Congenital Afibrinogenemia
A Study of Some Basic Aspects of Coagulation

By Benjamin Alexander, Robert Goldstein, Lester Rich, Anne G. Le Böloc'h, Louis K. Diamond and Wayne Borges

The coagulation mechanism has been arbitrarily divided into three stages: the first involving the elaboration of thromboplastin activity, the second concerned with the conversion of prothrombin to thrombin, and the third dealing with the interaction of thrombin with fibrinogen to yield the fibrin clot. Although the over-all reaction normally proceeds by a series of integrated mechanisms some of which are known to influence the others, considerable knowledge has been gained by study of each of these phases in isolated systems. The question always arises as to whether, under such circumstances, variables and artifacts may have been introduced which might lead to erroneous interpretations.

From the investigator's point of view, the most ideal conditions are those which, provided by nature, require minimal manipulation. Such conditions for investigating certain basic aspects of coagulation were afforded by three subjects who exhibited only one abnormality, namely, congenital afibrinogenemia.

The observations in this communication pertain to (1) the coagulation sequence with particular reference to changes in platelets, antihemophilic factor (AHF), prothrombin, Ac-globulin, and SPCA; (2) the relation between antithrombin, heparin co-factor and fibrinogen; (3) the relation between fibrinogen concentration and one-stage prothrombin activity. The studies elucidate many reactions of the clotting mechanism which are patently independent of the thrombin-fibrinogen-fibrin interaction.

Three subjects* were studied, a 4 1/2 year old boy (C. M., no. 333043), a 5 year old boy (E. J., no. 320138), and his 1 1/2 year old sister (D. C. no. 314690). They had had repeated spontaneous and post-traumatic hemorrhages into skin, muscles, joints, and loose tissue structures. The parents of E. J. and D. C. were first
Cousins, but there was no familial history of hemorrhagic disease. No abnormalities could be detected in the blood of any of the parents. The patients' bloods remained fluid indefinitely in ordinary glass clotting tubes and failed to clot upon the addition of thrombin or thromboplastin. Their plasmas exhibited no precipitate when heated at 52 C., and orthodox methods of measuring fibrinogen showed none since no clot could be obtained. Recently developed immunochemical tests, applied to the blood of two of the subjects (C. M. and E. J.), showed no more than 1.2 mg. of fibrinogen or fibrinogen-like material per 100 ml.1

Exhaustive study of the patients revealed no other hematologic or other clinical abnormalities. The infusion of normal blood, plasma, or plasma fraction I was repeatedly effective in restoring normal hemostasis and rectifying the clotting defect.

**Table 1.—Plasma Antihemophilic Activity in Congenital Afibrinogenemia (E. J.)**

<table>
<thead>
<tr>
<th>Amount plasma* added to 2.0 ml. hemoph. blood</th>
<th>Clotting time (Lee-White, 37 C.) (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>T. K.</td>
</tr>
<tr>
<td>Date: 7/21/47</td>
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<tr>
<td>0.001</td>
<td>50</td>
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<td>10</td>
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<td></td>
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<tr>
<td>0.0005</td>
<td>210</td>
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<tr>
<td>0.001</td>
<td>35</td>
</tr>
<tr>
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<td>26</td>
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<tr>
<td>0.10</td>
<td>19</td>
</tr>
<tr>
<td></td>
<td>16</td>
</tr>
</tbody>
</table>

* The test plasmas were suitably diluted in physiologic saline to provide the amount of plasma indicated in a volume of 0.1 ml.
LABORATORY STUDIES

Antihemophilic Factor (AHF), Relationship to Fibrinogen

Aim. The antihemophilic principle of normal human plasma is found in substantial amounts in plasma protein fractions rich in fibrinogen. Attempts to separate these moieties have invariably failed or resulted in almost complete loss of antihemophilic activity. The availability of plasma virtually devoid of fibrinogen presented a unique opportunity to establish whether these two clotting factors are actually distinct, or whether the inability to separate the two entities indicated that AHF of plasma was but another physiologic function of normal fibrinogen and, accordingly, inseparable.

The data include quantitation of the AHF of human plasma congenitally afibrinogenemic or defibrinogenated by heat or by addition of thrombin, and of plasma from dogs with experimentally induced afibrinogenemia.

