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INTERACTION BETWEEN PROTEASES AND BOVINE ERYTHROCYTE MEMBRANES

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SUMMARY

1. Bovine erythrocyte ghosts bind trypsin, chymotrypsin, [^{3}H]chymotrypsin and chymotrypsinogen, while exhibiting no consequential binding of trypsinogen or totally acetylated trypsin.

2. In the presence of erythrocyte ghosts, the tryptic activation of trypsinogen and the tryptic hydrolysis of benzoyl-DL-arginine-β-naphthylamide is inhibited, whereas the tryptic and chymotryptic hydrolysis of casein and the tryptic activation of chymotrypsinogen are not inhibited.

3. A 2-fold increase over expected casein hydrolysis upon activation of chymotrypsinogen A by trypsin in the presence of ghosts is observed in combined supernatant and sediment fractions after centrifugation, suggesting the presence of chymotrypsin of a higher magnitude of activity than α-chymotrypsin.

4. A selectivity in substrate discrimination is suggested by the ability of ghosts to preferentially bind chymotrypsinogen while having little effect on trypsinogen and by the ability of trypsin to preferentially activate chymotrypsinogen in the presence of ghosts.

5. A role for the regulation of physiological events by membranes in binding proteases is suggested.

INTRODUCTION

Particulate inhibitors of the tryptic hydrolysis of benzoyl-DL-arginine-β-naphthylamide (BANA) have been reported in rat liver[1], kidney homogenates[2], and normal human and bovine brain homogenates[3]. The binding and inhibition of the chymotryptic hydrolysis of glutaryl-L-phenylalanine-β-naphthylamide (GPNA) and casein has been reported in human and bovine brain cell fractions as well as erythrocyte membranes[4]. It has been suggested that the binding and inhibition of proteases by membranes may serve in part to regulate proteolytic activity in the cell, thus potentially serving an important function in the overall control mechanisms of enzymatic activity[4].

Abbreviations: BANA, benzoyl-DL-arginine-β-naphthylamide; GPNA, glutaryl-L-phenylalanine-β-naphthylamide.

Several soluble proteolytic inhibitors have been reported in blood, many neutralizing more than one proteolytic enzyme. The α_1-antitrypsin of Bundy and Mehl[5] has been shown to inhibit trypsin, chymotrypsin, plasmin, plasma kallikrein, and elastase, although α_1-antichymotrypsin neutralizes only chymotrypsin (N. Heimburger, H. Haupt and H. G. Schwick, personal communication). A delicate balance must be maintained between the proteolytic enzymes and inhibitors of the blood clotting system to maintain normal blood flow. The inactivation of trypsin and chymotrypsin may be particularly important since trypsin has been reported to activate blood clotting factor X (ref. 6), prothrombin[7], and plasminogen[8], while α-chymotrypsin has been reported to activate plasminogen[8]. Since the action and interaction of several inhibitors is necessary to control blood clotting it seems possible that inactivation of enzymes by binding to ghosts may be important, and that it may provide a rapid available inhibitory mechanism under circumstances where high levels of proteases might be present.

The purpose of this study is to explore the possibility of the binding and inhibition of proteolytic enzymes by erythrocyte membranes as a means of suggesting how membranes may affect hydrolytic activity involved in the formation and dissolution of clots. A preliminary report has appeared elsewhere[9].

MATERIALS AND METHODS

Crystalline trypsin (2 ×), chymotrypsin (3 ×), trypsinogen (1 ×), chymotrypsinogen (5 ×) and [^{3}H]chymotrypsin (6 ×) were obtained from Worthington Biochemical Corp., Freehold, N. J. Crystalline acetylated trypsin (1 ×) and BANA ("Mann Assayed" grade) were purchased from Schwarz/Mann Research Laboratories, Orangeburg, N.Y. Hammersten casein was prepared by the method of Moll *et al.*[10].

Bovine red blood cell ghosts were prepared by the method of Green *et al.*[11]. Protein determinations of ghosts were performed by the method of Lowry *et al.*[12] after solubilization with 0.25 % sodium deoxycholate.

Assay of trypsin activity with BANA

To 10 μl of trypsin (10 μg in 1 mM HCl) was added sufficient 0.1 M phosphate buffer, pH 7.8, to give a total volume of 2.0 ml. 1 ml of BANA (0.8 mg in 0.1 M phosphate buffer, pH 7.8) was added thereto. After incubation at 37 °C for 30 min in a shaking water bath, the reaction was stopped with 1.5 ml of 40 % trichloroacetic acid and the mixtures were centrifuged at 27000 × *g* for 15 min. The β-naphthylamine liberated was determined in the trichloroacetic acid supernatant fractions by the Bratton–Marshall[13] reaction, as described by Blackwood and Mandl[1].

