THE CLOTTING BEHAVIOR OF HUMAN "PLATELET-FREE" PLASMA: EVIDENCE FOR THE EXISTENCE OF A "PLASMA THROMBOPLASTIN"

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(Received for publication October 8, 1948)

The precise role of platelets in the coagulation of blood has long been a matter of debate. According to the classical theory of blood coagulation, platelets are the most important source of the thromboplastic substance necessary to convert prothrombin to thrombin (1). On the other hand Lenggenhager (2, 3) believes that platelets play no part in the initiation of coagulation. It is a well-known fact that patients with thrombocytopenia rarely have defective blood coagulation as measured by the usual laboratory tests. Aggeler and his associates (4) in a recent study of 404 patients found no statistically significant correlation between platelet counts and the clotting times of whole blood. While in most cases of thrombocytopenia the clotting time of whole blood is normal, there may be a diminished consumption of prothrombin during clotting (5). Quick (6) has shown that there is a decreased consumption of prothrombin during the clotting of plasma in which the platelet concentration has been reduced by centrifugation.

Studies on the function of platelets in coagulation have been hampered by the difficulty of rendering plasma to which no anticoagulant has been added platelet-free before spontaneous coagulation occurs. However, the addition of anticoagulants has not simplified the problem. Quick (7) has called attention to the acceleration of clotting produced by decalcification and subsequent recalcification, presumably as a result of physico-chemical alterations in which platelet rupture may play a major part.

Numerous studies have been undertaken using decalcified blood. Eagle (8) has shown that the clotting time of oxalated plasma upon recalcification varies inversely with the concentration of platelets. Cramer and Pringle (9) and Eagle (8) found that oxalated plasmas after Berkefeld filtration were incoagulable upon recalcification. However, in view of our present knowledge it seems quite likely that the prothrombin was removed in the process of filtration.

Brinkhous (10) studied the recalcified plasma clotting time of citrated human plasma collected in silicone-treated apparatus. After 22½ hours of centrifugation at 14,000 RPM (about 14,000 g), this plasma was incoagulable upon recalcification. Centrifugation for 165 minutes failed to produce incoagulable plasma. This difference in clotting behavior was presumed to be due to the complete removal of platelets after the prolonged centrifugation. The author concluded that the clotting of normal plasma depends on the presence of formed elements. No platelet counts were recorded. In our experience platelets can be completely removed at 14,000 g in much less time than 165 minutes. In Brinkhous' experiment the failure of the plasma to clot might well be explained by changes not related to platelets.

A number of attempts have been made to prepare platelet-free plasma without anticoagulants. Bordet and Gengou (11, 12) collected blood in paraffin-coated vessels and removed the formed elements by centrifugation. The supernatant plasma was found to clot promptly when placed in glass tubes, but the clotting time in paraffined tubes was greatly prolonged. These authors believed that initiation of coagulation was brought about by a physico-chemical change induced in platelet-free plasma by contact with glass surfaces. Whether or not their plasmas were actually platelet-free cannot be determined.

Fuchs (13, 14) on the other hand reported the preparation of a "Stable Plasma" without the use of anticoagulants by means of special venipuncture technique, paraffin-lined apparatus, low temperatures and rapidly attained high-speed centrifugation. Although the characteristics of this "Stable Plasma" were not clearly described, Fuchs stated

that it was spontaneously incoagulable in glass and paraffined tubes. Feissly (15) and Smith, Warner and Brinkhous (16) have been unable to confirm Fuchs' observations using similar techniques.

Jaques and his co-workers (17) have introduced into blood coagulation studies a method for coating laboratory apparatus with a non-wettable surface of silicone.[1] With this apparatus it is possible to obtain a fluid plasma free of platelets without the use of anticoagulants. Jaques and his associates presented experimental data to show that as the number of platelets is reduced by centrifugation the spontaneous clotting time of this native (undecalcified) plasma becomes longer. However, their lowest reported platelet counts were in the neighborhood of 2500 per cmm., a level not approaching platelet-free. These authors believe that in silicone-coated containers the initiation of coagulation is independent of platelet action.

Quick (6) using silicone-treated apparatus found a decreased consumption of prothrombin during the process of clotting when native plasmas were subjected to "high-speed" centrifugation (3500 RPM for seven minutes) as compared to "low-speed" centrifugation. This finding was attributed to the removal of all but a few of the platelets. However, no platelet counts were recorded. Quick did not obtain spontaneously incoagulable plasmas, but he believed it highly probable that with removal of all the platelets before any disintegration occurs, a spontaneously incoagulable plasma could be obtained.

Patton, Ware and Seegers (18) studied the clotting behavior of normal dog plasma by means of silicone-treated apparatus and high-speed centrifugation. They were unable regularly to obtain spontaneously incoagulable plasma, and found that platelet-free plasma clotted as promptly in silicone-treated as in glass tubes. For this reason they concluded that there was no evidence for the view that plasma contains a soluble factor which can initiate clotting independent of platelet action. In view of the high-speed centrifugation employed (22,000–23,000 RPM) it seems possible that the plasma thromboplastic protein described by Chargaff and West (19) could have been removed in these experiments.

The purpose of our studies was to determine the clotting behavior of human platelet-free plasma prepared by the use of silicone-treated apparatus and high-speed centrifugation.

