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Clotting Defect in Hemophilia: Deficiency in a Plasma Factor Required for Platelet Utilization.

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Previous studies[1] have shown that in hemophilic blood there is a delayed conversion of prothrombin into thrombin, apparently because liberation of thromboplastin from the formed elements of the blood is slow. Following removal of formed elements by centrifugation, normal plasma showed a delayed prothrombin conversion rate, but the delay was much less than in hemophilic blood. It was postulated that further centrifugation would produce in normal blood a slow clotting time and a delayed prothrombin conversion rate identical with those in hemophilic blood. It was suggested also that normal plasma, rendered spontaneously incoagulable by freeing it of its formed elements, was needed for a crucial test of the clotting defect in hemophilia.

Fuchs[2] reported having obtained spontaneously incoagulable human plasma by high speed centrifugation of carefully collected blood in paraffined glassware. Neither Feissly[3] nor Smith, Warner and Brinkhous[4] were able to confirm this work, despite the fact that equipment of Fuch's design was used. Recently Jaques and coworkers[5] reported the use of non-wettable surfaces treated with silicone to delay the clotting time of normal blood. In the experiments reported below this method has been adapted to obtain from normal individuals plasmas which clotted slowly or not at all in contact with *ordinary* glass. Such plasmas may be termed quasi-hemophilic since, like hemophilic plasma, they had a delayed prothrombin conversion rate but clotted promptly after addition of thrombin or thromboplastin. They were tested for their corrective effect on the clotting of true hemophilic blood and plasma. A preliminary report of these data was made recently.[6]

The technic used to obtain quasi-hemophilic and hemophilic plasmas was briefly as follows: Syringes, needles and glassware were treated with a methylchlorosilane, General Electric Dri-Film.[5] Blood was drawn from the antecubital vein directly into sodium citrate solution (3.2% $Na_3C_6H_5O_7 \cdot 2H_2O$) in the ratio of 8 ml blood to 1 ml anticoagulant. The blood was centrifuged initially up to 30 minutes in an angle centrifuge at 5700 r.p.m. (about 3300 g). The supernatant plasma then was subjected to a series of recentrifugations of 30-90 minutes each at the same speed. In instances in which the total centrifugation time exceeded 2 hours, the plasma was centrifuged for a period of 10-20 minutes at 14,000 r.p.m. (about 14,000 g) immediately after the initial centrifugation. Platelet-free plasmas were centrifuged for 165-1350 minutes, platelet-rich plasmas for 5 minutes. Collection and centrifugation of the blood were carried out in a constant temperature room (2°C).

Clotting time determinations were made after recalcification with $CaCl_2$ (1.2%) at 28°C in ordinary glassware. Each clotting tube contained 0.15 ml citrated plasma. Calcium, sufficient to give the shortest clotting time, and saline (0.9% NaCl) were added to make a total volume of 0.25 ml. In testing its effect on the clotting of hemophilic samples, the normal plasma was added in the ratio of 1 part to 10 parts hemophilic plasma. The

1 Brinkhous, K. M., *Am. J. Med. Sci.*, 1939, **198**, 509.

2 Fuchs, H. J., *Arch. exp. Zellforsch.*, 1933, **14**, 334.

3 Feissly, R., *Helv. Med. Acta*, 1940, **7**, 583.

4 Smith, H. P., Warner, E. D., and Brinkhous, K. M., unpublished data.

5 Jaques, L. B., Fidlar, E., Feldsted, E. T., and Macdonald, A. G., *Canadian Med. Assn. J.*, 1946, **55**, 26.

6 Brinkhous, K. M., *Fed. Proc.*, 1947, **6**, 389.

TABLE I.
Effect of Centrifugation Time on Clotting of Recalcified Normal Blood or Plasma.

Centrifugation time min.	Clotting time min.
0	6½
10	8½
30	10
60	18½
90	20
120	31
165	37
1350	no clot 1800

actual volume added was adjusted to correct for the citrate content. When whole blood was used, the volume was adjusted in addition for cell volume as indicated by the hematocrit.

Platelet suspensions were prepared by differential centrifugation of blood in silicone glassware. The platelet sediment was washed by resuspension in citrate-saline solution (1 part citrate solution, 9 parts saline) and recentrifuged. This was repeated 3 times. Microscopic examination showed the platelets to be well preserved. Platelet suspensions were added to provide 25,000-30,000 platelets per mm^3 in the final clotting mixture.