Assay of trypsin, totally acetylated trypsin, and chymotrypsin activity with casein

Aliquots of enzyme, generally 0.4–1.0 μg, were diluted to 1 ml with 0.1 M phosphate buffer, pH 7.6. 1 ml of 2 % casein was added thereto, and the mixture was incubated at 37 °C for 30 min after which time, the reaction was terminated with 4.0 ml of 2.5 % trichloroacetic acid. The increase in phenolic tyrosine residues in the trichloroacetic acid supernatant fraction was determined according to the procedure of Lowry *et al.*[12].

Determination of binding and inhibition of trypsin, chymotrypsin, and totally acetylated trypsin

To two 10-μl aliquots of trypsin (5 μg in 1.0 mM HCl) were added 1.99 ml of ghosts (1.13 mg protein). After standing at 0–5 °C for 15 min, one aliquot was centrifuged at 27000 × *g* for 15 min. The sediment was taken up in 0.1 M phosphate buffer, pH 7.8. The mixture, supernatant and sediment fractions were tested for activity on BANA as described above.

To 10-μl aliquots of each of the enzymes (10 μg in 1 mM HCl) was added 3.99 ml of ghosts (2.26 mg protein). After standing at 0–5 °C for 15 min, a 1 ml aliquot was removed and the remainder was centrifuged at 27000 × *g* for 15 min. The mixture, supernatant and resuspended sediment fractions were tested for caseinolytic activity as described above.

Binding and dissociability of [^{3}H]chymotrypsin

To two 0.1-ml aliquots of [^{3}H]chymotrypsin (10 μg in 1 mM HCl) were added 3.90 ml of ghosts (2.21 mg protein) or 3.90 ml of 0.1 M Tris–HCl buffer, pH 7.6. The ghosts–[^{3}H]chymotrypsin mixtures were allowed to stand at 0–5 °C for 15 min, after which time they were centrifuged at 27000 × *g* for 15 min. The sediment was resuspended in Tris buffer.

Equivalent volumes of supernatant and sediment were added to 15.0 ml Aquasol and the radioactivity was measured in a Packard Tri-Carb scintillation spectrometer (Model No. 3375).

Binding and inhibition of the tryptic activation of trypsinogen and chymotrypsinogen

To 0.1 ml of zymogen (100 μg in 1 mM HCl) was added 3.82 ml ghosts (2.17 mg protein) and 0.08 ml of 0.05 M $CaCl_2$. After standing at 0–5 °C for 15 min, a 1-ml aliquot was removed and the remainder was centrifuged at 27000 × *g* for 15 min. The sediment was taken up in 0.1 M Tris–HCl buffer, pH 7.6, containing 0.01 M $CaCl_2$. To 1 ml of the mixture, supernatant, sediment and control trypsinogen or chymotrypsinogen (0.1 ml zymogen + 0.08 ml $CaCl_2$ + 3.82 ml of 0.1 M Tris–HCl buffer, pH 7.6) was added 25 μl trypsin (25 μg in 1 mM HCl) and the mixtures were allowed to stand at room temperature. At 2, 4 and 6 h intervals, 0.02 ml of each mixture was withdrawn and tested for caseinolytic activity as previously described.

RESULTS

The data in Table I indicate that the presence of higher levels of erythrocyte ghosts effect an increasing inhibition of the tryptic hydrolysis of BANA. While the hydrolysis of the model substrate is impaired in the presence of ghosts, the hydrolysis of casein by trypsin is not affected materially (Table II). It is also seen that trypsin is bound with the membranes since they may be centrifuged and the major portion of the tryptic activity in a trypsin–ghosts mixture may be found associated with the particulate fraction. Chymotrypsin behaves in an analogous manner to trypsin in that essentially no significant inhibition of caseinolytic activity by the enzyme is observed in the presence of ghosts (Table III). Furthermore, chymotrypsin distributes itself approximately evenly between the supernatant fraction and the packed membranous sediment which occupies less than 2 % of the total volume (before resuspension). The

TABLE I

INHIBITION OF THE TRYPTIC HYDROLYSIS OF BENZOYL-DL-ARGININE-β-NAPHTHYLAMIDE BY BOVINE RED BLOOD CELL GHOSTS

To 10 μg trypsin was added various volumes of bovine red blood cell ghosts suspension and 0.1 M phosphate buffer to a volume of 2.0 ml. After standing 15 min at 0–5 °C, 1 ml of BANA substrate was added. The mixture was incubated at 37 °C in a shaking water bath for 30 min, after which time 1.5 ml of 40% trichloroacetic acid was added to stop the reaction. The β-naphthylamine liberated was determined in the trichloroacetic acid supernatant fraction by the Bratton–Marshall reaction.

