STUDIES ON THE ISOLATION, PURIFICATION AND CHARACTERIZATION OF PLASMINOGEN FROM HUMAN PLASMA FRACTION III

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By

Gerda Mootse

A THESIS

Submitted to the School of Advanced Graduate Studies of Michigan State University of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

DOCTOR OF PHILOSOPHY

Department of Chemistry

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AN ABSTRACT

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ABSTRACT

Purification of the plasma enzymes, enzyme activators, and enzyme inhibitors should give material with which many fundamental questions can be answered. Therefore, methods that give only reliable and repeatable preparations have practical significance. Such was the object of this study.

The starting material used was Cohn's Fraction III obtained from human plasma which contains two known precursors of proteolytic enzymes, namely, plasminogen and prothrombin.

An extensive purification study prior to applying the method of Kline was carried out on Fraction III. Thereafter an improved modification of the Kline procedure was developed.

The initial purification consisted of the elimination of the two main components from the Fraction III, fibrinogen and lipid material. Fibrinogen was first extracted into phosphate buffer at pH 6.4, ionic strength = 0.05. Plasminogen was next dissolved from the remaining precipitate into 0.1 M acetate buffer of pH 4.6, leaving lipid material with the residue. Adjusting the solution to pH 7.4 precipitated plasminogen. This precipitate was now ready for final purification by a modified Kline procedure.

The principal change in the Kline method developed in this study was the fractionation of Solution A (modified) by ammonium sulfate. The precipitate obtained at 0.20 - 0.34 saturation gave the highest activity yet reported, i.e. 120-140 P.U./mg.N with 30 - 40 per cent yield.

The high purity plasminogen appeared homogeneous according to ultracentrifuge sedimentation patterns, but various components showed up in

electrophoretic patterns obtained by runs in glycine buffer at pH 2.1.

Caseinolytic, fibrinolytic and p-toluene-sulfonyl-L-arginine methyl ester esterolytic activity by the plasminogen preparation was found to be inhibited by cysteine. L-lysine ethyl and methyl ester activities were not inhibited by this agent.

The presumed importance of the -S-S- linkage in the plasminogen molecule is indicated. Cysteine inhibition is suggested to be the result of reduction of -S-S- bond(s) or a disulfide interchange reaction in presence of thiol compounds.

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INTRODUCTION

Recognition of the physiological significance of the fibrinolytic system during thrombotic conditions has promoted a growing interest in the problems concerning the proteolytic enzymes in human blood.

There have been many observations on the similarity of the mechanisms of clotting and fibrinolysis, both having parallel systems of plasma and tissue factors.

It is possible that recent advances in the identification of clotting factors may reveal links between these systems.

As is usual, the extensive work done has revealed a greater complexity than was suspected.

Factors separated in the laboratory may in life form part of a single complex, the activity of which depends on its integrity.

Mammalian plasma proteolytic enzyme system is comprised of many individual components.

Plasminogen, the precursor of the fibrinolytic enzyme found in the blood and active at neutral pH, can be converted to its active enzyme, plasmin, by a variety of activators.

The means by which active enzyme is released from the precursor in the blood and the mechanism of this activation, remain obscure.

Inconsistent behavior of precursor samples during fractionation procedures, suggesting coprecipitation with other proteins, has in general resulted in distribution of the proenzyme into every fraction separated, and too frequently, in proportions similar to those in which total protein was distributed.

Changes in apparent solubility by factors of several fold have

been encountered frequently under supposedly fixed conditions, but at different stages of purification.

The principal objects of this study were to discover methods for further purifying plasminogen and to elucidate the properties of both the active and inactive forms of the enzyme.

In the following experiments to be described, attempts have been made to study the purification of plasminogen in a more or less systematic manner step by step using Cohn's Fraction III from human plasma, as the starting material.

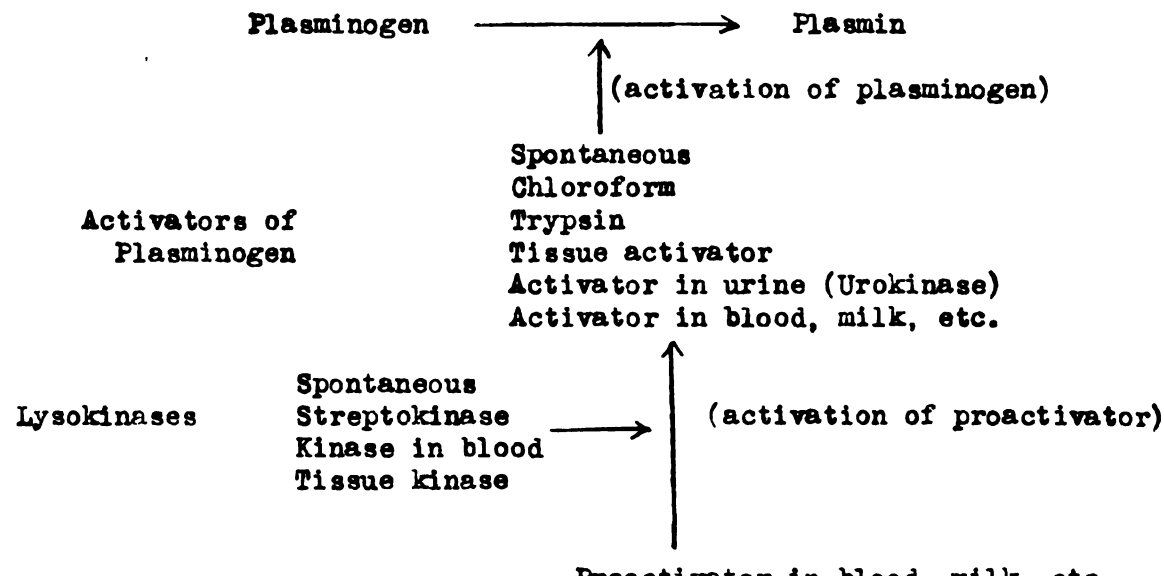
HISTORICAL

That mamallian blood contains a proteolytic enzyme which causes clotted blood to redissolve was first reported by Dastre, 1893[1].

Reviews of the subsequent literature pertaining to the proteolytic enzyme system have been published by Christensen[2], by Kaplan[3], by Rocha E. Silva and co-workers[4], by Mac Farlane and Biggs[5], and in 1956 by Astrup[6].

The historical background reveals that the development of fibrinolytic activity in the organism is the result of a very complicated process.

Experimental findings and conclusions related to fibrinolysis may be summarized in an activation scheme as presented by Astrup[6] in 1954.



Spontaneous activation in vitro may occur during the process of plasma fractionation[7], after the removal of a plasma inhibitor by chloroform[2,4], or under conditions where stabilization of newly formed plasmin has been assured[8].

An investigation by Alkjaersig and co-workers[8] of the spontaneous activation of plasminogen prepared by the Kline[9] procedure has shown that the early methods described were unreliable and unsatisfactory, since both the enzyme and its precursor were unstable. They found that 50 per cent glycerol stabilized plasminogen and plasmin, and under defined conditions also induced the complete conversion of plasminogen to plasmin which remained stable. The appearance of trichloroacetic acid (TCA), soluble moieties in the activation mixture indicated that proteolysis was involved in the activation.

The kinetics of the activation of plasminogen by trypsin, streptokinase (S.K.) and urokinase[10,11] has been measured by the same authors[10]. Their results showed that the activators exerted their effect through an enzymatic process, since Lineweaver-Burk plots[12] were linear in each case. During the activation process there was a release of a TCA-soluble moiety, equivalent in each case to some 25 per cent of the original TCA-precipitable material.

Concerning the role of fibrinolysis in several physiological and pathological conditions one may refer to the article by Müllertz[13]. Preparations containing plasminogen or plasmin are very often contaminated with an activator or with its precursor (proactivator) of plasminogen.

Human blood contains large amounts of proactivator and relatively small amounts of plasminogen, by addition of streptokinase (S.K., bacterial product of hemolytic streptococci) large amounts of activator and small amounts of plasmin are formed. Bovine blood has much plasminogen, and little or no proactivator. Bovine plasmin is not formed with S.K.[14]. Activation of plasminogen from various animal species by S.K.

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was studied by Takayoshi[15] with the results that dog and human responded the greatest, thus showing species difference.

There exist various procedures for estimating the activity of plasmin. The choice of the method has a profound influence upon the results obtained.

Casein, or any other plasminogen-free substrate gives an expression of proteolytic activity (usually evaluated by measuring the absorbancy of the deproteinized filtrate at 280 mμ). In less pure preparations inhibitory agents are usually present and the results will not give a direct estimate of plasmin. The casein method is rather sensitive to the presence of inhibitory agents. The activators of plasminogen can also be determined by their effect on purified plasminogen with casein[14].

The lytic effect of less pure plasmin can be followed on certain synthetic amino acid esters such as p-toluenesulfonylarginine methyl-ester (TAMe) and lysine methyl- or ethyl-ester (LMe or LEe). The affinity of plasmin for these synthetic substrates is very high. Therefore this estimation is only slightly influenced by inhibitory agents, so that less pure plasmin preparations can be assayed compared to the casein method[6].

The esterase activity of plasmin has been determined: (a) titrimetrically[16,17], (b) manometrically[18], (c) determining the free lysine formed turbidimetrically in acetone[19], and colorimetrically[20,21].

Roberts in 1958 reported the poor reproducibility (specifically with LEe substrate) by the titrimetric method and suggested a modified Hestrin's[20] colorimetric method for esterase activity[21]. The method is based on the formation of a ferric complex of the hydroxamic acid resulting from the reaction of an ester with alkaline hydroxylamine.

The tendency of fibrinogen and fibrin to retain large amounts of plasminogen causes fibrinolytic and fibrinogenolytic methods to be essentially a means of estimating the activators of plasminogen. Only when the absence of activating agents in the test solution has been secured can the results be read in terms of proteolytic activity with respect to plasmin present. This phenomenon explains the discrepancies encountered in estimation of lytic activity when results obtained on fibrin or fibrinogen are compared with those obtained on casein. A great amount of work has been done to prepare fibrinogen (and fibrin) free from plasminogen for the estimation of fibrinolytic enzymes in the presence of activators of plasminogen.

Lassen[22] found that the fibrin layer in the fibrin plate method of assay[23] could be heated for 30 - 45 minutes at 80° C. without seriously interfering with the structure of the clot. This heat-denatured fibrin contained no plasminogen, and inhibitory agents had also been destroyed. This method, the so called heated fibrin-plate method, has been very useful in differentiating between activating agents and proteolytic enzyme activity. Factors influencing susceptibility of fibrin and fibrinogen to proteolysis by plasmin has been discussed by Celander et al[24].

Methods for the detection and measurement of human fibrinolysis have been evaluated by V. Kaulla and Schultz[25]. They could not find any correlation between esterase activity and fibrinolytic activity when the activation of human-plasma plasminogen was brought about by human activator, such as urokinase. This is contrary to the results obtained after activation of plasminogen by S.K.[16].

Other methods of activity determination, used by previous workers

include change in viscometric measurement on casein or gelatin[26] substrate solutions.

Protaminase activity of plasmin has been demonstrated by Kjeldgaard and Plou̇g[19] on the heparin-protamine complex and by Brunfeldt and Poulsen[27] on insulin-protamine complex. Limited proteolysis by plasmin on corticotropin A has been shown by White[28].

Plasmin effects the non-Newtonian viscosity and the character of the mucin clot of solution containing the hyaluronic-acid-protein complex[29] (similar to papain).

N-terminal amino acids formed during digestion of bovine fibrinogen by urokinase activated plasmin were determined by Wallén et al[30]. Wallén and Bergstrom showed, that plasmin split at least 150 bonds at maximal fibrinogen digestion[31]. The same peptide bonds were attacked by thrombin whether it was digested before by plasmin or not.

Upon digestion of pure fibrin and fibrinogen by plasmin, there appeared antithrombin activity in the digestion mixture[32].

Similarly it has been found that the mechanism of inhibition is very complicated. Inhibitory systems consist of antiplasmin (different from antitrypsin) and inhibitors against activators and lysokinases.

Heat labile trypsin inhibitor in plasma and serum is probably responsible also for a large part of the plasmin inhibiting effect of blood. Jacobsson[33] separated by electrophoresis two different trypsin inhibitors. One inhibitor migrated with the α_1 globulins, and the other with α_2 globulins. Alpha-2 inhibitor had a potent effect on S.K. activated human plasminogen tested on fibrin. The α_1 fraction was separated by the anion exchange method as described by Moll et al[34] and it failed to inhibit plasmin.

Norman[35] reported the α-1 and α-2 anti-plasmins of human plasma. The α-2 inhibitor dissociated readily from the plasmin-inhibitor complex and only 50 per cent was inactivated after 90 minutes at 60° C. The α-1 inhibitor did not dissociate readily and was destroyed at 60° C for 30 minutes. Both inhibitors were inactivated at 25° C at a pH below 5.5 or above 11.0.

A proteolytic inhibitor of plasmin with anticoagulant activity has been separated from human urine and from isoelectrically precipitated serum globulin by Shulman[36]. The reaction of the inhibitor with plasma proteolytic enzymes was reversible.

The inhibitory effects of a number of inorganic and organic compounds, including dyes, have been studied.

Metallic Ions. Copper and zinc ions in 0.01 M final concentrations completely inhibited spontaneous activation in glycerol[8]. Calcium, magnesium and manganese had no such effect[8].

Calcium inhibition of active fibrinolytic enzyme in normal plasma has been shown by Fearnly[37] to be maximal at the lowest plasma dilution. Thromboelastographic studies of S.K.-induced fibrinolysis by O'Neal and Tillman[38] showed the same inhibitory effect by $CaCl_2$. Ratnoff[39] reported rapid activation of precursor (plasminogen) by calcium ions in human plasma. However, partially purified plasminogen was not activated by calcium ions.

As the investigations presented by various authors are still of a rather preliminary nature, there are controversial statements concerning the effect of various ions and various organic compounds.

Astrup[6] found the activation or inhibition effect of heparin to depend upon pH and ionic strength of the fibrin substrate and on the

plasmin sample. Therefore it appears possible to bring into harmony previous discordant observations about heparin.

Plasminogen is unaffected by diisopropylfluorophosphate (DFP), but plasmin was inhibited non-competitively[40].

Plasmin inhibition by toxic phosphorus compounds has been studied by Mounter and Shipley[40]. Such protein combining dyes as congo-red, trypan-red, trypan-blue did not inhibit fibrinolysis[41].

Ambrus et al.[42] reported severe allergic reactions of living organisms in response to injection of S.K. or S.K. activated plasmin. However, no anti-bodies were produced upon introduction of urokinase or urokinase activated plasmin.

A report by Ferguson et al.[43] ascribes a weak trypsin-like thromboplastic activity to the fibrinolytic enzyme present in normal human blood, suggesting participation of this proteolytic enzyme in blood coagulation. This observation was questioned by Seegers et al[44]. The findings reported by V. Kaulla[45] in 1958 raise anew the question of the interaction of fibrinolytic enzyme and clotting factor. It was observed that induction of <u>in vitro</u> fibrinolysis in human blood either by S.K. or bovine fibrinolysin was always preceded by earlier onset of fibrin formation.

<u>Plasminogen</u>. Preparations containing plasminogen have been usually purified by isoelectric precipitation or by ammonium sulfate fractionation of blood plasma or serum. Partial purification of the enzyme was reported by Edsall et al.[46], Loomis et al.[47], Rocha E. Silva et al.[4], and Remmert et al[48]. The principal obstacle to purification has been the intense co-precipitating properties of plasminogen. For this reason, the classical techniques of salt, alcohol, and isoelectric fractionation have proved of limited value.

The extreme stability of plasminogen toward acid was used for basis of purification by Christensen[49].

Tagnon[50] in 1942 showed that the activity of the fibrinolytic enzyme was connected with the euglobulin fraction of the blood. In 1951 Christensen and Smith[49] were the first investigators to utilize the Cohn Fraction III[51] as a starting material for plasminogen of human origin. Plasminogen is one of the least soluble of plasma proteins, being found in the residues after extracting the other components of Cohn's Fractions I and II + III[7].

Kline[9] by using Christensen's acid extraction in connection with selectively denaturing the interacting contaminants at high pH, obtained the concentration of enzyme more than 400 times that of serum.

Few attempts have been made to purify the activated enzyme. In 1956 Kline and Fishman[52] reported 2-5 times purification over that of high purity plasminogen by fractionating the active enzyme with alcohol. Physico-chemical properties of plasminogen and plasmin have been investigated by Shulman and co-workers[53], and some of their data may be summarized as follows:

A Summary of Properties[53]

	Plasminogen(Human)	Plasmin(Human)
Casein units per mg. of N[a]		60-100
Molecular Weight	143,000	108,000(spontaneous)
Isoelectric point	5.6	6.2
Electrophoretic mobilities in glycine buffer pH 2.1	8.2×10^{-5} cm^2 per v per sec. 7.3×10^{-5} cm^2 per v per sec.	8.9×10^{-5} cm^2 per v per sec.(spontaneous)
Tryosine	5.91%	6.32%
Tryptophan	3.78%	4.04%
Ratio Tryosine to Tryptophane	1.56	1.56
Nitrogen	15.8 %	14.1 %
Phosphorus	0.19%	
Hexose	0.98%	1.51%

a) Proteolytic or caseinolytic unit--1 proteolytic unit was arbitrarily taken as the amount of enzyme producing an increase of 450γ of acid-soluble tyrosine in a medium of 4 per cent casein in 1 hour[48].

Ultracentrifugal information about changes during activation of human plasminogen has been reported by Markus et al[54]. All of these constitutional data are at present open to some degree of uncertainty because of the heterogeneity of the preparations. For instance, the physicochemical data of plasminogen by Heitzenhoffer et al[55], who reported 70 per cent purity as determined electrophoretically, does not compare with Shulman's data.

Enzymatic properties of bovine plasmin have been characterized by Ronwin[56] and compared with those of thrombin and trypsin[57]. He emphasized an extraordinary similarity: witness their action on the same substrates, identical pH optimum curves, etc. However, plasmin and trypsin showed marked resistance to combined acid and heat treatment while thrombin rapidly deteriorated under the same conditions[56].

EXPERIMENTAL

A. Apparatus:

Temperature control-- A constant temperature bath sold by the American Instrument Co. and equipped with a Thryratron relay Aminco thermoregulator unit which controlled the temperature at $37.5^{\circ} \pm 0.05^{\circ}$ C was used. For low temperature control, constant low temperature thermostat tanks and refrigerated centrifuges controlled to any required temperature were employed. (The Servall refrigerated centrifuge with size SS-1 rotor for 50 ml tubes and International Refr igerated centrifuge Model PR-2 were utilized.)

pH Meter-- A Beckman Model G or Model H2, line operated pH meter was used for hydrogen ion activity measurements. Stability for the Model H2 was improved by use of a 115 volt Sola constant-voltage transformer between the line and the instrument. Values of pH were determined with glass electrode. As reference electrode the calomel cell was used, and for calibration at pH 7.0 Beckman standard buffer was properly diluted or at pH 4.0, 0.0500 M acid phthalate was employed.

