STUDIES OF THE PLASMIN SYSTEM

II. INHIBITION OF PLASMIN BY SERUM OR PLASMA*

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Plasma exhibits an ability to neutralize or destroy the characteristic proteolytic action of active plasmin. The importance of inhibitors has been realized in evaluating the function of the plasmin system; but the nature of blood inhibitors is poorly understood and has been the subject of differing observations.

A rather slow, temperature-dependent inhibition was described by Guest, Daly, Ware, and Seegers (1), as well as by Ungar and Damgaard (2). On the other hand, Shulman (3), Jacobsson (4), and Thomas and Dingle (5) have reported rapid and apparently stoichiometric reactions that did not particularly depend on temperature. Grob (6) found a rapid but reversible inhibition of plasmin by serum and, more recently, Shulman (7) isolated a low molecular weight inhibitor from plasma and urine which also reacted rapidly and reversibly, but which accounted for only a small portion of inhibitor activity of whole plasma. Meyers and Burdon found that the variations in proteolytic activity of dilutions of whole serum activated by streptokinase suggested a dissociable inhibitor (8). These apparently conflicting observations may be reconciled by the suggestion of Ratnoff that there are several plasmin inhibitors in blood with differing actions (9). Subsequently, Ratnoff, Lepow, and Pillemer have given evidence for at least three inhibitors in plasma. In their experiments, one plasma component was destroyed by heating at 56° for 30 minutes. The remaining heat-stable inhibitor they further subdivided on the basis that part of the inhibitory power was inactivated by ammonia and primary amines, leaving a component stable to both heat and these chemicals (10).

The present investigation restudies the mode of action of plasma inhibitors on plasmin, using the recently described caseinolytic assay (11). By several methods kinetic evidence was found for two inhibitory components for plasmin, one acting rapidly but reversibly, the other acting more slowly and at a

rate that depends on temperature.[1] The methods of study were more direct than those used by previous investigators, in that a partially purified human plasmin was prepared free of activators (12), so that a known amount of plasmin was added to plasma in order to follow the inactivation of plasmin under varying conditions. Part of this work was done before a purified active plasmin was available and a more complicated system was used, wherein streptokinase-activated plasminogen reacted with serum that had had its plasminogen removed. In a subsequent paper, the physical separation of the two proposed inhibitory components will be described and methods given for their individual estimation in whole plasma.

Materials and Methods

Plasminogen.—Human plasma fraction III was used as the starting material[2] in preparation of plasminogen by Kline's method (13). The plasminogen powder was dried with alcohol and ether according to Cliffton and Cannamela (14).

Streptokinase.—Varidase (lot #2200-10173), a lyophilized mixture of streptococcal enzymes consisting of streptokinase, streptodornase, other protein, and phosphate buffer was kindly furnished by the Lederle Laboratories Division, American Cyanamid Company. This material contained about 4000 Christensen units of streptokinase per mg.

Plasmin.—Preparation of active plasmin from plasminogen has been described in detail elsewhere (12). Briefly, 400 mg. human plasminogen was dissolved in 200 ml. borate-saline buffer, pH 7.4, and 3.0 mg. streptokinase added. After incubation for 30 minutes at 25°C., 200 ml. 2 M NaCl was added and the pH adjusted to 2.0 with N HCl. The resulting precipitate was separated by centrifugation, washed twice with 1 M NaCl at pH 2.0, and finally dried with alcohol and ether. The resulting white powder was stable at 5°C. for at least a year. It was highly soluble and stable in 0.0025 M HCl, but poorly soluble and unstable in neutral salt solutions. The plasmin preparations used in these experiments had specific activities of more than 80×10^{-3} caseinolytic units per mg. When solutions of plasmin in buffer were needed, a concentrated stock solution was made in 0.0025 M HCl and diluted to the desired strength with buffer at the beginning of the experiment.

Plasminogen-Free Serum.—Normal guinea pig or human serum was dialyzed against distilled water for 24 hours at 5°C. The dialyzed serum was diluted to twice original volume with distilled water, and the reaction adjusted to pH 5.2 by glass electrode with 5 per cent acetic acid. The resulting euglobulin precipitate was allowed to ripen 30 minutes at 5°C. and separated by centrifugation at 1500 R.P.M. for 15 minutes and discarded. The reaction of the clear supernatant was readjusted to pH 7.4 with N NaOH. Serum so prepared was discarded after 1 week's storage at 5°C. if not used.

Human Plasma.—Citrated whole human blood rejected for reasons of insufficient collection or positive serologic test for syphilis was obtained from the blood bank of The Johns Hopkins Hospital. Using aseptic technique, the plasma was drawn off from the settled red cells and any remaining cells and precipitated fibrin separated by centrifugation. Several units of plasma so prepared were pooled and dispensed into sterile 10 ml. screw top vials and stored at 5°C. At the time of use any new fibrin precipitate which had formed was removed by centrifugation.

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[1] Preliminary reports of parts of these studies appeared in *Fed. Proc.*, 1956, **15**, 606, and in *Clin. Research Proc.* 1957, **5**, 155.

[2] Human fraction III supplied by E. R. Squibb and Sons, Corporation, through the courtesy of Dr. J. N. Ashworth, American National Red Cross.

As an alternative method, fresh blood was collected by venipuncture, and each 10 ml. blood added to a centrifuge tube containing 0.3 ml. 5 per cent disodium versenate (disodium ethylene-diamine tetra-acetate) as an anticoagulant. Plasma was separated by centrifugation and used on the day of collection. Fresh versenated plasma showed slightly greater plasmin inhibitory capacity than stored citrated plasma, but the nature of the inhibition was not significantly different.