Addition to 10 μg trypsin	*nmoles of β-naphthylamine released*	*% of control activity*
	45.4	100.0
Erythrocyte ghosts, 0.11 mg	49.5	108.9
0.23 mg	42.3	93.1
0.45 mg	41.9	92.1
0.68 mg	35.6	78.2
0.91 mg	26.1	57.4
1.13 mg	28.4	62.4

TABLE II

BINDING AND INHIBITION OF TRYPSIN BY BOVINE RED BLOOD CELL GHOSTS

To 10-μl aliquots of trypsin (5 μg in 1 mM HCl) was added 1.99 ml of bovine red blood cell ghosts suspension (1.13 mg protein). After standing at 0–5 °C for 15 min, one aliquot was centrifuged at 27000 × *g* for 15 min. The sediment was taken up in 0.1 M phosphate buffer, pH 7.8. The mixture, supernatant and sediment fractions were tested for tryptic activity with BANA, as described in the text. To 10 μl of trypsin (10 μg in 1 mM HCl) was added 3.99 ml of bovine red blood cell ghosts suspension (2.26 mg protein). After standing at 0–5 °C for 15 min, a 1 ml aliquot was removed and the remainder was centrifuged at 27000 × *g* for 15 min. The sediment was taken up in 0.1 M phosphate buffer, pH 7.6. The mixture, supernatant and sediment fractions were tested for activity with casein, as described in the text.

Preparation	*Hydrolysis of benzoyl-DL-arginine-β-naphthylamide*		*Casein hydrolysis*	
	nmoles of β-naphthylamine liberated	*% of control activity*	*mequiv of tyrosine solubilized, × 10³*	*% of control activity*
Trypsin	23.85	100.0	1.15	100.0
Trypsin + erythrocyte membranes	9.45	39.6	1.22	106.1
Supernatant after centrifugation	2.70	11.3	0.29	24.9
Sediment after centrifugation	11.25	47.2	0.72	62.3

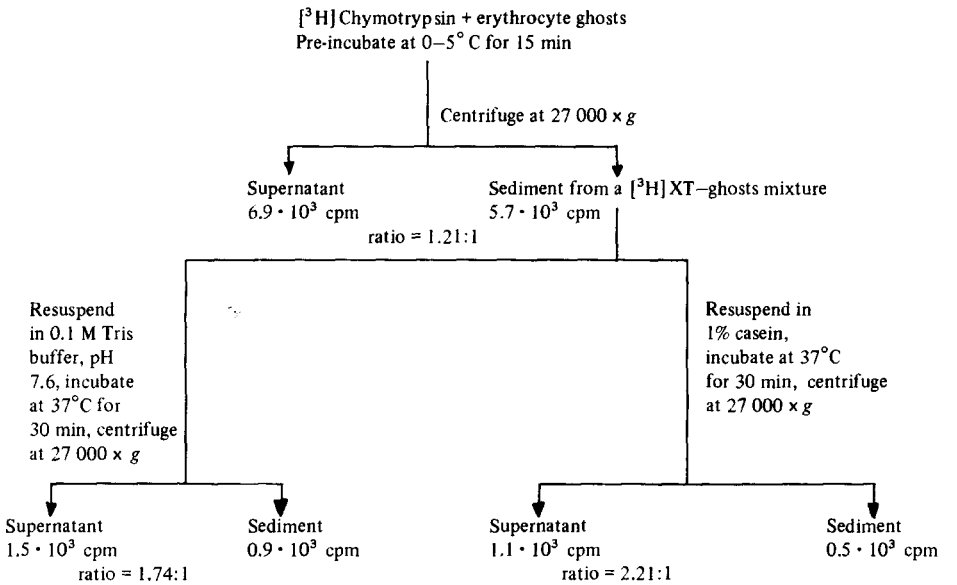
binding of chymotrypsin to ghosts is also confirmed non-enzymatically in counting the distribution of tritiated chymotrypsin (Table III). Although an initial distribution of chymotrypsin between particulate and supernatant fractions varying from 1:1 to as much as 1.2:1 may be observed, the enzyme more readily dissociates from the membranes in the presence of added casein (to a 1 % final concentration) since a ratio of 1100 cpm to 500 cpm is obtained for supernatant and sediment fractions, respectively (2.2:1). The bound enzyme does not quite as readily dissociate when resuspended in Tris buffer (Scheme 1).