Methods. AHF was measured in some experiments by a method previously described, in which the clot accelerating effect of plasma was observed on freshly shed blood from well standardized hemophiliacs. In other experiments, assay was accomplished by measuring the improved prothrombin consumption of hemophilic blood induced by the in vitro addition of the test material, com-

<table>
<thead>
<tr>
<th>Material added to 2.0 ml. hem. blood*</th>
<th>Clotting time (Lee-White, 37 C.) (min.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>hemoph. R. R.</td>
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<tr>
<td>0.10 ml saline</td>
<td>96</td>
</tr>
<tr>
<td>0.001 ml norm. plas.</td>
<td>25</td>
</tr>
<tr>
<td>0.001 ml afib. plas.</td>
<td>23</td>
</tr>
<tr>
<td>0.001 ml afib. ser.</td>
<td>63</td>
</tr>
<tr>
<td>0.005 ml norm. plas.</td>
<td>19</td>
</tr>
<tr>
<td>0.005 ml afib. plas.</td>
<td>17</td>
</tr>
<tr>
<td>0.005 ml afib. ser.</td>
<td>44</td>
</tr>
<tr>
<td>0.010 ml norm. plas.</td>
<td>15</td>
</tr>
<tr>
<td>0.010 ml afib. plas.</td>
<td>10</td>
</tr>
<tr>
<td>0.010 ml afib. ser.</td>
<td>32</td>
</tr>
<tr>
<td>0.001 ml heated† norm. plas.</td>
<td>38</td>
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<tr>
<td>0.001 ml heated† afib. plas.</td>
<td>30</td>
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<tr>
<td>0.005 ml heated norm. plas.</td>
<td>28</td>
</tr>
<tr>
<td>0.005 ml heated afib. plas.</td>
<td>23</td>
</tr>
<tr>
<td>0.010 ml heated norm. plas.</td>
<td>28</td>
</tr>
<tr>
<td>0.010 ml heated afib. plas.</td>
<td>21</td>
</tr>
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</table>

* Material suitably diluted in physiologic saline to contain the amounts specified in a volume of 0.1 ml.
† Plasma heated at 56 C. for 3 min., centrifuged, and the supernatant suitably diluted with physiologic saline. Heating of the afibrinogenemic plasma induced no precipitation.
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pared with the effect of normal human plasma simultaneously tested, according to the method of Graham, et al.,7,8 slightly modified by us.

Results. It is evident from the data (tables 1, 2, 3) that congenital afibrino-

**Table 3.—Antihemophilic Activity of Afibrinogenemic (C.M.) Blood Determined by Prothrombin Consumption Technic**

Hemophilic blood, drawn with an Arquad (Armour Co.) coated needle and a siliconized syringe replacing a regular glass syringe after free flow of blood was assured. Two ml. of blood were placed in a clotting time ordinary glass test tube containing test material, and a stopper was inserted consisting of rubber covered with Parafilm (a plastic film obtained from Marathon Corp, Menasha, Wisc.). The mixture was gently inverted thrice and kept at 37 C. for one hour. Citrate was then added (1 vol. of 2.5 per cent sod. cit. to 9 vol. blood), and the mixture stirred with a silicone coated glass rod. The prothrombin content of the plasma and serum was determined by both the one-stage16 and the two-stage procedure.17 All tests were done in duplicate.

**Table 4.—Antihemophilic Activity of Normal Plasma Defibrinated by Heat**

Citrated plasma heated at 55 to 56 C. for 2½ minutes. Three minutes elapsed before the plasma, in the waterbath, attained the desired temperature. After heating, the plasma was chilled to 10 C. (within ½ min.) by immersion in an ice bath, and then was centrifuged at 1000 rpm for 5 minutes. The supernatant was separated and promptly tested.

**Material suitably diluted with physiologic saline to contain the amounts of material indicated, in 0.1 ml.

† The afibrinogenemic plas. had 247 units proth./ml. by the two-stage method, 83% by the one-stage procedure. The afib. oxal. ser. had 9 units proth./ml.; 15%, one-stage. This serum was derived from the afibrinogenemic blood allowed to stand in a glass test tube for 1 hr. at which time it was oxalated.

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The afibrinogenemic plasma was normal in its ability to correct the clotting defect of human hemophilic blood. It may therefore be concluded that the plasma contained normal amounts of AHF. These observations, in agreement with the findings of Graham and his associates it that afibrinogenemic plasma from dogs with in-
duced liver disease are fully corrective of the clotting defect in canine hemophilia, support the concept that antihemophilic globulin is distinct from fibrinogen.