[1] General Electric Dri-Film 9987.

METHODS

Preparation of "platelet-free" plasma: Needles, syringes, test-tubes and pipets were treated with silicone after the method described by Jaques (17). When drawing blood, in order to avoid contamination with tissue juice, we employed a multiple syringe technique, discarding the contents of the first syringe. Thirty to 40 ml. of blood from the second syringe were carefully placed in iced silicone-treated tubes and centrifuged at 7000 RPM (about 6000 g) at 4° C for five minutes to remove the cells and most of the platelets. The upper portion of the plasma was removed with a silicone-treated pipet and re-centrifuged at 12,000 to 14,000 RPM (17,500–22,000 g) for 10 minutes. Occasionally another centrifugation at this speed was performed. Thereafter the upper portion of this plasma was removed and stored in silicone-treated tubes in an ice bath. Normal plasma obtained in this manner remains fluid for at least several days at 4° C.

Clotting times: All clotting times were performed by a modified Lee-White three tube method at 37° C using scrupulously clean glass or silicone-treated tubes. With this technique normal individuals occasionally have a whole blood clotting time in glass tubes as long as 30 minutes, and the clotting time in silicone tubes may be much longer. For the sake of brevity only the clotting time in the third and most significant tube is recorded, although it is ordinarily wise to consider the results in all three tubes. Clotting tubes were observed for a minimum period of 24 hours.

Prothrombin times: Prothrombin times were determined by the one-stage method of Quick using a highly standardized rabbit-brain thromboplastin prepared according to the technique of Brambel (20). Since the native "platelet-free" plasma had not been decalcified, in the prothrombin estimations on this plasma 0.1 ml. of physiological saline was used in place of 0.1 ml. of M/40 calcium chloride.

Anticoagulant assays: Tests for the presence of circulating anticoagulants were performed by adding freshly drawn normal blood to the subject's "platelet-free" plasma, and noting the effect on the clotting time of the former. Details on the use of this method and its sensitivity to heparin have been reported (21).

Platelet counts: Platelet counts on whole blood were performed by a routine method employing Rees-Ecker diluent. Normal values in our laboratory are in excess of 200,000 per cmm. Platelet counts on "platelet-free" plasma were performed by introducing the undiluted plasma into a Spencer "Bright-Line" counting chamber. The use of silicone-treated cover-slips delayed clotting 30 minutes or more, enabling one to complete accurate counts. By using the fine adjustment of the microscope the entire area between the cover-slip and chamber was visualized so that platelets which had not settled to the counting chamber surface could be counted. All refractile bodies resembling platelets in any manner whatsoever

were counted, so that reported values are maximal and correct values are probably somewhat lower.

Preparation of plasmas with varying platelet concentrations: In order to study further the role of platelets in clotting, different specimens of the same native plasma were rendered "platelet-free" by high-speed centrifugation and platelet-rich by very low speed centrifugation. Platelet counts were performed, and the two plasmas then mixed in varying proportions.

RESULTS

Eighty-six human subjects were studied. Of these, 41 were normal individuals and the remainder were patients with various diseases.

NORMALS

Studies of the clotting behavior of normal "platelet-free" plasma correlated with the whole

TABLE I

The clotting behavior of normal "platelet-free" and "platelet-poor" plasmas correlated with the whole blood clotting times and prothrombin times of oxalated plasmas

Subject	Whole blood clotting times in glass tubes	Prothrombin times of oxalated plasma	Native "platelet-free" plasma			
			Platelets	Glass tube clotting times	Silicone tube clotting times	Prothrombin times
	min.	*sec.*	*per cmm.*	*min.*	*min.*	*sec.*
1	11	—	60	13	—	—
2	10	—	60	15	—	—
3	16	17	20	77	—	—
			20	31	—	—
4	14	17	20	29	—	—
5	21	15	20	74	—	—
6	15	15	>1000	14	—	—
7	17	16	20	12	—	—
8	24	16	96	35	—	15
9	29	17	55	42	—	16
10	23	18	500	53	—	14
11	27	16	230	27	—	13
12	10	—	None	9	—	14
13	14	17	None	17	—	15
14	15	15	40	34	—	11
15	18	—	200	24	—	13
16	23	18	12	20	—	16
17	17	21	8	—	23	18
18	24	—	3	—	45	—
19	17	—	>1000	24	34	—
20	14	—	None	19	60	—
			9	24	34	—
21	12	—	4	31	75	—
			None	36	126	—
22	24	22	None	48	183	20
			None	41	75	23
23	11	18	None	14	16	19
24	14	19	3	35	46	19
25	15	18	50	15	15	17
26	19	—	21	8	10	—
27	32	19	11	140	>210	17
28	19	19	8	26	28	19
29	17	22	6	47	>70	19
30	19	—	16	14	30*	18
31	16	19	2	26	47*	20
32	22	21	11	62	320*	—
33	22	21	10	39	No clots 72 hrs.*	18
34	21	—	13	60	>130*	23
35	17	21	1	42	140*	20
36	15	21	1	17	No clots 48 hrs.*	19
37	17	20	3	54	No clots 72 hrs.*	21
38	19	18	5	47	165*	19
39	18	18	1	70	No clots 24 hrs.*	23
40	16	16	3	27	63*	18
41	22	16	4	33	Partial clot 24 hrs.*	—

Note: All determinations on each subject were made on a specimen obtained from the same venipuncture. All procedures were carried out at 37° C.