Timer-- A Meylan stop watch was used to time the reaction periods.

Spectrophotometers-- Absorbancy measurements at 280 mμ were made using the Beckman Model DU spectrophotometer. The Beckman Model B spectrophotometer was used in the determination of Biuret nitrogen and for esterase activity according to modified Hestrin's colorimetric method.

Dialysis-- All the dialyses were made in Visking cellophane tubing using an external rotating liquied dialyzer constructed according to

Djang, ~~Lillevik and Bell~~[58].

Electrophoretic Analyses-- were made with the Tiselius electrophoresis apparatus Model 138 (Perkin Elmer Corp.). For conductivity measurements, the Model RC-IB resistance bridge (Industrial Instruments Inc.) attached to a conductivity cell (Perkin Elmer) of cell constant = 0.4893 was used.

Electromagnetic Stirrer-- An electromagnetic stirrer (Labline Inc,) was used in the formol, alcohol, and water media titration work.

Glassware-- Volumetric glassware were of Pyrex glass brand.

For large scale preparations the stainless steel equipment in the Michigan Department of Health Laboratories was used.

Analytical Ultracentrifuge-- The Spinco Model E (Specialized Instruments Corp.) was utilized for studying the sedimentation behavior of proteins.

B. Materials and Reagents:

Chemicals-- All inorganic and organic chemicals were either c.p. or reagent grade unless otherwise specified.

Buffers- Phosphate buffers-- proportions were calculated using both the Henderson-Hasselbach equation and the Lewis ionic strength equation. Proportions for phosphate buffers used in Michigan Department of Health Laboratories were calculated by using industrial nomograms. In every case the pH was checked and adjusted with the aid of a pH-meter.

Sodium acetate buffers-- Stock solutions of about 10 M acetic acid and about 4 M sodium acetate proved convenient in the preparation of the buffers.

Citrate buffers-- Stock solution of pH 6.0 ionic strength 0.3 buffer was prepared by adjusting 0.055 M sodium citrate solution to pH 6.0 with concentrated hydrochloric acid. Different pH-s were obtained by adjusting the stock solution with concentrated hydrochloric acid or 4N sodium hydroxide.

Sodium glycinate buffer pH 9.5-- 75 gm. glycine and 20 gm. sodium hydroxide per liter.

Glycine-acetate buffer pH 5.5-- 150 ml. of 95 per cent ethanol, 2.0 ml. of M sodium acetate, 1.4 ml. of M acetic acid and 45 g. of glycine per liter[59].

Glycine-phosphate buffer pH 6.8 - 6.9-- 160 ml. of 95 per cent ethanol, 45 g. of glycine, 2.5 ml. of sodium glycinate buffer, 3.2 ml. of 0.5 M disodium phosphate and 2.4 ml. of 0.5 M monosodium phosphate brought to 1 liter.[59].

Buffers for electrophoresis:

Glycine buffer pH 2.1-- ionic strengths 0.05 and 0.1. 0.05 M and 0.1 M hydrochloric acid respectively adjusted to pH 2.1 with glycine.

Veronal buffer-- pH 8.6 ionic strength 0.1 was prepared by dissolving 21.197 gm. of Veronal (Barbital, N.F., Fisher Scientific Company) and 0.1 mole of sodium hydroxide in distilled water and making the volume to 1 liter.

Borate buffers-- proportions were calculated using the Henderson - Hasselbach equation.

0.05 N Alcoholic Potassium hydroxide-- 3.75 gm. potassium hydroxide was dissolved in 62.5 ml. distilled water and diluted to one liter with 95 per cent ethanol. The reagent was standarized against 0.1067N hydrochloric acid with phenolphthalein as indicator.

Thymolphthalein Indicator-- The indicator solution for the Willstatter and Waldschmidt - Leitz(1921)[60] titration was prepared by diluting 6 ml. of a 0.5 per cent thymolphthalein in 95 per cent ethanol to 100 ml with absolute alcohol.

Saturated ammonium sulfate solution-- prepared by shaking at about 23° C until definite precipitate stayed on the bottom of the flask, then filtered. Since saturation depends on the temperature, 4 Molar solutions were used later.

Proenzyme source-- Fractions III were received as frozen pastes from the American National Red Cross from stocks maintained at Squibb and Sons. The procedure for alcohol fractionation of pooled human plasma is summarized in Scheme 1.

The purified proenzyme was stored frozen in 0.05 or 0.1 M acetate buffer pH 4.6 or in water, acidified with a couple of drops of N hydrochloric acid (pH around 4.0).

Casein Stock solution was prepared of Hammarsten casein or Sheffield, devitaminized 80A3 casein. About 6 percent casein was suspended in 0.1 M phosphate buffer pH 7.4, 0.9 per cent sodium chloride, stirred about 3 hours and pH adjusted to 7.4 with 4 N sodium hydroxide. The suspension was heated in boiling water bath at 95° C 15 minutes, then cooled and nitrogen determined by semi-micro Kjeldahl method. The

dispersion was now diluted to 6 per cent casein with the same buffer. (6.38 was used for conversion factor) The stock solution was stored frozen in 50 ml rubber stoppered bottles for immediate use.

Streptokinase-- Streptodornase, Lederle Varidase, was dissolved in distilled water 3000 units per ml.and kept as stock solution in refrigerator +4° C.

Crude lyophilized thrombin preparation (lot # 204, M.D.H. labs. 1951).

Protamine, used in purification studies was from Eli Lilly Co. E-696-B preparation.

Reagents for inhibition and inactivation experiments:

DL-Methionine (Nutritional Biochemicals Corp.)

L-Cysteine (free base, Nutritional Biochemicals Corp.) 0.1 M solution was freshly prepared every day.

Cystine, C.P. Pfanstiehl Chemical Co.

Heparin Sodium (Abbott). Each cc. contained 1000 U.S.P. (approx 10 mg) Heparin Sodium and 0.02 per cent propylparaben.

Na-ascor bate (Nutritional Biochemical Corp.)

Urea (Baker analyzed reagent)

1M Calcium chloride solution.

Reagents for modified Hestrin's colorimetric method were made as described by Roberts[21].

L-lysine methylester (LMe) di-hydrochloride (Nutritional Biochemicals Corp.) 0.2 M solution was freshly prepared every other day.

p-Toluene sulfonyl L-arginine methylester hydrochloride (TAMe) (H. M. Chemical Company Ltd, Santa Monica, California) 0.1 M solution was also freshly prepared every other day.

Reagent for Biuret nitrogen[61] A modification worked out in M.D.H. Laboratories. Two times concentrated Rosenthal-Cundiff reagent was made as follows:[66]

6.0 gm.of cupric sulfate pentahydrate,
24.0 gm.of ethylenediaminetetraacetic acid disodium salt and
4.0 gm.potassium iodide were dissolved in 500 ml.distilled water. Then 165 ml.of 18.2 M sodium hydroxide added and filled to 2 liters.

0.15 N sodium chloride was used for blank.

Reagents for esterase activity titration[16,17]

L-lysine ethylester (LEe) (Bios Laboratories)

Imidazole buffer 0.1 M, pH adjusted to 6.5

Formaldehyde solution approximately 37 per cent, pH adjusted to 8.0

Indicator 0.01 per cent phenol red

Redistilled acetone

0.0508 N sodium hydroxide

The other reagents used were the same as in Roberts[21] method.

Folin-Ciocalteu Phenol reagents for protein estimation[62] were made in the M.D.H. Laboratories.

20 per cent TCA

10 per cent sodium hydroxide

25 per cent sodium carbonate

Folin Ciocalteu reagent[63]

Stock standard tyrosine: 20.0 mg. L-tyrosine dissolved in 0.1 N hydrochloric acid and made to 100 ml. with 0.1 N hydrochloric acid.

Semi-micro Kjeldahl reagents were those used in M.D.H. Laboratories.

Reagents for adsorption studies:

Kaolin NF colloidal, Mallinkrodt

Barium sulfate, Baker's Chemical

Aluminum hydroxide

Calcium hydroxyapatite prepared according to Tiselius et al[64].

Other Miscellaneous Reagents:

Trichloroacetic acid (TCA), 10 per cent for protein precipitation

0.05 N sulfuric acid for Kline's[9] extraction was prepared by diluting 1.39 ml. concentrated sulfuric acid to one liter.

Acid acetone (2 drops of N hydrochloric acid added to 100 ml. of acetone).

Approximate N hydrochloric acid, N sodium hydroxide and N sulfuric acid were used to adjust the hydrogen ion concentration.

C. Experimental Procedures:

Determination of proteolytic activity by the method of Remmert and Cohen[48]

An appropriate amount (0.5 ml.) of plasminogen suspension was placed in a test tube and diluted to 1.8 ml. with 0.1 M phosphate - 0.9 per cent saline buffer pH 7.4. Next 0.3 ml. of 6 per cent casein in the same buffer

and predetermined units* of S.K. contained in 0.2 ml.(3000 S.K.U./ml) water solution were added. The mixture was incubated at 37.5° C for 10 minutes to permit complete activation of the plasminogen after which 3.7 ml.of 6 per cent casein were then added, and the combination mixed thoroughly. An aliquot (2 ml) was withdrawn and added to an equal volume of 10 per cent TCA. Incubation of the remaining sample was continued at 37.5° C for 1 hour, when a second aliquot was withdrawn and treated in the same manner. After 30 minutes 3 ml water was added to the aliquot-TCA mixture, and the precipitated protein was removed by filtration through Whatman 2 paper. The absorbancy of duplicate filtrates was read in a Beckman DU spectrophotometer at 280 mμ against a blank of water. Absorbancy readings were converted into micrograms of acid-soluble tyrosine by reference to the standard tyrosine curve. Units of acid-soluble tyrosine per complete digest were calculated from the results for zero and 60 minute aliquots, the increase being taken as a measure of the proteolytic activity. The enzyme and S.K. concentration effect on proteolytic activity is shown in the following Table I and Figures 1 and 2.

TABLE I

Effect of Plasminogen Dilution on Proteolytic Activity[48]

Enzyme Suspension	Dilution	O.D.[a]	P.U./ml.	Mg.N/ml.	P.U./mg.N	% Recovery of Activity
.25-.34 saturated $(NH_4)_2SO_4$ fraction from Sol. A[9]	1 : 10	.355 1.02 .365 1.03	9.7	0.079	123	50
	1 : 20	.329 .681	10.3		131	53
	1: 25	.310 .621	11.5		145	59

[a]Absorbancies (Optical densities) are from zero and 1 hour digestion mixtures.

* Determined by finding amount needed to produce optimum caseinolytic activity[48] See Figure 2.

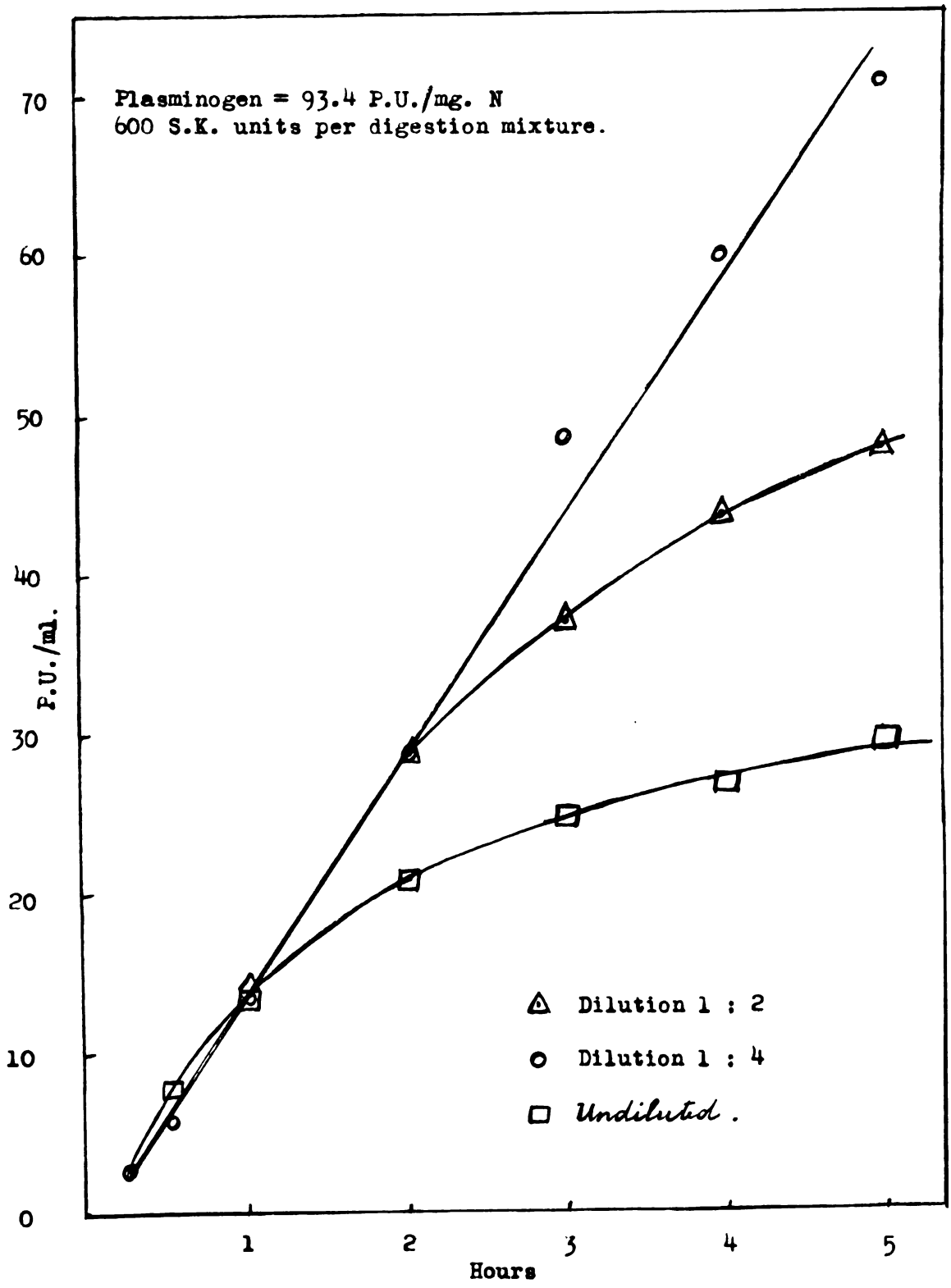


Fig. 1.The Effect of Enzyme Concentration on Proteolytic Activity according to the procedure of Remmert and Cohen[48].

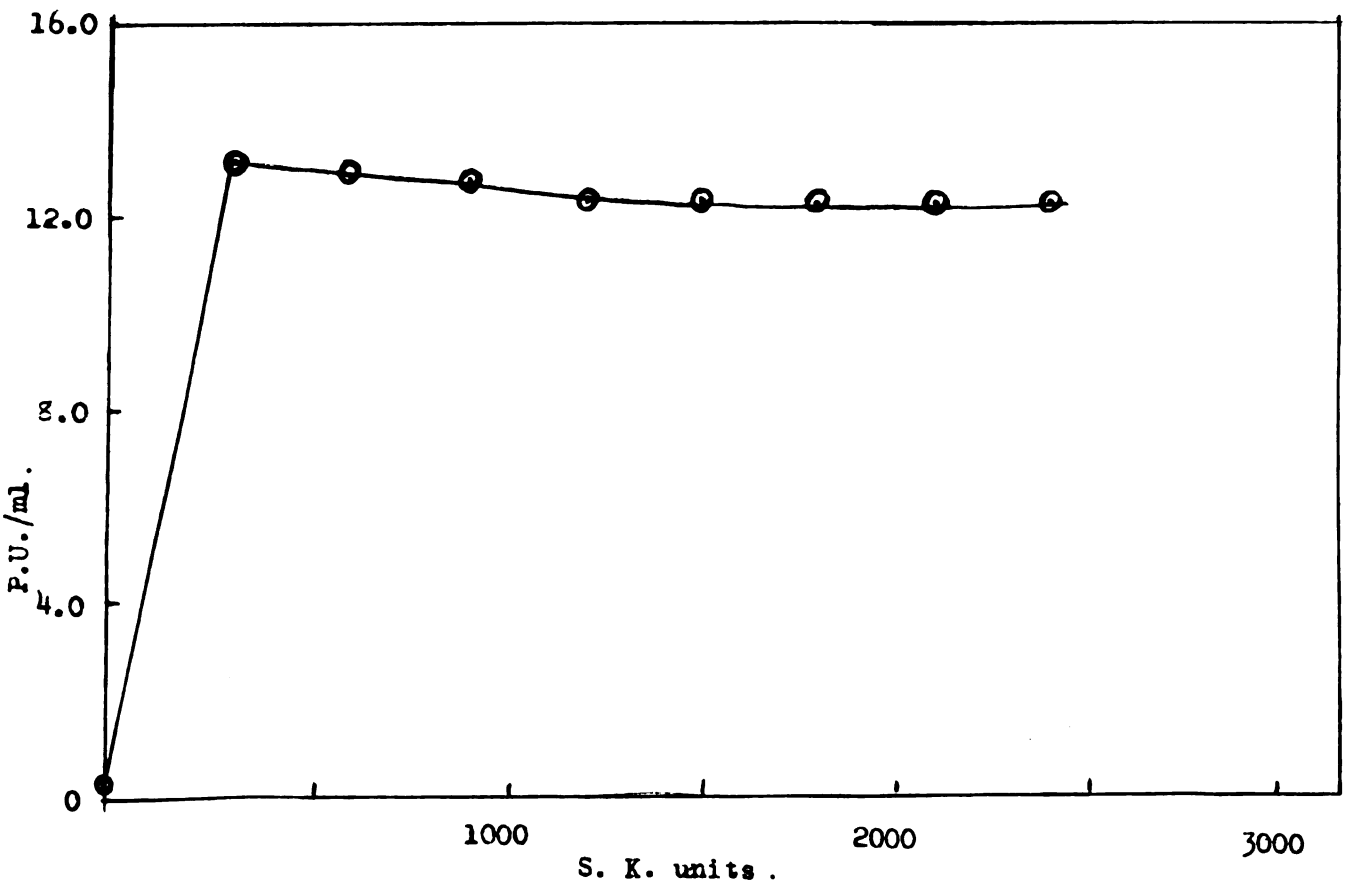
Esterase activity determinations:

1. Modified Hestrin's colorimeteric method[21] --

One ml. samples of LMe substrate, 2.8 ml. of imidazole buffer, 0.2ml. of S.K. (3000 U./ml) or water for unactivated runs, were placed in a bath at 37.5° C for 5 minutes. One ml. of enzyme solution was then added, the tubes were thoroughly mixed and a 1 ml. of digest was removed and added to 1 ml. each of reagent 1 and 2. The time the sample was added to alkaline hydroxylamine was called zero time. A final sample was removed for a blank and added to a tube containing 1 ml. each of reagents 1, 2 and 3. After 2 or more minutes 1 ml. of reagent 3 was

Figure 2

Effect of Streptokinase Concentration on Proteolytic Activity[48]



added to each tube and after 30 minutes to 24 hours of standing at room temperature 1 ml.was then added to 4 ml.of reagent 4 and the absorbancy was read against its blank at 525 mμ within 2-3 minutes of color formation. The rate of disappearance of the ester in the tube containing S.K. minus the rate in the tube containing no S.K. (spontaneous activity) is equal to the rate of ester hydrolysis.

