STUDIES OF THE PLASMIN SYSTEM

III. PHYSICAL PROPERTIES OF THE TWO PLASMIN INHIBITORS IN PLASMA*

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In the preceding paper of this series kinetic evidence was presented that indicated the presence of two inhibitory components for plasmin in normal plasma (1). These were distinguished by their relative speed of action as observed by *in vitro* tests and the two postulated components were tentatively referred to as the "immediate" inhibitor and the "slow" inhibitor.

This paper reports further identification of the two inhibitors on the basis of their physical properties. The immediate inhibitor was found to be more stable to extremes of pH and more stable to heating than the slow inhibitor. More important, by zone electrophoresis of human plasma in a starch supporting medium the slow inhibitor was found to migrate as an $\alpha 1$-globulin while the immediate inhibitor was an $\alpha 2$-globulin. For this reason it is suggested that the designations "$\alpha 1$-antiplasmin" and "$\alpha 2$-antiplasmin" be adopted for these two substances. A number of normal human plasmas were assayed for their content of the two inhibitors.

Materials and Methods

Materials.—Plasminogen, streptokinase, plasmin, human plasma, borate-saline buffer, and methylamine have been described in the previous paper of this series (1). All dilutions of plasma were made with borate-saline buffer, pH 7.4. Cohn's fractions IV-1 and IV-4 of human plasma were kindly supplied by E. R. Squibb and Sons through the courtesy of Dr. J. N. Ashworth, American National Red Cross.

Plasmin Assay.—Residual plasmin after inhibition was determined by the previously described caseinolytic assay as modified (1, 2). A standard curve constructed from determinations with purified plasmin was used to convert optical density readings to plasmin units. Determinations of slow inhibitor were done with 0.2 M methylamine present; consequently, it was necessary to use a different standard curve because of the mild inhibitory effect of methylamine (2). This curve was prepared as follows: Protease determinations were done on a series of concentrations of plasmin both with, and without 0.2 M methylamine. From the assays done without methylamine, the specific activity in units per milligram was determined by the use of a standard curve as already described (2). A new standard curve was made

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by using the unit values for each concentration of plasmin as abscissae and, as ordinates, the optical densities representing digestion with 0.2 M methylamine present. Units of plasmin could then be read directly from this curve when determinations with 0.2 M methylamine were done.

Assay of Immediate Inhibitor.—Duplicate determinations were usually done on 0.5 ml. aliquots of a dilution of plasma with buffer or on 0.5 ml. of eluates from starch electrophoresis. The dilution actually used in each experiment is given below. The aliquots were pipetted into 16 x 100 mm. test tubes and 1.0 ml. 4 per cent casein, pH 7.4, was added to each. When there was but little inhibitor in a sample, a 1.0 ml. aliquot of plasma or eluate was used with 0.5 ml. of 8 per cent casein, giving the same final concentration of casein for digestion. The samples were then warmed to 37°C. in a constant temperature water bath and 0.5 ml. 0.0025 M HCl containing 0.2 mg. plasmin (80 × 10^{-3} units per mg.) was added to each in timed sequence. Control observations on the activity of plasmin alone were done by replacing plasma with buffer. After 30 minutes' incubation, the reaction was stopped with 0.5 ml. 15 per cent trichloroacetic acid. An additional 2.5 ml. 15 per cent trichloroacetic acid was added 5 to 10 minutes later. Duplicate blanks for each test material were made by adding trichloroacetic acid before the plasmin. After standing overnight at 4°C. the precipitates were separated by centrifugation for 1 hour at 2700 R.P.M. The optical density of each supernatant was read in a Beckman DU Spectrophotometer at 280 mμ and the reading translated into plasmin units by reference to the standard curve. The units of plasmin inhibited by an aliquot of plasma was the difference between the plasmin activity without plasma and the activity in the presence of plasma. The units of inhibitor per milliliter of plasma could then be calculated from the dilution of plasma. Because the amount of inhibition (in units) depends upon the amount of added plasmin (1), only observations made the same day with same amount of plasmin are presented for comparison. A technique for comparing assays performed on different days is presented in the section on determination of inhibitor in normal plasma.