Assay of Plasmin.—The previously described caseinolytic method was used for determination of residual plasmin (11). The method originally described employed a single addition of 3.0 ml. 10 per cent trichloroacetic acid; it now has been modified slightly in that 0.5 ml. 15 per cent trichloroacetic acid is used to stop the reaction, and after about 10 minutes, an additional 2.5 ml. 15 per cent trichloroacetic acid is added. The precipitates are allowed to stand overnight at 5°C. to permit complete precipitation. The two stage precipitation is needed when whole plasma or serum is present, in order to obtain consistent values for reagent blanks.

Buffer.—Borate-saline buffer, pH 7.4, as described by Palitzsch (15) was used throughout. This was prepared by mixing 0.05 M sodium borate (19.108 gm. $Na_2B_4O_7 \cdot 10H_2O$ per liter of distilled water) with 0.2 M boric acid-salt solution (12.404 gm. H_3BO_3 and 2.925 gm. NaCl per liter of distilled water) until pH 7.4 was obtained by glass electrode. Approximately 1.2 parts borate solution to 8.8 parts boric acid-salt solution were required.

Methylamine.—C.P. methylamine hydrochloride was dissolved in borate-saline buffer and pH adjusted to 7.4 with N HCl or N NaOH.

EXPERIMENTAL

In a preliminary experiment, whole human plasma was warmed to 37°C. and 0.1 mg. streptokinase was added per ml. of plasma. The appearance and disappearance of proteolytic activity was followed by removal of 1.0 ml. aliquots for casein digestion. The appearance of protease was apparently instantaneous and was followed by a progressive loss of activity so that it was barely detectable after 5 minutes. Furthermore, the maximum activity from whole plasma was only about one-half that obtained from the separated euglobulin of the same plasma. Two important facts are obtained from this simple experiment. First, despite the rapid action of streptokinase, inhibitors prevent the appearance of the full potential protease in the plasma. Second, at body temperature, plasma is capable of inactivating all of its own content of plasmin after a brief period.

The following experiments were designed to explore the nature of rapid and effective plasmin inhibition by whole plasma. As it was difficult to determine accurately the plasmin content of whole serum, a procedure that gave better results was employed: a predetermined amount of plasmin was added to serum or plasma, and the decay of proteolytic activity was observed. In the experiments to be described next, this was done with purified plasminogen activated with streptokinase. The streptokinase in the mixture, however, caused activation of plasminogen in whole serum and plasma, so it was necessary to prepare serum free of plasminogen by removing the "globulin," in order to test the inhibitory mechanism without the simultaneous occurrence of activation reactions.

After equilibration in a constant temperature water bath, 1.6 ml. borate-saline buffer containing 0.16 mg. streptokinase was added to 8.0 ml. buffer containing 5.6 mg. purified plasminogen. Depending upon the temperature to be tested, activation was allowed to occur for either 5 minutes at 37°C. or 20 minutes at 25°C. Then, 8.0 ml. plasminogen-free guinea pig or hu-
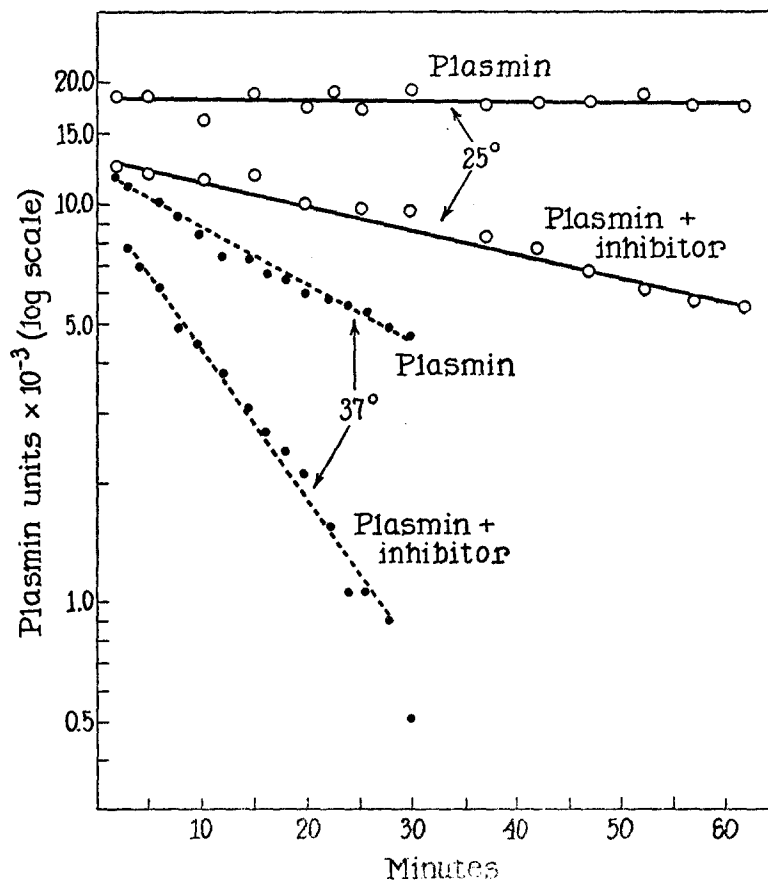

Fig. 1. Deterioration of purified human plasminogen activated with streptokinase and held at the indicated temperatures at pH 7.4. The upper line of each pair shows the enzyme in buffer alone, while the lower line shows the same amount of plasmin in the presence of euglobulin-free guinea pig serum.

man serum diluted 1/4 with buffer was added, and timing begun. Control reactions were done with the serum replaced by buffer. During incubation, 1.1 ml. aliquots were removed and added to 1.0 ml. previously warmed 4.0 per cent casein at 37°C. for determination of residual plasmin. Fig. 1 illustrates the results of two such experiments with guinea pig serum.