TABLE III

BINDING OF CHYMOTRYPSIN BY BOVINE RED BLOOD CELL GHOSTS

To 0.01 ml chymotrypsin (10 μg in 1 mM HCl) was added 3.99 ml of bovine red blood cell ghosts suspension containing 2.26 mg protein. After standing at 0–5 °C for 15 min, an aliquot of the mixture was removed, and the remainder was centrifuged at 27 000 × *g* for 15 min. The sediment was taken up in phosphate buffer. The mixture, supernatant and sediment fractions were tested for activity with casein, as described in the text. To 3.90 ml of ghosts (2.21 mg protein) was added 0.1 ml of [^{3}H]chymotrypsin (10 μg in 1 mM HCl). The mixture was treated as outlined above and aliquots of supernatant and sediment fractions were added to 15.0 ml of Aquasol and counted in a Packard TriCarb scintillation counter.

Preparation	*mequiv tyrosine solubilized/h per μg chymotrypsin, × 10^3*	*% of control activity*	*cpm of chymotrypsin, × 10^{-3}*	*Corrected total % cpm*
Chymotrypsin	1.84	100.0		
Chymotrypsin + erythrocyte ghosts	1.60	86.5		
Supernatant after centrifugation	0.90	48.8	2.2	51.1
Sediment after centrifugation	1.07	58.6	2.1	48.9



Scheme 1. Dissociability of [^{3}H]chymotrypsin from erythrocyte membranes. XT = chymotrypsin.

Totally acetylated trypsin, which retains its enzymic activity upon acylation of the ε-amino and phenolic hydroxy residues, does not effectively bind to ghosts nor is it considerably inhibited in their presence, as determined using casein as a substrate (Table IV). The tryptic activation of trypsinogen is inhibited by the erythrocyte membranes, as noted in Table V. It is further observed that more than two thirds of the potential tryptic activity in the mixture is found in the supernatant fraction upon centrifugation of the mixture, suggesting that most of the trypsinogen does not bind

TABLE IV

BINDING OF ACETYLATED TRYPSIN BY BOVINE RED BLOOD CELL GHOSTS

To 10 μl acetylated trypsin (10 μg in 1 mM HCl) was added 3.99 ml of bovine red blood cell ghosts suspension containing 2.26 mg protein. After standing at 0–5 °C for 15 min, an aliquot of the mixture was removed, and the remainder was centrifuged at 27000 × *g* for 15 min. The sediment was taken up in phosphate buffer. The mixture, supernatant and sediment fractions were tested for activity with casein, as described in the text.

Preparation	*mequiv tyrosine solubilized/h per μg trypsin, × 10^3*	% *of control activity*
Trypsin	1.69	—
Acetylated trypsin, alone	1.22	100.0
Acetylated trypsin + erythrocyte ghosts	0.96	78.8
Supernatant after centrifugation	0.98	80.8
Sediment after centrifugation	0.17	13.6

TABLE V

INHIBITION OF THE TRYPTIC ACTIVATION OF TRYPSINOGEN BY BOVINE ERYTHROCYTE GHOSTS

To 0.10 ml trypsinogen (100 μg in 1 mM HCl) was added 3.82 ml red blood cell ghosts suspension containing 2.17 mg protein and 0.08 ml of 0.05 M $CaCl_2$. After standing at 0–5 °C for 15 min, an aliquot of the mixture was removed, and the remainder was centrifuged at 27000 × *g* for 15 min. The sediment was taken up in Tris–HCl buffer. To 1 ml of mixture, supernatant, sediment and control trypsinogen was added 0.025 ml trypsin (25 μg in 1 mM HCl) and the mixtures allowed to stand at room temperature. At 2, 4 and 6 h, 20 μl of each mixture was withdrawn and tested for activity using casein as substrate, as described in the text.

