This investigation was supported by U.S.P.H.S. Grant 5 RO1 AM11073-03.

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Received Sept. 4, 1968. P.S.E.B.M., 1969, Vol. 130.

One-State "Factor V" Bioassays: Specific and Other Determinants in Blood-Clotting Test Systems* (33586)

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Recent studies with blood-clotting assay systems, using three types of activator, namely, (1) tissue thromboplastin, (2) Stypven (Russell's viper venom), (3) thrombokinase, convincingly show that 2-stage thrombin-generation systems (4) fail to yield more than a simple and immediate summation of factor V (V) and thrombin (T) effects, whereas all three types of 1-stage factor V assay systems indicate large apparent potentiations of the (T + V) mixtures. Since the first of these seemingly incompatible data renders untenable the current popular idea of a V → Va activation, it is necessary to find some new explanations for the potentiations in the 1-stage systems. Because many possible reactions and interactions could be proceeding simultaneously or successively in so complicated a mixture as is provided by the deceptively simple 1-stage tests, reinterpretations may not be easy but could require a very sophisticated analysis. An important new step in this direction is offered in the present study, which attempts to assess the specific and the combined (or derived) effects of the five basic components (II, TK, V, PL, Ca^{2+}) in a thrombokinase (TK)-activated thrombin-generation mixture when added to the above test systems. This communication will deal chiefly with the extrinsic (PT) and intrinsic (TK) 1-stage factor V assay methods.

* Supported by USPHS Research Grant No. HE-01510, National Institutes of Health.

Materials and Methods. Reagents are fully described in recent publications[1] (1) and may be briefly identified as follows: *V-deficient substrate* (—V sub) is factor V-poor aged normal human oxalated plasma. *Tissue thromboplastin* (Tpln, factor III?) is commercial Simplastin (Warner-Chilcott). This contains an adequate amount of *calcium* (Ca^{2+}), of which an equivalent, in the form of 0.1 ml of 25 m*M* $CaCl_2$, is supplied in all other 1-stage tests, unless otherwise specified. The *additive* (incubate) mixtures, described under "Results," contain 0.2 vol of 0.1 *M* $CaCl_2$ whenever Ca^{2+} is indicated. *Buffer* (IBS) is 0.9 vol of saline (0.85% NaCl) with 0.1 vol of imidazole, buffered at pH 7.35. *Phospholipid* (PL) is commercial Thrombofax (Ortho Lab.). *Thrombokinase* (TK, factor Xa?), courtesy of Dr. J. H. Milstone, is from "step 7" in the current method of purification (5). The TK_5, TK_{10} indicate, by the subscript, actual protein concentrations as μg/ml, by which the enzyme test solutions are quantitated. *Factor V* (V) is purified from $BaSO_4$-adsorbed bovine oxalated plasma and assayed as recently described (1, 6). *Prothrombin* (factor II) is a barium citrate–citrate eluate (El) of bovine citrated plasma, which is a very potent II and low in factors V, VII, X[1] (7).

Assays. These are by the routine 1-stage

[1] Methodologies, which are not considered in this paper, will be published elsewhere: Thromb. Diath. Haemorrhag., in press.

TABLE I. Effects of Preincubates, Composed of Additives Noted (+), on 1-Stage "Factor V" Assays, by (A) PT, or (B) TK, Methods (37°).

	Preincubate					(A) PT method			(B) TK method		
No.	II	V	TK	PL	Ca^{2+}	0.5 min	End pt.	(Opt) (min)	0.5 min	End pt.	(Opt) (min)
1	—	—	—	—	—	~0	—		~0	—	
2	—	+	—	—	—	5	—		15	—	
3	+	+	+	+	+	22	100	(3)	16	42	(2)
4[a]	+	+	+	+	+	4.5	50	(4)	16	37	(4)
5[b]	+	+	+	+	+	tr.	4	(5)	5.5	12.5	(3)
6	—	+	+	+	+	15	—		25	—	
7[a]	—	+	+	+	+	50	—		19.5	—	
8[b]	—	+	+	+	+	2	—		4.5	—	
9	+	+	+	+	—	7	—		16.5	—	
10	—	+	+	+	—	9.5	—		18.5	—	
11[a]	—	+	+	+	—	3	—		19	—	
12	—	+	+	—	—	6	—		8.5	—	
13[a]	—	+	+	—	—	~0	—		~0	—	
14	+	+	—	+	+	3.5	—		22	—	
15	—	+	—	+	+	3.5	—		22	—	

