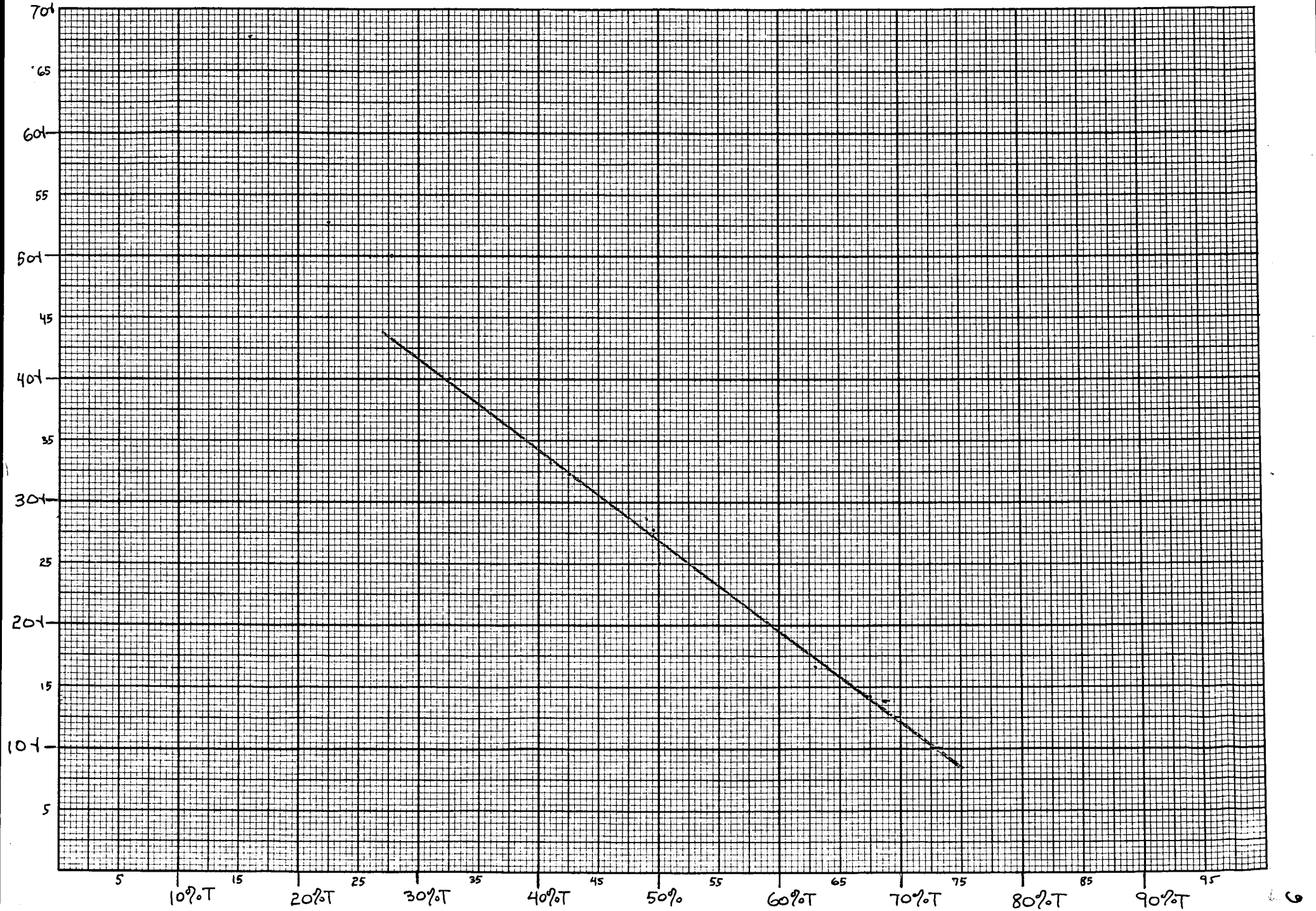

2. Fibrin Clot

a. Fibrinogen

220 mg of bovine fibrinogen, fraction I, was dissolved in .85% NaCl. The fibrinogen was brought under a high vacuum and 50 ml of .85% saline were introduced via a 50cc syringe. This procedure was necessary as the fibrinogen is not readily soluble at atmospheric pressure.

b. Thrombin

Powdered bovine topical thrombin concentrate, diluted just before use with isotonic saline and containing 1000 N.I.H. units per ml was used. Two to three drops were used to produce a solid clot.

