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Research Article

SURFACE AS A QUANTITATIVE FACTOR IN PROTHROMBIN UTILIZATION [1]

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The accelerating effect of foreign surfaces on coagulation of whole blood has been recognized for many years. In 1863, Lister (1) was ". . . aware of the great influence exerted upon the blood by exposure for a very short time to a foreign solid . . . ," and concluded, "The real cause of the coagulation of the blood, when shed from the body, is the influence exerted upon it by ordinary matter. . . ." Since the original observations of Lister, many subsequent investigators have studied this phenomenon (2–15).

In the experiments to be described, the rate of prothrombin utilization was employed as a measure of the effect of foreign surface on the blood coagulation mechanism. Blood and plasma from normal human subjects and from patients with hemophilia and thrombocytopenia were exposed to differing glass surface areas, and prothrombin utilization was measured.

METHODS

Methods of obtaining and handling blood: Approximately 60 ml. of blood were drawn from the antecubital vein of human subjects using sterile, non-silicone treated 18 gauge needles and silicone [4] treated syringes lubricated with silicone oil.[5] In order to expose this blood to various areas of foreign surface, 2.0 ml. portions of the blood were measured in duplicate directly from the syringe into 13 × 100 mm. test tubes which had been prepared in different ways. Some tubes were silicone-treated, others were plain glass (*i.e.*, chemically clean, dry, "Pyrex" glass), and still others were plain glass tubes containing weighed amounts of crushed glass.[6] The silicone treated tubes were left undisturbed. Some of the plain glass tubes were left undisturbed, others were completely inverted 25 times with a parafilm [7] stopper by the use of a gentle rocking motion, and still others were vigorously shaken for two minutes with a 00 rubber stopper. The tubes containing crushed glass were vigorously shaken for two minutes with a 00 rubber stopper. All of the tubes were then incubated in a water bath maintained at 37° C. for measured periods of time. The time of incubation was considered to have begun when the blood was first seen entering the syringe. At the end of the desired period of incubation, coagulation was terminated by the addition of 0.2 ml. of M/10 sodium oxalate. The clot, if any, was completely broken and the tubes shaken. The supernatant plasma or serum was obtained by centrifugation at 2,500 rpm. for 5 to 10 minutes. The residual prothrombin was determined by the one-stage procedure, and the remaining plasma or serum was stored at −20° C. until analyzed by the two-stage method. To serve as a control, 4.5 ml. of the original blood specimen were mixed with 0.5 ml. of M/10 sodium oxalate immediately after withdrawal, and the plasma was obtained by centrifugation at 2,500 rpm. for 5 to 10 minutes. The one-stage prothrombin time was determined, and the remaining plasma was stored at −20° C. until analyzed by the two-stage procedure.

Preparation of plasma with varying platelet levels: Using silicone-treated syringes lubricated with silicone oil, blood was obtained from normal human subjects without the use of an anticoagulant. Plasma with varying platelet levels was prepared by differential centrifugation at low temperatures as previously described (16).

Two-stage prothrombin determinations were performed by a modification of the procedure of Ware and Seegers (17). This procedure was modified as follows: 1) M/10 sodium oxalate was used as an anticoagulant instead of 3.2 per cent sodium citrate; 2) the procedure was carried out at 37° C.; and 3) the fibrinogen used was prepared from human plasma by the cold precipitation method of Ware, Guest, and Seegers (18). This fibrinogen was further purified by modifying the method of

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[4] General Electric Company, Dri-film, SC–87 (formerly 9987).

[5] General Electric Company, Silicone Oil, SF–96 (200) (formerly 9996–200).

[6] Crushed glass was prepared from chemically clean, dry, "Pyrex" glass ground in a mortar and pestle. The crushed glass used had a particle size that passed a No. 50 mesh screen and that was retained by a No. 100 mesh screen.

[7] Parafilm grade "M", Marathon Corporation.

Laki (19). The additional purification consisted of washing the product with oxalated saline four to five times in the cold, dissolving at room temperature in oxalated saline, precipitating with 25 per cent saturated ammonium sulfate, re-dissolving in buffer at pH 7.3, dialyzing against barbital buffer at 5° C., filtering with pressure through a Seitz filter using 20 per cent asbestos pads, and storing at − 20° C. At the beginning of these experiments, 250 ml. of citrated plasma were obtained from a normal subject and stored in 2.0 ml. portions at − 20° C. This plasma served as a reference standard for the two-stage procedure, and daily checks for accuracy of this procedure were performed on this plasma. In these experiments appropriate correction was made for dilution of the plasma or serum with anticoagulant as determined by the hematocrit values.

