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THE INTERACTION IN HUMAN PLASMA OF ANTIPLASMIN, THE FAST-REACTING PLASMIN INHIBITOR, WITH PLASMIN, THROMBIN, TRYPSIN AND CHYMOTRYPSIN

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Summary

The inhibition of plasmin, (EC 3.4.21.7), thrombin (EC 3.4.21.5), trypsin (EC 3.4.21.4) and chymotrypsin (EC 3.4.21.1) by antiplasmin, the recently described fast-reacting plasmin inhibitor of human plasma, was studied.

To determine the quantitative importance of antiplasmin relative to the other plasma protease inhibitors, enzyme inhibition assays were performed on whole plasma and on plasma specifically depleted in antiplasmin, after addition of excess enzyme. Plasmin was the only enzyme for which the inhibitory capacity of antiplasmin-depleted plasma was lower than that of normal plasma.

To determine the affinity of the enzymes for antiplasmin, as compared to the other inhibitors, various amounts of enzymes were added to normal plasma and the formation of enzyme-antiplasmin complexes studied by crossed immunoelectrophoresis using specific antisera against antiplasmin. Plasmin and trypsin, but not thrombin or chymotrypsin formed complexes with antiplasmin.

It is concluded that antiplasmin is the only fast-reacting plasmin inhibitor of human plasma. It is also a fast-reacting inhibitor of trypsin but only accounts for a very small part of the fast-reacting trypsin-inhibitory activity of plasma. This can be explained by the low concentration of antiplasmin (1 μM) in normal plasma, compared to the other inhibitors (e.g. α_1-antitrypsin: 40—80 μM).

Introduction

Recently, a fast-reacting plasmin inhibitor has been discovered in human plasma [1—4]. Whether this protein, which we have called antiplasmin, is

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Abbreviations: CTA unit, Committee on Thrombolytic Agents. 1 CTA unit of plasmin releases 0.1 μequiv. of tyrosine/min from a standardized solution of α-casein at 37.5°C. NIH unit, 1 NIH unit of thrombin will clot 1.0 ml of a standardized fibrinogen solution in 15 ± 0.5 s at 28 ± 1.0°C.

specific for plasmin or whether it plays a role in the inhibition of other proteases remained to be established. All of the other major protease inhibitors of human plasma, with the exception of α_1-antichymotrypsin, show a wide spectrum of protease inhibiting activity, at least when the pure, or partially purified, inhibitor is considered [5]. The interaction of an inhibitor and enzyme in pure system, however, gives little information about the relative affinity of the inhibitor for proteases in the presence of other inhibitors. For this reason we have used techniques in this study which allowed us to examine the interaction of enzymes with antiplasmin in the presence of normal amounts of the other plasma protease inhibitors i.e. in plasma.

Materials and Methods

Normal plasma

Pooled citrated human plasma from three or more normal subjects was used fresh, or stored at −20° C until use.

Antiplasmin-depleted plasma

Human plasma was depleted in antiplasmin by immunoabsorption, as described elsewhere [2]. Such plasma contained less than 5 percent of the normal amount of material reacting with antiserum against human antiplasmin, but unchanged quantities of α_2-macroglobulin, α_1-antitrypsin, C_1-esterase inhibitor and antithrombin III as determined by an immunoelectrophoretic method [6].

α_1-antitrypsin-deficient serum.

This was the kind gift of Dr. M. Kahn, Département de Pneumologie-Cardiologie, Clinique Médico-Chirurgicale Dr. Derscheid, Waterloo, Belgium. This serum was completely deficient in α_1-antitrypsin.

Enzymes, antisera and reagents

Trypsin (bovine pancreatic, 2 times crystallized, specific activity 12 000 units benzoyl-DL-arginine ethyl ester (BzArgOEE) esterase activity per mg protein), and α-chymotrypsin (bovine pancreatic, 3 times crystallized, specific activity 54 units benzoyl-DL-tyrosine ethyl ester (BzTyrOEE) esterase activity per mg protein) were purchased from Sigma Chemical Co., St. Louis, Mo. U.S.A.; thrombin (bovine, Topostasine) from Roche, Brussels, Belgium; plasmin (human, specific activity approximately 15 CTA units per mg protein) from AB Kabi, Stockholm, Sweden; and Reptilase (a preparation of Bothrops atrox venom) from Laboratoire Stago, Asnières-sur-Seine, France.