Most of the data on hemophilic blood was obtained from one patient, although the experiments were repeated on at least one of 3 other hemophilic patients. The normal blood samples were obtained from 7 different individuals.

Quasi-hemophilic plasma from normal blood. Table I shows that centrifugation of normal blood for ½ hour or less produced plasmas with clotting times within the range commonly accepted as normal, but that longer centrifugation produced progressively longer clotting times. Finally, plasma was obtained which did not clot within 30 hours after recalcification.

The prothrombin content[7,8] of several samples of quasi-hemophilic plasma, including those spontaneously incoagulable, was within the normal range of 90% to 110%. The prothrombin conversion rate of these samples, determined as previously described,[1] was delayed. The longer the clotting time, the greater was the delay.

Plasmas, similar to those shown in Table I, and whole blood were clotted with the same rapidity by thrombin solutions (Parke Davis "Thrombin Topical") and by thromboplastic extract of beef lung.[8] Tests for antithrombin activity gave normal values. All of the plasmas, when incubated with thrombin solutions, destroyed thrombin in the same amount and at the same rate.

The addition of platelet suspensions to the quasi-hemophilic plasmas reduced the clotting time to 6 to 9 minutes. This is in contrast to the behaviour of platelet-free hemophilic plasma described later (see Table III).

Failure of quasi-hemophilic plasma to correct the clotting defect of platelet-free hemophilic plasma (see Table II). Normal citrated whole blood reduced the clotting times both of whole hemophilic blood and of platelet-free hemophilic plasma to 6 minutes. The effectiveness of the normal plasmas in correcting the clotting defect of platelet-free hemophilic plasma decreased as their clotting times became prolonged. The spontaneously incoagulable normal plasma was entirely without effect. Regardless of the degree of prolongation of their clotting times, however, the normal plasmas retained their corrective action on the clotting of whole hemophilic blood.

Corrective action of normal plasma on the clotting of hemophilic plasma in the presence of platelets. In a series of further tests, quasi-hemophilic plasma was added to platelet-rich hemophilic plasma, and platelet-rich normal plasma was added to platelet-free hemophilic plasma. In the presence of numerous platelets, either normal or hemophilic, the hemophilic clotting time was reduced to the normal range. The results of one test follow. When a sample of platelet-poor normal plasma which had a clotting time of 27 minutes was added to platelet-free hemophilic plasma, the clotting time was 25 minutes; when it was added to platelet-rich hemophilic plasma the clotting time was 7 minutes, and when added to whole hemophilic blood, clotting occurred in 7½ minutes.

Effect of platelet suspensions on clotting of

[7] Warner, E. D., Brinkhous, K. M., and Smith, H. P., *Am. J. Physiol.*, 1936, **114**, 667.

[8] Smith, H. P., Warner, E. D., and Brinkhous, K. M., *J. Exp. Med.*, 1937, **66**, 801.

TABLE II.
Effect of Addition of Normal Blood or Plasmas on Clotting of Hemophilic Blood or Plasmas.

Clotting time Recalcified normal plasma (to be added to hemophilic plasma), min.	Clotting time Recalcified hemophilic blood or plasma, after addition of normal plasma	
	Whole hemophilic blood, min.	Platelet-free hemophilic plasma, min.
6*	6	6
8	7	18
17	6½	19
31	6	26
40	7¾	30
no clot 1800	6	no clot 1800
	45-120†	no clot 1800†

* Whole blood.
† Controls, showing clotting times of various hemophilic specimens after recalcification, without normal plasma addition.

TABLE III.
Effect of Platelet Suspensions and Normal Plasma* on Clotting of Platelet-free Hemophilic Plasma.*

Supplement	Clotting time Recalcified hemophilic plasma, after addition of platelets or plasma, min.
Normal platelet suspension	32
Hemophilic platelet suspension	28
Normal platelet-poor plasma†	30
Normal plasma + normal platelet susp.	8½
Normal plasma + hemophilic platelet susp.	6

* Plasmas used in this experiment had been centrifuged for 120 minutes.
† Clotting time of recalcified normal plasma was 32 minutes.

platelet-free hemophilic plasma (see Table III). With the platelets alone the clotting time remained prolonged, but in the presence of normal plasma, which by itself did not correct the clotting defect, the platelets reduced the clotting time to the normal range.

Discussion. The data indicate that the clotting of normal plasma depends upon the presence of formed elements. Plasma deprived of formed elements or their disintegration products is stable, even in the presence of a wettable surface and optimal calcium in concentration. The ability to clot is restored by addition of platelets. Unless formed elements are present, normal stable plasma is without effect on the clotting of hemophilic plasma. These data indicate that platelets and perhaps other formed elements cannot be utilized in the absence of a plasma factor present in normal blood.

