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THROMBOSIS ET DIATHESIS HAEMORRHAGICA

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Effects of Thorium Dioxide upon Blood Clotting and Platelets. I. *In Vitro* Studies*

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Colloidal thorium dioxide is stored in the reticulo-endothelial system (RES) and is widely used in experimental medicine to induce "reticulo-endothelial blockade". Using it for this purpose to prepare rabbits for the generalized Shwartzman reaction, we observed serious hemostatic defects following the standard intravenous dose of 3 ml/kg. This observation may have at least two implications. First, the hemostatic defects could be related to the mechanism, as yet poorly understood, whereby large colloidal particles "block" the granulopectic function of the RES. Second, "blockade" is often induced to prepare an animal for another experimental purpose, e. g., for the generalized Shwartzman reaction, and the hemostatic defects may influence this part of the experiment.

These considerations prompted us to examine the hemorrhagic diathesis induced by thorium dioxide in greater detail. Our main goal has been to answer the practical question: what changes in clotting factors and platelets do thorium dioxide suspensions produce as they are commonly used to "block" the RES in the rabbit? This paper is a report of *in vitro* studies; *in vivo* studies are presented in a companion paper.

Materials

1. Thorium Dioxide Suspensions

These are available as a sterile, stabilized 24–26% solution of thorium dioxide in a carrier solution of 25% dextrin and 0.15% methylparaben. They contain 19–20% thorium dioxide by

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weight (7). Two varieties can be obtained in the United States from Fellows-Testagar, Detroit, Michigan, namely:

1. Unfiltered thorium dioxide suspension, obtainable only for animal research.

2. Filtered thorium dioxide suspension, available for clinical use as a radiographic contrast medium.

These preparations are said to differ only in particle size. However, we have found that the unfiltered preparation contains about 10% more radioactivity than the filtered preparation.

It is often impossible to be sure which preparation an investigator has used, for both have been called Thorotrast. Before 1955, Thorotrast was not filtered, but since that date all new preparations labelled Thorotrast have been the filtered product. I 1955, Hyman and co-workers (18) reported that this material was less effective in "blocking" the RES than the unfiltered material. Consequently, some investigators have obtained the old material, which is now sold under the name Unfiltered Thorium Dioxide Suspension (Fellows-Testagar, Detroit, Michigan). Unfortunately, some investigators who use this unfiltered product still refer to it as Thorotrast.

Since we used the unfiltered suspension in our experiments on the generalized Shwartzman reaction, we have used this material, referred to hereafter by the initials TDS (thorium dioxide suspension), for most of our coagulation studies. Supplementary studies were also carried out with the filtered preparation, i. e., with the suspension which is now properly called Thorotrast.

In some of the data to follow the concentration of TDS or Thorotrast in plasma or serum is given in per cent. This value is: (volume of undiluted TDS or Thorotrast/final volume of the mixture) $\times$ 100. The standard "blocking" dose of TDS in the rabbit is 3 ml/kg body weight. With a blood volume of 7% of body weight and a hematocrit of 43% (if all of the injected material remained in the circulation), this *in vivo* dose would be equivalent to adding 1 ml of TDS to 13 ml of plasma *in vitro*, i. e., to a TDS concentration of 7.1%.

2. Other Materials

Venous blood was obtained with "silicone technique" from normal humans and from the inferior vena cava of New Zealand rabbits anaesthetized with pentobarbital. Three *anticoagulants* were used: 1. citrate anticoagulant, 0.06 M in trisodium citrate and 0.04 M in citric acid, 2. heparin (Upjohn, Kalamazoo, Michigan), 50 μg/ml, and 3. EDTA (disodium salt of ethylene-diamine-tetra-acetic acid), in a 1% solution. One part of anticoagulant was mixed with nine parts of blood or, in some experiments, with five parts of serum. *Platelet-rich plasma* was prepared by centrifuging blood at 700 rpm for 20 min at 4° C; *platelet-poor plasma* was prepared by centrifuging twice at 10,000 rpm for 10 min at 4° C. *Plasma was absorbed* by diluting an aluminum hydroxide suspension (Cutter Laboratories, Berkeley, California) 1:4 in distilled water and incubating one part of this diluted suspension with 10 parts of citrated, platelet-poor plasma for 3 min at 37° C. *Serum* was made by allowing blood to incubate for 2 hrs after clotting in a glass tube at 37° C and then separating the serum by centrifuging at 10,000 rpm for 10 min. Plasma and serum were stored in plastic vials at $-20°$ C.

Activated factor XI was prepared by column chromatography of human plasma on diatomaceous earth powder (24) and was dialyzed against isotonic saline. *ADP* (adenosine diphosphate, sodium salt) from Pabst Laboratory, Milwaukee, Wisconsin, was dissolved in saline and stored at $-20°$ C. *Barbital buffer* was prepared as described elsewhere (21). *Citrated diluting fluid* contained 1 part of 0.1 M sodium citrate plus 5 parts of 0.9 g per cent sodium chloride solution. *Dextrin* (Fellows-Testagar, Detroit, Michigan) is the complete carrier solution for the thorium dioxide suspension. *Endotoxin* (E. coli 0111: B4, Type W, Difco Laboratories, Detroit, Michigan) was dissolved in saline and used immediately. "*Exhausted plasma*", plasma deficient in both factor XII and factor XI, was prepared as described elsewhere (21). *Fibrinogen* from human plasma

was obtained from Merck, Sharp and Dohme, West Point, Pennsylvania. *Liquoid* (sodium polyanetholsulphonate), supplied by Hoffmann-La Roche, Basel, Switzerland, was dissolved in saline. *Protamine sulphate* (Lilly, Indianapolis, Indiana) was diluted in saline. *Thrombin* of bovine origin (Parke-Davis, Detroit, Michigan) was prepared and stored as described earlier (21).

Other reagents used in the clotting assays are described in the references cited with the assays.

Methods

Clotting factors were measured as follows:

Fibrinogen: slight modification of the method of Blombäck & Blombäck (2).

Prothrombin: modification of the method of Hjort et al. (13) in which a "proconvertin reagent" (12) supplied factor X.

Factor V: one-stage assay with human brain thromboplastin and stored human plasma substrate prepared according to Stormorken (28).

Factor VII: one-stage assay with human brain thromboplastin, absorbed ox plasma to supply added factor V, and hereditary factor VII deficiency plasma (12).

Factor VIII: an activated partial thromboplastin time assay with a cephalin-kaolin reagent and hereditary factor VIII deficiency substrate plasma (24).

Factor IX: partial thromboplastin time assays with hereditary factor IX deficiency substrate plasma and a cephalin reagent. Two types were used: 1. a "contact" assay with kaolin powder providing optimal contact-active surface, and 2. an "intact" assay without exposure of reagents to contact-active surface. Details are given elsewhere (24).

Factor X: modification of Hougie's method (15) in which cephalin serves as the lipid and the incubation time from addition of the venom is exactly 3 min.

