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INTERACTION BETWEEN PROTEASES AND BOVINE ERYTHROCYTE MEMBRANES

II. BINDING OF FIBRINOLYTIC COMPONENTS*

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SUMMARY

1. Bovine erythrocyte ghosts bind plasminogen and plasmin, but do not inhibit the caseinolytic activity of plasmin.

2. Urokinase, which is not bound to ghosts, readily activates plasminogen in the presence and absence of ghosts without an apparent loss in efficiency.

3. Inactivation of trypsin solutions at physiological pH is prevented by the presence of ghosts having the capacity to bind the enzyme.

4. Incomplete activation of plasminogen by trypsin is observed upon addition of trypsin to ghosts–plasminogen mixtures or upon addition of plasminogen to ghosts–trypsin mixtures.

5. Increased binding of [^{3}H]trypsin to ghosts is seen in the presence of plasminogen regardless of the order of addition of the proteins to the ghosts, suggesting that there are separate binding sites on the surface of the membranes for trypsin and plasminogen, and that trypsin might be bound both directly to ghosts and to plasminogen which binds to ghosts.

6. Totally acetylated trypsin incompletely activates plasminogen in the presence of ghosts in a manner identical to that of trypsin. These results are interpreted to suggest that acetylated sites on trypsin, which reduce binding directly to ghosts, are in no way influential in plasminogen activation, and that the binding of plasminogen to ghosts must be the step which, in an unknown manner, affects conformation of the former thereby reducing the efficiency of native trypsin and acetyltrypsin, while exerting no noticeable influence on urokinase activation.

7. A potential role for the possible transport by membranes of zymogens and enzymes involved in fibrinolysis in the blood is suggested.

Abbreviations: CTA unit, standard unit of urokinase activity adopted by the Committee on Thrombolytic Agents, National Heart Institute; saline–phosphate buffer, 0.06 M phosphate buffer, pH 7.6, containing 0.07 M NaCl.

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INTRODUCTION

Burkholder and Brecher [1] have recently reported that bovine red blood cell membranes selectively bind and inhibit certain proteolytic enzymes and inhibit activation of their zymogens. Based upon these observations, it was postulated that selective binding and inhibition of proteases, particularly of the blood coagulation system, may support functions of other known regulatory mechanisms. This work has now been extended to include interaction of bovine erythrocyte membranes with proteases of the fibrinolytic system. The fibrinolytic system is extremely important in haemostasis as a direct antagonist of the blood coagulation system. Fibrinolysis involves the activation of plasminogen to plasmin by one of several activator substances which may be found in the blood. Plasmin is a serine proteinase capable of hydrolyzing fibrin, thereby dissolving clots. During the formation of fibrin clots, approximately 20–30% of the plasma content of plasminogen is entrapped in the clot [2]. Hence, plasminogen activators may act on both fibrin-bound and freely circulating plasminogen [2]. Plasminogen activators include urokinase, tissue kinases, streptokinase, staphylokinase, trypsin [2] and a recently characterized blood proactivator [3]. One or more of these substances may function in controlling plasmin levels under normal circumstances or under varying pathological conditions.

This study reports the interaction of bovine erythrocyte membranes with plasmin, as well as effects of the membranes on the activation of plasminogen by two specific activators, urokinase and trypsin. Possible consequences of such interactions on transport, localization and regulation are discussed. A preliminary report has appeared elsewhere [4].

MATERIALS AND METHODS

Crystalline trypsin (2×), [^{3}H]trypsin (2×), and human plasminogen were obtained from Worthington Biochemical Corporation, Freehold, N. J. Crystalline acetylated trypsin (1×) and Hammersten casein, which was prepared by the method of Bruhn et al. [5], were purchased from Schwarz/Mann Research Laboratories, Orangeburg, N.Y. Urokinase was obtained from Nutritional Biochemical Corp., Cleveland, Ohio. Cohn Fraction V bovine serum albumin (Nutritional Biochemical Corp.) was added to the urokinase solutions at 1 mg/10000 standard units of urokinase activity (CTA units) to assure stabilization of the enzyme during storage, after solubilization.

Bovine red blood cell ghosts were prepared by the method of Green et al. [6]. The final pellet was homogeneous and white and consisted of intact dimpled ghosts as viewed by interference contrast microscopy. The ghosts were centrifuged and resuspended in saline phosphate buffer before use. Protein determinations were performed as previously described [1].

Assay of trypsin, acetylated trypsin, and plasmin activity with casein

Caseinolytic activity was measured by a modification of the method of Bruhn et al. [5]. Aliquots of enzyme, 0.5–10.0 μg, were diluted to 0.6 ml with saline phosphate buffer. 0.4 ml of 1% casein was added thereto, and the mixture was incubated at 37 °C for 60 min, after which time the reaction was terminated with 1.0 ml of

15% trichloroacetic acid. The mixture was filtered and the increase in phenolic tyrosine residues in the trichloroacetic acid supernatant fraction was determined according to the method of Lowry et al. [7].