IV Procedure and Tabulation of Results

A. Caseinolysis

1. Introduction

The hydrolysis of casein was measured by the liberation of tyrosine and determined colormetrically by the method of Folin and Ciocalteu (13). The procedures employed were modified from Shinowara (33), Remmert and Cohen (30), Ferguson and Lewis (11), Heidelberger and MacPherson (18) and Ratnoff (28). The plasminogen was incubated with a predetermined excess of the staphylokinase for 1 1/2 to 2 hours at 37°C. for the complete activation of the lytic factor. At the end of this period the .9% to 1% casein solution was added and five minutes from the time of this addition, a 2 ml aliquot was withdrawn and the hydrolysis stopped by the precipitation of the protein in 2 ml 10% trichloroacetic acid. Hiller and Van Slyke (19) found that increasing the concentration of trichloroacetic acid increases the precipitation of non-protein nitrogen compounds. The untreated solution was placed in the incubator at 37°C. for a 60-minute period, while the precipitated aliquot, whose time of withdrawal was recorded as "zero minutes", was placed in the frigerator for 20 to 30 minutes, for the complete precipitation of the casein. The casein was next removed via filtration through Whatman folded filter papers #12, and a 1 ml aliquot of the digestion filtrate was placed in a Coleman Junior Spectrophotometer cuvette containing 1 ml 5% trichloroacetic acid. These tubes were allowed to stand till the "60-minute" aliquots were similarly prepared.

At the end of the one hour incubation at 37° C., a second 2 ml aliquot was withdrawn and pipetted into 2 mls of 10% trichloroacetic acid and treated in the same manner as the "zero minute" aliquot above. With the two sets of tubes all prepared, 6 ml of 12.5% Na_2CO_3 and 1 ml of the Folin-Ciocalteu reagent (diluted 1:2) respectively were added to each tube and the color value of the digestion filtrate was then read at λ = 650 in terms of mgms. of pure tyrosine as determined from the calibration curve in figure 3.

Two control tubes were simultaneously run with each set of determinations. The necessity for two controls was demonstrated by Shinowara (33). One control contained the 4 ml of the casein solution and the second contained the kinase and plasminogen; both were diluted up to volume with $PO_4^{\equiv}$ - saline buffer, pH=7.4. The enzymatic activity in all runs was expressed as mg of tyrosine liberated per digestion mixture by substrating the amounts found in the enzyme and substrate controls from that found in the enzyme-substrate mixture (33). The zero minute result thus obtained subtracted from the 60-minute run would then indicate the proteolytic activity exhibited by the "plasmin" preparation upon the casein substrate.

2. Procedure

The first caseinolysis experiment was performed on a sample of the unfractionated horse serum to determine its initial proteolytic activity. Three control and three test runs were performed simultaneously in a series of Wasserman tubes as follows: Control # 1 initially contained .3 ml of

the .9 to 1% casein solution, 1.0 ml $PO_4^{\equiv}$-saline buffer [pH-7.4], but neither staphylokinase nor plasminogen was added --this control allowed for the determination of the hydrolysis of the casein solution during the one hour incubation test period; Control #2 initially contained 4.6 ml of the buffer, .2 ml of the unfractionated serum (source of the plasminogen) and .2 ml of the kinase--the hydrolysis products of the kinase and the serum protein during the one-hour incubation may now be determined. The third and final control tube contained the casein and the plasminogen source but no kinase was added, in order to determine any caseinolytic activity due to the spontaneous activation of the plasminogen (in the absence of any activator).

The three test runs were simultaneously prepared as follows: the first and second Wasserman tubes contained .3 ml of the casein solution, .6 ml buffer followed by.2 ml horse serum (plasminogen source) and .2 ml staphylokinase. In order to determine if the .2 ml of staphylokinase was actually in excess of the plasminogen present (to insure complete activation), the third test run was performed with the same volumes of the casein solution and plasminogen source, but with .4 ml of the kinase and .4 ml of buffer to dilute to volume. The final volume in all tubes was now 1.3 ml, except for control #2, which anticipated the final volume of 5 mls.

All tubes were simultaneously incubated at 37°C. for 1 1/2 hours to allow for plasminogen activation, and then, but for control #2, 3.7 ml of the casein solution was added to each. The volume in each tube was now 5 ml, which meant that the

"plasmin" would be acting in a .7% casein medium.