Attempts to explore this question further with normal human blood defibrinogenated artificially have thus far failed. Defibrination of plasma by heat resulted in a marked loss of antihemophilic activity (tables 2, 4), findings which are in agreement with those of Lewis and colleagues. Apparently the decrease was not entirely due to precipitation of denatured fibrinogen since considerable (although perhaps not as much) destruction occurred also in heated afibrinogenemic plasma.

Defibrination accomplished by addition of thrombin to whole plasma (5 U. per ml.), also results in almost complete loss of antihemophilic activity (table 5). The same supervened when the plasma was diluted tenfold prior to the thrombin addition (1 U. to 10 ml. of diluted plasma), a step introduced to minimize absorption or inclusion of AHF by the fibrin clot. The losses resulting from de-

<table>
<thead>
<tr>
<th>Added to 2.0 ml. hemophilic blood</th>
<th>Resid. Serum proth. (units per ml.)</th>
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<tr>
<td>0.1 ml. saline</td>
<td>210</td>
</tr>
<tr>
<td>0.05 norm. plas.</td>
<td>39</td>
</tr>
<tr>
<td>0.05 norm. plas. defib.*</td>
<td>180</td>
</tr>
<tr>
<td>0.05 afib. plas. plus thrombin.*</td>
<td>210</td>
</tr>
</tbody>
</table>

* Oxalated plasma, pooled from five normal subjects, was defibrinated by the addition of 5 units (in 0.1 ml. physiologic saline solution) of Parke, Davis Topical Thrombin to 1.0 ml. of plasma. The supernatant was separated from the clot, incubated at 37 C. for 1/2 hour, and subsequently tested. Oxalated plasma from afibrinogenemic C. J. was handled similarly. No clot formed upon the addition of thrombin. The same plasma, not treated with thrombin, contained normal antihemophilic activity (fig. 1).

That this discrepancy is not referable to species difference is indicated by the following experiment: A medium-sized dog was rendered afibrinogenemic by the slow (1 1/2 hr.) intravenous infusion of about 2500 units of bovine thrombin (Parke, Davis, Co.) in 150 ml. of physiologic saline solution. At the end of the infusion the blood was incoagulable. A sample of citrated plasma obtained immediately before and after the thrombin infusion was added to freshly shed human hemophilic blood (T. K.) which had a control clotting time of 42 minutes. A mixture of 2.0 ml. hemophilic blood with 0.001 ml. of canine plasma (before thrombin) clotted in 12 minutes; a mixture with 0.001 ml. of afibrinogenemic canine plasma clotted in 36 minutes. These data indicate a profound loss in antihemophilic activity (against human hemophilia) consequent to the thrombin infusion.
Disappearance of AHF during Coagulation

Thus, defibrination of human plasma by thrombin or heat induces substantial losses in AHF. Normal plasma which undergoes spontaneous coagulation, thereby becoming afibrinogenemic, similarly loses most of its antihemophilic activity. One cu. mm. of normal serum, separated from clotted blood 10 minutes after blood was shed and tested 35 minutes later, lowered the clotting time of 2 ml. of known hemophilic blood (R. R.) from 140 minutes to 69 minutes,* in contrast to the parent plasma which reduced it to 26 minutes. Oxalation of the serum promptly after separation made no difference.

![Graph A](image1.png)

**Fig. 1.—Fate of antihemophilic factor during exposure of congenital afibrinogenemic (C.M.) blood to glass surface.**

Chart A indicates improvement in the prothrombin consumption of hemophilic blood, induced by the in vitro addition of varying amounts of normal plasma. Chart B shows the prothrombin consumption of the same hemophilic blood to which has been added a fixed amount (0.05 ml.) of afibrinogenemic plasma, or "serum" obtained at intervals during exposure of the afibrinogenemic blood to glass. The antihemophilic activity of the specimens plotted in B was calculated by interpolating the observed residual serum prothrombin values (of the hemophilic blood-afibrinogenemic serum mixtures) from chart A. It should be noted that 0.05 ml. of the afibrinogenemic plasma had the same clot-promoting effect on hemophilic blood as an equivalent amount of normal plasma.

Prothrombin determined by the two-stage method.17

Although it is possible that the disappearance of AHF was referable to deposition of fibrin, this seems most unlikely in view of the observations on afibrinogenemic blood. One hour after such blood was shed, the "serum" contained a negligible amount of AHF compared with its parent plasma (table 3). This is in agreement with the findings of Graham, Penick, and Brinkhous10 in the canine species. The same was true of serum obtained from citrated afibrinogenemic plasma which was recalcified (table 2). It may therefore be concluded that the disappearance of AHF during coagulation is not related to fibrinogen-fibrin

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* The slight amount of residual activity is readily explained by the SPCA in the serum which is known to accelerate the coagulation of hemophilic blood.10a
conversions. On the other hand, losses induced by heating plasma to 56 C. may be due in part to precipitation of denatured fibrinogen since heated afibrinogenemic plasma appears to retain more antihemophilic activity than normal plasma similarly treated (table 2).