2. Titration method for esterase activity.

a) TAMe as substrate[16]

One ml.of 0.1 M TAMe, 2.5 ml.of tris (hydroxymethyl) amino methane buffer, 0.5 M pH 9.0 and 0.2 ml.S.K. (3000 u/ml) or water are incubated at 37.5° C for 5 minutes. One ml.enzyme solution is then added and thoroughly mixed. A 1 ml.aliquot is immediately withdrawn and added to a tube containing 1 ml.of formaldehyde. After 30 minutes of incubation additional 1 ml.aliquot is withdrawn and added to 1 ml.of formaldehyde. The aliquots containing 0.2 ml.of o.o1 per cent phenol red as an indicator are titrated with 0.05 N sodium hydroxide. Alkali consumed by the samples containing water was subtracted from the alkali consumed by the samples containing S.K. The results were expressed as the mean increase in micromoles of acid liberated per 30 minutes per mg.N.

b) LEe as substrate[17]

One ml.of 0.2 M LEe, 2.5 ml.0.1 M imidazole buffer, pH 6.5 and 0.2 ml.S.K. or water are incubated at 37.5° C for 5 minutes. One ml. of enzyme solution is then added, thoroughly mixed and 1 ml.of mixture immediately withdrawn and added to 10 ml.redistilled acetone. After 30 minutes, the lysine precipitate is centrifuged and dissolved in 2 ml. of water, treated with 2 ml.formaldehyde and the mixture titrated with 0.05 N sodium hydroxide to phenolphthalein pink.

Modified Kline Fibrinolytic Method[52]:

The plasminogen suspension, contained in a volume of 0.9 ml.of 0.05 M phosphate buffer of pH 7.6 was activated for 3 minutes at 37.5° C with 0.1 ml.of S.K. (300 u). After activation 0.04 ml.of bovine thrombin solution (commercial thrombin, Parke, Davis & Co., dissolved in equal mixture of saline and glycerol--100 u per ml) were added. Immediately (zero time) 5 ml.of 0.4 per cent human fibrinogen, (59 per cent clottable, M.D.H. lab.) dissolved in 0.05 M phosphate buffer of pH 7.6, was blown into the tube, thoroughly mixed, and placed in a water bath at 37.5° C. The time of clot lysis was determined.

Nitrogen Estimations:

1. Usually made by semi-micro Kjeldahl procedure[65] by Biophysics Department of M.D.H. Laboratories.
2. Determination of proteins by Folin-Ciocalteu Phenol reagent[62].

To 1 ml.samples (0.01 to 0.3 mg.N or 0.06 to 1.8 mg.protein) was added 0.5 ml.10 per cent sodium hydroxide and placed into boiling water bath for 10 minutes. Then it was cooled to room temperature and 6.5 ml. of distilled water were added and shaken thoroughly. After mixing first with 1 ml.Folin-Ciocalteu reagent, then with 3 ml.of 25 per cent sodium carbonate, the tubes were left at room temperature for 15 minutes. Absorbancy was read at 540 mμ with a Beckman model B spectrophotometer. The absorbancy of the standard tyrosine 0.1 mg.should be about 0.182 mg. N (1.0 mg.tyrosine is equivalent to 11.4 mg.protein — varies with different proteins). Protein samples that contained ammonium sulfate were precipitated at first with 4 per cent (total) TCA, the precipitate centrifuged and then dissolved in 1 ml.water.

3. Biuret Nitrogen determination[66] modified for plasma proteins by Michigan Department of Health Laboratories.

For Biuret color formation, two standard curves were worked out in the M.D.H. Laboratories. To 5 ml.aliquots containing 0.01-0.18 mg.N/ml, 1 ml.Biuret reagent was added and after 2 hours color formation the absorbancy was read at 540 mμ with a Beckman model B spectrophotometer. To 1 ml.aliquots containing 0.16-1.6 mg.N/ ml.5 ml.of Biuret reagent were added, and absorbancy read after half an hour. Each series of readings was standardized against a standard preparation of normal human serum albumin. For a blank a 0.15 N sodium chloride solution was used.

Lipid Analysis:

Total cholesterol[67], total and inorganic phosphorus[68] (difference assumed to be lipid phosphorus) were determined by Biophysics Department of the Michigan Department of Health Laboratories.

Alcoholic potassium hydroxide titration for total acidity change:

The specific activity of the enzyme used was 88 P.U./mg.N. The digestion conditions described under "Determination of proteolytic activity[48]" were followed. One ml.aliquots were removed from the digestion mixture at intervals, cooled to 0° C and immediately titrated in alcohol according to the method of Willstatter and Waldschmidt-Leitz[60]. This method is a modification of Foreman's[69] original alcoholic sodium hydroxide titration. The aliquots removed were directly pipetted into 3 ml.of absolute alcohol-indicator mixture contained in 25 x 100 mm.test tubes. Each sample was then titrated against 0.05 N alcoholic potassium hydroxide solution to a distinct blue color; six ml.of absolute alcohol was added and the sample again titrated to the appearance of permanent blue color. A five ml.burette calibrated to 0.02 ml.

was used. The sample was kept well stirred during the process of titration with the aid of electromagnetic stirrer which also aids in keeping minimum time for minimum carbon dioxide interference. The initial titer obtained from the aliquot taken immediately after mixing (Zero time) was subtracted from subsequent titers to get the increment in ml. (Δ ml.) of standard alcoholic potassium hydroxide required for titration of the acid groups produced during digestion, per ml.of the digest. The results obtained are reported in Table II and shown in Figure 3.

TABLE II

Liberation of Acidic Groups During Proteolysis of 4 Per Cent Casein Solution, Using 88 P.U./mg.N Plasminogen, as Determined by Titration in Alcohol and Water Media

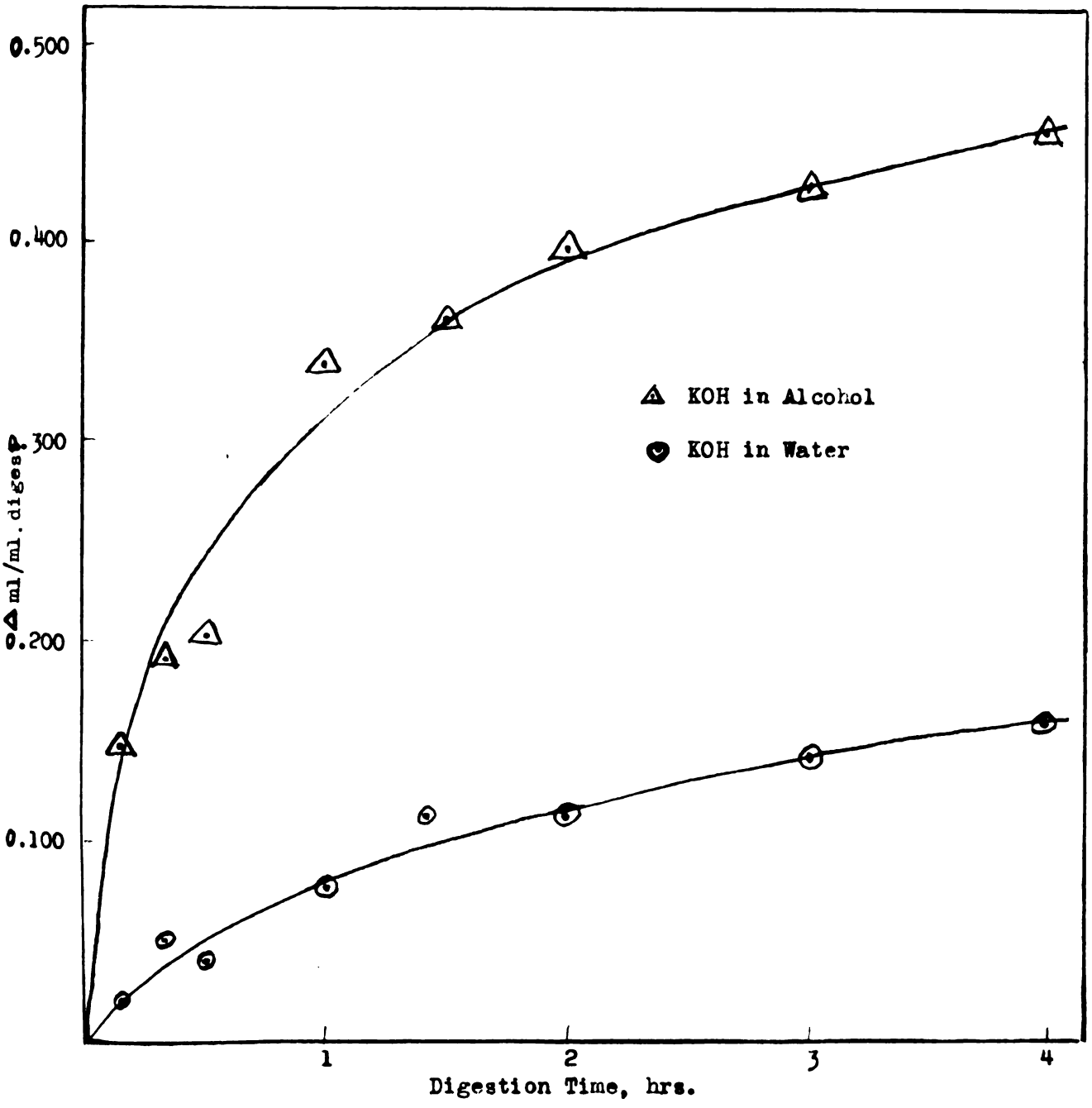
Digestion Time (minutes)	Alcohol Medium Δ ml.of 0.045 N KOH/ml.	Water Medium Δ ml.of 0.045 N KOH/ml.
0	—	--
10	0.146	0.020
20	0.195	0.053
30	0.203	0.040
60	0.340	0.078
90	0.361	0.118
120	0.399	0.115
180	0.430	0.143
240	0.456	0.158

Aqueous Potassium Hydroxide Titration:

For aqueous titrations, 1 ml.aliquots were removed from the same digestion mixture at specified intervals of time and added directly to 9 ml.of distilled water containing thymolphthalein indicator, already placed in 25 x 100 mm.test tubes. Each aliquot thus removed was immediately titrated against the same standard alcoholic potassium hydroxide, using the same burette as mentioned above, to the appearance of blue color. The solution was kept well stirred during titration with

Figure 3

Consumption of Base in Alcohol and Water Media, During Digestion of 4 Per Cent Casein Solution, Plasminogen = 88 P.U./mg.N



the help of an electromagnetic stirrer. The initial titer obtained from the aliquot taken immediately after mixing was subtracted from the subsequent titers as mentioned in alcoholic potassium hydroxide titration. The results were also treated and expressed in the same manner as in alcoholic potassium hydroxide titration. See Table II and Figure 3.

Inhibition in Activation Systems:

The proenzyme suspension was diluted with an inhibitor solution (or with water for control) just before the 10 minute activation period in proteolytic assay method[48]. In esterase inhibition experiments the inhibitor was added to the enzyme suspension just before the enzyme addition to the 5 minute equilibrated substrate mixture[16, 17].

Electrophoresis:

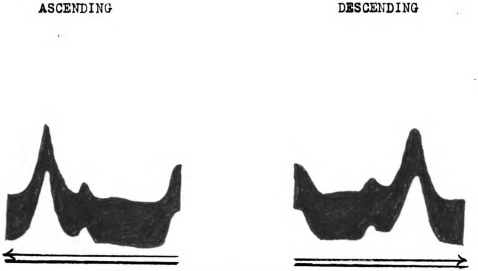
The procedure described in the instruction manual for the Perkin-Elmer electrophoresis instrument was used. Usually, about one per cent protein solution was equilibrated by dialysis, against 300 ml. of the selected buffer at 5° C. For electrophoresis of enzyme digestion mixture on casein, the mixture was inactivated at first by heating 10 minutes in boiling water-bath, then immediately cooled to 0° C and dialyzed overnight against Veronal buffer, pH 8.6 at 4° C. The results are shown in Figure 4.

Analysis in the Ultracentrifuge:

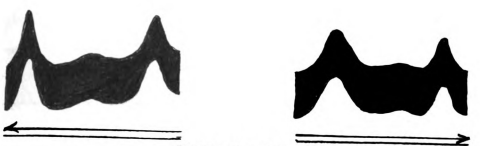
The sedimentation behavior of the plasminogen when dialyzed overnight against glycine buffer pH 2.1, ionic strength 0.05 was studied using the Spinco analytical ultracentrifuge run at 0.5° - 1.5° C and 59,780 r.p.m. The results are given in Table XXXIII and shown in Figure 6. (Shown under the properties of plasminogen).

Figure 4

Electrophoretic Patterns of 4 Per Cent Casein at Zero Time and After 30 Minute Digestion. In Veronal Buffer pH 8.6, $\Gamma/2 = 0.1$



0 Time Digest
5160 Sec.; 1.3 % Protein



30 Minutes Digest
5160 Sec.; 1.3 % Protein

Preparative Procedures for Plasminogen:

The development of a method for the preparation of partially purified plasminogen, which would prove suitable for subsequent purification by Kline procedure, was investigated. In the purification experiments to be reported, the caseinolytic activity per mg of nitrogen (P.U. per mg of N) was used as the sole criterion for the degree of purification attained. All the data given are calculated for 100 g of starting material Fraction III.

A. Studies on Method 9

1) The activity of the starting material, Fraction III:

Table III shows the activity of various samples made by suspending 100 g.of Fraction III in acetate buffer as shown in Scheme 1 and following the exact procedure of Method 9[7].

TABLE III

Proteolytic Activity and Nitrogen in Various Samples of Fraction III Suspended in 0.008 M Acetate, pH 5.4[a]

Sample	P.U./ml.	Mg.N/ml.	P.U./mg.N	P.U.[b]	Total N[b]
1	7.6	2.82	2.7	9232	3412
2	8.1	2.74	3.0	9234	3123
3	7.4	2.72	2.7	8793	3223
4	8.6	3.17	2.7	10535	3883
5	8.1	3.00	2.7	9234	3123

a) 100 gm.of Fraction III suspended according to method 9 shown in Scheme I.

b) The values in the last two columns are calculated from measured suspension volume times values in columns one and two respectively.

2) An idea of the amount of activity retained in the supernatant of the suspension of Fraction III in acetate buffer is given by results in Table IV.

SCHEME 1

METHOD 6

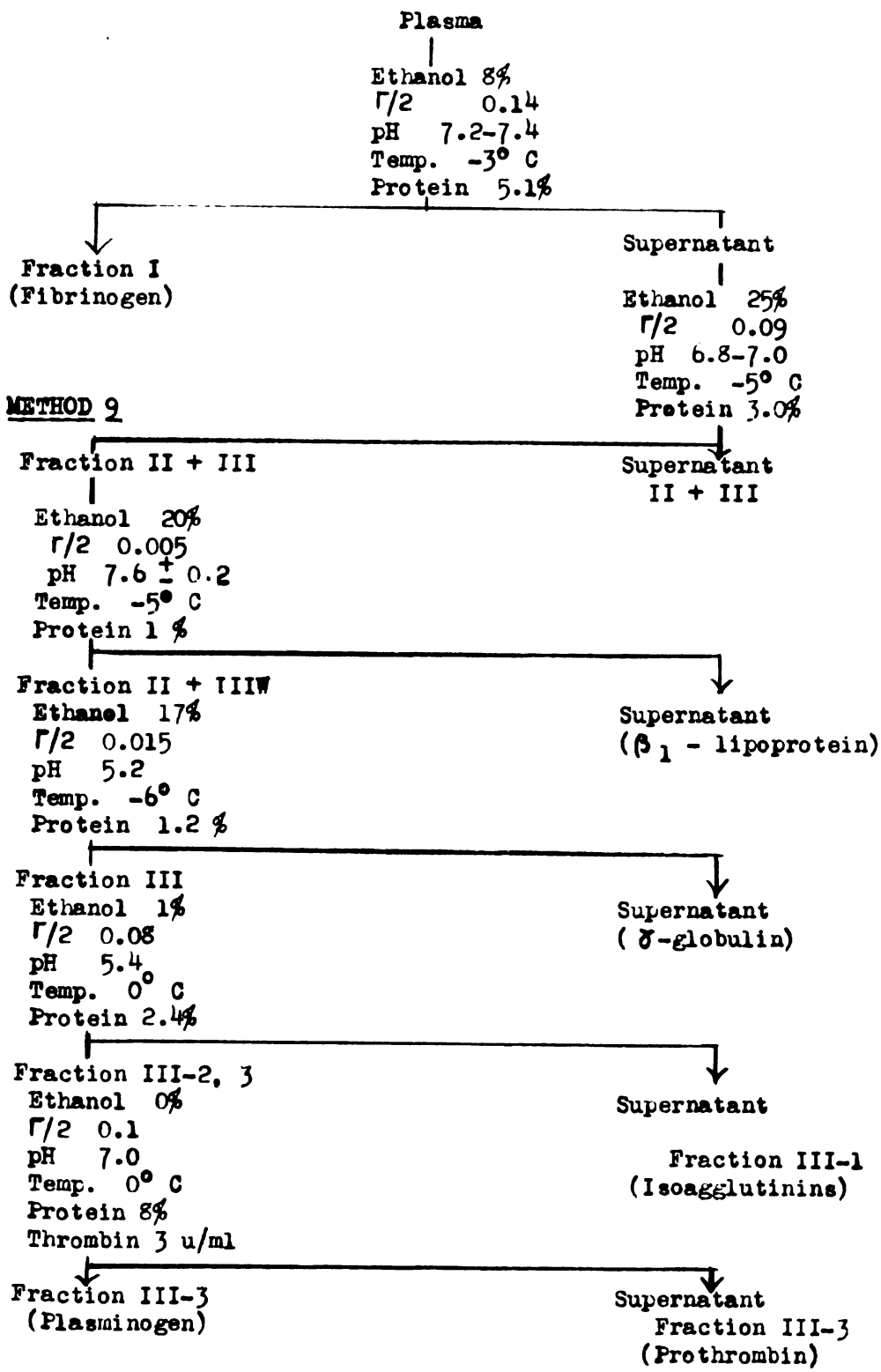


TABLE IV

Proteolytic Activity and Nitrogen in the Supernatant From Fraction III Suspension[a]

Sample	P.U./ml.	Mg.N/ml.	P.U./mg.N	% of Total Activity	% of Total N
1	0.5	1.74	0.3	3	31
2	0.5	1.66	0.3	5	58
3	0.4	1.64	0.3	5	57
4	0.4	1.66	0.2	5	60
5	0.3	1.61	0.2	3	46

a) Refers to supernatant of Fraction III-2,3 in Scheme 1. These values are determined on supernatants of samples listed in Table III.