At the end of five minutes a 2 ml aliquot was withdrawn from each tube and treated with 2 ml 10% trichloroacetic to stop the reaction. The resulting ppt. and its untreated mother liquor were treated and tested as outlined in fig. 4.

fig. 4 Caseinolytic Activity Assays

- .3 ml .9→1% casein sol.
- .2 ml plasminogen source
- .2 ml Staphylokinase
- .6 ml $PO_4^{=}$ - NaCl buffer, pH 7.4

total vol. = 1.0 ml
can titrate plasminogen vs. staphylokinase by varying buffer vol. : staphylokinase vol., etc.

↓

incubate at 37°C for 1½ hrs. for complete plasminogen activation; then add 3.7 ml .9→1% casein sol., stir thoroughly, and allow to stand 5 min.; withdraw a 2 ml aliquot.

↓

incubate untreated solution at 37°C for 60-min.
↓ 1-hr.

withdraw a 2 ml aliquot, pipett same into 2 ml 10% trichloroacetic acid & place in refrig. 20→30 min. Filter via Whatman folded filter papers # 12.

filtrate: 1 ml of the digestion filtrate analyzed colorimetrically for acid soluble tyrosine, via the Folin phenol reagent, as outlined at right.

ppt.: discard

2 ml aliquot: pipett same into 2 ml 10% trichloroacetic acid and place in refrig. 20→30 min.; filter via Whatman folded filter papers # 12.

filtrate:

- 1 ml 5% trichloroacetic acid
- 1 ml digestion filtrate
- 6 ml 12.5% Na_2CO_3

and incubate at 37°C for 5 min. Then add 1 ml of Folin-Ciocalteu reagent, incubate 10→15 min. for maximum color development & read against a blank at $\lambda = 650$.

ppt.: discard

Other caseinolysis experiments were similarly performed with the Remmert and Cohen preparation as the plasminogen source. The results are all shown in figure 5 below.

3. Caseinolytic Results

fig. 5

a) Analysis of the Unfractionated Horse Serum

mls added	C#1	C#2	C#3	T#1	T#2	T#3
.9%→1% casein	.3 3.7	0 0	.3 3.7	.3 3.7	.3 3.7	.3 3.7
$PO_4^{\equiv}$-NaCl buffer, pH 7.4	.2 | .6 .2	.3 | .6 3.7	.2 | .6 0	0 | .6	0 | .6	0 | .4
Staphylo-Kinase	0	.2	0	.2	.2	.4
Plasminogen	0	.2	.2	.2	.2	.2
Zero min.	64.6	90.8	62.8	59.0	59.5	59.5
60 min.	59.3	89.0	62.8	57.6	58.0	56.2

b) Plasminogen prepared via R+Cohen immediately prior to Kaolin adsorption—prepared from a different horse serum sample

mls added	C#1	C#2	C#3	T#1	T#2
.9%→1% casein	.3 3.7	0 0	.3 3.7	.3 3.7	.3 3.7
$PO_4^{\equiv}$-NaCl buffer, pH 7.4	.2 | 0 .2	.3 | 0 3.7	.2 | 0	0 | 0	0 | 0
Staphylo-Kinase	0	.2	0	.2	.2
Plasminogen	0	.2	.2	.2	.2
Zero min.	76.0	99.5	74.5	72.6	70.0
60 min.	75.0	98.6)2 96.0	72.0	70.3	67.1

c) [R+Cohen] Action of the dialysate from .3 M NaCl. Same fraction as 6b but from horse serum used in all other tests.

mls added	C#1	C#2	C#3	T#1	T#2	T#3
.9%→1% casein	.3 3.7	0 0	.3 3.7	.3 3.7	.3 3.7	.3 3.7
$PO_4^{\equiv}$-NaCl buffer, pH 7.4	.2 | .6 .2	.3 | .6 3.7	.2 | .6 0	0 | .6	0 | .6	0 | .4
Staphylo-Kinase	0	.2	0	.2	.2	.4
Plasminogen	0	.2	.2	.2	.2	.2
Zero min.	56±1	85.8	56±1	55±.5	55.0	54±.5
60 min.	56±1	91.0	56±1	55±.5	55±.5	54±.5

d) Action of the final fraction obtained via Remmert+Cohen procedure.