Residual prothrombin by the two-stage procedure was determined without modification of the above procedure. It should be noted that when the residual prothrombin was less than 10 per cent of the control, accurate measurement of prothrombin could not be made by this technique.

One-stage prothrombin times were determined in duplicate at 37° C. by a modification of the procedure of Quick (20), using 0.1 ml. of the control oxalated plasma, 0.1 ml. of a standardized rabbit brain thromboplastin, and 0.1 ml. of M/10 calcium chloride. The normal range is 16 to 18 seconds.

Residual prothrombin by the one-stage procedure was determined in duplicate at 37° C. by a modification of the procedure of Quick and Favre-Gilly (21). The clotting time of a mixture of 0.1 ml. of the test oxalated plasma or serum, 0.1 ml. of barium sulfate adsorbed plasma from a normal subject, 0.1 ml. of a standardized rabbit brain thromboplastin, and 0.1 ml. of M/25 calcium chloride was determined. Prothrombin time of unclotted plasma using this technique did not differ significantly from the results of the one-stage prothrombin test described above.

The volume of packed cells, expressed in per cent, was determined in Wintrobe hematocrit tubes.

Platelet counts were performed by the phase contrast technique of Brecher and Cronkite (22) in which 1 per cent ammonium oxalate is used as the diluent. A dilution of 1:100 was used when the platelet level was approximately normal, whereas thrombocytopenic specimens were diluted 1:20. Platelet counts on specimens of platelet-poor plasma were performed by observing the undiluted plasma in the counting chamber using the phase contrast microscope.

Whole blood clotting times were determined by a modification of the method of Lee and White as previously described (23).

Tests for the detection of circulating anticoagulants were performed by a previously described method (24).

RESULTS

Effects of varying glass surface area on the rate of prothrombin utilization in whole blood

Normal blood: In 11 experiments on normal human subjects, blood was obtained by a single venipuncture and distributed into a series of duplicate tubes prepared to provide various areas of glass surface. These tubes were incubated at 37°

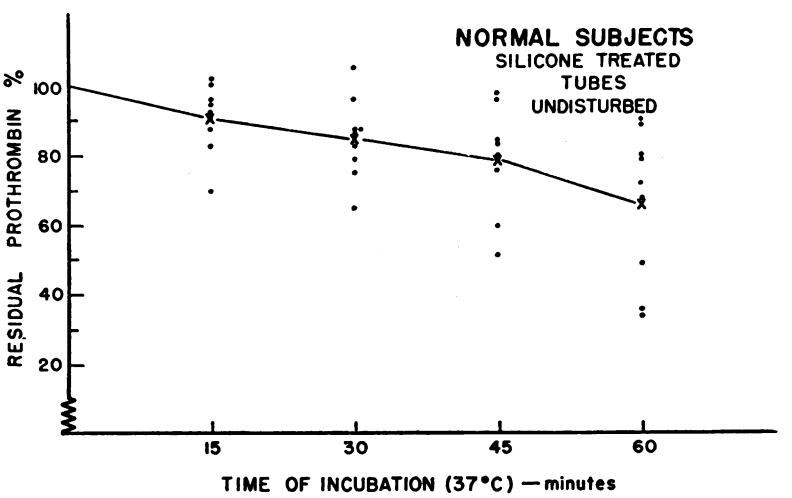

FIG. 1. RESIDUAL PROTHROMBIN AS DETERMINED BY THE TWO-STAGE PROCEDURE EXPRESSED IN PER CENT OF THE PRE-INCUBATION VALUE FOR EACH SUBJECT

Each point in the graph represents the mean of duplicate determinations. The crosses connected by the solid line represent the mean of all the determinations at each time interval.