* In these experiments the improved technique of re-coating the silicone-treated tubes before each use was employed.

blood clotting time in glass tubes and the prothrombin time of oxalated plasma are shown in Table I.

We abandoned the routine use of silicone-treated tubes for determination of the whole blood clotting time, since the results were too variable, poor clots were formed, and it was difficult to recognize end-points. The clotting time of whole blood in silicone-treated tubes seems to be more a measure of imperfection in technique than of the inherent properties of the blood. Theoretically blood should not clot in silicone-treated tubes if technique were perfect.

It was not always possible to obtain a plasma entirely free of refractile bodies even when repeated high-speed centrifugations were used. It seemed unlikely that all of these refractile bodies were platelets, although they must be presumed to be and were recorded as such. In some instances platelets appeared to be completely absent. To emphasize the extremely small number of platelets remaining after centrifugation we have called these plasmas "platelet-free" in contrast to the "deplateletized" plasmas of Tocantins (22) which contained as many as 20,000 platelets per cmm. Furthermore, we found no difference in behavior of plasmas containing 0 to 100 platelets per cmm.

Normal "platelet-free" plasmas invariably clotted in glass tubes at 37° C in a relatively short time. There was considerable variation in the time required for this clotting. In most instances, however, the clotting time of normal "platelet-free" plasma in glass tubes was longer than that of the whole blood from which it was derived. Even with careful technique it was rarely possible to demonstrate a perfect correlation between platelet counts and clotting times (Table II). With platelet counts under 1000 per cmm. there appeared to be no correlation between platelets and clotting time. Completely platelet-free plasmas did not have clotting times longer than plasmas containing a few platelets.

TABLE II

The relationship of the spontaneous clotting times of native (undecalcified) plasma to the number of platelets present

Platelets (*per cmm.*)	6	28,000	56,000	140,000	280,000	420,000	560,000
Clotting time in glass tubes at 37° C. (*in minutes*)	48	33	24	19	15	9	11

Note: Normal native "platelet-free" and "platelet-rich" plasmas were obtained from pooled sources by high- and low-speed centrifugations respectively. Platelet counts were performed, and the plasmas mixed in varying proportions.

Clotting of normal platelet-free plasma in glass tubes could be prevented by the addition of sodium oxalate. This indicates that thrombin did not exist in the platelet-free plasma prior to its introduction into glass tubes. As a matter of fact, no thrombin was demonstrable until immediately prior to the onset of visible clotting.

In our early experiments we did not recoat the silicone-treated clotting tubes before each use. Under these conditions the clotting times of "platelet-free" plasmas in silicone-treated tubes were not always significantly longer than the clotting times in glass tubes. When freshly prepared silicone-treated tubes were used, a significant difference was constantly observed. In fact, in four cases (cases Nos. 33, 36, 37, 39) *the plasmas formed no clots in silicone-treated tubes at 37° C.* In two of these there was a very slight precipitation of some amorphous material but no true clot formation. In the other two cases, although the plasmas became slightly cloudy after several hours, there was not the slightest evidence of precipitation or fibrin formation. It seems possible that with constantly perfect technique one could regularly obtain platelet-free plasmas spontaneously incoagulable in silicone-treated tubes at 37° C.

When thromboplastin was added to these "platelet-free" plasmas, prompt clotting occurred in glass or silicone-treated tubes. The prothrombin times of these plasmas were almost the same as the prothrombin times of oxalated plasmas obtained from the same venipuncture. Native "platelet-free" plasmas maintained their prothrombin activity as measured by the one-stage method when they were stored in a frozen state, whereas the prothrombin times of frozen oxalated plasmas became a few seconds longer. Freezing and thawing of "platelet-free" plasma in silicone-treated tubes resulted in shortened clotting times.

The mechanism of clotting of "platelet-free" plasma was observed in Spencer "Bright-Line" counting chambers. Clotting occurred first on the cover-slip and not until later on the silvered counting chamber surface. Clotting definitely did not

begin around platelets when they were present. As clotting occurred small refractile structures appeared at the crossings of the fibers. Although no platelets may have been present before clotting occurred, thousands of these refractile structures could be seen at the fiber crossings once clotting began. If a few platelets were present, these were seen to settle to the chamber surface and disappear only after clotting was well under way.

By altering the platelet concentrations of plasmas we were able to demonstrate that the degree and speed of clot retraction varied directly with the number of platelets present. This correlation was poor and irregular in glass tubes but excellent in silicone-treated tubes. Clot retraction occurred promptly with platelet-counts as low as 20,000 per cmm. in silicone-treated but not in glass tubes. The clots of platelet-free plasmas did not retract either in glass or in silicone-treated tubes.

The dynamics of the coagulation of platelet-free plasma in glass tubes was studied by determining the time of thrombin appearance, fibrinogen utilization, and prothrombin consumption. Results of a representative experiment are shown in Table III. In general there was minimal prothrombin consumption even over a period of several hours.