mls added	C#1	C#2	C#3	T#1	T#2	T#3
.9%→1% casein	.3 3.7	0 0	.3 3.7	.3 3.7	.3 3.7	.3 3.7
$PO_4^{\equiv}$-NaCl buffer, pH 7.4	.2 | .6 .2	.3 | .6 3.7	.2 | .6 0	0 | .6	0 | .6	0 | .4
Staphylo-Kinase	0	.2	0	.2	.2	.4
Plasminogen	0	.2	.2	.2	.2	.2
Zero min.	61.5	93.5	58.0	60.9	61.0	51±1
60 min.	59.0	91.5	53.5	59.0	59.5	50±1

These results indicated that a two-fold increase in the kinase volume added did not result in any increase in the acid-soluble tyrosine produced in a .7% casein medium, thus demonstrating that the kinase was already present in excess. The caseinolytic activity of the "plasmin", as assayed via the acid-soluble tyrosine produced after a 1 1/2 to 2 hour preliminary activation period with its kinase followed by a one hour test period of "plasmin" proteolytic activity in a .7% casein medium, has not been demonstrated. The possibility that either the kinase or the plasminogen source (Remmert and Cohen prep.) or both were inactive may be ruled out, as these same preparations were later demonstrated to completely lyse fibrin clots in from 3 to 4 hours. The spontaneous activation of plasminogen in C # 3 cannot be demonstrated either. This lack of "plasmin" caseinolytic activity when contrasted with its potent fibrinolytic activity seems to indicate that the two processes are not due to the same enzyme activity.

B. Fibrinolysis

The methods employed for the demonstration of fibrinolysis were slightly modified from Milstone (24).

1. Methods, Procedure and Tabulation of Results

The fibrin clots were prepared in Wasserman tubes via .2 ml of the bovine fibrinogen solution to which was added 2 drops of a bovine thrombin concentrate containing 1000 N.I.H. units per ml. The plasminogen and fibrinogen were added to .5 ml of the staphylokinase prepared in serial dilution before the

formation of the clot (i.e., before the addition of the thrombin fraction). The Wasserman tubes were thoroughly shaken, the thrombinconcentrate added and the time of clot formation (a few seconds) noted. The time of fibrinolysis was recorded as the time from initial clot formation to that of complete clot lysis as noted by the complete liquefaction of the clot [4 +lysis].

The plasminogen preparations used, the volumes added, the order of the addition and the results are tabulated in figures 5 and 6 below. The degree of clot lysis is recorded as follows:

(1) [+] = sliding clot;
(2) [++] =1/2 tube liquid;
(3) [+++] =tiny floating;
(4) [++++] =no visible clot.

Fig. 5 Method for fibrinolytic determinations using four plasminogen sources activated via the same staphylokinase preparation.

a) horse serum

.5 ml staphlykinase..serial dil.
.2 ml horse serum
.6 ml $PO_4^{\equiv}$-saline buffer,pH=7.4
.2 ml bovine fibrinogen
220 mg bovine fraction I + 50 ml .85% NaCl
2 to 3 drops bovine topical thrombin...1000 NIH units/ml

b) human plasma

.5 ml staphylokinase..... serial dil.
.2 ml human plasma
[*.2+.6]=.8 ml $PO_4^{\equiv}$-saline buffer
2 to 3 drops thrombin

*The further addition of fibrinogen is unnecessary, as human plasma is used here.

c) Remmert and Cohen

.5 ml staphylokinase... serial dil.
.2 ml Remmert and Cohen prep.
.6 ml $PO_4^{\equiv}$-saline buffer, pH=7.4
.2 ml bovine fibrinogen
2 to 3 drops thrombin

d) Kaulla (ppt. from pH=6)

.5 ml staphylokinase
.2 ml Kaulla(a partial suspension)
.6 ml $PO_4^{\equiv}$-saline buffer, pH=7.4
.2 ml bovine fibrinogen, fraction I
2 to 3 drops thrombin