[a] Ca^{2+}, but no *activator*, in assay.
[b] No Ca^{2+}, nor *activator*, in assay.

systems[1] (1), using either (A) the well-known modified prothrombin time (PT) method of factor V (labile factor) bioassay, originally introduced by Quick and Stefanini (8), or (B) our[1] new substitute test with TK (thrombokinase) as follows: In order, mix 0.1 ml of PL, 0.1 ml of TK_5, 0.1 ml of additive mixture (see Table I), 0.1 ml of Ca (25 m*M*), 0.1 ml of —V sub, and time clotting (CT) at 37°. It is important to add the substrate last to minimize certain possibilities of premature reactions, some of which are inhibitory. Assays (%) are obtained by referring these CT's to standard rectilinear log–log plots of data secured with serial percentage dilutions of the same factor V preparation, for each method of assay. Methods (A) and (B) have different standards and must not be directly compared.

Results. Table I shows effects of preincubates, composed of additives noted (+), on 1-stage factor V assays, by (A) PT, or (B) TK, methods. All combinations of factors which gave significant results are included in Table I. No assays were obtainable whenever factor V was omitted. Tests no. 1 are the substrate controls, virtually zero.

(A) *PT test data.* Test no. 2 is factor V alone (5%). In no. 3, the additive is the complete five-component thrombic (T) mixture, originally with an equivalent factor V content. The T is tested (a) after 0.5-min preincubation, and repeatedly until (b) the end point (End pt.), with note of the optimal incubation period (Opt), here 3 min. At this end point, the activity increase in no. 3, as compared with no. 2, is twentyfold. The same procedure is followed in the other test systems, but it is only with the complete thrombin-generating mixtures that any progressive enhancement can be detected, e.g., nos. 4, 5. In all other cases, the maximal activity requires no preincubation, but, in fact, such treatment leads to some decay in most instances. In no. 4, the Simplastin is omitted in the final assay, but is replaced by an equivalent solution of $CaCl_2$ (25 m*M*). It is very interesting that apparently 50%, or one-half, of the activity previously noted in no. 3 can now (no. 4) be obtained in the absence of Simplastin. The obvious fact that this result must depend on a different activator mechanism raises doubts about the precise meaning of the assay. The same question applies to no. 5, where Ca^{2+} as well as Simplastin is omitted in the final assay. Here the new

activity is greatly reduced, to a mere 4%, if we concede validity of the assay. The inability to obtain a marked activity in no. 5, as compared to no. 4, must somehow depend on an insufficiency of available Ca^{2+}, since all other particulars are identical in the two series. That there is little, if any, direct clotting activity due to the carry-over thrombin in the T mixture could be due to action of antithrombin(s) in the —V sub plasma. The most logical, and new, explanation of the activity in nos. 3 and 4 assays is that it depends, not on thrombin, but on the carry-over of *thrombokinase,* whether in its original state, or, possibly, enhanced by reactions in the preincubation mixture (see "Discussion"). The foregoing conclusion is confirmed in no. 7 where no II, and hence no thrombin generation, occurs in the additive mixture. The 50% value in no. 7 is the same as the end point in no. 4. The other TK test results are not nearly so striking, but do supply suggestive evidence of the need for factor V and PL as well as Ca^{2+}, for the enzyme to continue functioning in any subsequent thrombin generation. Such new thrombin formation must occur from prothrombin in the substrate plasma to clot its fibrinogen and thus give a clotting-*time*, which permits us to attempt a bioassay. Availability of essential cofactors is clearly of the greatest importance in accounting for the marked influence which TK has been shown to exercise in the 1-stage PT test. Besides this, there is suggestive, if not decisive, evidence of (a) multiple, and possibly competitive, reactions, e.g., no. 6 vs no. 7; (b) factor (and product) instabilities during the incubations, as noted above; and (c) certain inhibitory phenomena, including antithrombin(s). All of these reactions greatly complicate interpretations of the alleged bioassays.