According to Graham, et al., bon the rate of AHF disappearance during the coagulation of canine blood is closely related to the velocity of prothrombin consumption. This suggests that elaborated thrombin may destroy, or otherwise inactivate, AHF. This possibility, explored by the addition of thrombin to afibrinogenemic plasma, is supported by the data (table 5).

It was of interest to determine the speed with which human AHF disappears during coagulation. Venous blood was drawn from the afibrinogenemic subjects with an Arquad* coated needle and silicone coated syringe. After the needle was inserted into the vein directly, the first few milliliters of blood were discarded by disengaging the syringe, and another coated syringe was applied to withdraw the sample tested. Aliquots of blood were placed in uncoated glass clotting tubes, and at specified intervals citrate was added (1 vol. 2.5 per cent sodium citrate to 9 vol. blood) to stop the clotting process. Within 5 minutes after the afibrinogenemic blood was exposed to a foreign surface, approximately 80 per cent of its antihemophilic activity disappeared (fig. 1); in 10 minutes it was practically all consumed. Rapid disappearance of AHF during the coagulation of canine blood has been described also by Graham and colleagues, but apparently it proceeds much more rapidly in man.

**Platelet Changes during “Coagulation” of Afibrinogenemic Blood**

Aim: It is well known that when blood is exposed to a foreign surface, the platelets progressively become adhesive, agglutinate, and lyse. The factors which initiate or influence this reaction, known as “viscous metamorphosis,” remain obscure. According to some investigators the conversion of fibrinogen to fibrin plays an important role in these changes. There is considerable evidence, however, to indicate that platelet agglutination is independent of this step. Piniger and Prunty describe platelet metamorphosis and white thrombi (platelet agglutinates) in a congenital afibrinogenemic blood which was exposed to glass surfaces.

Method and results: This question was explored by making observations on platelet morphology and number after afibrinogenemic blood was shed. In figure 2 are presented drawings of sequential changes in platelet morphology at specified intervals during exposure to a glass surface.

Observations were also made following recalcification of citrated plasma derived from the same blood by the same technic. Within 3 minutes some of the platelets became swollen, showed tails and cobwebs. Some clumping was also evident but these changes were not pronounced. At 5 minutes many platelets vanished, and there suddenly appeared some free dark spots (granulomeres?) exhibiting Brownian movement. At 8 minutes the platelets appeared coalesced into large clumps (comparable to the 20’ drawing of figure 2), and a few very faint shreds (trace fibrin?) appeared in the plasma. These findings agree entirely

* A nonwetting agent supplied by Armour Co., Chicago.
with those described by Pinniger and Prunty. The sequence seemed to have proceeded somewhat faster in the recalcified citrated plasma than in the whole freshly shed blood.

In figures 3 and 4 are presented platelet counts at intervals after the blood was withdrawn. Citrate was added to aliquots of the freshly shed blood to stop

"coagulation" at specific intervals. Figure 3 also includes observations simultaneously obtained on hemophilic blood exposed to glass tubes, and on normal blood in uncoated as well as siliconized tubes. It is evident that the platelets agglutinate, and most of them disappear, when afibrinogenemic blood is exposed

Fig. 2.—Platelet alterations during "coagulation" of afibrinogenemic (E.J.) blood. Drawings of platelets as visualized through phase contrast microscope. Magnification 97X.

Blood taken with Arquad coated needle, silicone coated syringe, syringe disengaged and first few ml. of blood discarded, and fresh siliconized syringe used. Aliquots (2 ml.) of blood placed in clean ordinary glass clotting tubes (100 mm. x 11 mm.) and anticoagulant (sodium citrate, 1 vol. to 9 vol. of blood) added at specified intervals. Tubes kept at 37 C. for 1 hour, then refrigerated, and centrifuged 5 hours later at 500 rpm. The supernatant platelet-rich plasma were then visualized on siliconized slides and cover slips. Drawings are of forms showing most marked alterations. C represents plasma citrated immediately upon withdrawal of blood; 2', citrate added 2 min. after blood exposed to glass; 4', citrate added 4 min. after blood exposed to glass, etc., for 6', 10', 15', 20', 30', and 60'.