All dilution made with .1M $PO_4^{\equiv}$ ~.9% saline buffer, pH =7.4
Final volume in all fibrinolysis determinations is 1.5 m.l

fig. 6a Results of Fibrinolytic Determinations

Dilutions of the Staphylokinase	HORSE SERUM 2	3	4	5	over-night	HUMAN PLASMA 2	3	4	5	over-night	REMMERT and COHEN (.2 ml) 2	3	4	5	over-night	REMMERT and COHEN (.4 ml) 2	3	4	5	over-night	KAULLA 2	3	4	5	over-night
0	+	+	+	+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	±	+	+	+	++ 2 +++
2	+	+	+	+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	—	±	+	+	++ 2 +++
4	+	+	+	+	+2 ++	+	+	+	+	+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	—	±	+	+	++ 2 +++
8	+	+	+	+	+	+	+	+	+	+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	—	—	+	+	++ 2 +++
16	+	+	+	+	+	+	+	+	+	+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	—	—	+	+	++ 2 +++
32	+	+	+	+	+	±	±	±	±	+	+	++	++ 2 +++	+++	4+	4+	4+	4+	4+	4+	—	—	—	—	++
0	+	+	+	+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+						±	±	+	+	++ 2 +++
2	+	+	+	+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+	4+						—	±	+	+	++ 2 +++
4	+	+	+	+	+2 ++	+	+	+	+	+	4+	4+	4+	4+	4+						—	—	—	—	++ 2 +++
8	+	+	+	+	+	+	+	+	+	+	4+	4+	4+	4+	4+						—	—	—	—	++ 2 +++
16	+	+	+	+	+	+	+	+	+	+	4+	4+	4+	4+	4+						—	—	—	—	++
32	+	+	+	+	+	+	+	+	+	+	+	++	++ 2 +++	+++	4+						—	—	—	—	+++
0	+	+	+	+	4+																±	±	+	+	++ 2 +++
2	+	+	+	+	4+																—	±	±	+	++ 2 +++
4	+	+	+	+	+2 ++																—	—	—	+	++ 2 +++
8	+	+	+	+	++ 2 ++																—	—	—	±	+++
16	+	+	+	+	+																—	—	—	±	++ 2 +++
32	+	+	+	+	+																—	—	—	—	++
1.1 ml buffer + .2 ml horse serum + .2 ml fibrinogen + 2 dr. thrombin																					±	+	+	+	+
1.1 ml " + .2 ml + .2 ml " + 2 dr. "																					—	+	+	+	+
1.1 ml " + .2 ml Remmert + Cohen + .2 ml " + 2 dr. "																					—	±	+	+	4+
1.1 ml " + .2 ml " " " + .2 ml " + 2 dr. "																					—	±	+	+	4+
1.1 ml " + .2 ml Kaulla + .2 ml " + 2 dr. "																					—	±	+	+	4+
1.1 ml " + .2 ml " + .2 ml " + 2 dr. "																					—	±	+	+	4+

(*) The Remmert + Cohen and the Kaulla plasminogen preparations, in the absence of the kinase, liquefied the control clots on overnight incubation. This may either be due to the spontaneous activation of the plasminogen (3, 4, 5) or to bacterial contamination or to both, as the preparations were not sterile.

fig. 6b Comparative purification of Plasminogen via the two methods employed.

Dilutions of Staphylokinase	HORSE SERUM 1	2	3	4	5	over-night	REMMERT + COHEN (.2ml used) 1	2	3	4	5	over-night	KAULLA 1	2	3	4	5	over-night
0	±	±	±	+	+	+++	+++	+++	4+	4+	4+	4+	—	—	—	—	—	+
2	±	±	±	±	+	++	+++	+++	4+	4+	4+	4+	—	—	—	—	—	++
4	±	±	±	±	±	+++	+++	+++	4+	4+	4+	4+	—	—	—	—	—	+++
8	±	±	±	±	+	+	+++	+++	+++	4+	4+	4+	—	—	—	—	—	+++
16	—	—	±	±	±	±	+	+++	+++	+++	+++	4+	—	—	—	—	—	+++
32	±	±	±	±	±	±	—	+++	+++	+++	+++	4+	—	—	—	—	—	+++
64	±	+	+	+	+	+	—	—	±	++	+++	4+	—	—	—	—	—	++
128	±	±	±	±	±	±	—	—	—	—	—	4+	—	—	—	—	—	++
256	±	±	±	±	±	±	—	—	—	—	—	4+	—	—	—	—	—	+++
0							+++ 2 +++	+++	4+	4+	4+	4+	—	—	—	—	—	±
2							4+	4+	4+	4+	4+	4+	—	—	—	—	—	++
4							+++	+++	+++	4+	4+	4+	—	—	—	—	—	+++ 2 +++
8							+++	+++	+++	4+	4+	4+	—	—	—	—	—	4+
16							++	+++	+++	+++	+++	4+	—	—	—	—	—	4+
32							—	—	+++ 2 +++	+++	+++	4+	—	—	—	—	—	+++
64							—	—	—	—	—	4+	—	—	—	—	—	+++
128							—	—	—	—	—	4+	—	—	—	—	—	++
256							—	—	—	—	—	4+	—	—	—	—	—	4+
	1.1ml buffer + .2ml horse serum + .2ml fibrinogen + 2dr. thrombin												—	+	+	+++ 2	++	++
	1.1ml " + .2ml " " + .2ml " + 2dr. "												—	±	+	+++ 2	+++ 2	++
	1.1ml " + .2ml R+Cohen + .2ml " + 2dr. "												—	—	—	—	—	4+
	1.1ml " + .2ml R+Cohen + .2ml " + 2dr. "												—	—	—	—	—	4+
	1.1ml " + .2ml Kaulla + .2ml " + 2dr. "												—	—	—	—	—	4+
	1.1ml " + .2ml Kaulla + .2ml " + 2dr. "												—	—	—	—	—	4+
	1.3ml " + .2ml fibrinogen + 2dr. thrombin												—	—	—	—	—	4+