Activation of plasminogen with urokinase

To 0.1-ml aliquots of plasminogen (5 μg in 0.06 M phosphate buffer, pH 7.6, containing 0.07 M NaCl (saline–phosphate buffer)) were added 0.025 ml (5 CTA units), 0.050 ml (10 CTA units), or 0.1 ml (20 CTA units) of urokinase (in saline–phosphate buffer). The volumes were adjusted to 0.6 ml with the same buffer. After standing at room temperature for 5, 10, 20, 30, or 60 min, the mixtures were tested for caseinolytic activity as described above.

Determination of binding and inhibition of plasmin, plasminogen and urokinase

To 1.5 ml of plasminogen (75 μg in saline–phosphate buffer) was added 4.5 ml of ghosts (2.84 mg protein) or 4.5 ml of saline–phosphate buffer. After standing at 0–5 °C for 15 min an aliquot was removed and the remainder was centrifuged at $27000 \times g$ for 15 min. The sediment was resuspended in saline–phosphate buffer. To 2.0 ml of the original mixture, supernatant fraction, resuspended sediment and control plasminogen was added 0.5 ml of urokinase (100 CTA units in saline–phosphate buffer). The mixtures were allowed to stand for 5 min at room temperature, after which time 0.5 ml of each preparation was withdrawn and tested for caseinolytic activity as described above.

To 0.5 ml of plasminogen (50 μg in saline–phosphate buffer) was added 0.5 ml of urokinase (200 CTA units in saline–phosphate buffer). After standing at room temperature for 5 min, 3.0 ml of ghosts (1.89 mg protein) was added thereto, and the mixture was allowed to stand at 0–5 °C for 15 min. An aliquot was removed, and the remainder was centrifuged at $27000 \times g$ for 15 min. The mixture, supernatant and resuspended sediment fractions were tested for caseinolytic activity as described above.

To 1.5 ml of urokinase (300 CTA units in saline–phosphate buffer) was added 4.5 ml of red blood cell ghosts suspension (2.84 mg protein) and 1.5 ml of saline–phosphate buffer. After standing at 0–5 °C for 15 min an aliquot was removed, and the remainder was centrifuged at $27000 \times g$ for 15 min. The sediment was resuspended in saline phosphate buffer. To 2.5 ml of mixture, supernatant fraction, resuspended sediment, and control urokinase (0.5 ml urokinase (100 CTA units) + 2.0 ml of saline–phosphate buffer) was added 0.5 ml plasminogen (25 μg in saline–phosphate buffer). After standing at room temperature for 5 min, 0.6 ml of each mixture was withdrawn and tested for caseinolytic activity as described above.

Activation of plasminogen with trypsin and acetylated trypsin in the presence and absence of ghosts

To 0.2-ml aliquots of trypsin (15 μg in 1.0 mM HCl) or totally acetylated trypsin (15 μg in 1.0 mM HCl) were added 2.5 ml of ghosts (1.89 mg protein) or 2.5 ml of saline–phosphate buffer. After standing at 0–5 °C for 15 min, 0.3 ml of plasminogen (300 μg in saline–phosphate buffer) was added to each mixture. Control mixtures containing 15 μg of trypsin or 15 μg of totally acetylated trypsin plus 2.5 ml of ghosts were also prepared. The mixtures were allowed to stand at room

temperature. At 5-, 15-, 30-, 60-, 90-, and 120-min intervals, a 0.1-ml aliquot of each mixture was withdrawn and tested for caseinolytic activity as described above.

To 0.3-ml aliquots of plasminogen (300 μg in saline–phosphate buffer) and to 0.3-ml aliquots of saline–phosphate buffer were added 2.5 ml of ghosts (1.89 mg protein) or 2.5 ml of saline–phosphate buffer. After standing at 0–5 °C for 15 min, 0.2 ml of trypsin (15 μg in 1.0 mM HCl) or totally acetylated trypsin (15 μg in 1.0 mM HCl) was added to each mixture. The mixtures were allowed to stand at room temperature. At 5-, 15-, 30-, 60-, 90-, and 120-min intervals a 0.1-ml aliquot of each mixture was withdrawn and tested for caseinolytic activity as described above.

Binding of [^3H]trypsin to ghosts in the presence and absence of plasminogen

To a 7.65-ml aliquot of ghosts (5.67 mg protein) was added 0.9 ml of plasminogen (900 μg in saline–phosphate buffer). To two 7.65-ml aliquots of ghosts were added 0.45 ml of [^{3}H]trypsin (45 μg in 1.0 mM HCl). The mixtures were allowed to stand for 15 min at 0–5 °C after which time 0.45 ml of [^{3}H]trypsin was added to the first mixture, 0.9 ml of plasminogen (900 μg in saline–phosphate buffer) to the second mixture and 0.9 ml of saline–phosphate buffer to the third mixture, respectively. The third mixture was centrifuged immediately at 27000 $\times g$ for 15 min and the other mixtures were allowed to stand at room temperature. At 5, 30, and 90 min, aliquots were removed from the first two mixtures and centrifuged as above. All sediments were resuspended in saline–phosphate buffer. Aliquots of supernatant and resuspended sediment fractions were added to 10.0 ml of Aquasol and counted in a Packard TriCarb scintillation counter.