2'—platelets generally larger than in C.
4'—no changes over 2' sample.
6'—more swelling of platelets and more spicule formation.
20'—distinct and massive clumping and some very fine strands of what might be trace fibrin deposit.
30', 60'—marked agglutination and loss of distinct platelet outline.

Fig. 3.—Clumping and disappearance of platelets during coagulation. Blood samples handled as described in figure 2. Platelets enumerated on blood samples at specified intervals by direct Rees-Ecker method. Degree of platelet clumping as observed in the counting chamber designated roughly from 1+ to 4+.
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to a glass surface,* just as in normal blood. These changes, becoming first evident within a few minutes after blood is shed, are considerably retarded by siliconed surfaces and are markedly delayed in hemophilia. It is of interest that a substantial number of platelets, most of them agglutinated, are still demonstrable even after 50 minutes.

Role of Thrombin in Platelet Agglutination and Lysis

It may now be concluded that viscous metamorphosis and lysis of platelets proceeds via mechanisms independent of the fibrinogen-fibrin conversion. According to Quick,15 thrombin is the sole agent responsible for these changes. This concept is still unsupported by experimental proof. The availability of afibrinogenemic blood permitted investigation of this point since the addition of thrombin would not be complicated by activation of fibrinogen and its conversion to fibrin.

Oxalated plasma was unsatisfactory for this purpose because of marked refringence of calcium oxalate crystals under the phase microscope. Accordingly, platelet-rich citrated plasma, obtained as described above, was mixed with purified bovine thrombin,† and observations under the phase microscope were promptly begun. Prior to thrombin addition, the platelets were mostly dispersed

* Considerable platelet clumping was also observed in a sample of the same blood kept without anticoagulant in a silicone coated tube for six hours at 5°C. Discrete platelets showed remarkably little morphologic distortion.
† Generously supplied by Dr. Walter Seegers of Wayne University.
with a very few small agglutinates. As in normal citrated plasma, some platelets were oblong or round in shape; a few exhibited spicules or tails.

Shortly after the addition of thrombin (10 units in 0.1 ml. of sal. to 0.1 ml. of plasma) the platelets coalesced into large aggregates, and again there appeared some fine strands which suggested a trace of fibrin. Many platelets became more oval with big long tails. Twenty-five minutes later, almost suddenly, most of the platelets disappeared with the simultaneous appearance of numerous small dense dark spots exhibiting intense Brownian movement. This has been repeatedly observed also in normal plasma. Whether the dark spots arose directly from the disappearing platelets and represented the platelet granulomeres could not be ascertained.

Similar studies were made on platelets separated from normal blood, drawn as described above. It was promptly placed in a siliconized glass tube containing 0.1 vol. of a 1 per cent solution of sodium sequestrene in 0.7 per cent sodium chloride solution. The mixture was lightly spun to remove most of the erythrocytes and leukocytes, the supernatant platelet-rich plasma was then centrifuged for 10 min. at 4000 rpm, and the platelet sediment was washed thrice with physiologic saline. Whereas purified bovine thrombin added to a saline suspension of once-washed platelets induced platelet agglutination and lysis (as well as deposition of fibrin, indicating incomplete separation of plasma proteins), no changes occurred in the twice- and thrice-washed platelet suspensions. The evidence therefore indicates that while thrombin is probably instrumental in causing platelet agglutination and lysis, other plasma factors (excluding fibrinogen) must also be involved, since the changes cannot be induced on platelets separated from their plasma milieu.

Prothrombin Consumption during Coagulation

Although there is little reason to suspect that fibrinogen should affect the rate of prothrombin disappearance from freshly shed blood, the availability of afibrinogenemic blood permitted a definitive answer to this question. Aliquots of incoagulable blood, drawn as described above, were placed in uncoated glass clotting tubes, and sufficient oxalate was added at specified intervals to stop the clotting process. The plasma or sera prothrombic activities were determined by both the one-stage and two-stage procedures.

In the instance of C. M. (fig. 5), the prothrombin (two-stage) began to disappear 5 minutes after exposure of the blood to a glass surface; within 30 minutes, more than half had been consumed. It seems that the velocity of disappearance appears slower when citrate is used as anticoagulant instead of oxalate. In E. J. (fig. 6) prothrombin consumption seemed to proceed a little faster. Nevertheless, in both instances it was somewhat more rapid than occurs in normal blood. The marked discrepancy between the one-stage and two-stage values for prothrombic activity during coagulation, in accordance with what has been repeatedly ob-

* It is also of interest that the platelets in afibrinogenemic plasma treated with sodium sequestrene (1 vol. of 1 per cent sod. sequestrene in 0.7 per cent sod. chloride solution, to 9 vol. of blood) appear the same as in normal plasma so collected. They show more spicules and appear larger than in citrated plasma, normal or afibrinogenemic.
served in normal blood, is attributable to the elaboration of accelerators (serum Ac-globulin and SPCA) during the clotting process from their relatively inert plasma precursors.