* Once more the controls containing the plasminogen preparations in the absence of Kinase lysed the clots upon overnight incubation at 37°C. However, a control tube containing .2ml fibrinogen diluted up to volume with buffer and then clotted with thrombin was also lysed upon overnight incubation. Bacterial contamination is evident here, but the possibility of spontaneous activation of the plasminogen (3, 4, 5) preparation is still present. Both factors probably take part in the lysis. As all determinations were made at 3→4 hrs. for the assay the factor of bacterial contamination may be discarded, as all controls were negative at this time.

2. Fibrinolytic activity of a fraction precipitated at ph=5 from Kaulla procedure

One fraction precipitated at pH=5 in Kaulla (22) was not discarded, as called for in the original procedure, but was dried in a dessicator and set aside in the cold at -4° C. to -6° C. Since the final active fraction was precipitated from the supernatant layer of the fraction precipitated above, and especially since this active fraction was obtained at pH=6 (a difference of but one pH unit and yet the separation of an active from an inactive fraction), it was felt advisable to test the fibrinolytic activity of both fractions.

The fraction obtained at pH=5 was taken up in 1/10 volume buffer, pH=7.4 (20 to 22 mls), and its fibrinolytic activity tested in duplicate. The same staphylokinase preparation in the identical dilutions used previously and the same volumes of buffer, thrombin and fibrinogen from the prepared stock solutions used previously were employed. The only new factor introduced was the plasminogen source. In one set of tubes no clots appeared upon the addition of thrombin, while in the duplicate a solid clot formed in dilutions up to 1:4 of the kinase, while at the higher dilutions of the kinase, sliding clots and 1/2 liquefied clots appeared and at the highest dilutions used, no clot formation took place at all. The tubes were allowed to stand at room temperature and it was noted that the lysis was directly proportional to the kinase concentration [or inversely proportional to kinase dilution]. At the end of 1 to 1 1/2 hours all clots were completely liquefied.

As the above were preliminary tests and the stock prepar-

ations used (i.e., fibrinogen, thrombin and buffer) were the same as employed earlier, no controls were run. Consequently, the only new material introduced was the source of plasminogen. In an attempt to repeat these results, the identical procedure was run the following day with the same solutions. The earlier results could not be duplicated, however, and a second and third attempt at verification on following days similarly failed. This would seem to indicate that either the first results were totally incorrect or that the particular source of plasminogen employed was exceedingly unstable in solution. Kaulla (22) found that "acid-plasmin" is very unstable in solution and is rapidly inactivated.

A possible explanation for the earlier findings would be the presence of staphylocoagulase in the kinase preparation. Consequently, a series of Wasserman tubes were set up with the same kinase in serial dilutions, the identical buffer, fibrinogen and plasminogen source, but no thrombin was added to either the series containing the Kaulla fraction (from pH=5.0) or the Remmert and Cohen preparation. There was no indication of clot formation in any tube over a test period of six hours. The action of coagulase may therefore be disregarded in the series of fibrinolytic experiments performed here. Therefore the possibility that the earlier observation (i.e., the higher the concentration of kinase, the longer the clot lysis time) was due to the higher concentration of coagulases in the tube containing the higher kinase concentration, may be discarded. These results also indicate that the staphylokinase may be isolated free of the staphylocoagulase.