RESULTS

The data in Table I indicate that increasing levels of urokinase cause an increase in the activation of plasminogen in the model system, using casein as a substrate for plasmin. The increase is essentially linear through the highest level of urokinase tested. Furthermore, a maximum level of plasminogen activation at the level of 20 CTA units of urokinase to 5 μg of plasminogen is reached within 5 min after

TABLE I

EFFECT OF VARIOUS LEVELS OF UROKINASE ON THE ACTIVATION OF PLASMINOGEN

To 0.1-ml aliquots of plasminogen (5 μg in saline–phosphate buffer) was added 0.025 ml (5 CTA units), 0.050 ml (10 CTA units) or 0.10 ml (20 CTA units) of urokinase (in saline–phosphate buffer), and saline–phosphate buffer to 0.6 ml. The mixtures were allowed to stand at room temperature for 20 min, and were tested for caseinolytic activity as described in the text.

Addition of 5 μg plasminogen	Trichloroacetic acid-precipitable casein solubilized per h (mg)
5 CTA units of urokinase	0.14
10 CTA units of urokinase	0.23
20 CTA units of urokinase	0.38

TABLE II

EFFECT OF ACTIVATION TIME ON THE ACTIVATION OF PLASMINOGEN BY UROKINASE

To 0.1-ml aliquots of plasminogen (5 μg in saline–phosphate buffer) was added 0.1 ml urokinase (20 CTA units in saline–phosphate buffer) and 0.4 ml saline–phosphate buffer. After standing at room temperature for 5, 10, 20, 30 and 60 min, the mixtures were tested for caseinolytic activity as described in the text.

Activation time after mixing 5 μg plasminogen with 20 CTA units of urokinase (min)	Trichloroacetic acid-precipitable casein solubilized per h (mg)
5	0.43
10	0.45
20	0.44
30	0.33
60	0.41

addition of the urokinase (Table II). Activation may be complete in considerably less than 5 min.

Upon the prior incubation of human plasminogen with bovine erythrocyte ghosts, full activation of plasminogen by urokinase is observed (Table III). It is also seen that plasminogen is bound to the membranes since they may be centrifuged and about half of the activatable plasminogen in a plasminogen–ghosts mixture is found associated with the particulate fraction which occupies less than 2% of the

TABLE III

ACTIVATION OF PLASMINOGEN BY UROKINASE IN THE PRESENCE OF BOVINE RED BLOOD CELL GHOSTS

To 1.5 ml of plasminogen (75 μg in saline–phosphate buffer) was added 4.5 ml of red blood cell ghosts suspension (2.84 mg protein) or 4.5 ml of saline–phosphate buffer. After standing at 0–5 °C for 15 min, an aliquot was removed and the remainder was centrifuged at 27000 $\times$ g for 15 min. To 2.0 ml of mixture, supernatant, resuspended sediment and control plasminogen was added 0.5 ml urokinase (100 CTA units in saline–phosphate buffer). The mixtures were allowed to stand at room temperature for 5 min after which time each was tested for caseinolytic activity as described in the text. The number of observations is given in parenthesis.

Preparation	Trichloroacetic acid-precipitable casein solubilized per h (mean $\pm$ S.E.) (mg)	Per cent of control activity
Urokinase	0 (4)	0
Plasminogen + urokinase	0.33 $\pm$ 0.021 (4)	100
Plasminogen + red blood cell ghosts	0.40 $\pm$ 0.019	0
Plasminogen + urokinase + red blood cell ghosts	0.40 $\pm$ 0.018 (4)	121.2
Supernatant after centrifugation and activation with urokinase	0.22 $\pm$ 0.019 (4)	63.6
Sediment after centrifugation and activation with urokinase	0.18 $\pm$ 0.006 (4)	54.5

total volume before resuspension (Table III). Under conditions in which maximal activation of plasminogen to plasmin is allowed to occur prior to the addition of ghosts, the ability of plasmin to hydrolyze casein in the presence of ghosts is not effected (Table IV). Furthermore, plasmin distributes itself approximately evenly between the packed membranous sediment and the supernatant fraction upon centrifugation of plasmin–ghosts mixtures.

