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# THE COAGULATION DEFECT IN HEMOPHILIA: THE CLOT PROMOTING ACTIVITY IN HEMOPHILIA OF BERKEFELDED NORMAL HUMAN PLASMA FREE FROM FIBRINOGEN AND PROTHROMBIN

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Research Article



# THE COAGULATION DEFECT IN HEMOPHILIA: THE CLOT PROMOTING ACTIVITY IN HEMOPHILIA OF BERKEFELDED NORMAL HUMAN PLASMA FREE FROM FIBRINOGEN AND PROTHROMBIN [1]

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Previous investigations in this laboratory have shown that normal human plasma rendered free from cellular elements, by Berkefeld filtration, is capable of reducing the coagulation time of the blood of patients with hemophilia both *in vitro* and *in vivo* (1). It has also been demonstrated that this coagulation activity of the plasma was *associated* with the globulin fraction of the plasma proteins (2, 3). The published data do not however identify the active material as a protein, nor do they differentiate it from fibrinogen and prothrombin, constituents of the globulin fraction which also play a rôle in blood coagulation. Indeed "globulin substance" as prepared by previous methods was known to contain both prothrombin and fibrinogen (2). The present communication concerns a study of the clot promoting activity of normal human plasma, after the removal of prothrombin and fibrinogen.

## PREPARATION OF MATERIALS

Several authors (4, 5, 6) have described methods for the removal of prothrombin from blood plasma. These methods consist essentially of adsorption of this substance on the hydroxides of aluminum, calcium, or magnesium. Chew (7) has suggested recently that following filtration through Seitz filters (calcium magnesium aluminum iron silicate) a prothrombin free filtrate of plasma can be obtained.

By the use of the Quick *et al.* technique (8) it is possible to obtain a measure of the amount of prothrombin contained in preparations made from plasma.[2] Table I shows the "prothrombin times" of certain of these preparations. It will be observed that while preparations with commercial aluminum hydroxide were quite inefficient the use of aluminum hydroxide C-gamma (9) gave preparations relatively free from prothrombin. The amount of prothrombin remaining depended upon the number of times the adsorption was repeated. More striking results were obtained however by passing Berkefeld filtrates of citrated normal human plasma 5 times through Seitz filters using fresh pads for each filtration. In addition to being prothrombin free these preparations had the advantage of being sterile. On the basis of these observations the Seitz filtration method was used routinely for the removal of prothrombin from plasma. Each batch of plasma treated in this manner was tested for prothrombin by the Quick technique and none was found. The presence of fibrinogen in the Seitz filtrate was demonstrated by the formation of a clot upon the addition of fresh serum as a source of thrombin. In one typical experiment one-tenth ml. of such serum clotted one-tenth ml. of the Seitz filtrate in 29 seconds.

Plasma was rendered free from fibrinogen by heat coagulation. The Berkefeld filtrate from *fresh* citrated normal human plasma was heated to 56° C. in the water bath. It was held at this temperature for 2 minutes and the copious precipitate containing the fibrinogen was removed by filtration. The absence of fibrinogen in the filtrate was demonstrated by the failure of clot formation when fresh serum acting as a source of thrombin was added.

TABLE I

*The effect of certain adsorbents on the removal of prothrombin from normal citrated plasma as measured by the Quick "prothrombin time"*

| Method of preparation of Berkefeld plasma | "Prothrombin time." Average of three determinations |
|---|---|
| | *seconds* |
| None (control) | 29 |
| Commercial aluminum hydroxide 50 cc. plasma + 50 cc. alumina cream | 36 |
| Above procedure repeated 3 times | 40 |
| Aluminum hydroxide C-gamma (1 gram) to 50 cc. plasma | 73 |
| Above procedure repeated twice | 217 |
| Above procedure repeated 3 times | 510 |
| Five times through Seitz pads | No clot |

[1] The expenses of this investigation were defrayed in part by a gift to Harvard University from Smith, Kline, and French Laboratories, Philadelphia; and by a grant given in honor of Francis Weld Peabody by the Ella Sachs Plotz Foundation.

[2] All plasmas studied were derived from normal human 0.25 per cent citrated blood centrifuged 30 minutes at 2000 r.p.m., filtered through 2 thicknesses of Number 2 Whatman paper and then through a Berkefeld V filter.

For the preparation of plasma free from both fibrinogen and prothrombin, the fibrinogen was removed by the above technique and the filtrate passed 5 times through Seitz filters to remove the prothrombin. When any of the preparations were to be stored they were dried by the lyophile method to avoid chemical change during desiccation.

