

**THE COAGULATION DEFECT IN HEMOPHILIA. STUDIES ON THE REFRACTORY PHASE FOLLOWING REPEATED INJECTIONS OF GLOBULIN SUBSTANCE DERIVED FROM NORMAL HUMAN PLASMA IN HEMOPHILIA**

Frederick J. Pohle, F. H. L. Taylor

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THE COAGULATION DEFECT IN HEMOPHILIA. STUDIES ON THE  
REFRACTORY PHASE FOLLOWING REPEATED INJECTIONS  
OF GLOBULIN SUBSTANCE DERIVED FROM  
NORMAL HUMAN PLASMA IN HEMOPHILIA<sup>1</sup>

By FREDERICK J. POHLE AND F. H. L. TAYLOR

(From the Thorndike Memorial Laboratory, Second and Fourth Medical Services (Harvard),  
Boston City Hospital, and the Department of Medicine,  
Harvard Medical School, Boston)

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It has been suggested that the defect in coagulation of blood in hemophilia resides in a globulin fraction of the plasma (1, 2). Globulin substance, prepared by isoelectric precipitation from citrated, cellular-free, normal human plasma, possesses marked clot-accelerating properties for hemophilic blood *in vitro* and *in vivo*. There is a prompt decrease in the blood coagulation time when a saline suspension of this material is injected either intravenously or intramuscularly into hemophilic subjects. The coagulation time remains low following such injections for approximately 6 hours, when it commences to return toward the pre-injection level. Attempts to *maintain* a *reduction* of the coagulation time with repeated injections of globulin substance led to the discovery that a refractory phase occurred after several injections had been given (2). During this phase the coagulation time was little affected by repeated injections although it was demonstrated that the concentration of this clot-accelerating material progressively increased in the circulating blood of the patient. Recovery from this refractory state was always complete within 24 hours after the last injection of globulin substance. The present communication reports further studies concerning the refractory period and offers an explanation of certain aspects of this phenomenon.

METHODS

*Coagulation time.* The method by which the coagulation time of venous blood was determined has been described elsewhere (2).

<sup>1</sup> The expenses of this research were defrayed in part by a gift to Harvard University from Smith, Kline, and French Laboratories of Philadelphia.

*Control period.* The investigation was carried out on four hemophilic patients between the ages of 18 and 48 years who had been under observation in this clinic for several years. The shortest blood coagulation time for these four patients was 40 minutes and the longest was 180 minutes. Prior to each set of observations a control period of 48 hours was established. If the coagulation time of the blood fluctuated appreciably during this period no test observations were made of that subject. Patients who had had recent hemorrhages were excluded from this study.

*Preparation of globulin substance.* The globulin substance was prepared from citrated normal human plasma in the same manner as previously described (2). In all experiments the dried material was suspended in the same volume of 0.85 per cent sodium chloride solution as the volume of plasma from which it was derived, rendered sterile and free of particulate matter by Berkefeld filtration. Each lot of globulin substance, so prepared, was shown to have maximum clot-accelerating properties for hemophilic blood *in vitro* (2) before it was used parenterally.

*Standard test dose.* A standard test dose of 65 cc. of plasma or an equivalent amount of saline suspension of globulin substance was used for *in vivo* studies. The standard test dose of globulin substance contained approximately 300 mgm. of the dried material.

*Preparation of dried material by the Flosdorf-Mudd technique.* In certain instances, plasma or the freshly prepared, moist precipitate of globulin substance was dried by the lyophilic process as described by Flosdorf and Mudd (3). This porous dry material prepared from either source was redissolved in an amount of distilled water equal in volume to that of the original plasma.

## EXPERIMENTAL

*The effect of repeated intravenous injections of plasma.* In view of the fact, shown by many observations, that a refractory phase followed repeated injections of globulin substance into hemophilic patients (Figure 1), it was necessary to observe the effects of repeated injections of the parent plasma.<sup>2</sup> Three hemophilic subjects were given a standard test dose of citrated normal human plasma intravenously, repeated four times at 6-hour intervals. The results obtained in one typical set of observations are presented in Figure 2. As shown in Figure 2, the blood coagulation time was maintained near normal values, and throughout the entire period of observation there was *no suggestion of the development of a refractory period following any injection of plasma.*

*The effect of dried plasma upon the coagulation time of hemophilic blood in vitro.* Normal citrated human plasma was dried in open dishes at room temperature in a current of air created by an electric fan. The dry material, so obtained, was suspended in the same volume of distilled water as the original volume of the plasma, centrifuged, and then its clot-accelerating properties

<sup>2</sup> All plasma referred to in this communication has been passed through a Berkefeld V filter.