TABLE IV

BINDING OF PLASMIN BY BOVINE RED BLOOD CELL GHOSTS

To 0.5 ml plasminogen (50 μg in saline–phosphate buffer) was added 0.5 ml of urokinase (200 CTA units in saline–phosphate buffer). After standing 5 min at room temperature, 3.0 ml of red cell ghosts suspension (1.89 mg protein) was added and the mixture allowed to stand at 0–5 °C for 15 minutes. A 1.6-ml aliquot was removed and the remainder was centrifuged at 27000 $\times g$ for 15 min. The mixture, supernatant and resuspended sediment fractions were tested for caseinolytic activity as described in the text. Between parentheses the number of observations.

Preparation	Trichloroacetic acid-precipitable casein solubilized per h (mean ± S.E.) (mg)	Per cent of control activity
Urokinase	0 (3)	0
Plasmin	0.32 ± 0.017 (3)	100
Plasmin + red blood cell ghosts	0.31 ± 0.007 (3)	96.9
Supernatant after centrifugation	0.14 ± 0.010 (2)	43.8
Sediment after centrifugation	0.15 ± 0.023 (3)	46.9

TABLE V

BINDING OF UROKINASE BY BOVINE RED BLOOD CELL GHOSTS

To 1.5 ml of urokinase (300 CTA units in saline–phosphate buffer) was added 4.5 ml of red blood cell ghosts suspension (2.84 mg protein) and 1.5 ml saline–phosphate buffer. After standing at 0–5 °C for 15 min an aliquot was removed and the remainder was centrifuged at 27000 × g for 15 min. The sediment was resuspended in saline-phosphate buffer. To 2.5 ml of mixture, supernatant, resuspended sediment and control urokinase was added 0.5 ml plasminogen (25 μg in saline–phosphate buffer). After standing at room temperature for 5 min, 0.6 ml of each mixture was withdrawn and tested for caseinolytic activity as described in the text. Between parentheses is the number of observations.

Preparation	Trichloroacetic acid-precipitable casein solubilized per h (mean ± S.E.) (mg)	Per cent of control activity
Plasminogen + urokinase	0.26 ± 0.013 (2)	100
Urokinase, alone	0 (2)	0
Urokinase + red blood cell ghosts + plasminogen	0.31 ± 0.11 (4)	119.2
Supernatant after centrifugation and addition of plasminogen	0.35 ± 0.020 (4)	134.6
Sediment after centrifugation and addition of plasminogen	0 (2)	0
Urokinase + ghosts	0 (2)	0
Plasminogen + ghosts	0 (2)	0

TABLE VI

EFFECT OF A TRYPSIN–RED BLOOD CELL GHOSTS MIXTURE ON THE ACTIVATION OF PLASMINOGEN

To 0.2-ml aliquots of trypsin (15 μg in 1.0 mM HCl) were added 2.5 ml red blood cell ghosts suspension (1.89 mg protein) or 2.5 ml of saline–phosphate buffer. After standing at 0–5 °C for 15 min, 0.3 ml of plasminogen (300 μg in saline phosphate buffer) or 0.3 ml saline–phosphate buffer was added to each mixture. Control mixtures containing 15 μg trypsin plus 2.5 ml ghosts or 15 μg trypsin plus 2.5 ml saline–phosphate buffer were also prepared. The mixtures were allowed to stand at room temperature. At 5-, 15-, 30-, 60-, 90-, and 120-min intervals, 0.1 ml of each mixture was withdrawn and tested for caseinolytic activity as described in the text. The significance level was calculated using a *t*-test with respect to the trypsin + plasminogen mixture. NS, not significant.

Preparation	Activation time (min)	Number of observations	Precipitable casein solubilized per h (mean ± S.E.) (mg)	Significance level	Per cent of control activity
Trypsin + plasminogen	5	10	1.09 ± 0.016		100.0
	15	10	1.16 ± 0.016		100.0
	30	10	1.27 ± 0.011		100.0
	60	10	1.35 ± 0.012		100.0
	90	7	1.34 ± 0.036		100.0
	120	8	1.43 ± 0.016		100.0
Addition of plasminogen to a trypsin–ghosts mixture	5	10	1.06 ± 0.015	NS	97.2
	15	10	1.11 ± 0.013	$0.01 < P < 0.02$	95.7
	30	10	1.16 ± 0.013	$P < 0.001$	91.3
	60	10	1.23 ± 0.011	$P < 0.001$	91.1
	90	8	1.30 ± 0.029	NS	97.0
	120	8	1.26 ± 0.033	$P < 0.001$	88.1
Trypsin + buffer	5	8	1.01 ± 0.021		100.0
	15	8	1.01 ± 0.017		100.0
	30	8	0.97 ± 0.010		100.0
	60	8	0.89 ± 0.027		100.0
	90	8	0.81 ± 0.035		100.0
	120	8	0.66 ± 0.030		100.0
Trypsin + ghosts	5	8	0.97 ± 0.018		96.0
	15	8	1.00 ± 0.014		99.0
	30	8	0.97 ± 0.016		100.0
	60	8	0.95 ± 0.030		106.7
	90	8	0.95 ± 0.040		117.3
	120	8	0.98 ± 0.016		148.5

In contrast to the binding of plasmin and plasminogen, urokinase is apparently not bound to ghosts since no detectable urokinase activity is observed in the sediment fractions after centrifugation of urokinase–ghosts mixtures (Table V). It is further observed that ghosts preparations do not interfere with the capacity of urokinase to activate plasminogen.

