(SFFV) and which probably is responsible for inducing polycythemia when present in high titers, and another which, when active in the presence of little or no SFFV, induces anemia. The role of either virus in initiating leukemia remains to be determined.

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Effects of Thorotrast upon the Reactivity and Intravascular Disappearance Rate of Fibrinogen in the Rabbit* (32832)

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The experiments reported herein are an extension of earlier studies from this laboratory of the *in vitro* (1) and the *in vivo* (2) effects of Thorotrast upon hemostatic factors in the rabbit. In those studies Thorotrast was found to prolong the thrombin time of plasma but not to lower its fibrinogen level. Further evidence of the effect of Thorotrast upon fibrinogen reactivity is presented below along with measurements of the effect of Thorotrast upon the intravascular disappearance rate of ^{131}I-labeled fibrinogen. One purpose was to determine if the return of the thrombin time to normal after Thorotrast was associated with the removal from the circulation of increased amounts of fibrinogen altered by Thorotrast.

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A second purpose was to look for evidence of intravascular clotting after Thorotrast. Our recent studies indicated that measurement of the disappearance rate of ^{131}I-labeled fibrinogen may detect intravascular clotting after endotoxin in rabbits whose fibrinogen levels do not fall. Both the injection of Thorotrast (2) and of tissue thromboplastin (3) lower Factor V, Factor VIII, and platelet levels in the rabbit. The possibility existed that the falls induced by Thorotrast might stem not only from its direct action upon these factors but also from intravascular clotting.

Thorotrast is used to prepare rabbits for the deposition of fibrin in glomerular capillaries and renal cortical necrosis (the generalized Shwartzman reaction) by agents known to promote intravascular clotting, e.g., endotoxin (4), activated Factor X (5), and by agents under study for their ability to promote intravascular clotting (6). It has been assumed that the intravascular clotting is induced by only the second agent and that

Thorotrast acts to prevent cellular clearance of fibrin in the liver (7). Yet, Thorotrast alone has been reported to produce renal cortical necrosis in rabbits (8). Therefore, it seemed important to us to determine if Thorotrast, in the dosage used to produce "reticuloendothelial blockade" in the rabbit (3 ml/kg) was itself capable of inducing intravascular clotting measurable by an increased rate of disappearance of labeled intravascular fibrinogen.

Materials and Methods. Thorotrast R, a sterile, filtered, stabilized 24–26% solution of thorium dioxide in a carrier solution of 25% dextrin and 0.15% methylparasept (Fellows-Testagar, Detroit, Michigan) was given intravenously in a dosage of 3 ml/kg. Liquid R, sodium polyanethol sulfonate (Hoffmann-La-Roche, Nutley, New Jersey) was dissolved in saline. Endotoxin—Bacto Lipopolysaccharide W *E. coli* 0111:B4, (DIFCO Laboratories, Detroit, Michigan) was dissolved in saline. Rabbits—New Zealand females were fed Purina rabbit chow with tetracycline and allowed water *ad libitum*. For at least 3 days before and during experiments, potassium iodide (200 mg/liter) and sodium chloride (1.8 gm/liter) were added to the drinking water to prevent accumulation of iodide-^{131}I.

Rabbit fibrinogen, 97% clottable, was prepared from plasma according to McFarlane (9) using 25 and 23% saturated ammonium sulfate, and was stored at —20°C until labeled with ^{131}I by Abbott Laboratories, North Chicago, Illinois. The initial activity was 198 μC/mg. The preparation was stored frozen and used within 3 weeks. The first experimental sample, which was taken 30 hours after the injection of the labeled material (see below), had the following mean distribution of radioactivity expressed as percentage of whole plasma radioactivity: fibrin clot, 85% (range 77–94%); trichloroacetic acid precipitate of the supernatant of the fibrin clot, 7%; radioactivity remaining in the supernatant after treatment with trichloracetic acid, 8%. Plots of whole plasma radioactivity and of fibrin clot radioactivity in serial samples against time gave lines with parallel slopes (Fig. 4).

Ear veins were dilated with xylene and warm water, a 20-gauge siliconized needle was inserted, and blood was allowed to drip directly from the hub, 3 ml onto 6 mg of EDTA (disodium salt of ethylenediaminetetraacetic acid) powder in a glass tube, or 1.8 ml into 0.2 ml of citrate anticoagulant (0.06 *M* in trisodium citrate and 0.04 *M* in citric acid) in plastic. Platelet-poor plasma was prepared by centrifuging at 10,000 rpm for 10 min, and was stored in plastic vials at —20°C.

