Influence of Soybean Phosphatide on Blood Coagulation and its Use in the Thromboplastin Generation Test. (23640)

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For many years a lipid substance has been postulated to be a factor necessary for the physiological clotting of blood(1). The essential lipid component probably is a phospholipid supplied by platelets(2). In the early phases of coagulation, platelets, or certain phospholipid substitutes, interact with plasma factors and calcium to form plasma thromboplastin(3). Knowledge gained from the use of the thromboplastin generation test (4) has strengthened this concept. Several workers have found that phospholipid derived from brain(5) or from soybeans(6) will provide complete substitution for platelets in this test. It is the purpose of this report to show the effect of soybean phosphatide in certain clotting systems and to illustrate its use in a simplified thromboplastin generation test.

Materials and methods. All blood was drawn by the 2-syringe technic. Normal oxalated plasma was obtained by adding 7 ml of whole blood to 1.0 ml of 1.85% potassium oxalate solution and centrifuging the mixture 10 min. at 4,000 rpm and 4°C. Platelet-rich plasma was prepared by centrifugation at only 700 rpm for 10 min. It was kept frozen and thawed just before use. A saline or aqueous soybean phosphatide† emulsion was made in various concentrations (water appeared to provide a better emulsion). A 0.1% concentration was used in all tests except where specifically noted. This emulsion remained fully active for at least 3 months when stored at −26°C. Barium sulfate adsorbed plasma was provided by mixing 1 ml of oxalated plasma with 100 mg $BaSO_4$ for 30 min. with a magnetic stirrer and then centrifuging. For the "aged serum" reagent, blood was allowed to clot and then was incubated for 2 hours at 37°C. The resulting serum was aged an additional hour at 37°C before use and was always free from demonstrable thrombin. Crude lung thromboplastin was prepared from beef lung(7). The *thromboplastin generation test* of Biggs and Douglas(4) was modified as follows: 0.3 ml of barium sulfate adsorbed plasma (diluted 1/5), 0.3 ml aged serum (diluted 1/5), 0.3 ml of 0.1% soybean phosphatide, and 0.1 ml of 0.015 M $CaCl_2$ were incubated at 37°C. A stopwatch was started upon the addition of the $CaCl_2$. The incubation times were 2, 4, 6, 8, 10, and 12 min. Thirty seconds before each of the above times 0.1 ml of the incubation mixture was added to a clotting tube containing 0.1 ml of 0.025 M $CaCl_2$. At precisely each incubation time, 0.1 ml of oxalated plasma was added to the clotting tube, a second stopwatch was started, and the time required for the formation of a fibrin clot was recorded. With normal plasma, thromboplastin was generated in from 6-8 min. of incubation and substrate plasma was clotted in 10-12 sec. *Prothrombin consumption test.* Three ml of whole blood in each of 2 conical centrifuge tubes was incubated at 37°C for periods of 15 and 45 min. respectively and then centrifuged for 10 min. at 4,000 rpm at 4°C. At exactly 30 and 60 min., respectively, from the time that the blood was drawn, 0.1 ml of 2.85% sodium citrate was added to each 0.7 ml specimen of the serum. After 10 min. of further incubation residual prothrombin then was estimated by the two-stage method(7) and related to the prothrombin concentration of the plasma. The percentage of residual prothrombin in normal subjects was usually below 40% in the 30-min. specimen and below 10% in the 60-min. specimen. For *one-stage prothrombin times,* 0.1 ml of rabbit brain thromboplastin‡ was added to 0.1 ml of oxalated plasma. The mixture then was clotted by 0.1 ml 0.025 M $CaCl_2$. In the determina-

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† Gliddex "O" (Glidden Co.)

‡ Permaplastin (C. W. Alban and Co.)