Although trypsin has been observed to activate human plasminogen in a manner comparable to urokinase [8], activation of the zymogen by erythrocyte-bound trypsin mixtures is partially inhibited (Table VI). The data in Table VI further suggest that erythrocyte membranes effect a stabilization of trypsin to inactivation at room temperature over the 2-h activation period. A similar inhibition of activation of plas-

TABLE VII

EFFECT OF TRYPSIN ON THE ACTIVATION OF A PLASMINOGEN–RED BLOOD CELL GHOSTS MIXTURE

To 0.3 ml plasminogen (300 μg in saline–phosphate buffer) and to 0.3 ml saline–phosphate buffer was added 2.5 ml red blood cell ghosts suspension (1.89 mg protein) or 2.5 ml saline–phosphate buffer. After standing at 0–5 °C for 15 min, 0.2 ml trypsin (15 μg in 1.0 mM HCl) was added to each mixture. Control mixtures containing 15 μg trypsin plus 2.5 ml ghosts or 15 μg trypsin plus 2.5 ml saline–phosphate buffer were also prepared. The mixtures were allowed to stand at room temperature. At 5-, 15-, 30-, 60-, 90- and 120-min intervals, 0.1 ml of each mixture was withdrawn and tested for caseinolytic activity as described in the text. The significance level was calculated using a *t*-test with respect to the trypsin+plasminogen mixture.

Preparation	Activation time (min)	Number of observations	Precipitable casein solubilized per h (mean ± S.E.) (mg)	Significance level	Per cent of control activity
Trypsin+plasminogen	5	8	1.32 ± 0.054		100.0
	15	8	1.40 ± 0.056		100.0
	30	8	1.54 ± 0.056		100.0
	60	8	1.54 ± 0.054		100.0
	90	6	1.66 ± 0.076		100.0
	120	6	1.73 ± 0.057		100.0
Addition of trypsin to a plasminogen-ghosts mixture	5	8	1.21 ± 0.070	NS	91.7
	15	8	1.26 ± 0.068	NS	90.0
	30	8	1.30 ± 0.060	$0.01 < P < 0.02$	84.4
	60	8	1.33 ± 0.065	$0.02 < P < 0.05$	86.4
	90	6	1.35 ± 0.077	$0.01 < P < 0.02$	81.3
	120	6	1.39 ± 0.079	$0.001 < P < 0.01$	80.3
Trypsin+buffer	5	6	1.25 ± 0.071		100.0
	15	6	1.18 ± 0.071		100.0
	30	6	1.15 ± 0.067		100.0
	60	6	1.09 ± 0.071		100.0
	90	6	0.96 ± 0.074		100.0
	120	6	0.80 ± 0.075		100.0
Trypsin+ghosts	5	6	1.14 ± 0.070		91.2
	15	6	1.17 ± 0.077		99.2
	30	6	1.11 ± 0.079		96.5
	60	6	1.15 ± 0.066		105.5
	90	6	1.15 ± 0.068		119.8
	120	6	1.09 ± 0.081		136.3

minogen is observed upon incubation of trypsin with plasminogen–ghosts mixtures (Table VII).

In order to study the effect of plasminogen on the binding of trypsin, to ghosts, (and vice versa), [^{3}H]trypsin was incubated with ghosts in the presence of plasminogen. An initial distribution of [^{3}H]trypsin between supernatant and sediment fractions of 60:40 was observed before the addition of plasminogen (Table VIII). However, upon the addition of [^{3}H]trypsin to a plasminogen–ghosts mixture, or upon the addition of plasminogen to a tritiated trypsin–ghosts mixture, an increased level of sediment-associated counts is observed up to at least 90 min after addition of enzyme or zymogen to the mixture (Table VIII). These data suggest that the pre-

TABLE VIII

BINDING OF [^{3}H]TRYPSIN TO ERYTHROCYTE GHOSTS IN THE PRESENCE AND ABSENCE OF PLASMINOGEN

To one 7.65-ml aliquot of ghosts (5.67 mg protein) was added 0.9 ml plasminogen (900 μg in saline–phosphate buffer) and to two 7.6-ml aliquots of ghosts was added 0.45 ml [^{3}H]trypsin (45 μg in 1.0 mM HCl). The mixtures were allowed to stand for 15 min at 0–4 °C after which time 0.45 ml [^{3}H]trypsin (45 μg in 1.0 mM HCl) was added to the first mixture, 0.9 ml plasminogen (900 μg in saline–phosphate buffer) to the second and 0.9 ml saline–phosphate buffer to the third respectively. The last mixture was centrifuged immediately at 27000 $\times g$ for 15 min and the other mixtures were allowed to stand at room temperature. At 5, 30 and 90 min, an aliquot was removed from the first two mixtures and centrifuged as above. All sediments were resuspended in saline-phosphate buffer. Aliquots of supernatant and resuspended sediment fractions were added to 10.0 ml of Aquasol and counted in a Packard TriCarb scintillation counter. The significance level was calculated using a t-test with respect to the [^{3}H]trypsin+ghosts sediment. Between parentheses is the number of observations.

