

PROTEOLYTIC ENZYMES AND PLATELETS IN RELATION TO BLOOD COAGULATION¹

By BURTON L. TRAVIS AND JOHN H. FERGUSON

(From the Department of Physiology, University of North Carolina, Chapel Hill)

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Ferguson's (1, 2) concept of a role for proteolytic enzymes (3) in the mechanisms of blood clotting was that of a weak digestive action "disaggregating" (Pope, 1938) the complex unions of plasma proteins with calcium and thromboplastic phospholipid (probably cephalin), thereby mobilizing or making available these activators and thus, in a sense, "catalyzing" the prothrombin activation (4). Observations believed to support such a theory included: 1) the clot-aiding effects, particularly referable to the conversion of prothrombin into active thrombin, on adding (crystalline) trypsin (2, 4); and 2) the evidence, not altogether conclusive (4) nor confirmed (5-7), that preparations of serum protease (tryptase, plasmin, or fibrinolysin) resemble pancreatic trypsin in many ways although differing significantly in others (3).

The establishment of crucial data in this, as in all work on the problem of the conversion of prothrombin to thrombin, is technically difficult because of the biochemical complexities, the presence in the natural clotting system (*e.g.* plasma) of unrecognized factors (some of which have recently been revealed), and questions as to contaminants (the known ones being demonstrable) in even the purest of the isolated reagents available to date (4). From the start (1939) of our work with the enzyme problem we have accepted the current, and still valid, working hypothesis that, under all ordinary ("physiological") circumstances, prothrombin requires for its activation free Ca-ions and a phospholipid-containing material for which the terms "thromboplastin" or "thrombokinas" are commonly used. It has more recently been shown by a number of workers that other factors are also involved and evidently must have been unwittingly provided in the test materials of all early experiments. Our own observations (8)

that, with very carefully purified and treated reagents, no thrombin could be detected in over 24 hours in mixtures of prothrombin, brain thromboplastin, and calcium salt, but required the addition of another factor or factors which could be supplied in the form of Ware and Seegers' (9) "serum accelerator globulin" (AcG) preparation, caused us to add this reagent to the purified test systems in the present studies. Under these circumstances, as in the past, we feel that our studies are concerned primarily with the basic "thromboplastic" mechanisms.

The present work commences with the confirmation of the clot-aiding effects of trypsin and of serum fibrinolysin on ordinary plasma and supplements this with an experiment on platelet-free plasma. Proceeding thence to the study of thrombin formation from highly purified prothrombin (with added AcG), a number of enzyme preparations, activators, and inhibitors have been tested, in various combinations, in an effort to shed further light upon the possible mechanisms of action of the proteolytic enzymes in such clotting systems. The thromboplastic role of platelets in relation to these phenomena has been given special attention. The weight of evidence eventually leads to the conclusion that whereas trypsin does seem to act with parallelism of its clot-aiding and proteolytic effects, serum fibrinolysin can still aid thrombin formation (in these systems) even when the proteolytic action is reduced greatly or abolished by appropriate enzyme inhibitors. This must suggest another factor in the serum enzyme preparation, the possible nature and mode of action of which are investigated and discussed.

MATERIALS

1. Borate *buffer* solution, pH = 7.75, specific resistance 170 ohms (at 21°C), effective ionic strength 0.055, contained (per liter): 11.25 gm. H_2BO_3 , 4 gm. $Na_2B_4O_7$, 12 H_2O , 2.25 gm. NaCl.
2. *Plasma*, oxalated or citrated, was prepared from dog or beef blood. In special studies, cited in the text,

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- (a) "deplateletized" plasma (PFP) was prepared by the silicone technic (10) and long centrifugation (multispeed) in the refrigerated centrifuge; (b) "defibrinated" plasma (FFP) was obtained by heating at 51°C for one-half hour and centrifugation; (c) "prothrombin-free" plasma (S.P.I.) was obtained by five successive filtrations through Seitz pads, according to the method of Owren (11).
3. *Serum*, obtained from beef blood, was rendered prothrombin-free by adsorption with BaSO₄ (12).
4. *Fibrinogen*: (F₁) 0.5–1.0% solution in borate buffer of Armour's bovine fibrinogen (Fraction I). This was free from fibrinolysin but contained small traces of prothrombin which, for many tests, was removed by BaSO₄ adsorption (F₂).
5. Purified *prothrombin* (Pro), bovine, preparations of Dr. W. H. Seegers (Wayne University), stock solution (0.1%) in borate buffer. The solutions used in most of the cited experiments contained an insignificant trace of thrombin (clotting times with F₂ > 30 min.) and some "accelerator" factor(s), but the latter was always supplemented with added AcG.
6. *Accelerator globulin* (AcG), bovine "serum type," preparation of Drs. A. G. Ware and W. H. Seegers, stock solution (0.1%) in borate buffer. It contained traces of prothrombin and a minute amount of thrombin, considered non-significant in the dilutions employed for the cited experiments.
7. *Thromboplastins*: Tpln. A, a highly active thromboplastin prepared from acetone-dried dog brain; Tpln. B, Schieffelin's stabilized (horse brain) thromboplastin, courtesy Drs. E. W. Blanchard and C. F. Gerber.
8. *Fibrinolysin* (Lysin): purified preparation from beef serum, courtesy Dr. E. C. Loomis (Parke Davis Co., Detroit). 1% stock solution freshly prepared immediately before use.
9. *Antifibrinolysin* (AFIn): purified bovine preparation, courtesy Dr. Loomis.
10. *Trypsin* (pancreatic): (Tryp.), commercial preparation (Fairchild Bros., Foster, N. Y.); *Crystalline trypsin* (XT), courtesy Dr. M. Kunitz (Rockefeller Institute, Princeton).
11. *Soybean inhibitor* (antiprotease): crystalline preparation (SBI), courtesy Dr. Kunitz.
12. *Pancreatic inhibitor* (antiprotease) (PI): Sharp and Dohme (Lot P. A-97-1), courtesy Dr. L. A. Kazal.
13. *Washed platelets* (Plat.): 100 ml. dog blood collected into 13 ml. 3.4% sod. citrate. Repeated centrifuging for a few minutes at 1000 r.p.m. until red cells no longer visible in sediment. Platelets then recovered by one-half hour centrifuging at 5000 r.p.m., resuspended and well washed three times by thorough mixing with successive 50 ml. saline (containing 1/8 vol. 3.4% citrate) and a fourth washing in plain 0.9% NaCl. Final sediment suspended in 1 ml. saline gave a concentrated preparation, Wright-stained smears of which showed only granular platelet material with no leukocytes and only a rare erythrocyte. Grinding the platelet sediment with distilled water on a number of