Thrombin time. Citrated plasma, 0.2 ml, was warmed for 3 min, and then 0.1 ml of thrombin (5 NIH-units/ml) was added. Clotting time was recorded in seconds.

Radioactivity measurement. EDTA plasma, 0.3 ml was mixed with 5.5 ml of barbital buffer [2.93 gm of NaCl, 5.71 gm of Nabarbital, 22.3 ml of 1 *N* HCl, made up to 1 liter with distilled water] and 0.05 ml of calcium-thrombin (equal parts 1 *M* $CaCl_2$ and 1:100 dilution in saline of 1000 units/ml stock bovine thrombin prepared as described elsewhere (10)). A glass rod was placed in the mixture. After 60 min the clot was wound out on the glass rod, washed in 5 ml of saline and 5 ml of distilled water for 30 min and dissolved in 5 ml of alkaline urea (0.2 *N* NaOH added to 400 gm of urea to make 1 liter). Aliquots of plasma, nonclottable supernatant buffer, and alkaline urea fibrin solution were counted for radioactivity, which was expressed as a percentage of plasma radioactivity of the first sample. Thorotrast radioactivity did not interfere with counting of ^{131}I radioactivity. When Thorotrast prevented the fibrin clot from adhering to the glass rod, separation was made by filtration.

Results. 1. Additional evidence that Thorotrast alters fibrinogen reactivity. (a) The microscopic appearance of the clot. Four tenths ml of rabbit or human citrated plasma were recalcified with 0.1 ml of 40 m*M* $CaCl_2$, a drop was placed between a glass microscope slide and a cover slip, and observed under phase microscopy. Fibrin was first seen on the surface of tiny particles as a fine network which radiated from different particles to meet and intermesh (Fig. 1A). With plasma that had incubated at 37°C with one-twentieth its volume of Thorotrast for 30–90