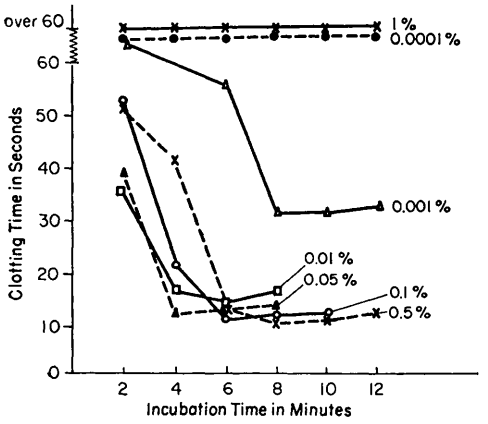

FIG. 1. Effect of soybean phosphatide concentration on thromboplastin generation test. Clotting times are those of recalcified oxalated plasma with the addition of thromboplastin generated at the indicated incubation time.

tion of the *Stypven*§ *time,* 0.1 ml of Stypven was used as thromboplastin in the one-stage prothrombin time. The *clotting time of recalcified plasma* was ascertained by mixing 0.1 ml of oxalated plasma and 0.1 ml of normal saline, and recording the clotting time at 37°C produced by the addition of 0.1 ml of 0.025 M $CaCl_2$. The same procedure was used in the *"accelerated" plasma clotting time* except that 0.1 ml of soybean phosphatide solution was used in place of saline. The *heparin tolerance test* was performed by adding 0.1 ml of varying concentrations of heparin‖ to 0.1 ml of oxalated plasma. The mixture was then clotted at 37°C by 0.1 ml of 0.025 M $CaCl_2$. The *protamine tolerance test* was performed similarly with protamine¶ substituted for heparin. *Whole blood clotting times* were carried out by the Lee White technic. All of the above clotting asays were carried out in plain glass tubes.

Results. The effect of phosphatide concentration on thromboplastin generation test. Five normal subjects were studied with comparable results in each instance. Fig. 1, with the data from one subject, relates the generation of thromboplastin to various concentrations of phosphatide emulsion. At low concentrations (0.0001%) and at high concentrations (1%), no thromboplastin was generated. Maximal thromboplastin generation occurred when the concentration was between 0.01% and 0.1%. Saline and water dilutions of the phosphatide resulted in comparable clotting activity.

§ Stypven (Russell's Viper Venom 1:10,000, Burroughs Wellcome and Co.)

‖ Sodium Heparin (Abbott)

¶ Protamine Sulfate (Eli Lilly)

TABLE I. Effect of Varying Concentrations of Soybean Phosphatide upon Accelerated Plasma Clotting Time.

Phosphatide	5%	1%	0.1%	0.01%	0.001%	0%
Clotting time in sec.	281	90	55	68	110	130

Shortening of clotting time of recalcified plasma. In over 100 normal subjects, the addition of 0.1% soybean phosphatide always shortened the clotting time of recalcified plasma when plasma was obtained after centrifugation at 4,000 rpm. The "accelerated" clotting time was from two-fifths to three-fifths of the saline control. A typical acceleration was from 135 sec. to 62 sec. Phosphatide effected considerable shortening of prolonged clotting times in patients with hemophilia or circulating anticoagulants. Phosphatide did, however, fail to alter the clotting time of previously frozen platelet-rich plasma. Such plasma already had an "accelerated" clotting time.

Table I, showing data from one of 5 normal subjects, indicates that the concentration of soybean phosphatide greatly influences the accelerated plasma clotting time. At low concentrations (0.001%), there was little acceleration of clotting; at high concentrations (5%), there was a marked anticoagulant effect. Similar results were obtained in the other 4 subjects.

Effect on the "one-stage prothrombin time" using different sources of thromboplastin. Soybean phosphatide did not shorten the clotting time when brain or lung thromboplastins were used (Table II). Only the "Stypven" time was shortened by the addition of the phosphatide. In this test platelet-rich plasma and soybean phosphatide behaved similarly in accelerating the Stypven time.

Alteration of prothrombin consumption. Fresh blood was added to tubes containing

TABLE II. Influence of Soybean Phosphatide on "One-Stage Prothrombin Time."*

	Clotting time (sec.)	
	Saline control	Phosphatide
Rabbit brain thromboplastin	20	19
Heated lung " †	22	25
Stypven (1:10,000)	21	9
Stypven using platelet-rich plasma	8	9

* 0.1 ml plasma, 0.1 ml of a thromboplastin, 0.1 ml saline or phosphatide, and 0.1 ml 0.025 M $CaCl_2$ were added together.
† 56°C for 30 min.