Preparation	Incubation at room temperature (min)	cpm of [^{3}H]trypsin (mean ± S.E. · 10^{-3})	Significance level	Corrected total percentage cpm
[^{3}H]Trypsin+ghosts				
Supernatant after centrifugation	–	72.6 ± 6.3 (3)		61.3
Sediment after centrifugation	–	45.9 ± 7.1 (3)		38.7
[^{3}H]Trypsin–ghosts+plasminogen				
Supernatant after centrifugation	5	153.3 ± 32.4 (3)		42.5
Sediment after centrifugation	5	207.4 ± 22.2 (3)	$0.001 < P < 0.01$	57.5
Supernatant after centrifugation	30	163.2 ± 26.1 (3)		44.1
Sediment after centrifugation	30	206.5 ± 34.2 (3)	$0.001 < P < 0.01$	55.9
Supernatant after centrifugation	90	191.2 ± 10.5 (3)		49.5
Sediment after centrifugation	90	195.4 ± 26.6 (3)	$0.001 < P < 0.01$	50.5
Plasminogen–ghosts+[^{3}H]trypsin				
Supernatant after centrifugation	5	155.4 ± 39.4 (3)		42.3
Sediment after centrifugation	5	211.9 ± 10.9 (3)	$P < 0.001$	57.7
Supernatant after centrifugation	30	169.4 ± 35.9 (3)		46.6
Sediment after centrifugation	30	194.5 ± 16.9 (3)	$0.001 < P < 0.01$	53.4
Supernatant after centrifugation	90	181.0 ± 35.5 (3)		48.5
Sediment after centrifugation	90	192.3 ± 22.5 (3)	$0.001 < P < 0.01$	51.5

sence of plasminogen affects an increase in binding of the trypsin to the membranes.

It has previously been reported that totally acetylated trypsin does not effectively bind to red blood cell membranes nor is it considerably inhibited in their presence, as measured using casein as a substrate [1]. The data in Tables IX and X indicate that totally acetylated trypsin also retains its ability to activate plasminogen. It is further seen that upon the addition of plasminogen to a totally acetylated trypsin–ghosts mixture (Table IX) or upon the addition of totally acetylated trypsin to a plasminogen–ghosts mixture (Table X), inhibition of plasminogen activation occurs, as with native trypsin. These data appear to suggest that the binding of plasminogen to membranes interferes with plasminogen activation by acetylated trypsin.

TABLE IX

EFFECT OF AN ACETYLATED TRYPSIN–RED BLOOD CELLS GHOSTS MIXTURE ON THE ACTIVATION OF PLASMINOGEN

To 0.2-ml aliquots of totally acetylated trypsin (15 μg in 1.0 mM HCl) was added 2.5 ml red blood cell ghosts suspension (1.89 mg protein) or 2.5 ml saline–phosphate buffer. After standing at 0–5 °C for 15 min, 0.3 ml plasminogen (300 μg in saline–phosphate buffer) or 0.3 ml saline–phosphate buffer was added to each mixture. Control mixtures containing 15 μg acetylated trypsin and 2.5 ml ghosts, 15 μg acetylated trypsin plus saline–phosphate buffer, or 15 μg trypsin plus saline–phosphate buffer were also prepared. The mixtures were allowed to stand at room temperature. At 5, 15, 30, 60, 90 and 120 min, 0.1 ml of each mixture was withdrawn and tested for caseinolytic activity as described in the text. The significance level was calculated using a *t*-test with respect to the acetylated trypsin + plasminogen mixture.