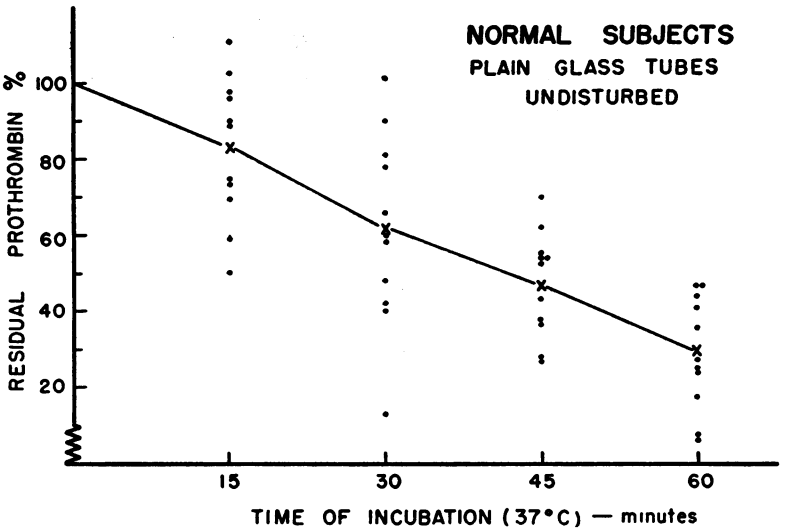

FIGURE 2

The points on this graph represent the mean of duplicate determinations on blood specimens from the same venipuncture as those shown on Figures 1 and 3. Only the surface to which the blood was exposed was changed. The residual prothrombin was measured as in Figure 1.

C. for periods up to 60 minutes. Coagulation was terminated at the end of the desired interval by the addition of oxalate, and the residual prothrombin was determined by the two-stage procedure.

When normal blood was incubated in undisturbed silicone-treated tubes, the rate of prothrombin utilization tended to be slow (Figure 1). However, there was considerable variation in the results of the individual determinations, and after 60 minutes of incubation, the residual prothrombin varied between 34 and 90 per cent. It is apparent that under these conditions, normal blood may fail to show significant prothrombin utilization.

When normal blood was incubated in undisturbed plain glass tubes, the rate of prothrombin utilization was increased (Figure 2). Again, there was considerable variation in the results of the individual determinations, and after 60 minutes of incubation, the residual prothrombin varied from less than 10 to 47 per cent.

Gentle inversion of the plain glass tubes at the beginning of the period of incubation greatly accelerated the rate of prothrombin utilization (Figure 3). Under these conditions, utilization of prothrombin was virtually complete in every instance in 45 minutes of incubation. In many other experiments, agitation of normal blood in plain glass tubes or in tubes containing crushed glass invariably led to complete utilization of prothrombin after 60 minutes of incubation.

Other experiments were designed to determine whether transient exposure of blood to glass surface would lead to continuing utilization of prothrombin. Two ml. portions of whole blood from a normal individual were measured into a series of plain glass and silicone-treated tubes. Some of the glass tubes were inverted 25 times with a parafilm stopper, and others were shaken for two minutes with a rubber stopper. Immediately following this agitation, and within three minutes after initial exposure of the blood to glass, duplicate samples were transferred to silicone treated tubes. Other duplicate samples were left in the original glass and silicone-treated tubes. All tubes were then incubated, undisturbed for 60 minutes at 37° C. Coagulation was terminated at various times by the addition of oxalate, and residual prothrombin was determined by the two-stage procedure. Blood transiently exposed to glass showed little or no change in the level of prothrombin immediately after exposure to glass, but during subsequent incubation in silicone-treated tubes, prothrombin utilization was as rapid as in blood stored in contact with glass throughout the pe-

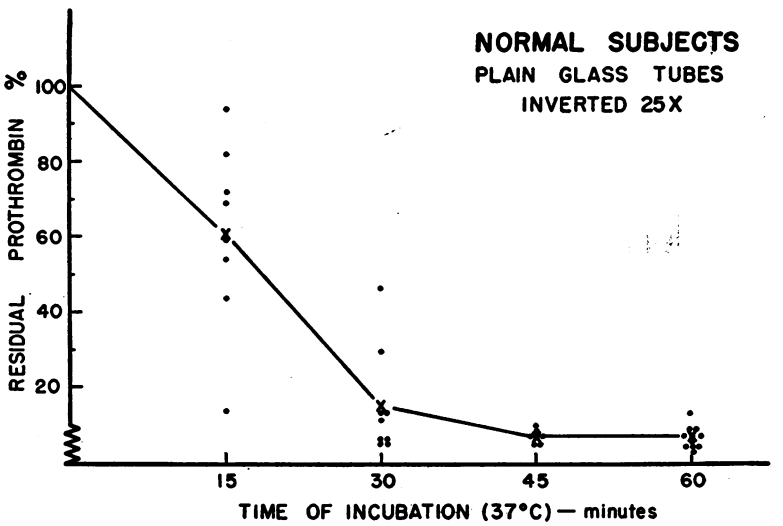

FIGURE 3

Blood specimens were obtained from the same venipunctures as in the studies shown in Figures 1 and 2. The glass tubes were gently inverted 25 times after the introduction of the blood, and then were allowed to stand undisturbed during the remainder of the incubation period. The undulating portion of the ordinate indicates that values of less than 10 per cent generally could not be accurately measured by the two-stage procedure.