TABLE I

*Effect of a solution of air-dried, citrated, normal human plasma on the coagulation time of hemophilic blood in vitro*

	Coagulation time
	<i>minutes</i>
2 cc. hemophilic blood . . . . .	43.5
2 cc. hemophilic blood + 0.01 cc. suspension of dried plasma . . . . .	43.5
2 cc. hemophilic blood + 0.03 cc. suspension of dried plasma . . . . .	41.0
2 cc. hemophilic blood + 0.05 cc. suspension of dried plasma . . . . .	42.0
2 cc. hemophilic blood + 0.1 cc. suspension of dried plasma . . . . .	35.0
2 cc. hemophilic blood + 0.2 cc. suspension of dried plasma . . . . .	28.5

for hemophilic blood tested *in vitro*. Normal plasma has been shown to reduce the coagulation time of hemophilic blood in a quantitative manner (1, 2). There was a marked loss of these properties associated with the type of desiccation described, as shown by Table I.

Normal citrated plasma was then dried by the lyophilic process. This material when redissolved in distilled water showed maximum clot-promoting properties for hemophilic blood *in vitro* (Table II). These observations indicated that the clot-accelerating properties of plasma

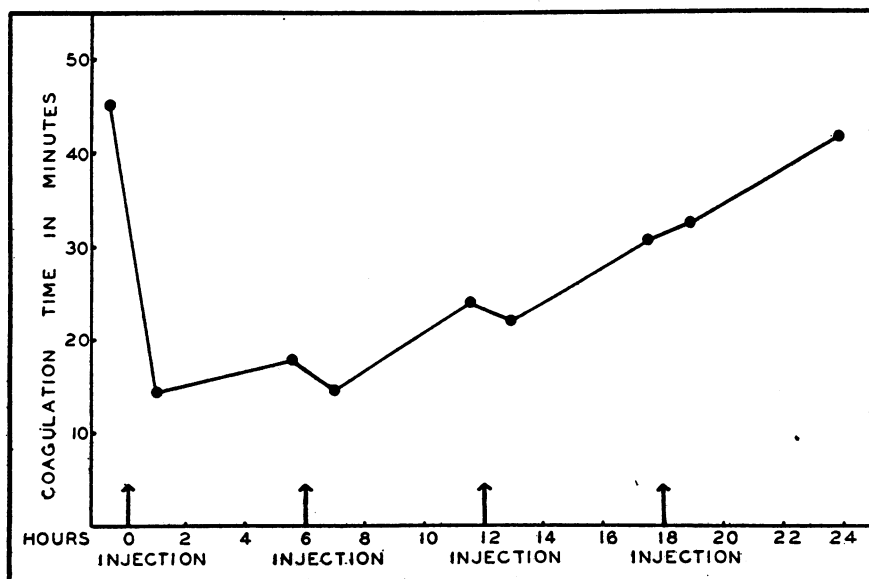


FIG. 1. EFFECT OF MULTIPLE INTRAMUSCULAR INJECTIONS OF GLOBULIN SUBSTANCE ON THE COAGULATION TIME OF THE BLOOD IN HEMOPHILIA

TABLE II

*Effect of lyophilized, citrated, normal human plasma on the coagulation time of hemophilic blood in vitro*

	Coagulation time
	minutes
2 cc. hemophilic blood.....	70.0
2 cc. hemophilic blood + 0.01 cc. lyophilic plasma.....	21.0
2 cc. hemophilic blood + 0.03 cc. lyophilic plasma.....	12.0
2 cc. hemophilic blood + 0.05 cc. lyophilic plasma.....	10.5
2 cc. hemophilic blood + 0.1 cc. lyophilic plasma.....	6.5
2 cc. hemophilic blood + 0.2 cc. lyophilic plasma.....	4.5

were not disturbed by the freezing and drying process used in this technique.

*The effect of parenteral injections of lyophilized plasma.* A single intravenous or intramuscular injection of lyophilized plasma redissolved in 65 cc. of distilled water was given to the same hemophilic patient on four different occasions. In each instance there was a prompt reduction in the coagulation time of the blood to normal or near normal values within one-half hour. This reduction in the coagulation time was maintained for approximately six hours after which it gradually returned to the pre-injection level. Occa-

sionally, the patients experienced some local pain following the intramuscular injection but no hematomas or general systemic reactions occurred.

A standard test dose (65 cc.) of a solution of lyophilized plasma was given intravenously four times at 6-hour intervals to a hemophilic patient. The results were entirely comparable to those shown in Figure 2. The shortened coagulation time was maintained and *a refractory period did not develop* following any of the injections. The observations were repeated except that the plasma was administered intramuscularly. The results were again entirely similar.

