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Blood Thromboplastin: Its Preparation and Properties.* (25100)

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The concept of blood thromboplastin has been accepted by most workers in the clotting field since publication by Biggs and associates (1) of their classical studies on initial stages of blood coagulation. Bergsagel and Hougie (2) have shown that an activity corresponding to that of whole blood thromboplastin can be sedimented on platelets incubated in serum and recalcified plasma reagent, and they designated this activity "product 2." Similar activity has been sedimented on crude cephalin by Streuli(3); the present study deals with properties of such a thromboplastic preparation.

Materials and methods. Thromboplastin generation reagents were prepared as previously described(4), except that dilutions of all reagents were made with isotonic saline brought to pH 7.3 with imidazol buffer. Plasma reagent of human origin was diluted to 20% and bovine to 10%. The basic steps in preparation of blood thromboplastin were adapted from the method of Bergsagel and Hougie(2) as follows: one volume each of plasma reagent, serum reagent, and 0.025 M $CaCl_2$ were mixed and allowed to incubate at 37°C for 15 minutes. The clot which invariably formed was carefully removed, and an additional volume of cephalin reagent was added. Another 5 minutes incubation at 37°C gave a thromboplastin reagent which clotted recalcified human substrate plasma in 9-11 seconds. This was immediately centrifuged for 30 minutes and about 20,000 rpm at 4°C in an International PR-2 Refrigerated Centrifuge equipped with multi-speed attachment. The supernatant was discarded, and the sediment was resuspended in buffered isotonic saline containing 0.025 M $CaCl_2$. The mixture was again centrifuged as before, and the procedure repeated to give 2 washings. The last supernatant had no demonstrable thromboplastic activity. The final sediment was suspended in a volume of buffered calcium-saline equal to ¼ the volume of original generating mixture. By subsequent dilution this reagent was adjusted so that it clotted substrate human plasma in about 12 seconds. It was found that "cephalin" preparations from human brain or platelets(5) gave identical results. Plasma deficient in factor V was prepared by aging oxalated human plasma at 37°C for 3 days; plasma deficient in Stuart factor was kindly supplied by Dr. J. B. Graham from University of North Carolina.

Results. Blood thromboplastin obtained in the sediment was highly active, and had clotting properties described by others in whole thromboplastin generation mixtures(6). The reagent clotted normal, factor V-deficient, and Stuart factor-deficient plasmas equally well thereby demonstrating properties different from those of tissue thromboplastin (Table I). Blood thromboplastin preparation failed to clot 0.5% concentrations of Cohn's fraction I (Cutter) or substrate plasma with 0.1 cc of 0.38% sodium citrate substituted for calcium reagent, indicating absence of demonstrable thrombin activity.

TABLE I. Clotting Times in Seconds of Substrate Plasmas.

	Thromboplastin	
	Tissue	Blood
Normal	12	12
Factor V—deficient	37	13
Stuart factor—deficient	59	13

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TABLE II. Properties of Blood Thromboplastin.

Thromboplastin preparation	Human plasma substrate clotting time (sec.)
Fresh*	12
Incubated 1 hr at 37°C*	12
" 5 min. at 56°C*	28
" 10 min. at 56°C*	180
" 18 hr at 4°C*	20
" 18 hr at 4°C†	13
" 18 hr at 37°C†	60
Stored at 20°C for 24 hr*	23
Idem 3 wk*	56
Incubated 30 min. with equal vol of buffered saline*	17
Incubated 30 min. with equal vol of human serum*	40
Resuspended and washed sediment recovered from thromboplastin-serum incubation*	41

* Human plasma reagent.
† Bovine plasma reagent.

Some properties of blood thromboplastin are given in Table II. Storage stability was considerably greater than activity developed in the usual thromboplastin generation mixtures where there is rapid deterioration at 37°C(7). This increased storage stability probably results from removal of thromboplastin inactivator(s) in the washing process. Addition of citrated serum to the thromboplastin preparation resulted in rapid inactivation; and activity was not restored by resedimentation and washing twice as described above. Further increased storage stability was obtained when bovine instead of human plasma reagent was used. Activity was rapidly destroyed at 56°C and disappeared in the frozen state.