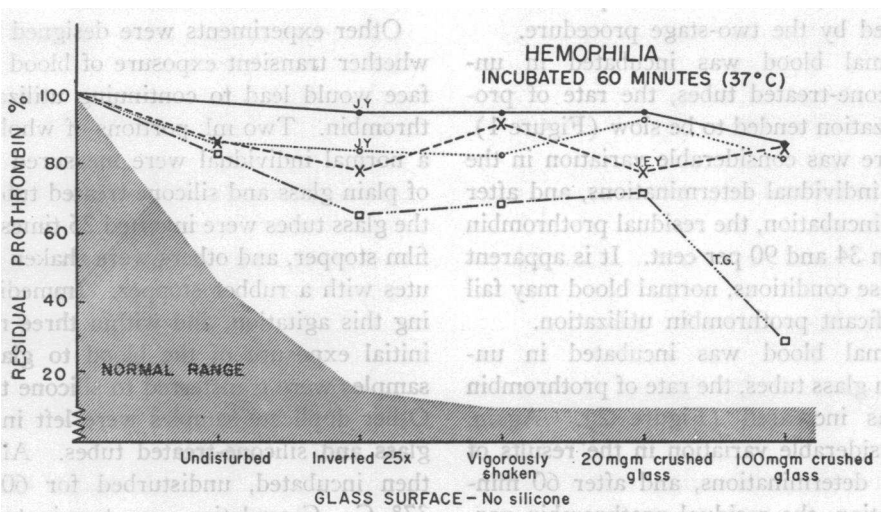

FIG. 4. THE RESULTS OF FOUR EXPERIMENTS ON THE BLOOD OF THREE HEMOPHILIC PATIENTS

Each point on the graph represents the mean of duplicate determinations of residual prothrombin as measured by the two-stage procedure. The time of incubation was held constant at 60 minutes. The degree of exposure of the blood to glass surface area was varied as indicated on the abscissa. In this and in Figures 5–8 the units on the abscissa have no numerical significance, since surface exposure was not measured in absolute terms.

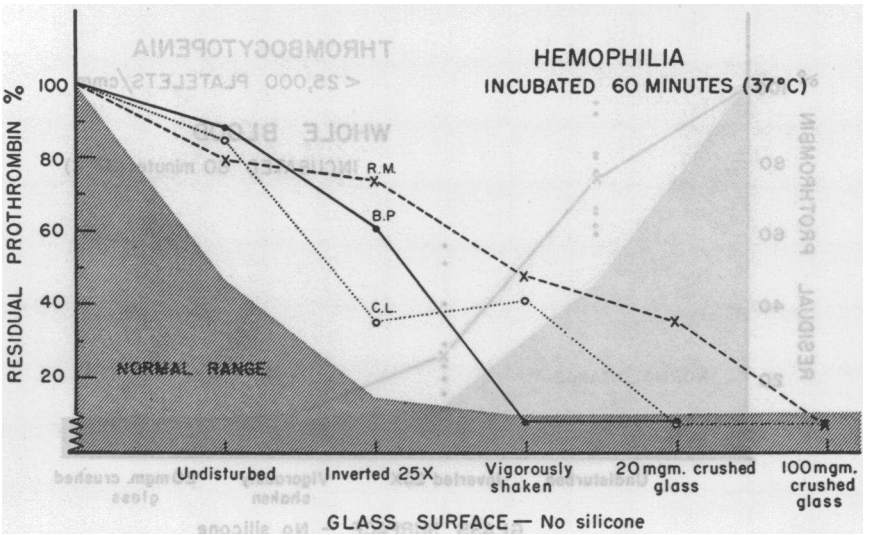

FIG. 5. THE RESULTS OBTAINED ON THE BLOOD OF THREE ADDITIONAL HEMOPHILIC PATIENTS

The procedures employed were identical to those used in obtaining the data for Figure 4.

riod of incubation. The blood that was exposed only to the silicone-treated tubes, without any exposure to glass, utilized much less prothrombin.

It is apparent from these data that the rate of prothrombin utilization in normal blood is directly related to glass surface area to which the blood is exposed. It is interesting to note that a mean residual prothrombin value of approximately 70 per cent was found in normal blood incubated in the silicone-treated tubes for 60 minutes, in the undisturbed plain glass tubes for 30 minutes, and in the inverted plain glass tubes for 15 minutes.

Hemophilic blood: Blood was obtained from six hemophilic patients and distributed into a series of duplicate glass tubes which were incubated at 37° C. for 60 minutes. Some of the tubes were left undisturbed throughout the period of incubation. Others were inverted 25 times or were vigorously shaken for two minutes and then left undisturbed. Some of the tubes that were vigorously shaken contained weighed amounts of crushed glass. After 60 minutes of incubation, coagulation was terminated by the addition of oxalate and the residual prothrombin determined by the two-stage procedure.

