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THE THROMBOTIC ACTIVITY OF ACTIVATION PRODUCT *

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A complete thrombus requires up to 8 hours to form when blood is held motionless within an isolated venous segment (1). In striking contrast, blood clots within 15 minutes when it is put into a glass test tube.

Surfaces such as glass, kaolin powder, or diatomaceous earth enhance blood coagulability by promoting the generation of an intermediate clotting activity which has been named activation product (AP) (2). [Others have referred to this activity as the "contact" factor (3) and as the third prothromboplastic factor (4).] Formation of activation product requires the interaction of at least two plasma clotting factors, Hageman factor (HF) and plasma thromboplastin antecedent (PTA) (2, 4–6). Ratnoff, Davie and Mallett (6) have recently presented evidence that contact with a foreign surface first activates HF which in turn activates PTA; i.e., that activation product is "activated" plasma thromboplastin antecedent. Although knowledge of the chemistry of activation product is still meager, it is important to emphasize that *its generation does not require calcium ions, platelets, or any of the known plasma clotting factors except HF and PTA.*

Intrinsic coagulation [1] *in vitro* proceeds rapidly in the presence of activation product. For example, Waaler has shown that normal native platelet-rich plasma may be kept fluid for more than 1 hour at 37.5° C in a perfectly siliconized test tube; in striking contrast, the recalcification time of citrated plasma in which maximum activation product activity has formed is only 40 to 50 seconds (2). Gaston and DeOrsay (7) have reported that the rate of formation of thromboplastin in the throboplastin generation test increases in parallel with the activation product content of the incubation mixture.

Thus, formation of activation product may be thought of as the trigger mechanism for intrinsic coagulation *in vitro*. The ability of activation product, isolated from all other measurable *in vitro* clotting activities, to trigger clotting *in vivo* has not been studied previously.

Wessler (8) has shown that the intravenous injection of normal serum induces a fleeting, generalized hypercoagulable state, during which a thrombus will form in an isolated venous segment within 10 minutes. The presumed single moiety in normal human serum responsible for this thrombosis-inducing activity was named the serum thrombotic accelerator (STA) (9).

Reimer, Wessler and Deykin (9) have shown that serum must contact glass to develop thrombotic activity. Moreover, Wessler and Reimer (10) could not demonstrate thrombotic activity in serum lacking Hageman factor and found inconstant activity in serum lacking PTA. These observations suggested that STA activity might be an *in vivo* manifestation of activation product.

Other observations of Wessler and his associates have raised doubts as to the identity of STA activity and activation product. Thus, Reimer and Wessler stated (3) that serum depleted of "contact" factor retained thrombotic activity. Moreover, the formation of activation product does not require plasma thromboplastin component (PTC, Factor IX); yet, Wessler and Reimer (10) found no thrombotic activity in hereditary PTC-deficiency serum and Reimer and associates (9) found no thrombotic activity in normal human serum fractions prepared free of PTC.

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[1] Intrinsic coagulation refers to clotting resulting from the generation of the blood's own thromboplastic activity. Extrinsic coagulation refers to clotting induced by tissue products with thromboplastic activity.

The experiments resported herein were designed to examine the thrombotic activity of activation product. Activation product was prepared free of other known *in vitro* clotting factor activities both from human serum and from human plasma. Its thrombotic activity was tested in rabbits using a modification of the biological assay for thrombosis-inducing activity in human serum and human serum fractions designed by Wessler, Reimer and Sheps (11). The data clearly show that activation product possesses powerful thrombotic activity; they also strongly suggest that activation product is the moiety in normal human serum responsible for its thrombosis-inducing activity.

MATERIALS

A. *Materials tested for thrombotic activity*

Normal serum was prepared by incubating clotted blood in glass tubes for 2 hours at room temperature and for an additional 18 hours at 4° C. The serum was separated by centrifuging at 2,500 G and stored frozen in stoppered, glass tubes at approximately − 20° C.

Speedex-adsorbed normal serum was prepared by shaking normal serum vigorously for 10 minutes at room temperature with 120 mg of diatomaceous earth powder (Dicalite Speedex, Great Lakes Carbon Co., Walteria, Calif.) per ml of serum. The Speedex powder was then removed by centrifuging twice for 20 minutes at 14,450 G at 4° C. The supernate was incubated for 5 hours at 37° C in glass tubes to allow residual activation product activity to decay (2, 4) and was stored frozen at − 20° C in stoppered, glass tubes.

