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# THE COAGULATION DEFECT IN HEMOPHILIA. THE EFFECT IN HEMOPHILIA OF INTRAMUSCULAR ADMINISTRATION. OF A GLOBULIN SUBSTANCE DERIVED FROM NORMAL HUMAN PLASMA

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Research Article



# THE COAGULATION DEFECT IN HEMOPHILIA. THE EFFECT IN HEMOPHILIA OF INTRAMUSCULAR ADMINISTRATION OF A GLOBULIN SUBSTANCE DERIVED FROM NORMAL HUMAN PLASMA[1]

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The recent investigations of Patek and Stetson (1)[2] indicate that the defect in coagulation of blood in hemophilia resides in the plasma rather than in the platelets. Patek and Taylor (2, 3)[2] isolated a substance from citrated normal cellular-free plasma, by isoelectric precipitation, with which was associated the clot-promoting factor of normal plasma. This substance was effective, both in vitro and after intravenous injection, in reducing the coagulation time of hemophilic blood. By the same method, a similar substance in approximately the same quantity was obtained from citrated hemophilic plasma, but its clot-accelerating power on hemophilic blood was much less marked. In the absence of definite knowledge concerning the nature of the effective material it was called "globulin substance" which is the nomenclature that will be retained in this communication. The material was thermolabile, insoluble in water, partially soluble in physiological saline solution, and non-diffusible. It was precipitated in optimal amounts between pH 5.9 and 6.4. The material passed readily through a Berkefeld filter without loss of potency and was almost completely inactivated by small excesses of alkali (3). Bendien and Creveld (4) have isolated a substance, by dilution and acidification, from normal serum which they injected intramuscularly and intravenously and report favorable results.

Patek and Taylor's (3) studies imply that in some respects hemophilia is a deficiency disease in which certain factors present in normal cellular-free plasma are either reduced or modified. The present communication reports a study of the effects of intramuscular administration of globulin substance on the blood coagulation time of hemophilic subjects, and on the content of a clot-promoting factor in the blood following such administration.

## METHODS

*Coagulation time.* In general the standard procedure followed was the same as that used in previous studies (1, 3) made in this laboratory. All coagulation times were determined on venous blood. Hypodermic syringes and number 20 steel needles for venepuncture were freely rinsed with physiological saline solution immediately before use. The blood was taken under stasis from arm veins, the tourniquet being removed after the sample was withdrawn. If venepuncture was not immediately successful, another vein was used and another syringe and needle employed. Two cc. of blood so drawn were transferred, after removal of the needle from the syringe, with minimum agitation, to 100 × 13 mm. test tubes having an inside diameter of 11.5 to 12 mm. The tubes were cleansed with concentrated bichromate-sulphuric acid solution and thoroughly rinsed free from this material with distilled water and finally with physiological saline solution. The presence of air bubbles in the blood sample was avoided because they tend to shorten the coagulation time.

When test substances were to be assayed they were pipetted into the test tubes prior to the addition of the blood. Further mixing was not found necessary.

Immediately after the addition of whole blood, duplicate tubes were placed in a water bath at 37.5° C. Agitation of the tubes was avoided. Only one of the duplicate tubes was read from time to time by gently tilting until just before the end point, when both tubes were read. The coagulation time was the interval elapsing from withdrawal of blood to the time when the tubes could be slowly inverted without loss of contents. The times for the two tubes usually checked very closely, and the average time was taken as the true coagulation time. When there was a considerable discrepancy, the longer coagulation time only was recorded. Normal controls by this method, independent of sex, gave values for the coagulation time of venous blood of from 6 to 12 minutes.

*Control period.* The investigation was carried out on seven hemophilic patients between the ages of 16 and 47 years who had been under observation in this clinic for

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

[2] Review of literature here.

some years. Prior to any series of observations the coagulation time was always determined several times for 48 hours. If the coagulation time fluctuated during the control period, no test observations were made upon that subject. Patients with active or recent hemorrhage were not included in this study because the resulting spontaneous reduction of the coagulation time is often considerable.

*Preparation of globulin substance.* Blood was collected from normal individuals by sterile technique. The blood was citrated to a final concentration of 0.25 per cent sodium citrate and allowed to stand in the ice box at 5° to 10° C. for 48 hours. The plasma was pipetted off, and filtered through one thickness of Number 5 Whatman's filter paper by Buchner filtration, and finally any remaining cellular elements were removed from the filtrate by passage through a Berkefeld V filter. Plasma so treated was found to retain its full coagulating potency for at least six weeks.