In order to investigate a possible quantitative relationship between platelet concentration and the amount of prothrombin converted in the process of clotting, prothrombin times of the residual serum after clotting were determined using plasmas containing varying numbers of platelets. The results in Table IV show a striking correlation even at the higher platelet levels when glass tubes were used. In silicone-treated tubes prothrombin consumption was minimal regardless of the number of platelets present.

High-speed centrifugation did not prolong the prothrombin time of native plasma when rabbit brain extract was used as a source of thromboplastin. However, prothrombin times with Russell's viper venom [2] were greatly prolonged by high-speed centrifugation. Similar observations on oxalated plasmas were reported by Macfarlane (23).

Plasmas from fasting individuals gave the best results in our experiments. In grossly fatty plas-

[2] Stypven, Wellcome Physiological Research Laboratories, Beckenham, England.

TABLE III

The dynamics of the coagulation of normal platelet-free plasma: tests for the rate of thrombin formation, fibrinogen utilization, and prothrombin consumption

Time after plasma transferred to glass tube at 37° C.	Gross appearance of plasma	*Assay for thrombin* Clotting time of the mixture: 0.2 ml. oxalated plasma* 0.2 ml. platelet-free plasma	*Assay for fibrinogen* Clotting time of the mixture: 0.2 ml. thrombin† solution 0.2 ml. platelet-free plasma	*Assay for prothrombin* Clotting time of the mixture: 0.1 ml. thromboplastin 0.1 ml. prothrombin-free plasma‡ 0.1 ml. platelet-free plasma
min.		*sec.*	*sec.*	*sec.*
0	Fluid	No clots	7	16
1	Fluid	No clots	—	—
5	Fluid	No clots	—	—
10	Fluid	No clots	—	—
13	Small fiber	No clots	—	—
15	Definite fibrin formation	420	10	—
17	Solid clot	240	7	—
20	(Clot compressed to remove "plasma")	75	11	—
23	Re-appearance of solid clot	60	15	—
24	—	—	—	16
28	—	74	No clots	—
31	—	85	No clots	—
35	—	120	No clots	17
45	—	No clots	No clots	—
55	—	No clots	No clots	15
75	—	No clots	—	15
135	—	—	—	16

* Eight parts prothrombin-free plasma (barium sulfate adsorption) and 2 parts M/10 sodium oxalate.
† Thrombin, Upjohn Company, Kalamazoo, Michigan.
‡ Three parts prothrombin-free plasma (barium sulfate adsorption) and one part of M/40 calcium chloride.

TABLE IV

The relationship of prothrombin consumption in the clotting of native plasma in glass tubes to the number of platelets present, as measured by the determination of prothrombin times on serum expressed from the clots four hours after clotting

Platelets (*per cmm.*)	6	175,000	262,500	350,000
Clotting times in glass tubes at 37° C. (*min.*)	14	10	7	10
Prothrombin time of expressed serum 4 hours after clotting (*sec.*)	20	63	80	141

The plasmas with various platelet concentrations were prepared by mixing platelet-free and platelet-rich plasmas from the same source.

The prothrombin times were determined by adding 0.1 cc. of the expressed serum to a mixture of 0.1cc. thromboplastin and 0.1 cc. prothrombin-free plasma at 37° C. and measuring the time required for clotting.

mas coagulation often occurred in the lipoid layer at the top of the tube after centrifugation.

Hemophilia

Seven patients with hemophilia were studied. Results are shown in Table V. In every instance the "platelet-free" plasma was spontaneously incoagulable in glass and silicone-treated tubes at 37° C regardless of the length of the whole blood clotting time. No trace of fibrin was noted in any of the tubes even after a period of days or weeks. Yet the addition of rabbit brain thromboplastin produced prompt clotting, with prothrombin times comparable to those of oxalated plasmas. When hemophilic blood was centrifuged in lusteroid tubes untreated with silicone, the resultant platelet-free plasma clotted. The silicone surface therefore appeared to be necessary for the preparation of spontaneously incoagulable hemophilic platelet-free plasma. When whole blood clotting times on hemophilic blood were performed in silicone-treated tubes, the clotting times were tremendously prolonged and in some instances no clotting occurred regardless of the clotting time in glass tubes.

One untreated hemophilic patient (Case No. 42) had a normal whole blood clotting time ranging from 12 to 25 minutes during an active bleeding episode. A platelet-rich plasma obtained by low-speed centrifugation had approximately the same clotting time. However, the "platelet-free" plasma was spontaneously incoagulable in glass and silicone tubes. Thus it appears that platelets are necessary for the clotting of hemophilic plasma, although our present results are compatible with the

TABLE V

The clotting behavior of hemophilic "platelet-free" plasma

Patient	Whole blood clotting times in glass tubes	Prothrombin times of oxalated plasma	Native "platelet-free" plasma			
			Platelets	Glass tube clotting times	Silicone tube clotting times	Prothrombin times
	min.	*sec.*	*per cmm.*	*min.*	*min.*	*sec.*
42	25	—	None	No clots	—	14
	16		>1000	No clots	—	—
43*	225	15	60	No clots	—	—
	390	21	20	No clots	—	—
	300	21	20	No clots	—	15
44	325	19	60	No clots	—	19
45	600	21	1700	No clots	—	20
			None	No clots	—	20
46	33	19	290	No clots	—	17
	46	22	30	No clots	—	—
	74	—	18	—	No clots	—
47	240	—	None	No clots	No clots	—
			None	No clots	No clots	18
48	212	21	None	No clots	No clots	22
	180	23	70	No clots	No clots	24

* This patient had a positive test for the presence of circulating anticoagulant.

view that they are not necessary for the initiation of coagulation of normal plasma.

