A Simple Assay Method for Factor X (Stuart-Prower Factor).* (27328)

CECIL HOUGIE (With technical assistance of Mabel Kadeschuk and R. Clarke)
Department of Pathology, University of Washington, Seattle

It is possible by filtration of plasma through asbestos pads to adsorb factor VII and factor X (Stuart-Prower factor) while maintaining a relatively high concentration of prothrombin and other clotting factors in the filtrate. Asbestos-filtered ox or human plasma has therefore been widely used as a substrate for assay of combined factor VII and factor X activity. The observation that Russell's viper venom restores to normal the clotting defect of factor VII-deficient but not factor X-deficient blood provided the basis of a specific assay for factor X. Seitz-filtered ox plasma is used as substrate and the thromboplastin source is Russell's viper venom and 'cephalin'(1). However asbestos filtration gives variable results and the yield of prothrombin-rich plasma is poor(2). Adamis (3) found that wood charcoals are capable of adsorbing factors VII and X from ox plasma, the filtrate retaining a high proportion of prothrombin. They used plasma filtered in this way in preference to asbestos filtered plasma as the substrate for a specific factor X assay (4). Although charcoal adsorption of plasma results in good yields of factors VII- and X-deficient plasma, the method is too time-consuming for general use.

Soulier(5) studied the properties of bentonite and found that at a certain concentration, combined factors VII and X activity were adsorbed from plasma while the supernatant after centrifugation contains almost all of the original prothrombin. Such a preparation of bentonite-treated plasma was shown by these workers to contain little factor V or fibrinogen. These activities may be restored by addition of $BaSO_4$ or $Al(OH)_3$-adsorbed plasma which is free of factors VII and X. It will be shown in this communication that this mixture forms an excellent and easily prepared substrate for specific assay of factor X activity.

Methods and materials. Veronal buffer (Michaelis) pH 7.35 was used to make plasma and serum dilutions. Blood was collected from normal subjects using glass syringes; 0.13 M trisodium citrate was used as the anticoagulant (1 part to 9 parts blood). Plasma was separated after centrifugation at 3000 rpm for 20 minutes. Russell's viper venom ("Stypven")† was diluted with veronal buffer to 1 in 200,000 and then frozen and stored at −20°C in aliquots sufficient for one day's use. $Al(OH)_3$ was prepared and used according to the method of Biggs and Macfarlane(6).

Bentonite was obtained from Pro-Labo‡; bentonite obtained from another source has been found to be unsatisfactory (communication from Dr. J. P. Soulier). The bentonite was added to fresh citrated normal plasma at room temperature and the mixture stirred with glass rods for 10 minutes. After centrifugation at 3000 rpm for 20 minutes, the supernatant was frozen in small lots. Normal citrated plasma was treated with $Al(OH)_3$ (0.5 ml gel/10 ml plasma); if the one-stage prothrombin time after the first absorption was less than 5 minutes, a second absorption was carried out. The $Al(OH)_3$-treated plasma, unless it was to be used the same day, was frozen and stored in small lots at −20°C; it could be used for at least 14 days following collection. The factor X-deficient plasma was obtained from a patient with a congenital deficiency of factor X; the original factor X assay method using this plasma has been described(7). Factor VII assays were carried out using plasma from a patient congenitally deficient in factor VII. Prothrombin assays were carried out by the 2-stage method of Wagner(8). Factor X assays using the bentonite-treated plasma were performed by mixing equal parts of bentonite-treated plasma (170 mg/10 ml) and Al-$(OH)_3$-treated plasma. To 0.1 ml of this mixture was added in rapid succession 0.1 ml

* This work was done during the tenure of an established investigatorship of Am. Heart Assn. The investigation was supported by a grant from Nat. Inst. Health, U.S.P.H.S.

† Burroughs Wellcome & Co., Tuckahoe, N. Y.

‡ 12, Rue Pelée, Paris 11, France.