## EXPERIMENTAL

### In vitro *studies*

*In vitro* determinations of the clot promoting activity of plasma freed from prothrombin or both prothrombin and fibrinogen were made. Varying amounts of the preparations to be tested were added to 2 ml. of hemophilic blood, using the standard technique previously described (3). In some instances the results obtained were compared with the Berkefeld plasma from which the preparation was derived. Precise quantitative relationships were not possible since there was a loss of plasma to the filter pads and some evaporation occurred during the time required for Seitz filtration. The results however were sufficiently clear cut to permit conclusions to be drawn. A summary of the data from typical experiments is given in Table II.

These data indicate that the greater part of the clot promoting activity of cellular free normal human plasma is not dependent on the presence of either fibrinogen or prothrombin, and remains in the filtrate from which these proteins are removed.

### *The effect,* in vivo, *of a single injection of normal human plasma free from prothrombin on the coagulation time of the blood of a patient with hemophilia*

Two patients with hemophilia served for *in vivo* studies. In one, a single intravenous injection of 50 ml. of normal human plasma free from prothrombin was administered. The coagulation time fell from 108 to 28 minutes 1 hour after the injection was given. It remained at this low level for 7 hours, after which it returned slowly to a value of 128 minutes in the course of 69 hours. The results are shown graphically in Figure 1. The observations were repeated on the

TABLE II

*The effect of preparations of citrated normal human plasma free from prothrombin and fibrinogen on the coagulation time of hemophilic blood* in vitro

| Control coagulation time of 2 ml. of blood from patient with hemophilia | Preparation employed | Coagulation time of 2 ml. of blood from patient with hemophilia after the addition of preparations in the amounts shown below. | | |
|---|---|---|---|---|
| | | 0.01 ml. | 0.05 ml. | 0.10 ml. |
| *minutes* 129 | Original Berkefeld plasma | *minutes* | *minutes* | *minutes* 14 |
| 129 | Original after 5 times through Seitz filter | | | 16 |
| 25 | Original Berkefeld plasma | | | 10 |
| 25 | Original after 5 times through Seitz filter | | | 13 |
| 36 | Filtered 5 times through Seitz filter | 23 | 21 | 20 |
| 42 | Fibrinogen free plasma. Filtered 5 times through Seitz filter | 25 | 18 | 17 |
| 81 | Fibrinogen free plasma. Filtered 5 times through Seitz filter | | 21 | 16 |
| 136 | Fibrinogen free plasma. Filtered 5 times through Seitz filter | 33 | 26 | 18 |
| 136 | Fibrinogen free plasma. Filtered 5 times through Seitz filter | 59 | 30 | 24 |
| 35 | Fibrinogen free plasma. Filtered 5 times through Seitz filter | 28 | 23 | 18 |