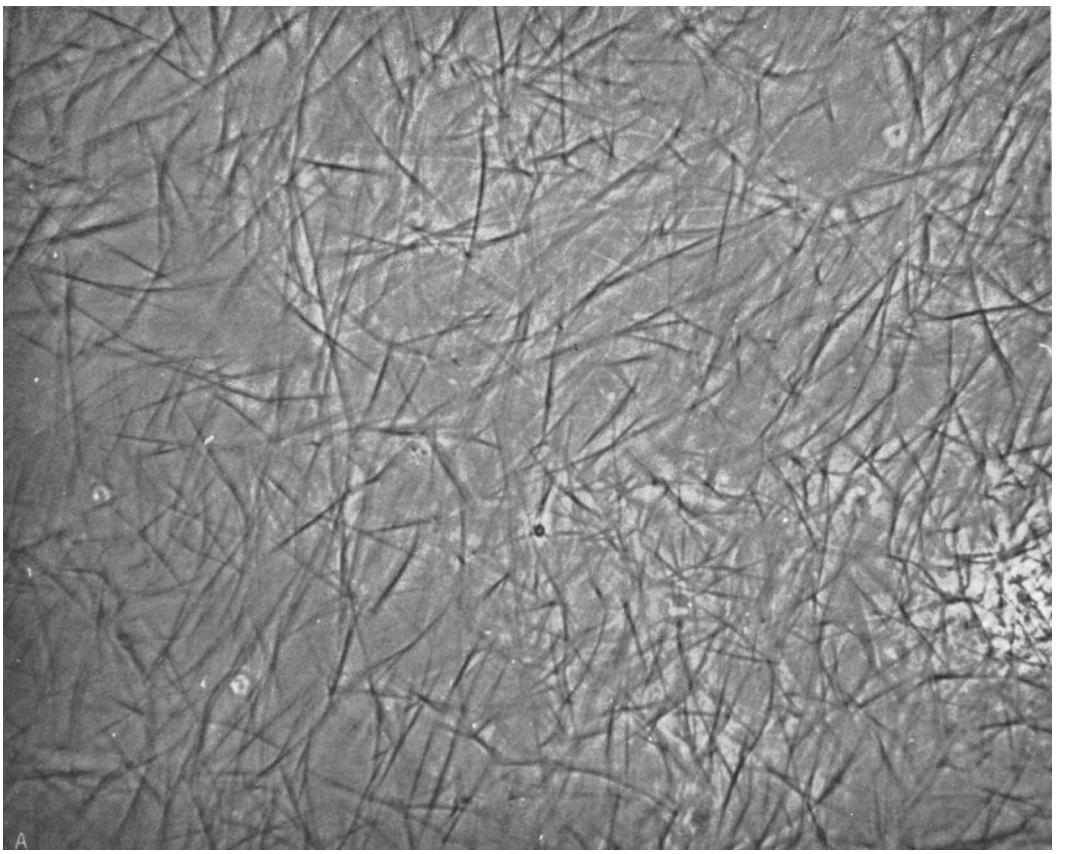


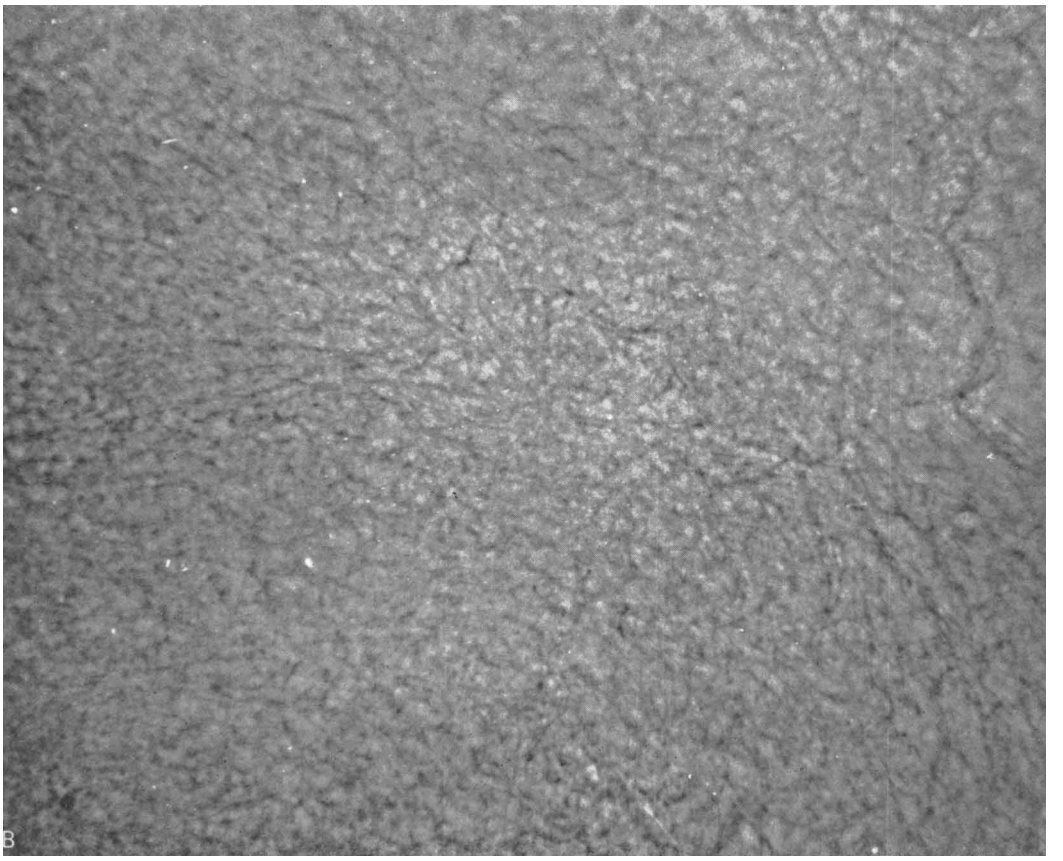
FIG. 1. Phase micrograph (original magification × 450) of fibrin clot formed by recalcifying 0.4 ml of plasma with 0.1 ml of 40 m*M* $CaCl_2$. (a) normal plasma, (b) plasma incubated with Thorotrast (0.95 ml of plasma + 0.05 ml of Thorotrast at 37°C) for 90 min.

min, fibrin appeared as a mixture of amorphous precipitate and very short strands (Fig. 1B).

(b) Effect on thrombin time. The striking effect of the injection of Thorotrast on the thrombin time was confirmed (2) (Fig. 2). Since there was at least a 30-min delay before the thrombin time lengthened, a time-consuming reaction, presumably between Thorotrast and fibrinogen, must occur. The thrombin time remained markedly prolonged as long as significant amounts of Thorotrast radioactivity could be detected in the plasma. Some prolongation was still evident 10 hours after the injection, but values had returned to normal at 23 hours.

