Determinants of the So-called "Prothrombin Time".* (22705)

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A recent study(1), assaying the blood-clotting components in normal newborn infants, emphasizes the lack of bleeding disorders and the essentially normal values of the unmodified "prothrombin time" test(2), despite significantly low levels of prothrombin and proconvertin (SPCA, stable factor, factor VII, etc.). It was noted, however, that proaccelerin (AcG, labile factor, factor V, etc.) in the infants' bloods, contrary to previous reports(3,4) was usually *hyper*normal, with values up to 300% of standard normal adults' levels. Dr. Quick(5), and others, have recognized the influence upon the one-stage prothrombin time test of *hypo*normal levels of all 3 of the above thrombin-forming factors. Recently, Quick and Hussey(6) attempted to test effects of adding "excess" of (a) AcG (labile factor) or (b) proconvertin (stable factor) to (1) normal and (2) prothr.-deficient,(3) AcG-deficient, and (4) proconv.-deficient plasmas. They concluded that " . . . a deficiency of any one is *not* compensated by an excess of the other two . . ." (*i.e.* of the 3 thrombin-forming factors). " . . . The determinant of the prothrombin time is the component which decreases below a minimum critical level . . ." As these authors do not give analyses to show the exact amounts of the 3 components in each test, there is considerable question as to the degrees of the deficiencies and "excesses" stated. The following experiments, therefore, were designed to retest various artificial plasma systems, whose assayed composition with reference to the 3 factors under consideration was defined, and could be correlated with the clotting-times after adding calcium and tissue thromboplastin essentially in the manner of the Quick test.

Methods and reagents. Assays: I. (pro) thrombin, (pro) convertin, and (pro) accelerin—the method does not distinguish precursors from the activated forms—are assayed by specific onestage procedures, which follow a common pattern, thus: 0.1 ml of suitable dilution of agent to be tested is mixed with 0.1 ml appropriate "substrate" and the clotting timed at 37°C on adding 0.2 ml *Catpln.* (equal (0.1 ml) vols., preincubated, of 0.02 M $CaCl_2$ and 'Soluplastin', courtesy of Schieffelin & Co.). The *substrates* are modified plasmas lacking the factor to be tested, but complete with regard to the other two(1). Clotting-times are converted into *percentage* units by reference to standard dilution curves obtained with normal adult human plasmas. These "units" (per ml) are employed throughout the present work. Confirmatory prothrombin analyses by the 'improved' 2-stage method(7) are also performed, but give no significant differences in the assay values.

II. *Thrombin* test: 0.2 ml fibrinogen + 0.2 ml agent tested. Observe for clotting.

III. *Antithrombin test*: Incubate 1 ml agent + 1 ml thrombin (bovine, Upjohn's, 20 units/ml) in siliconed tubes, at 25°C, and test 0.2 ml samples with 0.2 ml fibrinogen, at intervals, to observe any loss of thrombic activity.

Materials. Oxalated dog plasma is adsorbed with $BaSO_4$, 100 mg/ml, for 10 min. at 25°C. The supernatant is used to prepare *fibrinogen* by ¼ sat. $(NH_4)_2SO_4$, redissolving the precipitate in ⅓ original vol. of 0.85% NaCl and dialysing against saline containing 0.05 M trisod. citrate. This stock fibrinogen solution is stored frozen at —20°C for many months and is free from prothr., proconv., proaccel., by the above assays.

The $BaSO_4$ sediment is washed (x 2) with dist. water and eluted with ⅓ original (plasma) vol. of 0.2 M trisod. citrate and the *eluate* dialysed against 0.85% NaCl. *Eluate assays. Prep. I.* Prothr. 180, proconv. 100; AcG trace (±), thrombin 0, antithrombin 0. *Prep. II.* Prothr. 120; proconv. 174; AcG

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TABLE I. Varying AcG, at Several Eluate (Prothrombin + Proconvertin) Dilutions.

Eluate (I)	Pro-thrombin	Procon-vertin	AcG ±	15	30	60	100	200	300
			c.t. (sec.)						
1 : 1	60	33.3	38.2	20.2	18.1	13.7	11.5	*10.6*	*10.2*
1 : 2	30	16.7	48.1	28.1	22.6	16.4	13.9	11.8	*10.4*
1 : 4	15	8.3	64.4	—	23.1	21.6	15.7	14.9	13.7
1 : 8	7.5	4.1	92.	—	—	28.5	23.6	18.5	17.9