Preparation	*Activation time (h)*	*mg casein solubilized per h*	*μg active trypsin*	*μg increase in trypsin due to activation of trypsinogen*	% *increase in tryptic activity*
Trypsin	0	2.71	0.49	—	—
Trypsinogen + trypsin	2	6.07	1.09	0.60	123.5
	4	7.37	1.33	0.84	172.5
	6	3.40	0.61	0.12	25.4
Trypsinogen + ghosts + trypsin	2	3.70	0.67	0.18	36.5
	4	3.75	0.68	0.19	38.13
	6	3.85	0.69	0.21	42.0
Supernatant after centrifugation	2	5.89	1.06	0.57	117.2
	4	7.10	1.28	0.79	162.3
	6	3.60	0.65	0.16	32.8
Sediment after centrifugation	2	4.11	0.74	0.25	51.6
	4	4.21	0.76	0.27	55.3
	6	3.50	0.63	0.14	29.1

to ghosts. In the absence of ghosts, under the experimental conditions employed, trypsinogen appears to be activated within 4 h, after which time autolysis occurs.

In contrast to the inhibition of the tryptic activation of trypsinogen by ghosts over a 6-h period, the particulate membranes permit activation of chymotrypsinogen

A after a short delay (Table VI). Upon centrifugation of chymotrypsinogen–ghosts mixtures and activation of supernatant and sediment fractions with trypsin, considerably higher caseinolytic activity is obtained in the combined fractions than is expected, suggesting that the activity represented in the mixture might possibly be that of a form of chymotrypsin: either π or δ which is bound to membranes and inhibited by them, but is capable of partially dissociating from them in a centrifugal field. The authors are at a loss to explain the phenomenon of repeatedly lower caseinolytic activity in the supernatant fraction at 60 min after initiation of activation of chymotrypsinogen by trypsin.

TABLE VI

THE TRYPTIC ACTIVATION OF CHYMOTRYPSINOGEN

To 0.10 ml chymotrypsinogen A (100 μg in 1 mM HCl) was added 3.82 ml of erythrocyte ghosts (2.17 mg protein) suspension and 0.08 ml of 0.05 M $CaCl_2$. 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 taken up in 0.1 M Tris buffer, pH 7.6, containing 10 mM $CaCl_2$. To 1 ml of mixture, supernatant and sediment fractions, as well as control chymotrypsinogen, was added 0.025 ml trypsin (25 μg in 1 mM HCl). The mixture was allowed to pre-incubate at room temperature. Aliquots of 20 μl were taken at the indicated times for assay of proteolytic activity, using casein as the substrate.

Preparation	*Activation time (min)*	*mg casein solubilized per h*	*Increase in casein hydrolyzed over that of added trypsin*	*% increase*
Trypsin (0.49 μg)	0	3.47	—	—
Chymotrypsinogen + trypsin	30	5.66	2.19	63.1
	60	5.66	2.19	63.1
	90	5.88	2.41	69.5
Chymotrypsinogen + ghosts + trypsin	30	3.82	0.35	10.1
	60	6.32	2.85	82.1
	90	6.51	3.04	87.6
Supernatant + trypsin	30	7.06	3.59	103.5
	60	4.13	0.66	19.0
	90	6.76	3.29	94.8
Sediment + trypsin	30	6.63	3.16	91.1
	60	7.26	3.79	109.2
	90	3.87	0.40	11.5

In the course of studying the activation of trypsinogen and chymotrypsinogen by trypsin, it was observed that trypsin solutions which were freshly prepared in 1 mM HCl and diluted with 0.1 M Tris buffer, pH 7.6, containing 10 mM $CaCl_2$ as a stabilizing factor, were susceptible to 50 % inactivation upon standing at room temperature for less than 2h. In contradistinction, trypsin which was prepared in 1 mM HCl and was refrigerated for 7–11 days prior to dilution with Tris buffer in a similar manner, remained quite stable in activity for more than 4 h at room temperature (Table VII). These data appear to suggest the possibility that trypsin which is stored refrigerated in 1 mM HCl for a period of time might assume a more stable conformation prior to any presumed subsequent alterations as a result of a pH change on dilution with Tris buffer.

TABLE VII

EFFECT OF STORAGE OF TRYPSIN SOLUTIONS ON THE STABILITY OF TRYPSIN TO AUTOLYSIS

50 μl trypsin (50 μg in 1 mM HCl) stored refrigerated for 7–11 days and 50 μl of freshly prepared trypsin (50 μg in 1 mM HCl) were diluted to 1.025 ml with 0.1 M Tris–HCl buffer, pH 7.6, containing 0.01 M $CaCl_2$. The mixtures were then allowed to stand at room temperature. At 0, 2, 4 and 6 h, 0.98 μg of trypsin was removed and tested for activity using casein as a substrate, as described in the text.