Normal serum eluate was prepared from the Speedex powder with which normal serum had been adsorbed. The powder was washed 3 times with volumes of 0.05 M sodium chloride solution equal to the original serum volume and was then eluted by shaking vigorously for 20 minutes at room temperature with the same volume of 7 per cent (wt/vol) calcium chloride dihydride. The powder was removed by centrifuging twice for 20 minutes at 14,450 G at 4° C. The supernate was dialyzed for 48 to 64 hours against multiple changes of isotonic, citrated saline solutions (diluting fluid I followed by diluting fluid II) to remove calcium ions. It was then stored frozen at − 20° C in tightly capped, polyethylene tubes. The eluates were used within 3 weeks.

Although the eluate looked water-clear, traces of residual Speedex powder were found in a lyophilized sample. (Since our technique of lyophilization completely inactivated activation product, only "liquid frozen" preparations were assayed for thrombotic activity.) The degree of contamination, as estimated in subsequent samples by comparing the absorbance of the eluates with that of standard suspensions of Speedex powder in diluting fluid II, using a Weston colorimeter at setting A, was found to be 0.039 to 0.078 mg of Speedex powder per ml of eluate.

HF-, PTA-, and PTC-deficiency serum eluates were prepared in an identical manner from blood of donors with these specific deficiencies. The diagnosis of Hageman factor deficiency in our donor was confirmed by cross-correction experiments kindly performed by Dr. Oscar Ratnoff. The detailed studies on our donor with PTA deficiency have been reported earlier [E.G., Case 3 (12)]. Our three PTC-deficiency donors had classical clinical findings of severe hemophilia and PTC levels of 0.5, 5, and 11 per cent of normal, respectively, in our quantitative assay for PTC.

Normal plasma eluate was prepared as described by Waaler (2). Nine parts of normal blood, taken with needles coated with Monocote-E (Armour and Co.) and syringes coated with silicone (Dri-film, GE SC-87), were added to 1 part of a buffered citrate anticoagulant (made by combining 2 parts of 0.1 M citric acid and 3 parts of 0.1 M sodium citrate) in polyethylene tubes. The blood was centrifuged for 20 minutes at 14,500 G at 4° C and the resultant platelet-poor plasma was removed using "silicone technique." The plasma was shaken with Speedex powder and the powder separated, washed, and eluted as described above, except that hypertonic sodium chloride solution was used as the eluting fluid. This was prepared by adding 7 G of sodium chloride to 100 ml of a fluid consisting of 1 part barbital buffer (a solution of 0.028 M sodium chloride plus 0.125 M sodium barbital, total ionic strength 0.15, whose pH was adjusted to pH 7.25 with 0.1 M hydrochloric acid) and 4 parts distilled water. The resultant eluate was dialyzed for approximately 24 hours against 3 changes of isotonic sodium chloride and frozen at − 20° C in polyethylene tubes. Plasma eluate was thereby prepared without exposure to calcium ions at any step.

Rabbit brain thromboplastin (Bacto Thromboplastin, Difco Laboratories) was prepared according to the manufacturer's instructions. Dilute suspensions for injection were made with diluting fluid II.

Bovine thrombin (Topical Thrombin, Parke, Davis and Co.) was adsorbed with barium sulfate and prepared in a stock solution as described elsewhere (13). Dilutions for injection were prepared with diluting fluid II in polyethylene tubes and used within 30 minutes.

Speedex powder suspensions were made by serial dilution of weighed amounts of powder in diluting fluid II. They were stored in capped tubes at 4° C until used.

Diluting fluid II was prepared by combining 1 part of 0.025 M sodium citrate, 1 part of barbital buffer, and 3 parts of 0.85 per cent sodium chloride solution.

B. *Materials used for in vitro clotting factor assays*

Exhausted plasma is the name for plasma depleted of its HF, PTA, and activation product by adsorption with diatomaceous earth powder and subsequent incubation at 37° C (2). It was prepared from normal platelet-

TABLE I

Clotting factor activities in exhausted plasma

Assay	Per cent of standard reference activity
HF	1
PTA	5
PTC	30
AHG	76
Prothrombin-proconvertin-Stuart factor complex	125
Proaccelerin	65
Proconvertin	160
Thrombin time	100

poor plasma, using Speedex powder as described above. After adsorption, the pH of the plasma was adjusted to 7.35 U with 0.1 N HCl and the plasma was incubated for 5 hours at 37° C to destroy residual activation product. It was stored frozen at − 20° C in capped, polyethylene tubes.

Exhausted plasma prepared in this manner did not shorten significantly the partial thromboplastin time of normal plasma which had not been exposed to an activating surface, of HF-deficiency plasma, or of PTA-deficiency plasma. As shown in Table I, its content of those clotting factors not involved in activation product formation sufficed for its use as a substrate for measuring activation product.