The hemophilic "platelet-free" plasmas were tested for antithromboplastic properties by determining prothrombin times with progressively diluted thromboplastin solutions. For this we used silicone-treated tubes and native "platelet-free" plasma. The results shown in Table VI reveal no definite evidence for the presence of antithromboplastic activity in hemophilic plasma. In one experiment the native plasmas were first oxalated, then incubated several minutes with thromboplastin solutions and finally recalcified. Results were the same in this experiment. Variations in the clotting times obtained with highly diluted thromboplastin solutions must be interpreted with great caution since under such circumstances it is technically difficult to reduplicate results.

Heparinized blood

"Platelet-free" plasmas were obtained from normal individuals after the intravenous injection of small quantities of heparin. In other experiments heparin in small amounts was added *in vitro* to normal "platelet-free" plasma. Results shown in Table VII demonstrate that even after minute doses of heparin, so small that the whole blood clotting times were not affected, the "platelet-free" plasmas would not clot in glass or silicone-treated tubes at 37° C. The *in vitro* addition of minute amounts of heparin likewise resulted in the production of incoagulable plasma. The authors (24) have previously shown that there is an inverse relationship between the action of heparin and platelet concentration. With low concentrations of heparin, prothrombin determinations on the "platelet-free" plasmas gave normal results.

Dicumarolized patients

Five patients with hypoprothrombinemia due to dicumarol were studied. Results are shown in Table VIII. While the findings are somewhat variable, the "platelet-free" plasmas of these patients were often spontaneously incoagulable in glass or silicone-treated tubes at 37° C. On two occasions these plasmas gave suggestively positive tests for the presence of circulating anticoagulant. The addition of solutions of thrombin to oxalated plasmas of dicumarolized patients resulted in longer clotting times than the addition of the same thrombin solution to normal plasmas. Further studies are in progress regarding this finding.

Thrombocytopenia

Studies were performed on 12 patients with hemorrhagic diathesis associated with thrombo-

TABLE VI

Tests for the presence of antithromboplastic activity in native "platelet-free" hemophilic plasma
Prothrombin times were determined using serial dilutions of thromboplastin.

	Prothrombin times (*in seconds*) using serial dilutions of thromboplastin										
	Undiluted	1:10	1:50	1:100	1:200	1:400	1:600	1:800	1:1600	1:3200	Saline
Patient No. 43*	15	21	65	170	307	416	—	—	—	—	—
Control	15	19	56	108	240	320	—	—	—	—	—
Patient No. 48	16	24	—	49	65	109	—	260	345	—	>24 hrs.
Control	16	21	—	40	57	135	—	151	205	—	660
Patient No. 46	19	22	35	50	62	83	93	133	—	—	—
Control	17	21	32	44	59	77	91	112	—	—	—
Patient No. 46	17	20	29	36	51	75	—	—	—	—	—
Control	15	19	30	37	50	—	—	—	—	—	—
Patient No. 46†	22	22	32	42	64	73	96	116	165	290	—
Control	20	22	33	44	60	64	78	94	161	325	—

* This patient had a positive test for circulating anticoagulant.

† In this experiment the native "platelet-free" plasma was first oxalated by the addition of 10% by volume of M/10 sodium oxalate. 0.1 ml. of this plasma was added to 0.1 ml. of thromboplastin. After several minutes of incubation at 37° C., this mixture was recalcified by the addition of 0.1 ml. of M/40 calcium chloride solution.

Note: All tests performed at 37° C.

TABLE VII

The clotting behavior of "platelet-free" plasmas obtained from normal subjects after the intravenous injection of varying amounts of heparin as indicated

Subject		Whole blood clotting times in glass tubes	Prothrombin time of oxalated plasma	Native "platelet-free" plasma			
				Platelets	Glass tube clotting times	Silicone tube clotting times	Prothrombin times
		min.	*sec.*	*per cmm.*	*min.*	*min.*	*sec.*
49	41 minutes after intravenous injection of 50 mgms. of heparin	102	—	230	No clots	—	19
50	40 minutes after intravenous injection of 15 mgms. of heparin	105	16	None	No clots	—	15
51	30 minutes after intravenous injection of 10 mgms. of heparin	27	21	36	No clots	No clots	18
52	16 minutes after intravenous injection of 10 mgms. of heparin	31	18	3	No clots	No clots	19
53	12 minutes after intravenous injection of 7.5 mgms. of heparin	19	17	3	No clots	No clots	24
54	18 minutes after intravenous injection of 5 mgms. of heparin	30	19	7	No clots	No clots	18
55	12 minutes after intravenous injection of 5 mgms. of heparin	17	21	2	No clots	No clots	19
56	16 minutes after intravenous injection of 3 mgms. of heparin	20	18	24	No clots	No clots	16
57	16 minutes after intravenous injection of 3 mgms. of heparin	17	22	16	No clots	No clots	19
58	*In vitro* dilutions of "platelet-free" plasma to:						
	0.01 mgms. heparin/cc.	—	—	5	No clots	No clots	18
	0.001 mgm. heparin/cc.	—	—	5	No clots	No clots	—
	0.0005 mgm. heparin/cc.	—	—	5	No clots	No clots	—

