Factor V and Thrombin in the Extrinsic Clotting System* (33574)

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Factor V (1), whose synonyms include labile factor (2), accelerator globulin (3), and other terms (4), has evoked an extensive modern literature (5) in which serious discrepancies of both fact and interpretation invite further investigation. Particularly questionable are ideas of a conversion of factor V to an "activated" material (Va?) and its possible relation to thrombin. The present contribution introduces a restudy of these problems and will be limited to 1-stage and 2-stage assay systems that depend on tissue thromboplastin. This agent is also called factor III and is considered as an *extrinsic* activator system (6).

Materials and Methods. Buffers are either imidazole-buffered 0.85% NaCl (IBS) or 0.05 *M* Tris–0.05 *M* NaCl, both types adjusted to pH 7.3 ± 0.05. *V-deficient substrate* (7) is aged normal human oxalated plasma, stored at −20° and made factor V-deficient by repeated thawings and refreezings over a period of weeks. *Tissue thromboplastin* (Tpln) is commercial Simplastin (Warner-Chilcott). This contains ionized calcium (CA^{2+}) adequate for 1-stage tests, but needing supplementation for the 2-stage tests. *Factor V* (V) is purified from $BaSO_4$-adsorbed bovine oxalated plasma by a recent method (8), which is now improved by use of an isoelectric precipitation (6.3>pH>5.2) between the prechromatographic 18–39% saturation with ammonium sulfate (SAS) and the Sephadex G-200 gel filtration. *Thrombin-treated factor V* (V_T) is obtained by treating 9 vol of the above SAS fraction with 1 vol of purified thrombin (Tp, see below) for 30 min at 37° in Tris–NaCl buffer. After subsequent centrifuging at 1500*g* for 10 min at 0–4°, the filtered supernatant is subjected to the usual Sephadex G-200 chromatography. The new elution pattern and fraction test activities are restudied. Some lots of the tubes containing active treated factor V are pooled and lyophilized. *Untreated thrombin* (T_U) is commercial (Upjohn's) bovine thrombin, courtesy Dr. J. T. Correll. It is diluted with Tris-NaCl to 100 manufacturer's units/ml for stock solutions, which are stored at −20°. From such stocks of T_U, a *purified thrombin* (T_P) is obtained by chromatography on DEAE-cellulose, after step 1 of the procedure of Yin and Wessler (9), which is basically similar to that proposed by Kerwin and Milstone (10) for obtaining thrombin free from thrombokinase (Xa). *Prothrombin* (factor II) is a $BaSO_4$–citrate eluate (El) from oxalated canine plasma, which also provides essential factors VII and X (11). *Fibrinogen* (factor I) is prepared from Armour's bovine plasma fraction I as previously described (11). It contains 3–4 mg/ml of clottable protein. Stock *calcium chloride* (Ca) is 0.1 *M*.

1-stage tests for alleged "factor V" activity. These are by the well-known modified prothrombin time (PT) method (7), here using Simplastin. At 37°, in order, mix 0.1 ml of additive, 0.2 ml of Simplastin, 0.1 ml of factor V-deficient substrate. The substrate is added last to minimize certain unwanted reactions, some of which are inhibitory. Buffer (IBS) is used in the controls, and other additives are factor V (V), or thrombin (T), or mixtures (V + T) with or without a period of preincubation.

2-stage tests for quantitating thrombin generation (11–13). The first step, at 25 ± 1°, incubates a mixture (ml) of 1.1 IBS, 0.4 Ca (0.1 *M*), 0.2 Tpln (Simplastin), 0.1 factor V (or other test additive), and 0.2 ml of eluate prothrombin (supplying factors II, VII, X). Step 2 times the clotting of successive aliquots of this incubate when added to an equal (0.2 ml) volume of fibrinogen. Routine

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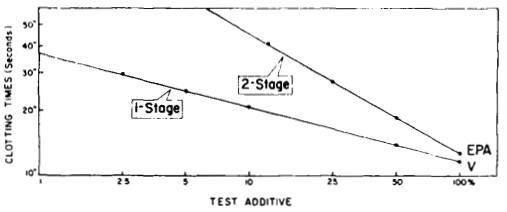

FIG. 1. Log–log reference standards for bioassays by 2-stage (EPA) and 1-stage ("factor V") methods; see text.

tests are made initially after 0.5 min and then at 1-min intervals until the shortest clotting-time (CT) end-pont, noting the optimal incubation period. This established method (13) is admittedly difficult to standardize, but conditions can be found which consistently give the validating rectilinear log–log reference plot, such as that illustrated in the upper line of Fig. 1.