Preparation	*Time standing at room temperature (h)*	*mg casein solubilized/h per 0.98 µg trypsin*	*% retention of activity*
Trypsin, freshly prepared in 1 mM HCl	0	6.63	100
	2	3.39	51.1
	4	3.58	54.0
	6	3.12	47.0
Trypsin, prepared in 1 mM HCl and stored refrigerated for 7–11 days	0	6.32	100
	2	6.15	97.4
	4	6.40	101.3
	6	3.02	47.8

DISCUSSION

Proteolytic degradation of the erythrocyte membrane has been extensively used to characterize its component proteins. The sialoglycoprotein components of right side out vesicles of erythrocyte membranes are readily attacked by trypsin and papain and exhibit a partial resistance to attack by chymotrypsin. Trypsin, papain, and chymotrypsin also readily digest sialoglycoproteins of intact erythrocytes[14]. Sialic acid has been shown to be localized on the outer surface of the membrane and to be mainly responsible for the negative charge of the outer surface of the membrane[15]. In contrast to the effect of proteolytic enzymes on erythrocyte membranes, this study was conducted to determine the effect on the availability of proteases for hydrolytic activity in the presence of ghosts.

The results presented herein show a definite interaction and affinity of bovine red blood cell ghosts for proteases and zymogens. Although the tryptic hydrolysis of trypsinogen and the model substrate BANA was inhibited in the presence of ghosts, the tryptic activity toward chymotrypsinogen and casein was not significantly affected, nor was the chymotryptic hydrolysis of casein. The lag in the tryptic activation of chymotrypsinogen may simply reflect a competition between sialoglycoprotein on the membrane surface and the chymotrypsinogen for the binding or active site of trypsin. The most intriguing and potentially significant results were those showing the ability of ghosts to preferentially bind chymotrypsinogen A as opposed to trypsinogen and the ability of trypsin to preferentially activate chymotrypsionogen A in a zymogen–ghosts mixture. Since trypsin is readily bound to ghosts, the binding of chymotrypsinogen may very well help to localize the zymogen in the general area of the highest enzyme concentration. Interaction between trypsin and membranes and between chymotrypsinogen and membranes may be looked upon as equilibrium reactions which favor association of the proteins with the membranes. In so doing,

there occurs a localization of these proteins in the neighborhood of the membranes. Dissociation of these proteins would result in a situation whereby interplay of trypsin with chymotrypsinogen would be favored as a result of their close proximity, in contrast to considerably greater intermolecular distances in solution in the absence of membranes. It is also possible that membrane-bound trypsin might act catalytically upon a substrate in a manner similar to the covalently bound, insolubilized trypsin[16]. In as much as trypsinogen is not as selectively bound to the membrane surface, it is more randomly distributed in the suspension and is thus less available to the bound trypsin as a substrate.

Abita *et al.*[17] have studied the kinetics of the activation of trypsinogen and chymotrypsinogen by trypsin and report similar K_m values for the hydrolysis of the Lys_6–Ile_7 bond of trypsinogen and the Arg_{15}–Ile_{16} bond of chymotrypsinogen A, namely 0.40 mM and 1.09 mM, respectively. MacLure and Edelman[18] noted a K_m for the chymotrypsinogen A activation of 0.56 mM, under slightly different conditions. On the other hand, the values for k_{cat} determined for trypsinogen and chymotrypsinogen are $2.5 \cdot 10^{-3}$ s^{-1} and 0.18 s^{-1}, respectively[17],indicating a much larger turnover of enzyme with chymotrypsinogen as a substrate as compared with trypsinogen. The data reported herein suggest that adsorption of trypsin to membranes does not materially affect the activation of chymotrypsinogen while apparently exerting a significant negative influence on the activation of trypsinogen. Either affinity of trypsin for the membranes or subtle conformation changes on the enzyme may account for this phenomenon. Indeed, covalently bound trypsin is no longer inhibited by soybean trypsin inhibitor while still retaining enzymic activity, as followed by esterolysis[16]. Such effects suggest that alterations in conformation will effect binding capabilities.