Plasmin had an inactivation rate of its own that was considerable at 37°C., but very small at 25°C. In either case, upon the addition of serum, there was

an immediate loss of activity which occurred too rapidly for rate measure-
ment, followed by a slower but progressive inhibition which proceeded over
the entire period measured. The second reaction appeared to be more rapid at
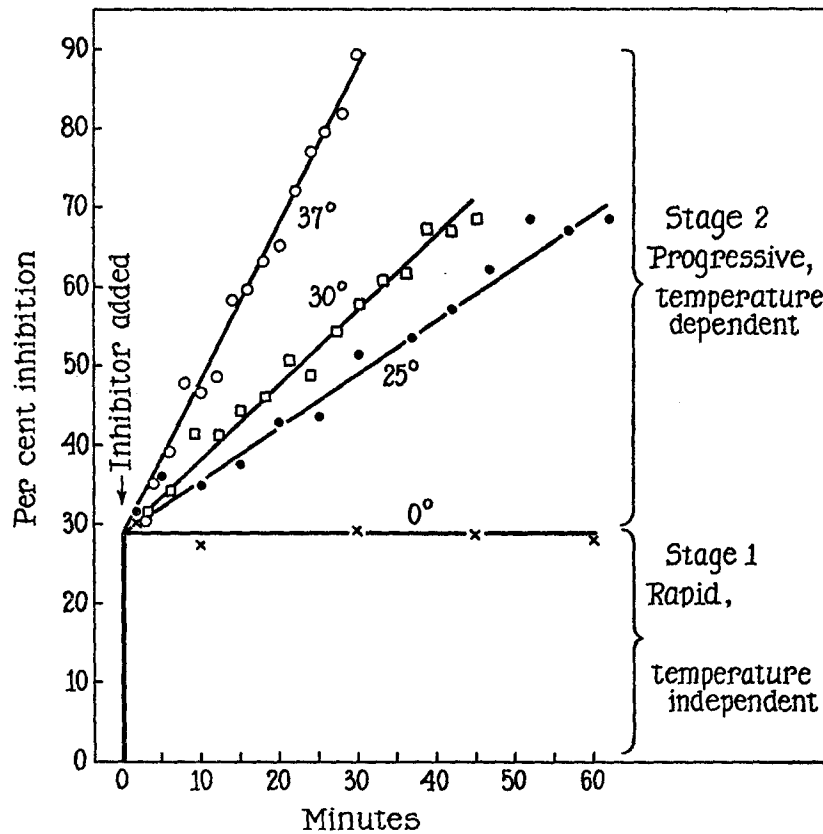37°C., and this fact suggested that it depended on temperature. The depend-

FIG. 2. Rate of inhibition of samples of purified human plasminogen activated with strep-
tokinase and held at different temperatures. At 0 time euglobulin-free guinea pig serum added.
Per cent inhibition calculated from the difference between the partially inhibited plasmin and
plasmin incubated at the same temperature in buffer alone.

ence of the reaction on temperature was further tested by doing similar experi-
ments at 0°C. and 30°C., with a result that demonstrated the suggested two
stage reaction more fully.

Fig. 2 illustrates the per cent plasmin inhibited with time at each tempera-
ture. Percentages were calculated using values of plasmin determined from
controls in which there was no serum. At all temperatures the immediate
inhibition was the same, but the slow reaction was highly temperature-de-

pendent, being nil at 0°C., and increasing rapidly with increase in temperature. The same experiments were also done with human serum and entirely commensurate results were obtained, with slightly less content of both inhibitors.

On the basis of these rather crude experiments, which were complicated by the concomitant occurrence of self-inactivation of plasmin, it seemed possible that there are two substances in guinea pig and human serum which have differing modes of action on plasmin. On the basis of their relative speed of action, these hypothetical substances are referred to in the remainder of the paper as the "immediate" inhibitor and the "slow" inhibitor.

Immediate Inhibitor

The following experiments examine the early reaction in more detail. For this work, a way of using unaltered serum or plasma was sought, as the removal of the globulin from the serum might change the remaining proteins. Active plasmin free of streptokinase was prepared by the method previously reported (12) and was found not to activate purified plasminogen during several hours at 25°C. This material could be added to plasma and the plasminogen in the plasma would not be activated, at least, not during the rather short time of observation. When plasmin without streptokinase was added to whole plasma, the twofold reaction just described was again seen. Plasma exhibited somewhat greater inhibition than serum, so plasma was used in preference to serum in the remaining experiments.

The evidence that there are two inhibitory reactions, one rapid and one slow, depends on the assumption that the second or slow reaction is stopped by the addition of substrate for the determination of residual plasmin. If the inhibitor continued to act on the enzyme during the course of the 30 minute digestion period necessary for assay, falsely high values for inhibition would be obtained, and the apparent immediate inhibition would be an artifact that results from the assay method. It seemed possible to assess such an effect by testing whether plasmin held partially inactivated by the presence of inhibitor would behave differently from plasmin used alone. Digestion of 2 per cent casein by plasmin is linear up to an optical density of 0.350 (11). If plasmin in the presence of inhibitor also digests casein in a linear fashion, it would be reasonable to conclude that no change in plasmin concentration occurs during the assay period. Experiments were undertaken to determine this point.