HF-, PTA-, PTC-, and antihemophilic globulin (AHG, Factor VIII)-deficiency substrate plasmas were obtained from donors with these hereditary deficiencies by means of the techniques described above for preparing normal platelet-poor plasma. These substrate plasmas were stored frozen at − 20° C in capped, polyethylene tubes until used. Lyophilized *Stuart factor (Factor X)-deficiency plasma* was obtained through the kindness of Dr. John B. Graham. Lyophilized *proconvertin (Factor VII)-deficiency plasma* was supplied through the courtesy of Dr. P. A. Owren's laboratory. *Proaccelerin (Factor V)-deficiency plasma* was prepared either by the method of Stormorken (14) or by the method of Borchgrevink, Pool and Stormorken (15).

"Cephalin," an acetone-insoluble, ether-soluble extract of human brain, was prepared as described elsewhere (16); the stock reagent, a 38 mg per ml suspension in barbital buffer, was stored frozen. Before use it was further diluted with barbital buffer to optimal concentration.

Kaolin powder (China Clay, Braun Chemical Co., Los Angeles) was added to some assays to provide a uniform activating surface. The kaolin was suspended in isotonic saline and mixed with an equal volume of "cephalin." The concentration of each reagent was adjusted so as to provide optimal activity in the final clotting mixture.

Diluting fluid I was made by mixing 1 part of 0.1 M sodium citrate and 6 parts of 0.85 per cent sodium chloride solution.

Calcium chloride was prepared in a stock 100 mM aqueous solution and diluted in distilled water to the correct concentration for each test system.

METHODS

A. *The assay for thrombotic activity*

At the suggestion of Dr. David Blankenhorn, the technique of Wessler and co-workers (11) was modified to use the palisading veins of the rabbit's small intestinal mesentery as a source of multiple, uniform, venous segments. This allowed several assays to be performed in one animal.

Fasting, female white rabbits weighing 1.5 to 2.5 kg were anesthetized with 9 ml per kg of a 20 per cent solution of ethyl carbamate (urethan, Merck) given subcutaneously. The small bowel mesentery was exposed and covered with gauze soaked in warm, isotonic saline. The test material was warmed to 37° C, and 3 ml per kg rabbit weight was rapidly injected into an ear vein. One minute and 10 seconds later a pair of veins was isolated between noncrushing vascular clamps (serrefine, 27 mm, Arthur H. Thomas Co.). Silk ligatures were placed as illustrated in Figure 1 to afford hemostasis when the isolated segments were removed. After exactly 10 minutes of stasis, the isolated segments were transferred to a Petri dish containing citrate anticoagulant and opened immediately. Thrombosis was scored (11): 0 indicated no macroscopic thrombus; 1 to 3+ indicated increasing degrees of incomplete thrombosis; 4+ indicated a complete thrombus cast of the vessel. After each positive assay, persisting hypercoagulability was ruled out by a negative control assay with known inert material (either diluting fluid II or isotonic sodium chloride) before further tests were performed.

To summarize, our technique differed from the original technique of Wessler and co-workers (11) in the follow-

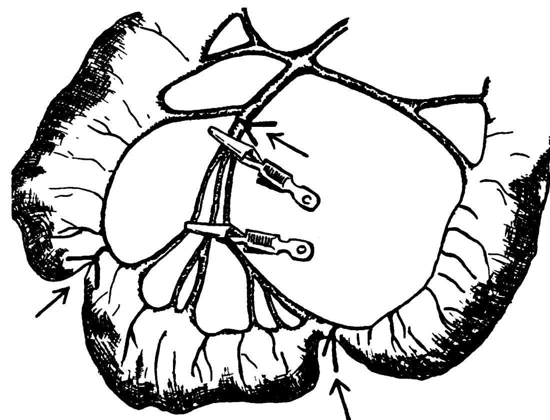

FIG. 1. METHOD OF ISOLATING VENOUS SEGMENTS FOR THE THROMBOTIC ACTIVITY ASSAY. Segments of the small intestinal veins of the test animal are isolated between small vascular clamps as shown. Silk ligatures are placed (arrows) to provide hemostasis when the segments are excised.