Factor XI: activated partial thromboplastin time assay with a "cephalin-kaolin reagent" and hereditary factor XI deficiency substrate plasma (22).

Factor XII: a similar system with Hageman factor deficiency substrate plasma. The test sample contacts the cephalin-kaolin reagent before the deficiency plasma is added to the clotting mixture (24).

Platelet factor 3: 0.1 ml of platelet-poor human plasma reagent prepared according to Husom (17), 0.1 ml of a dilution of test reagent, and 0.1 ml of Russell's viper venom were incubated together for exactly 3 min at 37°C and then recalcified with 0.1 ml of calcium chloride.

Unless stated otherwise, test samples were diluted in the citrated diluting fluid described above. Clotting times were converted to per cent activity from dilution curves prepared with citrated human or rabbit plasma.

Platelets were counted in duplicate by the method of Brecher and Cronkite (3). *Platelet adhesiveness* was measured in platelet-rich plasma as described by Stormorken et al. (29). *Clot retraction* was evaluated in platelet-rich plasma by the method of Voss (30).

Results

Effect of TDS upon the Whole Blood Clotting Time

High concentration of TDS (1 part plus 10 parts of blood) markedly prolonged the whole blood clotting time in plastic tubes but not in glass tubes (Table 1). The anticoagulant effect of TDS requires time to develop, and,

Table 1. *Effect of TDS upon the Whole Blood Clotting Time.* One ml of Rabbit Blood was Placed in a Warmed (37° C) Glass or Plastic Tube Containing 0.1 ml of either TDS or Saline.

Test material	Clotting time (min)	
	Glass tube	Plastic tube
Saline	6.7	12.14
TDS	5.8	<300

therefore, expresses itself *in vitro* only when clotting is delayed, e. g., by the lack of a negatively charged surface.

Effect of TDS and Thorotrast upon the Thrombin Time and upon Fibrinogen

When TDS was incubated with citrated plasma, it progressively prolonged the thrombin time. Thorotrast had a similar but smaller effect, whereas dextrin had no effect (see Fig. 1). As Fig. 2 illustrates, plots of thrombin times versus incubation times on double logarithmic paper gave nearly straight lines with a sharp bend after several minutes' incubation. The degree of prolongation varied with the temperature during incubation (Fig. 3) and with the concen-

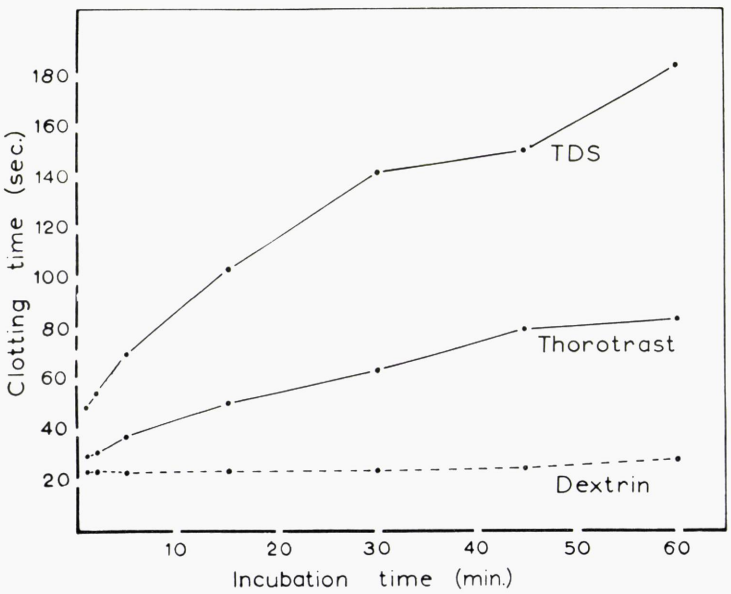

Fig. 1. *Prolongation of the thrombin time by TDS and Thorotrast.* Two ml of citrated, platelet-poor, rabbit plasma were incubated with 0.1 ml of dextrin, TDS, or Thorotrast at 37° C. At intervals, the thrombin time was measured as follows: 0.2 ml incubated plasma + 0.1 ml thrombin (5 NIH-u/ml).

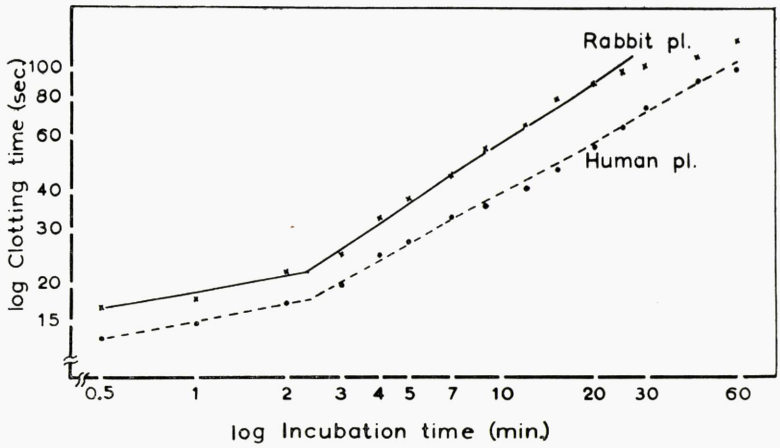

Fig. 2. *Double logarithmic plot of thrombin time against incubation time with TDS.* Three ml of citrated, platelet-poor rabbit and human plasma were incubated with 0.3 ml of TDS at 37° C. At intervals, the thrombin time of subsamples was determined as in Fig. 1 with thrombin containing 10 NIH-u/ml.

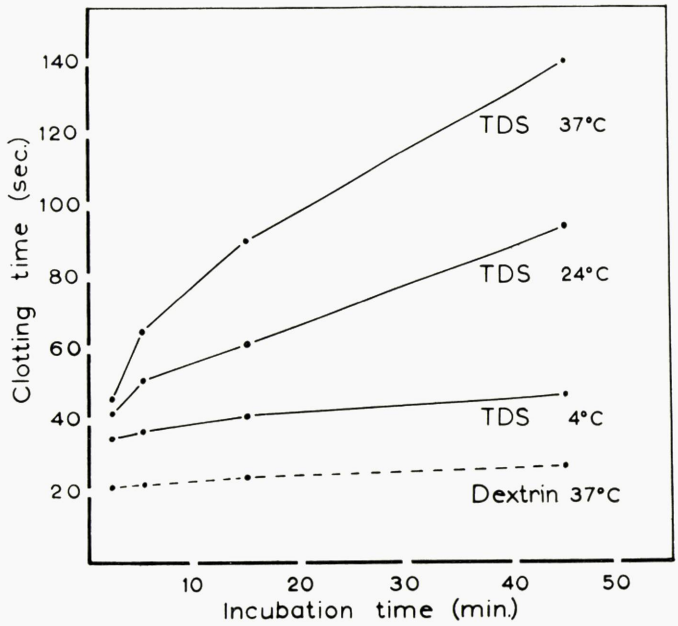

Fig. 3. *The effect of TDS upon the thrombin time: influence of the temperature during incubation.* Two ml of citrated, platelet-poor, rabbit plasma were incubated with 0.1 ml of dextrin or TDS at 4°, 24° or 37° C. At intervals, the thrombin time of subsamples was measured as in Fig. 1.