0.5 ml of phosphatide emulsion and prothrombin consumption was measured after 30 and 60 minutes of incubation. The results with and without phosphatide were compared in 3 normal subjects. A typical experiment is shown in Table III (A). Phosphatide apparently accelerated the prothrombin consumption since little residual prothrombin remained even at 30 min. In 4 patients with prolonged prothrombin consumption due to thrombocytopenia (platelet counts below 10,000 per cu mm), phosphatide completely corrected the abnormality. The results obtained with one patient are shown in Table III (B). The faulty clot retraction of blood from thrombocytopenic patients, however, was not altered.

Whole blood clotting time. Whole blood clotting times always were shortened by prior rinsing of the clotting tubes with the 0.1% phosphatide emulsion. Average results from 5 subjects were as follows:

Saline control	9.24 min.
Phosphatide	5.0 min.

Anti-heparin and anti-protamine action. When increasing amounts of heparin were added to normal oxalated plasma, the recalcified clotting time was lengthened progressively. Fig. 2 shows a typical normal heparin

TABLE III. Effect of Phosphatide upon Prothrombin Consumption.

	Residual prothrombin, %			
	Control		With phosphatide	
	30′	60′	30′	60′
A. Normal patient	31	7	6	6
B. Thrombocytopenic patient	83	49	1	1

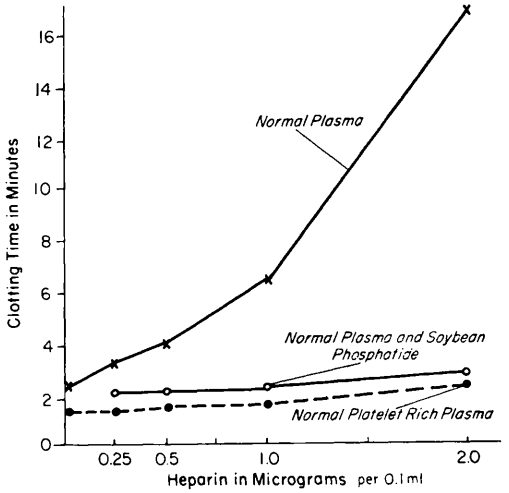

FIG. 2. Influence of soybean phosphatide and platelets upon heparin tolerance test. Clotting mixture consisted of 0.1 ml of oxalated plasma, 0.1 ml of heparin, 0.1 ml of 0.025 M $CaCl_2$ with and without 0.1 ml of 0.1% phosphatide.

tolerance curve and the anti-heparin effect of phosphatide. Soybean phosphatide nullified the typical heparin effect in each of the 5 subjects studied. This anti-heparin action is similar to that of platelet-rich plasma. Soybean phosphatide exhibited similar anti-heparin effects (Table IV) following administration of intravenous heparin in a dog. The heparin-induced effects in whole blood clotting time, plasma clotting time, Stypven time, and prothrombin consumption were abolished by addition of phosphatide. As expected, intravenous heparin changed greatly the *in vitro* heparin tolerance test, *e.g.*, the clotting time with 2.0 μg of heparin increased from 13 to 31 min. The addition of soybean phosphatide nullified this change and reduced the clotting time to 2.6 min. Protamine behaved as an anticoagulant when added to normal human plasma in concentrations from 0.25 to 2.0 μg per ml. In a clotting system analogous to that used in the heparin tolerance test 2.0 μg of protamine lengthened the clotting time from 120 sec. to 240 sec. The use of phosphatide together with this concentration of protamine reduced the clotting time to 105 sec.

Partial thromboplastic activity. Like human brain cephalin, soybean phosphatide is a partial thromboplastin. When it is used in

TABLE IV. Effect of Soybean Phosphatide on Clotting Tests before and 20 Min. after 10 mg of Intravenous Heparin in the Dog.

	Whole blood clotting time (min.)	Plasma clotting time (sec.)	Stypven time (sec.)	Prothrombin consumption (% of residual prothrombin)	Heparin tolerance test (Times in min.) (conc. of heparin in μg/0.1 ml) .25	.5	1	2
Before heparin								
Control	3	124	11.5	0	2.3	3.3	5.6	13.0
Plus phosphatide	3	44	3	0	1.1	1.2	1.5	2.6
After heparin								
Control	24	230	17.3	70	6.9	13.0	18.0	31.0
Plus phosphatide	9	60	3	0	1.2	1.2	2.0	2.6

the thromboplastin generation test on patients with classical hemophilia and with plasma thromboplastin component (PTC) deficiency, thromboplastin is not generated (Fig. 3 and 4). Only when the system is supplied with the appropriate factors present in either normal barium sulfate adsorbed plasma (Fig. 3) or in normal serum (Fig. 4) will plasma thromboplastin be generated adequately. Soybean phosphatide proved also to be a partial thromboplastin in the 2-stage prothrombin test. Substituted for the usual lung thromboplastin, the phosphatide converted little prothrombin to thrombin.