3) To determine activity loss by varying the conditions in the last step of Method 9 the supernatants of this step were assayed and results are given in Table V.

TABLE V

Proteolytic Activity and Nitrogen in Supernatants of Fraction III-3 After Treatments Affecting the Clot Formation Step of Method 9[a]

Treatment	P.U./ml.	mg.N/ml.	P.U./mg.N	% of Total Activity	% of Total N
1000 u Thrombin	11.6	6.87	1.7	33	21
Heated 56°C 10 min.	12.8	6.53	2.0	35	48
Heated 56°C 10 min. + 16 u Thrombin	13.0	7.29	1.8	31	52

a) 0.1 M NaCl, pH adjusted to 6.8.

Other unsuccessful experiments were tried which involved phosphate buffers of pH 7.4, or citrate pH 6.4 and ionic strengths from 0.1 - 0.5 to test for improving clottability as in the last step of Method 9.

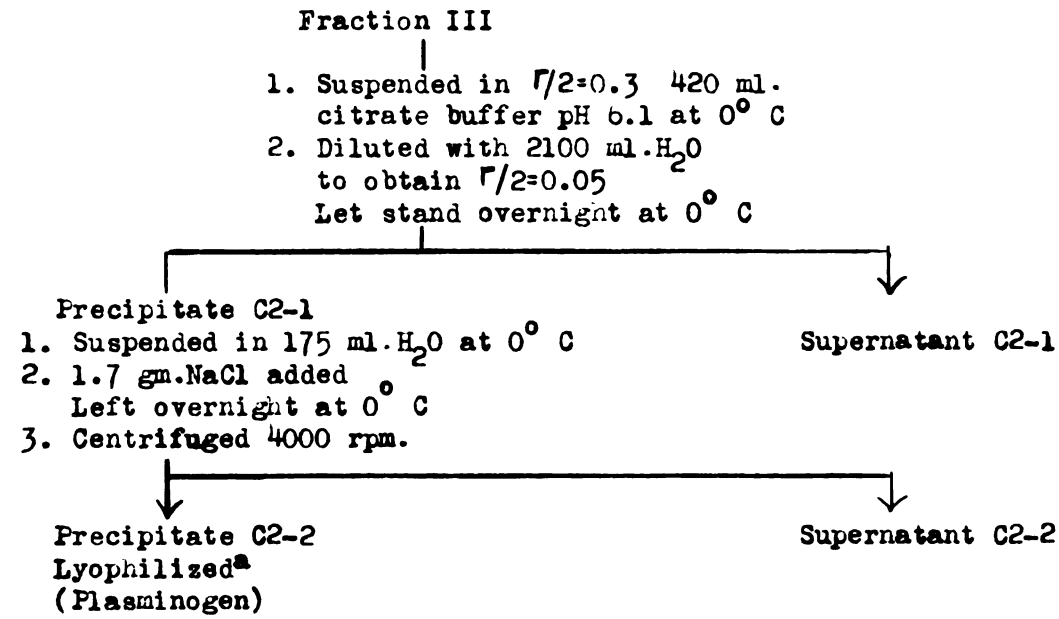
B. Modifications of Studies on Method 9.

1) As illustrated in Scheme 2, citrate (instead of acetate)[70] buffers of pH 6.1 to 6.4 and ionic strengths 0.05 - 0.3 were utilized

in making suspensions of Fraction III. Table VI, which includes various lots of Fraction III, shows the activity given by the starting materials when the citrate suspensions were made.

SCHEME 2*

A STUDY OF METHOD 9 USING CITRATE



a) This product was also frozen or used directly for Kline method[9] of purification, but the lyophilized product was superior.

*The system of naming supernatants and precipitates involves using the capitol letter to code the buffer, the number that follows identifies the scheme and the last number preceded by a dash indicates the step of the scheme.

Also an indication of activity and nitrogen that went into the supernatants of the citrate suspensions (Scheme 2) is given in Table VII.

2) The best conditions for precipitating plasminogen from resuspended precipitates (obtained by use of aforementioned citrate buffers) were investigated. First, various volumes of water were used for resuspending, then also various additions of sodium chloride with

TABLE VI

Proteolytic Activity and Nitrogen in Citrate Buffer Suspensions of Fraction III[a]

pH	Γ/2	P.U./ml.	mg.N/ml.	P.U./mg.N	RU.	Total N
6.16	0.05	4.2	1.36	3.1	11,367	3672
6.12	0.05	4.4	1.53	2.9	10,925	3825
6.12	0.05	4.5			11,610	
6.12	0.05	4.8	1.55	3.1	12,050	3875
6.1	0.05	5.1			12,979	
The above samples were from a preparation Fraction III labelled lot #1745 (Squibb)						
6.4	0.16	10.7	4.62	2.3	11,791	4620
6.4	0.16	10.7	4.78	2.2	10,777	4828
6.1	0.16	10.3	4.6	2.2	10,382	4646
6.1	0.16	10.4	4.97	2.1	9,644	4608
6.4	0.13	10.5	4.36	2.4	10,669	4447
6.1	0.1	9.0	3.67	2.4	12,110	4955
6.1	0.1	7.8	3.02	2.6	11,791	4589
6.4	0.08	6.5	2.37	2.7	13,219	4834
6.1	0.08	6.8	2.4	2.9	14,036	4925
The above samples were from the same preparation of Fraction III (Squibb)						
6.4	0.3	9.4	8.62	1.1	6,956	6370
6.16	0.3	5.5	4.16	1.3	6,475	4871
6.1	0.15	4.8	3.93	1.2	4,557	3715
6.1	0.15	9.5	8.1	1.2	4,615	3976
The above last four samples were from a preparation of Fraction III labelled lot # 1711 (Squibb)						

a) The procedure of Kekwick[70] was followed.

or without calcium chloride were made, and finally the pH was varied from 6.1 to 6.9. The optimal time for precipitation was also noted. The results are summarized in Tables VIII and IX. The procedure found for the best yield of plasminogen is shown in Scheme 2.

TABLE VII

Proteolytic Activity and Nitrogen in the Supernatants from Fraction III Suspensions Using Citrate Buffers[a]

Γ/2	P.U./ml.	mg.N/ml.	P.U./mg.N	% of Total Activity	% of Total N	Time of Precipitation
0.33	10.1	5.8	1.7	66	84	1 hour
0.33	6.4	5.37	1.2	30	56	overnight
0.05	1.4	1.12	1.3	31	63	1 hour
0.05	0.8	1.14	0.9	16	56	overnight
0.1	2.9	2.81	1.0	21	60	1 hour
0.05	0.9	1.0	0.9	22	52	1 hour
0.3	3.7	5.06	0.7	59	99	overnight
0.3	3.6	4.13	0.9	63	97	overnight
0.05	0.1	0.96	0.2	7	56	overnight
0.05	0.1	0.96	0.1	6	59	overnight

The above last four samples were from Fraction III labelled lot #1711 (Squibb).

a) Before dilution pH varied from 6.1-6.4. The first four suspensions had a pH of 6.4. The 5th and 6th samples had a pH of 6.1.

1.1	2.64	0.4	5	41
1.0	1.18	0.8	9	36
0.4	1.09	0.4	8	54
0.2	0.93	0.2	3	42
0.3	0.89	0.3	6	46
0.4	0.85	0.4	10	48
0.1	0.84	0.1	4	44
	0.86			58
--	0.91	--	--	58
0.1	0.88	0.1	2	57
0.1	0.87	0.1	3	60
	0.83			54
	0.79			48

a) The suspensions were made at room temperature and allowed to settle out at 0° C, except for lot #1745 (the last 6 samples) when suspensions were made and precipitated at 0° C.

TABLE VIII

Proteolytic Activity and Nitrogen in the Supernatants from the Second Precipitate of Scheme 2[a]

pH	H_2O ml.	NaCl gm.	$CaCl_2$ gm.	P.U./ml.	Mg.N/ml.	P.U./mg.N	% of Total Activity	% of Total N
	150	1.3	--	9.6	4.6	2.1	15	22
	200	1.7	--	9.2	4.28	2.1	18	26
	180	1.7	--	10.3	5.9	1.7	18	32
	136	1.27	--	2.9	5.31	0.5	11	24
	136	1.27	--	2.9	5.31	0.5	12	28
	146	1.22	0.11	0.9	4.17	0.2	1	18
	150	1.68	0.28	6.5	4.87	1.3	12	29
6.9	284	2.47	0.09	4.5	3.42	1.3	14	27
6.8	225	2.45	0.09	7.9	5.66	1.4	26	39
6.8	250	2.3	0.1	6.4	3.93	1.6	20	27
6.8	175	2.0	--	10.7	7.23	1.5	26	46
6.4	127	2.3	--	9.9	6.76	1.5	17	29
6.1	200	2.2	--	9.3	6.71	1.4	19	43

a) Optimal time for precipitation was overnight at 0° C. The first three samples were from lot #1745, the next two from lot #1711 and the last from a third lot of Fraction III.

TABLE IX

Proteolytic Activity per mg.Nitrogen in Supernatant C-2 of Scheme 2 as Influenced by pH, Salt and Precipitation Time[a]

pH	Time	P.U./mg.N
6.18	2 hours	0.9
6.4	2 hours	1.4
6.4	overnight	1.0
6.8	2 hours	1.7
6.8	overnight	1.2
6.8	overnight	1.0

a) 2.33 gm. NaCl per 100 gm. of Fraction III was added, suspensions were made with 250 ml. water, and 0.03% $CaCl_2$ was included in the last 3 samples. The last sample was also stirred up before centrifugation.

C. Other preliminary procedures for purification of plasminogen from Fraction III.

1) After the conditions, shown in Scheme 2 involving the citrate step, were worked out to produce partially purified plasminogen precipitate, this product (as shown in Scheme 3) was resuspended in either 0.05 or 0.1 M acetate buffer of pH 4.6. The residue was analyzed for proteolytic activity, nitrogen and cholesterol content and the results are given in Table X.

The suspension in acetate buffer was adjusted to pH 7.4 with 1 N sodium hydroxide and centrifuged. The new precipitate was collected and directly used for further purification according to the Kline[9] method (ppt. A3-3 of Scheme 3).

SCHEME 3

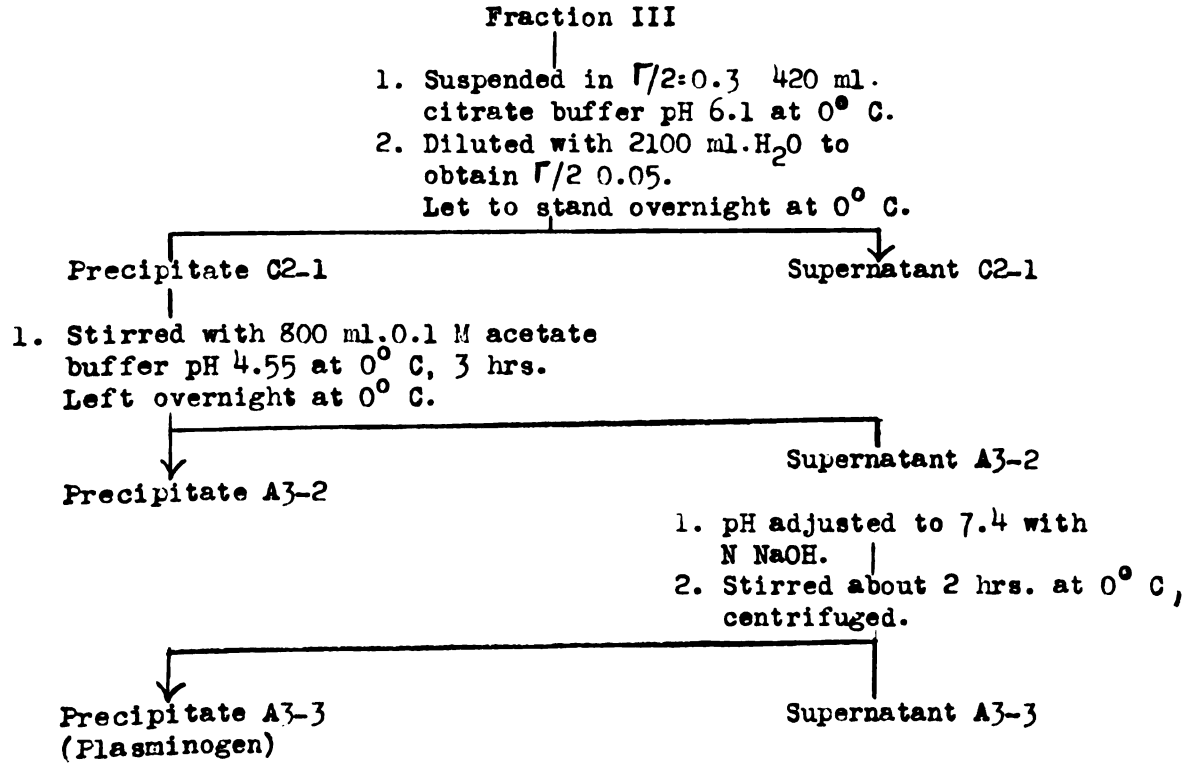


TABLE X

Proteolytic Activity and Nitrogen of Supernatants and Precipitates in Step 2 and 3 of Scheme 3

Product	P.U. ml.	mg.N ml.	P.U. mg.N	% of Total Activity	% of Total N	Total Cholesterol gm.
Ppt.A3-2	1.4	0.62	2.3	4	6	0.47
	1.3	0.36	3.6	3	2	
	3.8	0.624	6.1	12(pH 4.7)	6	
Super A3-2	1.9	0.75	2.5	31[a]	39	
	4.4	0.88	5.0	75	47	
	13.2	1.812	7.3	89	39	
Super A3-3[b]	--	0.388	--	--	20	
[c]	0.4	0.556	0.7	7	31	
[d]	0.6	1.087	0.5	4	25	
		0.432			19	
	0.2	0.490	0.5	4	27	
Ppt.A3-3	28.7	1.74	16.5	100	19	

a) The "Solution A" step of the Kline method yielded 49% of the total activity.

b) Precipitation time was 3 hours.

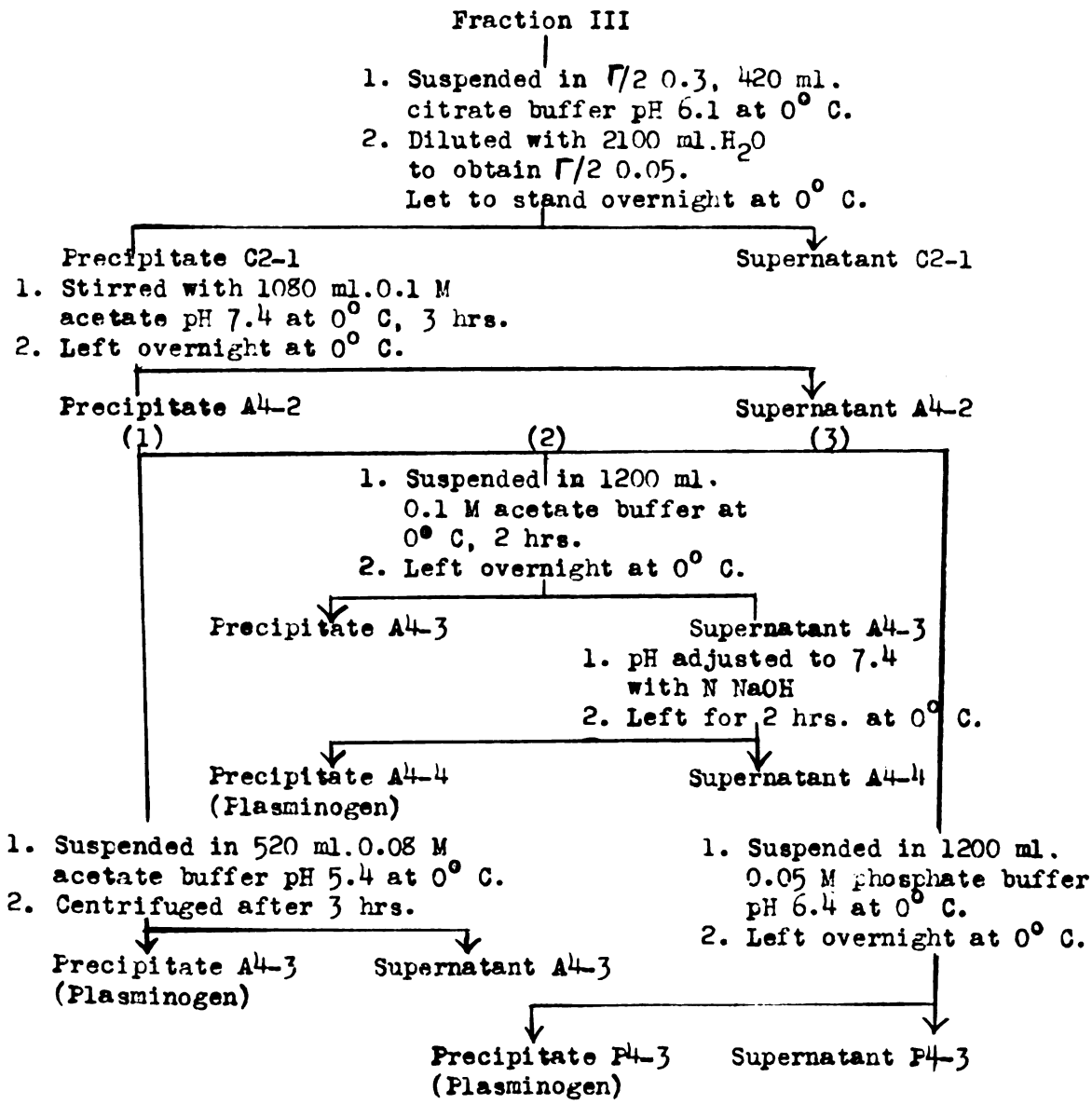
c) The sample was precipitated 1 1/2 hours and the ppt.A3-3 is from that sample.

d) The last three samples were from overnight standing.

