1951, v27, 25.
6. Smith, D. T., and Scott, N. B., *Am. Rev. Tuberc.*, 1950, v62, 121.
7. Follensby, E. M., and Hooker, S. B., *J. Immunol.*, 1948, v55, 205.
8. Bawden, F. C., and Kleczkowski, A., *Brit. J. Exp. Path.*, 1941, v22, 208.
9. Kleczkowski, A., *ibid.*, 1941, v22, 188.
10. ———, *ibid.*, 1941, v22, 192.
11. Jones, F. S., *J. Exp. Med.*, 1927, v46, 291.
12. ———, *ibid.*, 1928, v47, 254.
13. Shibley, G. S., *ibid.*, 1929, v50, 825.

Received July 26, 1954. P.S.E.B.M., 1954, v87.

Serum Accelerator Factors and Antihemophilic Factor (AHF) in Early Phases of Clotting.* (21280)

JOHN B. GRAHAM, R. D. LANGDELL, F. C. MORRISON, JR., AND K. M. BRINKHOUS.
From the Department of Pathology, University of North Carolina, Chapel Hill, N. C.

In recent years a number of plasma factors have been described which are required for the optimal conversion of prothrombin to thrombin. A simple, yet effective, partial separation of these substances can be achieved with $BaSO_4$ and other alkaline earth salts. At least two plasma factors, AHF and Ac-globulin, are adsorbed hardly at all with $BaSO_4$; others, particularly PTC and convertin (SPCA) are adsorbed almost completely. Prepared from plasma, the citrate eluate of the $BaSO_4$ adsorbent contains prothrombin; however, made from serum, it is free of prothrombic activity when the parent blood has been clotted with thromboplastin. The $BaSO_4$ eluate from such serum will be designated hereafter as serum accelerator factor (SA) with the understanding that it consists of at least 2, and possibly other, clotting components. A preliminary report of our findings has been published previously(1).

Materials and methods. Blood and *native plasma* were obtained from normal and hemophilic dogs, as described previously(2). *Platelet-poor hemophilic plasma* was prepared similarly by centrifugation at 15,000 *g* for 2 hours at 4°C in siliconed tubes. The source of the *antihemophilic factor* (*AHF*) was prothrombin-free plasma, prepared from normal oxalated dog plasma by $BaSO_4$ adsorption (40 mg/ml) and dialysis against .032% sodium citrate in 0.9% NaCl (2 hr, 4°C). Plasma from both normal and hemophilic dogs was adsorbed in a similar manner and used as the diluent in the experiments of Fig. 2. *Serum accelerator factor* (*SA*) was prepared from normal canine blood to which thromboplastin had been added. The resulting serum was adsorbed with $BaSO_4$, and the adsorbent eluted with 3.2% sodium citrate. This eluate contained no prothrombin, AHF, or Ac-globulin activity, and did not clot citrated plasma in 6 hours or fibrinogen in 10 minutes. The eluate, after dialysis against normal saline, was stored at −20°C at a concentration of 20-25 SPCA units per ml.(3). *Plasma and serum prothrombic activity* were determined by the one-stage method of Langdell, Graham and Brinkhous(4), after the samples had been diluted with prothrombin-free normal or hemophilic plasma. *Plasma and serum prothrombin concentration* were determined by the Iowa 2-stage method(5), modified by the addition of Factor V(6) to the diluting fluid.

Results. The effect of the addition of SA was determined in several types of clotting systems.

The reaction of *hemophilic whole blood* is shown in Table I, Tests 2-5. The addition of SA caused the clotting time to be reduced to the normal range of 5-8 minutes, but did not alter the defective prothrombin utilization (Test 3). When SA and AHF were added together, both clotting time and prothrombin utilization were more rapid than in normal blood (cp. Tests 1 and 5). The prothrombin

* This investigation was supported (in part) by research grant H-1333 from the National Heart Institute of the National Institutes of Health, Public Health Service.

TABLE I. Effect of Serum Accelerator (SA) and Antihemophilic Factor (AHF) on Clotting of Hemophilic Whole Blood and Hemophilic Platelet-Poor Plasma.