It is of interest that Quick found virtually complete prothrombin consumption, probably on C. M., within 15 minutes after the plasma was exposed to a foreign surface. This finding of apparently faster prothrombin disappearance than we obtained, probably was due to differences in technic. In Quick's experiment, longer intervals elapsed between the shedding of the blood and the time anticoagulant was added although the plasma was kept refrigerated in siliconed glass prior to exposure to uncoated glass. This would permit elaboration of the serum type of accelerators both of which markedly accelerate prothrombin conversion to thrombin. Moreover, it is not clear what anticoagulant he used.

**Ac-Globulin Disappearance in Coagulation of Afibrinogenemic Blood**

It has already been established that Ac-globulin rapidly disappears during the clotting of normal blood, even faster than prothrombin. That this is not related to fibrinogen conversion to fibrin is indicated by the data obtained on E. J. (fig. 6). As with prothrombin, the Ac-globulin seems to disappear at a faster rate from afibrinogenemem blood than from normal. Moreover, its disappearance closely parallels prothrombin consumption whereas in normal blood substantial Ac-globulin consumption precedes prothrombin disappearance by a significant interval.

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* Deduced from a note in the patient's record that during a visit to Boston, Dr. Quick had performed a prothrombin consumption test on him at the Boston City Hospital. In the reference cited, Dr. Quick states that he performed the experiment at the Boston City Hospital, although no specific mention is made of the subject whose blood was tested.
SPCA Mechanism

Aim: A unique opportunity was also afforded for exploring more precisely the formation of SPCA from its plasma precursor during the coagulation sequence, uncomplicated by the activation of fibrinogen.

![Graph](image)

**Fig. 6.—Prothrombin and Ac-globulin consumption during "coagulation" of afibrinogenemic blood (E.J.). Oxalate or citrate used as anticoagulant.**

**Table 6.—SPCA Activity in Afibrinogenemia**

<table>
<thead>
<tr>
<th>Subject</th>
<th>Spca (%)</th>
<th>Prothrombin (% of orig. plas.)</th>
</tr>
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<tbody>
<tr>
<td></td>
<td></td>
<td>one-stage</td>
</tr>
<tr>
<td>C. M.</td>
<td>100</td>
<td>15</td>
</tr>
<tr>
<td>C. M.</td>
<td>?</td>
<td>34</td>
</tr>
<tr>
<td>D. C.</td>
<td>110</td>
<td>10</td>
</tr>
<tr>
<td>E. J.</td>
<td>57</td>
<td>10</td>
</tr>
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</table>

* Assay by previously reported method. Figures refer to enhancement of prothrombic activity of normal plasma to which test serum was added.

**Methods and results:** Sera, separated 1 hour after afibrinogenemic blood was exposed to a glass surface, contained normal amounts of SPCA activity (table 6), as measured by previously described methods.

The elaboration of SPCA is also manifest by increases in one-stage prothrombic activity during the early phase of clotting. This was clearly evident in shed afibrinogenemic blood (figs. 5, 6). The evolution of SPCA from its relatively inert plasma precursor proceeded within minutes after the blood was exposed to...
a glass surface; substantial changes became evident before any detectable amount of prothrombin had disappeared.

SPCA formation was also evaluated by the technique of Owren \(^{26, 27}\) in which plasma proconvertin (pro-SPCA) is measured by its clot promoting effect on bovine plasma rendered free of proconvertin by filtration through a 25 per cent asbestos Seitz filter. Transformation of proconvertin into active convertin becomes reflected in greater clot-promoting effect of the sera than was present in the original parent plasmas. Here, too, within a few minutes after blood from E. J. was shed, substantial conversion of proconvertin to convertin occurred, even before significant amounts of prothrombin disappeared. Maximal conversion was reached within approximately 30 minutes.

Studies on Plasma Antithrombin Activity

Aim. The ability of plasma to inactivate or otherwise dispose of thrombin is largely referable to a plasma protein frequently referred to as "natural antithrombin." \(^{28-30}\) Antithrombic activity has also been attributed to fibrinogen-fibrin. There is little question that during the formation of fibrin, a significant amount of the thrombin is rapidly adsorbed by the clot, from which it later is slowly released. \(^{31}\) According to Quick and Favre-Gilly, \(^{32}\) fibrin is the important physiologic antithrombin.