Rabbit antiserum against α_1-antitrypsin was purchased from Behringwerke (Marburg/Lahn, West Germany). The preparation of the specific rabbit antisera against human antiplasmin and plasminogen has been described previously [1,2].

The chromogenic substrates S-2160 (Bz-Phe-Val-Arg-p-nitroanilide) and S-2251 (H-D-Val-Leu-Lys-p-nitroanilide) were the kind gifts of AB Kabi Peptide Research, Mölndal, Sweden, and fibrinogen (human, grade L) of AB Kabi, Stockholm, Sweden.

Enzyme inhibition assays

(a). The thrombin inhibition assay using the synthetic substrate S-2160 was performed according to Blombäck et al. [7] and *(b) the clotting assay for antithrombin* was essentially that of Howie et al. [8].

(c). Antiplasmin assay (S-2251) has been described elsewhere [2] but in this study the fast antiplasmin was measured using no pre-incubation of plasmin and plasma.

(d). Caseinolytic antiplasmin assay was performed as described by Amery et al. [9] but using no pre-incubation or 10 min pre-incubation of enzyme and plasma.

For the following assays, preliminary experiments were performed to determine a suitable combination of enzyme concentration and incubation time which would allow sufficient hydrolysis of the substrate to give at least a sevenfold increase in absorbance without causing exhaustion of the substrate. The amount of normal plasma which caused approximately 50% inhibition after 10 min pre-incubation with the enzyme was then determined, and the test was repeated using this same amount of antiplsmin-depleted plasma. Since antiplasmin is known to be a very rapid inhibitor of plasmin [10], a "fast-inhibitor" assay was performed in the same way but using 0 or 20 s pre-incubation of plasma and enzyme (as indicated in Table I).

(e). Trypsin inhibition assay (S-2160). This was performed basically as outlined by the manufacturers. 1 ml of Tris/imidazole buffer pH 8.1, I 0.15 containing 0.75 μl of plasma was prewarmed at 37°C. 50 μl of trypsin solution (0.04 mg/ml) was added and, after 20 s or 10 min, 100 μl of 1 mM S-2160 was added. After a further 90 s the reaction was stopped by the addition of 150 μl glacial acetic acid, and the absorbance of the sample (S) was read at 405 nm. Values were also obtained for an enzyme control (E) without plasma and a sample blank (S_B) and enzyme blank (E_B) using buffer in place of enzyme. The inhibition of enzyme activity was expressed as a percentage of the total enzyme activity using the formula:

$$\frac{(E - E_B) - (S - S_B)}{(E - E_B)} \times 100\%$$

(f). Trypsin inhibition assay (caseinolytic). This assay was based on the caseinolytic anti-plasmin assay described by Amery et al. [9]. 1 ml of pre-warmed trypsin solution (1.5 μg/ml in 0.07 M phosphate buffer, pH 7.4, containing 0.05 M NaCl) and 1 ml of prewarmed plasma dilution (0.4 μl/ml in the same buffer) were allowed to react for 10 min before the addition of 1 ml 1% casein. After a further 30 min incubation the reaction was stopped by the addition of 4 ml 4.9% trichloroacetic acid. After filtering, the non-precipitated peptide material was measured by the method of Lowry et al. [11]. Control and blank values were also measured and the percentage inhibition of enzyme activity determined as above. The "fast inhibitor" assay was performed with no preincubation of enzyme and plasma, the casein being added to the plasma dilution just prior to the addition of the enzyme.

(g). Chymotrypsin inhibitor assay (caseinolytic) was performed in the same

way as the trypsin inhibitor assay. The concentration of the chymotrypsin solution was 1.5 μg/ml and the plasma dilution was 0.5 μl/ml.