Discussion. Soybean phosphatide contains a mixture of phosphatides: lecithin, phosphatidyl ethanolamine, phosphatidyl serine, and inositol phosphatide. Rouser and co-workers have indicated recently that only phosphatidyl ethanolamine is an accelerator of blood clotting, whereas both phosphatidyl serine and inositol phosphatide behave as inhibitors of

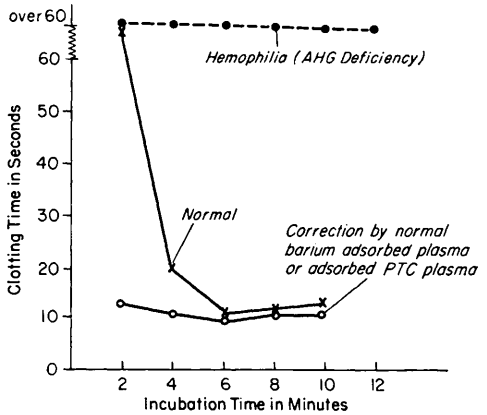

FIG. 3. Use of soybean phosphatide in thromboplastin generation test of hemophilia.

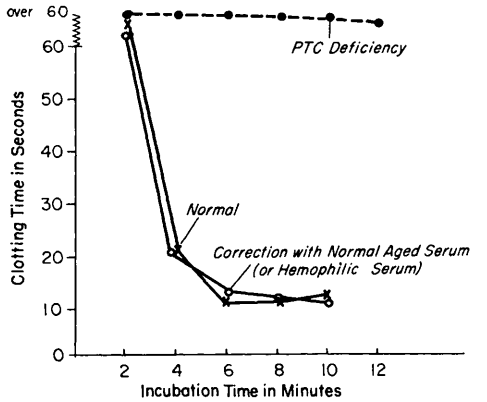

FIG. 4. Use of soybean phosphatide in thromboplastin generation test of PTC deficiency.

coagulation(8). Barkham obtained substantially the same results with the use of phosphatidyl ethanolamine and phosphatidyl serine, fractionated from human brain(9). Chemically pure lecithin has no activity in blood clotting systems(8). O'Brien has compared the similarities of platelets and phosphatidyl ethanolamine in blood coagulation (10). These similarities suggested to Macfarlane that the substance in platelets active in the generation of plasma thromboplastin possibly may be phosphatidyl ethanolamine (3). It therefore seems likely that a soybean phosphatide emulsion may influence blood clotting either as a lipid accelerator or as a lipid inhibitor depending on the concentration of the emulsion. White *et al.* found that concentrations of soybean phosphatide above 0.25% were inhibitory in the clotting of dog plasma(11). Our results, obtained by varying the concentration of soybean phosphatide in the thromboplastin generation test, have

indicated an optimal concentration range for maximal thromboplastin generation. High concentrations were inhibitory. Klein *et al.* had noted the same phenomena when the concentration of platelets was varied in the thromboplastin generation test(12). Concentrations of platelets above 1,000,000 per cu mm were inhibitory; concentrations below 50,000 per cu mm had little activity. Platelets at the equivalent physiological concentration had maximal effect in generating thromboplastin. Likewise, the effect of varying concentrations of platelets on the clotting time of recalcified plasma(13) is similar to our results indicated in Table II with differing concentrations of soybean phosphatide.

Soybean phosphatide failed to shorten the one-stage prothrombin time when either brain or lung thromboplastin was employed. Both of these moieties contain phosphatides(14). On the other hand, the "Stypven" time was shortened greatly by phosphatide and by platelet-rich plasma. The clotting activity of the Russell viper venom protein has been shown to be influenced greatly by either phosphatides or platelets(3).