The results of four experiments on the blood of three hemophilic patients are shown in Figure 4. In none of these studies did the prothrombin utilization reach the normal range even when the blood was exposed to large glass surface areas. Nevertheless, appreciable prothrombin utilization did occur in one instance.

In identical studies on the blood of three other hemophilic patients, prothrombin utilization was directly related to the glass surface area, and when glass surface area was sufficiently large, utilization of prothrombin was complete (Figure 5).

All six of these hemophilic patients had classical manifestations of the disease. Clinically all but one (B. P.) had severe hemophilia. None of these patients had recently been transfused or received antihemophilic preparations. In each case, tests for the detection of circulating anticoagulants gave negative results. The blood of patient J. Y. (Figure 4) failed to correct the prothrombin utilization defect of all of the other five patients. The coagulation defect in each case was corrected *in vitro* by a source of antihemophilic globulin believed to be deficient in PTC (Christmas) factor.

Thrombocytopenic blood: Blood was obtained from 10 patients with platelet counts ranging between 2,000 and 23,000 per cmm. The blood was handled in the same manner as that described for hemophilic blood.

The results of this study are shown in Figure

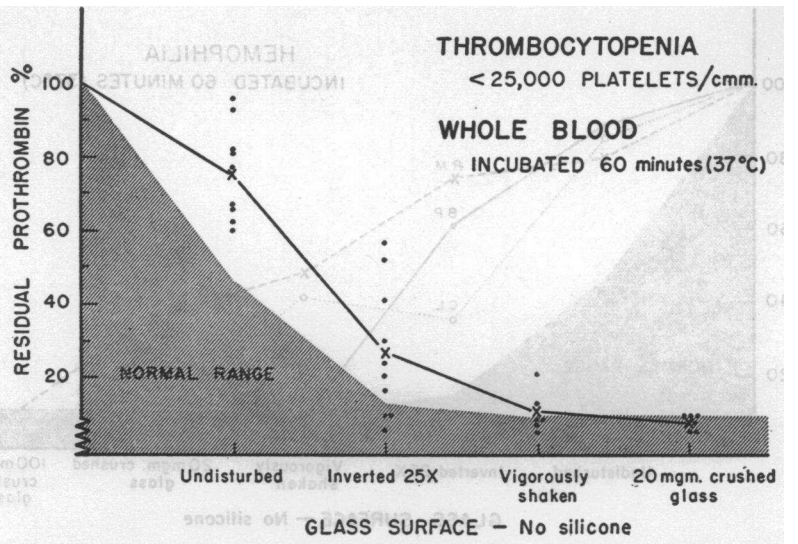

FIG. 6. THE RESULTS OBTAINED ON THE BLOOD OF 10 PATIENTS WITH THROMBOCYTOPENIA

Each point on the graph represents the mean of duplicate determinations of residual prothrombin as measured by the two-stage procedure. The time of incubation was 60 minutes. The degree of exposure of the blood to glass surface area was varied as indicated on the abscissa. The crosses connected by the solid line represent the mean of all of the determinations at each glass surface exposure.

6. It is apparent that only when the blood was left undisturbed in plain glass tubes were the results uniformly outside the normal range. Following simple inversion of the glass tubes 25 times, prothrombin utilization in three instances became as rapid as the normal. When exposure to glass surface was further increased, prothrombin utilization was complete in every instance.

Some of these thrombocytopenic patients were found to have prolonged whole blood clotting times. In these cases tests for the detection of circulating anticoagulants were negative.

Effect of varying glass surface area and platelet concentration on the rate of prothrombin utilization in plasma

Plasma with varying platelet levels was prepared from the blood of fasting normal subjects without the use of an anticoagulant. In each experiment, blood obtained by a single venipuncture from one subject was employed. Two ml. portions of the plasma were distributed in test tubes providing varying exposure to glass surface. After 60 minutes incubation at 37° C., coagulation was terminated by the addition of 0.3 ml. of M/10 sodium oxalate and the residual prothrombin was measured. In order to determine whether prothrombin utilization had occurred as a result of the manipulation involved in the preparation of the plasma, two types of controls were employed. The first control was plasma obtained from blood which was oxalated immediately following withdrawal from the vein. The second control was plasma which was oxalated after centrifugation had been performed but before the beginning of the period of incubation. In every experiment there was no significant difference between the prothrombin concentration of the two control specimens. The time of incubation was considered to have begun when the test plasma was introduced into the tubes at 37° C. Therefore, the period of incubation was considered to have begun in these experiments after the blood had been centrifuged.