Crossed immunoelectrophoresis

Crossed immunoelectrophoresis according to the method of Clarke and Freeman [12] was performed, using antiserum to antiplasmin, α_1-antitrypsin or plasminogen, on samples of normal plasma or α_1-antitrypsin-deficient serum containing various amounts of plasmin, thrombin, trypsin or chymotrypsin. The mixtures of plasma and enzyme solution (in Tris · HCl buffer, pH 7.4, I 0.15) were allowed to stand at 37°C for 10 min before application to the gel. 5-μl portions of the mixtures were applied to the agarose gel and the first dimension run for 2.5 h at a potential gradient of 40 V/cm. The second dimension was run for 4 h at a potential gradient of 30 V/cm into gel containing 0.02 ml antiserum per ml of gel.

For the experiment using ^{125}I-labelled trypsin the second dimension was run for 20 h to remove the radioactive material which had not been immunoprecipitated. The gel was then cut into sections which were chopped up finely and placed in tubes, and the tubes were measured for radioactivity. (The position of the sections was marked on a duplicate gel).

^{125}I-labelling of trypsin

This was performed as described by McFarlane [13].

Results

The enzyme inhibition assays revealed a significant difference between normal and antiplasmin-depleted plasma only in the case of plasmin (Table I). With thrombin, trypsin and chymotrypsin the removal of antiplasmin from plasma had no effect on the inhibition.

The results of crossed immunoelectrophoresis, using antiplasmin antiserum, of normal plasma and normal plasma containing plasmin, thrombin or chymotrypsin are shown in Fig. 1. When plasmin was added to plasma a second peak which reacted with antiplasmin antiserum appeared. This peak was due to the plasmin-antiplasmin complex which has a different electrophoretic mobility to free antiplasmin [1]. As might be expected, more antiplasmin had shifted to the position of this second peak at the higher plasmin concentration, which was similar to that used in the enzyme inhibition assays. Thrombin did not appear to bind to antiplasmin since the antiplasmin peak height and electrophoretic mobility remained unchanged. Chymotrypsin had no effect at the lower concentration, but at the higher concentration the antiplasmin peak was flattened and irregular, suggesting that chymotrypsin had degraded the antiplasmin.

Fig. 2 shows the results of crossed immunoelectrophoresis, using antiplasmin antiserum, of normal plasma samples containing increasing amounts of trypsin. At 5 μM trypsin a second peak was just visible, and as the concentration of trypsin was increased more antiplasmin was displaced to this position. At 100 μM trypsin, (a similar concentration to that used in the enzyme inhibition assays), however, this second peak had disappeared and a third peak had been

TABLE I

RESULTS OF ENZYME INHIBITION ASSAYS

Enzyme inhibition assays using protein and synthetic peptide substrates were performed as described in the methods section. The results are given as mean ± S.D. The statistical significance of the difference between the inhibitory activity of normal and antiplasmin-depleted plasma was determined using Student's *t*-test. For details see Methods

Enzyme	Substrate	Pre-incubation of enzyme and plasma	*n*	Enzyme: plasma ratio	% inhibition by:		*P*
					Normal plasma	Antiplasmin-depleted plasma	
Plasmin	S-2251	0	5	4 *	48 ± 4	1 ± 2	$<<0.001$
Plasmin	S-2251	5 min	4	4	73 ± 7	35 ± 7	<0.001
Plasmin	Casein	0	5	5 *	60 ± 7	28 ± 3	<0.001
Plasmin	Casein	10 min	5	5	86 ± 2	78 ± 3	$0.01 > P > 0.005$
Thrombin	S-2160	20 s	4	100 *	7 ± 5	7 ± 6	>0.05
Thrombin	S-2160	10 min	4	100	43 ± 4	37 ± 5	>0.05
Thrombin	Fibrinogen	5 min	4	80 *	49 ± 3	48 ± 3	>0.05
Trypsin	S-2160	20 s	6	2.7 **	4 ± 5	3 ± 4	>0.05
Trypsin	S-2160	10 min	6	2.7	43 ± 8	41 ± 14	>0.05
Trypsin	Casein	0	4	3.8 **	28 ± 3	26 ± 7	>0.05
Trypsin	Casein	10 min	4	3.8	54 ± 7	41 ± 13	>0.05
Chymotrypsin	Casein	0	4	3.0 **	51 ± 7	49 ± 12	>0.05
Chymotrypsin	Casein	10 min	4	3.0	53 ± 6	44 ± 5	>0.05

* Units of enzyme per ml plasma.