Trypsin contains 14 lysine residues, as well as 10 tyrosine residues[19]. The lack of significant binding of totally acetylated trypsin to membranes suggest the possible implication of ε-amino groups of lysine and phenolic hydroxy groups of tyrosine in the binding phenomenon. At pH 7.6, at which the interactions were studied, phenolic hydroxy groups would not be charged, but might be available to act in H-bonding. The proteases and zymogens studied would be positively charged, having protonated ε-amino groups, since the proteins have isoelectric pH values ranging from 8.1 for α-chymotrypsin[20] to 10.5 for trypsin[21] under various buffer conditions. The isoelectric point of erythrocyte membranes has been observed to be raised to pH 4–5 upon release of sialic acid[15], suggesting a strongly negatively charged surface at pH 7.6. These factors further suggest the possibility of an ionic interaction between the positively charged enzymes and the negatively charged membranes. The ε-amino groups of lysine have previously been implicated in binding mechanisms since acetylated trypsin is unresponsive to inhibition by the ovomucoid inhibitor, in contrast to native trypsin[22]. A similar essentiality for charged ε-amino groups of lysine in binding to membranes is offered here in view of the lack of binding of wholly acetylated trypsin to ghosts. However, the selectivity of the binding cannot be accounted for by the simple availability of the ε-amino groups for interaction. It has been observed that six of the thirteen ε-amino groups of both chymotrypsin and chymotrypsinogen are available for reaction with 1-fluoro dinitrobenzene[23] and that all 14 lysines of trypsin[24] and trypsinogen[25] have free amino groups available for reaction. Nonetheless only chymotrypsin, chymotrypsinogen and trypsin are bound (to the membranes) to a considerable extent. Although the data suggest that the ε-amino groups may be

involved in the binding phenomenon, other factors such as H-bonding between the phenolic hydroxy groups and the membranes should also be considered.

The 2-fold increase over expected caseinolytic activity found in combined supernatant and sediment fractions upon the addition of trypsin to a ghosts–chymotrypsinogen A mixture suggests possible binding of a chymotrypsin other than α-chymotrypsin. The activation of chymotrypsinogen has been shown to involve the formation of intermediates through the removal of dipeptides from the zymogen[26]. The initial intermediates are π- and δ-chymotrypsin which have been reported to have a higher activity than all other chymotrypsins[26]. A plausible explanation for the increased activity is that π- or δ-chymotrypsin is bound to the ghosts in a manner preventing further activation to α-chymotrypsin which has a lower enzymic activity. In the absence of ghosts, α-chymotrypsin would be the end product. There is no apparent reason for maintaining the chymotrypsins of higher activity in the supernatant fraction for a considerable time period.

As a consequence of these investigations, it would seem worthwhile to consider the physiological significance of selectivity in the capacity of erythrocyte membranes to bind enzymes. It has been observed that erythrocyte stroma can supply the same phosphatides as platelet factor 3 in promoting clot formation *via* the intrinsic pathway[27,28]. Phosphatides presumably promote the formation of complexes involving the phosphatides, blood coagulation proteins, and Ca^{2+}, the action of the phosphatides being dependent upon a micellar form and a negative Zeta potential[29–31]. This system is well adapted to the properties of the blood platelets since most blood coagulation proteins are loosely adsorbed on the platelet surface. Upon viscous metamorphis of the platelets at a site of trauma, the phosphatides are released thus being available for complex formation[32]. The involvement of erythrocyte membranes in this mechanism would seem to be a secondary effect due to incidental breakdown of the erythrocytes. However, in view of the fact that many coagulation factors seem to exhibit properties of proteolytic enzymes, the data herein may possibly suggest another role for the erythrocyte membrane, that is, as an anticoagulant. Continuous blood flow depends on an intricate balance between coagulant factors, fibrinolytic factors and inhibitors of these factors. It seems likely that coagulation enzymes would be bound at the interface of the highly ionic surface of the membrane, possibly in an analogous manner to the proteolytic enzymes studied. The binding of these enzymes may effectively reduce their concentration in the blood and help to maintain the balance of coagulants to anticoagulants. The mechanism may be particularly effective in situations of high levels of blood lipids or in cases of spontaneous tissue destruction such as vessel walls or platelets in which the coagulation mechanism could be triggered resulting in intravascular blood clotting. In view of the fact that trypsin activates blood coagulation, the role of membranes in regulating the tryptic hydrolysis of such specific substrates as factor X, prothrombin and plasminogen, and their consequent impact on the formation and dissolution of blood clots remains to be studied. A further outcome relates to the suggestion that outer membranes of particulate elements of cells and the endoplasmic reticulum might contribute to the regulation of overall hydrolytic activity in the cell by binding and immobilizing freely circulating hydrolases. Such a role for membranes would support the functions of soluble protease inhibitors as well as the mechanisms governing biosynthesis of enzymes and their inhibitors.