(B) *TK test data.* Here the situation may be simpler because the only prime activator is TK, which is the main assay reagent as well as a component of most of the additive mixtures. The enzymic reactivity, of course, must be considered in relation to other components of these systems, particularly with reference to its cofactors (9). Results of the (B) tests differ from the analogous (A) data in several significant respects. In (B) no. 2, the selected factor V, alone, assays 15%. In no. 3, with the complete thrombic mixture (T) added, the assay is questionably increased (16%) in the 0.5-min test but is unequivocally boosted (to 42%) at the end point. In no. 4, the *reagent* TK_5 is omitted while retaining the Ca^{2+}, and there is little difference from the no. 3 result. In fact, the 37% end point in no. 4, compared with the 42% end point in no. 3, might be correlated with computed differences in the *final* TK concentrations, namely, 0.1 and 1.1 μg/ml, for nos. 4 and 3, respectively. This simple computation, however, does not take into account any possible enhancement of TK activity as carried over in the T mixtures (cf[1]). A similar comparison may be made between nos. 6 and 7, but here without any possible complication due to thrombin in the additive mixtures, since these now lack prothrombin (II). No progressive activation occurs in these preincubates, but their immediate (0.5 min) assays, respectively, give 25 and 19.5%, which, again, show small differences that could possibly be accounted for on the basis of the above differences in available TK activity. In no. 11, where Ca^{2+} is omitted from the preincubate but retained in the final assay, the difference from no. 7 is nonsignificant (19 vs 19.5%). Simple computations, which tentatively ignore questions of the specific cation availability, place the *final* Ca concentrations at 4 m*M* in no. 11, *vs* 9 m*M* in no. 7. The indications are that both these concentrations are within the optimal range for the action of the 0.1 μg/ml carry-over TK on the substrate's prothrombin. However, when the sole source of cation is the 4 m*M* carry-over in the additive mixture, as in nos. 5 and 8, this is clearly insufficient, as shown by the corresponding reductions in the bioassays. This important finding raises serious questions about the state of ionized Ca^{2+} in the additive mixtures. In fact, we must enquire precisely about all cofactor availabilities. We note, for instance, in no. 13, the complete failure to obtain any assay when PL is omitted from the test system. The CT

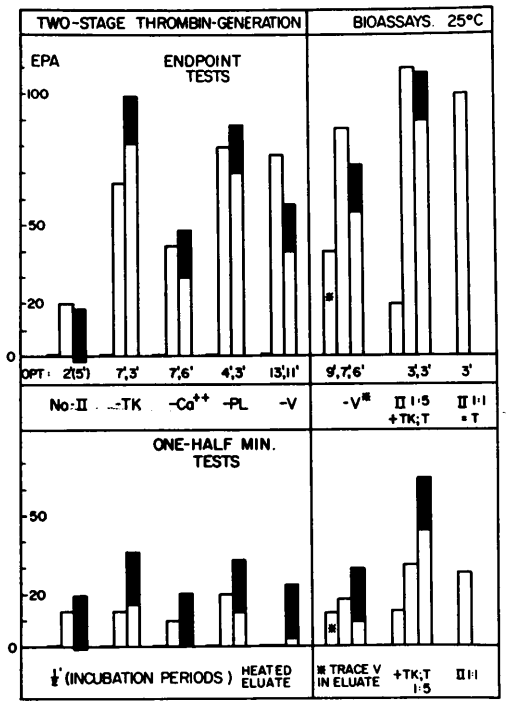

FIG. 1. Two-stage thrombin-generation bioassays (25 ± 1°). (top) endpoint tests; (bottom) 0.5-min preincubate tests, on the various mixtures indicated (see text). Optimal incubation times (min) are noted in columns two (0.2 vol of factor restoration) and three (0.2 vol of T mixture), of each tripartite series. In controls, column one, no thrombin appeared in >0.5 hr, when any of the five factors was absent.

here was 135 sec. Omitting the TK_{10} from the additive, with (no. 14) or without (no. 15) prothrombin (II), yields 22% assays in both cases. This result indicates that the 1 μg/ml assay TK_5 concentration is very adequate, and the value is quite similar to the 25% in no. 6, where 1.1 μg/ml (total) TK can be identified (without cognizance of "reactivities").