A mixture of 20 ml. 4.0 per cent casein, pH 7.4, 4.0 ml. human plasma, and 6.0 ml. borate-saline buffer was prepared and warmed to 37°C. Then, 10 ml. 0.0025 M HCl containing 6.0 mg. plasmin was added and timing begun. The chosen quantity of plasma is sufficient to inhibit this amount of plasmin partially, but not completely. A control was set up consisting of 20 ml. 4.0 per cent casein, 10 ml. buffer, and 10 ml. 0.0025 M HCl containing 4.0 mg. plasmin. During incubation 2.0 ml. aliquots were removed from each tube and precipitated with 3.0 ml. 15 per cent trichloroacetic acid added in 2 steps. Optical density of the clear supernatant was read at 280 mμ after centrifugation. Fig. 3 shows the increase in optical density obtained from the two digestion mixtures.

The course of digestion of casein by partially inhibited plasmin showed the same linear nature as that of plasmin without inhibitor. The initial lag in digestion has been observed by others (8) and is explained by Christensen as being due to plastein formation (16). This experiment and several others with the same result indicated that there was no further inactivation of plasmin during the assay procedure, and gave a sounder basis for the next experiments with early inhibitor.

FIG. 3. Digestion of casein by plasmin alone, and by plasmin partially inhibited with hu man plasma as measured by the incr ease in optical density of trichloroacetic acid filtrates

To a series of tubes containing 1.0 ml. 4.0 per cent casein, pH 7.4, was added various amounts, up to 0.5 ml., of whole fresh versenated human plasma. The volume was made up to 1.5 ml. with borate-saline buffer. The tubes were warmed to 37°C., and 0.5 ml. of plasmin in 0.0025 M HCl was added to each. Two series were made, one containing 15.3 × 10⁻³ units (0.175 mg.), and the other, 8.8 × 10⁻³ units (0.100 mg.) of plasmin per tube. After exactly 30 minutes of digestion, the reaction was stopped by adding 15 per cent trichloroacetic acid in the usual manner. Appropriate blanks were made by adding trichloracetic acid before plasmin. The residual uninhibited plasmin was determined from the optical densities of the clear super-natant fluids. The amount of plasmin inhibited was equal to the original plasmin (8.8 or 15.3 × 10⁻³ units) less the residual plasmin. The results are shown in Fig. 4.

The curves show decreasing increments of inhibition with each increment of plasma. Also, more inhibition occurred when more plasmin was presented for inhibition. These facts suggest a reversible reaction not going to comple-tion. The Kunitz formula (17) was used to calculate theoretical curves for a

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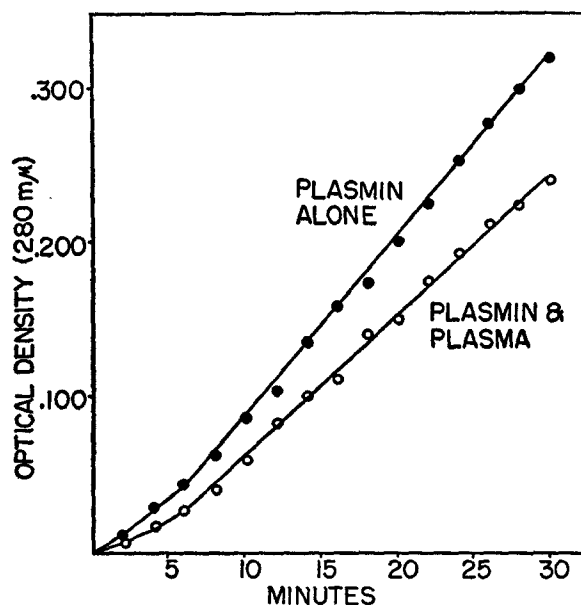

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dissociable combination under the above conditions, and only partial agree-
ment between the mass action curve and experimental values was obtained
which was not close enough to suggest a simple reversible reaction of the sort
observed by others with purified inhibitors (7, 17). It seems likely that the
reaction of whole plasma with plasmin is too complex for easy mathematical
analysis, but the reversibility of the combination may be tested in another
way. It may be shown that the inhibition becomes less with higher concentra-
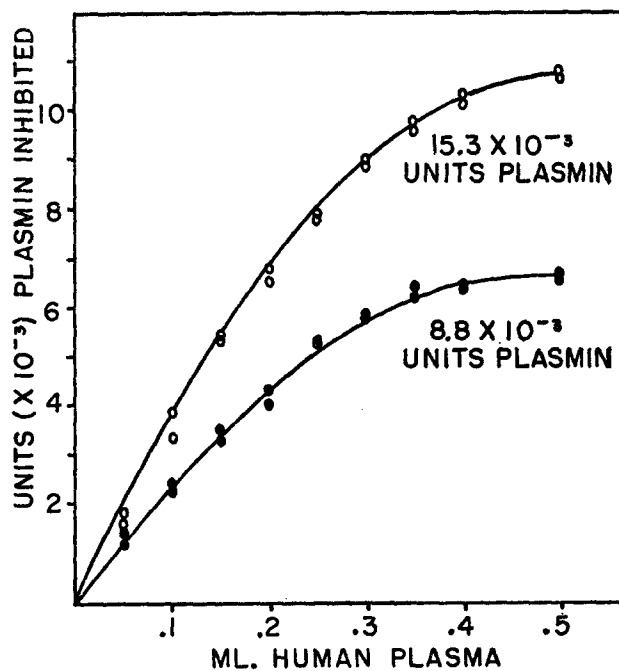
Fig. 4. Immediate inhibition of two concentrations of purified plasmin by varying amounts
of whole human plasma. Plasmin was added to plasma with casein already present.

tions of substrate, indicating that the substrate competes with inhibitor for
enzyme.