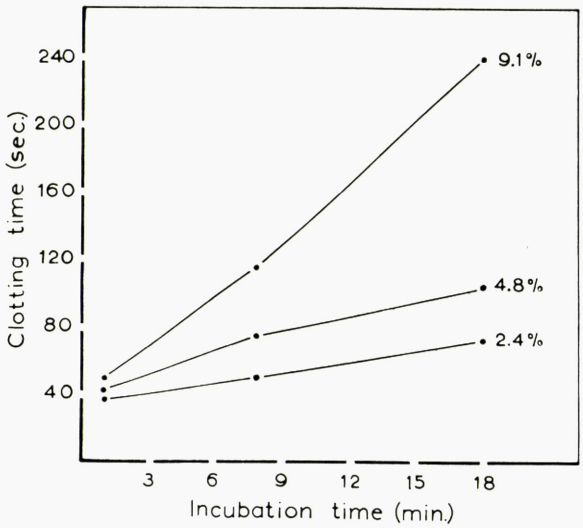

Fig. 4. *The effect of TDS upon the thrombin time: influence of the concentration of TDS.* Two ml of citrated, platelet-poor, rabbit plasma were incubated with 0.05, 0.1 or 0.2 ml of TDS at 37° C. At intervals, the thrombin time of subsamples was measured as in Fig. 1.

tration of TDS (Fig. 4). Similar effects were found when TDS was added to absorbed plasma, to EDTA-plasma, and to a human fibrinogen preparation.

When the thrombin time was only moderately prolonged, the amount of clotable fibrinogen in the plasma was not altered (see Table 2). However, as the defect increased, the clot became large, friable and difficult to wind onto a

Table 2. *The Effect of TDS upon the Thrombin Time and upon the Measurement of Clotable Fibrinogen.* Four ml of Citrated, Platelet-Poor, Rabbit Plasma were Incubated at 37° C with 0.1 ml of Dextrin or TDS. At Intervals, the Thrombin Time was Measured (See Fig. 1) and Fibrinogen was Determined (See Methods).

Material added	Incubation time (min)	Thrombin time (sec)	Fibrinogen (mg/100 ml)
Saline	0	20.5	177
Dextrin	1	19.0	174
	5	20.5	157
	20	21.5	157
	60	23.1	154
TDS	1	30.7	177
	5	48.2	180
	20	72	(232)
	60	93	(554)

glass rod. More and more plasma proteins became occluded in the clot, resulting in false elevations of fibrinogen values (Table 2). TDS also influenced the heat precipitability of fibrinogen at 56° C; the precipitate became large, fluffy and could not be packed by centrifugation. TDS did not, in itself, precipitate fibrinogen, except in an occasional heparinized rabbit plasma incubated for several hours at 37° C with TDS at a concentration of 9%. Moreover, TDS did not increase the precipitate produced by adding small amounts of Liquoid to plasma.

A moderately prolonged thrombin time produced by TDS could be shortened by large amounts of protamine sulphate, a material known to influence the physical properties of fibrinogen (10).

Table 3. *Occlusion of TDS in the Clot.* One ml of Citrated, Platelet-Poor Rabbit Plasma was Mixed with TDS to Give the Concentrations Listed, Incubated for 2 min at Room Temperature, and then Clotted with 0.05 ml of Thrombin (5 NIH-u/ml). After 30 min, the Clot was Removed with a Glass Rod and Dissolved in 1 ml of Alkaline Urea. The Radioactivity of the Dissolved Clot and the Serum was Then Determined Separately.

Test material	Net counts per minute with:	
	4.2% TDS	2.1% TDS
Unclotted plasma	402	232
Serum	239	133
Dissolved clot	133	101

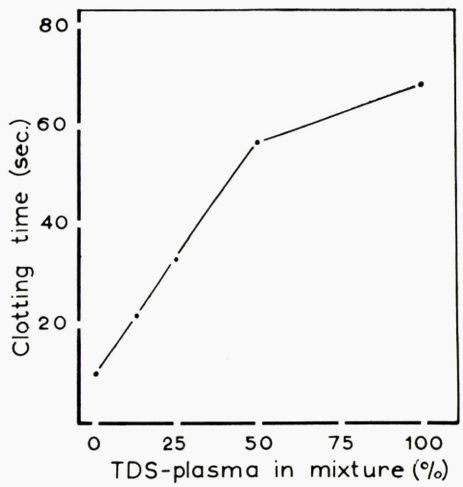

Fig. 5. *Thrombin times of mixtures of TDS-plasma and dextrin-plasma.* Two ml portions of citrated, platelet-poor rabbit plasma were incubated with 0.2 ml of dextrin or TDS for 20 min at 37° C and then mixed in various proportions. The thrombin time was determined as follows: 0.4 ml mixture + 0.2 ml thrombin (5 NIH-u/ml).

Since thorium dioxide is radioactive, we could also determine its distribution after coagulation. As Table 3 shows, TDS was concentrated in the clot to a remarkable degree.

Plasma incubated with TDS had anticoagulant activity that prolonged the thrombin time of normal plasma (see Fig. 5). The prolongation could be attributed to altered fibrinogen for, as the data of Table 4 illustrate, serum prepared from TDS-plasma had no anticoagulant effect.

Table 4. *Anticoagulant Activity in TDS-Plasma but Not in TDS-Serum.* A Rabbit Received an Intravenous Injection of 3 ml TDS/kg. Blood was Drawn 15 min Later, and 1 Part was Used to Prepare Citrated, Platelet-Poor Plasma and the Other to Prepare Citrated Serum. Normal Citrated, Platelet-Poor, Rabbit Plasma was Then Mixed with an Equal Part of Buffer, or TDS-Plasma, or TDS-Serum, and the Thrombin Times of the Mixtures were Determined as in Fig. 1.

Test material	Thrombin time (sec)
Normal plasma + buffer	19
Normal plasma + TDS-serum	21
Normal plasma + TDS-plasma	74
TDS-plasma + buffer	90

The above observations indicate that thorium dioxide suspensions incubated with plasma *in vitro* progressively and seriously damage its fibrinogen.