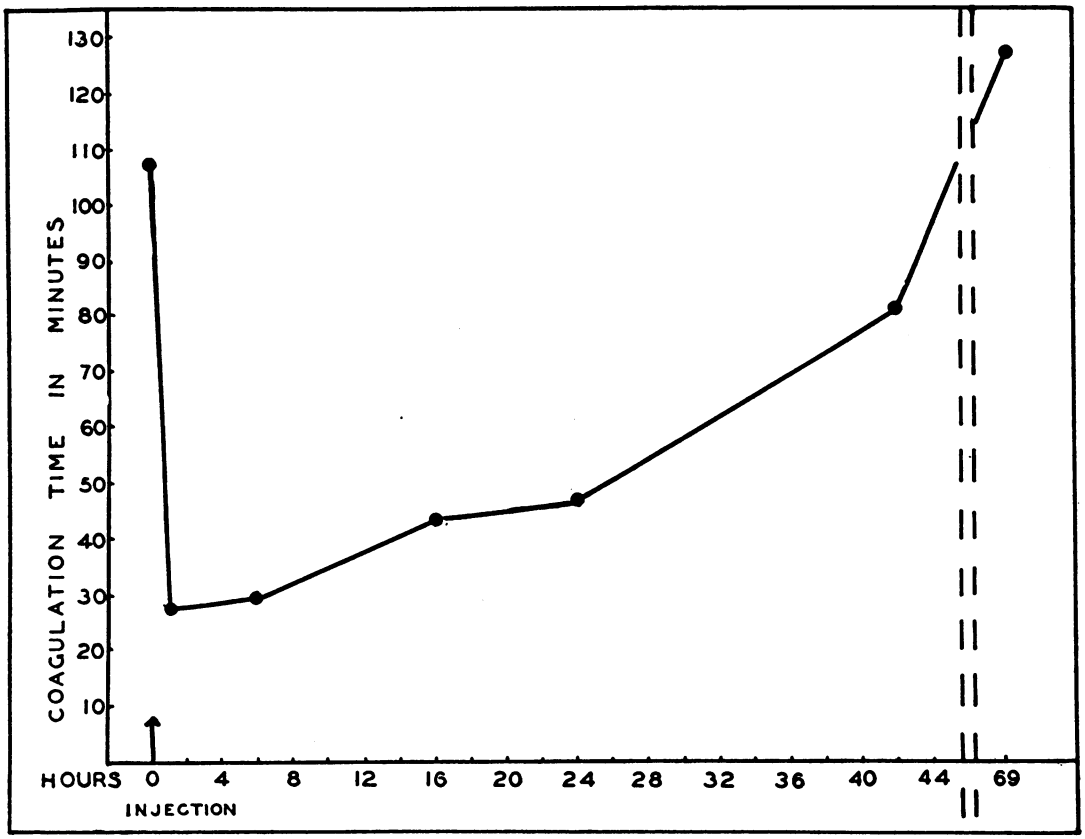


FIG. 1. EFFECT OF A SINGLE INTRAVENOUS INJECTION OF NORMAL HUMAN PLASMA FREE FROM *prothrombin* ON THE BLOOD COAGULATION TIME IN A PATIENT WITH HEMOPHILIA

second patient with hemophilia with entirely similar results.

*The effect,* in vivo, *of a single injection of normal human plasma free from fibrinogen and prothrombin on the coagulation time of the blood of a patient with hemophilia*

A patient with hemophilia was given a single intravenous injection of 50 ml. of normal human plasma free from fibrinogen and prothrombin. The coagulation time of his blood fell from 110 minutes to 26 minutes 1 hour after the injection. This low level persisted for 6 hours after which it slowly returned to the pre-injection level reaching 100 minutes in 52 hours after the injection. These results are shown graphically in Figure 2. A similar response to the administration of this material was obtained in a second patient with hemophilia. By a comparison of the data presented graphically in Figure 1 with the data previously published (1) on single intravenous injections of normal human citrated plasma into patients with hemophilia no essential difference can be discerned. Figures 1 and 2 are virtually super-imposable indicating that the removal of fibrinogen in addition to prothrombin did not diminish the effectiveness of the clot promoting activity of the filtrate.

*The effect,* in vivo, *of multiple injections of normal human plasma free from fibrinogen and prothrombin on the coagulation time of the blood of a patient with hemophilia*

A patient with hemophilia was given a total of 4 intravenous injections of 50 ml. of normal human plasma free from fibrinogen and prothrombin. The injections were given at intervals of 6 hours. In 1 hour following the initial injec-