To avoid the possible introduction of additional calcium, the Berkefeld filtrate was diluted with 10 volumes of distilled water rather than with tap water as previously described (3). The globulin substance was then precipitated by acidifying to pH 5.9 to 6.1 by the addition of one per cent acetic acid. The acid was added slowly with constant stirring and a final pH adjustment made with the aid of the quinhydrone electrode. Approximately 20 cc. of one per cent acetic acid are required to reduce the pH of 100 cc. of normal plasma, diluted 1 to 10 with water, to 6.0.

The precipitate of globulin substance was removed by centrifuging at 2,000 r.p.m. for 20 minutes and the supernatant liquid discarded. The globulin substance was removed without dilution to a vacuum desiccator and dried. The material was then finely ground and stored in weighing bottles in a desiccator over calcium chloride. Approximately 450 mgm. of the final dried product were obtained from each 100 cc. of plasma.

*Preparation of globulin substance for parenteral use.* When globulin substance was to be injected, a weighed amount of dried material was triturated with a small amount of isotonic saline and then diluted with isotonic saline to the calculated original volume of the plasma from which it was derived. This suspension was then centrifuged at 2,000 r.p.m. for 10 minutes and the supernatant fluid passed through a Berkefeld V filter. The final filtrate showed no loss of potency when checked against the original saline suspension.

*Test for potency of globulin substance.* Each lot of globulin substance was tested in vitro for clot-promoting powers. Ten milligrams of dried globulin substance were triturated with a few drops of isotonic saline to obtain an even suspension. This suspension was diluted to a total volume of 2 cc. with physiological saline solution after which it was centrifuged at 2,000 r.p.m. for 10 minutes. Various amounts of the clear supernatant fluid were then pipetted in duplicate into the bottom of the standard coagulation tubes and 2 cc. of hemophilic blood added, as described above. As a control, varying amounts of isotonic saline were used instead of the saline suspension of globulin substance. In all experiments the addition of pure saline solution was without effect.

## EXPERIMENTAL

*The quantitative nature of the action of globulin substance on the coagulation of hemophilic and normal blood in vitro.* In previous studies (3) there was some indication that there existed a quantitative relationship between the amount of globulin substance added to blood and its effect on coagulation time. These observations were based on the increase in coagulation time of hemophilic blood *in vitro* when suspensions of globulin substance were added in increasing dilutions. Using the technique described above for testing globulin substance, it has been possible to confirm and amplify these findings. Table I shows examples of the response repeatedly observed when globulin substance was added in various dilutions to hemophilic and normal blood *in vitro*. The addition of only 0.01 cc. of a suspension of globulin substance to 2 cc. of hemophilic blood resulted in a definite reduction of coagulation time. This corresponds to a dilution of 1:200. With increasing concentration of globulin substance, the effectiveness was increased. The results were similar with normal blood. In the example pre-

TABLE I

*Quantitative effect of globulin substance prepared from filtered normal plasma on normal and hemophilic blood in vitro*

| | Coagulation time | |
|---|---|---|
| | Normal | Hemophilic |
| | *minutes* | *minutes* |
| 2 cc. control blood | 8.0 | 85.0 |
| 2 cc. control blood + 0.01 cc. suspension of normal globulin substance | 8.0 | 19.0 |
| 2 cc. control blood + 0.03 cc. suspension of normal globulin substance | 7.0 | 9.0 |
| 2 cc. control blood + 0.05 cc. suspension of normal globulin substance | 5.5 | 8.0 |
| 2 cc. control blood + 0.1 cc. suspension of normal globulin substance | 3.5 | 4.5 |
| 2 cc. control blood + 0.15 cc. suspension of normal globulin substance | 3.5 | 3.5 |

sented, the coagulation time of the normal blood was reduced from 8 to 3.5 minutes, while with the same amount of globulin substance, the coagulation time of the hemophilic blood was reduced from 85 to 3.5 minutes.

Globulin substance prepared from hemophilic plasma had much less activity in accelerating clot formation of hemophilic blood than that derived from normal plasma. These observations confirm those already published (3), namely that globulin substance from hemophilic plasma is not without coagulation-promoting power, but that this property is reduced as compared to that of globulin substance prepared from normal plasma.

In an attempt to determine whether hemophilic patients with very long coagulation times had a lower titer of active factor in their blood than those with shorter coagulation times, tests were made with various dilutions of plasma from such patients. While such dilutions of plasma always showed an effect in reducing the coagulation time of another hemophilic blood, no conclusive data were obtained to show that the degree of reduction correlated with the coagulation time of the patient from which the plasma was obtained.