Assays. Figure 1 exemplifies the rectilinear log–log reference standards which validate both the 1-stage (14) and the 2-stage (12) methods. In the case of the 2-stage tests, the abscissa measures, logarithmically, the percentage concentrations of the prothrombin, obtained in a series of standard El (II) dilutions. In many cases, a 1:10 dilution by volume in the final incubation mixture conveniently serves as a standard "100%". We call such percentage measurements equivalents of prothrombin activated (EPA) (15), since the end-point clotting times, in seconds on the logarithmic ordinate scale, effectively measure the corresponding thrombin yields. Under favorable conditions, it is often possible also to quantitate the additive thrombins, especially T_P, from the same reference (EPA) plot. Such thrombin "percentages" will be used to quantitate the additive in some of the Tables. In others, the thrombin will be measured as manufacturer's units (u/ml), allowing for the final dilution.

In the 1-stage assays, giving the lower line of Fig. 1, the standard is obtained by dilutions of the same purified factor V preparation as is used in the comparative tests. Conveniently, "100%" again refers to a 1:10 dilution by volume in the final test mixture, when quantitating the factor V additive. "Factor V" *activity*, on the other hand, is the percentage value obtained from the standard plot by referring the actual test clotting time (CT) to it. The true meaning of such "activity" values is questionable, however, if the additive is thrombin or a (T + V) mixture.

Results. 1-stage tests. Table I shows "factor V" assay values (%) obtained as described above. The second vertical column notes % V added (final concentration) at zero incubation time or as reassayed after the 5- or 10-min preincubations in (V + IBS) controls. In parallel experiments, three small quantities (5, 2.5, 1.25%) of V were tested with the stated admixture of thrombin (T_U),

TABLE I. Effects of Thrombin (T_U) on 1-Stage 'Factor V' Assays (37°).[a]

(V + T_U) preincub. time (min)	% V added, or assayed	Added thrombin (u/ml): 0.005	0.05	0.5	2.5	5
—	0	Trace	Trace	1	10	72
Zero	5.0	5.8	6.8	22.8	37.3	>100
5	3.2	3.1	6.5	18.1	23.8	77
10	2.2	2.3	4.7	12.2	13.6	63
Zero	2.5	3.1	—	12.7	24.4	>100
5	1.8	2.4	—	5.5	11.0	52.5
10	1.3	1.8	—	5.2	7.5	30.5
Zero	1.25	1.47	—	4.6	16.4	>100
5	1.0	0.6	—	2.4	6.9	34.5
10	0.1	0.4	—	1.9	4.5	22

[a] Assay values, from standard rectilinear log-log plots, are obtained by the Simplastin (PT) method.

TABLE II. Effects of Purified Thrombin (T_P) on Simplastin 1-Stage 'Factor V' Assays (%; 37°).[a]

	Preincub. (min)	% V added, or assayed	Added thrombin[b] (u/ml): 0.05	0.5	2.5
	—	0	trace (tr)[b]	1 (1)[b]	22 (10)[b]
(A)	Zero	5	6 (6)	20.5 (22.5)	25 (37)
	5	3.5	4.5 (6.5)	13 (18)	19.5 (24)
(B)	Zero	5	5.5	17	—
	5	3.5	4	9	—

[a] Preincubations of T_P[b] with (A) untreated factor V; (B) V_T = product recovered by Sephadex G-200 gel filtration of T_P-treated factor V.

[b] Results with corresponding amounts of unpurified thrombin (T_U), for comparison, in parentheses.

the concentrations of which (0.005–5 u/ml) varied over a thousandfold range. The controls with T_U alone (0 factor V) show negligible effects below a thrombin concentration of 0.5 u/ml. This limit may depend upon antithrombin(s) in the substrate plasma. Significant "assay" values are obtained at higher T_U concentrations, however. At 0.5 u/ml thrombin, and above, apparent potentiation of the assay values is consistently observed, thus confirming many, if not all, reports in the previous literature (5, 16). This potentiating effect of crude thrombin is clearly immediate and does not increase with preincubation. In fact, the heightened activity decays, much as it does in the thrombin-free controls, but because it starts at a higher level the value does not usually fall to that in the controls during the period of these tests. It will do so, however, in a longer period. Much depends on the particular additive concentrations chosen for the experiment and especially on the stability of the factor V preparation. At high dilutions, stability of the thrombin may also be questioned.

Effects of purified thrombin (T_P) on 1-stage tests. Table II shows effects of purified thrombin (A) on untreated factor V; (B) on V_T, namely, the product recovered by Sephadex G-200 gel filtration of T_P-treated factor V. In parentheses are also shown results with corresponding amounts of unpurified thrombin (T_U) in the control and (A) test series. It is evident that the DEAE purification (see "Methods") seems to have little effect on the ability of thrombin to potentiate immediately with factor V in the 1-stage assays. Many other experiments using T_P and T_U preincubates with factor V failed to show any *progressive* enhancement, but only the variable decay, also encountered in IBS controls. The new observation in (B) is especially interesting since it shows that thrombin-treated V (V_T) potentiates anew with a second thrombin (T_P) addition, which would hardly be exptected if V_T was already "activated" (Va?). This finding was confirmed on several occasions, and again no preincubation is required.