** mg enzyme per ml plasma.

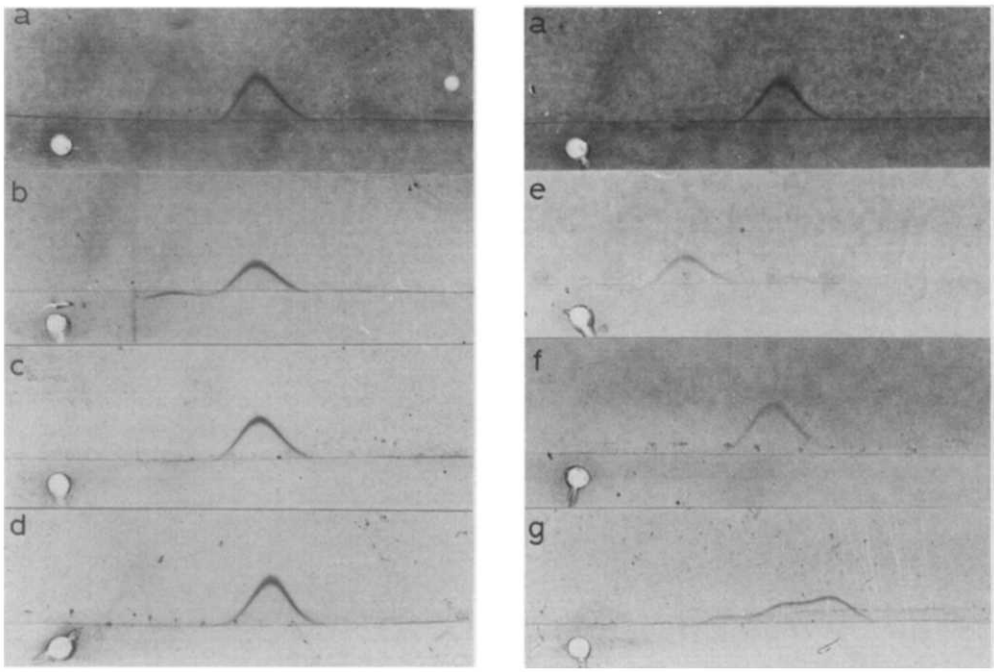

Fig. 1. Crossed immunoelectrophoresis, using antiplasmin antiserum of (a) normal plasma, and normal plasma containing the following concentrations of enzymes: (b) 0.4 CTA units plasmin per ml, (c) 10 NIH units thrombin per ml, (d) 0.3 mg chymotrypsin per ml, (e) 4 CTA units plasmin per ml, (f) 100 NIH units thrombin per ml, (g) 3 mg chymotrypsin per ml. The concentrations in (e), (f) and (g) are similar to those used in the enzyme inhibition assays. Crossed immunoelectrophoresis was performed as described in the methods section.

formed which was slightly more anodal than the first peak. Crossed immunoelectrophoresis of these samples using plasminogen antiserum showed no change in the plasminogen peak at any concentration of trypsin. Crossed immunoelectrophoresis, using antiplasmin antiserum, of normal plasma plus ^{125}I-labelled trypsin (in which the electrophoresis was run for 20 h in the second direction to remove any trypsin not associated with antiplasmin) revealed the presence of radioactivity in both the second and third peaks described above.

Crossed immunoelectrophoresis, using α_1-antitrypsin antiserum, was performed on samples of normal plasma containing increasing amounts of trypsin. These samples were the same ones used in the experiment with antiplasmin antiserum (Fig. 2). The results are shown in Fig. 3. At a concentration of 10 μM trypsin a small second peak had appeared, and by 40 μM trypsin the amount of α_1-antitrypsin which had shifted to this position (assumed to be trypsin-α_1-antitrypsin complex) was considerable. At 100 μM trypsin, two peaks still remained, but both had shifted in position relative to the peaks at 40 μM.