A representative experiment is shown in Figure 7. It is apparent that when the glass surface area was constant, the rate of prothrombin utilization was directly related to the platelet concentration.

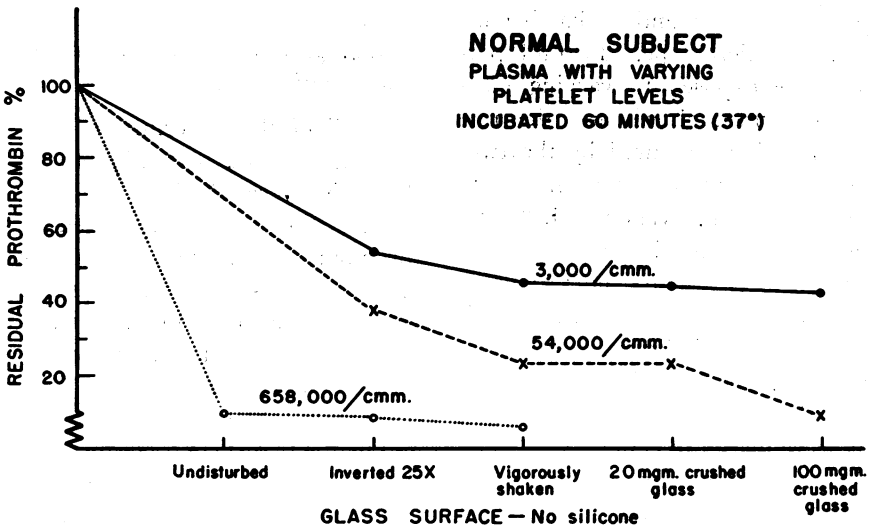

FIG. 7. PLASMA SAMPLES WITH VARYING PLATELET LEVELS PREPARED FROM BLOOD OBTAINED BY A SINGLE VENIPUNCTURE FROM A NORMAL FASTING SUBJECT

Residual prothrombin expressed in per cent of the control is plotted along the ordinate. The degree of exposure to glass surface area is plotted along the abscissa. All samples were incubated for 60 minutes at 37° C.

Conversely, when platelet concentration was constant, the rate of prothrombin utilization was directly related to glass surface area.

In other experiments, prothrombin utilization of plasma that was virtually platelet-free was studied. Platelet-poor plasma obtained as in the preceding experiments was re-centrifuged at 17,500 to 22,000 G for 10 minutes at 4°C. Plasma prepared in this way contained less than 100 platelets per cmm. as determined by direct observation of

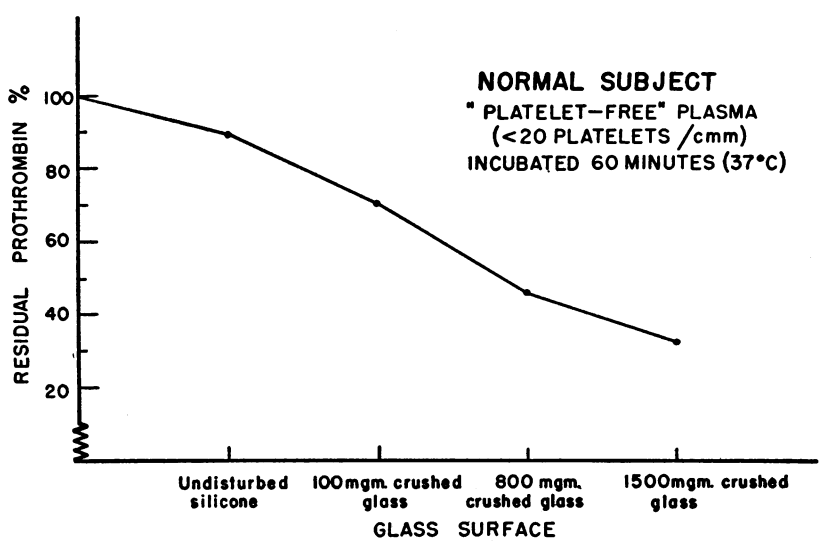

FIG. 8. "PLATELET-FREE" PLASMA PREPARED FROM BLOOD OF A NORMAL FASTING SUBJECT

Residual prothrombin expressed in per cent of the control is plotted along the ordinate. The degree of exposure to glass surface area is plotted along the abscissa. All samples were incubated for 60 minutes at 37° C.