The mechanism of the well known antithrombic action of heparin also is not clear. Whether it acts via a heparin co-factor (not fibrinogen) in the plasma, \(^{33}\) or by adversely influencing the thrombin-fibrinogen interaction, or both, is still debatable. Studies were made on congenital afibrinogenemic plasma in an attempt to clarify these points.

Methods and results: Antithrombic activity was determined by the method of Klein and Seegers. \(^{36}\) The ability of afibrinogenemic plasma to inactivate thrombin did not appear significantly different from that of normal plasma (table 7). In this experiment, the normal plasma was defibrinated by a small thrombin addition prior to its being loaded with a larger amount of thrombin. Since the afibrinogenemic plasma was studied under the same conditions, comparison of the data is valid. It may, therefore, be concluded that the natural antithrombic activity of the afibrinogenemic plasma was normal.

To explore the heparin co-factor question, two types of experiments were performed. In the first, human thrombin (Cutter Lab.) was added to citrated afibrinogenemic plasma with and without heparin. At specified intervals, aliquots of the mixtures were assayed for residual thrombic activity against a standard fibrinogen solution. The data (fig. 7) warrant the conclusion that the ability of afibrinogenemic plasma to inactivate thrombin was not appreciably influenced by heparin. This substantiates the conclusions of Astrup and colleagues \(^{29, 33}\) and Klein and Seegers \(^{36}\) that plasma contains a factor, not fibrinogen, which can inactivate thrombin and which is not influenced in this capacity by heparin.

The second type of experiment involved the use of much smaller amounts of thrombin as well as varying amounts of heparin (table 8). These were added to human fibrinogen (fraction I) dissolved in saline or in afibrinogenemic plasma. In the presence of heparin, coagulation of the fibrinogen was much slower in the plasma than in the fibrinogen-saline solution. To be sure, the heparin retarded the clotting of the purified fibrinogen also, but its ability to do so was
enormously enhanced when plasma (afibrinogenemic or normal) was incorporated in the system. Thus, a plasma co-factor for heparin, which is not fibrinogen, is clearly demonstrated, the combination somehow inactivating thrombin. An

Table 7.—Antithrombic Activity of Afibrinogenemic Plasma Compared with Normal Plasma

Subject: C. M.

Citrated plasma defibrinated* by addition of human thrombin (Cutter Lab.) (50 U./ml.) as follows: 0.2 ml. thrombin solution was added to 0.9 ml. plasma. The mixture was allowed to stand for 10 min., the clot was removed, and the supernatant was allowed to stand at room temperature for another 10 min.

Nine-tenths ml. of thrombin-treated plasma plus 0.1 ml. physiologic saline solution plus 1.0 ml. human thrombin solution (1395 U./ml.).

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<thead>
<tr>
<th>Time after addition of thrombin (min.†)</th>
<th>Amount Thrombin Inactivated (units)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>norm. plas.</td>
</tr>
<tr>
<td>10</td>
<td>585</td>
</tr>
<tr>
<td>60</td>
<td>705</td>
</tr>
</tbody>
</table>

* Both afibrinogenemic and normal plasma treated with thrombin. In the case of the afibrinogenemic plasma, no clot was obtained by thrombin addition.
† Aliquot removed, diluted 100 X or 150 X with physiologic saline solution, 1.0 ml. of diluted material added to 3.0 ml. of reaction mixture to measure residual thrombin, according to the method of Klein and Seegers.3

Fig. 7. Elaboration of convertin from proconvertin during "coagulation" of afibrinogenic blood (E.J). Blood drawn by technic (as described in text) minimizing exposure to foreign surface till placed in glass clotting tubes. Citrate added to aliquots at intervals specified.

equally valid interpretation would be that the entity influences fibrinogen in such a way as to make it less susceptible to thrombin in the presence of heparin. Regardless of the interpretation, the data indicate that the action of heparin in blocking the appearance of insoluble fibrin resulting from thrombin-fibrinogen
interaction is greatly increased by a plasma moiety, the combination producing
its effect by a mechanism distinctly different from that of the thrombin-inac-
tivating property of "natural" antithrombin.