2-stage tests. Table III shows effects of various thrombin (T_P) and factor V (V) mixtures on thrombin-generation assays by the Simplastin 2-stage method. Assays (% EPA) are cited for the 0.5 min and end-point tests, with the optimal incubation period (min) noted in parentheses. The *sum* of the value for thrombin alone (No V) and that for the V alone (0% T_P) is subtracted from the assay value for the total thrombin + factor V mixture, in each case, to yield the true increment, Δ, which is given below each test result. For example, with 5% V + 5% T_P, in 0.5 min tests, the mixture assays (EPA %) 7, whereas 5% V alone gives 4 and 5% T_P alone gives 3, whence $\Delta = 7 - (4 + 3) = 0$. Simily, for the end point tests of the same series: $\Delta = 39 - (36 + 3) = 0$. This is a "perfect" result, achieved on some occasions, but the

TABLE III. Effects of Various Thrombin (T_P) and Factor V (V) Mixtures on Thrombin Yields (EPA%) in Simplastin 2-Stage Tests (25°).

	No V		5% V		10% V		25% V	
% T_P	0.5 min	End pt.	0.5 min	End pt.[a]	0.5 min	End pt.[a]	0.5 min	End pt.[a]
0	tr	tr	4	36 (6)	5.5	45 (8)	5	47 (5)
5	3	—	7	39 (7)	8	49 (4)	10	56 (6)
			Δ: 0[b]	0	−0.5	+1	+2	+6
0	tr	tr	4	34 (6)	5.5	50 (5)	6.5	50 (6)
10	6	—	11	41 (7)	13.5	62 (5)	12.5	62 (6)
			Δ: +1	+1	+2	+6	0	+6
0	tr	tr	3	30 (8)	4	50 (6)	6	99 (5)
25	15	—	18	54 (6)	25	67 (5)	27	112 (8)
			Δ: 0	+9	+6	+2	+6	−2

[a] Opt. incubation period (min) in parentheses.

[b] Δ: increment obtained by subtracting *sum* of values for T_P and V, assayed separately, from assay of (T_P + V) mixture.

point is repeatedly established, namely, that in 2-stage tests Δ is nonsignificant, and only small (±) values occur for minor technical reasons. This is a very different result from the phenomenon observed in the 1-stage assays. The range of thrombin and factor V concentrations tested in the comparable experiments of Table III embraces fairly high concentrations of both agents. Many confirmatory results have also been obtained with still lower and higher T_P and V concentrations. In addition, preincubations up to 20 or 30 min were tried on many occasions. In no instance did this cause any development, let alone "enhancement," of the looked-for extra activity.

Comparison between 1-stage and 2-stage tests, using exactly the same factor V and thrombin mixtures, in parallel tests run simultaneously on another occasion, is shown in Table IV. The key point is that the final concentrations of V and T_P are the same in the two series, precisely allowing for the different dilution conditions in the two methods. For the simplest possible presentation, the data are here limited just to the increment (Δ) values in each case. The obvious difference, namely, that significant apparent potentiations are strictly limited to the 1-stage test system, is well illustrated. Not only does such potentiation fail to occur in the 2-stage tests, whether in the initial 0.5 min or in the end-point tests, but the optimal (opt.) incubation period is little, if at all, affected. This last is shown by including (in parentheses) the incubation period *differences* (Δ). All clotting-time shortenings in the 2-stage tests, within a small margin for experimental error,

TABLE IV. Comparison of Assay Increments, Δ, in Parallel 1-Stage and 2-Stage Simplastin Tests; (see text).

Method	% T +	5% V	10% V	25% V
1-stage	5	+11	+26	+9
2-stage[a]	5	−2.5 to −6(+3)	−0.5 to −3.5(0)	+4 to 0(0)
1-stage	10	+44	+57	+20
2-stage[a]	10	−2.5 to 0(+2)	−2 to 0(+1)	−3 to 0(0)
1-stage	25	+9	+20	++ (large)
2-stage[a]	25	−1 to +2(0)	−2 to −7(0)	−4 to −1(+1)

[a] The 2-stage test data show increments, Δ, in 0.5 min to end-point tests, and (in parentheses) any change in opt. incubation period (min).

can be quantitatively accounted for by a simple summation of the independent factor V and thrombin effects. This evidence argues most strongly against any idea of a V→"Va" activation.