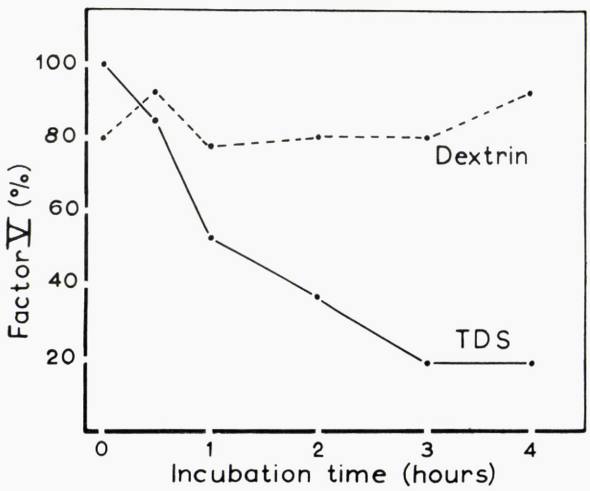

Fig. 6. *Progressive inactivation of factor V by TDS.* Two ml of citrated, platelet-poor, rabbit plasma were incubated with 0.2 ml of dextrin or TDS at 37° C. At intervals, factor V was measured in diluted subsamples.

Effect of TDS upon Factor V

Colman and Alexander (5) reported that thorium rapidly and progressively inactivated factor V in oxalated human plasma. Our findings on factor V in citrated plasma agree with their observations (see Fig. 6). The inactivation was somewhat more rapid in human than in rabbit plasma.

Effect of TDS upon Factor VIII

TDS did not inactivate factor VIII in either human or rabbit citrated, platelet-poor plasma. For example, in one experiment, 0.1 ml of TDS, of dextrin, or of saline was incubated at 37° C with 1.0 ml of rabbit plasma; after 1 hour, diluted subsamples from these incubation mixtures gave clotting times in a factor VIII assay of 48, 49, and 48 sec, respectively. Attempts to study the effect of TDS upon factor VIII in non-citrated, heparinized human plasma were unsuccessful because heparin interfered with the assay. In heparinized rabbit plasma, which can be assayed at a dilution that eliminates interference by heparin, incubation with TDS failed to produce an unequivocal effect upon factor VIII.

Effect of TDS upon Factors XI and XII

Because citrate may interfere with the effect of TDS (see later discussion), these experiments were carried out with heparinized human plasma. They were entirely negative and, therefore, are only briefly summarized.

One ml of platelet-poor plasma containing 5 μg of heparin was incubated at 37° C for 1 hr with 0.1 ml of TDS or dextrin. Diluted subsamples from both mixtures gave identical clotting times of 80 sec in a factor XII assay (blank time of assay, 330 sec). Clotting times of 115 and 118 sec were obtained in a factor XI assay for subsamples from the TDS-plasma and dextrin-plasma mixtures, respectively. This result is less reliable, because, to eliminate the effect of the heparin on the assay, we had to use high dilutions whose clotting times approached the blank time of 132 sec.

TDS and dextrin were incubated with a saline solution of activated factor XI prepared by a modification of Waaler's technique (21). Again, no evidence of a selective destruction by TDS could be demonstrated. As shown in Table 5, incubation with either TDS or dextrin for 1 hr at 37° C resulted in a loss of approximately 50% of the activity as compared with incubation at 4° C.

Effect of TDS and Thorotrast upon Prothrombin

Incubation with TDS markedly reduced the prothrombin activity of *heparinized* human or rabbit plasma, and incubation with Thorotrast had a similar but smaller effect (see Table 6). The prothrombin level varied with the concen-

Table 5. *Failure of TDS to Destroy Activated Factor XI (Activation Product).* One ml Portions of an Activated Factor XI Preparation were Mixed with 0.1 ml of either TDS or Dextrin and Incubated for 1 hr at either 4° C or 37° C. Dilutions in Saline were Then Assayed for Residual Activity by Incubating 0.1 ml of "Exhausted Plasma", 0.1 ml of Cephalin, and 0.1 ml of Diluted Test Material for 3 min in a Clear Plastic Tube at 37° C and Then Recalcifying with 0.1 ml of 30 mM Calcium Chloride (Blank Time of Assay, 165 sec).

Additive	Incubation temp. (°C)	Dilution in assay	Clotting time (sec)
Dextrin	4	1:10	120
		1:20	142
		1:40	154
TDS	4	1:10	120
Dextrin	37	1:10	146
TDS	37	1:10	144

tration of TDS or Thorotrast and with the manner in which the test plasma was diluted in the assay. Usually, the test plasma is diluted in a diluting fluid which is 0.0166 M in sodium citrate. However, as Table 6 illustrates, even a brief exposure of the test plasma to this concentration of citrate ion was sufficient to reverse completely a mild defect and to reverse partly a more pronounced defect. When the experiment summarized in Table 6 was repeated with *citrated* plasma instead of heparinized plasma, we found that the citrate completely prevented damage to prothrombin by either TDS or Thorotrast.

Serum from normal blood clotted in a glass tube at 37° C contains small amounts of residual prothrombin. Incubation of non-citrated human or rabbit

Table 6. *Inactivation of Prothrombin by TDS and Thorotrast.* One ml of Human Heparinized, Platelet-Poor Plasma was Incubated for 1 hr at 37° C with 0.1 ml of Dextrin, or of TDS Diluted in Dextrin, or of Thorotrast Diluted in Dextrin. Subsamples were Then Diluted *either* in Saline *or* in Citrated Diluting Fluid and Tested for Prothrombin Activity. The Test Sample was Added to the Assay Mixture just before Recalcification.

Additive	Concentration %	Prothrombin activity (%) with test sample diluted in:	
		Saline	Citr. dil. fluid
Dextrin	—	80	80
TDS	7.3	11	20
	3.0	25	65
	1.5	39	78
Thorotrast	7.3	38	68
	3.0	65	73
	1.5	73	78

serum with TDS reduced this residual activity, but the effect was less pronounced than in plasma. For example, in one experiment, incubation with 3.0% TDS for 1 hour at 37° C reduced the prothrombin activity from 4.7 to 4.2%.

TDS also inactivated thrombin. When a dilute solution of bovine thrombin (2 NIH-u/ml) was incubated with 2.5% TDS at 37° C, about 90% of the thrombin activity was lost after 15 min.

Effect of TDS and Thorotrast upon Factors VII, IX, and X

TDS markedly depressed the activity of these factors in rabbit and in human serum. As Table 7 illustrates, incubation of human serum for 1 hr at 37° C with 7.3% TDS reduced the level of factor VII to 10%, the level of factor IX to 4%, and the level of factor X to less than 1%. At lesser concentrations of TDS, factor X clearly was more sensitive than the other serum factors. Thorotrast exerted about one-fourth to one-half the effect of a comparable concentration of TDS.

Table 7. *Inactivation of Serum Factors by TDS and Thorotrast.* One ml of Non-Citrated Human Serum was Incubated for 1 hr at 37° C with 0.1 ml of Dextrin, or of TDS Diluted in Dextrin, or of Thorotrast Diluted in Dextrin. Test Samples were Then Diluted *either* in Saline *or* in Citrated Diluting Fluid and Tested in Clotting Factor Assays. The Diluted Test Sample was Added to the Clotting Mixture just before Recalcification.