Test No.	Coagulant added	Clotting time, min.	Residual prothrombin, %* Min. after drawing blood 15	30	60	120
	Normal whole blood (1 ml)					
1	0.9% NaCl (.1 ml)	7½	61	15	—	—
	Hemophilic whole blood (1 ml)					
2	0.9% NaCl (.1 ml)	30	100	100	100	100
3	SA (20 u/ml, .1 ml)	6	105	100	90	100
4	AHF (.1 ml normal pl)	8	78	10	—	—
5	SA + AHF (3 + 4)	4	22	10	—	—
	Hemophilic platelet-poor plasma (0.5 ml)					
6	0.9% NaCl (.1 ml)	84	—	90	—	92
7	SA (25 u/ml, .1 ml)	8	—	—	85	72
8	AHF (.1 ml normal pl)	24	—	85	85	72
9	SA + AHF (7 + 8)	8	—	78	78	74
10	AHF + platelets (.1 ml normal pl + 2×10^8 platelets)		10	—	—	—

* In all experiments the 100% prothrombin control was a sample of blood (1 ml) or plasma (0.5 ml), citrated with 0.15 ml of 3.2% sodium citrate. Residual prothrombin was determined by the 2-stage method.

utilization curves indicated that the prothrombin "half-life", time required for conversion of 50% of the prothrombin, was 18¾ minutes in normal blood (Test 1), 21 minutes in hemophilic blood with AHF alone (Test 4) and 10 minutes with both SA and AHF (Test 5).

Hemophilic platelet-poor plasma reacted quite differently (Table I, Tests 6-10). After addition of SA, either alone or with AHF, the clotting time became nearly normal, but utilization of prothrombin remained impaired. By contrast, addition of AHF and platelets (Test 10) corrected completely the defect in prothrombin utilization.

The results with *normal native plasma* containing platelets are shown in Fig. 1. On addition of SA, prothrombin was utilized more quickly than in the control plasma. This change is similar to that seen when comparing Tests 1 and 5 of Table I. The prothrombin "half-life", interpolated from Fig. 1, was 24 minutes for the control, 8½ minutes with the lesser quantity of SA and 5 minutes with the greater. The latent period, the period prior to detectible prothrombin loss(7), was 12-16 minutes in the control, 3½-5 minutes with the smaller SA addition and 0-2 minutes with the larger quantity.

The *prothrombic hyperactivity of hemophilic serum* was described in an earlier publication(4) and has been attributed to the presence of serum accelerators plus a high residual prothrombin concentration. The experiments shown in Fig. 2 duplicate and extend our previous findings. Repeating the earlier experiments, but using $BaSO_4$-treated hemophilic plasma in addition to similarly treated normal plasma as a diluent and source of fibrinogen, we found that the usual prothrombic hyperactivity could not be elicited in the absence of AHF.

Discussion. Several interesting facts emerge from the experiments described above. The SA corrects the prolonged clotting time of hemophilic blood without altering the fundamental defect, the block in prothrombin utilization. A somewhat similar experiment has been reported by Deutsch and Schaden(8). This finding implies that assays for antihemophilic activity should be regarded with skepticism when based on a clotting time type of procedure and when the serum factors have not been removed. It is apparent also from Table I that, even with AHF assays of the prothrombin utilization type, the serum factors may cause serious error, *provided* AHF is also present.

As shown in Fig. 1, the clotting time may be shortened markedly and the latent period almost abolished without significant effect on the rate of prothrombin utilization. The dissociation of latent period and reaction rate in