*The effect of intramuscular injection of globulin substance.* Patek and Taylor (3) have shown that the intravenous injection of normal globulin substance in amounts varying from 1 to 1.25 grams into hemophilic subjects was followed by a prompt fall in the coagulation time of the blood. In all subjects studied the coagulation time returned to pre-injection levels in from 24 to 48 hours after the injection. In order to carry out certain parts of the present investigation it became necessary to use other than the intravenous route of administration.

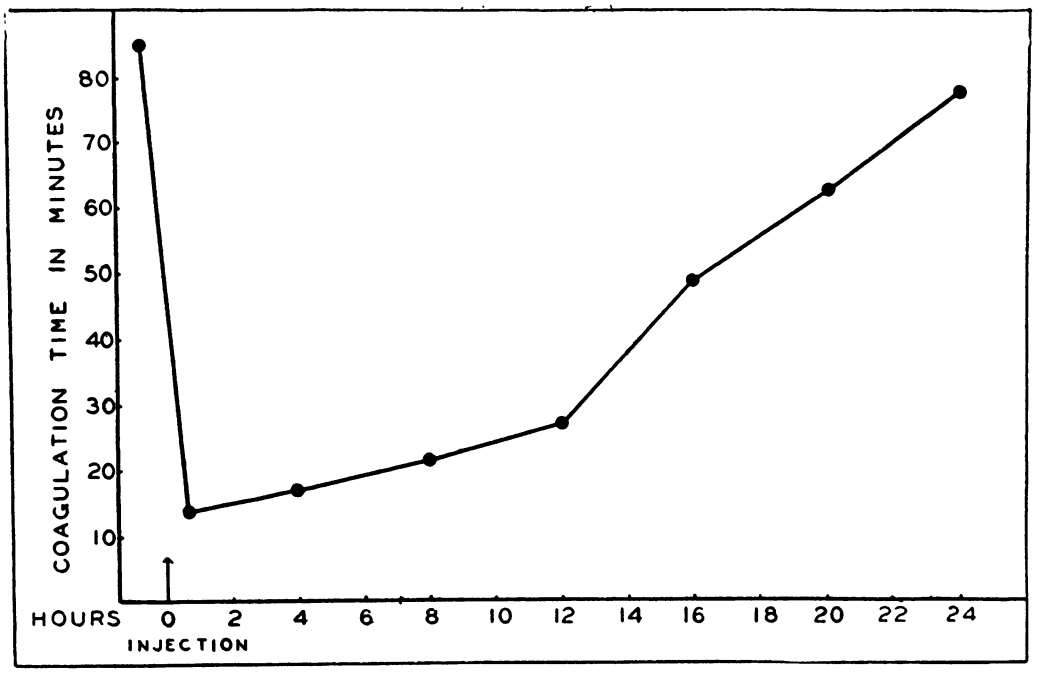


FIG. 1. EFFECT OF A SINGLE INTRAMUSCULAR INJECTION OF "GLOBULIN SUBSTANCE" ON COAGULATION TIME OF HEMOPHILIC BLOOD

The intramuscular administration of globulin substance was free from even the slight systemic effect that accompanies the intravenous administration (3). No local reactions or hematomas developed. All injections were made into the anterior thigh muscles. Intradermal skin tests with globulin substance were uniformly negative.

A *standard test dose* was decided upon after consideration for the effectiveness of the globulin substance and the inadvisability of giving too large a volume of fluid intramuscularly to a hemophilic patient. Three hundred milligrams of globulin substance was suspended in 75 cc. of

isotonic saline solution. After centrifuging and Berkefeld filtration, 65 cc. were available for injection. This standard test dose represented about one-third of the dosage previously given intravenously (3).

Four hemophilic patients with coagulation times between 35 and 85 minutes were injected intramuscularly with a single standard test dose of globulin substance. Figure 1 shows the results obtained in a case in which the coagulation time was followed at 4-hour intervals for a 24-hour period after injection. Comparable data were obtained on the remaining cases. There was a favorable response in all instances. There was a sharp fall in the coagulation times to minimum values in from 30 minutes to 1 hour after injection, which were sustained for several hours. The coagulation times returned to pre-injection levels in 24 hours.