occasions did not modify its behavior in our test systems. The thoroughness of the washing is believed satisfactory for the virtual absence of plasma contaminants.

14. *Thrombin* (Thr.): bovine thrombin (Upjohn's), courtesy Dr. J. T. Correll. Stock solution in borate buffer (100 u./ml.), diluted as required. For the tests of Table VII a special preparation was made and used as described in the text.

Effects of Fibrinolysin on Clotting of Recalcified Plasma

In numerous experiments on oxalated plasma (dog: beef), optimally recalcified, Loomis' fibrinolysin consistently reduced the clotting-times, e.g. beef plasma (26°C): 1) control, 330 sec.; 2) shorter clotting-times, with increasing lysin, to 135 sec. "optimum" at 1.5–2.0 mg./ml. lysin; 3) lessening effects with further amounts of enzyme, e.g. 205 sec. at 6.0 mg./ml. In the last test, the clot lysed in one-half hour, whereas at 1.5–2.0 mg./ml. (optimal for clotting), fibrinolysis was incomplete in 48 hours at room temperature. That the inhibitory effect of plasma antilysin is responsible is evident from clot-lysis times for any single concentration of lysin tested in a) plasma clots, b) pure fibrin clots—see Table I.

Effects of Proteases, etc. on Clotting of Recalcified Deplateletized Plasma

Deplateletized citrated dog plasma (PFP: see Materials) was used for the tests of Table I. Triplicate clotting tests immediately after preparation of PFP, using 0.9% NaCl diluent and simple recalcification at room temperature, showed no clotting until 32–36 hours. The marked clot-accelerating effects of 4 mg. Loomis' fibrinolysin, of 25–100 µg. crystalline trypsin, and of a diluted (suboptimal) brain thromboplastin, are evident. Five-tenths mg. soybean inhibitor reverses the clot acceleration in all cases, but particularly in the case of trypsin.

In these and other experiments we have made with deplateletized plasmas, clotting in times exceeding half an hour appeared to involve some unexplained questions of non-specific factors relating to colloidal stability, rather than specific ("thromboplastic") factors, like those in the data of Table I, which have a very marked influence in reducing the clotting time.

As in all plasma experiments, fibrinolytic effects are weak because of natural antilysin and prob-

TABLE I

Effects of proteases, etc. on clotting times of platelet-poor plasma

Clotting times and lysis times, at 38°C, of 0.5 ml. citrated dog plasma (platelet-poor: silicone technic), recalcified with 0.1 ml. M/10 CaCl₂, in presence of 1.0 ml. additional volume of borate buffer (pH = 7.75) containing the amounts of other reagents noted. ∞ = no lysis in 10 days.

	Inhibitor	Activator	Clotting time	Lysis-time	
				Plasma clot	Std. fibrin clot (13)
1.	0	0	2000 sec.	∞	
2.	SBI (0.5 mg.)	0	2880 sec.	∞	
3.	0	Lysin (4 mg.)	127 sec.	5 days (tr.)	5½ min.
4.	SBI (0.5 mg.)	Lysin (4 mg.)	525 sec.	∞	1½–2 days
5.	0	XT (tryp.) (25µg.)	365 sec.	4–5 days	9 min.
6.	SBI (0.5 mg.)	XT (tryp.) (25µg.)	2490 sec.	∞	5 days
7.	0	XT (tryp.) (50µg.)	168 sec.	2–3 days	5½ min.
8.	SBI (0.5 mg.)	XT (tryp.) (50µg.)	2400 sec.	∞	5 days
9.	0	XT (tryp.) (100µg.)	118 sec.	18 hours	3 min.
10.	SBI (0.5 mg.)	XT (tryp.) (100µg.)	1800 sec.	∞	4½ days
11.	0	Tpln. B (dil.)	65 sec.	∞	
12.	SBI (0.5 mg.)	Tpln. B (dil.)	352 sec.	∞	

ably also, in part, due to instability of the enzyme preparations over the long periods (days). The standard clot (fibrin) lysis times included in the last column of Table I afford a reliable measure of respective lytic potencies (13).

ting time and the fact that this is faster (12 sec.) with the lysin present than in the control (21 sec.), show that the enzyme acts by facilitating thrombin formation. Any tissue thromboplastin will give similar results.