Note: In the case of Subject No. 58 the "platelet-free" plasma was obtained first, and then varying amounts of heparin added *in vitro* to the final concentrations indicated.

cytopenia (Table IX). Three of these patients had marked prolongation of their whole blood clotting times and in each of these instances the "platelet-free" plasmas were spontaneously incoagulable in glass and silicone-treated tubes. Eight patients with essentially normal whole blood clotting times had "platelet-free" plasmas which clotted in glass tubes in a perfectly normal fashion. In all of our cases of thrombocytopenia a sensitive test for circulating anticoagulant was negative. In order to eliminate the possibility that failure of the three "platelet-free" plasmas to clot in glass tubes could be attributed to the presence of anticoagulant in quantities too small to be detected by our routine anticoagulant assay, a further study was carried out in one case. Spontaneously incoagulable "platelet-free" plasma from patient No. 73 was added to normal "platelet-free" plasma in glass tubes. Five tenths ml. of the patient's plasma failed to prevent coagulation of 1.0 ml. of normal "platelet-free" plasma, conclusively demonstrating the absence of anticoagulant.

TABLE VIII

The clotting behavior of "platelet-free" plasma from patients with hypoprothrombinemia due to dicumarol

Patient	Whole blood clotting time in glass tubes	Prothrombin time of oxalated plasma	"Platelet-free plasma"			
			Platelets	Glass tube clotting time	Silicone tube clotting time	Prothrombin time
	(*min.*)	(*sec.*)	(*per cmm.*)	(*min.*)	(*min.*)	(*sec.*)
59	32	40	5	40	45	44
			1	44	No clots	45
60	20	107	10	<800	<800	128
61	41	52	2	No clots	No clots	51
			1	No clots	No clots	52
	26	53	11	No clots	No clots	44
			2	No clots	No clots	43
	18	34	60	35	No clots	42
62	55	27	None	No clots	No clots	24
	15	23	4	No clots	No clots	23
63	55	117	8	No clots	No clots	109

All of the determinations were made at 37° C.

Miscellaneous patients

Table IX shows the results of studies on a group of patients with hemorrhagic diathesis associated with a variety of conditions. Detailed studies on the two patients with "undiagnosed disease due to circulating anticoagulant" have already been reported (25).

The patients with liver disease were of great interest. The two (Cases Nos. 81, 82) whose "platelet-free" plasma did not clot had positive tests for circulating anticoagulant as well as a severe hypoprothrombinemia. Furthermore, the clotting times of their plasmas after the addition of a thrombin solution were tremendously prolonged as compared to the clotting times of normal plasmas, although the fibrinogen concentrations were adequate. We therefore believe that these patients had circulating antithrombic anticoagulants. The rate of fibrinolysis of the sterile recalcified plasma clot was determined in these two cases by Dr. Oscar Ratnoff. The clot lysis time of patient No. 81 was normal, but that of patient No. 82 was shortened. There was no evidence that the coagulation defect was caused by increased proteolytic activity of the serum. Many patients with more markedly increased proteolytic activity of the serum showed no abnormality of blood coagulation.

In view of the reported occurrence of circulating heparin-like anticoagulant after treatment of Hodgkin's disease with nitrogen mustards [3] (26), three such patients were studied. None of these patients developed hemorrhagic diathesis and tests for circulating anticoagulant were negative. Their "platelet-free" plasmas were similar to those of normal individuals (Table X).

DISCUSSION

The use of silicone-treated apparatus has made possible the satisfactory preparation of a "platelet-free" plasma without the use of anticoagulants. Employing this technique we have been able to study the clotting behavior of human plasma. The most significant finding has been that *normal "platelet-free" plasma clots in a relatively short time in glass tubes at 37° C, but its clotting time in silicone-treated tubes is markedly prolonged and sometimes there is no clotting at all.* This observation suggests that contact with glass activates some plasma constituent which can initiate clotting. This factor is apparently activated slowly or not at all by contact with silicone-treated surfaces. We believe that with constantly perfect technique, platelet-free plasma would regularly be spontaneously incoagulable in silicone-treated tubes.

We have no information concerning the origin and nature of this plasma factor. It is quite possible that during the manipulations involved in our experiments a few platelets may have been broken up. However, if active thromboplastic substance were liberated in this manner, it seems reasonable to assume that the clotting time of platelet-free plasma would be the same in silicone-treated tubes as in glass tubes. There is no evidence that a silicone surface itself interferes with the clotting process, for on addition of highly diluted thromboplastin to platelet-free plasma, clotting occurs as promptly in silicone-treated tubes as in glass tubes. We are therefore unable to escape the conclusion that an inactive thromboplastin precursor in plasma is activated on contact with glass surfaces.

The existence of a plasma thromboplastin independent of platelets has been postulated by some workers (2, 3). Howell (27) believed that a continuous destruction of platelets *in vivo* led to the

[3] Methyl-bis-(β-chloroethyl) amine hydrochloride, Merck & Co., Inc.