Additive	Concentration (%)	Activity (%) of serum factors: VII		IX		X	
		Sal.	Cit.	Sal.	Cit.	Sal.	Cit.
Dextrin	—	380	440	66	96	114	120
TDS	7.3	10	184	4	64	1	24
	3.0	70	290	8	176	4	68
	1.5	160	390	30	100	8	100
Thorotrast	7.3	53	290	18	116	7	64
	3.0	280	400	41	116	20	112
	1.5	310	400	45	108	30	112

As with prothrombin, when the test sample was diluted in citrated diluting fluid for assay, milder defects were completely reversed and more severe defects were partially reversed. When serum was citrated (1 part of 0.1 M citrate to 5 parts of serum) prior to incubation with TDS, large amounts of TDS (a concentration of 9% or more) were required to reduce the activity of these factors.

Factors VII, IX, and X also fell on incubation of heparinized plasma with TDS but not on incubation of citrated plasma. This was true for both human and rabbit plasma. Like the inactivation of fibrinogen and factor V, the in-

activation of the Vitamin K dependent factors was also a time-consuming process. This is illustrated for factor X in Fig. 7.

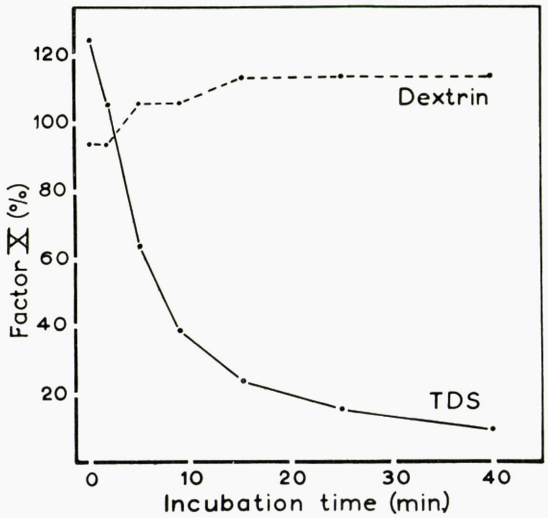

Fig. 7. *Progressive inactivation of factor X by TDS.* Two ml of non-citrated, human serum were incubated at 37° C with either 0.2 ml of TDS diluted in dextrin (final concentration of TDS in serum, 7.0%) or with dextrin. At intervals, subsamples were diluted in saline and assayed for factor X.

Lundblad and Davie (20) reported that thorium destroys activated factor IX (factor IX_a) but not native factor IX. We found (see Table 7) that TDS inactivated nearly all of the factor IX activity in serum prepared by allowing blood to clot in a glass tube and to stand at 37° C for 2 hrs. Such serum contains some factor IX_a but most of its factor IX is still in the native form (25). One may also use heparinized plasma to study this question because heparin blocks the activation of factor IX by activated factor XI (23). When we incubated heparinized plasma with TDS, factor IX activity (measured in a "contact" assay providing optimal contact-active surface for measuring native factor IX) consistently fell. For example, in one experiment factor IX activity dropped from an initial level of 48% to 8% after incubation of heparinized human plasma containing 9.1% TDS for 1 hr at 37° C. (The low initial value reflects a slight interference by heparin with the assay.) We believe, therefore, that native factor IX is not immune to thorium.

We have also examined the effect of TDS upon the activated form of factor X (factor X_a). An eluate from serum (12) was first fully activated with Russell's viper venom and calcium, and then incubated with TDS (final con-

centration 3%) or with dextrin. Far more activity (about 90%) was lost over 15 min at 37° C in the mixture containing TDS.

Effect of TDS upon the Behaviour of Serum in the Thromboplastin Generation Test (TGT)

In their extensive study on the effect of thorium upon thromboplastin generation, Colman and Alexander (5) reported that Thorotrast in a final concentration of 3.0% did not inactivate significant amounts of any of the known clotting factors in serum. Nevertheless, they found that such serum failed to support intrinsic prothrombinase generation in the TGT and postulated the existence of a previously unknown clotting factor, the "Thorium Vulnerable Factor" (TVF).

We have confirmed that serum incubated with 3% TDS behaves abnormally in the TGT (see Fig. 8) and that the defect is largely – but not quite completely – reversible by citrate (see Table 8). Such serum is also markedly deficient in factors IX and X when tested in specific assays (see Table 7). The significance of this in relation to the TVF is discussed later.

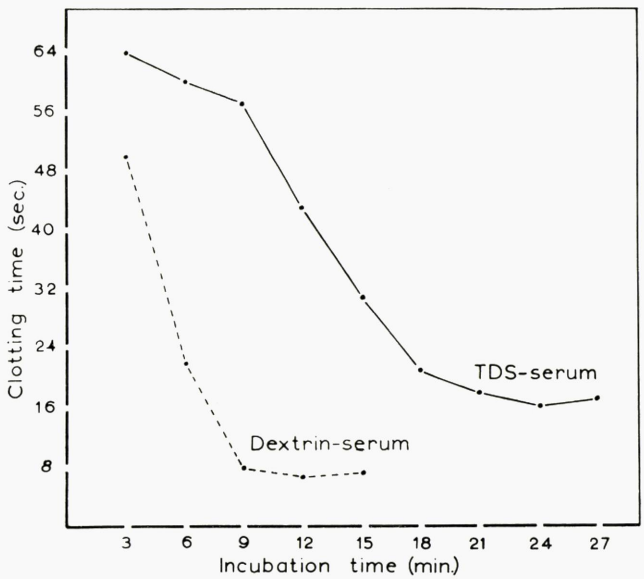

Fig. 8. *Defective Thromboplastin Generation Test of serum incubated with TDS (3.0%).* One ml of human serum was first incubated for 1 hr at 37° C with *either* 0.1 ml of dextrin *or* TDS diluted 1:3 in dextrin. The incubation mixture for the TGT contained equal parts of one of these sera diluted 1:10, adsorbed plasma diluted 1:5, cephalin, saline, and calcium chloride 30 mM. All dilutions were in barbital buffer.

Table 8. *Partial Correction by Citrate of the TDS-Induced Serum Defect in the TGT.* The Incubation Mixture for the TGT Contained Equal Parts of: *Either* Dextrin-Serum *Or* 3.0% TDS-Serum (See Fig. 8) Diluted 1:10, Adsorbed Plasma Diluted 1:5, Cephalin, Calcium Chloride 30 mM, and, as an Added Reagent, *Either* Saline *Or* Citrated-Saline. All Dilutions were in Barbital Buffer.

Incubation time in TGT (min)	Clotting time in TGT (sec) when incubation mixture contained:			
	Dextrin-serum plus:		TDS-serum plus:	
	Saline	Citrated saline	Saline	Citrated saline
3	29	7.2	—	35
6	8.4	8.6	>60	10.2
9	8.2	10.0	31	10.4
12	10.0	—	18	14.3
15	—	—	22	—

Effect of TDS upon Platelets

Incubation with TDS did not significantly reduce the platelet count of blood containing citrate or EDTA as the anticoagulant (Table 9). Platelets usually aggregate in heparinized rabbit blood, causing the count to fall on standing. This fall was actually less on incubation with added TDS or dextrin.