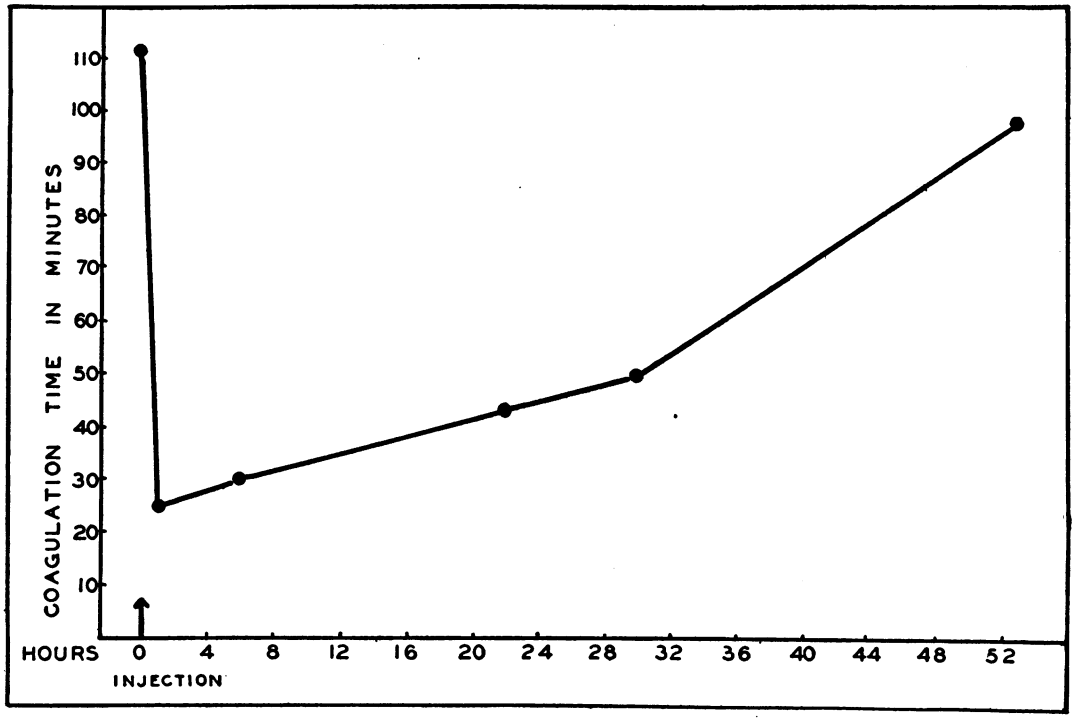


FIG. 2. EFFECT OF A SINGLE INTRAVENOUS INJECTION OF NORMAL HUMAN PLASMA FREE FROM *prothrombin* AND *fibrinogen* ON THE BLOOD COAGULATION TIME IN A PATIENT WITH HEMOPHILIA

tion the coagulation time of the blood of the patient fell from 135 to 40 minutes. It remained between 40 and 45 minutes for the next 6 hours. Following the second injection the coagulation time fell to 20 minutes and remained at a level of between 20 and 30 minutes after the subsequent injections. After the fourth and last injection the coagulation time of the blood remained between 30 and 40 minutes for a period of 25 hours after which it slowly returned in 3½ days to pre-injection levels. The data are presented graphically in Figure 3. A comparison of these data with those previously published for multiple injections of unmodified normal human plasma (10) shows a close correspondence. The maintenance of a lowered coagulation time by repeated injections of unmodified plasma and preparations of plasma free from prothrombin and fibrinogen is in sharp contrast to the refractory phase reported as accompanying multiple injections of acid-precipitated "globulin substance" (3).

## DISCUSSION

In an earlier publication (2) it was pointed out that against a calcium-fibrinogen system "globulin substance" behaved as prothrombin in the production of a fibrin clot. It was also shown that in this regard the preparations from the cellular free blood plasma of both normal persons and cases of hemophilia behaved in a similar manner. It was quite evident therefore that "globulin substance" contained prothrombin as defined by the clotting mechanism.

The "prothrombin time" as determined by the method of Quick *et al.* (8) of patients with hemophilia is normal. A fall of 20 per cent of the total amount of prothrombin may occur, however, without appreciable change in the "prothrombin time" (8). For this reason a normal "prothrombin time" would not necessarily mean that the addition of more prothrombin could not force the coagulation mechanism toward clot formation, and hence decrease the coagulation time of the blood. It becomes necessary therefore to know whether the clot promoting activity of plasma described in earlier investigations published from this laboratory (1, 2) can exist in the absence of prothrombin.

The present observations seem to answer this question. Both in the test tube and following in-