Assay of Slow Inhibitor.—0.5 ml. of a dilution of test plasma or of eluate from starch electrophoresis was pipetted into a 16 mm. test tube and 0.4 ml. 5 M methylamine, buffered at pH 7.4, was added. The sample was brought to 25°C. in a constant temperature water bath and 0.1 ml. of 0.0025 M HCl containing 1 mg. of purified plasmin was added.[1] A control tube in which the plasma was replaced by buffer was also prepared. The tubes were incubated for 180 minutes at 25°C. to allow the inhibition reaction to go to completion. A total of 4 aliquots of 0.2 ml. were then removed for 2 determinations and 2 blanks. Each aliquot for assay was added to 0.8 ml. buffer and 1.0 ml. 4 per cent casein already mixed and warmed to 37°C. Blanks were made by precipitating with trichloroacetic acid before the aliquots were added. After incubation for exactly 30 minutes at 37°, the protein was precipitated with 15 per cent trichloroacetic acid in the usual manner and, after separation, the optical densities of the clear supernatants were read at 280 mμ. Optical density was converted to plasmin activity in units by reference to the standard curve for determinations done with 0.2 M methylamine. The inhibition in units per aliquot of plasma was the difference between the activity in the presence of buffer and the activity in the presence of plasma. The inhibitor per ml. plasma was then found by multiplying the units inhibitor so obtained by the dilution of plasma in the casein digest mixture (usually 1:50 or 1:100). The slow inhibitor per ml. plasma did not vary with the amount of plasmin presented for inhibition if enough plasmin was used (1).

Zone Electrophoresis in Starch.—The method was essentially the method of Kunkel and Slater (3). Migrations were carried out in veronal buffer, pH 8.6, 0.1 ionic strength. Potato starch was washed 4 times with distilled water and 2 times with buffer. The blocks were 0.5

cm. thick, 13 cm. wide, and 40 cm. long. 5.0 ml. stored citrated plasma or fresh versene plasma was dyed with bromophenol blue and inoculated 10 cm. from the anodal end of the block. Migration was carried out in a cold room at 12°C. and was continued until the dyed portion (albumin) had moved about 20 to 25 cm. from the origin. For human plasma this was about 32 hours at 410 v. and 50 ma. or 50 hours at 210 v. and 22 ma. For guinea pig plasma, 44 hours at 350 v. and 50 ma. or 70 hours at 190 v. and 22 ma. were needed. The blocks were cut into 1 cm. sections and each section was eluted into 5 ml. borate-saline buffer. In the earlier experiments the eluates were dialyzed against borate-saline buffer, pH 7.4 for 48 hours in order to remove veronal. Later, this was found to be unnecessary. Protein determinations were done on 0.1 ml. aliquots of the eluates by a modified Folin-Ciocalteu method (4). Aliquots of 0.5 ml. of eluate were used for determination of immediate and slow inhibitors and the values expressed as units $\times$ 10^{-3} inhibition per ml. of eluate.
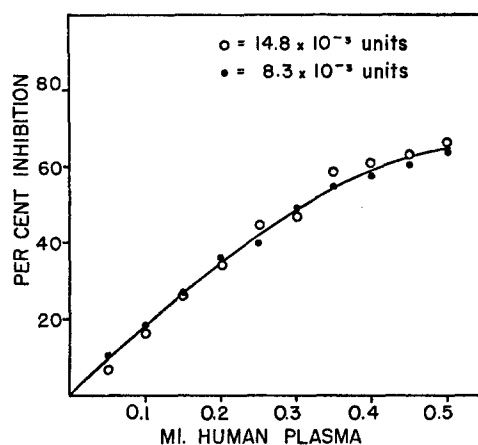

FIG. 1. The per cent immediate inhibition found from varying quantities of a normal human plasma when two different amounts of purified plasmin were presented for inhibition. Open circles: 14.8 $\times$ 10^{-3} units plasmin; closed circles: 8.3 $\times$ 10^{-3} units plasmin.

Stability to Changes in pH.—A pool of stored citrated normal human plasma was used. 15 ml. distilled water was added to 20 ml. plasma, and the reaction was adjusted to the desired pH by careful addition of 1 N KOH or 1 N HCl during continuous measurement with a glass electrode and continuous mixing by a magnetic stirring device. The plasma was transferred to a volumetric flask, diluted to 50 ml., and was allowed to stand for 60 minutes at room temperature. At this point, the reaction was readjusted to pH 7.4 and the volume brought to 100 ml. with borate-saline buffer. At reactions below pH 5.0 a precipitate formed which did not redissolve upon neutralization. The final dilution was 1:5 and the residual immediate and slow inhibitors were determined on 0.5 ml. aliquots of the treated plasma. When there was a precipitate in the treated plasma, it was included in the aliquot. The inhibitors were compared with those in a control sample of the same plasma diluted 1:5 with buffer and stored at 4°C. during the experiment. Values for remaining inhibitor were expressed as per cent of the inhibitor in the control plasma.