2. Since it was noted, that the separation of precipitate depended upon exact pH of 4.6 with acetate buffer, an effort was made to avoid this step by suspending the citrate precipitate (C-1 in Scheme 2) directly in 0.1 M sodium acetate of pH 7.4. The best conditions for this step are shown in Scheme 4. The subsequent precipitate was subjected to three different treatments using acetate buffers of 4.6 - 7.4 and phosphate buffer at pH 6.4. Table XI contains all the data resulting from steps in Scheme 4.

3. More experiments were carried out using citrate buffer in the first step of Scheme 2. Experiments on the isolation of the isoagglutinins in 0.08 M acetate buffer pH 5.4 (Method 9) then followed.

SCHEME 4



The collected precipitate was resuspended in 0.1 M acetate pH 7.4 and the resulting residue applied directly to Kline acid extraction. Scheme 5 gives the best conditions for each step. Table XII presents the data obtained, mostly for losses of impurities.

TABLE XI

Proteolytic Activity and Nitrogen of Supernatants and Precipitates in Step 2,3 and 4 of Scheme 4[a]

Product	P.U./ml.	mg.N/ml.	P.U./mg.N	% of Total Activity	% of Total N
Super A4-2	0.3	0.663	0.4	2.7	22.7
		0.745			21.7
	0.5	0.83	0.6	4.7	24.5
	0.5	0.80	0.6	4.7	23.5
	--	0.26			13.1[b]
Super P4-3	0.3	0.176	1.6	2.8	5.6
		0.174			5.5
Super A4-3 (1)	1.9	0.406	4.7	8.6	5.8
Super A4-3 (2)	6.4	0.591	10.8	68.2	19.9
Super A4-4	0.5	0.31		5.2	11.2
	0.2	0.305		2.8	10.8

a) All the precipitates were collected after 24 hours, except the last one which was centrifuged after 2 hours.

b) $\Gamma/2 = 0.05$.

SCHEME 5

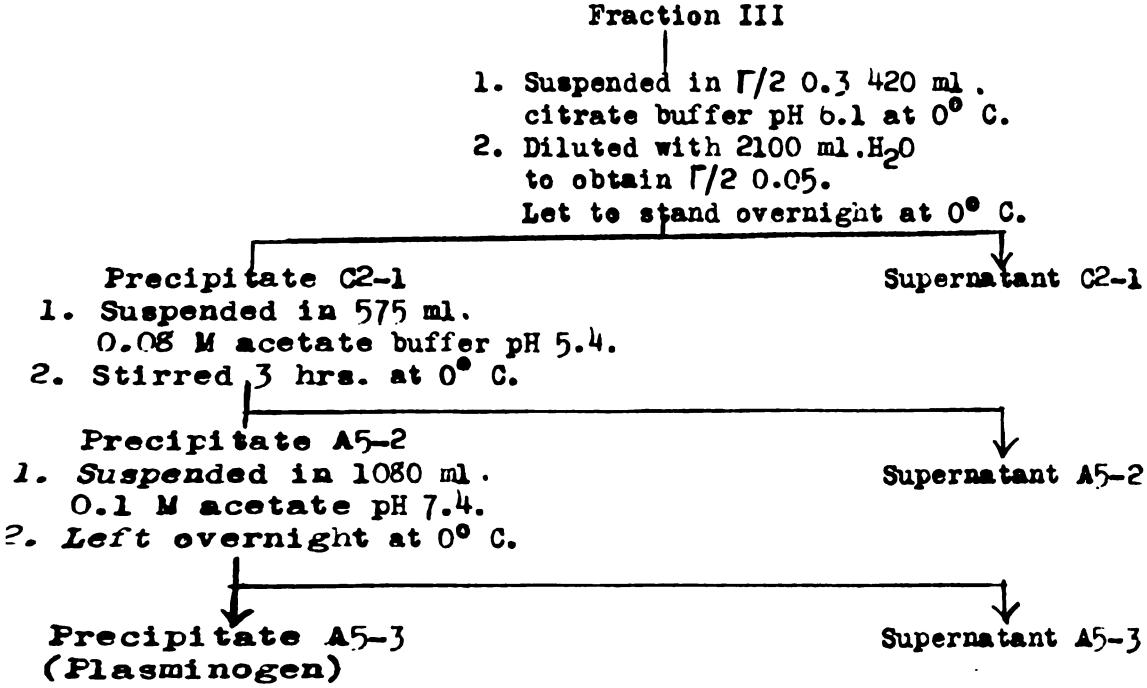


TABLE XII

Proteolytic Activity (of one sample) and Nitrogen of Supernatants in Step 2 and 3 of Scheme 5

Product	P.U./ml.	mg.N/ml.	P.U./mg.N	% of Total Activity	% of Total N
Super A5-2	0.1	0.67	0.1	0.3	10.4
		0.34			9.4
		0.327			9.1
		0.345			9.3
Super A5-3		0.585			14
		0.514			14.6

The last attempts to provide the best conditions for the prefractionation are given in Scheme 6. The citrate step in Scheme 3 was replaced by 0.05 M phosphate buffer of pH 6.4, with little modifications of steps 2 and 3. The experimental data are presented in Table XIII.

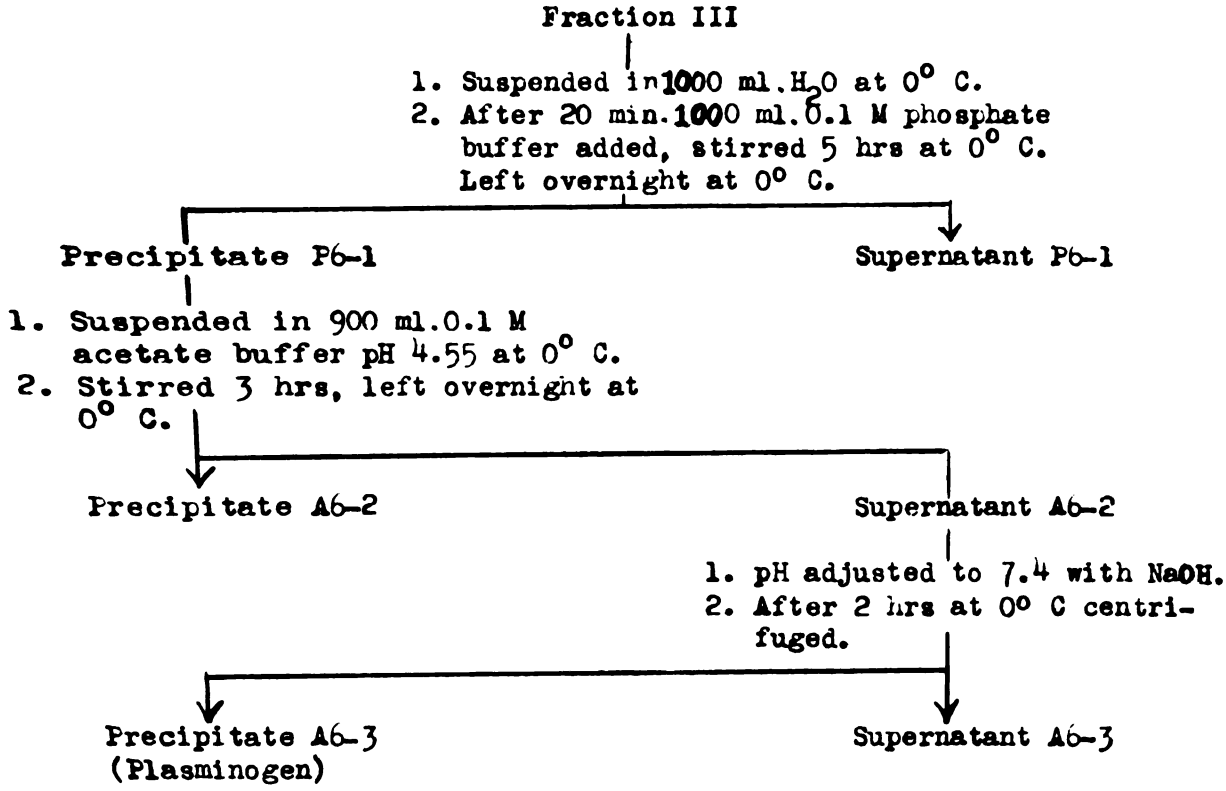
TABLE XIII

Proteolytic Activity and Nitrogen in Supernatants of Step 1 and 2 of Scheme 6

Product	P.U./ml.	mg.N/ml.	P.U./mg.N	% of Total Activity	% of Total N
Super P6-1	--	1.22	—		62
Super A6-2	6.9	0.852	8	52	20

D. Attempts to remove lipoidal material from the crude plasminogen preparation by extraction with glycine-phosphate buffer pH 6.8 or eliminate γ globulins (if present as impurities) with glycine-acetate buffer at pH 5.5 were unsuccessful[59]. Experiments involving the adsorption of proteins from Fraction III were made using Kaolin, aluminum hydroxide, barium sulfate and calcium-hydroxyapatite[64] and phosphate buffers of pH 6.4 - 7.4 for adsorption and elution. The result gave adsorption but no satisfactory elution.

SCHEME 6



E. Pastes obtained after various pretreatments and before Kline acid extraction[9] were used as follows:

1) Directly as such by suspension into 0.05 N sulfuric acid.

2) Frozen for at least 24 hours before thawing for 0.05 N sulfuric acid extraction.

3) Directly lyophilized and subsequently extracted by 0.05 N sulfuric acid.

Butanol-acetone (6 : 4) extraction at -15° C was carried out on the lyophilized paste obtained according to Scheme 2, e.i. Ppt.C2-2 (lot #1711). The extract was analyzed for proteolytic units, nitrogen, total and inorganic phosphorus and cholesterol. The results are shown in Table XIV.

TABLE XIV

Proteolytic Activity, Nitrogen, Total and Inorganic Phosphorus and cholesterol in Butanol-Acetone (6 : 4) Extract of Lyophilized Paste C2-2 of Scheme 2[a]

Proteolytic activity	--
Total Nitrogen	1.14 mg.
Total Phosphorus (lipid)	7.01 mg.
Inorganic Phosphorus	--
Cholesterol	130 mg.

a) Therefore: Lyophilized C2-2 has 4.04% Cholesterol and 0.22% lipid phosphorus.

F. Kline Method

1) The pretreated Fraction III as has just been described was further purified by Kline's[9] modification of Christensen's procedure[49] as shown in Scheme 7. Ten mimute extractions with 0.05 N sulfuric acid and mechanical stirring at room temperature, recommended by Kline, were not adequate. The yield and purity of plasminogen was increased by using the Potter-Elvehjem homogenizer to break the particles (especially necessary for lyophilized paste.) A second extraction, after precipitation of the proenzyme at pH 5.3, sometimes doubled the specific activity. The effect of the protein to acid ratio for extraction is given in Table XV. The yield and specific activity obtained by using prefractionated pastes of Fraction III were investigated and data are presented.

When the pastes were used:

1) directly, see Table XVI.

2) frozen pastes, Table XVII.

3) lyophilized pastes, Table XVII.

4) or butanol treated pastes, Table XV.

Each 0.05 N sulfuric acid suspension of a prementioned pretreated Fraction III was centrifuged and its supernatant adjusted to pH 11 with N sodium hydroxide for 3 minutes, then the pH was brought to 5.3 with N hydrochloric acid and the preparation placed in the refrigerator for a minimum of 3 hours. Then the pH of the suspension was readjusted to 2.0 with N hydrochloric acid and centrifuged for 1 hour. The plasminogen solution (Solution A) of Scheme 7 was adjusted to pH 8.6 with N sodium hydroxide and 1 ml. of 0.02 M phosphate buffer pH 6.0 was added for every 100 ml. of pH 8.6 solution. The suspension was left overnight at +4° C to settle. The next morning the plasminogen precipitate (Ppt. B of Scheme 7) was collected by centrifugation and dissolved in distilled water with the aid of a drop or two of N hydrochloric acid. Total activity and nitrogen loss remaining in supernatant B is given in Table XVIII.

SCHEME 7

The Kline Procedure for Preparation of Plasminogen[9]

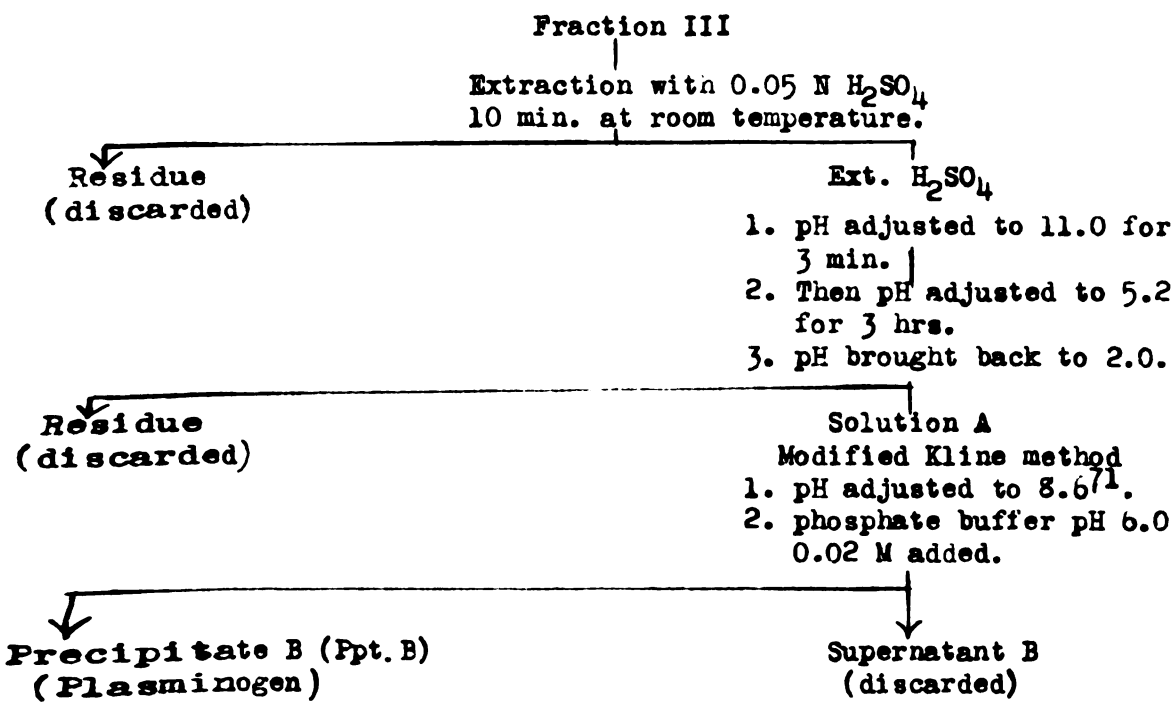


TABLE XV

Proteolytic Activity and Nitrogen of Lyophilized Paste C2-2 of Scheme 2 (lot #1711) after Treatment by Various Steps of Kline Method

Product	P.U./ml.	mg.N/ml.	P.U./mg.N	% Recovery of Activity
Sol.A	12.9	0.297	43	27
Ppt.B	12.4	0.395	31	7
Super B	4.5	0.154	29	10
3.23 gm.(100 gm.frozen paste) extracted with 100 ml.0.05 N H_2SO_4				
Extr. H_2SO_4	12.4	0.90	14	40
Sol.A	18.0	0.5	36	40
Ppt.B	10.5	0.212	49	23
Super B	5.5	0.23	24	13
Extr. H_2SO_4	16.3	0.64	25	36.6
Sol.A	14.3	0.462	31	33.2
Ppt.B	7.9	0.178	44	25.8
Super B	1.7	0.133	12	
Butanol treated lyophilized paste used for extraction				
Sol.A	14.8	0.450	33	31
Ppt.B	8.7	0.206	42	25
Super B	1.4	0.112	12	
Extr. H_2SO_4 was not adjusted to pH 11, but directly adjusted to pH 5.3. The last three samples were all extracted with 130 ml.of 0.05 N H_2SO_4.				
Sol.A	13.7	0.384	36	42
160 ml.0.05 N H_2SO_4 was used for extraction				
Sol.A	9.4	0.243	39	40
Ppt.B	13	0.25	52	36
Super B	1.4	0.114	13	6
225 ml.0.05 N H_2SO_4 was used for extraction				

TABLE XVI

Proteolytic Activity and Nitrogen of 0.05 N H_2SO_4 Extracts of the Last Precipitate of Scheme 3 Through 6[a]

Product		pH	P.U./ml.	mg.N/ml.	P.U./mg.N	% Recovery of Activity
Ext.H_2SO_4	A3-3	1.9	16.8	0.546	31	56
		1.9	12.8	0.516	23	52
	[b]		11.0	0.635	17	49
Sol.A	A3-3		15.0	0.24	63	41
			11.4	0.132	86	37
Ppt.B	A3-3		8.8	0.218	101	22
Ext.H_2SO_4	A5-3	1.9	12.9	1.11	12	70
Sol.A	A5-3		14.9	0.24	62	79
Ext.H_2SO_4	A4-2		14.5	1.191	12	80
		1.85	11.9	0.348	34	50
			9.5	0.387	25	62
Sol.A	A4-2		16.1	0.333	48	37
			7.5	0.087	86	25
Ext.H_2SO_4	A4-3(1)		10.4	0.711	15	56
Sol.A	A4-3(1)		6.8	0.108	63	31
Sol.A	P4-3		12.3	0.246	50	47
Ext.H_2SO_4	A6-3	2.0	12.2	0.46	26	42
Sol.A	A6-3		12.4	0.254	49	37
			10.5	0.11	96	54

[a] Difficultly separated precipitates were centrifuged 10,000 r.p.m.

[b] The sample was frozen before extraction, all the other samples were extracted directly.

TABLE XVII

Proteolytic Activity and Nitrogen of C2-2 Scheme 2 in Various Steps of Kline Method

Product	P.U./ml.	mg.N/ml.	P.U./mg.N	% Recovery of Activity
Ext. H_2SO_4	23.1	1.74	13	51
Sol. A	19.4	0.69	28	38
Ppt. B	1.9	0.042	45	16
Super B	6.4	0.357	18	13
Lyophilized paste was used for extraction.				
Ext. H_2SO_4	21.3	2.049	10	56
Sol. A	18.2	0.654	28	41
Ppt. B	5.2	0.128	41	32
Super B	2.5	0.234	11	9
300 ml. of 0.05 N H_2SO_4 was used for extraction of both of the pastes. pH was 1.78.				
Sol. A	14.4	0.45	32	46
Extraction with 400 ml. 0.05 N H_2SO_4 All the samples were from lot #1745.				
Ext. H_2SO_4	10.5	1.11	9	61
Sol. A	8.1	0.224	36	59
Ppt. B	3.7	0.061	60	34
Super B	2.0	0.125	16	15
275 ml. of 0.05 N H_2SO_4 used for extraction. The sample is from lot #1711. The last three samples were frozen pastes.				