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REFERENCES

1 C. Blackwood and I. Mandl, *Anal. Biochem.*, 2 (1961) 370.
2 C. Blackwood, B. F. Erlanger and I. Mandl, *Biochemistry*, 12 (1965) 128.
3 A. S. Brecher and N. M. Quinn, *Biochem. J.*, 102 (1967) 120.
4 A. S. Brecher, B. B. Oliphant and V. P. Wasilauskas, Jr, *Arch. Int. Physiol. Biochem.*, 76 (1968) 287.
5 H. F. Bundy and J. W. Mehl, *J. Biol. Chem.*, 234 (1959) 1124.
6 E. T. Yin, *Thromb. Diath. Haemorrh.*, 12 (1964) 307.
7 R. H. Landaburu, M. I. Barnhart and W. H. Seegers, *Am. J. Physiol.*, 201 (1961) 298.
8 A. A. Hakim, *Enzymologia*, 40 (1971) 15.
9 A. S. Brecher and D. E. Burkholder, *Abstr. 3rd Congr., Int. Soc. Thrombosis and Haemostasis, Washington, D.C., Aug. 22–26, 1972.*
10 F. C. Moll, S. F. Sunden and J. R. Brown, *J. Biol. Chem.*, 233 (1958) 121.
11 D. E. Green, E. Murer, H. O. Hulton, S. H. Richardson, B. Salmon, G. P. Brierley and H. Baum, *Arch. Biochem. Biophys.*, 112 (1965) 635.
12 O. H. Lowry, N. J. Rosebrough, A. L. Farr and R. J. Randall, *J. Biol. Chem.*, 193 (1951) 265.
13 C. A. Bratton and E. K. Marshall, Jr, *J. Biol. Chem.*, 128 (1939) 537.
14 T. L. Steck, G. Fairbanks and D. F. H. Wallach, *Biochemistry*, 10 (1971) 2617.
15 E. H. Eylar, M. A. Madoff, O. V. Brody and J. L. Oncley, *J. Biol. Chem.*, 237 (1962) 1992.
16 Y. Levin, M. Pecht, L. Goldstein and E. Katchalski, *Biochemistry*, 3 (1964) 1905.
17 J. P. Abita, M. Delaage and M. Lazdunski, *Eur. J. Biochem.*, 8 (1969) 314.
18 W. O. MacLure and G. M. Edelman, *Biochemistry*, 6 (1967) 567.
19 I. Inada, M. Kamata, A. Matsushima and K. Shibata, *Biochim. Biophys. Acta*, 81 (1964) 323.
20 E. Anderson and R. Alberty, *J. Phys. Colloid Chem.*, 52 (1948) 1345.
21 L. Cunningham, Jr, *J. Biol. Chem.*, 211 (1954) 13.
22 H. Fraenkel-Conrat, R. S. Bean and H. Lineweaver, *J. Biol. Chem.*, 177 (1949) 385.
23 V. Massey and B. S. Hartley, *Biochim. Biophys. Acta*, 21 (1956) 361.
24 J. Labouesse and M. Gervais, *Eur. J. Biochem.*, 2 (1967) 215.
25 J. F. Pechere and H. Neurath, *J. Biol. Chem.*, 229 (1957) 389.
26 P. Desnuelle, in P. D. Boyer, H. Lardy and K. Myrbäck, *The Enzymes*, Vol. 4, Academic Press, New York, 1960, p. 106.
27 G. Y. Shinowara, *J. Biol. Chem.*, 225 (1957) 63.
28 F. Jobin and M. P. Esnouf, *Biochem. J.*, 102 (1967) 666.
29 A. D. Bangham, *Nature*, 192 (1961) 1197.
30 D. Papahadjopoulos, C. Hougie and D. J. Hanahan, *Proc. Soc. Exp. Biol. Med.*, 111 (1962) 412.
31 M. J. Silver, D. L. Turner, I. Rodalewicz, N. Giardano, R. Holburn, S. F. Herb and F. E. Luddy, *Thromb. Diath. Haemorrh.*, 10 (1963) 164.
32 E. F. Mammen, in N. U. Bang, F. K. Beller, E. Deutsch and E. F. Mammen, *Thrombosis and Bleeding Disorders*, Academic Press, New York, 1971, p. 45.