Stability to Heating.—Aliquots of 5.0 ml. of a citrated human plasma were pipetted into a series of 13 x 100 mm. tubes and heated in a constant temperature water bath at 60°C. At various times a tube was withdrawn and plunged immediately into an ice bath for cooling.

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The heated samples were stored at 4°C. until tested. Any precipitated fibrinogen was removed by centrifugation. The samples were diluted 1:2 with borate-saline buffer for immediate inhibitor assay and 1:5 for slow inhibitor assay. Residual inhibitor was calculated as the per cent of activity in an unheated sample of plasma.

Assay of Normal Human Plasma.—10 ml. of blood was withdrawn by venipuncture from apparently normal men and women laboratory personnel and immediately transferred to bottles containing the dried residue of 1.2 ml. of 1 per cent disodium versenate (disodium ethylenediamine tetraacetate). The bottles were corked and shaken vigorously. The plasma was separated from cells by centrifugation for 10 minutes at 2500 R.P.M. Plasma so collected was stored at 4°C. for not more than 48 hours before use. Measurement of immediate inhibitor was done on a 1:2 dilution of each plasma, whereas slow inhibitor was assayed using a 1:10 dilution.

As already mentioned, determinations of immediate inhibitor done on different days with different amounts of plasmin are not directly comparable, because the inhibition, if expressed in units, varies with the amount of plasmin presented for inhibition. If inhibition is calculated as a percentage, however, values may be compared. Fig. 1 illustrates this point and shows that the per cent inhibition by several amounts of plasma was the same whether 8.3×10^{-3} units or 14.8×10^{-3} units plasmin was presented for inhibition. With most samples of untreated human plasma, 0.5 ml. of a 1:2 dilution inhibited about 50 per cent of 16×10^{-3} units of plasmin. These amounts of plasmin and plasma were in the best range for measurement by the caseinolytic method and were used for routine determination of the immediate inhibitor in plasma. Although from Fig. 1 the relationship between the amount of inhibitor present and the per cent inhibition is not linear, use of this method of calculation does provide a basis for comparison between plasmas. It is also likely that only in this middle range of plasmin concentrations does the per cent inhibition seem to be independent of concentration of the enzyme.

Unit values for slow inhibitor do not depend on the amount of plasmin added and, therefore may be compared directly.

EXPERIMENTAL

Effect of pH on Antiplasmins.—Aliquots of a normal human plasma were incubated at room temperature for 60 minutes at various pH's and were then neutralized. The samples were assayed for residual inhibitors after this treatment. Fig. 2 shows that both types of inhibitor were stable over a broad zone from pH 5.5 to 10.5 and showed a rapid loss of activity beyond either of these extremes. Immediate inhibitor showed slightly more stability at acid reactions but there was not a clear distinction between the two inhibitors in this physical property.

Effect of Heating on Antiplasmins.—Heating was found to be a more useful method of differentiating between plasma inhibitors. In a preliminary experiment, the antiplasmins were found to be stable at temperatures below 56°C. Consequently, 60°C. was used and aliquots of a normal human plasma were heated for various times and, after cooling, were analyzed for residual antiplasmin. Fig. 3 illustrates that slow inhibitor was destroyed rapidly and completely whereas immediate inhibitor was inactivated more slowly. After 60 minutes of heating no further immediate inhibitor was lost and about 50 per
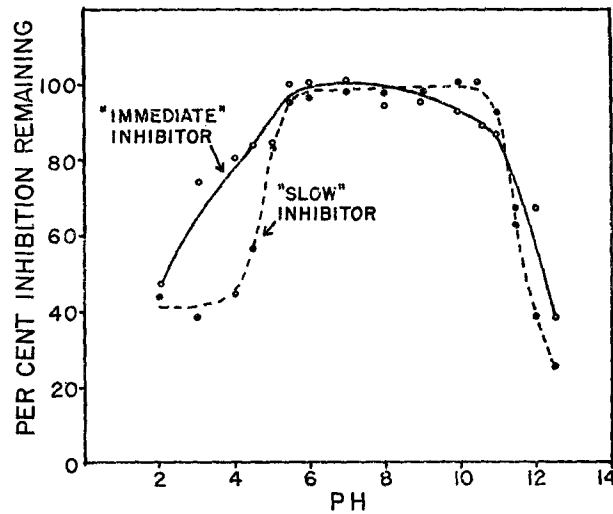
FIG. 2. The stability of antiplasmin to changes in pH. Aliquots of a normal human plasma were subjected to the indicated pH for 60 minutes at room temperature, neutralized, and then assayed for residual inhibitor activity.
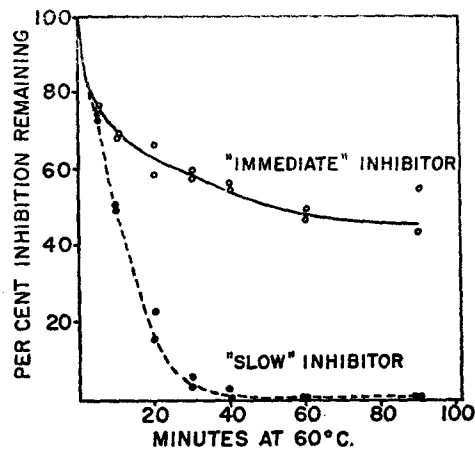