Effect of Fibrinolysin on Conversion to Thrombin of Prothrombin in Fibrinogen-Free Plasma

In several experiments, beef plasma heated to 51°C for one-half hour and centrifuged from the coagulated protein was obtained fibrinogen-free, by tests with purified thrombin. Table II shows, by means of the shortening clotting times of successive samples removed after the stated incubation periods and tested on fibrinogen, the progressive thrombin formation in such fibrinogen-free plasma after recalcification, with and without fibrinolysin added. The evident shortening of the time required to reach the best (*i.e.* quickest) clot-

Effects of Fibrinolysin and Other Activators on Thrombin Formation from Purified Prothrombin

Table III shows, by means of clotting times (sec.) of samples of various thrombic mixtures added to test fibrinogen after the stated incubation periods, the *relative* amounts of thrombin formation from a purified prothrombin solution. We have presented a critical analysis of this method in a recent publication (4). In brief, the shortest clotting time reached measures "complete" conversion of prothrombin to thrombin under the particular test conditions, while the incubation

TABLE II

"Thromboplastic" action of fibrinolysin in activation of prothrombin in recalcified fibrinogen-free plasma

Two and five-tenths ml. plasma (beef: defibrinated at 51°C for one-half hr.), recalcified with 0.25 ml. M/10 CaCl₂, with (1) 0.75 ml. borate buffer (control) or (2) 0.75 ml. 1% lysin. After stated incubation periods, 0.25 ml. samples tested with 0.5 ml. 0.5% fibrinogen (F₁), noting clotting times (sec.), at 26°C, pH = 7.75.

	Added agent	Incubation period (min.)											
		1'	2'	3'	4'	5'	6'	7'	8'	10'	11'	15'	20'
1.	Buffer (only)	200"	190"	150"	107"	60"	—	28"	24"	21"	—	21"	24"
2.	Lysin	100"	50"	19"	14"	12"	12"	—	—	—	12"	—	—

period needed for this is a measure of the effectiveness of the activator factors. Since calcium is optimal and a sufficiency of other known accelerators is supplied in the AcG added (perhaps presumptively), the "thromboplastic" factor is believed to be the significant variable. We have not included in the present work any attempt to define a thrombin "unit" or "percentage" (of prothrombin activated), since mere inspection of the data in each experiment suffices clearly to bring out the essential points of comparison. Thus, in the experiments of Table III: 1) No clots in one-half hour shows inadequacy of calcium and AcG alone to activate the purified prothrombin solution; 2) fibrinolysin, in addition, has so little effect as to be practically negligible; 3) platelets, on the other

TABLE III

Effects of fibrinolysin and other activators on thrombin formation from purified prothrombin

Clotting times (sec.), at 26°C, pH = 7.75 (borate buffer), for 0.5 ml. F₁ + 0.25 ml. thrombic mixture (after stated incubation periods), containing 0.2 ml. Pro. (0.1%), 0.1 ml. AcG (0.1%), 0.5 ml. M/20 CaCl₂, and other activators in amounts specified, with borate buffer solution to total volume 5 ml. ∞ = No clot in one-half hr.

	Activators	Incubation period (min.)						
		1'	3'	5'	10'	20'	40'	60'
1.	0	—	—	∞	∞	∞	∞	∞
2.	Lysin (3 mg.)	—	—	390"	380"	368"	335"	320"
3.	Plat. (0.5 ml.)	420"	155"	60"	21"	—	—	18"
4.	Lysin (3 mg.) Plat. (0.5 ml.)	75"	22"	16"	11"	11"	—	10"
5.	Tpln. A (0.5 ml.)	240"	120"	10"	10"	10"	10"	10"

hand, contribute a definite "thromboplastic" effect and result in a fairly satisfactory thrombin formation, although inferior to a typical potent tissue thromboplastin (tests 5). The fact that lysin and platelets, acting in conjunction (tests 4), produce the best activation, though not yielding more thrombin (10 sec. clotting time optimum) than in tests 5, argues strongly for a "platelet thromboplastin potentiating" (PTP) action of the enzyme.

Clot-Aiding Effects of Platelets

The designation of the mode of action of platelets as "thromboplastic" in the preceding section would not only be in line with the definition and

TABLE IV

Effect of washed platelets as an "accelerator" of the activation of AcG-poor prothrombin

Two and five-tenths ml. vol. "thrombic mixtures" (T) containing borate buffer (pH = 7.75), 0.1 ml. Pro. (0.1%), 0.25 ml. CaCl₂ (M/20), and activators noted. Clotting times (sec.) of 0.5 ml. F₁ + 0.25 ml. T (at stated incubation periods): 26°C

	Activators ml. (dilution)	Incubation time (min.)				
		5'	10'	20'	40'	60'
1.	Tpln., 0.25	420"	300"	202"	141"	110"
2.	Tpln., 0.25 Plat., 0.1	390"	240"	80"	22.4"	15"
3.	Tpln., 0.25 AcG, 0.1 (0.001%)	300"	140"	52"	25.8"	20.3"
4.	Tpln., 0.25 AcG, 0.1 (0.1%)	11.4"	9.4"	8.0"	7.8"	7.6"

working hypothesis presented in the introduction, but the presence of added AcG (ineffective, with Ca alone) would argue against the platelet activators being significantly of the type supplied by serum accelerator globulin. Since a paper by Ware, Fahey and Seegers (14), appearing while the present studies were in progress, claimed that platelets supplied very little thromboplastin, but significant amounts of AcG, and some factor (probably of the type we call "fibrinoplastic" [15]) which aids the thrombin-fibrinogen reaction, these claims were carefully and repeatedly investigated with our platelet preparations.

Table IV is a typical experiment to illustrate how little "AcG-like" action was found in these well-washed platelet preparations. The poor thrombin formation in tests 1 indicates the deficiency of AcG in the chosen prothrombin, so that optimal calcium and tissue thromboplastin are insufficient activators in the hour incubation period. Compare the excellent activation in 4, with plenty of AcG added. Platelets (tests 2) produced relatively little effect, roughly comparable to the weaker AcG addition in 3, which amounts to a concentration of only 1:2,500,000 in the final thrombic mixture.

Tested on thrombin-fibrinogen mixtures, our platelets showed, if anything, a few seconds' prolongation of clotting times and no "fibrinoplastic" enhancement of clotting at several dilutions. We did find some evidence that a commonly encountered instability of thrombin solutions was apt to be less in the presence of platelets, however.