A simple hypothesis based on these findings is offered: Normal plasma contains a factor necessary for thromboplastin liberation from the formed elements of the blood, presumably by platelet lysis. This platelet-lysin or thrombocytolysin is deficient in hemophilic plasma. When the lytic factor is supplied to hemophiliacs, as in blood or plasma transfusions, platelets rupture in the normal manner. Sufficient thromboplastin then becomes available, and the block in the clotting mechanism is removed.

Numerous workers, including Feissly,[9] have attempted to test the effect of normal plasma on hemophilic plasma in the absence of platelets. Their results have shown an acceleration of the clotting of hemophilic plasma in the apparent absence of platelets. These discordant results may be due to the presence of free thromboplastin in the normal plasma from breakdown of the formed elements during collection and handling of the plasma, and perhaps also from incomplete removal of formed elements by centrifugation.

In the experiments presented in this paper, the centrifugal forces used were so low that it appears unlikely that any macromolecular substance essential for clotting, such as the thromboplastic protein described by Chargaff

[9] Feissly, R., *Helv. Med. Acta*, 1945, **12**, 467.

and West,[10] could have been removed by the centrifugation.

Summary. 1. By special handling and prolonged centrifugation of normal blood, plasmas with a delayed clotting time can be obtained. If centrifugation is prolonged sufficiently, the plasmas become spontaneously incoagulable.

2. Normal plasmas of this type require the presence of platelets and perhaps other formed elements to correct the delayed clotting of hemophilic plasma.

3. These findings indicate that in hemophilia there is a deficiency in a plasma factor required for platelet utilization. It is suggested that this factor is a thrombocytolysin.

[10] Chargaff, E., and West, R., *J. Biol. Chem.*, 1946, **166**, 189.

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Effect of Penicillin on the Tubercle Bacillus *In vitro.*

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Reports concerning the effect of penicillin on the tubercle bacillus are contradictory. The Oxford group originally noted no inhibition of growth in glycerol broth with a penicillin concentration of 40 units per cc.[1] Subsequently, inhibition by as little as 20 units per cc was observed by one investigator,[2] whereas others reported no inhibition with concentrations of 20 to 30 units per cc.[3,4] Actual stimulation of growth by small concentrations of penicillin was described in one instance,[5] while in another, rapid destruction of large amounts of penicillin (800 units per cc) by culture filtrates of *M. tuberculosis* was observed.[6] These contradictory results, and the desirability of using penicillin to suppress the growth of contaminants in cultures for the primary isolation of tubercle bacilli, have made it appropriate to reinvestigate the effect of penicillin on the tubercle bacillus *in vitro.*

Materials. H37Rv, a standard virulent human strain of *M. tuberculosis,* was used throughout the experiments because of its similarity in virulence and response to antimicrobial agents to strains isolated from patients with tuberculosis.[7,8]

The experiments were performed in liquid and on solid media recently developed in this laboratory.[9,10] The penicillin employed was commercial crystalline penicillin G, obtained from various manufacturers.

Experimental. Lytic Action of Penicillin. A large inoculum of tubercle bacilli, 0.25 mg per cc, was added to test tubes 25 by 150 mm each containing 15 cc of Tween-albumin medium and penicillin in concentrations ranging from 100 to 1000 units per cc. The tubes were incubated at 37°C, and optical densities were recorded on the Coleman Spectrophotometer every day or two. Because of the possible destructive action of the tubercle bacillus on penicillin, the original concentrations were added to one set of tubes every 3 days on 4 occasions. To another set, no further penicillin was added to the amounts pres-

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[2] Hand, C. N., *J. Path. and Bact.*, 1946, **58**, 495.

[3] Smith, M. I., and Emmart, E. W., *Pub. Health Rep.*, 1944, **59**, 417.

[4] Friedmann, I., *Tubercle*, 1945, **26**, 75.

[5] Ungar, J., and Muggleton, P., *J. Path. and Bact.*, 1946, **58**, 501.

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[8] Feldman, W. H., and Hinshaw, H. C., *Am. Rev. Tuberc.*, 1947, **55**, 428.

[9] Dubos, R. J., and Davis, B. D., *J. Exp. Med.*, 1946, **83**, 409.

[10] Dubos, R. J., and Middlebrook, G., *Am. Rev. Tuberc.*, in press.