Preparation	Activation time (min)	Number of observations	Precipitable casein solubilized per h (mean ± S.E.) (mg)	Significance level	Per cent of control
Acetylated trypsin + plasminogen	5	6	0.87 ± 0.012		100.0
	15	6	0.94 ± 0.007		100.0
	30	5	1.01 ± 0.029		100.0
	60	6	1.16 ± 0.055		100.0
	90	4	1.31 ± 0.100		100.0
	120	4	1.34 ± 0.073		100.0
Addition of plasminogen to an acetylated trypsin–ghosts mixture	5	6	0.79 ± 0.017	$0.001 < P < 0.01$	90.8
	15	6	0.82 ± 0.008	$P < 0.001$	87.2
	30	6	0.88 ± 0.014	$0.001 \quad P < 0.01$	87.1
	60	6	0.95 ± 0.042	$0.001 < P < 0.01$	81.9
	90	4	1.05 ± 0.017	$0.02 < P < 0.05$	80.2
	120	4	1.08 ± 0.039	$0.01 < P < 0.02$	80.6
Acetylated trypsin + buffer	5	4	0.77 ± 0.019		100.0
	15	4	0.76 ± 0.027		100.0
	30	4	0.69 ± 0.010		100.0
	60	4	0.70 ± 0.028		100.0
	90	4	0.66 ± 0.040		100.0
	120	4	0.61 ± 0.041		100.0
Acetylated trypsin + ghosts	5	4	0.78 ± 0.041		101.3
	15	4	0.73 ± 0.016		96.1
	30	4	0.72 ± 0.009		104.3
	60	4	0.73 ± 0.006		104.3
	90	4	0.76 ± 0.019		115.2
	120	4	0.72 ± 0.028		118.0

DISCUSSION

The binding of proteins to cell surfaces has become an area of increasing interest and investigation. Many of these investigations involve the study of erythrocyte membranes and include such areas as antigenic reactions [9], agglutinin binding [10,11], binding of concanavalin A [12], immune adherence haemagglutination [13], and sugar transport [14,15]. Although the presence and activity of proteases found in the blood, particularly those involved in haemostasis [2,16], have also

TABLE X

EFFECT OF ACETYLATED TRYPSIN ON THE ACTIVATION OF A PLASMINOGEN–RED BLOOD CELL GHOSTS MIXTURE

To 0.3 ml plasminogen (300 μg in saline–phosphate buffer) was added 2.5 ml red blood cell ghosts suspension (1.89 mg protein) or 2.5 ml saline–phosphate buffer. After standing at 0–5 °C for 15 min, 0.2 ml of totally acetylated trypsin (15 μg in 1.0 mM HCl) was added to each mixture. Control mixtures containing 15 μg acetylated trypsin plus 2.5 ml ghosts, 15 μg acetylated trypsin plus saline–phosphate buffer, or 15 μg trypsin plus saline–phosphate buffer were also prepared. The mixtures were allowed to stand at room temperature. At 5, 15, 30, 60, 90 and 120 min, 0.1 ml of each mixture was withdrawn and tested for caseinolytic activity as described in the text. The significance level was calculated using a *t*-test with respect to the acetylated trypsin+plasminogen mixture.

Preparation	Activation time (min)	Number of observations	Precipitable casein solubilized per h (mean ± S.E.) (mg)	Significance level	Per cent of control activity
Acetylated trypsin+plasminogen	5	6	0.68 ± 0.013		100.0
	15	6	0.74 ± 0.030		100.0
	30	6	0.88 ± 0.021		100.0
	60	6	1.01 ± 0.007		100.0
	90	4	1.06 ± 0.016		100.0
	120	4	1.06 ± 0.016		100.0
Addition of acetylated trypsin to a plasminogen–ghosts mixture	5	6	0.59 ± 0.008	$P < 0.001$	86.8
	15	6	0.62 ± 0.010	$0.001 < P < 0.01$	83.8
	30	4	0.71 ± 0.011	$P < 0.001$	80.7
	60	6	0.79 ± 0.011	$P < 0.001$	78.2
	90	4	0.91 ± 0.041	$0.001 < P < 0.01$	85.8
	120	4	0.90 ± 0.015	$P < 0.001$	84.9
Acetylated trypsin+buffer	5	4	0.54 ± 0.029		100.0
	15	4	0.47 ± 0.040		100.0
	30	4	0.46 ± 0.021		100.0
	60	4	0.45 ± 0.023		100.0
	90	4	0.38 ± 0.020		100.0
	120	4	0.30 ± 0.011		100.0
Acetylated trypsin+ghosts	5	4	0.50 ± 0.007		92.6
	15	4	0.50 ± 0.008		106.4
	30	4	0.50 ± 0.017		108.7
	60	4	0.50 ± 0.010		111.1
	90	4	0.52 ± 0.010		136.8
	120	4	0.49 ± 0.014		163.3

been the subject of intense investigation, the possibility of the selective binding of these enzymes to the erythrocyte surface has received little attention. The objective of this study was to determine whether there may be a functional relationship between proteases found in the blood, particularly those of the fibrinolytic system, with erythrocyte membranes.

Previous investigations have indicated that the activation of plasminogen to plasmin by urokinase is an enzymatic transformation following first order kinetics

[8]. Although plasmin and plasminogen are bound to the membranes, no detectable binding of urokinase is observed. Neither prior incubation of plasminogen with membranes nor prior incubation of urokinase with membranes affect the ability of urokinase to activate plasminogen. The hydrolysis of casein by plasmin is also not affected by the membranes. In addition to the binding of plasminogen and plasmin to membranes, the binding of trypsin was also confirmed non-enzymatically with [^{3}H]trypsin. Since the enzymes and ghosts are in a relatively dispersed state in the 2% suspensions employed, attraction and binding between the enzymes and membranes must be the dominant feature, in contrast to random trapping during centrifugation.