Fig. 4 shows the results of crossed immunoelectrophoresis, using antiplasmin antiserum, of normal plasma and α_1-antitrypsin-deficient serum, to which had been added various amounts of trypsin. The amounts of new antiplasmin-con-

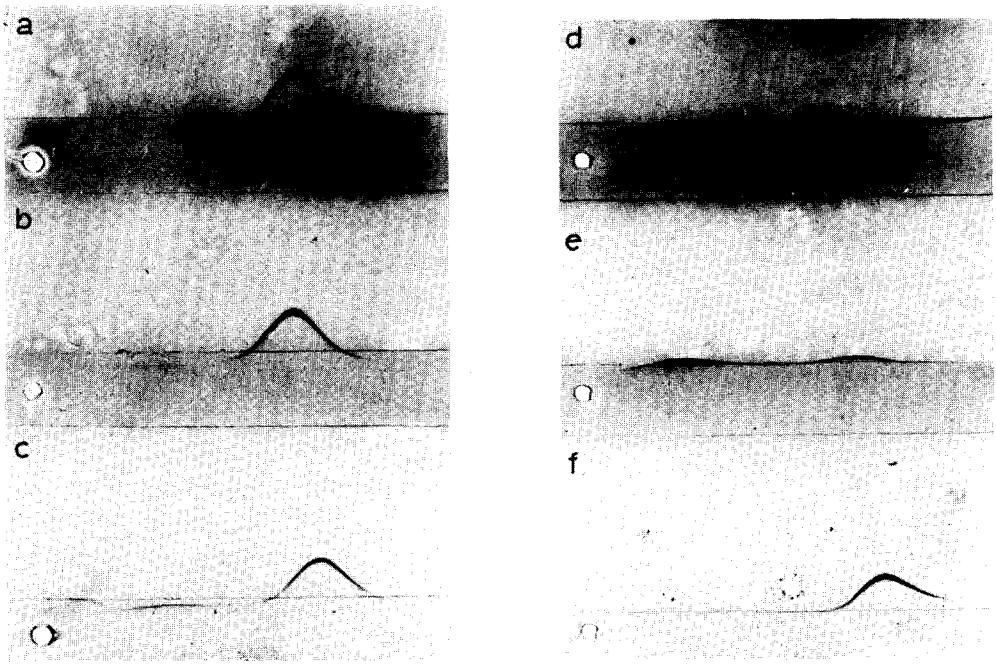

Fig. 2. Crossed immunoelectrophoresis, using antiplasmin antiserum, of (a) normal plasma, and normal plasma containing the following concentrations of trypsin (μM), (b) 2, (c) 5, (d) 10, (e) 40, (f) 100. The highest concentration is similar to that used in the trypsin inhibition assays. Crossed immunoelectrophoresis was performed as described in the methods section.