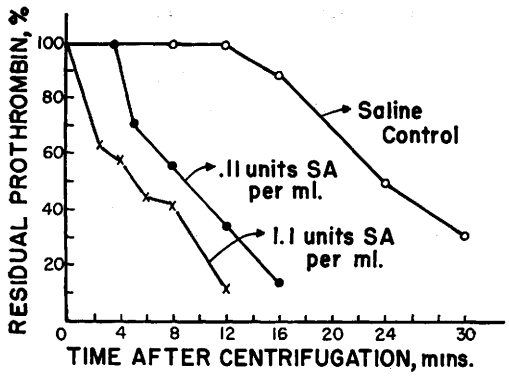

FIG. 1. Prothrombin utilization in native plasma from a normal dog. Eight experiments were performed, each similar to the one shown above. Platelet counts ranged from 5600 to 173600/mm³, but results were qualitatively similar in all. In the experiment above, native plasma (9800 platelets/mm³) was prepared from a normal dog by low-speed centrifugation in siliconed glassware at 4°C. Into each plain glass test tube at 28°C was placed 0.5 ml of the plasma. Also present in each was 0.1 ml of either 0.9% NaCl or SA. The SA concentrations, shown on the figure, are expressed in SPCA units per ml of reaction mixture. The clotting time of the saline series was 7½ min., with the smaller quantity of SA 2¾ min., with the larger quantity of SA 2¼ min.

both hemophilic and normal systems is of interest. It had been assumed that the length of the latent period in prothrombin conversion always varied inversely with the reaction rate as in many other biochemical reactions. More detailed experiments are indicated, but it would appear that the eluate operates very early in prothrombin conversion and lacks significant acceleratory effect once the reaction is underway. These results should not be interpreted as meaning that the eluate factors are inactive once thrombin appears.

Previous work had shown that AHF markedly affected the *rate* of prothrombin utilization(9). It is possible that SA and AHF operate sequentially during prothrombin conversion, SA acting earlier. This is contrary to the interpretation of Owren(10), if it is assumed that SA is equivalent to convertin. He believes AHF to act initially, and convertin, a serum factor, to be active later. Our suggestion is consistent, however, with the interpretation of Biggs, Douglas and Macfarlane(11), if one assumes that the latent period shortening by SA is the expression of its PTC rather than its convertin moiety. Our experiments do not show which specific factor of the eluate is responsible for the latent period effect. This answer awaits further chemical fractionation.

The necessity of platelets for prothrombin utilization is reemphasized by the data of Table I. From some of the literature (10,11), one would assume that platelets are not required once prothrombin begins disappearing. Our experiments suggest, on the contrary, that platelets are required throughout the period of prothrombin utilization, at least in blood and plasma. It is possible that the platelet, in combination with some reaction product perhaps, constitutes the "autocatalytic agent."

The basis of the hyperactive prothrombin time of serum shown in Fig. 2 (values >100%), is clarified by the experiments of Table I and Fig. 1. These data suggest that the increased "speed" of clotting is due primarily to a decreased latent period. This same effect may be produced by adding the eluate to normal plasma. The fact that this

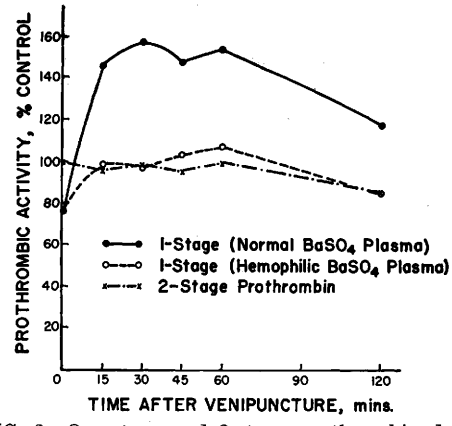

FIG. 2. One-stage and 2-stage prothrombin determinations in clotting hemophilic dog blood. At intervals 0.3 ml of 3.2% citrate was added to 2 ml of hemophilic blood clotting at 28°C, and the blood centrifuged immediately at 5000 *g* for 3 min. The serum was tested within 5 min. of citration by the one-stage technic. The actual reaction mixtures in the one-stage determination consisted of: 0.1 ml serum diluted 1-10 with $BaSO_4$ plasma + 0.1 ml Difco brain thromboplastin + 0.1 ml 0.02 M $CaCl_2$. Per cent prothrombic activity for each sample was determined by interpolating clotting time values on a dilution curve prepared by diluting the 100% control plasma with $BaSO_4$-adsorbed normal plasma. This control plasma had sat 35-40 min. at room temperature until its clotting time had reached a minimum. These minima varied from 11.5 to 14 sec. in different experiments. Two-stage determinations of prothrombin concentration were carried out later.

shortening in the prothrombin time, equivalent to a 50% increase in prothrombin, is accomplished by an eluate without prothrombin and containing an infinitesimal amount of thrombin, suggests that the substances responsible are accessory to the main reaction and not direct contributors to the thrombin pool.