*The effect of repeated parenteral injections of globulin substance.* Two intramuscular injections of a standard test dose of globulin substance were given to five subjects. Following the *initial* injection there was a prompt drop in the coagulation time in all cases similar to that recorded in Figure 1. However, when the *second* injection was given at any time within a 7-hour period there was not more than a slight further decrease in the coagulation time. A typical response is shown in Figure 2 (I. M.). In one instance a second injection failed to reduce the coagulation time to the point attained by the initial injection.

A hemophilic patient was injected with a standard test dose of globulin substance by the intravenous route. The results are plotted in Figure 2 (I. V.). There was an immediate abrupt fall in the coagulation time. A slight further drop followed a second intravenous injection 7 hours later. It may be observed (Figure 2) that the results obtained by the use of the intramuscular route are entirely similar to those obtained after intravenous injections.

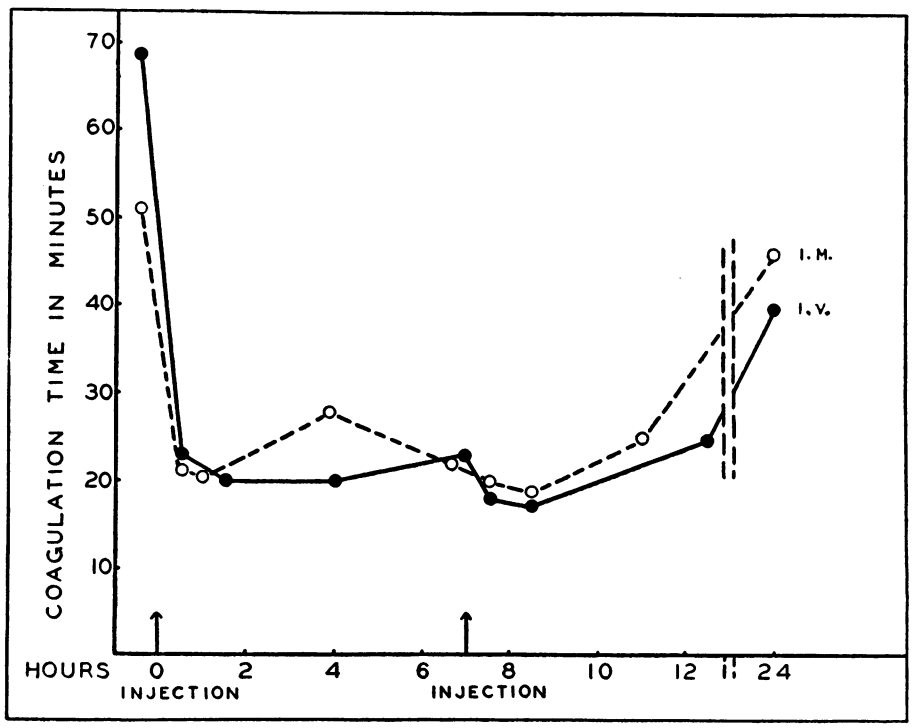


FIG. 2. EFFECT OF REPEATED INTRAMUSCULAR AND INTRAVENOUS INJECTIONS OF "GLOBULIN SUBSTANCE" ON COAGULATION TIME OF HEMOPHILIC BLOOD

Observations were made on one hemophilic subject to determine the effects of repeated intramuscular injections of standard test doses of globulin substance over a 24-hour period. Injections were given at 6-hour intervals. Following the *initial* injection there was the usual prompt

fall in the coagulation time (Figure 3). The *second* injection reduced the coagulation time to the level obtained by the initial injection. However, the *third* injection failed to reduce the coagulation time to the point attained by either of the first two injections. The *fourth* injection did not effect the coagulation time which continued to rise to the pre-injection level.

It is known from three sets of observations that if a hemophilic subject is re-injected either intramuscularly or intravenously with globulin substance 24 hours after the last injection, that the entire cycle of effect on his coagulation time repeats itself.

by observing its effect on a second hemophilic blood *in vitro*. Immediately before and 1 hour after each injection of the standard test dose of globulin substance, blood was drawn and citrated to a final concentration of 0.25 per cent sodium citrate. In each instance 0.1 cc. of the whole citrated blood was mixed with 2 cc. of blood from a second control hemophilic patient. This control patient had a coagulation time of 127 minutes which did not vary significantly during the period of observation. The results are given in Figure 4. This figure shows graphically the coagulation time of the control patient's blood after adding citrated whole blood obtained from the injected