Standard plasmin curves were prepared for final concentrations of 1.0 and 3.0 per cent
casein by the method described in the previous paper (11). Another early inhibition experiment
was done comparing inhibition by plasma in the presence of 1.0 and 3.0 per cent casein.
16 × 10⁻³ units plasmin was used per tube.

The curves in Fig. 5 show that inhibition by the same amount of plasma
was less when more substrate was present. They suggest competition between
a plasma component and casein substrate for the active site on the enzyme,
and support the conclusion that the reaction is reversible.

Meyers and Burdon were probably studying the same reaction when they activated a series of dilutions of human serum with streptokinase in the presence of casein or hemoglobin, and then followed the course of proteolysis. They also arrived at the conclusion that there is a dissociable inhibitor in serum, because, with increasing serum concentrations, proteolytic activity did not increase proportionately (8).
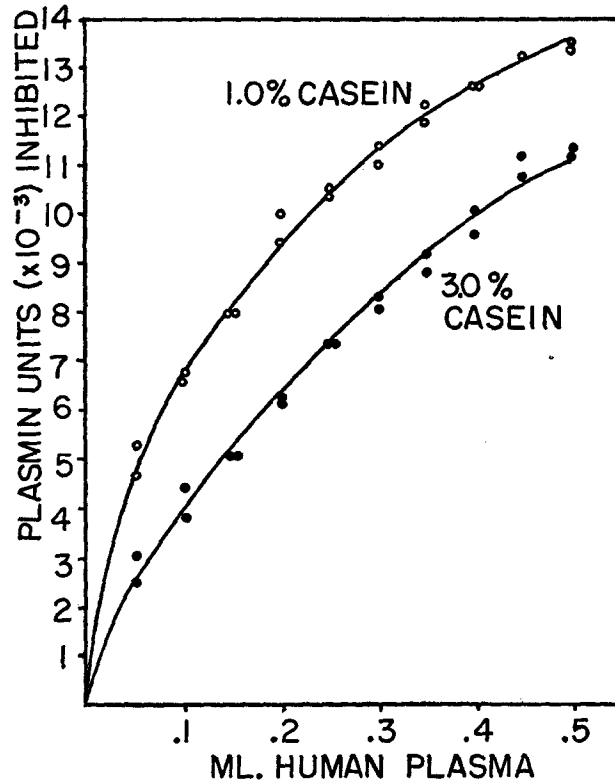

Fig. 5. Immediate inhibition of a single concentration of purified plasmin by whole human plasma in the presence of two different concentrations of casein.

Slow Inhibitor

Purified plasmin was next used in a study of the slow inhibitor of plasma. It has already been pointed out that plasmin is autodigestive at neutrality, and this process has been described in detail elsewhere (12). This represented a complicating side reaction which interfered with the observation of inhibition by plasma over a period of time. In the report on autoinactivation, it was shown that either methylamine or urea would check the autodigestion of plasmin by the ability of these substances to inhibit proteolysis. Subsequent

experiments demonstrated that plasmin incubated at 25°C. with a 2 M concentration of either compound was stable over many hours. It was also found that, although urea interfered with inhibition, 2 M methylamine allowed slow inhibition due to plasma to occur apparently unhindered. Thus, it was possible to follow the rate of slow inhibition at 25°C. while the *auto*-inhibition of plasmin was being suppressed by methylamine. Because methylamine inhibits the
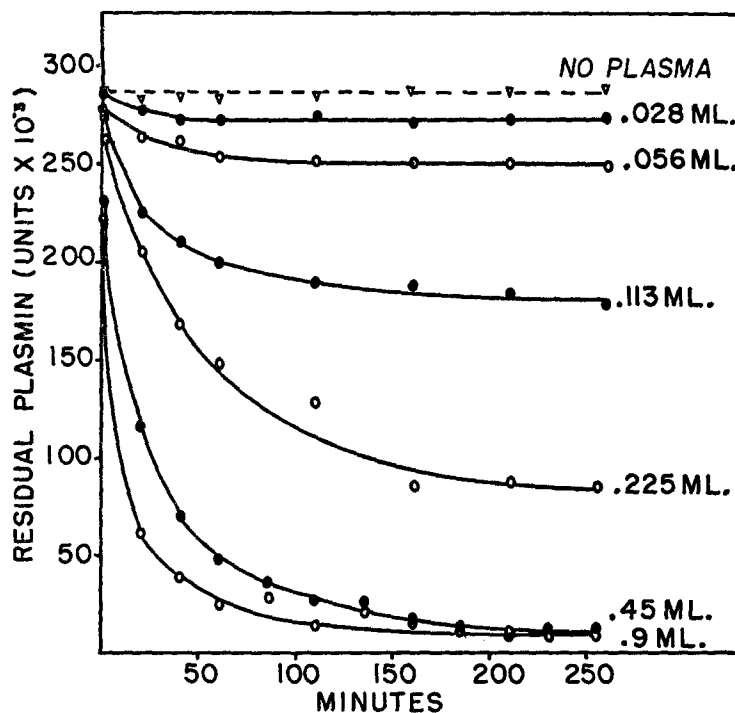

Fig. 6. Inhibition at 25°C. of 4.0 mg. plasmin stabilized with methylamine by varying quantities of whole human plasma, as indicated by measurements of residual plasmin in aliquots removed from the reaction mixture at various times.

proteolytic activity of plasmin, it was necessary to dilute it to 0.1 M for determinations of plasmin activity. This was done by allowing the reaction between plasma and a relatively concentrated solution of plasmin to proceed in 2 M methylamine. For purposes of measurement, aliquots of only 0.1 ml. were removed and diluted 20 times so that the concentration of methylamine would be 0.1 M during casein digestion. Low concentrations of methylamine were still slightly inhibitory to plasmin, but correction was made for this as described below.