*The effect of drying globulin substance by the Flosdorf-Mudd technique.* Globulin substance was prepared in the usual manner except that immediately after centrifugation the moist precipitate was frozen and dried under vacuum by the lyophilic process. This material was then ground, stored in a desiccator over calcium chloride, and as required suspended in the requisite amount of saline solution. The clot-accelerating properties of this preparation for hemophilic blood were tested *in vitro* (Table III) and *in vivo*. Globulin substance dried in this manner retained all of its potency.

One hemophilic subject was given intramuscularly a standard test dose of globulin substance

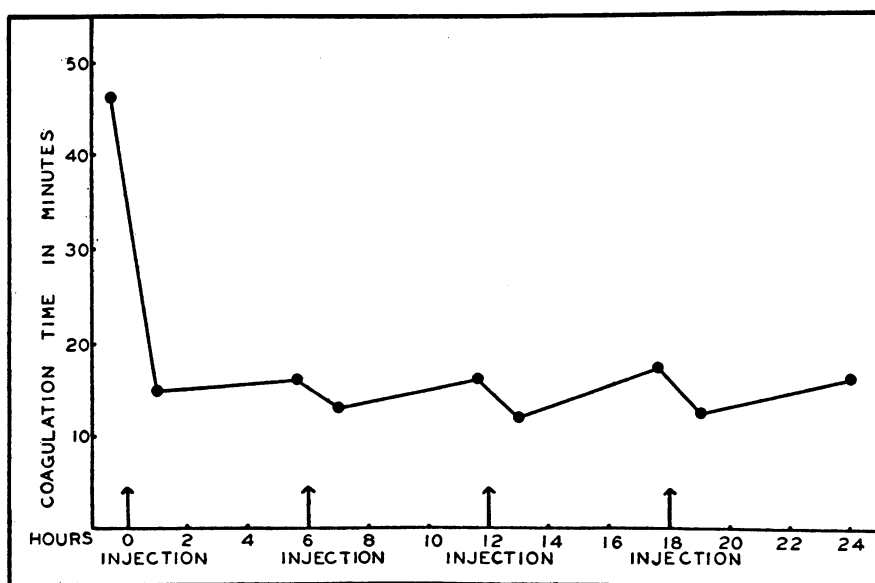


FIG. 2. EFFECT OF MULTIPLE INTRAVENOUS INJECTIONS OF NORMAL CITRATED PLASMA ON COAGULATION TIME OF THE BLOOD IN HEMOPHILIA

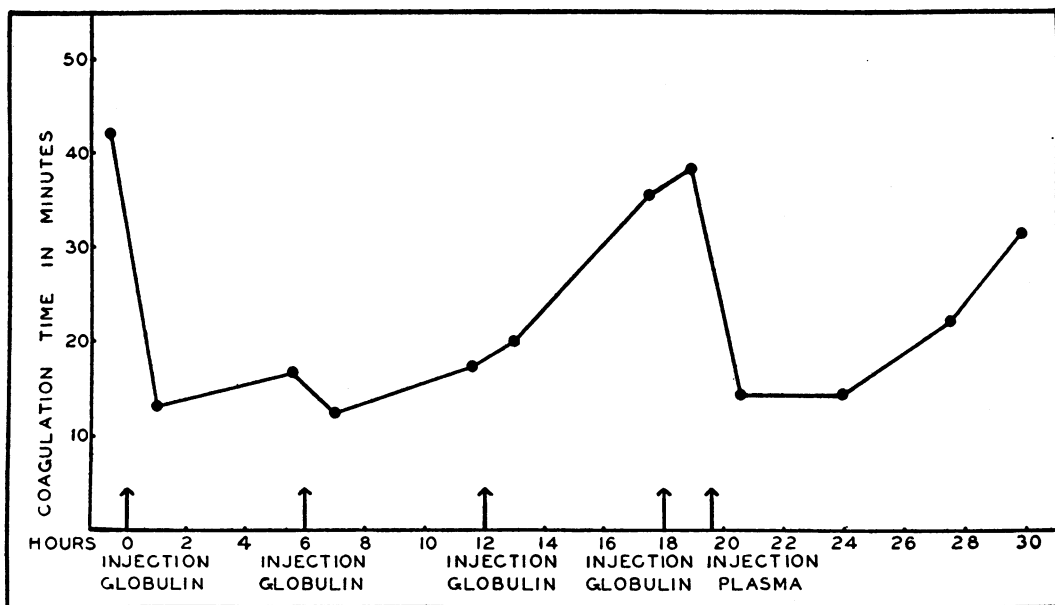


FIG. 3. EFFECT OF A SINGLE INJECTION OF LYOPHILE PLASMA ON THE REFRACTORY PHASE FOLLOWING MULTIPLE INJECTIONS OF GLOBULIN SUBSTANCE IN HEMOPHILIA (ALL INJECTIONS INTRAMUSCULAR)

dried in this manner every 6 hours for 24 hours. After the usual initial reduction in the coagulation time to normal values a refractory phase developed and further injections had little or no effect upon the coagulation time. Data obtained were entirely similar to those expressed graphically in Figure 1. Blood platelet counts taken every 3 hours during this set of observations showed no fluctuations greater than normal.