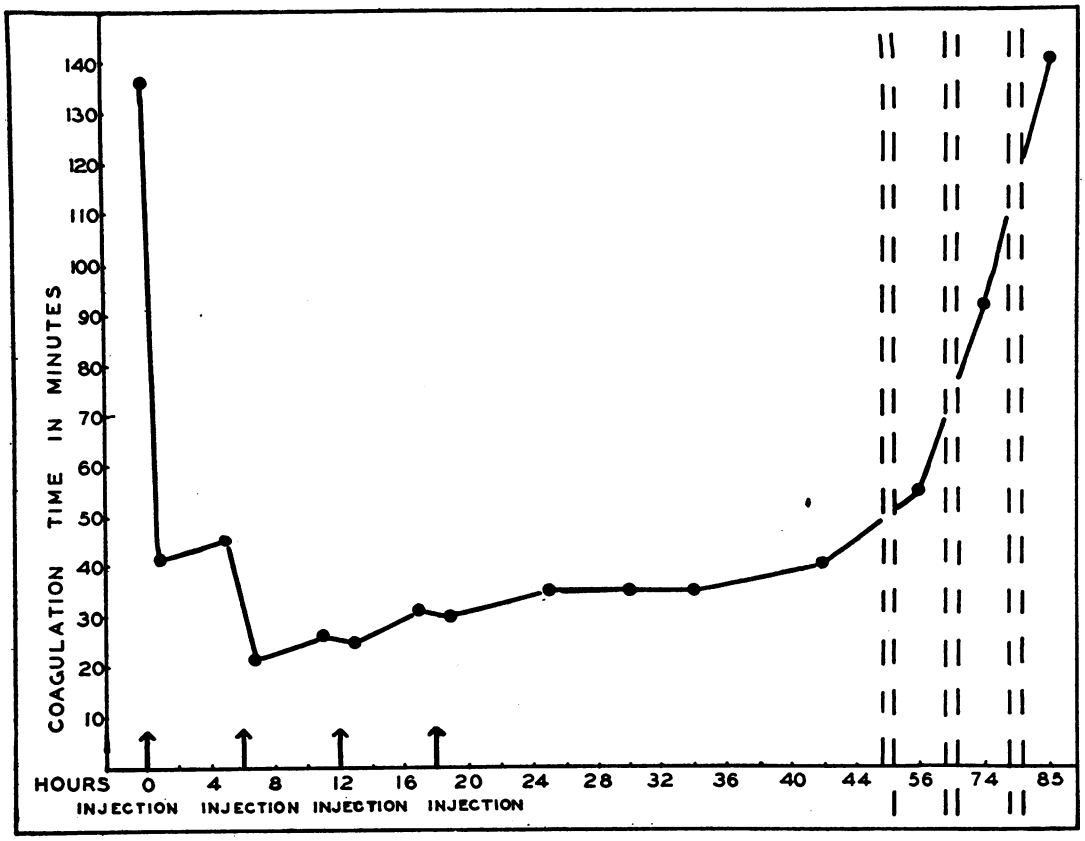


FIG. 3. EFFECT OF MULTIPLE INTRAVENOUS INJECTIONS OF NORMAL HUMAN PLASMA FREE FROM *prothrombin* AND *fibrinogen* ON THE BLOOD COAGULATION TIME IN A PATIENT WITH HEMOPHILIA

jection into patients with hemophilia the clot promoting activity occurs in plasma which contains no prothrombin. The same conclusions so far as *in vitro* experiments are concerned have been arrived at independently by Frank and Hartmann (6) and by Howell (11) who terms the active factor "plasma thromboplastin".

Recently Macfarlane has reported an individual who had a prolonged coagulation time and in whose blood no fibrinogen could be found (12). Since the presence of fibrinogen in "globulin substance" has been recorded (2), and since variations within "normal" limits in the fibrinogen content of the blood in hemophilia are known to occur it is again necessary to differentiate the clot promoting activity from this protein.

The data presented in this paper and also the experiences of Howell show that while fibrinogen is present in euglobulin (2) and in "globulin substance" prepared by acid precipitation, the removal of fibrinogen does not destroy the clot promoting activity of plasma. The *in vitro* observations of Howell and the data of the *in vitro* and *in vivo* observations of the present communication suggest, that so far as the patients studied are concerned, the coagulation defect in hemophilia does not lie in the prothrombin or fibrinogen fractions of the plasma proteins.

It is not implied, however, that deficiencies in fibrinogen and prothrombin may not modify the action of normal human plasma or derivatives of it on the blood of certain cases of hemophilia. Indeed Macfarlane's patient with congenital absence of fibrinogen and our own experiences with 2 atypical cases of hemophilia (13) would suggest that certain multiple deficiencies of factors controlling clotting may occur. Such interrelationships between the various substances present could be anticipated in a system of multiple components such as are concerned in the coagulation of the blood. The present report shows only that there exists in cellular-free normal human

plasma a factor other than fibrinogen or prothrombin which possesses clot promoting activity for the blood of patients with hemophilia.

## SUMMARY AND CONCLUSIONS

1. Standard methods for the removal of prothrombin and both prothrombin and fibrinogen from normal human Berkefelded citrated plasma are described.

2. Preparations obtained from normal human Berkefelded citrated plasma by the removal of prothrombin or both prothrombin and fibrinogen are capable of reducing the coagulation time of hemophilic blood *in vitro* in a quantitative manner.

3. Such preparations when injected intravenously into patients with hemophilia cause a prompt fall of their coagulation time.

4. Injections of such preparations repeated every 6 hours in patients with hemophilia maintain their coagulation time at this lowered level.

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