Two sets of proteolytic determinations were done on a series of dilutions of a preparation of plasmin, one set with 0.1 M methylamine in the digestion mixture, and one set without. From

the determinations done without methylamine the plasmin was found to have 83.4×10^{-3} caseinolytic units per mg. This value was used to plot a second standard curve, with abscissae expressed in units, 1 mg. plasmin being equal to 83.4×10^{-3} units, while the ordinates were
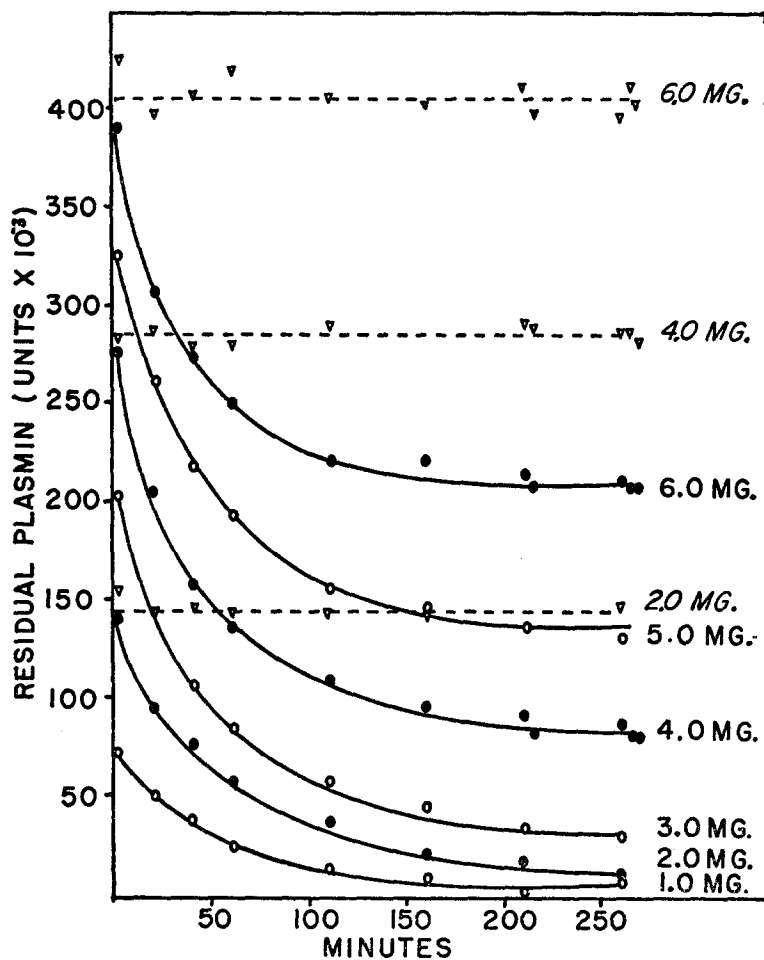

Fig. 7. Inhibition at 25°C. of varying quantities of plasmin stabilized with methylamine by 0.225 ml. whole human plasma, as indicated by assays of residual plasmin in aliquots removed at various times. Plasmin added indicated by the figures at right. Dotted lines are controls without plasma; solid lines are observations with plasma.

the optical densities obtained from digestion by the several amounts of plasmin in the presence of 0.1 M methylamine. When this curve was used, plasmin unknowns could be expressed in the same unit system used heretofore, although determinations were actually done in the presence of 0.1 M methylamine.

The rate of inactivation of plasmin by a series of dilutions of plasma was measured as follows: Dilutions of normal human citrated plasma in borate buffer were prepared in these pro-

portions: undiluted, 1/2, 1/4, 1/8, 1/16, 1/32, 1/64, and buffer alone. Tubes containing 0.9 ml. of the various dilutions were prepared and to each was added 0.9 ml. of 4.4 M methylamine. The tubes were warmed to 25°C. in a constant temperature water bath, and in carefully timed sequence 0.2 ml. 0.0025 M HCl containing 4.00 mg. plasmin was added to each.[3] This gave a final concentration of 2.0 M methylamine and 2.0 mg./ml. plasmin. At intervals, over a period of 260 minutes, 0.1 ml. aliquots were removed for determination of residual plasmin and added to a digestion mixture of 1.0 ml. 4.0 per cent casein and 0.9 ml. buffer already warmed to 37°C. After 30 minutes the caseinolytic reaction was stopped by trichloroacetic acid, as already described, and optical densities of the clear supernates were read at 280 mμ. The units of plasmin present was read from the calibration curve for 0.1 M methylamine described in the preceding paragraph.

In order to test the effect of *plasmin* concentration on the rate of inactivation, a similar experiment was done with a 1/4 dilution of plasma, and the added plasmin was varied between 1.0 and 6.0 mg. (final concentrations of plasmin, 0.5 to 3.0 mg./ml.).

The results of the two experiments are shown in Figs. 6 and 7. Residual plasmin is expressed as the units remaining in the 2.0 ml. mixture held at 25°C. and was calculated from protease determinations on the 0.1 ml. aliquots. Controls without plasma are indicated by dotted lines, while solid lines show inactivation in the presence of plasma. The amount of the varied component in the plasma-plasmin mixture is indicated by the figures at the right. The initial difference between the control and the experimental observation represents the effect of the "immediate" inhibitor. The difference between the first experimental observation on each curve and the remainder of the curve represents the progressive action of slow inhibitor.