the undiluted plasma in the counting chamber using the phase contrast microscope. Two ml. portions of plasma were exposed to varying glass surface areas as described above, and the rate of prothrombin utilization measured by the two-stage procedure. The results of a typical experiment are shown in Figure 8. When such "platelet-free" plasma was incubated in silicone-treated tubes for periods up to two hours, no significant utilization of prothrombin occurred, and the specimens remained fluid. This indicates that the coagulation process had not been initiated during the preparation of the plasma. When portions of the same plasma were placed in contact with glass surface, solid clots were formed, and prothrombin utilization was directly related to the degree of exposure to glass surface. When the glass surface area was very large, coagulation occurred within two minutes, and approximately two-thirds of the prothrombin was utilized within 60 minutes. In no experiment involving "platelet-free" plasma was complete prothrombin utilization observed. This was true even when the "platelet-free" plasma was vigorously shaken with 3,000 mgm. of crushed glass and incubated for periods up to two hours.

Comparison of the results of one-stage and two-stage procedures for the measurement of residual prothrombin

In all of the experiments that have been described, the data pertaining to the residual prothrombin were obtained by the two-stage procedure. In every experiment, one-stage prothrombin consumption tests also were performed on the same specimens. One-stage tests always were performed 30 minutes after the conclusion of the period of incubation and prior to freezing of the samples for storage. The relationship of the results obtained by the one-stage procedure and by the two-stage procedure is shown in Figure 9. It is apparent that regardless of the source of the specimen or the manner in which the specimen was handled, prothrombin utilization was detectible by the one-stage method only when the two-stage test indicated that less than 15 per cent of the prothrombin remained.

DISCUSSION

The degree of exposure of normal blood and plasma to a glass surface has been demonstrated to have a quantitative influence on the rate of pro-

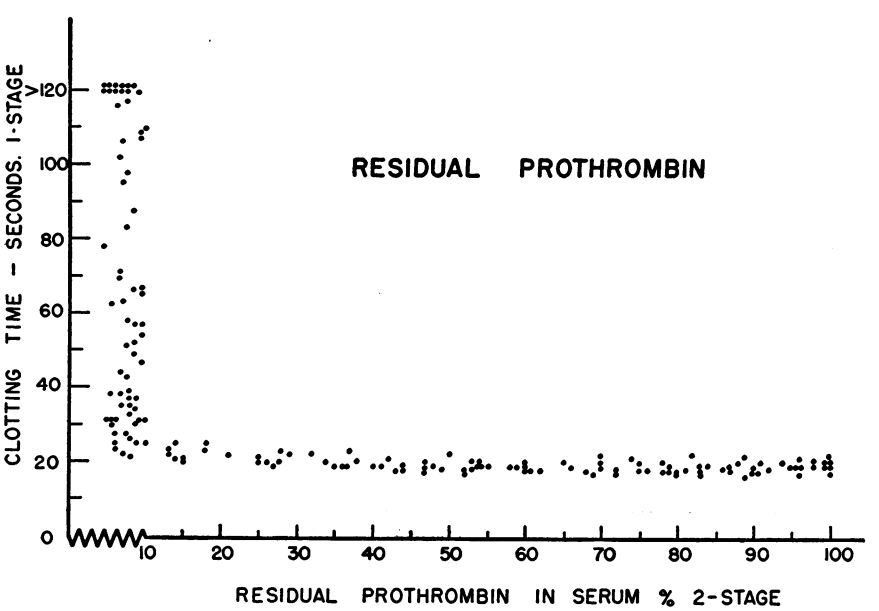

FIGURE 9

In all of the experiments described residual prothrombin was estimated both by the one-stage and two-stage procedures. This graph shows the relationship between the results obtained by the two methods.

thrombin utilization. Brief exposure of blood to glass surface initiates a process which leads to continuing prothrombin utilization even though little prothrombin has been utilized during the actual period of contact with glass. These studies confirm, in a precise manner, the observations of Lister (1).

Lister stated that contact of blood with a foreign surface induced ". . . a mutual reaction between its solid and fluid constituents, in which the corpuscles impart to the liquor sanguinis a disposition to coagulate." Most subsequent investigators, including many current workers, have agreed that the coagulant effect of foreign surface is mediated by the formed elements, particularly the platelets. An impressive array of recent evidence has shown that platelets are quantitatively concerned with the rate of prothrombin utilization in shed blood (13, 25–27). This relationship is confirmed by the present studies which, however, point out that at a given platelet level the rate of prothrombin utilization is directly related to the degree of exposure to a glass surface. The concept of a "critical platelet level" which is optimal for prothrombin utilization is not supported by the current work, since complete prothrombin utilization will occur in whole blood at any platelet level, when the blood is exposed to an appropriate foreign surface.