TABLE IX

The clotting behavior of "platelet-free" plasma from patients suffering from hemorrhagic diathesis due to a variety of conditions

	Subject	Whole blood platelets (*per cmm.*)	Whole blood clotting times in glass tubes (*min.*)	Prothrombin time of oxalated plasma (*sec.*)	Native "platelet-free" plasma			
					Platelets (*per cmm.*)	Glass tube clotting times (*min.*)	Silicone tube clotting times (*min.*)	Prothrombin times (*sec.*)
I. Hemorrhagic diathesis with thrombocytopenia								
Acute leukemia	64	18,000	36 hrs.	—	None	No clots	No clots	20
	65	44,000	19	22	9	27	32	20
		22,000	9	25	None	11	22	23
	66	12,000	88	22	None	No clots	No clots	21
	67	26,000	7	23	3	18	160	26
	68	22,000	27	23	2	180	14 hrs.	24
Idiopathic thrombocytopenic purpura	69	30,000	15	21	1	27	63	21
	70	40,000	8	18	6	15	23	17
	71	20,000	13	21	2	24	50	23
	72	32,000	31	20	4	24	37	21
Refractory anemia with thrombocytopenia	73	36,000	93	24	13	No clots	No clots	23
	74	90,000	25	21	11	90	No clots	18
					1	90	18 hrs.	18
Marchiafava-Micheli syndrome	75	68,000	29	—	3	No clots	No clots	18
II. Hemorrhagic diathesis without thrombocytopenia								
Non-thrombocytopenic purpura	76	200,000	14	18	5	11	11	18
Atypical hemorrhagic diathesis	77	226,000	21	21	13	60	No clots	22
Multiple myeloma	78	—	43	26	None	26	24	—
Undiagnosed disease with circulating anticoagulant	79†	—	120	30	None	No clots	No clots	24
	80†	—	68	20	—	No clots	No clots	15
III. Hypoprothrombinemia due to fatal liver disease								
	81†	—	109	54 (10%)*	10	No clots	No clots	54
	82†	—	24 hrs.	87 (5%)*	1000	No clots	No clots	—
	83	—	22	187 (<5%)*	45	55	78	155
					7	60	130	—

* Estimated percentage of normal prothrombin.
† These patients had positive assays for circulating anticoagulant.
Note: Patient No. 83 was not suffering from hemorrhagic diathesis, but is included for comparison with patients Nos. 81 and 82.

formation of a plasma thromboplastin, as was previously suggested by Morawitz (1). Lozner and Taylor (28) maintain that the effect of foreign surfaces such as glass is not due to lysis of platelets but rather to activation of a plasma thromboplastin. Owren (29) has presented data to refute Lenggenhager's (2, 3) conclusions concerning the existence of a plasma thromboplastic factor and considers that no substantial evidence has been presented. On the other hand, Chargaff and West (19) by ultracentrifugation of platelet-free plasma have obtained a sediment with potent thromboplastic properties. Quick (6) and Brinkhous (10) using techniques similar to those which we have employed have concluded that normal plasma contains a soluble factor whose interaction with platelets is necessary for normal blood coagulation. However, these authors do not believe

TABLE X

The clotting behavior of "platelet-free" plasma from patients with Hodgkin's disease treated with nitrogen mustards

Subject	Whole blood clotting times in glass tubes	Prothrombin time of oxalated plasma	Native "platelet-free" plasma				Time of determination in relation to treatment with nitrogen mustards*
			Platelets	Glass tube clotting times	Silicone tube clotting times	Prothrombin times	
	min.	*sec.*	*per cmm.*	*min.*	*min.*	*sec.*	
84	22	20	—	—	—	—	Before
	11	20	3	16	19†	19	One day after
	18	18	15	18	62	17	11 days after
	19	19	3	19	36	22	4 weeks after
85	16	24	4	52	No clots	30	Before
	27	22	5	37	No clots	21	One week after
	26	25	1	Partial clot in 24 hrs.	Partial clot in 24 hrs.	22	2 weeks after
86	14	31	6	25	60	30	During
	20	25	2	54	No clots	26	One week after
	31	26	None	180	No clots	25	2 weeks after

* Methyl-bis (β-chloroethyl) amine hydrochloride, Merck & Co., 0.1 mgm. per kilogram body weight on four successive days.

† Except for this experiment the improved technique of re-coating the silicone-treated tubes before each use was employed.

Note: All determinations were performed at 37° C.

that the plasma factor can initiate clotting in the absence of platelets.

Recent discovery of several patients with a curious type of circulating anticoagulant (25, 30) lends strong support to the theory of the existence of a plasma thromboplastin precursor. Although the plasmas of these patients contained a potent clotting inhibitor, they reacted in a perfectly normal way to highly diluted thromboplastin. It is apparent, therefore, that the action of the circulating anticoagulant in these cases preceded and in some way prevented the liberation of active thromboplastin. These observations can be explained only by assuming that the clotting inhibitor delays conversion of a thromboplastin precursor to an active thromboplastin.

While our studies show that platelets do not appear to be necessary for the initiation of clotting, it is clear that they increase the rate of clotting and the amount of prothrombin consumed in the process. There is a close correlation between the number of platelets present and the amount of prothrombin converted during clotting in glass tubes. Other observers (5, 6) have previously noted that in thrombocytopenic blood little prothrombin is consumed during clotting. The prothrombin consumed in the coagulation of platelet-free plasma is minimal.