It will be apparent that the rate of inactivation by slow inhibitor depended on the concentration of both plasma and of plasmin. When either substance was increased, the rate of inactivation as indicated by the initial slopes of the curves was increased. Furthermore, the reaction seemed to go almost to completion, that is, until one component or the other was used up. When there was an excess of plasma, the plasmin was virtually entirely inhibited; the points near the bottom of the graph represent the least amount of protease detectable by this method. On the other hand, when there was sufficient plasmin present, the inactivating capacity of plasma became exhausted in time and no further inhibition occurred.

Using those experiments in which there was an excess of plasmin, it was possible to calculate the inhibitory capacity of a given sample of plasma by the formula,

$$\frac{P_o - P}{S}$$

in which P_o equals plasmin activity at 0 time, P equals residual plasmin when inactivation is complete, and S equals ml. of plasma added. Table I shows

[3] Calibrated Lang-Levy micro pipettes (Microchemical Specialties Co., Berkeley, California) were used for delivery of volumes of 0.2 ml. or less.

calculations made from this formula with a single specimen of plasma.[4] Within experimental errors, the same value for the total inhibitory capacity of this plasma was obtained in several experiments in which a variety of concentrations of both plasma and plasmin were used.

If the reaction between two substances proceeds until one is entirely removed and at a rate which depends on the concentration of both reactants, and if the substances react in fixed ratio to each other as well, the circumstances suggest formation of a molecular combination which is only slightly dissociable and which follows second order kinetics. An attempt was made to fit the rate curves just described to the second order formula, but a different constant was obtained for each curve. Whether this difficulty is merely tech-

TABLE I

"Slow" Inhibitor Per Ml. of a Normal Human Citrated Plasma as Calculated from Several Experiments

Plasmin	Plasma	P_0	P	$P_0 - P$	Inhibitor per ml. plasma
mg.	*ml.*	*units* $\times$ 10^{-1}	*units* $\times$ 10^{-1}	*units* $\times$ 10^{-1}	*units* $\times$ 10^{-1}
6.0	0.225	390	206	184	819
5.0	0.225	324	132	192	855
4.0	0.225	276	80	196	872
3.0	0.225	204	30	174	774
4.0	0.028	288	266	22	786
4.0	0.056	282	240	42	750
4.0	0.113	272	176	96	849
4.0	0.225	260	84	176	784

nical or depends on interfering side reactions is not known. It is possible that homogeneous reaction rates might be obtained if a purified inhibitor were used. Nevertheless, the data thus far obtained suggest a firm intermolecular combination between enzyme and inhibitor which is formed at a measurable rate under appropriate conditions. It seems unlikely, therefore, that "slow" inhibitor is the peptidase described by Ungar and Adler (18) as correlating with the antiplasmin property of serum. The possibility still exists, however, that the reaction between plasmin and "slow" inhibitor is enzyme catalyzed.

DISCUSSION

It has not been noted previously that the plasmin-inhibiting capacity of the blood is so much greater than the potential plasmin. In a previous paper the plasminogen content of normal human serum or plasma was reported to

[4] The calculation of the inhibitor per ml. plasma did not regularly produce the same result when 0.028 or 0.056 ml. plasma was used, because any experimental error in the determination of either P_0 or P was so much magnified with small amounts of plasma.

be 25 to 30 × 10^{-3} caseinolytic units per ml. In the current study it can be seen that the antiplasmin content of normal plasma (at least that due to "slow" inhibitor) is around 800 × 10^{-3} units per ml. The term "slow" is, of course, a relative one. The dissociable inhibitor of blood acts too rapidly for rate measurement, whereas rate determinations are possible on the non-dissociable inhibitor at low temperatures in dilute solutions of plasma in the presence of a stabilizer for plasmin. Whereas the rate of combination under these conditions is indeed slow, under physiological conditions (37° and whole plasma), inactivation becomes quite rapid and plasma is capable of inhibiting its own content of plasmin within a few minutes.

It may be asked how plasmin can exert its characteristic proteolytic action in the body when so potent an inhibitor is always present. A possible answer to such a question is found in the experiments on the effect of a suitable substrate on inhibition. From these experiments, it is apparent that plasmin is protected and stabilized by a substrate, and this protection extends to the point of reversing the action of "immediate" inhibitor and halting the combination with "slow" inhibitor. According to present knowledge, fibrin is the most likely substance which may be acted on by plasmin *in vivo*, and in a recent publication, Celander and Guest showed that fibrin protects active plasmin from inhibitors the same as casein does in our experiments (19). Consequently, it seems reasonable to postulate that, once activated, plasmin at the site of a clot could continue its lytic action until the clot was digested and, only *after* lysis, would the non-dissociable inhibitor rapidly inactivate the enzyme. While lysis was going on, the dissociable inhibitor would be capable of preventing the digestion of proteins for which plasmin has a low affinity.

These attributes of the plasmin system which seem so ideal for the disposal of clots *in vivo* are suggestive. Up to this time, however, clot lysing activity has been demonstrated by *in vitro* observations only, and there is no direct experimental evidence that the system represents a mechanism that the body uses for removal of clots. Until such evidence is presented, the possibility remains that clot lysis is merely a laboratory phenomenon and that plasmin fulfils some other physiological function related to its ability to digest peptide or ester bonds.

In regard to the protective action of substrates, it can be said that, although fibrinogen is a substrate for plasmin, it was not possible to show that the fibrinogen in plasma was protective to plasmin, and inhibition proceeded as rapidly in plasma as in serum. Celander and Guest have also found that fibrinogen does not prevent inhibition of plasmin by serum (19). Apparently, this is no longer true once the fibrinogen is clotted.