FIG. 3. The stability of antiplasmins to heating. Aliquots of a normal human plasma were heated at 60°C. for various times and then assayed for residual inhibitors.

cent activity remained. On the other hand, the slow inhibitor was completely destroyed in 40 minutes. This experiment indicated a clear difference between the two inhibitors in the rate of inactivation at 60°C.

This result may be compared with that of Shulman who found that trypsin inhibitors were inactivated rapidly and almost completely at 60°, whereas

plasmin inhibitors were resistant to this temperature (5). It seems likely that his method for assay of antiplasmin was principally a measure of immediate inhibitor.

Partial Separation of Antiplasmins by Alcohol Precipitation.—Dried Cohn's fractions IV-1, IV-4, and V of human plasma obtained from the Red Cross

TABLE I

Plasmin Inhibitors in Fractions of Human Plasma Obtained by Cold Alcohol Precipitation

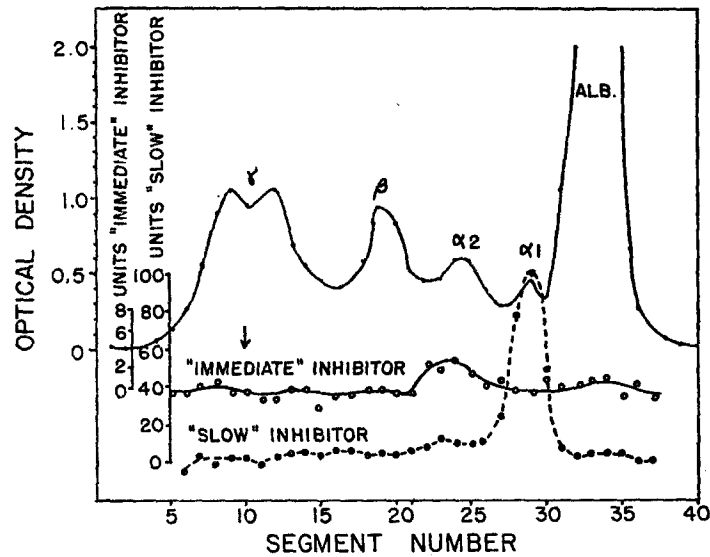| Fraction, 2% solution	Immediate inhibitor	Slow inhibitor
	units $\times$ *10⁻²/ml.*	*units* $\times$ *10⁻²/ml.*
IV-1	3.3	530
IV-4	0.8	710
V	0	0

FIG. 4. Electrophoretic mobility in starch of the antiplasmins in human plasma.

were dissolved in buffer to make 2.0 per cent solutions and aliquots were tested for antiplasmin activity. As indicated in Table I, the greater part of the immediate inhibitor was found in fraction IV-1, whereas slow inhibitor activity was found more in fraction IV-4. This indicates that fractionation of plasma with cold alcohol provides a partial separation of the two types of inhibitory activity.

Grob has studied the inhibitor content of Cohn's fractions of human plasma with a number of proteolytic enzymes. Fraction IV-1 contained almost all the inhibitor for serum proteinase (6). Although the data cannot be compared di-

rectly with our findings, it seems likely that he was measuring immediate inhibition, because it occurred in 20 minutes at room temperature and appeared to be reversible.