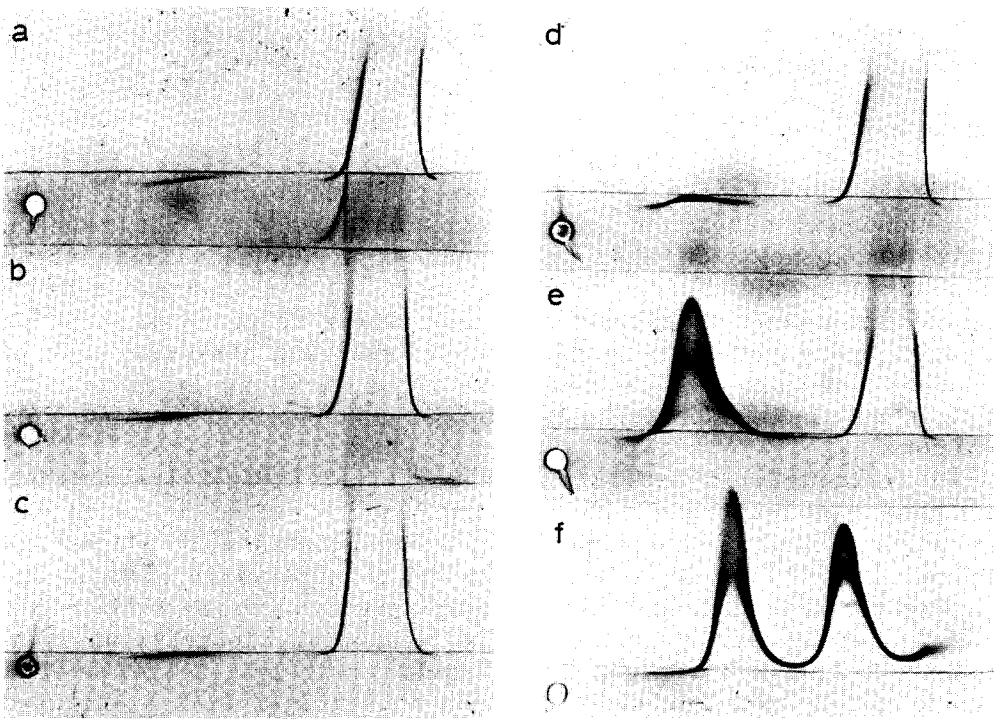

Fig. 3. Crossed immunoelectrophoresis, using α_1-antitrypsin antiserum of (a) normal plasma, and normal plasma containing the following concentrations of trypsin (μM): (b) 2, (c) 5, (d) 10, (e) 40, (f) 100. (These were the same samples used for crossed immunoelectrophoresis with antiplasmin antiserum, Fig. 2). Crossed immunoelectrophoresis was performed as described in the methods section.

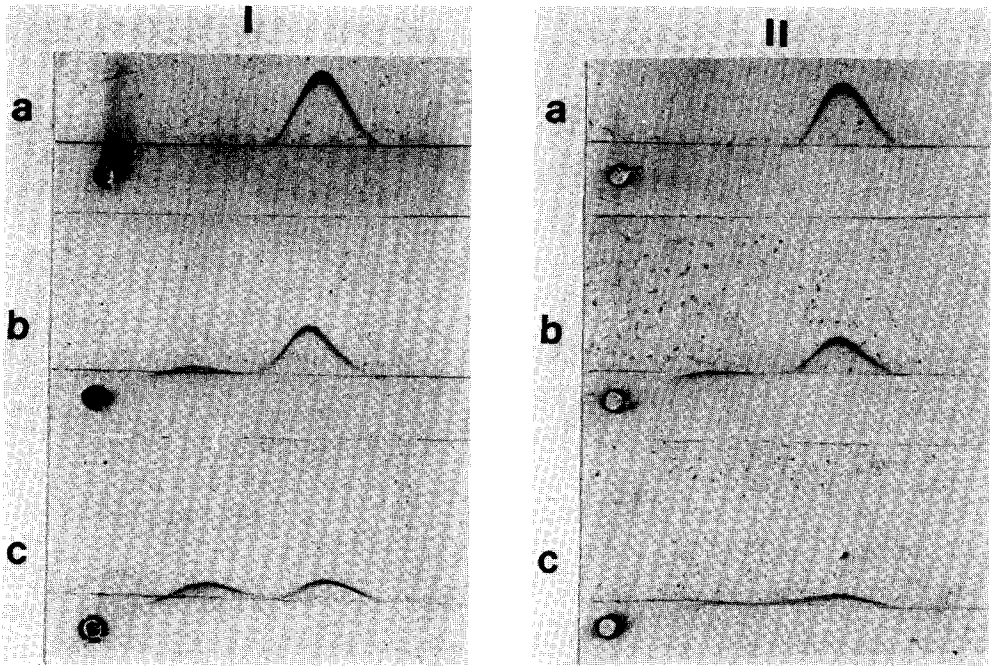

Fig. 4. Crossed immunoelectrophoresis, using antiplasmin antiserum, of I normal plasma and II α_1-antitrypsin-deficient serum containing (a) no trypsin and (b) 10 μM and (c) 20 μM trypsin. The technique was performed as described in the methods section.

taining peak which were produced at each trypsin concentration were very similar in the two series.