Unrefuted evidence has been presented by several investigators (3, 5–9, 13, 14) showing that cell-free plasma does contain all of the necessary constituents for coagulation, and that it will clot on contact with foreign surfaces. The current experiments again demonstrate that cell- and "platelet-free" plasma does clot, and furthermore, demonstrate that the rate of prothrombin utilization during clotting of such plasma is a function of the degree of exposure to glass surface. The earlier work has been criticized because the test plasma was not shown to be platelet-free since platelet counts were not performed (1, 3, 5–7), or the methods of counting platelets made it impossible to evaluate the actual platelet levels (8). In more recent work (14), and in the current experiments, direct observation of undiluted plasma samples using the phase contrast microscope have eliminated these objections. A further criticism has been that "platelet-free" plasma may clot because the manipulations involved in its preparation initiate prothrombin conversion by damaging the platelets. This is refuted by the demonstration that prothrombin utilization in such plasma does not occur if the plasma is protected from contact with glass surfaces.

Observations using the phase contrast microscope have been reported (14) showing that "platelet-free" plasma will form fibrin at the point of contact with glass particles in the absence of platelets. That this is true clotting and not defibrination by physical forces is shown by prothrombin utilization studies in such a system. That shaking blood or plasma with crushed glass does not *per se* denature or remove prothrombin is shown by the fact that hemophilic blood which has been incubated following vigorous shaking with crushed glass may show no change in the level of prothrombin.

The exact mechanism of action of glass surface in initiating coagulation is not known. Presumably contact with glass results in the development of thromboplastic activity. Whether this effect is due to an alteration of a specific plasma protein (8), to adsorption of an inhibitor (9), or to some other mechanism is not clear. It has been shown that coagulation of a globulin fraction of "platelet-free" plasma may be accelerated by contact with glass, but the component necessary for this effect was not identified (28). In hemophilia the elaboration of thromboplastic activity in blood and plasma after exposure to glass is impaired. This suggests that the antihemophilic globulin itself may be the site of the alteration. However, this has not been proved, and the blood of some patients with severe hemophilia may demonstrate complete prothrombin utilization when exposed to sufficient glass surface area. It seems likely that several plasma constituents are involved in the production of thromboplastic activity, and that a deficiency of any one might impair the rate of development of this activity. The fact that coagulation of blood or "platelet-free" plasma is hastened by dilution with saline or buffer solutions (14, 29) suggests that a clotting inhibitor is present, but it remains to be proved that the effect of glass surface is brought about by the removal of such an inhibitor.

The present observations have an important bearing on the use of "prothrombin consumption tests" for diagnostic purposes. In the perform-

ance of such tests it is clear that accurate control of exposure of blood to foreign surface is required, if results are to be meaningful. Normal blood may fail to utilize an appreciable amount of prothrombin, if exposure to foreign surface is minimal. On the other hand, in the presence of some coagulation defects prothrombin utilization may be complete if exposure to glass surface is large. When blood is permitted to stand in glass tubes, it is clear that even minor alterations in the manner of handling the tubes results in significant changes in the exposure of the blood to glass. The wide variation in prothrombin utilization in blood incubated in "undisturbed" glass tubes is probably accounted for by this fact. It was hoped in the present study that a single set of experimental conditions could be found which would exaggerate the differences between, and decrease the individual variations of normal and abnormal blood. By moderately increasing the glass surface exposure, hemophilic blood is more readily distinguished from normal blood, but such increased exposure may mask the defect in thrombocytopenic blood. In the present experiments prothrombin utilization could be detected by the one-stage method only when the two-stage procedure indicated that less than 15 per cent of the original prothrombin remained.

SUMMARY

1. The clot accelerating effect of exposure of blood and plasma to glass surfaces was measured quantitatively, employing a two-stage procedure to measure residual prothrombin.

2. Prothrombin utilization in normal human blood was shown to be quantitatively and directly related to the degree of exposure of the blood to glass.

3. The prothrombin utilization defect of hemophilic blood was in some instances completely overcome by exposure of the blood to large glass surface area.

4. The prothrombin utilization defect of thrombocytopenic blood was invariably overcome by exposure of the blood to large glass surface area.

5. Prothrombin utilization in plasma from normal subjects was shown to be directly related to the platelet level and to the degree of exposure of the plasma to glass surface.

6. Prothrombin utilization did occur in "platelet-free" plasma and was directly related to the degree of exposure of the plasma to glass surface.

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