±: thr. 0; antithr. 0. *AcG* ('serum'-type or accelerin): $BaCO_3$ adsorbed (4 g/100 ml) beef serum(8), assaying 1240-1380 AcG, and free from proconvertin and all but a very insignificant trace of (pro) thrombin. It contains antithrombin. *Proconvertin* (Prep. A). Serum obtained from recalcified oxalated beef plasma after 18 hrs at 4°C is treated with $BaSO_4$ (10 g/100 ml), the sediment washed (x 4) with dist. water and eluted with 1/5 original vol. of 0.2 M trisod. citrate and the eluate dialysed. This preparation assays 85 *pro*convertin, which special tests show to be mostly in the precursor form, as in the 'prothrombin'-eluates. Other assays: prothr. ±; AcG 0; thr. 0; antithr. ±. Many tests were made with this preparation, but the results were so similar to those with prep. B, in the cited experiments, that they will not be detailed. *Convertin* (Prep. B). Dr. R. H. Wagner *et al.*(9) of our Pathology Dept., kindly supplied us with this preparation, termed (SA) "serum accelerator" in their cited publication in this journal. Our assays show: convertin 81; prothrombin 1; AcG 0; thr. 0; antithr 0.

Test method. Using the above assay values and suitably diluting and mixing the reagents, various *artificial plasmas* are reconstituted. 0.15 ml (*e.g.* 0.05 ml eluate + 0.05 AcG or (pro) conv + 0.05 ml fibrinogen) is tested with 2 vols (*i.e.* 0.3 ml) Ca-tpln., at 37°C, in essentially the manner of the "prothrombin time" test. Such test on 0.15 ml normal dog plasma gives clotting-times of 7-8 seconds.

Results. Table I shows effects of varying the AcG, with 4 different dilutions of eluate (prothr. + proconv.). In each case clotting-times (c.t.) get shorter with increasing AcG. There is a significant failure to find any true "optimum" of AcG concentration. Thus c.t.'s continue to shorten above the 'normal' (100) AcG level even to 3 times that strength. When there is enough prothrombin (and proconvertin), the shortest c.t.'s tests, namely at 300 AcG in the 1:1 and 1:2 dilutions of eluate, are so similar as to be within the experimental error of reading. This is so despite the fact that one has twice the prothrombin (and proconvertin) content of the other. Under these circumstances, the test obviously ceases to be a measure of prothrombin (and proconvertin).

Table II shows effects of varying (pro) convertin (added prep. B), again at 4 different dilutions of eluate. Parallel tests are performed with added excess (300) AcG and with merely the trace (±) of AcG present in the eluates. Clotting-times are shorter, in each series, the higher the (pro) convertin level and the less dilute the prothrombin. The chief conclusion from this experiment is that increasing the (pro)-convertin, up to 85, fails to compensate for lack of AcG or diminishing amounts of prothrombin.

Table III is similar to the foregoing in varying the (pro) convertin and AcG, but the prothrombin is kept at a constant very low level. In each series, there is here a definite small improvement with each incre-

TABLE II. Varying Proconvertin, at Several Eluate Dilutions, with Excess or Trace Amounts of AcG.

Eluate (II)	Pro-thrombin	(Pro)con-vertin	AcG	Clot.-time
1 : 1	40	85	300	8.7″
"	"	"	±	17. "
1 : 2	20	56	300	9.3″
"	"	"	±	17.8″
1 : 4	10	41.5	300	11.5″
"	"	"	±	22.6″
1 : 8	5	34.3	300	13.4″
"	"	"	±	28.3″

TABLE III. Varying (Pro)convertin, at Constant Very Low Level Prothrombin (5), and with Excess or Trace Amounts of AcG.

	(Pro)convertin	AcG	Clot.-time
1 a	34.3	300	13.4″
b	34.3	±	28.3″
2 a	20.8	300	14.4″
b	20.8	±	30.6″
3 a	14	300	15.3″
b	14	±	34.2″
4 a	7.3	300	18.2″
b	7.3	±	40. ″

ment of (pro)convertin, in a range of rather low levels. However the large differences due to the major AcG variable persist.

Table IV shows effects of varying (pro) convertin (added prep. B), at constant low prothrombin level, with 4 different levels of AcG. In each case there is a definite inhibitory effect at the highest (69.5) (pro)convertin concentration and least effect at the lowest (29, proconvertin in eluate only), with an apparent optimum (at 56) in the first 3 series. At the highest (200) AcG levels, the effects of varying the (pro)convertin are abolished except for the minor inhibition. The differences between the 4 series which depend upon the AcG level are not significantly altered by varying the proconvertin. In fact, the effects due to the variation of the proconvertin level are quite minor in the range studied.