Separation of Antiplasmins by Zone Electrophoresis in Starch.—The electrophoretic mobilities of plasmin inhibitors in human plasma were sufficiently different for complete separation of the two types of inhibitory activity. Normal human plasma preserved with either citrate or versene was subjected to electrophoretic separation on starch blocks and the separated proteins were assayed for plasmin inhibitors. A typical curve of protein separation and inhibitory
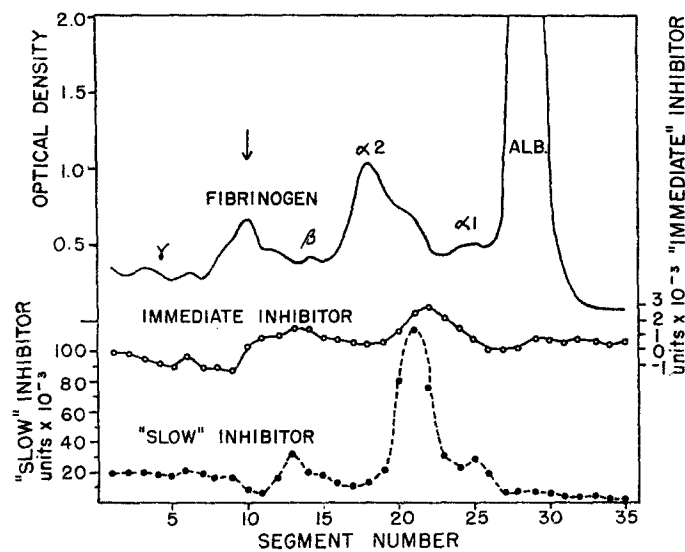

Fig. 5. Electrophoretic mobility in starch of the antiplasmins in guinea pig plasma.

activity is shown in Fig. 4. The results obtained from four experiments were quite consistent in that the immediate inhibitor invariably migrated as an $\alpha2$-globulin, whereas, the slow inhibitor was an $\alpha1$-globulin. In two experiments the $\alpha1$-globulin was not completely separated from the albumin but appeared as a "shoulder" on the anodal side of the peak due to albumin. In these cases, the slow inhibitor was associated with the "shoulder" and not with the main albumin peak. The two types of inhibitor, however, were always unequivocally separated and some fractions contained only immediate activity and others only slow activity. In the four experiments from 45 to 55 per cent of the slow activity of the plasma was recovered in the $\alpha1$-globulins. It is not possible to calculate recovery of the immediate inhibitor because of the reversible nature of reaction, but it seems likely that half or more of this activity appeared in $\alpha2$-globulins.

It is interesting to note that such complete separation could not be obtained when guinea pig plasma was used. Two different samples of normal guinea pig plasma were subjected to electrophoretic separation and a similar result was obtained with either sample. As shown in Fig. 5 the electrophoretic pattern of guinea pig plasma is quite different from that of human plasma. The inhibitors also displayed different mobilities, and in both experiments there was only a 1 cm. difference in the migration of the two peaks. Small secondary peaks of slow

TABLE II

α1-Antiplasmin, α2-Antiplasmin, and Plasminogen in Plasma from Normal Humans

Individual	α1-Antiplasmin	α2-Antiplasmin	Plasminogen
	units × 10⁻³/ml.	*per cent inhibition**	*units × 10⁻³/ml.*
R. F. (M)	800	54.5	31.4
A. L. (M)	990	60.4	26.2
R. P. (M)	760	54.0	37.9
J. S. (M)	960	58.0	28.8
R. W. (M)	660	49.5	24.4
R. W. (F)	900	58.0	20.0
P. N. (M)	700	54.5	31.0
P. S. (F)	950	57.2	21.0
J. M. (M)	775	52.0	30.3
T. W. (M)	940	54.8	24.2
J. R. (M)	785	55.5	30.5
C. R. (M)	800	55.6	27.6
Z. R. (F)	820	57.0	27.9
M. R. (F)	810	58.0	24.4
R. C. (F)	920	61.2	31.0
R. G. (M)	870	57.2	28.4
A. G. (M)	950	61.2	30.4
P. B. (M)	910	57.2	23.0

* Calculated as the per cent inhibition obtained in the standard test from 0.5 ml. of a 1/2 dilution of plasma (see Methods).

inhibitor were found in the α1- and β-regions and a small amount of immediate activity migrated as a β-globulin. It is, as yet, undetermined whether these represent other inhibitors of minor importance or whether they are portions of the principle inhibitors that are slightly altered or that have become attached to other proteins.