*Effects of Varying Fibrinolysin Concentrations
(Platelets Constant) and of Varying Platelet
Concentrations (Fibrinolysin Constant) on
Activation of Purified Prothrombin*

These experiments were performed by our usual technic of following the activation of prothrombin by means of the clotting times for fibrinogen (F_2) of samples of the various thrombic mixtures, removed after stated incubation periods. The 2.5 ml. mixtures contained 0.1 ml. Pro. (0.1%), 0.25 ml. $CaCl_2$ (50mM), 0.1 ml. AcG (0.1%), and the activators studied. From the series of data obtained, only the clotting-time values at a single incubation period were selected for comparing the effects of varying amounts of the fibrinolysin or platelet activators, and the accompanying graphs are plotted in this way.

In Figure 1 the three curves A, B, C are, respectively, the clotting times (sec.) of samples tested after one, five, and 60 min. incubation. The mixtures all contained 0.1 ml. platelet preparation but the fibrinolysin concentration was varied and is expressed as mg. per 2.5 ml. of the mixture. Curve B¹ is from another experiment and, therefore, not quantitatively comparable with B, but,

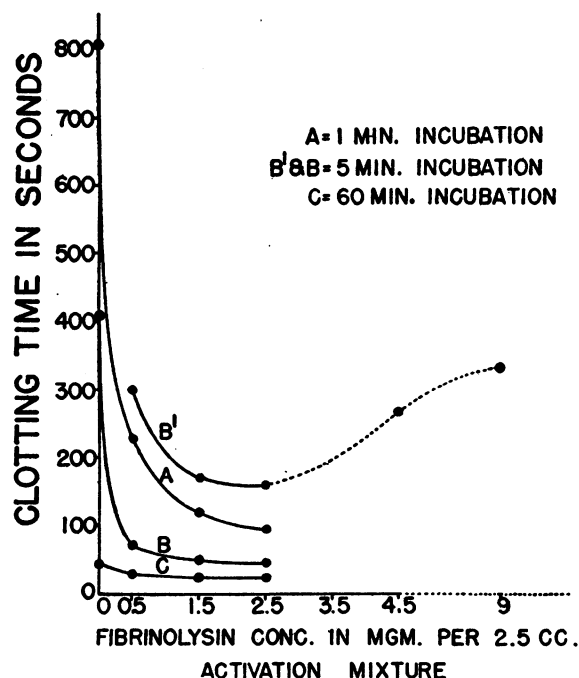


FIG. 1. EFFECT OF VARYING FIBRINOLYSIN CONCENTRATION WITH PLATELETS CONSTANT

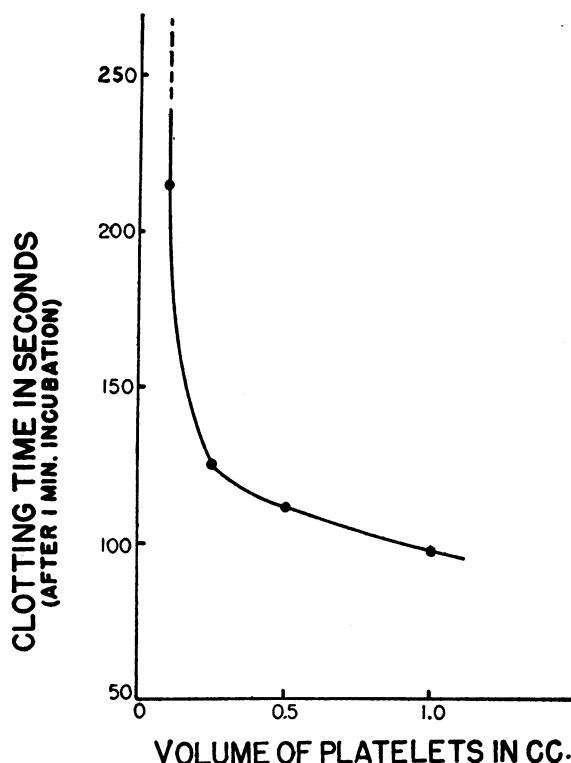


FIG. 2. EFFECT OF VARYING PLATELET CONCENTRATION WITH FIBRINOLYSIN CONSTANT

by extending the lysin concentration up to 9 mg. it brings out the additional point of an "optimum" for the enzyme effect at about 2.5 mg. (per 2.5 ml.) in this particular experiment. The data clearly show that the "potentiating" effect of fibrinolysin is more marked on the earlier phases of prothrombin activation and does depend upon the enzyme concentration, but, according to the 60 min. data of Figure 1, C, does not significantly influence the final thrombin yield (shortest clotting times).

Figure 2 is an experiment with fixed and approximately optimal fibrinolysin (2.5 mg./2.5 ml.) but varying the amount of platelet preparation between 0 and 1.0 ml./2.5 ml. of thrombic mixture. The one min. incubation samples are selected for the clotting-time test data plotted.

Figure 3 shows controls (five min. and 60 min. incubation samples) in an experiment with varying platelets (volumes plotted logarithmically) and no fibrinolysin present. The clotting times are significantly longer than when lysin is added and are expressed in minutes.