One might speculate endlessly over the synergism of SA and AHF shown in Fig. 2. We were surprised to find that supernormal prothrombic activity could not be obtained without AHF, despite the use of a potent brain thromboplastin. Possibly the simplest and least controversial inference which might be drawn from the experiments of Fig. 2 is that AHF, under certain conditions, is merely another of the numerous variables in the prothrombin time test.

Summary. 1. An eluate from $BaSO_4$-adsorption of normal serum is shown to have a marked effect on the clotting time in hemophilia without altering the defect in prothrombin utilization. 2. The eluate is shown to operate chiefly by reducing the latent period of prothrombin utilization. 3. The effect of this eluate on AHF assays is discussed. 4. The necessity of platelets for prothrombin conversion is demonstrated again under different experimental conditions. 5. AHF is shown to be required for the demonstration of maximal prothrombic activity in the prothrombin time test.

1. Graham, J. B., Langdell, R. D., Morrison, F. C., Jr., and Brinkhous, K. M., *Fed. Proc.*, 1953, v12, 388.

2. Buckwalter, J. A., Blythe, W. B., and Brinkhous, K. M., *Am. J. Physiol.*, 1949, v159, 316.

3. Alexander, B., Goldstein, R., and Landwehr, G., *J. Clin. Invest.*, 1950, v7, 881.

4. Langdell, R. D., Graham, J. B., and Brinkhous, K. M., PROC. SOC. EXP. BIOL. AND MED., 1950, v74, 424.

5. Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Exp. Med.*, 1937, v66, 801.

6. Owren, P. A., The Coagulation of Blood, J. Chr. Gunderson, Oslo, 1947, p82 ff.

7. Milstone, J. H., *J. Gen. Physiol.*, 1948, v31, 301.

8. Deutsch, E., and Schaden, W., *Biochem. Z.*, 1953, v324, 266.

9. Graham, J. B., Buckwalter, J. A., Hartley, L. J., and Brinkhous, K. M., *J. Exp. Med.*, 1949, v90, 97.

10. Owren, P. A., *Am. J. Med.*, 1953, v14, 201.

11. Biggs, R., Douglas, A. S., and Macfarlane, R. G., *J. Physiol.*, 1953, v122, 538.

Received July 26, 1954. P.S.E.B.M., 1954, v87.

Simplified Method for Determination of Total Adrenal Cholesterol.* (21281)

E. KNOBIL, M. G. HAGNEY, E. J. WILDER, AND F. N. BRIGGS.
(Introduced by E. M. Landis.)

From the Department of Physiology, Harvard Medical School and the Biological Research Laboratory, Harvard School of Dental Medicine, Boston, Mass.

The determination of total adrenal cholesterol by the method of Schoenheimer and Sperry(1) or modifications thereof has been a cumbersome and time-consuming procedure. The publication of a simple and rapid direct method for the determination of serum cholesterol by Zlatkis, Zak, and Boyle(2) prompted us to adapt their procedure to the determination of adrenal cholesterol. The modified Zlatkis, Zak, and Boyle procedure was subjected to comparison with that of Sperry and Webb(3) with good agreement between the 2 methods.

Methods. Reagents. With a few minor modifications, the reagents and following procedures are those previously described by Zlatkis, Zak, and Boyle(2) for the determination of total serum cholesterol. 1. *Standard cholesterol solution*: 0.2 mg pure, dry, ash-free cholesterol/ml of glacial acetic acid (C.P. 99.7%). 2. *Ferric chloride sol.*: Dissolve 10 g

* Aided by grant from the Milton Fund, Harvard University and by research grant from the National Institute of Arthritis and Metabolic Diseases of the National Institutes of Health, U. S. Public Health Service.