Jacobsson has done similar electrophoretic experiments separating the trypsin inhibitors of human serum (7). He found that there are also two inhibitors of trypsin; one is an α1-globulin and the other an α2-globulin. The α1-globulin contained more inhibitor for trypsin as it contains more for plasmin. In his experiments Jacobsson did not attempt to distinguish between trypsin inhibitors on a kinetic basis. The similarities in electrophoretic mobility and

relative potency of the trypsin and plasmin inhibitors of human plasma raise the possibility that the same proteins might inhibit both enzymes. Jacobsson also assayed his fractions for plasmin inhibitor and found activity only with the $\alpha2$-globulins. As he points out, his assay for plasmin inhibitor depends on inhibition of the ability of streptokinase-activated euglobulin to lyse a fibrin clot and may measure inhibition of an activator rather than of plasmin itself.

Plasminogen and Antiplasmin Content of Normal Human Plasmin.—Plasma was collected from a number of normal adult men and women and assayed for $\alpha1$- and $\alpha2$-antiplasmin by methods based on the kinetic difference between the two inhibitors. The same plasmas were also assayed for plasminogen by the previously described method (1). The data are shown in Table II and indicate that in the normal adult the substances vary within rather narrow limits. What pathological conditions that may cause them to vary further is, as yet, unknown.

DISCUSSION

The two plasmin inhibitors originally postulated from kinetic experiments have been shown to be different serum factors with different physical properties. The only *physical* property that provided a means for complete separation was electrophoretic mobility. Electrophoresis, unfortunately, is not a tool that will separate inhibitors in the plasma of any species, for the differences in mobility in guinea pig plasma were slight. The experiment with human plasma does, however, indicate that the methods of assay that have been developed are specific. The $\alpha1$-globulin fractions containing slow inhibitor showed no immediate activity; conversely, the $\alpha2$-globulin (immediate) inhibitor was only slightly active in the assay for slow inhibitor. The kinetic differences first noted, therefore, provide the most useful means for differentiating between the inhibitors and also allow the individual assay of either by simple methods.

The small activity of immediate inhibitor in the test for slow inhibitor could be eliminated by a change in the procedure wherein an aliquot is taken immediately upon adding plasma to the enzyme. The inhibition observed in this sample could be subtracted from the inhibition after 3 hours. The resulting difference would be a truer measure of slow inhibitor but, for routine purposes, the assay would be complicated unnecessarily in order to remove an error which has not been more than 5 per cent.

The names "$\alpha1$-antiplasmin" and "$\alpha2$-antiplasmin" are suggested for the two inhibitors described here and are based on electrophoretic mobility. These names are at best tentative and imperfect. Mobilities as two different α-globulins are properties of the inhibitors that may be found only in humans. As already noted (1), the previous terms, "immediate" and "slow," apply to properties that may be products of test tube study and which may have little to do with the speed of action *in vivo*. The possibility that trypsin inhibition and

plasmin inhibition might be performed by the same substances has again been raised by the similarity in electrophoretic mobility of the two kinds of activity as noted by Jacobsson and by us. Furthermore, thrombin is a proteolytic enzyme of limited and highly specific activity, and the kinetics of antithrombin action are similar to those found by us for "slow" or α1-antiplasmin (8, 9). It should be noted that purified inhibitors have each been found to inhibit a number of proteolytic enzymes, usually to a different degree (10, 11). Until it is known whether or not these plasma factors act specifically on plasmin and not on other enzymes, it may be premature to refer to them as "antiplasmins." There seems to be no choice, however, until more knowledge is obtained. Jacobsson has established a precedent for the terms suggested by giving trypsin inhibitors names according to their electrophoretic mobility.

Presumably the factors described here are protein in nature; they have a characteristic electrophoretic mobility, are precipitated by cold alcohol, and are inactivated by heat and by either acid or alkaline reaction. Proof that they are proteins depends, however, on their purification and subsequent chemical characterization. Furthermore, if protein is present, the possibility also exists that it is in combination with lipide or carbohydrate moieties.

CONCLUSIONS

There are two inhibitors of plasmin in the plasma proteins of the human.

α1-antiplasmin is heat-labile and migrates as an α1-globulin on electrophoresis. It combines non-dissociably with plasmin at a rate that depends on temperature.

α2-antiplasmin is relatively more heat-stable and migrates as an α2-globulin on electrophoresis. It combines dissociably with plasmin independently of temperature.

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