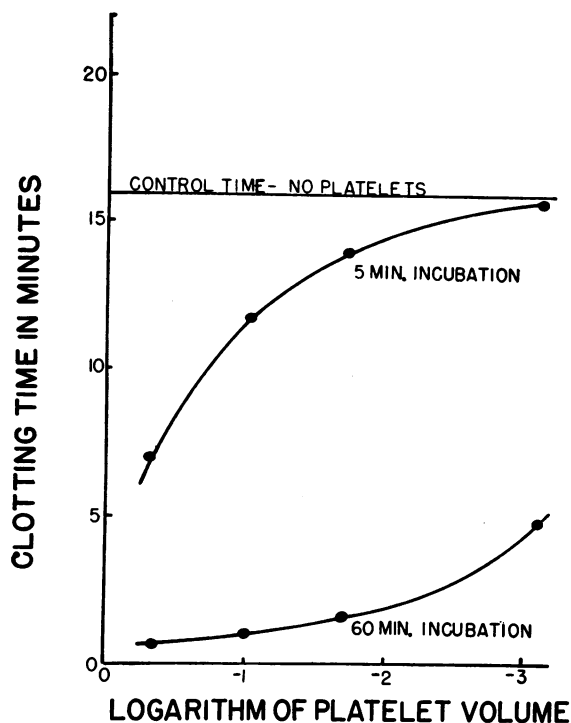


FIG. 3. EFFECT OF VARYING PLATELET CONCENTRATION; NO FIBRINOLYSIN PRESENT

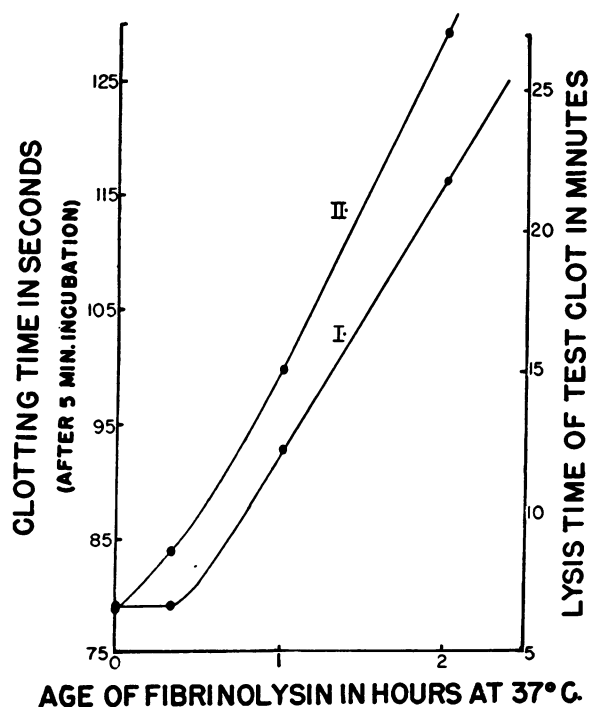


FIG. 4. LOSS OF THROMBOPLASTIC (I) AND LYTIC (II) ACTIVITY OF PROTEASE

Effects of Thermal Instability of Fibrinolysin on its Lytic Potency and on its Platelet Thromboplastin Potentiating Effects

Loomis' fibrinolysin (1% solution in borate buffer) was allowed to undergo deterioration at 37°C (water bath) and samples, removed at stated intervals of aging, tested simultaneously for 1) fibrinolytic potency, by the method of Lewis and Ferguson (13), and 2) platelet thromboplastin potentiating effect, in tests closely similar to those described above. Figure 4 plots, against age of lysin (up to two hours at 37°C), I. fibrinolysis times, which lengthen with weakening enzyme potency, and II. clotting times of five min. incubation samples, which, according to data of the preceding section, give a relative measure of "thromboplastic" potency. It would appear that the two activities of the enzyme preparation weaken at very similar rates. If the clotting-time data are plotted against residual fibrinolytic activity (units computed from a reference curve [13]), the figure will resemble the descending portion of curves B and B¹ of Figure 1, in which dilution, instead of deterioration, was the method of reducing the enzyme activity.

Effects of Protease-Inhibitors on Conversion of Prothrombin to Thrombin, in Presence of Brain Thromboplastin, Platelets, and Fibrinolysin

Preliminary to these studies, extensive experiments were carried out clearly to establish that 1) none of the antienzymes inhibited the thrombin-fibrinogen reaction, 2) fibrinolysin (0.1%) had little, if any, effect on strong thrombin (> 1 u./ml.) but could lengthen clotting times with weak thrombins (< 0.1 u./ml.) due to the opportunity afforded for fibrinogenolysis (cf. [4]).

Proceeding to the testing of thrombin formation from purified prothrombin with added AcG and optimal calcium and other reagents stated, the data of Table V record a typical series of experimental results. Interpreting the clotting-time tests in the usual way, the results indicate most significantly that pancreatic inhibitor (PI) and serum antifibrinolysin (AFIn) are *not* inhibitory to the cited thromboplastic agents (tplt. A or plat.-lysin mixtures) even though they prevented clot lysis in the fibrinolysin-containing tubes

TABLE V

Effects of antiproteases on thromboplastic agents

One-tenth ml. Pro. (0.1%), 0.1 ml. AcG (0.1%), 0.25 ml. Ca (M/20), activators and inhibitors stated, and borate buffer to 2.5 ml. vol. Clotting times (sec.) of 0.25 ml. mixture + 0.5 ml F₁, at stated incubation times, 25°C.

	Activators ml. (conc.)	Inhibitors ml. (conc.)	Incubation time (min.)				
			5'	10'	20'	40'	60'
1.	Tpln. A, 0.25	0	22.5"	17.1"	15.2"	15.0"	14.2"
2.	Tpln. A, 0.25	SBI, 0.1 (0.01%)	450"	420"	450"	450"	380"
3.	Plat., 0.1	0	49"	25.2"	21.8"	21.3"	21"
4.	Lysin, 0.2 (0.5%) Plat., 0.1 Lysin, 0.2 (0.5%)	SBI, 0.1 (0.1%)	630"	135"	42"	38.5"	35.4"
5.	Tpln. A, 0.25	0	25.5"	21.1"	19.2"	18.4"	19.6"
6.	Tpln. A, 0.25	PI, 0.1 (0.1%)	28.8"	23.3"	21.8"	21.5"	20"
7.	Plat., 0.1	0	29.1"	23"	22"	22"	22"
8.	Lysin, 0.15 (1%) Plat., 0.1 Lysin, 0.15 (1%)	PI, 0.1 (0.1%)	28"	22.5"	19.9"	20"	20"
9.	Tpln. A, 0.25	0	22.5"	15.4"	14.6"	13.1"	12.2"
10.	Tpln. A, 0.25	*AFln, 0.15 (0.5%)	28.5"	22.4"	20.6"	21.2"	21"
11.	Plat., 0.1	0	58.2"	29.3"	24.6"	24.3"	25"
12.	Lysin, 0.15 (1%) Plat., 0.1 Lysin, 0.15 (1%)	*AFln, 0.15 (0.5%)	37.6"	24.9"	22.4"	22.2"	20"