Table V shows effects of varying (pro)convertin (added prep. B), with constant 'normal' (100) AcG, at 2 fixed concentrations of prothrombin. In 1a the small improvement over 1b (at ½ the (pro)conv. conc.) is real. In 2b the improvement due to increase of (pro)conv. over that in 2c is again significant. In 2a the inhibitory effect of too much added convertin begins to appear (*cf.* Table IV).

TABLE IV. Varying (Pro)convertin, with Low Constant Prothrombin, at Different AcG Levels.

	AcG	Pro-thrombin	(Pro)convertin* 29	42.5	56	69.5
			c.t.			
1.	±	20	17.0	15.6	15.1	18.5
2.	13.3	20	13.5	11.6	11.2	14.0
3.	66.7	20	10.9	10.0	9.8	10.7
4.	200	20	*9.7*	*9.7*	*9.8*	10.3

* 29 units are *pro*convertin in the eluate, the rest is *convertin* added.

Discussion. Besides confirming the now well established fact that these one-stage clotting times (*cf.* "prothrombin time") depend upon the 3 known variables, prothrombin, AcG, and proconvertin, the present experiments add new information on the relative importance of each and on the results of varying their quantitative interrelationships upon the observed clotting-times.

Most significant is the evidence (Tables I and IV) that in a certain moderately low range of prothrombin (and proconvertin) concentrations a truly large excess, *i.e.* 200-300% of 'normal', AcG can abolish the clotting-time differences, within an experimental error of a few tenths of a second, which would be seen at 100% or lower AcG concentrations and, if shown, be an index of the varying prothrombin (and proconvertin). The AcG level is a very significant variable. On the other hand, the (pro)convertin, at any fixed prothrombin or AcG level, exerts relatively minor effects although these are not insignificant. Increasing amounts of (pro)convertin do compensate somewhat for reduced prothrombin levels, but very incompletely and they do not at all abolish the large differences due to varying AcG. There is an experimental limit to our tests on these points due to the appearance of an inhibitory phenomenon. This could not be attributed to antithrombin, since it was not seen with excessive amounts of the AcG preparation which was rich in that factor. It was seen even more in the non-cited experiments with our own *pro*convertin (prep. A), which did have a small amount of antithrombin. The antithrombin test with Dr. Wagner's convertin, however, was negative,

TABLE V. Varying (Pro)convertin, with Constant Normal (100) AcG, and 2 Fixed Concentrations of Prothrombin.

	AcG	Pro-thrombin	(Pro)con-vertin*	c.t.
1 a	100	40	94.8	9.3″
b	100	40	58	9.9″
2 a	100	20	80.3	10.9″
b	100	20	51.3	10.1″
c	100	20	29	12.0″

* In 1 series, 58 units are *pro*convertin in eluate; in 2 series, 29 units are *pro*convertin in eluate.

but it, too, gave inhibition (see Tables). Whether these inhibitory effects and the apparent "optimum," for concentrations of (pro)convertin, are due to (a) some unidentified inhibitor contaminating the present preparations, or (b) some type of competitive reaction in the complex sequence of the thrombin-forming processes, are matters which will require further investigation.

Further technical questions may arise concerning possible difference in one-stage systems, between *pro*-accelerin or *pro*convertin and their 'activated' forms, accelerin or convertin, respectively. Nothing in our test system suggests such a possibility and the determinants we have sought by adding the 'activated' forms, in all probability, hold also for their precursors. We believe, therefore, it is reasonable to transfer the results of these experiments to interpretations of "prothrombin times" encountered in ordinary plasmas. In particular, they offer an attractive explanation of the frequently normal "Quick" test on newborn infants' blood, namely, that the very considerable *hyper*-proaccelerinemia of such cases(1) can *compensate* for the normally low levels, in infants, of prothrombin and proconvertin. There is no need, in our opinion, to postulate any obscure "convertibility" factor or some hypothetical "prothrombinogen."

Summary. In analysed systems representing artificially reconstituted plasmas of known composition with respect to prothrombin, (pro)convertin, and AcG, one-stage clotting-times with Ca-thromboplastin, essentially in the manner of the "prothrombin time" (Quick) test, show: (1) prothrombin concentration is a major variable. (2) (pro) convertin concentration is a minor variable, which, except at very low(10) levels, affects the clotting-times significantly only at very low prothrombin levels. (3) High (pro)convertin levels, short of an unexplained inhibitory phenomenon (minor), do not compensate significantly for low levels of prothrombin or of AcG. (4) AcG levels are a major variable. (5) Excessively high (200-300% of 'normal') AcG levels *can compensate* for moderately low prothrombin and proconvertin, causing the one-stage test to lose significance as a measure of these other two factors. (6) It seems valid to apply this last result to explain the normal "prothrombin time" (and lack of bleeding) in normal healthy newborn infants(1).

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