Table 9. *Platelet Counts of Rabbit Blood Incubated with TDS and Dextrin.* Two ml of Blood Containing Anticoagulant were Mixed with 0.1 ml of Saline, Dextrin or TDS and Incubated for 1 hr at Room Temperature. The Figures are Means of Duplicate Counts.

Anticoagulant	Additive	Platelet count (per mm^3)	
		before incubation	after incubation
Citrate	Saline	280,00	—
	Dextrin	—	267,500
	TDS	—	282,500
EDTA	Saline	325,000	—
	Dextrin	—	270,000
	TDS	—	295,000

Because TDS is often used instead of endotoxin for the first or preparative injection in the experimental generalized Shwartzman reaction (11), we compared the effect of endotoxin and of TDS upon platelets *in vitro*. Clark and Batchelor (4) have shown that endotoxin causes rabbit platelets to aggregate and finally fuse. Using their technique and a final concentration of 100 μg of endotoxin per ml of blood, we confirmed their observation. However, when 1 part of TDS was incubated with 10 parts of rabbit blood, platelet clumping was not observed.

Endotoxin releases platelet factor 3 from intact rabbit platelets (8, 14). As Fig. 9 shows, this action of endotoxin was also confirmed. In contrast, TDS failed to release platelet factor 3 from rabbit platelets. When the experiment was repeated with human platelet-rich plasma (either citrated or heparinized) both TDS and endotoxin were without effect. Why endotoxin fails to release human platelet factor 3 is unknown.

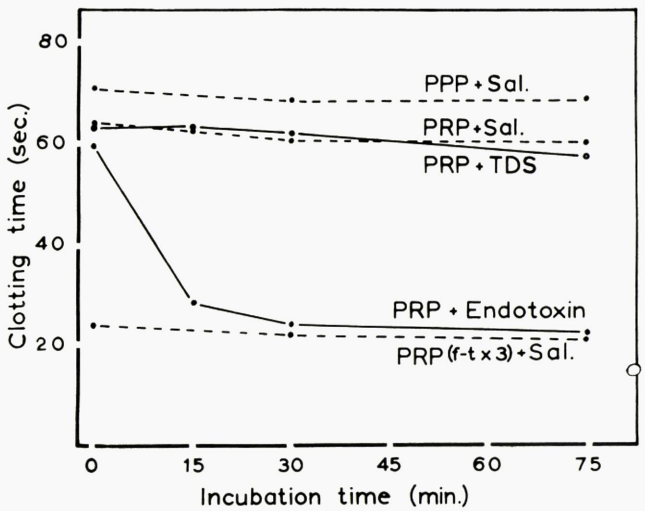

Fig. 9. *Failure of TDS to release platelet factor 3.* One ml of fresh, citrated, platelet-rich (368,000 platelets/mm^3) rabbit plasma (PRP), *or* 1 ml of the same platelet-rich plasma frozen-and-thawed 3 times [PRP (f-t $\times$ 3)], *or* 1 ml of fresh, citrated, platelet-poor (1,250 platelets/mm^3) rabbit plasma (PPP) was incubated in a plastic tube at 37° C with 0.1 ml of TDS, of endotoxin (1 mg/ml), or of saline. Each tube was then made up to 2 ml with saline. At intervals, subsamples were tested for platelet factor 3 (see Methods).

A small platelet button forms during high-speed centrifugation of normal, citrated, platelet-rich plasma and adheres firmly to the bottom of the tube. When the plasma contains TDS, the platelet button will look 2 to 3 times larger than normal and will float freely in the residual plasma at the bottom of the tube. This observation suggested that TDS altered platelet aggregation and led us to examine the effect of TDS upon platelet clumping induced by ADP. When platelets clump, the optical density of platelet-rich plasma falls. Using this indicator (see Fig. 10) we found that TDS moderately inhibited platelet aggregation in citrated rabbit plasma. A similar inhibition was observed when TDS was added to citrated or heparinized human platelet-rich plasma.

Platelet adhesivity was also examined in the system described by Stormorken et al. (29) in which an initial platelet count is made; a small dose of

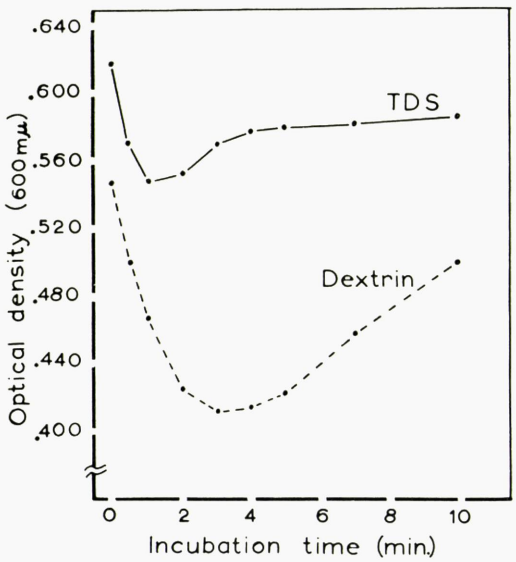

Fig. 10. *Inhibition of ADP-induced platelet aggregation by TDS.* In a siliconized cuvette 4.5 ml of citrated, platelet-rich, rabbit plasma (670,000 platelets/mm^3) were mixed with 0.5 ml of TDS or dextrin, and 0.5 ml of ADP (final conc. 1×10^{-6} M) was added immediately. The optical density of the mixture was read in a Weston colorimeter set at 600 mμ. The mixture was stirred with a magnetic stirrer between readings.

ADP is added to the citrated platelet-rich plasma; the plasma is then pushed through a standardized glass filter, and the platelets are again counted. Adhesiveness is expressed as the percentage of platelets adhering to the filter. Both TDS and dextrin decreased the adhesiveness of rabbit platelets as measured by this technique. Thus, in one experiment the adhesiveness after adding a 1 : 10 volume of test material was 76% for saline, 47% for dextrin and 31% for TDS. Similar results were obtained with human platelet-rich plasma.

It was difficult to distinguish between an effect of TDS upon clot formation and upon clot retraction. When citrated, platelet-rich plasma was incubated with TDS and clotted with thrombin, the poor clots that formed failed to retract. The addition of non-incubated, platelet-poor plasma failed to correct the abnormalities. In an effort to eliminate the effect upon clot formation, we incubated concentrated suspensions of washed rabbit platelets with TDS or saline for 1 hour at 37° C, diluted the incubated platelet suspension in saline, added platelet-poor plasma, and clotted the mixture with thrombin. The clots containing platelets incubated with TDS retracted as much as did the clots containing platelets incubated with saline. Apparently, therefore, TDS does not destroy the ability of platelets to induce clot retraction.