* A 15 min. incubation period, at 26°C, of antifibrinolysin with thromboplastic agent, was used to permit development of its antienzyme effect (13).

whereas inhibitor-free controls all lysed within 12–24 hours. Soybean inhibitor (SBI) was exceptional in inhibiting the brain thromboplastin (tests 2), so that it evidently has some "anti-thromboplastic" action, despite which its effect on activation with plat.-lysin (tests 4) is strongly inhibitory only in the earlier activation phases.

Effects of Antiproteases on Thromboplastic Action of Trypsin-Platelet Mixtures

Similar experiments to those in the preceding section were performed with pancreatic trypsin

(tryp.) instead of fibrinolysin, and with the antiproteases PI and AFln previously used. The results are given in Table VI. Here platelets alone (tests 1) and trypsin alone (tests 2) gave very slow and inadequate activation of the prothrombin-AcG-Ca mixture, whereas plat. + tryp. (tests 3) exerted a marked thromboplastic effect on the thrombin formation. In contrast to the previous results with fibrinolysin, the trypsin synergism with platelets was apparently completely blocked by both the tested inhibitors, PI (tests 4) and AFln (tests 5).

TABLE VI

Effects of antiproteases on thromboplastic action of trypsin-platelet mixtures

Two-tenths ml. Pro. (0.1%), 0.2 ml. AcG (0.1%), 0.5 ml. Ca (M/20), activators and inhibitors stated, and borate buffer to 5 ml. vol. Clotting times (sec.) for 0.25 mixture + 0.5 F₁ at stated incubation periods (25°C).

	Activators ml. (conc.)	Inhibitors ml. (conc.)	Incubation time (min.)				
			5'	10'	20'	40'	60'
1.	Plat., 0.1	0	720"	540"	270"	100"	65"
2.	Tryp., 0.3 (0.01%)	0	182"	140"	95"	58"	51"
3.	Plat., 0.1	0	47"	29.5"	26.5"	23.5"	23.2"
4.	Tryp., 0.3 (0.01%) Plat., 0.1	PI, 0.1 (0.1%)	660"	240"	175"	113"	96"
5.	Tryp., 0.3 (0.01%) Plat., 0.1 Tryp., 0.3 (0.01%)	AFln, 0.3 (4%)	710"	490"	205"	103"	71"

"Thromboplastic" Tests with Enzymes (sans Platelets) and Anti-Enzymes

Numerous tests on prothrombin, AcG, calcium mixtures, with fibrinolysin and with trypsin, but without any platelet or other added thromboplastin, merely confirmed the all but negligible "thromboplastic" effects of the enzymes alone. None of the inhibitors (SBI, PI, AFIn), acting alone, have significant effects except for some inhibition of the very minor thrombin formation (seen in controls) by SBI. The differences with enzyme + inhibitor combinations were slight and chiefly a matter of failure to inhibit the minor actions of fibrinolysin with PI and AFIn, and the blocking of minor actions of 0.01% trypsin. There is no need to cite these experimental data in detail.

Is There Thrombin in the Fibrinolysin Preparation?

It is extremely difficult to prepare fibrinolysin without significant contamination with thrombin. Loomis' serum enzyme preparation was selected for the present studies particularly because it appeared to fulfill the claim (16) to be free from thrombin and prothrombin. Owing to fibrinogenolysis, however, it is difficult to put this to an exacting test, but we sought to overcome this difficulty by neutralizing 0.3 ml. 1% lysin with 0.3 ml. 0.5% antilysin (Loomis') and testing on recalcified prothrombin-free fibrinogen (F_2). Even this test was inconclusive, however, as a small clot did appear in about two hours, persisting for at least four days (at 26°C). The antilysin alone caused no clot.

Effects of Very Weak Thrombin on the Thromboplastic Action of Platelet Preparations

We have repeatedly confirmed the ability to secure much better thrombin formation in purified prothrombins treated with calcium, AcG, and platelets, when a tiny trace of thrombin was added, far too little merely to add its own clotting-time contribution.

A special test of this phenomenon was made with the purest thrombin available to us. This reagent was obtained by allowing a 0.1% borate buffer solution of one of Seegers' highly purified prothrombins to activate for several weeks in the refrigerator (4°C) with added calcium salt only.

TABLE VII

Effects of weak thrombin on platelet thromboplastin

One-tenth ml. Pro. (0.1%), 0.1 ml. AcG (0.1%), 0.25 ml. Ca (M/20), activators cited, with borate buffer to 2.5 ml. vol. Clotting times (sec.) for 0.25 mixture + 0.5 ml. F_1 , at stated incubation times (26°C).

	Activators (ml.)	Incubation time (min.)				
		5'	10'	20'	40'	60'
0.	0	885"	913"	920"	970"	1010"
1.	Plat. (0.1)	810"	620"	330"	190"	97"
2.	Plat. (0.1) Thr. (0.25)	145"	52"	22"	12"	9"
3.	Plat. (0.1) 0.5% lysin (0.3)	60"	46"	15"	12"	9"

At the end of this time it had formed a potent thrombin, the activity of which was not increased by incubation with thromboplastin, indicating that unaltered prothrombin was no longer present. Several dilutions of the lysin- and thromboplastin-free product were examined for the platelet thromboplastin potentiating action. In tests 2 of Table VII an effect, qualitatively and approximately quantitatively similar to that obtained with 0.3 mg. fibrinolysin (tests 3), is noted. When freshly prepared, this particular thrombin dilution clotted the test fibrinogen in 750 seconds, but its potency decreased fairly rapidly on standing. Owing to this thrombin instability, exact control tests, *minus* the platelets, were impracticable. Such tests were attempted, however, and did show, in a reasonably satisfactory manner, that the thrombin itself caused only insignificant shortening of the control (tests 1) clotting times.