In the preliminary experiments conducted by Loomis, George and Ryder (23) it was found that the plasminogen preparations clotted fibrinogen. Tagnon (34) noted the same activity. However, later findings (23) showed that the clotting property of the lytic aiding fraction could be removed by the previous removal of the prothrombin from the unfractionated plasma by adsorption on $Mg(OH)_2$ and centrifugation. In none of the experiments conducted in this paper was there any evidence of clot formation in the presence of either the kinase or plasminogen or both, before the additon of the thrombin concentrate.

No explanation of the earlier findings is offered. However, two possibilities have been discarded.

3. Fibrinolysis vs. Caseinolysis

a. Methods and Tabulation of Results

The possible relationship between the fibrinolytic and caseinolytic activities of the most active plasminogen fraction; i.e., the Remmert and Cohen preparation, was next determined. The caseinolytic and fibrinolytic experiments (described previously) were run simultaneously (the plasminogen, kinase and buffer in both determinations were taken from the same stock preparations). Casein hydrolysis (i.e., as determined from the acid-soluble tyrosine assays run after a preliminary plasminogen-kinase activation period of 2 hours followed by a 1-hour test period) proved to be negligible (fig. 5d) despite the use of undiluted kinase. The fibrinolytic tests (fig. 7A), however, demonstrated that .5 ml of the kinase diluted 1:8 to 1:16 was capable of producing clot lysis in 3 hours,

a period in which both plasminogen-kinase activation and fibrinolysis had occurred. These findings agree with Shinowara (33), who suggested that caseinolytic and fibrinolytic activities are not the result of the same enzyme system.

fig. 7a Fibrinolytic & Caseinolytic Activity Compared; below are the fibrinolytic determinations. The caseinolytic activities run simultaneously are in fig. 5d above.

Dil. Kinase	1	2	3	4	5	6	7	overnight	1	2	3	4	5	6	7	overnight
0	−	4+	4+	4+	4+	4+	4+	4+	−	4+	4+	4+	4+	4+	4+	4+
2	−	4+	4+	4+	4+	4+	4+	4+	−	4+	4+	4+	4+	4+	4+	4+
4	−	4+	4+	4+	4+	4+	4+	4+	−	+	4+	4+	4+	4+	4+	4+
8	−	±	4+	4+	4+	4+	4+	4+	−	+	+++	+++	4+	4+	4+	4+
16	−	−	±	+	4+	4+	4+	4+	−	−	++	+++	4+	4+	4+	4+
32	−	−	−	−	±	+	++	4+	−	−	−	−	±	+	+++	4+
64	−	−	−	−	−	−	−	4+	−	−	−	−	−	−	−	4+
128	−	−	−	−	−	−	−	4+	−	−	−	−	−	−	−	4+
256	−	−	−	−	−	−	−	4+	−	−	−	−	−	−	−	4+
512	−	−	−	−	−	−	−	4+	−	−	−	−	−	−	−	4+
1024	−	−	−	−	−	−	−	4+	−	−	−	−	−	−	−	4+

fig. 7b Fibrinolytic Activity of the Material Precipitated at pH = 5 in Kaulla and Run at the Same Time as 7a & 5d.

Dilutions Staphyle.	1	2	3	4	5	6	7	over night	1	2	3	4	5	6	7	over night
0	−	−	−	−	−	−	−	+2 ++	−	−	−	−	−	−	−	4+
2	−	−	−	−	−	−	−	++	−	−	−	−	−	−	−	+
4	−	−	−	−	−	−	−	+2 +†	−	−	−	−	−	−	−	+
8	−	−	±	±	±	±	±	††	−	−	−	−	−	−	−	††
16	−	−	−	−	±	±	±	4†	−	−	−	−	±	±	±	+2 +++
32	−	−	±	±	±	±	±	††	−	−	−	−	±	±	±	4+
64	−	−	−	−	±	±	±	+	−	−	−	−	−	−	−	+
128	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+
256	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+
512	−	−	−	−	−	−	−	+	−	−	−	−	−	−	−	+

fig. 7c Controls were run at the same time as follows:

Control #1 (a + b)

1.1 ml buffer + .2 ml of the Kaulla fraction ppt. at pH=5 + .2 ml fibrinogen + 2 dr. thrombin

Control #2 (a + b)