The effect of an intramuscular injection of lyophilized plasma upon the refractory phase. One hemophilic subject was given a standard test dose of a saline suspension of globulin substance

TABLE III  
Effect of a saline suspension of globulin substance (dried by the lyophilic process) on the coagulation time of hemophilic blood in vitro

	Coagulation time
	minutes
2 cc. hemophilic blood . . . . .	175.0
2 cc. hemophilic blood + 0.01 cc. suspension of globulin substance . . . . .	19.0
2 cc. hemophilic blood + 0.03 cc. suspension of globulin substance . . . . .	8.0
2 cc. hemophilic blood + 0.05 cc. suspension of globulin substance . . . . .	6.5
2 cc. hemophilic blood + 0.1 cc. suspension of globulin substance . . . . .	5.0
2 cc. hemophilic blood + 0.2 cc. suspension of globulin substance . . . . .	3.5

repeated four times at 6-hour intervals. The usual refractory period developed after the first two injections and the blood coagulation time gradually returned to the pre-injection level. The third and fourth injections were entirely without effect on the coagulation time of the blood. Immediately after the fourth injection and at the height of the refractory period when further injections of globulin substance have been shown to be ineffective (2) this subject received a standard test dose of lyophilized plasma intramuscularly. There was a sharp reduction in the blood coagulation time, and it appeared that the refractory phase had been abolished by this single injection of plasma (Figure 3).

#### COMMENT

The fact that many observations have shown that repeated injections of globulin substance into hemophilic patients produced a refractory phase, as indicated by failure of further injections of globulin substance to reduce the coagulation time of hemophilic blood (2), necessitated further investigations to determine the nature of this reaction. Since the protein of the early preparations could have been denatured this condition might be considered a possible cause of the refractory phe-

nomenon. However, repeated injections of lyophilized globulin substance produced a similar refractory phase. Flosdorf and Mudd (3) have shown that the lyophilic process does not modify the protein complexes, and therefore it is not likely that denaturation is responsible for the development of the refractory period.

Earlier studies (2) demonstrated that the concentration of the clot-promoting factor progressively increased in the blood of the injected patient during the development of the refractory period. It was clear, therefore, that the concentration of this material in the blood was such that a reduction of the coagulation time should have taken place if globulin substance was the only material deficient or modified in hemophilic blood. From the rapidity of onset and recovery from the refractory state shown by the present and previous studies the phenomenon could not be a manifestation of an antigen-antibody reaction. Furthermore, there has been no evidence produced in any of our investigations to suggest that a non-coagulable phase occurs during the refractory phase or at any time after the administration of globulin substance.

The present observations show clearly that repeated injections of citrated normal human plasma or lyophilized plasma into hemophilic subjects maintains a reduced coagulation time of blood so long as the injections are continued (Figure 2). These data suggest that such plasmas contain certain substances active in blood coagulation not contained by preparations of globulin substance. This supposition is confirmed by the experience in one case in which lyophilized plasma was injected at the height of the refractory phase with a reduction of the blood coagulation time toward normal limits. The nature of this substance or these substances is at present under investigation. It is possible, however, from an inspection of the data presented in this and in a previous report (2), that a combination between globulin substance and this second substance or substances may play an essential rôle in the reduction of the coagulation time of hemophilic blood *in vivo*. Since globulin substance, like citrated normal plasma, exerts its clot-promoting effect on hemophilic blood in the test tube in a quantitative manner (Table III), it would appear

that under these circumstances the second substance or substances was present in sufficient quantity to satisfy the requirements of increasing amounts of globulin substance. However, during the development of the refractory phase in the patient with hemophilia it is possible that the increasing concentration of globulin substance in the blood finally results in the exhaustion of this second substance. The abolition of the refractory phase with a single injection of plasma is probably due to an increase in titer of this second substance. To what extent the injection of such normal plasma will quantitatively affect subsequent injection of globulin substance is at present under investigation.

#### CONCLUSIONS

1. In hemophilia, repeated injections of lyophilized globulin substance as well as normal globulin substance produced a refractory period after the usual initial reduction in the coagulation time of the blood.
2. Repeated injections of either normal human plasma or lyophilized plasma into hemophiles maintained a shortened blood coagulation time without, however, the development of a refractory phase.
3. The refractory phase can be abolished at its height by a single injection of plasma.
4. Both normal plasma and lyophilized plasma probably contain substances which play a rôle in the reduction of the coagulation time of blood *in vivo* and which are not present in globulin substance preparations.

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