Attempts at Isolation from Prothrombin-Free Plasma of a Factor Which Potentiates the Thromboplastic Effect of Platelets

Oxalated beef plasma was passed five times through Seitz filter pads to render it prothrombin-free by the method of Owren (11). Tests for thrombin and prothrombin being entirely negative, the Seitz plasma (S.Pl.) was then subjected to successive fractionation at (a) 20, (b) 30, (c) 40, and (d) 50% saturation with ammonium sulfate, at room temperature. All precipitates (redissolved in saline) and (e) the final supernatant ("albumin") were dialyzed until sulfate-free.

Tests on dialysates: with the usual prothrombin,

AcG, Ca system, with platelets, all proved negative. The fractions were then shaken with chloroform, following the old method (17, 18) for activating fibrinolysin.

Tests on CHCl_3 -treated dialysates: the usual testing for potentiation of platelet thromboplastin was again carried out, with the result that the only significant acceleration of thrombin conversion was found in Fraction C (30–40% $[\text{NH}_4]_2\text{SO}_4$).

Control tests: I. CHCl_3 -treated Fraction C, in same dilution as in preceding tests, showed (a) traces of thrombin, *e.g.*, 0.25 ml. C (dil.) + 0.5 ml. F_2 : clotting time (26°C) 575 sec.; (b) some fibrinolytic action, *e.g.*, lysis of foregoing clot in about 12 hours. II. The other CHCl_3 -treated fractions showed no evidence of thrombin or lysis.

In view of the data of the preceding section (Table VII), the trace of thrombin in CHCl_3 -treated Fraction C, as well as the fibrinolysin, and possibly other possible factors remaining to be detected, all need critical evaluation.

It was surprising to find any thrombin at all in the above material. Since CHCl_3 is known to inactivate antithrombin, we performed some tests which showed a considerable reactivation of thrombin which had lost practically all its activity after incubating 24 hours at room temperature with (a) Seitz-filtered plasma (S.Pl.) or with (b) BaSO_4 -adsorbed serum. A thrombin-destroying action of CHCl_3 , seen also with thrombin solution alone, partly (at least) explains the *partial* recovery. It was found, incidentally, that pancreatic inhibitor retards the antithrombic action and permits the subsequent CHCl_3 treatment to give better thrombin recoveries.

DISCUSSION

The present investigations confirm earlier observations that both trypsin and serum fibrinolysin (plasmin or tryptase) exert significant clot-aiding effects attributable to participation in the mechanisms of conversion of prothrombin to active thrombin. With purified prothrombin, optimal calcium, and such accessory factors as are supplied with added "serum accelerator globulin," but no added thromboplastin (cell- or tissue-factor), these enzyme preparations have little, if any, "thromboplastic" action of their own, the clotting times of the thrombic mixtures plus purified fibrinogen solution being too long to give significant results. In previous studies (4) with potent tissue thromboplastins, clotting times in control tests, without enzyme, were too short to give more than sug-

gestive evidence that addition of these proteases could "potentiate" the thromboplastic action, particularly in its earlier phases. In the present studies, well-washed platelet preparations, shown to be without significant effects other than those of a relatively weak "thromboplastic" character, were markedly potentiated by the enzyme materials in question.

This PTP ("platelet thromboplastin potentiating") effect of the serum fibrinolysin parallels its lytic activity both in dilution experiments and during deterioration at 37°C. However, protease inhibitors from pancreas (PI) and from serum (AFIn) do not abolish the PTP effect, although they do inhibit the enzymic fibrinolysis.

Soybean inhibitor (SBI) has additional "antithromboplastic" effects, even for tissue- and platelet-thromboplastins without proteolytic enzyme (Table V), so that its effect of at least partly inhibiting the PTP action may very well be due to its antithromboplastic instead of its antilytic effects.

In the case of trypsin, however, all the inhibitors abolish the two effects simultaneously. Thus, the arguments we have previously (4) built up for a "thromboplastic enzyme" mechanism accessory to the thrombin-forming phase of blood clotting, are now still valid only in the artificial test systems with pancreatic trypsin. Failure of correlation of the two effects in tests with appropriate inhibitors (serum antifibrinolysin especially) overthrows a large amount of evidence otherwise strongly suggesting a similar role for the natural serum protease fibrinolysin. The conclusion must be that some other factor must be sought for in the enzyme preparation to account for its thromboplastin potentiating effects.

The possibility that this may be a trace of thrombin is perhaps suggested by the discovery (Table VII) of a significant "PTP" effect of very small amounts of thrombin such as may frequently be found as contaminants of serum enzyme and many other blood preparations. However, the extremely minute and possibly doubtful traces of thrombin demonstrable in some special tests with the present lysis, would appear insufficient to invoke this explanation here. Nor is it probable that the minute traces of thrombin, present or appearing in the prothrombin-AcG mixtures (which are very dilute), can be implicated, particularly to explain the sharp *increase* in "thromboplastic" action when the enzyme-platelet activator is used and

compared with control tests. It is barely possible, however, that these sources of thrombin trace-contaminant may require some consideration in explaining (fully) the thromboplastic action of platelets alone (*i.e.*, without enzyme) as seen in the data of Tables III and IV.

Ware, Fahey, and Seegers (14) found some thrombin formation in platelet-activated mixtures containing stronger prothrombin (330 u./ml.) but none in an hour's incubation with much lower (1.34 u./ml.) prothrombin. If their prothrombin contained the usual trace of thrombin contaminant, these results at the higher concentrations would appear similar to ours, whereas at the lower concentrations the thrombin contaminant may very well have been diluted to insignificance.