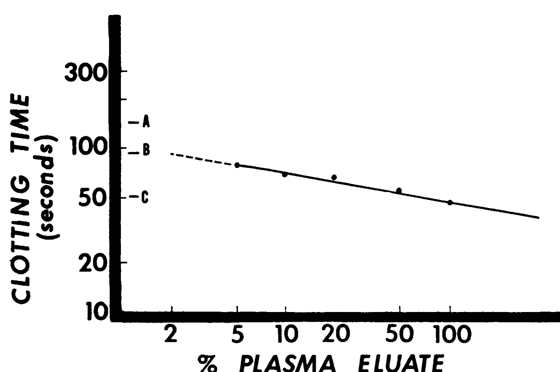

FIG. 2. PREFORMED ACTIVATION PRODUCT ACTIVITY OF DILUTIONS OF A PLASMA ELUATE. The eluate was diluted in diluting fluid II. At the left-hand margin of the graph are indicated the clotting times in this assay of the following test materials: A) normal plasma not exposed to a contact-activating surface; B) diluting fluid II; and C) normal plasma shaken for 1 minute with 5 mg per ml of kaolin powder just prior to testing.

ing respects: *1)* the veins of the rabbit's small intestinal mesentery were used as a source of isolated venous segments instead of the external jugular vein; *2)* the venous segment was isolated 1 minute and 10 seconds instead of 10 to 15 seconds after injecting the test material; *3)* 3 ml per kg of test material was injected instead of 1.32 ml per kg diluted to 5 ml with isotonic sodium chloride.

B. *In vitro clotting assays*

Substrate plasmas were thawed rapidly in warm water (approximately 37° C) and used promptly. Incubation and clotting were carried out at 37° C. Clotting times were converted to per cent of a reference standard from dilution curves plotted on log-log paper. An eluate of normal plasma prepared as described above was used as an arbitrary reference standard for preformed activation product. Normal plasma was used as a reference standard for the other assays.

The following assays are described because they are modifications of published techniques.

1. Preformed activation product assay. Waaler's activation product assay (2) was modified to measure only *preformed* activation product by preventing contact with an activating surface during the test. Pipets treated with Siliclad (Clay Adams, New York, N. Y.) and clear polystyrene clotting tubes (T 1275C, Falcon Plastics, Culver City, Calif.) were used. To minimize inhibition of preformed activation product, the test material was warmed separately and added as the final reagent. One-tenth ml of exhausted plasma and 0.1 ml of cephalin reagent (1/70) were incubated for 2 minutes and 45 seconds, then 0.1 ml of 30 mM calcium chloride was added. The prewarmed test material, 0.1 ml, was added 15 seconds later. The clotting tube was then shaken briefly, allowed to stand for 15 seconds, and then tilted at 5-second intervals. The reaction was timed from addition of the test material to appearance of the clot.

Figure 2 is a log-log plot of the clotting times of dilutions of a plasma eluate in this assay. The 100 per cent concentration represents the eluate at original plasma volume. Note also in Figure 2 that undiluted normal plasma did not shorten the clotting time until it had been exposed to an activating surface. Moreover, both HF- and PTA-deficiency plasma, that had been exposed to an activating surface (5 mg per ml kaolin powder), failed to shorten the clotting time. Thus, the assay measured only *preformed* activation product; it was insensitive to the precursors of activation product and to other plasma clotting factors.

2. PTC assay. Stapp's one stage technique (17) was modified by adding kaolin powder to the clotting mixture and by eliminating the adsorbed ox plasma reagent. One-tenth ml of PTC-deficiency plasma, 0.1 ml of cephalin (1/70)-kaolin (5 mg per ml) reagent, and test reagent (1:5 in diluting fluid I) were incubated for 10 minutes. Then 0.1 ml of 30 mM calcium chloride was added and the reaction timed from this point. The clotting tube was shaken briefly, allowed to stand for 30 seconds, and then tilted at 5-second intervals. The endpoint was flocculation of kaolin powder. In later experiments, the substrate plasma was increased to 0.2 ml and the kaolin concentration to 8 mg per ml to increase the fibrinogen content of the clotting mixture.

The kaolin powder generated enough activation product in the substrate plasma to make the assay independent of activation product in the test reagent. Thus, a plasma eluate with powerful activity, as measured in the preformed activation product assay, was unable to shorten the clotting time in the PTC assay (blank time, 116 seconds; plasma eluate, 110 seconds; normal plasma, 56 seconds).