Discussion

Our *in vitro* studies indicate that exposure to thorium dioxide suspensions damages most of the clotting factors and also interferes with platelet functions. The degree of damage depends upon the *type* of thorium dioxide (TDS produces more damage than Thorotrast), upon its *concentration*, and upon the *condition* of incubation (temperature, duration, presence or absence of citrate). It is convenient to arrange the observations into four groups.

Fibrinogen and factor V were progressively changed during incubation with thorium dioxide and finally became unable to function in clotting. Citrate or EDTA, in the concentrations used, did not prevent this damage. TDS and, to a lesser extent, Thorotrast were concentrated in the clot, which suggests that both materials bind to fibrinogen. Our observations of damage to fibrinogen agree with the earlier studies of Häusler and Vogel (19) and of Glazko and Greenberg (9). The latter reported that thorium may precipitate fibrinogen; we occasionally also observed a precipitate in heparinized rabbit plasma incubated with large doses of TDS. Colman and Alexander (5) reported that thorium alters factor V but not fibrinogen. Details of their observations on fibrinogen are not given; possibly, some of the technical factors mentioned above may account for their failure to observe an effect of thorium upon fibrinogen.

Prothrombin and the "serum factors" (factors VII, IX, and X) were all inactivated by thorium dioxide. Factor X was particularly sensitive to both TDS and Thorotrast. Citrate completely prevented or reversed the defects produced by moderate concentrations of thorium dioxide and partially prevented or reversed the defects produced by higher concentrations. The possibility of rapid reversal of a moderate defect by citrate makes the technique of assaying a test sample critical. A defect may be overlooked because it is reversed in the assay system before recalcification. Slätis' report (26) that Thorotrast does not affect prothrombin and factor VII in rat plasma can be questioned for this reason.

Colman and Alexander (5) have stated that thorium does not damage the known serum factors but inactivates a proposed new clotting factor in serum, the "thorium vulnerable factor" (TVF). Using specific assay systems modified to minimize exposure to citrate and oxalate ions during testing, we repeatedly and consistently demonstrated that thorium dioxide suspensions inactivated the three "old" serum factors, factors VII, IX, and X. Although we used the same range of concentrations of thorium dioxide suspensions as they did, we usually incubated for 1 hr before testing, whereas they added Thorotrast to serum just before using it in the thromboplastin generation test (5). Thus, the opportunity for damage to other factors in serum was greater under our con-

ditions, and, conceivably, this could account for the difference between our findings. What yet remains to be determined, is whether serum exposed to thorium just long enough to induce only a minimal lesion in the thromboplastin generation test still contains normal amounts of factors IX and X *as measured in specific assays modified to eliminate exposure of the test system to citrate or oxalate prior to recalcification.*

If such serum is found to exhibit moderate defects of factors IX and X, the further question arises: can the *combination* of these defects completely account for the TGT lesion ? Colman and Alexander (6) state that the TVF is decreased in the newborn, in patients receiving oral anticoagulant therapy, and in patients with liver disease – clinical conditions in which combined defects of factors IX and X are found. Their most convincing evidence for the new factor is the correction of the abnormal TGT of thorium-treated serum by hereditary factor IX deficiency serum and by hereditary factor X deficiency serum. What needs clarification, is whether or not a mixture of 2 sera – one thorium-treated to produce partial defects in both factors IX and X and the other with a single, complete defect of either factor IX or factor X – will pass as normal in the TGT. We believe that the hypothesis of a special "thorium vulnerable factor" needs further testing along such lines before its acceptance.

Factors VIII, XI, and XII did not appear to be inactivated by thorium dioxide under our conditions of incubation and testing.

Platelets exposed to thorium dioxide became less adhesive and less subject to aggregation. Possibly, this reflects an alteration in fibrinogen adsorbed onto the platelet surface. We failed to demonstrate destruction of platelets or release of platelet factor 3 during incubation of platelet-rich plasma with thorium dioxide suspensions *in vitro.*

It is tempting to postulate a common mechanism for the defects observed. Glazko and Greenberg (9) suggested that thorium, a polyvalent cation, reduces the negative charge of fibrinogen. Colman and Alexander (5) suggested that thorium inactivates clotting factors by forming complexes with them. The damage to clotting factors produced by other rare earth metals, (1, 16, 27) and the protection afforded by citrate support this concept, which fits the presently available facts.

The possible significance of these *in vitro* findings in relation to our *in vivo* observations are discussed in the next paper.

Summary

Thorium dioxide was found to damage clotting factors and to interfere with platelet functions *in vitro:*

1. Fibrinogen and factor V were progressively inactivated. Citrate did not prevent damage to these factors. Thorium dioxide was concentrated in the clot, which suggests that it binds to fibrinogen.

2. Factors II, VII, IX, and X were also inactivated. Inactivation of these factors could be prevented or reversed by citrate.

3. Factors VIII, XI, and XII were not altered.

4. Platelets became less adhesive and less subject to aggregation.

These effects were more marked with unfiltered suspensions of thorium dioxide (TDS) than with filtered suspensions (Thorotrast). They were also increased by increasing the concentration of thorium dioxide, and the time and temperature of incubation. Thorium dioxide probably forms complexes with the clotting proteins, thereby making them less reactive.

Résumé

Le dioxyde de thorium altère les facteurs de coagulation et interfère avec les fonctions plaquettaires in vitro:

1. Le fibrinogène et le facteur V sont progressivement inactivés. Le citrate ne peut prévenir l'altération de ces facteurs. Le dioxyde de thorium est concentré dans le caillot, ce qui suggère qu'il se lie au fibrinogène.

2. Les facteurs II, VII, IX et X sont également inactivés. Cette inactivation peut être prévenue ou est réversible en présence de citrate.

3. Les facteurs VIII, XI et XII ne sont pas altérés.

4. Les plaquettes deviennent moins adhésives et sont moins sujettes à l'aggrégation.

Ces effets sont plus marqués avec les suspensions non filtrées de dioxyde de thorium (TDS) qu'avec les suspensions filtrées (Thorotrast). Ils sont également augmentés par les concentrations croissantes de dioxyde de thorium et par la durée et la température d'incubation. Le dioxyde de thorium forme probablement des complexes avec les protéines du système de coagulation les rendant ainsi moins réactives.

Zusammenfassung

Es wurde erwiesen, daß Thorium-dioxyd *in vitro* die Gerinnungsfaktoren schädigte und daß es auch in die Funktionen der Blutplättchen eingriff.

1. Fibrinogen und Faktor V wurden fortschreitend inaktiviert. Zitrat konnte die Beschädigung dieser Faktoren nicht verhindern. Thorium-dioxyd war in dem Gerinnsel konzentriert, welches darauf deutet, daß es sich mit Fibrinogen verbindet.