Discussion

The results of the enzyme inhibition assays (Table I) show that, under the conditions used, antiplasmin significantly inhibits plasmin, but not thrombin, trypsin or chymotrypsin, when normal amounts of the other plasma protease inhibitors are present. The high residual activity of antiplasmin-depleted plasma in the fast-reacting antiplasmin assay using casein, compared to the value obtained in the synthetic substrate assay, is probably due to slow inhibition of the remaining plasmin by other inhibitors during the 60 min incubation time with casein. The corresponding incubation time in the synthetic substrate assay is 2 min.

The concentration of antiplasmin in plasma (about 1 μM [3,4]) is fairly low compared to the other major protease inhibitors. For instance, α_1-antitrypsin, the major trypsin inhibitor, is present at a serum concentration which varies between about 40 and 80 μM [5,14]. So, even if antiplasmin inhibits trypsin, its contribution to the total trypsin inhibitory capacity of plasma will be negligible. Crossed immunoelectrophoresis was therefore used to detect the interaction of antiplasmin with the enzymes studied, in the presence of the other plasma protease inhibitors. When crossed immunoelectrophoresis is performed on a mixture of an inhibitor and an enzyme, a decrease in size of the normal

free inhibitor peak and the concomitant appearance of a second inhibitor-containing peak with a different electrophoretic mobility indicate the formation of an enzyme · inhibitor complex. However, a new peak which appears only at an enzyme concentration which is high enough to saturate all the inhibitors in the system is probably due to a breakdown product of the inhibitor being studied, or of the enzyme · inhibitor complex if this has been formed previously.

From the results shown in Fig. 1 we can conclude that antiplasmin forms a complex with plasmin but not with thrombin or chymotrypsin, the flattened, irregular peak at high chymotrypsin concentration being due to degradation of antiplasmin by free enzyme. It has also been shown that antiplasmin forms a complex with trypsin (Fig. 2). The new antiplasmin-containing peak which appears when trypsin is added to plasma is not merely plasmin-antiplasmin produced upon trypsin activation of plasminogen, since ancillary experiments showed that the plasminogen peak remained unaltered at all concentrations of trypsin. Furthermore, the presence of trypsin in the new antiplasmin-containing peak is demonstrated by the incorporation of radioactivity when ^{125}I-labelled trypsin is used.

The third antiplasmin-containing peak which is formed at high trypsin concentration (Fig. 2f) also contained radioactivity, suggesting that it is a breakdown product of the trypsin · antiplasmin complex and not just of antiplasmin. At 100 μM trypsin (2.4 mg/ml plasma), free enzyme would certainly be present since the total trypsin inhibitory capacity of normal human serum has been estimated to be about 1 mg trypsin per ml serum [15,16]. This free trypsin activity probably also explains the peak shift seen in Fig. 3f. Ohlsson and Collen (unpublished) have recently used crossed immunoelectrophoresis to study the interaction of human neutral granulocyte collagenase, elastase and chymotrypsin-like enzyme with serum protease inhibitors. They found no complex formation between antiplasmin and these proteases but at enzyme concentrations high enough to saturate the serum inhibitors the antiplasmin peak shifted to a slightly more anodal position, a phenomenon which they considered was due to degradation of the antiplasmin by free proteolytic activity.

Although no quantitative conclusions can be drawn from the results shown in Figs. 2 and 3, it appears that the trypsin-antiplasmin complex is formed at least as quickly as the trypsin · α_1-antitrypsin complex. At low concentration of trypsin, antiplasmin might therefore be an important inhibitor, even though its contribution to the total trypsin inhibiting capacity of plasma will be very small. Fig. 4 indicates that the presence or absence of α_1-antitrypsin does not affect the rate or degree of formation of trypsin-antiplasmin complex. This finding suggests that there is at least one other inhibitor in human plasma which plays an important role in the inhibition of trypsin. This is probably α_2-macroglobulin, which has been shown to have a high affinity for trypsin, even in the presence of the other plasma protease inhibitors [17].

In conclusion, antiplasmin, apart from being a significant inhibitor of plasmin, also seems to be an inhibitor of trypsin (although quantitatively insignificant in plasma), but plays no part in the inhibition of chymotrypsin or thrombin when these are added to plasma.

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