3. AHG assay. The technique of Langdell, Wagner and Brinkhous (18) was also modified by adding kaolin powder to the clotting mixture. One-tenth ml of AHG-deficiency plasma, 0.1 ml of cephalin (1/70)-kaolin (12.5 mg per ml) reagent, and 0.1 ml of test reagent (1:5 in diluting fluid I) were incubated for 3 minutes. Then 0.1 ml of 30 mM calcium chloride was added and the reaction timed from this point. The clotting tube was shaken briefly, allowed to stand for 30 seconds, and then tilted

TABLE II

The thrombotic activity of normal serum, normal serum eluate, and Speedex-adsorbed normal serum

Test material	No. of tests*	Frequency of each thrombosis score 0	1+	2+	3+	4+
Normal serum	12			2	4	6
Normal serum eluate	15		1	2	1	11
Speedex-adsorbed serum	16	10	4	1	1	
Citrated-saline control	15	11	4			

* Represents the sum of tests performed on 3 sets of reagents prepared from different normal donors.

TABLE III

Clotting factor activities in normal serum, normal serum eluate, and Speedex-adsorbed normal serum

Clotting factor assay	Per cent of standard reference activity in:		
	Normal serum	Serum eluate	Adsorbed serum
Preformed activation product*	42 11 – 140	158 46 – 320	<1
AHG	8	2	11
PTC	200	7	8
Stuart factor	155	2	155
Proaccelerin	39	2	4
Proconvertin	210	15	130
Thrombin activity		0†	

* The values for preformed activation product are the means and ranges of 12 to 16 individual tests done just before the reagents were assayed for STA activity.

† No clot formed when 0.1 ml of serum eluate was added to 0.4 ml of barium sulfate-adsorbed oxalated bovine plasma.

at 5-second intervals. The endpoint was flocculation of kaolin powder.

This assay was also independent of activation product in the test reagent (blank time, 115 seconds; eluate full strength, 106 seconds; normal plasma diluted 1:5, 65 seconds).

The remainder of the assays were carried out as described elsewhere: HF activity (5), PTA activity (19), Stuart factor activity (20), proconvertin activity (21), proaccelerin activity (14, 15), prothrombin-proconvertin-Stuart factor complex (22), prothrombin-Stuart complex (23), thrombin time (13).

RESULTS

1. The thrombotic activity of normal serum reagents. Table II is a summary of our data on the thrombotic activity of normal serum, normal serum eluate, and Speedex-adsorbed normal serum. It shows that both normal serum and normal serum eluate possessed powerful, comparable thrombosis-inducing activity. In contrast, Speedex-adsorbed normal serum had no more thrombotic activity than an inert control fluid. The difference in thrombotic activity between normal serum eluate and Speedex-adsorbed normal serum is highly significant on statistical analysis by the binomial method (24) ($p > 10^{-6}$).

2. The in vitro clotting factor activities in normal serum reagents. Table III is a summary of our measurements of the *in vitro* clotting factor activities in normal serum, normal serum eluate, and Speedex-adsorbed normal serum. It shows that preformed activation product was the only clotting activity present in significant amounts in normal serum eluate. The presence of *preformed* activation product in the eluate was confirmed by demonstrating that the eluate shortened the partial thromboplastin times of both HF-deficiency plasma and PTA-deficiency plasma from blank times of greater than 300 seconds to normal times of less than 50 seconds *in the absence of contact with an activating surface*. This could not have occurred had the eluate contained primarily the precursors of activation product. (Of course, HF, "activated" HF (6), and PTA may have been present *in addition* to preformed activation product in the eluate, but could not be measured in this circumstance.)

The absence of significant AHG or PTC activity in the eluate made contamination with an intermediate beyond the stage of activation product, such as "intermediate product I" (25), highly unlikely. It also eliminated the possibility of contamination with activity resembling tissue thromboplastin.

As can be seen in Table III, adsorption with Speedex powder removed both activation product and PTC activity from serum. However, the lack of thrombotic activity in adsorbed serum cannot be attributed to lack of PTC, for the normal serum eluate was equally deficient in PTC activity. Thus, preformed activation product was the only recognized *in vitro* activity whose presence in or absence from serum could be related to the presence or absence of serum thrombotic activity.

3. The thrombotic activity of suspensions of Speedex powder. As noted earlier, the normal

TABLE IV

The thrombotic activity of suspensions of Speedex powder

Speedex concentration	Degree of STA activity (0 to 4+)
mg/ml	
0.00	0, 0, 0, 1+, 0
0.039*	0, 0, 0
0.078*	0, 0
0.156	0, 0, 1+
0.313	0, 1+, 1+
0.625	2+, 2+, 3+
1.25	0, 3+
2,50	2+, 2+
5.00	3+, 4+

* Eluate contained 0.039 to 0.078 mg/ml of Speedex powder.