Our preliminary attempts to prepare a "platelet thromboplastin potentiating" factor, from apparently thrombin- and prothrombin-free Seitz filtered ($\times 5$) plasma, succeeded only in the case of a CHCl_3 -treated 30–40% saturated $(\text{NH}_4)_2\text{SO}_4$ Fraction C, in which definite traces of both fibrinolysin and thrombin were demonstrated. The surprising finding of some thrombin here raises some new practical questions. Our suggested explanation is that some thrombin formation in artificially treated plasmas may not be detected, yet result in a "metathrombin" (combination of thrombin with natural antithrombin) from which thrombinic activity is recoverable under certain circumstances, *e.g.*, following treatment with chloroform. Many questions as to the state of both prothrombin and thrombin in plasma and in serum are opened up by these new facts and some special studies on these matters are being pursued and have already been mentioned in a preliminary communication (19).

On casual observation, our PTP factor might appear to be acting like the so-called "accelerator" agents, two and possibly more types of which have occupied the attention of a number of recent investigators. A recent paper (20) in this JOURNAL adequately reviews the current status of these factors and particularly gives experimental evidence that Seegers' "serum accelerator globulin" preparation contains both Alexander's SPCA ("serum prothrombin conversion accelerator") and "labile factor." As we have mentioned earlier, the routine addition of a reasonable amount of serum AcG in our test systems should, we believe, ensure a sufficiency of these "accelerator" factors and make it unnecessary to enter into any discussion of them in the present connection. Rather, we may proceed to consideration of our results in terms of the "thromboplastic" problem.

This does bring the present work into relation with some other studies in the coagulation literature, particularly those which give attention to the idea of a thromboplastin precursor or "prothrombokinase" (Collingwood and MacMahon, 1912; Widenbauer, 1943) requiring activation in order to provide the "thromboplastic" (thrombokinase) factor for the clotting mechanism. In a series of recent papers Milstone reviews this literature and presents his new experimental data. His first paper appeared while our present studies were nearing completion and offered evidence of the enhancement of prothrombin conversion to thrombin in the presence of platelets when a (crude) plasma "globulin" preparation was added. In discussion Milstone (21) states: "Conceivably, platelets might contain prothrombokinase as suggested by Collingwood and MacMahon, or a platelet enzyme might activate the prothrombokinase ('thromboplastinogen') of plasma as suggested by Quick (1947). The present experiments do not decide between these hypotheses, nor do they eliminate other possibilities." Milstone's second paper (22) contributes some new technics for analyzing the mechanisms of blood coagulation into three phases. A "prothrombokinase" preparation from a euglobulin precipitate of bovine plasma served for the study of *plasma* thrombokinase (thromboplastin) formation, with the aid of calcium. A noteworthy point in discussion is . . . "thrombin does not catalyze the activation of prothrombin; but its possible effect on the activation of prothrombokinase remains undetermined." Milstone (23) prepared from the BaSO_4 adsorbate of bovine plasma globulins a material ("converter fraction") which . . . "hastened both the activation of prothrombin and the conversion of crude prothrombokinase. . . . The relation of . . . converter to thrombokinase activity resembles that reported for platelets plus 'globulin' (21)." In a still more recent paper (24) appearing after this present work was submitted for publication, Milstone reinvestigated the experimental activation of purified prothrombin by platelets, brain thromboplastin, lipid thromboplastin, and crystalline trypsin, and emphasized the ability of the enzyme to act (*a*) alone, (*b*) as potentiator of lipid thromboplastin, and (*c*) together with platelets (even when amounts of trypsin and platelets were selected neither of which was demonstrably active alone). There

are obviously many points of confirmation and parallel interest between Milstone's work and ours, both the present and previously published (4). However, these serve only to emphasize the complexities of the problem and the final solution must await much future investigation.

CONCLUSIONS

1. Crystalline trypsin and "purified" serum fibrinolysin accelerate clotting of ordinary recalcified plasma and platelet-free plasma obtained with the silicone technic.

2. These enzymes accelerate and increase thrombin yields from heat-defibrinated plasma or other crude prothrombins.

3. They have no significant effects on the thrombin-fibrinogen reaction (apart from lytic interference) nor upon the activation of purified prothrombin with Ca and AcG but negligible thromboplastin.

4. They markedly potentiate platelet thromboplastic action.

5. *Well washed* platelets are thromboplastic, but negligibly AcG-like, and nonfibrinoplastic.

6. The platelet thromboplastin potentiating (PTP) effect of fibrinolysin shows an optimal enzyme concentration and lessens roughly parallel to weakening of lytic potency by dilution or by thermal deterioration.

7. It is, however, not abolished by effective antiproteases (antilytic) from pancreas (PI) and serum (antifibrinolysin), and only incompletely by soybean inhibitor (SBI), which, however, is anti-thromboplastic.

8. The PTP effects of trypsin, on the other hand, are completely abolished by the antilytic agents.

9. Very small amounts of thrombin (down to a limit) also have a PTP action, but the fibrinolysin used was practically thrombin-free.

10. A crude plasma protein fraction obtained at 30-40% $(\text{NH}_4)_2\text{SO}_4$ saturation of "prothrombin-free" (Seitz-filtered, $\times 5$) plasma, when treated with CHCl_3 , had PTP effects but contained some thrombin as well as fibrinolysin.

11. While many of these data support Ferguson's theory (1) of a clot-aiding role of "thromboplastic enzymes," perhaps still valid for trypsin, the inhibitor data controvert this for fibrinolysin.

After considering the possible significance of thrombin, there remains the possibility of a new PTP factor, possibly the "prothrombokinase converter factor" postulated by Milstone (*op. cit.*), but apparently unrelated to the "accelerator" factors about which much work has appeared in the recent coagulation literature.

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