TABLE XVIII

Proteolytic Activity and Nitrogen Remaining in Super B in the Last Step of the Kline Method[a]

pH of Super B	P.U./ml.	mg.N/ml.	P.U./mg.N	% Total Activity	% Total N
	3.2	0.109	29	11	1.1
7.5	2.4	0.11	22	8	1.2
7.4	2.1	0.106	20	7	1.1
7.2	1.9	0.102	17	6	1.1
7.2	2.3	0.096	24	8	1.0
6.98	1.2	0.081	15	4	0.9

a) Lot #1745.

Pretreated Fraction III according to Method 9 (in Scheme 1) was subjected directly to Kline's procedure. The purification results are shown in Table XIX.

TABLE XIX

Proteolytic Activity and Nitrogen of the Extract of Lyophilized Paste Fraction III-3 from Scheme 1 Method 9

Product	P.U./ml.	mg.N/ml.	P.U./mg.N	% Recovery of Activity
Ext.H_2SO_4	2.3	0.165	13.6	
Ext.H_2SO_4	14.8	1.446	10.3	10
Sol.A			22.2	9
Ppt.B			35.8	0.4
Ppt.B[a]			118.3	1.1

a) Ppt.B was obtained from the extract of Fraction III-3 precipitate which was formed applying at first heat, and after cooling Thrombin added.

To test the reproducibility of the steps in Scheme 2 plus the Kline method, three 100 gm.samples of Fraction III (lot #1745) were carried through removing in every step samples for proteolytic activity and nitrogen determination. The third sample was kept strictly at 0° C

throughout the experiment. The results are summarized in Table XX.

TABLE XX

Proteolytic Activity and Nitrogen in Precipitates and Supernatants of Fraction III Carried Through the Procedure of Scheme 2 and the Kline Purification Method

Product	P.U./ml.	mg.N/ml.	P.U./mg.N	Proteolytic Activity	Total Nitrogen
Fr. III #1745[a]	4.4	1.53	2.9	10,925	3825
	4.2	1.36	3.1	11,367	3672
	4.8	1.55	3.1	12,050	3875
Super C2-1	--	0.79	--	--	48%
	0.1	0.87	0.1	2.7%	60%
	--	0.91	--	--	58%
Super C2-2	9.6	4.7	2.0	16 %	22%
	9.6	4.6	2.1	15 %	22%
	10.3	5.9	1.7	18 %	32%
Extract H_2SO_4[a] from lyophilized pastes[b]	9.8	0.696	14.1	49 %	10%
	23.1	1.74	13.3	51 %	6%
	15.9	0.456	34.9	47 %	4%
Solution A	8.8	0.26	34.0	43 %	3.6%
	19.4	0.69	28.2	38 %	4.2%
	10.4	0.198	52.6	31 %	1.8%
Precipitate B	8.8	0.115	76.4	41 %	0.9%
	1.9	0.042	45.0	29 %	1.1%
	10.3	0.103	100.0	37 %	1 %
Super B	3.4	0.192	18.0	17 %	2.7%
	6.4	0.357	17.9	13 %	2.6%
	1.9	0.093	20.3	5.3%	1.7%

a) The first two samples in the first step were suspended at room temperature. All the samples were extracted with 0.05 N H_2SO_4 at room temperature.

b) Lyophilized pastes weighed 10 gm, 6 gm. and 2.5 gm. respectively. For extraction 600 ml, 300 ml. and 400 ml. of 0.05 N H_2SO_4 were used.

Attempts to show the applicability of the method for any starting material of Fraction III and the results with four different lots are shown in Table XXI.

TABLE XXI

Proteolytic Activity and Nitrogen from Four Different Starting Materials (Fraction III) According to Scheme 2 and the Kline Method (Scheme7)

Starting Material	Fr. III	Super C2-2	Super C2-2	Lyophilized Paste gm.	Extract H_2SO_4	Solution A	Ppt. B	% Recovery of Activity
II + III W reworked III	8635 PU. 2.2 PU./mgN	4% PU. 54% TN.	5.6% PU. 3.1% TN.	15.5	--	49% PU. 131 TN. 29.4 PU./mgN	70 PU./mgN	26%
II + III 112	6475 PU. 2.2 PU./mgN	6.1% PU. 48% TN.	5.7% PU. 2.8% TN.	13.0	--	75% PU. 138 TN. 35.3 PU./mgN	79 PU./mgN	36%
Squibb Fr. III Received 11-13-57	8250 PU. 2.2 PU./mgN	11.4% PU. 59% TN.	--	10.0	35.6% PU. 7.6 PU./mgN	24% PU. 128 TN. 15.3 PU./mgN	71 PU./mgN.	13%
Squibb #17145 Fr. III	10,925 PU. 2.9 PU/mgN	-- 48.3% TN.	16% PU. 22.4% TN.	10.0	49% PU. 14.1 PU/mgN	43% PU. 137 TN. 34 PU./mgN	76 PU./mgN	24%

P.U. = Proteolytic units.

T.N. = Total nitrogen in mg.

2) Modification of the Kline method[71].

a) Modified first step, buffer extractions compared with those of acid extracts.-- citrate buffer of pH 8.6, $\Gamma/2 = 0.3$ was used instead of 0.05 N sulfuric acid for 45 minute extraction of lyophilized C2-2 at room temperature and the data is given in Table XXII. The same lyophilized paste was extracted at room temperature with 260 ml.of 0.05 M acetate buffer of pH 4.6 for 45 minutes and centrifuged also at room temperature. Data showing effects of these buffers instead of 0.05 N sulfuric acid are given in Table XXII. When 500 ml.of 0.05 M acetate buffer pH 4.53 at 0° C was used for extraction of C2-2 (lot #1745) the results are given also in Table XXII.

b) Partition of the proteins in 0.05 N sulfuric acid extract with saturated ammonium sulfate solution and subsequent activity is shown in Table XXIII.

TABLE XXII

Proteolytic Activity and Nitrogen in Buffer Extracts of Lyophilized Paste C2-2 Scheme 2

Product	P.U./ml.	mg.N/ml.	P.U./mg.N	% Recovery of Activity	% Total N
190 ml. citrate buffer pH 8.6 $\Gamma/2 = 0.3$	23(1:5)[a]	1.67	13.8	80	7.3
260 ml. acetate buffer pH 4.6 M = 0.05	10.1	1.029	9.8	52.4	
Ext. H_2SO_4	23.9	1.21	19.7	44.7	
Sol. A	15.7	0.435	36.1	26.3	
Ppt. B	9.0	0.159	56.3	21.2	
Super B	3.8	0.141	27.1	6.2	
Both samples were from lot #1711					
500 ml. acetate buffer pH 4.53 M= 0.05	19.9(1:5)[a]	0.46	43	80	
Ext. H_2SO_4	13.3	0.371	36	41	
The sample in acetate buffer pH 4.53 was left overnight. 300 ml. of 0.05 N H_2SO_4 was used for extraction, pH 1.8.					

a) The proteolytic activity was determined in 1 : 5 dilution.

TABLE XXIII

Partition of Proteins with Saturated Ammonium Sulfate in Acid Extract of A4-2 Scheme 4

Starting Material	pH	PU/ml.	mg.N/ml.	PU./mg.N	$(NH_4)_2SO_4$ saturation	PU./mg.N	% Recovery of Activity	Time ppt.
Ext.H_2SO_4	1.9	14.5	1.191	12.21	0-.20 .20-.34	60	19	1 hr. overnight

3) Modified second step in Kline Method.

a) At pH 5.3 the precipitate after 3 hours standing was centrifuged, resuspended in 0.05 N sulfuric acid, and left at 0° C overnight. The next morning it was centrifuged in refrigerated centrifuge at 0° C to give the supernatant containing plasminogen called Solution A. The proteolytic activity and nitrogen loss in supernatant at pH 5.3 is given in Table XXIV. Specific activity of plasminogen in re-extracted 0.05 N sulfuric acid is presented in Table XXV.

TABLE XXIV

Proteolytic Activity and Nitrogen in Supernatant of Modified Kline Method at pH 5.3

PU/ml.	mg.N/ml.	PU./mgN	% Total Activity	% of Total N
0.5	0.072	6.3	1.4	0.7
0.4	0.073	5.0	1.2	0.6
0.8	0.085	9.9	2.7	0.9

b) When solution A of high specific activity (obtained by centrifuging the precipitate in second step at pH 5.3 in Kline method and redissolving in 0.05 N sulfuric acid), was adjusted to pH 8.6 and precipitated with 0.02 M phosphate buffer pH 6.0, a new precipitate resulted with no increase of proteolytic activity as seen from Table XXVI.

TABLE XXV

Proteolytic Activity and Nitrogen in Solution A of Modified Kline Method

PU/ml.	mg.N/ml.	PU/mg.N	% Total Activity	% of Total N
15.3	0.141	108.5	43	1.5
21.5	0.197	109.4	44	1.3

TABLE XXVI

Proteolytic Activity and Nitrogen in Precipitates B and Supernatants B in Modified Kline Method

Product	pH	PU/ml.	mg.N/ml.	PU/mg.N	% Total Activity	% Total N	Time Ppt.
Ppt. B	7.5	11.2	0.142	101	21	0.9	overnight
Ppt. B	7.2	8.2	0.078	105.5	40	1.2	1 1/2 hrs.
Super B	7.5	1.5	0.038	40.5	4.2	0.3	overnight
Super B	7.2	1.3	0.026	48.5	1.7	0.1	1 1/2 hrs.

Therefore ammonium sulfate fractionation was tried to increase the specific activity at the Solution A stage. The specific activities of starting solutions A and ammonium sulfate fractions derived therefrom are given in Table XXVII.

c) Experiments were made to further purify the precipitate B of plasminogen. Tables XXVIII and XXIX show the conditions of starting solutions subjected to various concentrations of ammonium sulfate to produce precipitate with resulting proteolytic activity of the products.

Precipitation of plasminogen (or formation of ppt. B) in the last step of the Kline method was investigated in presence of 1 M and 0.1 M sodium chloride. In both cases the specific activity was decreased without increasing the yield. Centrifugation at pH 9.0 before precipitation of plasminogen, as suggested by Kline, increased the activity at the expense of the yield.

TABLE XXVII

Specific Activities of Solutions A and of the Ammonium Sulfate Fractions of Them

Solution A	pH	PU/ml.	mg.N / ml.	PU. / mg.N	$(NH_4)_2SO_4$ Saturation	PU. / mg.N	% Recovery Activity	Time of ppt.
Scheme 2 (lot #1745)	2.0	21.5	0.197	109	0 -.20	47	6.9	45 min.
					.20-.34	129	89	overnight
Scheme 2				109	0 -.20	48	7.4	1 hour
		10.7	0.098		.20-.34	117	83	2 hours
Scheme 2	8.6			109	0 -.10	89	33.7	1 hour
					.10-.34	131	59	overnight
Scheme 2	2.0				0 -.25			1 hour
					.25-.34	145	59	overnight
					.34 super		2.6	
Scheme 2	2.0				0 -.20	32.4		1 hour
					.20-.28	119.8		1 hour
					.28-.34	154.0		overnight
(lot #1711)	7.0	8.1	0.224	36.2	0 -.25		59.7	overnight
					.25-.28			
					.28-.34	95.6	20	
					Super .34		8	
A3-3	1.71	11.4	0.132	86	0 -.20	21.8	8.2	overnight
					.20-.28	123.8	55	3 hours
					.20-.34	124.7	89	overnight
					.28-.34	133.0	38	overnight
Recovery of activity is given as per cent of Solution A								
A4-2[a]	2.0	7.5	0.087	86	0 -.20			2 hours
					.20-.34	127.9		
A4-3(1)		6.8	0.108	63	0 -.20			overnight
					.20-.34	115.3		overnight
A4-4					.20-.34	121.0	36	overnight
A6-3					0 -.20			1 1/2 hours
					.20-.34	136	34	overnight
A6-3	1.85				0 -.20			2 hours
					.20-.34	128		
A6-3		10.3	0.11	95.5	0 -.20			4 hours
	2.2				.20-.34	106.7	43	
A6-3		12.4	0.254	49.0	0 -.20			1 hour
					.20-.34	143.4	33	overnight

[a] From A4-2 on, the recovery is given as per cent of Fr. III. 4 M $(NH_4)_2SO_4$ was used throughout the experiment.

TABLE XXVIII

Specific Activities of Precipitates B and Ammonium Sulfate Fractions of Them

Starting Material	pH	pH of $(NH_4)_2SO_4$	PU. mg.N	$(NH_4)_2SO_4$ Saturation	PU. mg.N	% Recovery Activity	Time ppt.
Ppt.B(lot #1711)	8.6	7.0	49.3	Super .29	59.5	57	room t°
Ppt.B				Super .25	55.4	28	left at 0° C
Ppt.B	7.45	7.0		Super .20	56.0	33	
Ppt.B				Super .25	39.4	13	
Ppt.B	6.0	5.2		Super .20	63.1	49	
Ppt.B				Super .25	57.9	27	
Ppt.B	2.0	5.2		Super .20	59.8	60	
Ppt.B	7.0	7.0	45.6	Super .20	47.3	51	overnight
Ppt.B				.20-.28	85.0	33	
Ppt.B				Super .28	71.4	12	
Ppt.B			60.3	Super .20	53	47	
Ppt.B				.20-.28	75.1	25	
Ppt.B				Super .28		20	
Ppt.B			59.8	Super .20	67.3	46	
Ppt.B				.20-.28	81.4	24	
Ppt.B				Super .28	16.5	16	
Ppt.B			51.2	Super .20	76.4	73	
Ppt.B				.20-.28	85.4	29	
Ppt.B				Super .28		38	
Ppt.B	7.0	7.0	34.3	0 -.10		83	overnignt
Ppt.B				0 -.15		83	
Ppt.B				0 -.20		61	
Ppt.B				0 -.25		21	
Ppt.B				0 -.28		7.1	
Ppt.B				0 -.34		1.5	
Ppt.B	Taken to 2.5		76.0	0 -.20	36.4	21	3 hours
Ppt.B	Taken to 7.2			.20-.28	82.4	47	overnight
Ppt.B				.28-.34	30.6	2	
Ppt.B				Super .34		2.6	
Ppt.B 6 mo. in deep freeze	2.0 HCl used		73.7	0 -.20	64	30	1 hour
				.20-.28	104	53	overnight
				.28-.34	64.7	15	overnight
Ppt.B 6 mo. in deep freeze	2.0			0 -.20			1 hour
				.20-.34	88		5 hours
Ppt.B 6 mo. in deep freeze	2.0 H_2SO_4 used			0 -.20	68.2	PU. 3701	1 hour
				.20-.28	114.2	PU. 9901	1 1/2 hrs.
				.28-.34	101.2	PU. 1569	

TABLE XXIX

Proteolytic Activity in Various Ammonium Sulfate Fractions Precipitated from Acetate Buffer

Starting Material						$(NH_4)_2SO_4$ Saturation	FU. mg.N	% Recovery Activity	Time ppt.
	r/2	FU. ml.	mgN/ml.	pH	FU. mg.N				
Acetate buffer	0.05			4.53	92	0 -.05		gelatinous mass	2 hrs.
Ppt.B 1Yr in deep freeze						.05-.24	57.1	7.4	overnight
						.24-.34	105		
Acetate buffer	0.025			4.6		0 -.25	123	9.2	
						0 -.34	122	22.5	
						.25-.34	148	14.2	
						Super .34		1.7	
Acetate buffer	0.05	16.11	0.15	4.6	107	0 -.20	32.7	5.2	1 1/2 hrs.
						0 -.25	81.1	43	1 1/2 hrs.
						.20-.25	78.1	25	
						.20-.28	93.2	50	
						.20-.34	98.2	67	
						.25-.28	57.6	16	
						.25-.34	54.3	4.7	
						.28-.34	66.3	6.5	
						.28-.34	66.6	12.5	
						.25-.28	48	8.8	
						.25-.34	93.8	34	
						.28-.34	63.7	14.5	
Acetate buffer	0.05	17.16	0.256	4.55	67.0	0-.15	36	13	3 hrs.
						.15-.28	45.6	43	over weekend
						.28-.34	94	18	4 hrs.
						.34-.50	46.2	2.6	
Acetate buffer	0.05			8.6	125	pH 8.6 0 -.20	78.8	42	1 1/2 hrs.
						.20-.34	144	29	overnight

To concentrate the activity, 2.5 per cent TCA precipitation was tried but without success. Noteworthy is the observation that when dissolved frozen precipitate B was thawed slowly at +0.5° C, with 0.1 M acetate buffer pH 4.6 (equal amount added), and left overnight near 0° C, the centrifuged supernatant increased in specific activity as shown in Table XXX.

TABLE XXX

The Yield and Purity of Precipitate B Dissolved in Acetate Buffer pH 4.6

Starting Material			PU/mg.N	% Recovery[a] of Activity
PU/ml.	mg.N/ml.	PU/mg.N		
26.3	0.345	76	106	27
30.9	0.309	100	107	29
		56	68	

a) Per cent recovery of starting material Fraction III.

When a sample of frozen A3-3 (Scheme 3) was dissolved in 0.1 M acetate buffer pH 4.6, 16.5 P.U./mg.N was increased to 44.6 P.U./mg.N.

Sometimes P.U./mg.N were increased also by adjusting the slowly thawed dissolved precipitate B (78.8 P.U./mg.N) to pH 2.0; then the centrifuged supernatant showed 86.3 P.U./mg.N.

In an attempt to recover specific activity of redissolved precipitate B in 0.05 M acetate buffer of pH 4.6, experiments were carried out with the results shown in Table XXXI.

A solution of Precipitate B in 0.05 M acetate buffer of pH 4.6, having an activity of 125 P.U./mg.N, gave a precipitate with hydrochloric acid-acetone (1 : 5) with 60.8 P.U./mg.N, and representing a 49 per cent recovery of activity. With 2.5 per cent TCA the specific activity obtained was 65.9 P.U./mg.N and indicated a 53 per cent recovery of activity.

TABLE XXXI

Specific Activity and Recovery of Activity in Acetate Buffer Near the Neutrality

Starting Material				Product	pH	M	P.U./mg.N	% Recovery of Activity
pH	P.U./ml.	mg.N/ml.	P.U./mg.N					
4.6	23	0.183	125	Ppt.	7.4	0.05	67	50
			125	Ppt.	5.4	0.05	66	22
			125	Super	5.4	0.05	92	43
	17.2	0.162	106	Ppt.	7.4	0.2	100	65
				Super	7.4	0.2	55	18
				Ppt.	7.4	0.14	93	65
				Super	7.4	0.14	46	13
				Ppt.	7.4	0.05	79	62
				Ppt.	7.4	0.013	61	45
				Ppt.	7.4	0.005	66	50
		0.1	70	Ppt.	7.4	0.47	74	70
				Super		0.47	41	25
				Ppt.	7.4	0.37	95	75
				Super		0.37	42	25
				Ppt.	7.4	0.23	72	68
				Super		0.23	37	21
5.1	5.8	0.116	49.7	Super	5.4	0.006	51	20
5.4	0.6	0.012	51.0	Ppt.	7.4	0.006	94	100
4.6		0.256	77.3	Ppt.	5.4	0.05	59	44
				Super	5.4	0.05	78	47

G. Plasminogen activity on TAMe and LEe hydrolysis.

Results of investigation to separate TAMe (p-toluene-sulfonyl-L-arginine methyl ester) and LEe (lysine ethyl ester) activities by various treatments of plasminogen preparations are shown in Table XXXII. The activities were determined by titration in formaldehyde[16,17]. Spontaneous and S.K. activated TAMe esterase activity of plasminogen is shown in Figure 5.