Discussion. Considerations of TK reactivity, in relation to cofactors (Ca^{2+}, V), and also needing PL for the conversion of prothrombin to thrombin, are fully investigated in other publications (3, 9). Details of extensive new results with our 2-stage methods will be made available elsewhere[1]. They are so significant in supporting some of the findings of the present 1-stage tests, however, that we are including, in Fig. 1, a simplified bar-graph summarization of some typical 2-stage results. These illustrate simply (a) the total thrombin yield (EPA = equivalents of prothrombin activated), in each of the systems identified, as represented by the full column height, (b) the correction for the *thrombin* carry-over, which is the blacked-out section at the top of the third column, in each tripartite series. The first of these three columns is the control, without additive. Whenever any one of the five components of the thrombic (T) mixture is omitted, in turn, in the first five experimental series, no thrombin generation is detectible even after a preincubation period of 0.5 hr. Restoration of 0.2 vol of the missing factor, while retaining the other four at optimal (routine) strength, as shown in the second column of each series, yields good, but *sub*optimal, thrombin generation, hence constituting a sensitive comparison for effects of an equivalent 0.2 vol of freshly prepared and maximally activated complete T mixture. Data on the last test constitute the third column of the respective series and the comparison is made after deduction of the top section for the thrombin alone. This novel experimental analysis makes it possible to determine whether each factor's activity is retained, enhanced, or diminished (for whatever reason!) when it is only supplied in the form of the T mixture. Very briefly, significant differences in the results support the conclusions that (a) TK gains some reactivity, but (b) all three cofactors (Ca^{2+}, V, PL) lose some activity, when supplied in the thrombic mixtures. The losses (b) may be interpreted as some complexing of the three cofactors, which lessens their respective availabilities. It requires optimal new cofactors, therefore, to show the full TK enhancement (a). It is just such considerations of precise TK reactivity and its relation to availability of the three specified cofactors, which can account for the type of results demonstrated in the present 1-stage tests.

In conclusion, it may be strongly suggested that a major effect of thrombokinase, which is thus dependent on cofactor availabilities,

offers a satisfactory ultimate explanation for the apparent potentiations in 1-stage tests. These results do not depend on (extrinsic) thrombin nor on any hypothetical "factor Va" (1). In ascribing the potentiation mechanism to TK, it still relates to factor V (as such) in one important particular, namely, that factor V is an essential cofactor for small amounts of theombokinase. In other particulars, very small concentrations of factor V suffice for a potent TK, and hence mere use of a relatively V-deficient substrate plasma is no guarantee of a genuinely specific *factor V* activity assay, since combined activation effects are easily proved possible.

Nothing in the present investigations is incompatible with the previously promulgated view (9), namely, that factor V has a dual role. First, it is a specific cofactor determinant (along with Ca^{2+}) of the prothrombin-converter enzymic activity. Second, it seems to serve, somewhat like an amboceptor, to bring the enzymic activator complex (TK + Ca^{2+} + V) into steric juxtaposition to the reactive groups of prothrombin (II), as this thrombin precursor is held, with PL, in the micelles of a postulated *colloidal* substrate system. This explanation of thrombin generation and the present new conclusions as to the true meanings of clotting test bioassays are, we believe, helpful new concepts of the basic mechanisms of blood coagulation. Our 30-year old *thromboplastic enzyme* theory (10) has thus been modernized (3), as pointed out in a recent historical review (11).

Summary. Bioassays of effects on clotting tests, due to preincubates of various combinations of the five basic factors involved in enzymic (TK) thrombin generation, support a new idea that such assays are very sensitive to small amounts of thrombokinase, and that the reactivities of TK are determined by availabilities of the three cofactors, Ca^{2+}, factor V, and phospholipid (PL). The untenable theory of a V → Va activation can now be reinterpreted in the light of these new data.

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Received Sept. 11, 1968. P.S.E.B.M., 1969, Vol. 130.