Hemophilic platelet-free plasmas were invariably spontaneously incoagulable in glass tubes at 37° C although they clotted promptly on the addition of thromboplastin. This suggests that the defect in hemophilia is a deficiency of the plasma thromboplastic factor. It is apparent that hemophilia is not caused by any defect in platelets, but rather that the presence of platelets is what makes hemophilic blood clot at all. Even when an untreated hemophilic patient (*e.g.* Case No. 42) has a normal whole blood clotting time, the hemorrhagic diathesis may persist. This indicates that the plasma thromboplastin is necessary for normal hemostasis regardless of the clotting time of the blood *in vitro*. Presumably the "antihemophilic globulin" (31) is identical with the plasma thromboplastin precursor.

Whether or not the plasma thromboplastic factor is totally absent from hemophilic blood has not been established. Lenggenhager (32) believed that it was present in hemophilia but abnormally resistant to activation. Craddock and Lawrence (33) have presented data to indicate that "antihemophilic globulin" may be antigenic in hemophilic subjects. If their observations are correct, it must be presumed that hemophiliacs are entirely devoid of this plasma component.

Quick (34) observed that high-speed centrifugation of oxalated hemophilic plasma caused marked prolongation of the recalcified clotting time.

Quick originally believed that platelets were abnormally resistant in hemophilia, and that they could therefore be removed by centrifugation before thromboplastin was liberated. Rather, it seems to us that the results of Quick's experiment again demonstrate that platelets are critically necessary for the clotting of hemophilic plasma.

Hemophilic blood, presumably lacking in plasma thromboplastic factor, will clot if platelets are present, although clotting is delayed and incomplete. Likewise, normal plasma will clot in the absence of platelets, but in this instance also coagulation is incomplete. It appears that both the platelet and plasma factors are necessary for normal coagulation, although either one alone will suffice to initiate coagulation. The nature of the interaction between platelets and the plasma factor remains to be elucidated.

No evidence was obtained to support the theory that the primary defect in hemophilia is an increased antithromboplastic activity of the plasma. We were unable to demonstrate any antithromboplastic activity of the plasma of our hemophiliac patients even when a potent clotting inhibitor was present (Case No. 43).

Normal platelet-free plasma containing an exceedingly low concentration of heparin is spontaneously incoagulable at 37° C even though the prothrombin time upon the addition of thromboplastin is normal. The inverse relationship between heparin activity and platelet concentration has already been reported (24). Studies on the precise mode of action of heparin in platelet-free plasma will be published later.

Experiments on the plasmas of patients with hypoprothrombinemia due to dicumarol gave variable results. In most cases the "platelet-free" plasmas were spontaneously incoagulable in glass tubes. We are unable to account for this on the basis of hypoprothrombinemia alone. Two patients temporarily showed suggestive evidence of circulating anticoagulant. The oxalated plasmas of dicumarolized patients clotted more slowly on the addition of thrombin solution than did normal plasmas. Further studies will be necessary to explain these results.

The "platelet-free" plasmas of most of the patients with thrombocytopenia behaved in a normal manner. However, in each of three cases with prolonged whole blood clotting times the "platelet-free" plasma was spontaneously incoagulable. We are unable to explain the prolonged whole blood clotting time and associated failure of platelet-free plasma to clot in three cases. It is apparent that these deviations from normal were caused by a deficiency of a thromboplastic factor rather than by the presence of a clotting inhibitor. The numerical reduction of platelets alone is not sufficient to account for the coagulation defect, and we can only surmise that there must have been a deficiency also of a plasma thromboplastin. These plasmas could not be distinguished from those of the hemophiliacs. Other cases of thrombocytopenia with unaccountably prolonged whole blood clotting times have been reported (35).

Two of the three patients with fatal liver disease (Cases Nos. 81 and 82) had positive anticoagulant assays and their "platelet-free" plasmas did not clot. We have presented evidence that these patients had plasma antithrombic anticoagulants in addition to severe hypoprothrombinemia. By contrast, the "platelet-free" plasma of the third patient (Case No. 83) clotted in glass tubes in spite of a severe hypoprothrombinemia, and this patient had no evidence of circulating anticoagulant.

Three patients with Hodgkin's disease treated with nitrogen mustard showed no evidence of a disturbance of blood coagulation, and their "platelet-free" plasmas were similar to those of normal individuals. The heparin-like anticoagulant reported by Smith *et al.* (26) to occur following nitrogen mustard therapy was not demonstrable in our cases.

SUMMARY

1. By means of silicone-treated apparatus and high-speed centrifugation at low temperature it has been possible to prepare fluid platelet-free plasma without the use of anticoagulants.

2. Studies on the "platelet-free" plasmas of 86 human subjects with and without hemorrhagic diathesis are presented.

3. Evidence is submitted for the existence of a soluble plasma thromboplastin precursor which on contact with glass surfaces is converted to an active state. Both this plasma factor and platelets are necessary for normal coagulation, but clotting can be initiated by either alone.

4. The coagulation defect in hemophilia appears to be a deficiency of this plasma thromboplastin precursor.

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