H. The Fibrinolytic Activity of Plasminogen.

The experiment was carried out as previously described[52]. When plasminogen of 136 P.U./mg.N (3.7 P.U./ml) was mixed with 0.4 per cent protein (59 per cent clottable protein) no clot was formed. A control sample without enzyme but with the same amount of S.K. as in previous experiment, produced hydrolysis of the clot in 2 hours.

In the second run of samples all the other conditions were kept the same as above but in place of 5 ml.of 0.4 per cent protein, 1 ml. of higher clottability and higher fibrinogen content protein was used. With plasminogen of the above stated specific activity the clot lysed in 2 min. and 45 sec. Whereas the control lysed in 2 hours.

When a clot was formed in presence of cysteine, then there was very little hydrolysis even after 24 hours. Clot produced in the absence of S.K. and enzyme showed very little hydrolysis during 3 days.

J. Physico-Chemical properties of the Highly Active Plasminogen.

Ultracentrifuge sedimentation coefficients of the final plasminogen preparation are given in Table XXXIII; the untracentrifuge patterns are seen in Figure 6.

Electrophoresis patterns of plasminogen preparations with increasing proteolytic activity are presented in Figure 7.

TABLE XXXII

Plasmin Activity on TAMe and LEe

Preparation			P.U./mg.N	μM TAMe per mg.N hydrolyzed	Per cent spontaneous TAMe Activity	μM LEe per mg.N hydrolyzed
Acetate buffer pH 4.6	0.05 M		107	860	34	
Acetate buffer pH 4.6	0.05-.34 $(NH_4)_2SO_4$ in presence of protamine		102	605		396
Acetate buffer pH 4.6	pH taken to 5.5	super	95			129
Acetate buffer pH 4.6	pH taken to 5.5	ppt.	70			58
Acetate buffer pH 4.6	pH taken to 5.0	ppt.	73	520	--	
	7.4	ppt.	49	336	16	
Acetate buffer pH 4.6			125	540	25	258
Acetate buffer pH 4.6	0-.20 $(NH_4)_2SO_4$	pH 8.6	53	277	17	100
	.20-.34 $(NH_4)_2SO_4$	pH 8.6	119	465	20	206
	0-.34 $(NH_4)_2SO_4$		122	579	11	183
	.25-.34 $(NH_4)_2SO_4$		148	816	15	381
.10-.34 $(NH_4)_2SO_4$	precipitated at pH 7.4	0.01 M acetate	129	756		275
	precipitate dissolved in acetate pH 4.6		114			169

FIGURE 5

Spontaneous and S.K. Activated TAMe Esterase Activity of Plasminogen. 125 P.U./mg.N Plasminogen.

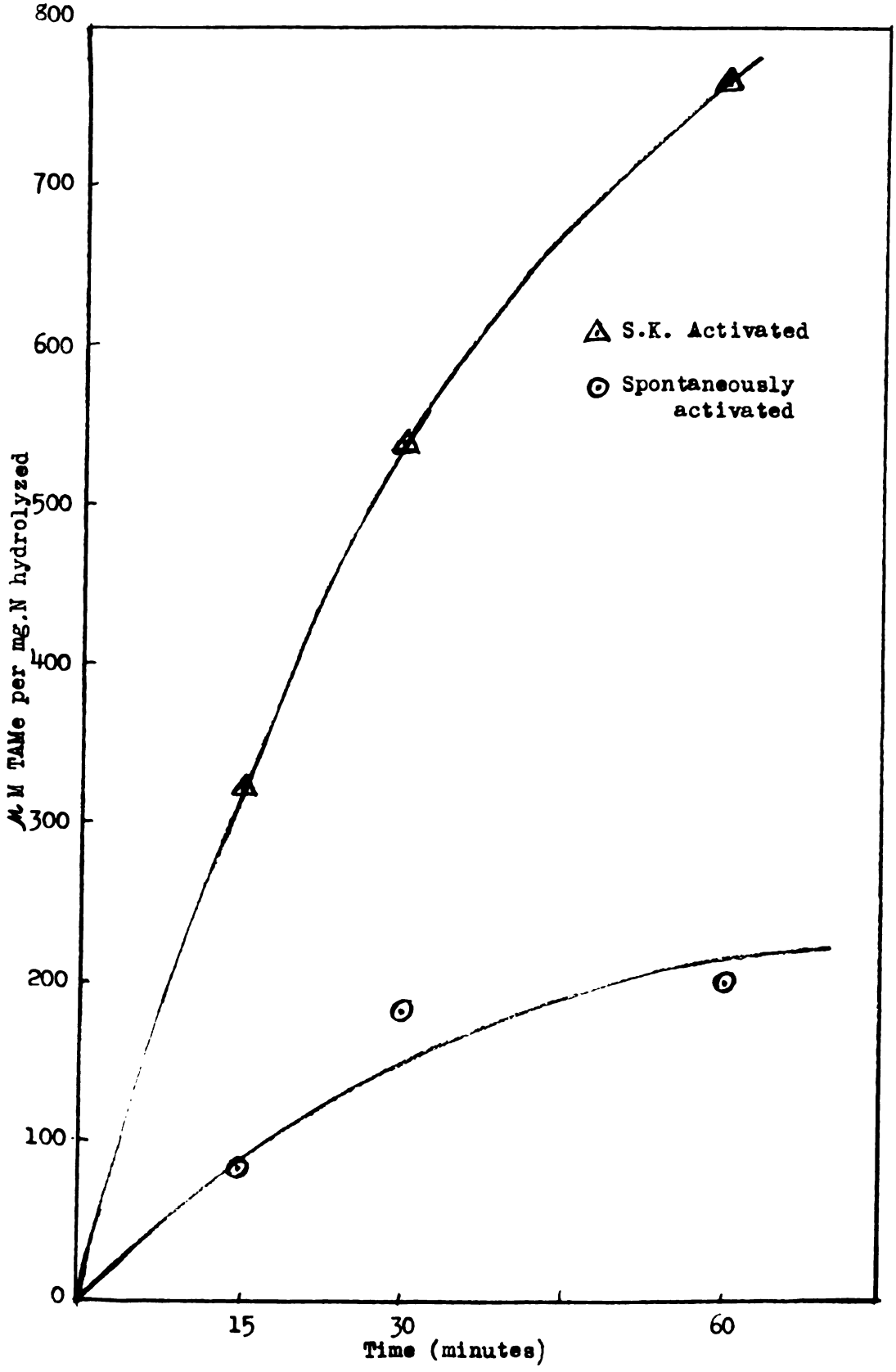


TABLE XXXIII

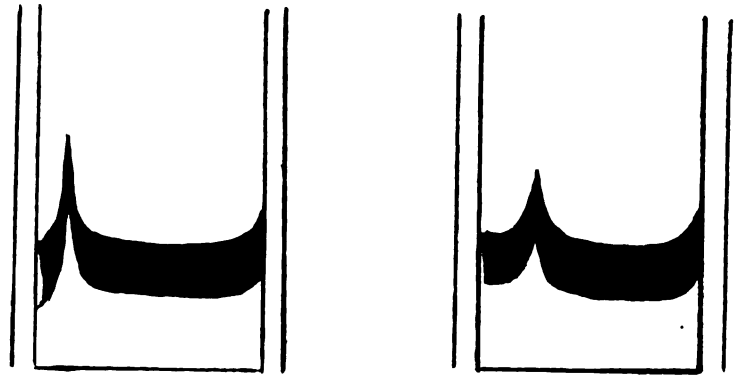
Approximate Sedimentation Coefficients of Plasminogen at 0.5° - 1.5° C in Glycine Buffer pH 2.1; $\Gamma/2 = 0.05$.

Concentration	Sedimentation Coefficient (Svedbergs)
0.9%	5.5
0.45%	5.9

Different plasminogen preparations and even different conditions of preparation and assay showed widely variable activity behavior in citrate buffers. In fact spontaneous activation, encountered occasionally, has generally led to avoidance of two subsequent treatments of the preparation with citrate buffers. During the course of reprecipitation in citrate, some of the plasminogen was activated to plasmin; or else, plasmin inhibitor was removed. The behavior of plasminogen in citrate buffers is demonstrated in the following experiments: (1) A dilution effect seemed apparent when Fraction III was suspended in citrate buffer of pH 6.1, ionic strength 0.05, since the total activity was 17 per cent higher as compared to after dilution two and one half times. (2) A spontaneous activation effect was apparent when Fraction III was suspended for 45 minutes at room temperature in pH 6.16 citrate buffer, ionic strength 0.3 (4.16 mg.N/ml) with the result that the preparation showed 3 per cent spontaneous (without S.K.) activity. (3) The same suspension with 0.1 ionic strength and half of the protein concentration became activated only 1.8 per cent in the first hour at room temperature and then increased to 2.7 per cent at the end of two hours. (4) Two subsequent citrate buffer treatments at pH 6.4 increased the spontaneous activity up to 5.6 per cent.

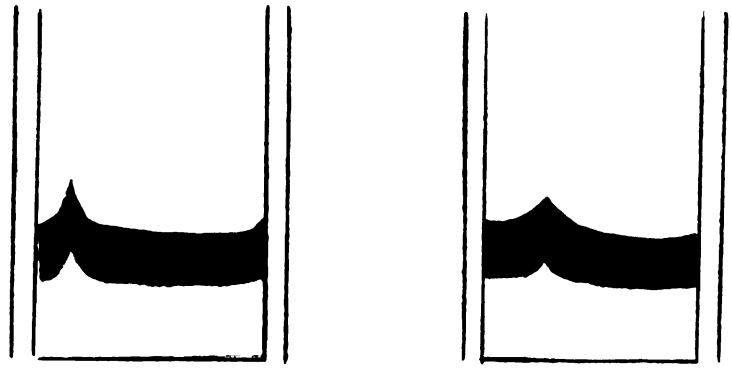
FIGURE 6

Ultracentrifuge Patterns of Plasminogen in Glycine Buffer pH 2.1;
$\Gamma/2 = 0.05$, at $0.5^{\circ} - 1.5^{\circ}$ C and 59,780 r.p.m.
Plasminogen 143 P.U./mg.N



32 minutes 64 minutes

Protein conc. 0.9 %

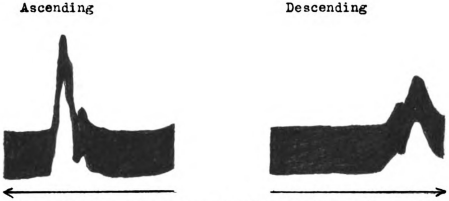


32 minutes 64 minutes

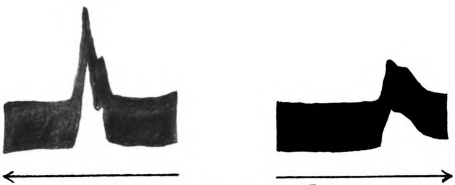
Protein conc. 0.45%

FIGURE 7

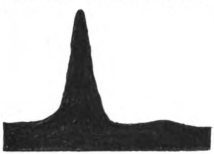
Electrophoretic Patterns of Plasminogen



4500 sec.; 115 volts, 15.8 milliamperes; 0.9 % protein in glycine buffer, pH 2.1; $\Gamma/2 = 0.05$ Plasminogen 143 P.U./mg.N.



1% protein in glycine buffer pH 2.1; $\Gamma/2 = 0.05$ Plasminogen approx. 85 P.U./mg.N.



Plasminogen from 0.28-0.34 $(NH_4)_2SO_4$ fraction in glycine buffer pH 2.1 $\Gamma/2 = 0.1$ 1% protein.

TABLE XXXIV

The Activation Behavior of Fraction III (lot #1711) in Citrate Buffer pH 6.4 $\Gamma/2 = 0.3$ during Various Intervals of Time at Room Temperature

Time of Standing	mg.N/ml.	% of Spontaneous Activity	% Recovery of Activity
1 hr.	8.62	3.5	100
3 hrs.		3.5	100
20 hrs.		8.5	100
4 days		20.3	67

After 4 days standing the zero time tyrosine estimated in TCA - soluble moiety of digestion mixture was increased by 10.5% tyrosine (with S.K.) and during spontaneous activation the increase was 21.3% tyrosine.

When precipitate B by the Kline procedure with 60 P.U./mg.N was suspended in 25 per cent sodium citrate (mg.N/ml. = 0.075) there was no change in proteolytic activity, no change in TCA precipitable nitrogen and no spontaneous activity in 20 hours. The semi-micro Kjeldahl nitrogen[65] was 15.7 per cent lower than the nitrogen according to Folin-Ciocalteu[62] determination and 19 per cent of "Folin-Ciocalteu" nitrogen was not precipitated by TCA.

When Precipitate B with 41.3 P.U./mg.N was suspended in citrate buffer pH 6.12, ionic strength 0.15 and pH lowered to 2.0 with 1 N hydrochloric acid, the suspension showed following activities:

Time of Standing	mg.N/ml.	% of spontaneous activity	% recovery of activity
0	0.03	19	100
30 min.		16	100
20 hrs.		—	116

Absorbancy (E_{280}) of casein digestion mixture at zero time also increased with time.

Spontaneous activation was also noticed occasionally with other buffers, especially when high specific activity preparations were investigated: 116 P.U./mg.N plasminogen in acetate buffer pH 4.6 gave 4.9 per cent activity without S.K. TCA precipitation of 26.5 P.U./mg.N plasminogen gave a precipitate with 56.4 P.U./mg.N plasminogen which indicated 8.7 per cent spontaneous change.

The effect of heating seems to depend also upon the previous history of the plasminogen preparation. Some results are shown from heating Fraction III in citrate buffer pH 6.4, ionic strength 0.3 at 54° C as follows:

Time Heated	P.U./ml.	mg.N/ml.	P.U./mg.N	% Recovery of Activity
14 min.	21.3	9.56	2.2	83
20 min.	17.6		1.9	62
The results at pH 7.02 with FrIII2,3 are:				
	27.5	5.85	4.7	
20 min.	19.4		3.2	70

A plasminogen preparation with 24 P.U./mg.N (mg.N/ml.= 0.37) when heated at 54° C 15 minutes in hydrochloric acid pH 4.3 retained its full activity. On the other hand, surprisingly, solution A with 63 P.U./mg.N placed into a boiling water bath for 20 minutes lost 70 per cent of its activity.

Plasminogen preparations for some unknown reason very often showed spontaneous activation on dialyses and loss of specific activity occurred as seen from the following data:

$(NH_4)_2SO_4$ Saturation	Starting P.U./mg.N	After Dialyses P.U./mg.N	% Spontaneous Activity	% Recovery of Activity
0 -.20	79	53	1.8	66
.20-.34[a]	144	119	4.4	59

a) There was also 25 per cent loss of nitrogen.

The total nitrogen content of impure samples of plasminogen was determined by the semi-micro Kjeldahl procedure[65]. Samples of lower nitrogen content were analyzed by Folin-Ciocalteu[62] or Biuret (M.D.H. lab.)[66], it was noticed that Folin-Ciocalteu method always gave higher nitrogen results. Interestingly enough, extracts of plasminogen in 0.05 N sulfuric acid showed by the Folin-Ciocalteu method an average of 9 per cent higher nitrogen than by Kjeldahl analysis and at the solution A stage about 24 per cent higher nitrogen values were noted. Another comparison of nitrogen results was made by precipitating the plasminogen from acetate buffer pH 4.6 at various pH's and analyzing for nitrogen by the two procedures. The results are as follows:

	% of Nitrogen higher by F. C.
Precipitate at pH 5.4	17
Supernatant pH 5.4	13
Precipitate at pH 6.1	24
Supernatant pH 6.1	18
Precipitate at pH 7.4	23
Supernatant (centrifuged after)	
1 hr.	9.1
2 hrs.	12.5
3 hrs.	18.4
overnight	36.3

J. Inhibition of Plasminogen Activation by Cysteine and other Substances:

Table XXXV presents the molarities of cysteine and calcium chloride and per cent activity obtained with S. K. in the presence or absence of cysteine or calcium.

TABLE XXXV

The Inhibitory Effect of Cysteine and Calcium Chloride

Molarity[a]	P.U./ml.	P.U./mg.N	% Activity Obtained
0.	10.4	131	100
0.0008	9.6		93
0.0017	8.1		78
0.0033	5.2		50
0.004	3.6		35
0	17.5	117	100
0.003	17.0		97
0.006	14.6		83.4
0.033	7.0		40
0		115	100
0.02			50.9
0.03			44.2

a) The first 5 molarities indicate cysteine, all the following ones are $CaCl_2$.

Other preliminary experiments included the finding of inhibition by thioglycolic acid, methionine, heparin, and sodium ascorbate.

The influence of urea on the activated enzyme (not the proenzyme activation process) was tried as Viswanatha et al[72] did on trypsin. There was no inhibition after 30 minutes in 8 M urea.

IV DISCUSSION

The aim of this work has been to present well founded experiments which add to the understanding of the characteristics of plasminogen or have important practical applications. What must be omitted are isolated experiments and speculations, the significance of which cannot at present be judged. Even these omissions cannot abolish the sense of confusion derived from what remains.

A. Some General Observations on Plasminogen Preparation:

Extensive investigations on the purification of plasminogen led Kline in 1953[9] to the crystallization of the proenzyme with 51 P.U./mg N. He concluded that the variation in purity obtained between various lots of Fraction III depended upon the amount of impurity which plasminogen coprecipitated in the final step.

Mounter and Shipley in 1958[40] also reported on the crystallization of plasminogen by the Kline procedure without giving any expression of activity of their crystalline proenzyme.

According to Alkjaersig, et al.[8] in 1958, it was stated as follows:

> The purity of the preparations of plasminogen that are purified according to Kline's modification is dependent in some yet poorly understood manner upon the characteristics of the Fraction III product from which it is prepared. With certain batches of Fraction III starting material, only relatively impure preparations of plasminogen could be obtained.

Many difficulties presented themselves in the purification experiments. Various factors and various experimental approaches were considered in order to determine the most satisfactory and economical method of obtaining relatively large amounts of purified plasminogen. Two large-scale experiments were performed and the results of these appeared

to be technically satisfactory. The recoveries of the activity and the specific activities of plasminogen in experiments employing from 2-4 kg Fraction III were even somewhat higher than those of the small-scale laboratory preparations described herein.