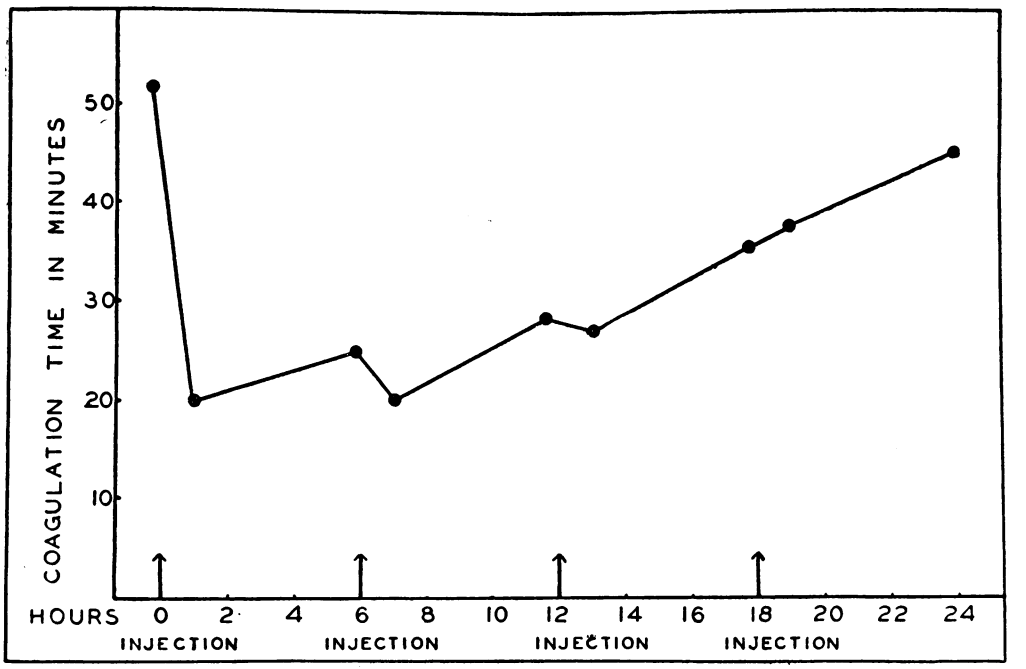


FIG. 3. EFFECT OF MULTIPLE INTRAMUSCULAR INJECTIONS OF "GLOBULIN SUBSTANCE" ON COAGULATION TIME OF HEMOPHILIC BLOOD

*Changes in the clot-promoting power of hemophilic blood and plasma following the intramuscular injection of globulin substance.* Because of the evidence that frequently repeated injections of globulin substance had a diminishing effect in reducing the coagulation time of hemophilic blood, it became desirable to attempt to determine the amount of clot-promoting factor in the circulating blood under these circumstances.

An approximation of the coagulant activity of the whole blood in the subject receiving repeated intramuscular injections (Figure 3) was obtained

patient. In other words, it may be considered that *the data indicate the changes in concentration of clot-promoting factor* present in the injected patient's blood. Figure 4 shows that the clot-accelerating power of the blood was increased after injections of globulin substance. In comparing Figure 3 with Figure 4 the important conclusions are that the coagulation time has risen in spite of repeated injections, while the concentration of clot-promoting material has increased in the patient's blood.

Since relatively large amounts of blood are re-

quired to prepare globulin substance, the actual preparation of the material from the patient every few hours is not feasible. However, it has been shown by dialysis experiments that the coagulant activity of plasma resides in the globulin substance

philic plasma possessed a slight clot-accelerating power when added to another hemophilic blood. However, it was not possible to demonstrate that the severity of the case as judged by the length of the coagulation time, could be correlated with