TABLE I. Adsorption of Clotting Factors by Bentonite.

	Amount of bentonite (mg) added to 10 ml plasma				
	25	100	150	200	300
	% of initial concentration				
Prothrombin	100	100	75	72	15
Factor X	100	50	6	0	0
" VII	100	12½	0	0	0

of "DIFCO" brain extract, 0.1 ml of test material (diluted 1:10), 0.1 ml of Russell's viper venom (diluted 1 in 200,000). These reagents were then placed in a water bath at 37° for 30 seconds; 0.1 ml 0.025 M $CaCl_2$ was added and clotting times recorded. Serial dilutions 1:10 to 1:160 of pooled normal serum were substituted for the test sample and a straight line calibration curve was obtained by plotting log of clotting times against log of concentration of factor X, *i.e.*, the per cent normal serum in the mixture. The 1:10 normal pooled serum dilution was usually arbitrarily chosen as the 100% reference point. A calibration curve was prepared for each batch of assays since the Russell's viper venom sometimes deteriorated on storage. Samples of serum obtained from 10 normal subjects assayed between 65 to 180% of the mean value suggesting a wide normal range but this wide scatter may be due, in part, to technic.

Results. The following experiment was performed to determine the concentration of bentonite sufficient to adsorb all factors VII and X yet leaving unadsorbed at least half of the original prothrombin. A sample of fresh plasma was divided into six 10-ml aliquots and each of these was treated with different amounts of bentonite. The supernatants were then assayed for factor X using plasma from a patient with congenital factor X deficiency (see methods) and prothrombin. Factor VII was also assayed although the concentration of this factor is unimportant with respect to the factor X assay. The results (Table I) show that optimal concentration of bentonite is between 150 to 200 mg per 10 ml plasma.

The assay system should be insensitive to variations in concentrations of prothrombin and factor VII in the test samples. The factor X content of plasma and serum prepared from the same blood sample are identical (9,10) while the prothrombin content of serum separated after 2 hours is usually below 10% of the original plasma value. Calibration curves obtained using plasma and serum from the same blood sample were very similar after correcting for the volume of sodium citrate initially present in the plasma (Fig. 1); thus differences in prothrombin concentrations do not significantly affect the assay.

Bachmann and co-workers(1,11) advocate a 1:200,000 dilution of "Stypven" in their assay; they found that this concentration was sufficient to make their assay insensitive to changes in factor VII. We have found this to be true with respect to our assay method. Using this concentration of "Stypven", serum from a patient with factor VII deficiency assayed at 100% factor X showing that the assay is insensitive to variations in concentration of this factor.

The original factor X assay method using plasma from a patient with a congenital deficiency of this factor and the new bentonite method were each used to assay the factor X content of plasma samples from patients under treatment with dicoumarol. The results (Fig. 2) show good agreement between the two methods.

Discussion. The main advantages of the new bentonite method compared to other methods using charcoal or asbestos filtration are the simplicity and reproducibility of the method. In the original assay method(7), plasma from a patient with a hereditary deficiency of factor X was used as the substrate. This patient appeared to be normal with respect to the other coagulation factors and it seems most unlikely that there was, in fact, a deficiency of another hitherto undescribed clotting factor closely akin to factor X. This possibly, however, is considerably greater when an artificially-depleted substrate is used. It is worth pointing out that the original factor VII assay method using asbestos absorbed plasma(12) was believed to measure only factor VII but this assumption was subsequently shown to be false.