Purification procedures seem still more complicated when it is considered that the starting material (Fraction III) contains two proteolytic and esterolytic enzymes (plasminogen and prothrombin). In addition to the preceding there is one esterolytic (the midpiece, component C'1, of complement) enzyme described by Boyd[73].

Characteristics of the esterase derived from preparations of the first component of complement has been given by Ratnoff and Lepow[74]. Substrates for this esterase are N-acetyl-L-tyrosine ethyl ester and TAMe. Plasminogen purified by the Kline procedure was regarded by Troll and Sherry[16] to be free of this enzyme.

Fraction III, plasma euglobulins, contain all of the factors essential for clotting and fibrinolysis, but the greatest part of antifibrinolysin is eliminated during preparation. It has been observed, that the clotted fraction of human euglobulin undergoes spontaneous fibrinolysis during incubation.

A factor closely associated with fibrinogen conversion has been also reported by Loewy et al[75]. In the presence of calcium it converts a weakly urea-soluble clot into a tough urea-insoluble gel. The mechanism of the conversion is regarded as a disulfide exchange reaction and the factor is capable of crosslinking at least 20,000 times its own weight of soluble fibrin.

Fraction III used in this study as starting material contained plasminogen in a concentration of about ten to twenty times greater than

that of the serum (0.14 P.U./mg.N)[76].

1. Development of the Method for Plasminogen Production.

All the purification experiments are outlined by Schemes 1 - 8. Inconsistant behavior of plasminogen samples derived during fractionation procedures in Method 9 is indicated by the activity and nitrogen determination data (Table IV and V). The fibrin formation step appears to be dependent upon the previous history of the sample. Attempts to work out the conditions in this step of Method 9 to yield a good starting material for the Kline procedure were unsuccessful. Loss of activity in Fraction III-3 was too high to give any improvement in applicability to prefractionation.

The research work was eventually extended to include the adaptation of a variety of buffers at various hydrogen ion concentrations to obtain a good prefractionation method. It turned out that the most favorable conditions for plasminogen prefractionation were given by use of citrate buffer at pH 6.4, ionic strength 0.05. This step increased the specific activity of plasminogen about a hundred per cent.

The application of citrate buffer as shown in Scheme 2, followed by the modified Kline method, was investigated in detail. Table XXI shows very constant nitrogen content in Solution A as obtained from various lots of Fraction III by following Scheme 2. On the other hand, the proteolytic activities differ greatly. For the moment, it is not possible to explain this phenomenon. It shows namely, that even if the proenzyme is inactivated, (or denatured) its nitrogen goes into Solution A. It is emphasized again, that when Scheme 2 is followed by the modified Kline method, it has good applicability provided the temperature is kept at 0° C in the citrate step and the prefractionated paste is

lyophilized before Kline treatment.

During later purification experiments an investigation was started to find the conditions where the prefractionated paste from Fraction III could be directly applied to the Kline method. Schemes 3 - 6 summarize experiments described herein, the optimum conditions for prefractionation.

Schemes 3 - 5 represent the experiments where citrate precipitate C2-1 was treated with acetate buffers. Acetate buffers were used as extractant for plasminogen (at pH 4.6) in Scheme 3 or as extractant for impurities in Schemes 4 and 5.

A factor of critical importance for success of the Scheme 3 extraction of plasminogen at pH 4.6, is the maintenance of exact hydrogen ion concentration. It is recommended that pH 4.5 used on occasions where there is loss of activity at pH 4.6. This solubility behavior of plasminogen at pH 4.6 with acetate buffer (Ionic strengths 0.05 - 0.1 do not make difference) is not readily understood. That solubility could be so dependent on exact pH 4.6 in acetate is hard to comprehend. In view of the results found by analyzing the precipitate at pH 4.6 (cholesterol content approx. 25 per cent) the simplest explanation could be the following assumption: the dissociation of an inhibitor-plasminogen complex may come about at this particular hydrogen ion concentration, followed by separation of the inhibitor with the lipids.

This hypothesis will agree with the finding of plasminogen inhibitor in β-lipoprotein, (Fr. III-0) rich in cholesterol, by other workers[7]. This assumption is also in accord with the finding, that even though only little protein impurities are eliminated at pH 4.6, it has great influence on the final purity and stability of plasminogen.

The procedure in Scheme 5, steps 2 and 3 showed no advantage over

second step of Scheme 4. One can conclude that the impurities (after the citrate step) which are soluble at pH 5.4 in acetate buffer are also extracted by pH 7.4 acetate buffer at the same protein concentration.

Other modifications adopted included an improved prefractionation procedure by use of 0.05 M phosphate buffer of pH 6.4 in place of citrate. Laki[77] used the aforementioned buffer for extraction of fibrinogen. Since one of the main impurities of the starting material, namely Fraction III, is fibrinogen, it seemed likely that its use would aid in purification. The precipitate upon phosphate buffer treatment was subsequently extracted by 0.1 M acetate buffer of pH 4.5 - 4.6. Washing the above mentioned precipitate with 700 ml.of water before acetate extraction on some occasions increased the final yield from 33 per cent to 43 per cent. The specific activity of the final product from Scheme 6, when followed by the modified Kline procedure (see Scheme 8) varied from 120 P.U./mg.N to 143 P.U./mg.N.

The Kline procedure was also investigated step by step to improve the specific activity and yield of the final plasminogen preparation.

The optimum volume of 0.05 N sulfuric acid for the extraction of lyophilized prefractionated paste (calculated for 100 g.of Fr. III) was not adequate for the direct extraction of unlyophilized paste. Also, of decisive importance for the success of extraction is that a homogeneous suspension be produced in the acid.

It is noteworthy that a 0.1 M acetate pH 4.6 buffer extract of lyophilized paste of Scheme 2 had higher specific and total activity than a 0.05 N sulfuric acid extract by the Kline method. This difference may probably be attributable to instability at pH 2.0 as shown by caseinolytic activity.

Extraction of the lipoidal material from lyophilized paste of Scheme 2 with butanol and subsequent application of the Kline method, gave an 0.05 N acid extract with higher specific activity but low yield. However, the final specific activity in precipitate B dropped considerably.

It was noted also that when the Kline method was modified by centrifuging the precipitate at pH 5.3 the supernatant contained very little impurities, but subsequent 0.05 N sulfuric acid extraction of the precipitate increased its specific activity considerably.

When precipitate B with 100 P.U./mg.N is formed from Solution A, there results very little increase of specific activity and very likely a loss in total activity.

Ammonium sulfate fractionation of Solution A as developed in this study gave the plasminogen fraction of highest purity at 0.20 - 0.34 saturation with 105 - 145 P.U./mg.N activity and an adequate yield (33 - 43 per cent of starting activity of Fraction III).

It was found that ammonium sulfate fractionation is best carried out at the Solution A stage.

Ammonium sulfate fractionation of dissolved precipitate B produced a precipitate with its highest specific activity at 0.20 - 0.28 saturation, but with loss of total activity. When precipitate B was kept frozen over a long period of time, this loss was particularly noted.

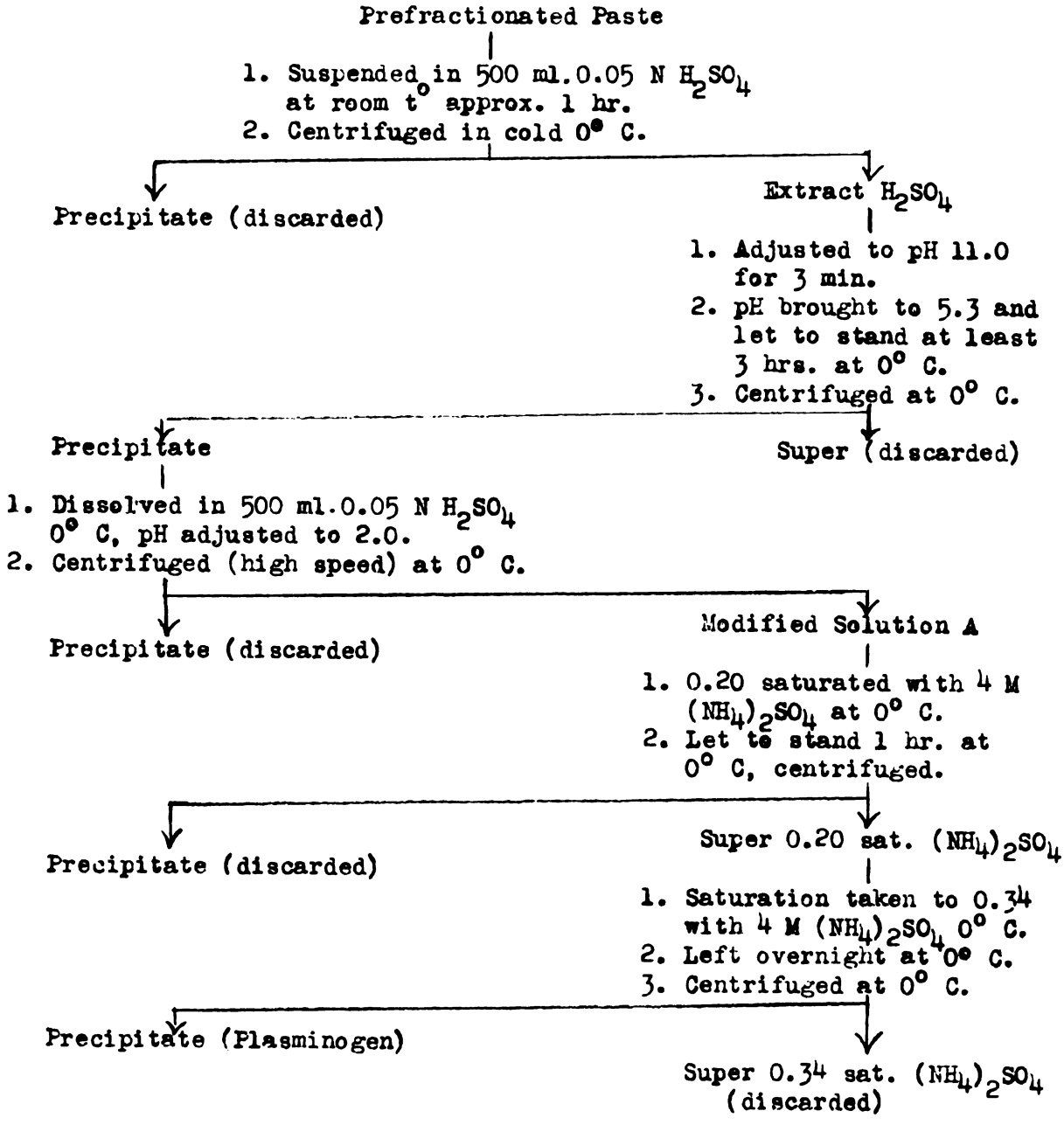
Thus procedure based on Scheme 6 followed by the modified Kline method (Scheme 8) was developed that permits a concentration of plasminogen of about 850 times greater than that of serum.

B. Problems Associated with Purification of Plasminogen.

Although an ultimate final purification of the enzyme was not attained, the following observations may assist a further development of the problem.

SCHEME 8

Modified Kline Method



Difficulties arose in the preparation of plasminogen owing to spontaneous activation to plasmin, and an unstable product in neutral or alkaline reaction resulted during the isolation procedure. The susceptability to spontaneous activation appeared even more acute with purification and when specific activity was increased (approx. 90 P.U./mg.N) or when citrate buffers were used. Plasminogen behavior in citrate buffers was so paradoxical that a further study of this phenomenon is desirable. For the time being, however, one can accept the explanation that plasminogen was activated in citrate buffers at room temperature because caseinolytic activity appeared without S.K. activation.

The drop-off of fibrinolytic power of stored blood at 4° C with 0.1 M sodium oxalate (1 : 10) to zero activity in seven days has been reported by a group of Italian workers[78] and this may be related to the above observations. It is also interesting that formation of active trypsin has been shown to be accelerated by citrate, oxalate and sulfate ions[79].

C. The Enzymatic Specificities of Plasmin and Thrombin.

The specificities of plasmin and thrombin seem to be narrower than that of the other proteolytic enzymes. Digestion of synthetic substrates (TAMe and LEe) have shown that they both hydrolyze peptide bonds in which the carbonyl group is contributed by arginine or lysine[16,17]. Ehrenpreis, et al[80], speculate that the primary action of thrombin on fibrinogen may be toward arginyl bonds and a slower secondary action of thrombin might take place at lysyl bonds. A similar basis could be proposed for the action of fibrinolysin from the finding reported by White[28] that bovine fibrinolysin splits the peptide bonds in corticotropin-A after arginine in position 8 and after lysine in position 15, but

fails to split this bond after lysine in position 21.

TAMe and LEe esterase activities, we found in this study, do not parallel each other. The plasmin product of this investigation showed high fibrinolytic activity on freshly formed (with bovine thrombin) human fibrin clots.

D. Changes in Total Acidity of Casein Digested by Plasmin.

Alcoholic-potassium hydroxide titration determines the total acid groups liberated during proteolysis. To minimize the error caused from carbon dioxide contamination, all the aliquots were titrated as quickly as possible.

From theoretical consideration, if during proteolysis there is only peptide bond cleavage, there should be practically no consumption of base in water medium when using the same indicator.

Titration data show that there was noticeable consumption of base in aqueous medium. Plasmin action was not limited to the hydrolysis of peptide bonds only, but in addition some other acid groups were also liberated. However, the action does not seem similar to the chymotrypsin digestion on casein.

E. Physico-chemical Data of the Plasminogen Preparation.

In Figure 6 are shown the sedimentation patterns of human plasminogen produced in this study. It shows only one peak. This preparation assayed 143 casein units per mg. of N. The sedimentation constants of the 0.9 per cent protein pattern is 5.5 S, and for 0.45 per cent protein 5.9 S.

Figure 7 represents the electrophoretic patterns of plasminogen. The first pattern shows the same preparation which was used for

ultracentrifuge. Of most interest, it was noticed that at the beginning of electrophoresis one of the components removed toward opposite direction indicating its opposite charge under the conditions used.

The second pattern shows plasminogen with approximately 85 P.U./mg.N. Average content estimated from ascending and descending patterns, indicates the main component to be about 62 per cent of the whole protein. The other three components are 25.8, 6.5 and 5.7 per cent.

The last pattern shows the ascending component of one preparation of plasminogen, which was fractionated twice with ammonium sulfate. The 0.28-0.34 saturated fraction with 154 P.U./mg.N was immediately dissolved in glycine buffer and analyzed.

The electrophoretic patterns are much more complex than anticipated and this suggests an investigation in greater detail of the effect of various buffers and various preparations of plasminogen. One might propose that the above discrepancies might be accounted for by the presence of various components present in starting preparation. It appears unlikely that preparation with approximately 85 P.U./mg.N will have as many components as a preparation with 143 P.U./mg.N. At this time, it is not possible to explain this observation. One might consider the unsuitability of amino acids as buffers at low hydrogen ion concentration for determination of the heterogeneity of proteins as reported by Woods[81]. One might also assume the possible activation of plasminogen during dialysis as introducing the new components.

F. Inhibition Studies.

As reported by Guest et al.[82] and confirmed in the present studies, cysteine strongly inhibits fibrinolysis by plasmin. In addition, it was also found, that cysteine inhibited caseinolytic and TAMe esterase activity.

On the other hand, the LEe was not inhibited, and on some occasions it was actually accelerated by cysteine. Furthermore, methionine too showed a weak inhibitory effect on caseinolysis, but cystine had no effect. The inhibitory effect of L-lysine was investigated by Müllertz[83] and recently Ablondi et al.[84] reported that epsilon-amino caproic acid inhibits fibrinolysis. The interpretation of these data might lead to *in vivo* inhibition of fibrinolysis. Thus more amino acids should be tested for their possible inhibitory effect on fibrinolysis.

Cysteine, reduced glutathione[83] and thioglycolate[28] might reduce the -S-S- bonds, perhaps required for enzyme activity. Another possible mode of action may involve the disulfide interchange reaction as reported by Green[85] to be the basis for thrombin inhibition by heparin.

SUMMARY

1. Studies on the purification of plasminogen from human Fraction III have been made by the development of prefractionation procedures for Fraction III, which is followed by modifications of the Kline method.

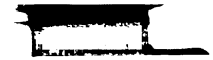
2. For prefractionation studies, citrate, acetate and phosphate buffers were used. Hydrogen ion concentration and ionic strengths were varied to obtain optimal conditions for the best yield and separation of the proenzyme. The best conditions were worked out in each case and summarized in schemes.

3. Removal of lipoidal material from the paste resulting from prefractionation, did not increase the specific activity of the final plasminogen precipitate B. However, lipoid removal enhanced extraction of activity into the 0.05 N acid of the Kline procedure.

4. The Kline method of isolating plasminogen was studied in detail. Ammonium sulfate fractionation technique was applied at each step after varying buffer anion and hydrogen ion concentration. The optimum conditions for fractionation and production of fractions with highest purity and activity from each step are reported.

5. A method for plasminogen preparation was developed which resulted in the highest proteolytic activity yet reported. This was accomplished by a combination of the best prefractionation Scheme worked out and the adaptation of a modified Kline method. The final product showed 120-143 P.U./mg.N of activity and gave 33-43 per cent yield.

6. Esterase activities of the high purity plasminogen on p-toluene sulfonyl L-arginine methylester (TAMe) and L-lysine ethylester (LEe) were determined after conversion to plasmin and compared with caseinolytic activity. They do not parallel each other.



7. Converted plasminogen action on human fibrin clots demonstrated that the final preparation of this investigation had high fibrinolytic activity.

8. Experiments on the action of activated plasminogen upon casein by electrophoretic pattern changes showed that the β-casein fraction is initially attacked. Alcoholic and aqueous titration changes during digestion indicated that acids other than those resulting from peptide bond cleavage may be liberated.

9. Spontaneous activation of plasminogen accounted occasionally for the loss of plasmin activity. The instability of plasmin was especially noticed upon the use of citrate buffers at room temperature.

10. Preliminary ultracentrifuge studies suggest that the plasminogen of this investigation to be a single component. Electrophoretic pattern differ and vary which is probably due to spontaneous activation during dialysis.

11. Inhibition studies showed cysteine to be inhibitory for fibrinolytic, caseinolytic and TAMe esterolytic activity. However, LEe esterase activity was not inhibited.

12. Some other substances tested for inhibitory effect were calcium chloride, thioglycolic acid, and methionine. These inhibited the activation process of plasminogen as determined by caseinolytic assays.

13. Attention was drawn to the cysteine inhibition investigations and further studies on this phenomenon are indicated. It is suggested that the possibilities of either reduction of the -S-S- bonds or interchange of disulfide be considered in connection with the inhibition process.

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