White previously had reported that soybean phosphatide, administered intravenously, restored to normal the deficient prothrombin consumption in thrombocytopenic dogs(11). Our results show that *in vitro* addition of soybean phosphatide to blood of thrombocytopenic patients will correct their abnormal prothrombin consumption. Even in normal blood the prothrombin consumption is considerably accelerated. This presumably occurs because relatively large amounts of the phosphatide thromboplastin factor are supplied at the instant blood begins to clot. When blood is allowed to clot in a plain dry glass tube, the gradual breakup of platelets probably provides the phosphatide thromboplastin factor at a much slower rate. The shortening of whole blood clotting time by phosphatide may be due to the same reason. Platelet-rich plasma thawed from the frozen state and soybean phosphatide each accelerated the clotting time of recalcified plasma. The use of these reagents together did not have a synergistic effect.

The lipid brain extract used by certain workers(5,15,16) as a substitute for platelets would appear to be analogous in thromboplastin activity to the "cephalin" preparations of earlier investigators. It is now known that cephalin is a mixture of the phosphatides; namely, phosphatidyl ethanolamine, phosphatidyl serine, and inositol phosphatide(17). Crude brain cephalin inhibited coagulation in high concentrations but in lower concentrations had clotting accelerating effects(15) similar to those described above for soybean phosphatide. Both Wolf and Rapaport have found that cephalin from brain does not have anti-heparin properties found in platelets(16, 18). The results in Fig. 2 and Table IV, however, indicate that soybean phosphatide has definite anti-heparin properties. The phosphatide abolished the usual anticoagulant effects of heparin in both human and dog plasma. Platelet-rich plasma behaved similarly in the heparin tolerance test. Protamine, a heparin ionic antagonist, is known to be an anticoagulant *in vitro*(19). Soybean phosphatide then appears to have the capacity of opposing the anticoagulant action of both protamine, a basic protein, and heparin, an acidic substance.

The use of soybean phosphatide as a platelet substitute has simplified the thromboplastin generation test in the study of patients with hemorrhagic disorders. It might appear that the use of a platelet suspension in this test would be more physiological. However, the preparation of platelets by freezing and thawing(20) or by repeated centrifugation and stirring with a wooden applicator stick(4) is hardly physiological. Intact platelets as such would seem to have little value in the thromboplastin generation test. The use of soybean phosphatide in this test insures a convenient uniform source of an apparently essential component. The siliconizing of glassware and needles is not necessary. Platelet defects such as occur in thrombasthenia or thrombocytopenia can be detected readily by the prothrombin consumption test. The complete correction of any abnormal prothrombin consumption by the *in vitro* use of soybean phosphatide would appear to confirm the diagnosis of such a platelet deficiency.

Summary. By its use in a variety of blood

clotting systems, soybean phosphatide has been shown to act as a partial thromboplastin in blood coagulation. *In vitro,* this substance displayed anti-heparin activity in human and dog blood and anti-protamine activity in human plasma. A simplified thromboplastin generation test is described for the study of hemorrhagic disorders. Soybean phosphatide serves as a complete substitute for platelets in this test.

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Inclusion Bodies of the Vaccinia Virus.* (23641)

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Kato *et al.*(1) presented evidence that ectromelia virus can propagate in the Ehrlich ascites tumor and in the Yoshida sarcoma cells, forming inclusion bodies of two kinds. Kamahora *et al.*(2) found that the fowl pox virus also produces 2 kinds of inclusion bodies, one of which corresponds to the Bollinger body, and the other, newly discovered by them, is similar to the "B" type body of the ectromelia virus. They stated that the "B" type body plays the chief role in the process of multiplication of these viruses, and apparently is the site providing the nucleoproteins needed for the synthesis of the viral elementary bodies. The Marchal and Bollinger bodies, which had been considered by other investigators as virus colonies, are merely reservoirs of mature virus. They noticed that the "B" type bodies are similar to the Guarnieri bodies observed in Giemsa-stained smear preparations of tissue infected with vaccinia virus.

In the present study, the vaccinia virus inclusion body was investigated in an attempt to establish the nature of the Guarnieri body.

Materials and methods. The refined vaccinia virus vaccine made in the Research Institute for Microbial Diseases, Osaka University, was used throughout this investigation. Adult rabbits and incubating eggs (12-day-old) were used as hosts of the virus. In the case of the rabbits the vaccine ID_{50} titer 6, mixed with penicillin, was pasted on the cor-

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