The present study confirms the suggestion of Ratnoff that there is more than one plasmin inhibitor in blood (9). The methods of study used here give evidence for only two inhibitors rather than the three postulated by Ratnoff, Lepow, and Pillemer (10).

They found two classes of inhibitors on the basis of relative heat stability and, as reported in the next paper of this series, the "immediate" inhibitor is more stable to heating than the "slow" inhibitor. These workers also observed that more plasmin activity was found when any primary amine (including methylamine) was present in an inhibitory system, and they suggested that a third inhibitor is inactivated by these chemicals. Neither of the inhibitors described herein is destroyed by methylamine or hydrazine. The findings of Ratnoff *et al.* can better be explained by the known ability of primary amines to preserve plasmin from auto-inactivation rather than by destruction of yet another inhibitor in plasma.

SUMMARY

The inactivation of purified plasmin by serum or plasma was studied using a caseinolytic assay for determination of plasmin. On the basis of kinetic evidence, the presence of two inhibitors in normal human or guinea pig serum or plasma is suggested. The first reacts immediately at any temperature, but is easily dissociable and does not combine with plasmin in a fixed ratio. The second combines more slowly with plasmin at a rate which depends on temperature and on the concentration of both plasmin and inhibitor. There is a fixed ratio of reaction between plasmin and the second inhibitor and the combination does not readily dissociate. There is sufficient of the "slow" inhibitor in normal plasma to inactivate more than 30 times its own content of potential plasmin. The presence of suitable substrate for plasmin tends to reverse the combination with "immediate" inhibitor and to stop the further action of "slow" inhibitor.

BIBLIOGRAPHY

1. Guest, M. M., Daly, B. M., Ware, A. G., and Seegers, W. H., A study of the antifibrinolysin activity in the plasmas of various animal species, *J. Clin. Inv.*, 1948, **27**, 785.
2. Ungar, G., and Damgaard, E., Studies on the fibrinolysin-antifibrinolysin system in serum. I. Action of the anterior pituitary, adrenal cortex, and spleen, *J. Exp. Med.*, 1951, **93**, 89.
3. Shulman, N. R., Studies on the inhibition of proteolytic enzymes by serum. I. The mechanism of the inhibition of trypsin, plasmin, and chymotrypsin by serum using fibrin tagged with I[131] as a substrate, *J. Exp. Med.*, 1952, **95**, 571.
4. Jacobsson, K., Studies on the trypsin and plasmin inhibitors in human blood serum, *Scand. J. Clin. and Lab. Inv.*, 1955, **7**, suppl. 14, 55.
5. Thomas, D. P. P., and Dingle, J. T. M., Serum antiplasmin and plasmin in rheumatoid arthritis, *Ann. Rheumatic Dis.*, 1955, **14**, 195.
6. Grob, D., Proteolytic enzymes. III. Further studies on protein, polypeptide, and other inhibitors of serum proteinase, leucoproteinase, trypsin, and papain, *J. Gen. Physiol.*, 1949, **33**, 103.

7. Shulman, N. R., A proteolytic inhibitor with anticoagulant activity separated from human urine and plasma, *J. Biol. Chem.*, 1955, **213**, 655.

8. Meyers, W. M., and Burdon, K. L., Proteolytic activity of whole human serum activated by streptokinase, *Arch. Biochem. and Biophysics*, 1956, **62**, 6.

9. Ratnoff, O. D., Studies on a proteolytic enzyme in human plasma. III. Some factors controlling the rate of fibrinolysis, *J. Exp. Med.*, 1948, **88**, 401.

10. Ratnoff, O. D., Lepow, I. H., and Pillemer, L., The multiplicity of plasmin inhibitors in human serum, demonstrated by the effect of primary amino compounds, *Bull. Johns Hopkins Hosp.*, 1954, **94**, 169.

11. Norman, P. S., Studies of the plasmin system. I. Measurement of human and animal plasminogen. Measurement of an activator in human serum, *J. Exp. Med.*, 1957, **106**, 423.

12. Norman, P. S., Effect of urea and methylamine on plasmin, *Proc. Soc. Exp. Biol. and Med.*, 1957, **96**, 709.

13. Kline, D. L., The purification and crystallization of plasminogen (profibrinolysin), *J. Biol. Chem.*, 1953, **204**, 949.

14. Cliffton, E. E., and Cannamela, D., Fibrinolytic and proteolytic activity of a human 'plasminogen' prepared from fraction III of human plasma, *J. Appl. Physiol.*, 1953, **6**, 42.

15. Palitzsch, S., Über die Anwendung von Borax- und Borsäurelösungen bei der colorimetrischen Messung der Wasserstoffionenkonzentration des Meerwassers, *Biochem. Z.*, 1915, **70**, 333.

16. Christensen, L. R., The action of proteolytic enzymes on casein proteins, *Arch. Biochem. and Biophysics*, 1954, **53**, 128.

17. Kunitz, M., Crystalline soybean trypsin inhibitor. II. General properties, *J. Gen. Physiol.*, 1947, **30**, 291.

18. Ungar, G., and Adler, B., A serum peptidase and its possible role in protease inhibition, *Proc. Soc. Exp. Biol. and Med.*, 1956, **91**, 231.

19. Celander, D. R., and Guest, M. M., Factors influencing susceptibility of fibrin and fibrinogen to proteolysis by fibrinolysin, *Arch. Biochem. and Biophysics*, 1957, **72**, 176.