When the cephalin reagent was incubated with either plasma reagent or serum reagent in presence of calcium ion, the resulting washed sediment was unable to act as a combined reagent when used in a thromboplastin generation test, as shown in Table III. Thus, there is no evidence that the cephalin reagent was capable of adsorbing separate clotting factors in nonspecific fashion. Moreover, if the washing process was accomplished in calcium-free buffered saline, a preparation of poor activity was obtained. Activity was not restored by subsequent addition of Ca^{++}.

An experiment was performed in which plasma and serum reagents were used without dilution, and calcium and cephalin reagents were used in 10-fold concentration. Thromboplastin formation was performed as above, except that final thromboplastic mixture was kept incubating in 37°C water bath until all activity was gone, as measured by inability to accelerate clotting time of recalcified substrate plasma. This incubation took about 45 minutes. The mixture was then centrifuged and the sediment washed as described above, after which the sediment was suspended in a volume of buffered saline equal to that of starting volume of added cephalin reagent. A second mixture was prepared in identical manner except that calcium reagent was omitted: this preparation failed to clot, and no thromboplastic activity developed. The final suspensions of both preparations were used as cephalin reagent in the standard thromboplastin generation test, comparing their activity by serial dilution with an aliquot of the cephalin reagent originally used which had concurrently incubated at 37°C. Sediments resuspended from both mixtures showed identical activity, which was about half that of the free cephalin reagent. The loss probably represented incomplete sedimentation of cephalin, and no change could be attributed to inactivation by clotting. These findings are of interest in view of the recent report by Alexander *et al.*(8) that "serum platelets" poorly support thromboplastin generation. It is also evident that the sedimented material is phosphatide-containing.

Discussion. The thromboplastic material

TABLE III. Properties of Variously Prepared Thromboplastin Sediments.

Thromboplastin preparation	Human plasma substrate clotting time (sec.)
Plasma reagent, cephalin, Ca^{++}—Preincubated, washed, used with serum reagent in TGT	30*
Serum reagent, cephalin, Ca^{++}—Preincubated, washed, used with plasma reagent in TGT	28*
Blood thromboplastin washed with Ca^{++}	12
Blood thromboplastin washed without Ca^{++}	32
Idem, incubated 10 min. with added Ca^{++}	33

* After 6 min. incubation of generating mixture.

reported here appears identical in its properties to "product 2" of Bergsagel and Hougie (2). Our studies and those of Streuli(3) indicate that the only necessary component provided by platelets is their phosphatide. Since the thromboplastic material is probably a phosphatide-protein complex, and because of its sedimentation properties, it has characteristics resembling those of tissue thromboplastin prepared by Chargaff(9). It differs from the latter in its ability to convert prothrombin to thrombin in the absence of factor V or Stuart factor, and in its dissociation in the absence of calcium ion.

Our studies support the concept of blood thromboplastin formation as a chain reaction, since there did not appear to be haphazard complexing of clotting components to the phosphatide emulsion. This was demonstrated by failure of new activity to form when the cephalin reagent was incubated with either plasma or serum reagents separately. The data do not contribute to the question as to which clotting factors are complexed or whether any act in enzymatic manner. However, the present technics may provide a means by which such problems may be more effectively handled. As purified clotting factors become available, it would be of considerable interest to determine which are sedimented with appropriate clotting phosphatide.

Summary. A reagent with activity resembling that of blood thromboplastin was sedimented on crude cephalin. Activity was lost if washing of sediment was undertaken in absence of calcium ion. The thromboplastic reagent normally clotted plasmas deficient in Stuart factor or factor V; activity was irreversibly destroyed following incubation with serum. Certain properties similar to those of tissue thromboplastin are indicated.

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Action of Rabies Vaccine Derived from Embryonated Duck Eggs Against Street Virus. (25101)

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Two alternatives appeared possible in attempts toward decreasing the chances of encephalomyelitis following use of rabies vaccine derived from rabbit brain or other species of brain. These are: (1) chemical or physical treatment of the vaccine to remove as far as possible factors contributing to this type of side reaction, and (2) preparation of vaccine from virus infected tissue initially devoid of such factors. We experimented mainly along the latter line since some early results indicated fixed rabies virus could be grown reasonably well in embryonated duck eggs(1). Such duck embryo (DE) killed rabies vaccine suffices as a useful agent for treatment of persons bitten by animals or otherwise exposed to rabies street virus, and degree of antibody response and minimal extent of side reactions in such human patients have been reported(2). Although DE rabies vaccine fulfills requirements of potency and safety for human usage, we have thus far reported only