TABLE V

Activation product and thrombotic activity of eluates derived from hereditary PTC-, HF-, and PTA-deficiency sera

Defect	Activation product	No. of tests	Frequency of each thrombosis score				
			0	1+	2+	3+	4+
	%						
PTC	99*	13†			1	6	6
HF	1	6	6				
PTA	<1	3	2	1			

* Mean of 6 determinations.
† Represents the sum of tests on 3 eluates prepared from different PTC-deficiency sera.

serum eluates contained slight traces of contaminating Speedex powder (0.039 to 0.078 mg per ml). This raised the possibility that their thrombotic activity might result simply from the intravenous injection of an activating surface. Therefore, suspensions of Speedex powder which had not been exposed to blood were tested for thrombotic activity.

Speedex powder was found to have thrombotic activity, but only at concentrations far greater than those present in the serum eluates (see Table IV). Complete thrombosis, which was usually obtained with eluate, required a concentration of powder approximately 50 times that present in the eluate. Suspensions comparable to the contamination in eluates were devoid of thrombotic activity. Thus, the thrombotic activity of serum eluate cannot be attributed to the injection of trace amounts of an activating surface. It is tempting to believe that the thrombi induced by large amounts of powder resulted from the generation of activation product *in vivo*.

4. The activation product and thrombotic activity of eluates of PTC-, HF-, and PTA-deficiency sera. Since Wessler and Reimer could not find STA activity in PTC-deficiency serum (10), and since PTC does not enter into activation product formation (2), it seemed critical to test eluates of PTC-deficiency serum for thrombotic activity. As can be seen in Table V, eluates of PTC-deficiency serum possessed both activation product and thrombotic activity. These experiments strengthen the evidence obtained with normal serum eluates that the thrombotic activity of such eluates is independent of PTC or of blood thromboplastin intermediates which require PTC for their formation.

As expected, activation product could not be demonstrated in an eluate prepared from HF-deficiency serum or in an eluate prepared from PTA-deficiency serum. *Neither eluate possessed thrombotic activity.* These data, which are also shown in Table V, deserve particular emphasis, for they confirm the dependence of the thrombotic activity in normal serum eluate upon its content of activation product.

5. The thrombotic activity and the in vitro clotting factor activities of plasma eluates. Any reagent prepared from serum has the inherent disadvantage of possible contamination with platelet products and with as yet uncharacterized serum thromboplastin intermediates. Our serum eluate had the added disadvantage of exposure to calcium ions during elution. Waaler (2) and Egli and Busch (26) have shown that activation product forms in the absence of calcium ions.

Therefore, to obtain further evidence that the thrombotic activity in serum and serum eluate was the result of activation product alone, eluates were prepared from *platelet-poor citrated plasma in the strict absence of calcium ions.* These plasma eluates (see Table VI) were found to possess thrombotic activity comparable to that of serum eluate.

The experiments summarized in Table VII compare the clotting factor activities of a plasma eluate, made up to original plasma volume, with those of an undiluted normal plasma which was exposed to an activating surface during testing. The data show that the plasma eluate contained powerful preformed activation product which shortened to normal the long clotting time of exhausted plasma, of HF-deficiency plasma and of PTA-deficiency plasma in the absence of an activating surface. However, in contrast to the normal plasma, the eluate was without significant

TABLE VI

The thrombotic activity of plasma eluate

Test material	No. of tests	Frequency of each thrombosis score				
		0	1+	2+	3+	4+
Plasma eluate	12*		1	1	4	6
Citrated-saline control	13	13				
Serum eluate	15		1	2	1	11

* Represents the sum of tests performed on 2 plasma eluates derived from different normal donors.

TABLE VII

The clotting factor activities in plasma eluate and in "activated" whole normal plasma

Substrate plasma	Clotting times (sec) on adding: Plasma eluate	Normal plasma	Saline
Exhausted plasma*	41, 41		118, 119
HF-deficient†	44, 45	41, 41	>400
PTA-deficient†	43, 45	43, 42	>500
AHG-deficient†	168, 177	45, 46	>500
PTC-deficient†	136, 155	41, 41	>500
Proaccelerin-deficient*	176, 156	14, 20	147, 148
Proconvertin-deficient*	157, 149	16, 16	197, 202
Prothrombin-Stuart-deficient*	600, 600	8, 8	

* See Methods for technique.