The distribution of Thorotrast radioactivity between fibrin clot and supernatant was determined in plasma from blood drawn 0.5 and 1 hour after rabbits were given Thorotrast. Approximately 75% of the radioactivity was associated with the fibrin clot and 25% with the supernatant (Table I). This raises the strong possibility that Thorotrast circulates largely bound to fibrinogen, but does not prove this since Thorotrast could have been occluded in the fibrin clot as it formed. Interestingly, the distribution of Thorotrast between the clot and the supernatant was the same in the 0.5 hour samples, when the thrombin time was still normal (30, 32, and 35 sec), as in the 1 hour samples, when the thrombin time was markedly prolonged (119, 194, 229 sec).

2. Clearance of ^{131}I-labeled fibrinogen. In preliminary studies with ^{131}I-labeled fibrinogen, plots of either plasma or fibrin-clot radioactivity against time on semilogarithmic paper consistently gave lines which were



curved from the injection time to between 20 and 30 hours and straight thereafter. Since earlier investigators (9, 11) have shown that the straight line portion of such curves is a valid measure of the disappearance of fibrinogen from the circulation, we adopted the following procedure: An initial sample was collected at least 30 hours after injection of labeled fibrinogen and a second sample was taken 16–18 hours later. A line drawn through these 2 points then defined the control labeled fibrinogen disappearance curve for that particular animal. Downward deviation of subsequent observed points from an extrapolation of the control line indicated excess disappearance of intravascular fibrinogen, which could

TABLE I. The Distribution of Radioactivity between Fibrin Clot and Supernatant in Plasma from Blood Taken 0.5 Hour and 1 Hour after Injection of Thorotrast.

	Net counts in 1 hour					
	Animal 1		Animal 2		Animal 3	
Sample	0.5 hour	1 hour	0.5 hour	1 hour	0.5 hour	1 hour
Fibrin clot	27,796	28,023	25,286	15,132	31,462	24,898
Supernatant	11,657	10,441	9406	5465	9632	7400

0.3 ml of plasma mixed with 5.5 ml of buffer and clotted with 0.05 ml of calcium-thrombin. After 1 hour the mixture is filtered. The filtrate is counted as the supernatant, and the clot on the filter paper is dissolved in 5 ml of alkaline urea and counted.

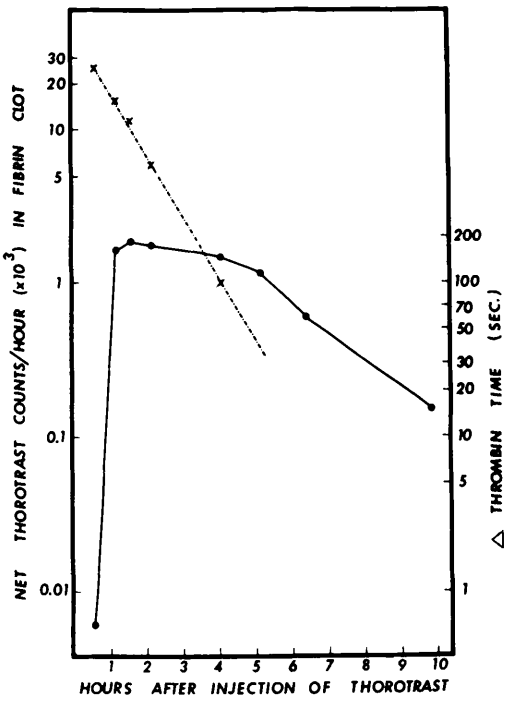


FIG. 2. Thorotrast radioactivity (×) in fibrin clot and change in thrombin time (●) following injection of Thorotrast (3 ml/kg) into a rabbit.

be expressed quantitatively as illustrated in Fig. 3.

Figure 3 shows that a decrease in the concentration of fibrinogen-^{131}I is a more sensitive index of intravascular clotting than the fibrinogen concentration. In this animal, given 50 μg/kg of endotoxin, a marked input of fibrinogen into the plasma caused fibrinogen levels to rise despite a significant increase in the rate of disappearance of fibrinogen from the plasma after endotoxin.

In 7 rabbits (wt. 1.3–1.8 kg) control samples were taken at 31 hours and at 47 hours after the injection of labeled fibrinogen and the animals were then given Thorotrast. Serial samples were collected at intervals up to 24 hours. In no animal did the observed whole plasma or fibrin-clot radioactivities deviate from the extrapolated line. A composite line constructed from the log mean of the data from all animals is shown in Fig. 4. Clearly, a significant increase in the removal of fibrinogen by any mechanism does not occur after Thorotrast.