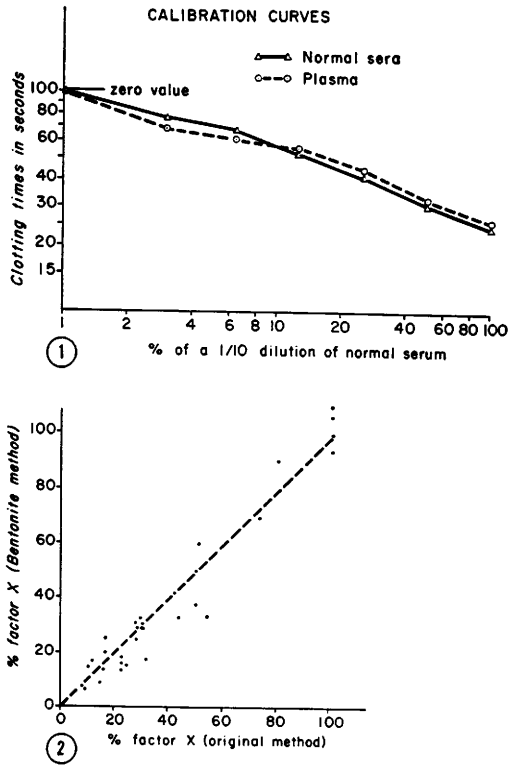

FIG. 1 and 2

In the bentonite assay method factor V and fibrinogen are adsorbed by the bentonite and must be replaced. In the method described here an equal part of human Al(OH)$_3$-treated plasma containing no prothrombin is added to the prothrombin-rich bentonite-treated plasma so that the prothrombin content of the final substrate is half that of the initial bentonite-treated plasma. Nevertheless the assay is insensitive to changes in prothrombin concentration. Although a relatively smaller amount of $BaSO_4$-treated ox plasma could be used instead of human Al(OH)$_3$-treated plasma to replace factor V and fibrinogen, human plasma is preferred because it is always available. The factor X assay method has been extensively used by us to test fractions obtained during the purification of the vit. K-dependent clotting factors. When we have compared results using this technic with those obtained by the original method with congenitally deficient plasma we have found the results to be very similar.

Conclusion. A new assay method for factor X is described. Human plasma is treated with bentonite resulting in a substrate rich in prothrombin but deficient in factors VII and X. Factor V and fibrinogen are also depleted but these factors are replaced by addition of Al(OH)$_3$-treated plasma. Russell viper venom is used to compensate for the factor VII deficiency. The method is a simple and reproducible one. The results obtained compare well with the original method using plasma from a patient with a congenital deficiency of factor X.

Addendum: The "cephalin"-RVV mixture used in the Bachmann assay(1) also gives satisfactory results in this assay method.

1. Bachmann, F., Duckert, F., Koller, F., *Thromb. et Diath. Haem.*, 1958, v2, 2.

2. Denson, K. W., *Acta Hemat.*, 1961, v25, 105.

3. Adamis, D., Sise, H. S., Kimball, D., *J. Lab. Clin. Med.*, 1956, v47, 320.

4. Sise, H. S., Lavelle, S. E., Becker, R., PROC. SOC. EXP. BIOL. AND MED., 1957, v96, 662.

5. Soulier, J. P., *J. Clin. Path.*, 1959, v12, 303.

6. Biggs, R., Macfarlane, R. G., *Human Blood Coagulation*, Blackwell Scientific Publications, 1957, 2nd Ed., 387.

7. Graham, J. B., Barrow, E. M., Hougie, C., *J. Clin. Invest.*, 1957, v36, 497.

8. Wagner, R. H., Graham, J. B., Penick, G. D., Brinkhous, K. M., in *The Coagulation of Blood*, Tocantins, L. M., Ed., Grune & Stratton, New York, 1955, p105.

9. Johnson, C. L., Jr., Ferguson, J. H., O'Hanlon, F. A., Black, W. L., *Thromb. et Diath. Haem.*, 1959, v3, 36.

10. Hougie, C., *ibid.*, 1959, v3, 578.

11. Bachmann, F., Duckert, F., Geiger, M., Baer, P., Koller, F., *ibid.*, 1957, v1, 2.

12. Koller, F., Loeliger, A., Duckert, F., *Acta Hemat.*, 1951, v6, 1.

Received December 27, 1961. P.S.E.B.M., 1962, v109.