Elution patterns. In the Sephadex G-200 chromatography, the elution patterns for thrombin-treated factor V (V_T), compared with those for untreated V, as tested in the 1-stage assay system, were basically similar overall, but with a significant tendency of the peak activity to shift to one or two collecting tubes later. Even more noticeable was the occurrence of extra activities in the V_T tubes following the peak, which could suggest greater gel retention. When a concentrate of *lyophilized* V_T was reconstituted, dialyzed against Tris–NaCl, treated with a second amount of ~5 u/ml of T_P, and then rechromatographed, the new elution pattern could be compared with a control consisting of the same V_T plus buffer only. It was most interesting that the retreated preparation again showed a shift in the elution peak and extension of factor V activity into the following tubes, very similar to the pattern change observed on the first thrombin treatment.

Stability studies, up to 7 days at −20°, and for several minutes at 37°, with a somewhat labile factor V and V_T prepared from it, showed a remarkable degree of parallelism in the decay of the 1-stage values. For both materials of the respective test series, the time required to reach 50% of the initial assay level was identical. This finding would seem to be inconsistent with reports of instability (17, 18) or "reversibility" (19) of V ⇄ Va, specifically attributable to thrombin. Other incidental observations also seem to oppose the idea that thrombin genuinely alters factor V activity in any reversible reaction. The present preliminary data, which include some immunochemical studies, are insufficient to suggest any biochemical answer to these problems. Since *factors VII and X* in our eluate prothrombin are essential participants in thrombin generation by tissue thromboplastin, it may be logically surmised that the thrombin treatment fails to activate them also.

Discussion. In the extensive modern literature (5, 16), which provides conflicting evidence concerning possible reactions between factor V and thrombin, a number of authors, including ourselves (11), have raised serious doubts about the theory that thrombin activates V to a "Va," with its various implications concerning roles of each in the conversion of prothrombin to thrombin. Assay results are affected by the absolute and relative amounts of V and T tested, but the apparent potentiation of V by T_P, in the 1-stage system is easily confirmed. Any genuine increment of factor V activity, however, should be equally easy to demonstrate in the 2-stage thrombin generation system. With a range of V and T_P concentrations separately able to affect the sensitive 2-stage assays and to be accurately quantitated by the validated test system, the failure to find any evidence of potentiation, in the 0.5 min test, at the endpoint, or in the optimal incubation time (particularly susceptible to an "accelerator" globulin) must be extremely significant. Moreover, the potentiations in the assay values in the 1-stage tests are reproducible when a thrombin-treated V is reexposed to additional thrombin. The same is true of the elution patterns of V + T_P and V_T + T_P in Sephadex G-200 chromatography. There are undoubtedly complex relationships in mixtures of factor V and thrombin, which these new studies only begin to examine. It does seem unwise, at present, to argue for a V→Va transformation when other explanations are waiting to be explored. Studies, similar to the present, but using *intrinsic* (and related) clotting systems, will be the topic of future presentations. A companion paper (20) will suggest an alternative explanation for the 1-stage test results. In the meantime, the current conventional extrinsic system data serve to reopen the topic and further to delineate the background and complexity of these problems.

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The Effect of Exogenous Dopamine on ACTH Secretion (33575)

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Exogenous dopamine has been shown to increase corticotropin (ACTH) secretion as measured by adrenal ascorbic acid depletion (1). Since a dichotomy has been demonstrated between adrenal ascorbic acid depletion and plasma corticosterone elevation in some conditions (2, 3), the effect of dopamine on plasma corticosterone levels was determined.

Epinephrine, closely related in biological action and chemical structure to dopamine, is thought to raise ACTH blood levels by acting as a nonspecific peripheral stress (4). Therefore, dopamine and epinephrine in equivalent ACTH-stimulating doses were compared in their effect on blood pressure as a measurement of peripheral stress.

Dexamethasone inhibits the nervous pathways of corticotropin releasing factor (CRF) release (5). Since dopamine is thought to influence these same pathways (1), the influence of pretreatment with dexamethasone on the ACTH-raising ability of dopamine was studied.

Methods. Male albino rats (Holtzman Company) weighing about 200 g were used in all experiments. Animals received food and water *ad libitum*, and were housed in individual cages under a constant illumination schedule (7 a.m. to 7 p.m.) for more than 6 days. All experiments were carried out between 10 a.m. to 12 noon. The substance to be tested was injected s.c. in a volume of 0.2 ml 15 min before a plasma sample was obtained from the unanesthetized rats. Plasma samples were obtained by rapid decapitation, collection of the blood in heparinized tubes, and then aspiration of the plasma after centrifugation. The plasma samples were frozen until used. A solution containing ascorbic

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