2. Die Faktoren II, VII, IX und X wurden auch inaktiviert. Dies konnte durch Zitrat verhindert oder reversiert werden.

3. Die Faktoren VIII, XI und XII wurden nicht beeinflußt.

4. Die Blutplättchen wurden weniger adhäsiv und aggregierten deshalb weniger als normal.

Diese Wirkungen waren stärker mit unfiltrierten Suspensionen von Thoriumdioxyd (TDS) als mit filtrierten Suspensionen (Thorotrast). Die Wirkungen wurden auch verstärkt durch erhöhte Konzentration von Thorium-dioxyd und durch Verlängerung der Zeit und Erhöhung der Temperatur der Inkubation. Es wird angenommen, daß Thorium-dioxyd sich mit den Gerinnungsfaktoren verbindet; dadurch werden diese Faktoren weniger reaktiv.

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References

(1) *Beller, F. K., E. Mammen:* Der Angriffspunkt der seltenen Erden Neodym im Gerinnungssystem. Arch. Gynäk. *187:* 319–336 (1956).

(2) *Blombäck, B., M. Blombäck:* Purification of human and bovine fibrinogen. Ark. Kemi *10:* 415–443 (1956).

(3) *Brecher, G., E. P. Cronkite:* Morphology and enumeration of human blood platelets. J. appl. Physiol. *3:* 365–377 (1950).

(4) *Clark, S. L., W. H. Batchelor:* The behavior of the formed elements of blood incubated with bacterial endotoxins. Naval med. Res. Inst. *15:* 361–374 (1957).

(5) *Colman, R., B. Alexander:* The effect of lanthanides and actinides on blood coagulation. I. Evidence for and properties of a new serum thromboplastic factor. J. clin. Invest. *43:* 705–719 (1964).

(6) *Colman, R., B. Alexander:* The effect of lanthanides and actinides on blood coagulation. II. Assay of a new serum thromboplastic factor vulnerable to these elements and its variations in health and disease. J. clin. Invest. *43:* 720–727 (1964).

(7) *Council on Pharmacy and Chemistry:* Thorotrast. J. Amer. med. Ass. *99:* 2183–2185 (1932).

(8) *Des Prez, R. M., H. I. Horowitz, E. W. Hook:* Effects of bacterial endotoxin on rabbit platelets. I. Platelet aggregation and release of platelet factors in vitro. J. exp. Med. *114:* 857–874 (1961).

(9) *Glazko, A. J., D. M. Greenberg:* The mechanism of the inhibiting effect of electrolytes and heparin on blood coagulation. Amer. J. Physiol. *128:* 399–407 (1940).

(10) *Godal, H. C.:* A comparison of two heparin-neutralizing agents: protamine and polybrene. Scand. J. clin. Lab. Invest. *12:* 446–457 (1960).

(11) *Good, R. A., L. Thomas:* Studies on the generalized Shwartzman reaction II. The production of bilateral cortical necrosis of the kidneys by a single injection of bacterial endotoxin in rabbits previously treated with Thorotrast or Trypan blue. J. exp. Med. *96:* 625–641 (1952).

(12) *Hjort, P. F.:* Intermediate reactions in the coagulation of blood with tissue thromboplastin. Scand. J. clin. Lab. Invest. *9* (suppl. 27): 183 pp. (1957).

(13) *Hjort, P. F., S. I. Rapaport, P. A. Owren:* A simple, specific one-stage prothrombin assay using Russell's viper venom in cephalin suspension. J. Lab. clin. Med. *46:* 89–97 (1955).

(14) *Horowitz, H. I., R. M. Des Prez, E. W. Hook:* Effects of bacterial endotoxin on rabbit platelets. II. Enhancement of platelet factor 3 activity in vitro and in vivo. J. exp. Med. *116:* 619–633 (1962).

(15) *Hougie, C.:* A simple assay method for Factor X (Stuart-Prower Factor). Proc. Soc. exp. Biol. (N. Y.) *109:* 754–756 (1962).

(16) *Hunter, R. B., W. Walker:* Neodymium 3-sulpho-isonicotinate and blood coagulation. Brit. med. J. *2:* 1214–1216 (1956).

(17) *Husom, O.:* A one stage method for the assay of platelet factor 3. Scand. J. clin. Lab. Invest. *13:* 609–618 (1961).

(18) *Hyman, C., R. Paldino, S. Bernick:* Thorotrast which does not block the RES. R. E. S. Bull. *1:* 81 (1955).

(19) *Häusler, H., L. Vogel:* Über Metallwirkungen. IV. Mitteilung: Weiteres über die Beeinflussung der Blutgerinnung durch Metalle. Biochem. Z. *287:* 405–410 (1936).

(20) *Lundblad, R. L., E. W. Davie:* The activation of Antihemophilic Factor (Factor VIII) by activated Christmas Factor (activated Factor IX). Biochemistry *3:* 1720–1725 (1964).

(21) *Rapaport, S. I., S. Schiffman, M. J. Patch, S. B. Ames:* The importance of activation of antihemophilic globulin and proaccelerin by traces of thrombin in the generation of intrinsic prothrombinase activity. Blood *21:* 221–236 (1963).

(22) *Rapaport, S. I., S. Schiffman, M. J. Patch, A. G. Ware:* A simple, specific one-stage assay for the plasma thromboplastin antecedent activity. J. Lab. clin. Med. *57:* 771–780 (1961).

(23) *Ratnoff, O. D., E. W. Davie:* The activation of Christmas Factor (Factor IX) by activated plasma thromboplastin antecedent (activated Factor XI). Biochemistry *1:* 677–685 (1962).

(24) *Schiffman, S., S. I. Rapaport, M. J. Patch:* The identification and synthesis of activated plasma thromboplastin component (PTC'). Blood *22:* 733–749 (1963).

(25) *Schiffman, S., S. I. Rapaport, M. J. Patch:* Starch block electrophoresis of plasma and serum clotting factors. Separation of activated PTC (PTC'). Blood *25:* 724–735 (1965).

(26) *Slätis, P.:* The synthesis of prothrombin and proconvertin in the reticulo-endothelial system. Scand. J. clin. Lab. Invest. *10* (suppl. 33): 83 pp. (1958).

(27) *Soulier, J. P., C. Weilland:* Données nouvelles concernant l'action anticoagulante d'un sel de néodyme. Rev. Hémat. *11:* 265–278 (1965).

(28) *Stormorken, H.:* The preparation of proaccelerin deficient (parahemophilia) plasma for the assay of proaccelerin. Scand. J. clin. Lab. Invest. *9:* 273–276 (1957).

(29) *Stormorken, H., Å. Lund-Riise, T. O. Rörvik:* Platelet adhesiveness to glass beads: methodological investigations using automatic platelet counting. Scand. J. clin. Lab. Invest. *17* (suppl. 84): 183–194 (1965).

(30) *Voss, D.:* Untersuchungen zur Gerinnsel-retraktion. Proc. VIIth Int. Congr. Soc. Hemat., Rome 1957.

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