The charge characteristics of the membranes and enzymes should also be considered. We have previously suggested the possibility that an ionic interaction between the negatively charged membranes [17] and the positively charged trypsin, chymotrypsin and chymotrypsinogen molecules may contribute to the overall binding of the enzymes to the membranes [1]. In view of the observation that plasmin and plasminogen are also adsorbed to membranes, while reported to have isoelectric pH values ranging from 6.3–8.6 [18–20], thereby being potentially negatively charged at pH 7.6, factors other than charge difference may play an essential role in the binding. The actual mechanism involved in the binding must be of sufficient strength to overcome the repulsion effect due to the like charges of the enzymes and membranes. Intercellular entrapment including that resulting from enzyme–substrate interactions on the inner surface of the membranes is another potential means by which proteins may become associated with the sediment fraction. However, evidence counterindicates these mechanisms since acetylated trypsin is apparently not "trapped" by the membranes while the inactive precursors of chymotrypsin [1] and plasmin are adsorbed to the membranes at levels equivalent to the active enzymes.

Trypsin has been observed to activate plasminogen in a manner comparable to that of urokinase [8]. Since membranes have been observed to adsorb trypsin as well as plasminogen, it seemed desirable to study the activation of plasminogen by trypsin in the presence of ghosts. A stabilization of trypsin to inactivation was observed over the 2-h activation periods at room temperature. In the process of stabilizing trypsin to inactivation, membranes inhibit the activation of plasminogen by trypsin. Inhibition of activation was observed regardless of whether trypsin or plasminogen was initially incubated with (and allowed to bind with) membranes. Since trypsin is known to hydrolyze ghosts [21,22], the membranes may serve as a preferential substrate for trypsin and thus act as a competitive inhibitor to plasminogen activation. In contrast to the previous suggestion that a lack of localization of the zymogen to the membranes may account for inhibition of the tryptic activation of trypsinogen [1], plasminogen and trypsin are both localized (bound) at the membranes while inhibition is still observed. The effect of the binding of the enzyme and zymogen to the membranes in a manner other than an enzyme–substrate association might also cause the observed inhibitions. This could include physical separation of the enzyme and zymogen or alterations in conformation in one or both of the molecules due to adsorption which interferes with activation.

We have previously reported that totally acetylated trypsin does not bind to erythrocyte ghosts nor is it considerably inhibited in their presence [1]. We now report that acetylated trypsin also retains its ability to activate plasminogen. It was

postulated that if the binding of trypsin to membranes limited its ability to activate plasminogen, the ability of acetylated trypsin to affect activation should not be effected since it is not bound to the membranes. However inhibition of plasminogen activation by acetylated trypsin was observed regardless of whether plasminogen or acetylated trypsin is initially added to membranes. These results suggest that the binding of plasminogen to membranes may sufficiently alter the plasminogen conformation or mask a susceptible site of attack by acetyl trypsin to inhibit activation.

The most striking comparison between the effects of trypsin, acetylated trypsin, and urokinase on plasminogen relates to the apparent specificities of binding and inhibition whereby plasminogen is readily activated by urokinase in the presence of ghosts while trypsin and acetylated trypsin do not affect full activation under similar conditions.

In order to study the possible effects that the binding of plasminogen might have on the binding of trypsin to the membranes, [^{3}H]trypsin and plasminogen were each incubated with ghosts prior to the addition of the other protein. The presence of plasminogen in the ghosts suspension resulted in increased adsorption of [^{3}H]trypsin to the membranes. Trypsin and plasminogen might well be bound at different sites on the membranes although supportive data for this suggestion is not yet available. Maximum potential binding of [^{3}H]trypsin could occur since there would be no competition for binding sites. The increased binding of [^{3}H]trypsin to the membranes observed in the presence of plasminogen may be the result of additional binding of the enzyme to bound plasminogen.

Conclusive evidence of specific changes in protein conformation and structure occurring during isolation of erythrocyte membranes has not been attained. Therefore it seems desirable to consider possible physiological consequences of the observations presented herein. Various investigations [2,3,23] have suggested that control of the fibrinolytic system is very important in maintaining normal body metabolism. Since red blood cells comprise 45% of the blood volume [24], it seems highly likely that interaction between blood proteases and the large membrane surface area represented by the erythrocytes does occur though the mechanism is unknown at this time. It is of particular interest that much of the plasminogen content of blood has been reported to be trapped in the clot during coagulation [2]. Part of this localization may possibly be due to plasminogen being associated with the red cells which are also trapped during clot formation. Thus the binding of proteases might be important in transport and localization of the enzymes in the blood as well as control of activity. Naturally the investigations reported herein must be extended to include whole bovine and human cells prior to extrapolation of the results to the in situ conditions.

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