† When the test substance was plasma eluate or saline control the assays were carried out without exposure to an activating surface in clear plastic tubes as follows: 0.1 ml of substrate plasma and 0.1 ml of cephalin reagent (1/70) were incubated together for 3 minutes, then 0.1 ml of prewarmed test substance and 0.1 ml of 30 mM calcium chloride were added and the clotting time noted. When the test substance was normal plasma it was activated by exposure to kaolin powder during the incubation period as follows: 0.1 ml of substrate plasma, 0.1 ml of a cephalin (1/70)-kaolin (8 mg per ml) reagent, and 0.1 ml of normal plasma were incubated together for 6 minutes, then 0.1 ml of 30 mM calcium chloride was added and the clotting time noted.

AHG, PTC, proaccelerin, proconvertin, or prothrombin-Stuart factor activities. The slight shortening of the clotting time of the AHG- and PTC-deficiency plasmas as compared with saline was an effect of activation product. Thus, when tested in our specific AHG and PTC assays, which provide optimal activation product from the substrate plasma, the eluate contained only about 1 per cent AHG and PTC activity, respectively. These data show that activation product, prepared from plasma with no chance for contamination with succeeding thromboplastin intermediates, possesses potent thrombotic activity.

6. *The thrombotic activity of brain thromboplastin and of thrombin.* Table VIII is a summary of experiments in two animals in which the thrombotic activity of plasma eluate was compared with the thrombotic activity of rabbit brain thromboplastin. The brain thromboplastin was diluted to give the clotting times listed when used as the "test material" in the preformed activation product assay. This permitted a rough quantitative comparison between the *in vitro* coagulant activity of the dilutions of tissue thromboplastin and of the plasma eluate. (It must be remembered that the mechanism of clotting differed; clotting with tissue thromboplastin invokes the extrinsic coagulation reactions whereas clotting with plasma eluate invokes only the intrinsic coagulation reactions.)

It can be seen from Table VIII that dilutions of brain thromboplastin which gave the same clotting times *in vitro* as plasma eluate had no thrombotic activity. Thrombotic activity was found only when a much stronger suspension of brain thromboplastin, capable of clotting exhausted plasma in 19 seconds, was injected.

These data confirm Wessler's earlier observations in dogs (8). They imply that the thrombotic potential of brain thromboplastin depends upon the thromboplastic activity of the material itself; i.e., that brain thromboplastin, unlike activation product, cannot activate the animal's *intrinsic* coagulation process.

Comparable experiments were performed in two rabbits which were injected with dilutions of bovine thrombin. Thrombin capable of clotting bovine plasma within 35 seconds (0.1 ml of bovine thrombin added to 0.4 ml of barium sulfate-adsorbed oxalated bovine plasma) did not exhibit thrombotic activity. However, thrombotic activity was found in 2 of 4 instances in which bovine thrombin, with a clotting time of 22 seconds, was injected. As noted in Table III, serum eluate with potent thrombotic activity failed to clot adsorbed bovine plasma.

DISCUSSION

The data presented above prove that activation product possesses potent thrombotic activity. The evidence may be summarized as follows.

1. Normal serum possesses thrombosis-inducing activity and activation product activity. Adsorption with Speedex powder and subsequent incubation at 37° C destroys both the thrombosis-in-

TABLE VIII

A comparison of the thrombotic activity of plasma eluate and various dilutions of rabbit brain thromboplastin

Test material	Clotting time (sec) in activation product assay	Degree of thrombotic activity (0 − 4+)
Plasma eluate	45, 44, 49, 51	4+, 4+, 3+, 4+
Brain thromboplastin:		
1:800	45, 41, 41	0, 0, 0, 0
1:100	29, 26, 28	0, 0, 0, 0
1:10	19	4+

ducing and the activation product activity of normal serum.

2. Eluates prepared from the Speedex powder contain powerful thrombotic activity. These eluates are rich in activation product activity but do not contain other measurable *in vitro* clotting factor activities.

3. Similar eluates prepared from PTC-deficiency serum possess both activation product and thrombotic activity. This confirms the independence of serum thrombotic activity from PTC.

4. Eluates prepared from hereditary HF- and PTA- (the precursors of activation product) deficiency sera possess neither activation product nor thrombotic activity.

5. Eluates prepared from platelet-poor citrated *plasma* possess potent thrombotic activity. They contain no measurable *in vitro* clotting activity except activation product. The unequivocal thrombotic activity of these eluates, which are made without exposure to calcium ions, proves that thrombotic activity does not require the presence of traces of thromboplastin intermediates beyond the stage of activation product.

In recent publications (3, 9, 10, 27) Wessler and his associates have referred to the moiety in normal mammalian serum that induces thrombosis in areas of vascular stasis as the serum thrombotic accelerator (STA). The evidence summarized above leads us to conclude that this moiety is activation product—i.e., that STA activity is an *in vivo* manifestation of activation product.