3. Precipitation of fibrinogen by Liquoid. Lee and co-workers (12) recently reported that a dose of Liquoid insufficient in itself to provoke renal cortical necrosis did so in rabbits given Thorotrast 3 hours earlier. Although Liquoid, unlike other agents with this effect, precipitates fibrinogen directly, incubating plasma *in vitro* with unfiltered thorium dioxide suspension did not visibly increase the precipitate formed on adding small amounts of Liquoid to rabbit plasma (1).

To determine whether Thorotrast alters the precipitability of fibrinogen when given *in vivo*, we drew blood into citrate from 4 rabbits 3 hours after injecting Thorotrast and incubated the plasma with one-tenth volume of Liquoid (0.02–10 mg/ml) for 60 min at 37°C. The quantity of precipitate was the same as that noted with plasma from a preinjection sample.

Summary and Conclusions. The data presented above confirm and extend previous observations (1, 2, 13) that Thorotrast markedly alters the reactivity of fibrinogen. Microscopic examination of clots from plasma incubated *in vitro* with Thorotrast revealed a progressive defect in fibrin strand formation. Thrombin times were prolonged for at least 10 hours after the intravenous injection into rabbits of the dosage (3 ml/kg) used to

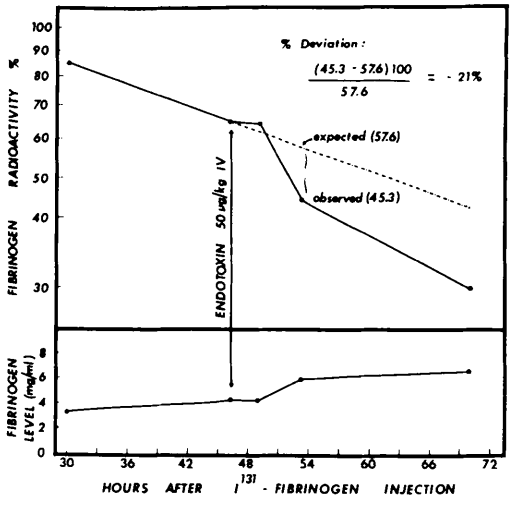


FIG. 3. Radioactivity of fibrin clot, and fibrinogen level in a rabbit given firinogen-^{131}I and then endotoxin (50 μg/kg). Each point is the mean of two determinations.

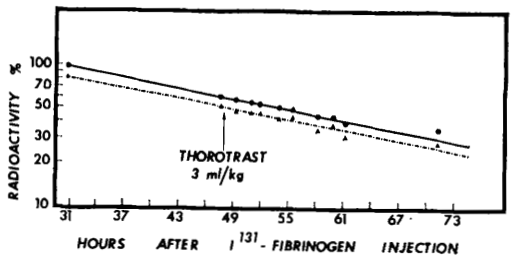


FIG. 4. ^{131}I-radioactivity in whole plasma (●) and fibrin clot (▲) in 7 rabbits given fibrinogen-^{131}I and then Thorotrast (3 ml/kg). Points are log means of from 2 to 7 animals.

produce "reticuloendothelial blockade." When plasma from blood drawn after the administration of Thorotrast was clotted, most of the radioactivity of the Thorotrast was found in the clot. This suggests that Thorotrast may circulate largely as a complex with fibrinogen.

Despite these marked effects, Thorotrast did not increase the rate of intravascular disappearance of ^{131}I-labeled fibrinogen. Thus, the return of the thrombin time to normal within 1 day after Thorotrast was not associated with the removal of measurable amounts of a Thorotrast–fibrinogen complex or of excess fibrinogen damaged directly or indirectly by Thorotrast. Apparently, as Thorotrast is removed from the circulation, fibrinogen altered by Thorotrast recovers its normal reactivity.

The fibrinogen present in the circulation 3 hours after the administration of Thorotrast was no more susceptible to precipitation *in vitro* by Liquoid than was normal fibrinogen. This supports the view of Lee and co-workers (12) that Thorotrast has no role other than altering reticuloendothelial function in preparing animals for renal cortical necrosis after Liquoid.

Thorotrast does not induce measurable intravascular clotting. Therefore, the decrease in platelets, Factor V, and Factor VIII observed earlier (2) must represent a direct effect of Thorotrast upon these factors. Endotoxin is normally absorbed from the gut and removed by the reticuloendothelial system (8). In our rabbits, which received a feed containing tetracycline, insufficient endotoxin was absorbed to change the disappearance rate of intravascular fibrinogen after "reticuloendothelial blockade." This suggests that in healthy animals, fed a diet supplemented with antibiotic, evidence of increased fibrinogen disappearance after Thorotrast and a second agent may be attributed to an action of the second agent, provided that this agent does not also increase absorption of endotoxin from the gut.

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