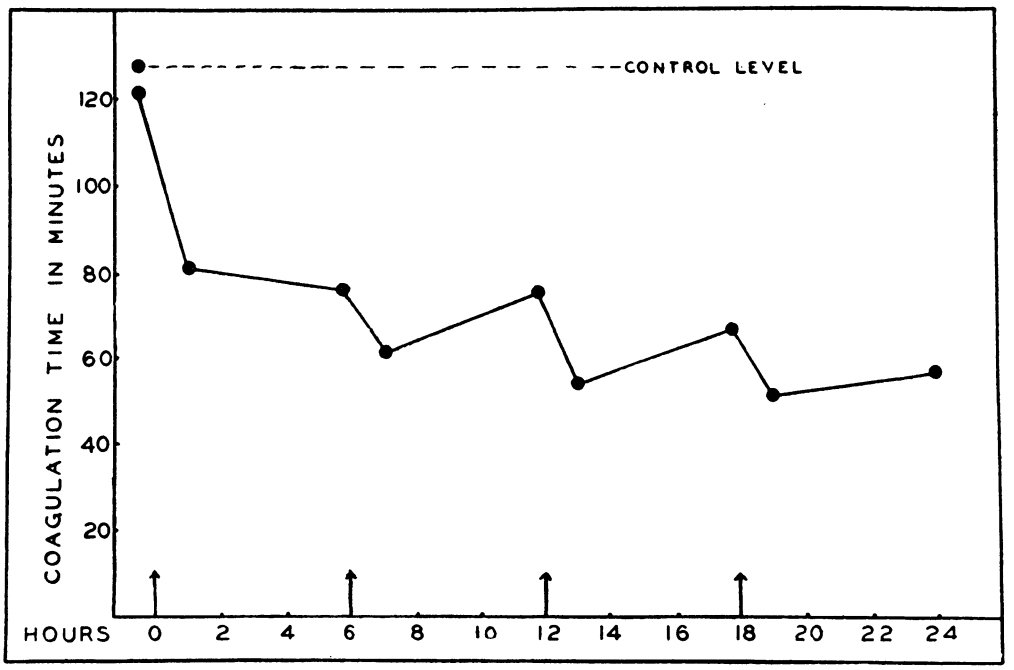


FIG. 4. EFFECT OF ADDING WHOLE BLOOD FROM A HEMOPHILIC SUBJECT INJECTED WITH "GLOBULIN SUBSTANCE" AT TIMES INDICATED, ON COAGULATION TIME OF CONTROL HEMOPHILIC BLOOD IN VITRO

(3). Hence the clot-promoting activity of plasma is an indirect measure of globulin substance. By the use of a similar technique of titration against a control hemophilic blood *in vitro* mentioned above, the increase in concentration of the clot-promoting factor was demonstrated in the cellular-free plasma following the intramuscular injection of globulin substance in three additional cases of hemophilia. In each instance the rise in coagulation time of the injected patient's blood, following the favorable response, occurred before the concentration of the coagulant factor in the plasma reached its maximum.

## SUMMARY AND DISCUSSION

The quantitative nature of the action of normal globulin substance on hemophilic blood *in vitro* has been indicated (3) and is confirmed by the studies presented here. In all instances hemo-

the degree of the clot-promoting activity of the plasma.

When a suspension of globulin substance derived from 300 mgm. of dried material was injected intramuscularly in hemophilia, the response was a sharp drop in the coagulation time which was sustained at low levels for approximately 8 hours and returned to the pre-injection level within 24 hours. The effectiveness of globulin substance is not increased by intravenous administration.

The observations with repeated intramuscular or intravenous injections of globulin substance strongly indicates that there is a refractory phase for repeated injections. It appears that during such a phase, the coagulation time of the blood in hemophilia increases although the concentration of globulin substance in the circulating plasma is not diminished. The refractory period is not

longer than 24 hours since an injection at that time again gives the optimal effect.

The refractory period cannot be associated with tissue fixation of the injected material since similar effects are experienced when second injections are made intravenously. Nor is there failure of absorption as proven by the method described for measuring the concentration of clot-promoting factor in the blood stream. The time factor would indicate that the phenomenon is not one of an antigen-antibody reaction. The actual cause of the refractory period and its nature awaits further investigation.

Mellanby (5) showed that the slow injection of small quantities of certain clot-accelerating snake venoms in animals produced a non-coagulable period. Mills (6) gave repeated small injections of tissue extracts to animals and observed a negative phase. These investigators ascribed the non-coagulable phase to defibrination of the blood. Eley, Green and McKhann (7) working with placental extract report no non-coagulable period in animals with the dose employed although they quote Sakurai as having noted such a phenomenon. However, there is no evidence in our data to indicate that a non-coagulable phase follows the administration of globulin substance in hemophilia. In no instance did the coagulation time of the blood rise above the initial level.

## CONCLUSIONS

1. Globulin substance prepared from normal human plasma accelerates clot-formation of hemophilic and normal blood *in vitro* in a quantitative manner.

2. Globulin substance may be administered intramuscularly in hemophilia with reduction of the coagulation time similar to that produced by its intravenous injection.

3. Following the *initial* injection of globulin substance in hemophilic subjects, a refractory phase is established. During this period the coagulation time is little affected by subsequent injections although it has been shown that the concentration of the clot-accelerating material is progressively increased in the circulating blood of the injected patient. Recovery from this state is complete in 24 hours.

4. There is no evidence that a non-coagulable phase occurs following the administration of globulin substance.

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