However, it must be pointed out that our results differ from those of Wessler and his associates in two important respects. Reimer, Wessler and Deykin (9) found STA activity in serum after adsorption with diatomaceous earth powder, whereas our adsorbed serum was devoid of thrombotic activity. One reason for this discrepancy may be that we used more powder—120 mg per ml of serum instead of the 30 mg per ml of serum which they used. Moreover, our adsorbed serum was incubated for 5 hours at 37° C; Waaler has shown (2) that an incubation period is mandatory to exhaust adsorbed serum of significant residual activation product activity.

The second important difference is that Wessler and Reimer (10) could not demonstrate thrombotic activity in PTC-deficiency serum, whereas our eluates prepared from PTC-deficiency serum possessed powerful thrombotic activity. We have no ready explanation for this difference. It is of interest that Reimer and Wessler (3) have more recently reported that sera whose PTC activity had been depressed either by the administration of bishydroxycoumarin (Dicumarol) or by adsorption retained STA activity.

The clear demonstration of the thrombotic potential of activation product has implications of importance for human thrombotic disease. The injection of the activation product from only 6 ml of human plasma can trigger thrombosis in a 2 kg rabbit with an estimated plasma volume of 70 to 110 ml (28). Thus, the injected activity must be the equivalent of only a small fraction of the animal's own activation product potential.

The *in vivo* evidence of the potency of activation product has its parallel in the *in vitro* experiments of Waaler (2). He showed that a perfectly taken, native, platelet-rich plasma could be kept fluid in a siliconized tube at body temperature for more than an hour; however, only a small defect in the silicone layer coating the test tube was sufficient to shorten the clotting time to less than 5 minutes. Apparently, inhibitors within stagnant blood cannot block clotting initiated by only a small amount of activation product, either *in vivo* or *in vitro*.

Wessler and colleagues (29) noted that serum does not possess thrombotic activity when it is injected into the portal vein. Spaet and Cintron (30) have shown that a later intrinsic thromboplastin intermediate, intermediate product I, is cleared rapidly from blood by the reticuloendothelial system, particularly in the liver. Thus, reticuloendothelial clearance appears to be the primary defense against active intrinsic coagulation intermediates. When stasis prevents or delays reticuloendothelial clearance, small amounts of the first known active intermediate of intrinsic coagulation, activation product, can induce thrombosis in the experimental animal. This could explain why stasis predisposes to venous thrombosis in humans.

The association between venous thrombosis and clinical disorders in which tissue materials with thromboplastic activity can enter the blood (e.g., metastatic malignancy, extensive surgery, certain obstetrical situations) is well established. The question has been raised (31) as to whether such tissue products induce thrombosis solely because

of their thromboplastic activity or because they also trigger the reactions by which blood generates its own thromboplastic activity. Biggs and Nossel (32) have presented evidence which suggests that dilute brain extract thromboplastin may initiate the contact phase of intrinsic coagulation *in vitro*. However, our experiments on the thrombotic activity of brain thromboplastin cast doubt upon the ability of this tissue product to trigger the intrinsic coagulation process *in vivo*. Moreover, they suggest that inhibitors within the blood can block the extrinsic coagulation reactions more effectively than they can block the intrinsic coagulation reactions.

Loeliger and co-workers (33) have suggested that tissue thromboplastin sets off blood thromboplastin generation by causing a small thrombus to form which then acts as an activating surface for the intrinsic coagulation process. It is difficult for us to reconcile this hypothesis with the observation that prothrombin consumption is markedly retarded in blood clotted in silicone-coated tubes and with the observation that serum from such blood does not contain potent activation product or STA activity (2, 9).

Moreover, no data are available to support the obvious possibility that a diseased vessel wall may act as an activating surface. At present, therefore, the mechanism or mechanisms whereby activation product may arise *in vivo* remain obscure. This should not divert attention from the significance of the observation that the injection of preformed activation product can trigger thrombosis in the experimental animal. The formation of activation product *in vivo* deserves serious consideration as one possible mechanism for the pathogenesis of thrombotic disorders in man.

SUMMARY

1. Activation product, the first known intermediate in the formation of intrinsic (blood) thromboplastin *in vitro,* has powerful thrombotic activity when injected intravenously into rabbits.

2. Evidence is presented which strongly suggests that activation product is the moiety in normal human serum responsible for inducing thrombosis in areas of vascular stasis; i.e., that the serum thrombotic accelerator (STA) phenomenon is an *in vivo* manifestation of activation product.

3. Activation product may play an important role in the pathogenesis of clinical venous thrombosis.

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