1.1 ml buffer + .2 R+Cohen + .2 ml fibrinogen + 2 dr. thrombin

Control #3

1.1 ml buffer + .2 ml fibrinogen + 2 dr. thrombin

	1	2	3	4	5	6	over night
C#1a	−	−	−	−	−	−	††
b	−	−	−	−	−	−	††
C#2a	−	−	−	−	−	−	4+
b	−	−	−	−	−	−	4+
C#3a	−	−	−	−	−	−	+2 ++
b	−	−	−	−	−	−	+2 ++

b. Conclusions

The controls (fig. 7c) indicate that the assay period used, i.e., 3 to 4 hours, eliminated possible bacterial contamination from consideration. It was evident that fibrinolysis took place in all the tubes with activated plasminogen, since the clots in all these tubes lysed more rapidly than the control clots. This demonstration of complete clot lysis but negligible caseinolysis (as assayed via the acid-soluble tyrosine production), agrees with Shinowara (33), who suggests "that the liquefaction of a fibrin clot...and the increase in acid soluble nitrogen are not representative of an identical enzyme system."

C. Lack of Demonstrable Coagulase Activity in the Staphylokinase Preparation

The unit of lytic activity in all fibrinolytic assays performed here was defined as the highest dilution of staphylokinase producing complete lysis 4+ in the respective plasminogen preparations; the same staphylokinase preparation was used in all the assays performed. As the coagulative-aiding property of staphylococci would tend toward tight clot formation, the activity of the lytic aiding fraction might be masked completely during the four-hour assay period. It would be difficult to evaluate the results, as the kinetics of the coagulative and lytic aiding properties of staphylococci, when both are present in unknown concentrations during the fibrinolysis assay, has not been determined. It has been demonstrated that there was no demonstrable coagulase activity in the previous fibrinolytic

assays. Therefore, tight clots in the presence of plasminogen and high kinase titers cannot be associated with a correspondingly high coagulase titer. The previous coagulase tests were performed merely in the presence of the bovine fibrinogen. Gerheim, Ferguson, et al (15), however, concluded that staphylocoagulation is produced by prostaphylocoagulase (the bacterial extoxin) plus a plasma cofactor other than thrombin. Consequently, further tests were performed to ascertain the effect of the undiluted kinase upon several samples of freshly prepared human plasma.

Demonstration that the kinase preparation lacks coagulative aiding activity on human plasma

(The method employed was modified from Walker, Derow and Schaefer (41)0

.5 ml of the staphylokinase preparation were added respectively to six Wasserman test tubes containing .5 ml of freshly prepared human plasma. The tubes were then incubated at 37°C. and checked every 30 seconds for a period of 15 minutes, and at longer intervals over a period of six hours. Examination for any longer period would be useless, as the plasminogen-kinase activation would have lysed the fibrinogen and/or fibrin.

At no time was there any evidence of clot formation in any tube. These findings indicate that it is possible to isolate the kinase free of the procoagulase. Gerheim, Ferguson et al (15) demonstrated that the two factors could successfully be separated by incubation, as the staphlocoagulase is thermolabile. It was shown that incubation at 75°C. for 2 hours

destroyed all coagulase-aiding properties while the clot lysis time of the same material was little altered. These findings indicate the separability of the coagulative and lytic aiding properties associated with staphylococci.

V. Summary

Plasminogen fractionated from normal horse serum and activated by a staphylokinase preparation has been shown to exhibit considerable fibrinolytic activity. The material obtained via a modified Remmert and Cohen procedure possessed considerably greater activity than the unfractionated serum, but the "acid-plasmin" obtained from a sample of the same serum via the modified Kaulla fractionation procedure exhibited negligible, if any, fibrinolytic activity. It is apparent that in the modified Kaulla procedure employed the active material was either lost in one of the discarded fractions, or was completely inactivated. (3,4,5). Neither plasminogen preparation produced any demonstrable increase in the acid-soluble tyrosine produced in a .7% casein solution. Here we are in agreement with Shinowara (33), in suggesting "that the liquefaction of a fibrin clot... and the increase in acid-soluble nitrogen are not representative of an identical enzyme system." The staphylokinase preparation, used as plasminogen activator throughout, has been shown to be free of the coagulative-aiding fraction. Ferguson, Travis and Gerheim (15) previously demonstrated that these two properties associated with staphylococci were distinct by destroying the wk activity of the staphylocoagulase at 75° C for 2 hours; the clot lysis time was little altered. It has been found that plasminogen fractionated from horse serum possesses considerable fibrinolytic but little caseinolytic activity.

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