Relationship of Plasma Fibrinogen Concentration to One-Stage Prothrombic Activity

Obviously the addition of thromboplastin and calcium to decalcified afi-

Table 8.—Antithrombic Activity in Congenital Afibrinogenemia

<table>
<thead>
<tr>
<th>Subject E. J.</th>
<th>Blood incoagulable, spontaneously or after added thrombin</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxal. plasma-fibrinogen* (ml.)</td>
<td>Saline-fb.* (ml.)</td>
</tr>
<tr>
<td>---------------</td>
<td>-----------------</td>
</tr>
<tr>
<td>6/25/52</td>
<td></td>
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<tr>
<td>—</td>
<td>0.1</td>
</tr>
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<td>0.1</td>
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<td>0.1</td>
</tr>
<tr>
<td>0.1</td>
<td>—</td>
</tr>
<tr>
<td>6/27/52</td>
<td></td>
</tr>
<tr>
<td>Cit. Plasma-fibrinogen*</td>
<td>0.1</td>
</tr>
<tr>
<td>—</td>
<td>0.1</td>
</tr>
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<td>0.1</td>
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<tr>
<td>0.1</td>
<td>—</td>
</tr>
<tr>
<td>Oxal. Norm. Plas.</td>
<td>0.1</td>
</tr>
<tr>
<td>0.1</td>
<td>—</td>
</tr>
</tbody>
</table>

* 0.25 per cent solution of human fraction I in afibrinogenemic plasma or saline.
† 0.1 ml. solution of heparin (Parke Davis), containing amount heparin indicated.
‡ 0.1 ml. bovine thrombin (Seegers), containing amount thrombin indicated.
§ Excesses of thrombin subsequently added; clotting occurred in all instances in less than 10 seconds.

brinogenemic plasma would fail to induce coagulation. According to the orthodox
one-stage method of measuring prothrombin, this could be erroneously inter-
preted as indicating zero prothrombin despite the fact that the prothrombin
content might be normal. Study of the relationship between fibrinogen concentra-
Fig. 8. – Antithrombic activity of afibrinogenemic plasma (C.M.). Human thrombin (Cutter) added to citrated plasma in the presence or absence of heparin. Residual thrombin determined, at intervals specified, by titration against a standard fibrinogen solution, as in the two-stage prothrombin method. 17

Fig. 9. – Relationship between plasma fibrinogen concentration and one-stage prothrombin time. Normal citrated plasma was mixed in various proportions with citrated congenital afibrinogenemic plasma (E.J.) or with citrated normal plasma defibrinated by addition of thrombin (5 units per ml.), and the prothrombin times determined on the mixtures. Fibrinogen concentrations calculated on the basis of the known proportions of normal plasma in each mixture, the fibrinogen concentration of the whole normal plasma (determined in the usual manner), and with the assumption that the incoagulable afibrinogenemic plasmas contained zero fibrinogen.
tion and prothrombin time was made possible by varying the proportions of afibrinogenemic to normal plasma in plasma mixtures. When the fibrinogen concentration is reduced below approximately 120 mg. per cent, the prothrombin time progressively rises (fig. 8). Below concentrations of 85 mg. per cent the clot becomes progressively smaller and more translucent. At extremely low levels, the end point of clotting fails to be sharp. These findings are in remarkable agreement with those reported by Pinniger and Prunty.14

Discussion

The studies delineated above permit unequivocal conclusions regarding certain basic aspects of the coagulation mechanism. Contrary to the concept of Lenggenhager,12 Owren,13 Wright,13 and Tocantins,14 the conversion of fibrinogen to fibrin plays little role, if any, in the agglutination and lysis of platelets during coagulation. In shed afibrinogenemic blood, morphologic alterations, clumping, and disappearance of platelets are unimpaired, appearing as one of the earliest phenomena detectable.

The factors responsible for these changes are still obscure. From our studies it appears likely that thrombin is one of the active agents although caution is indicated in drawing this conclusion since the responsible agent may not have been thrombin but a contaminant in the thrombin preparation used. In any event, factors other than thrombin must also be involved, since the same effects cannot be obtained on platelets separated from the plasma. This interpretation is supported also by the work of others.37, 38 Final clarification must await separation of the clotting constituents in pure form.

It can now be concluded that AHF is distinct from fibrinogen. That this is clearly established is important in view of some similarity in their physical and chemical properties, alluded to by Graham, et al.9 Both are preponderantly concentrated in plasma fraction I. Their separation in the laboratory has not yet been achieved, although a globulin fraction with clot accelerating activity on hemophilic blood has been obtained by Pavlovsky and Simonetti39 from congenitally afibrinogenemic plasma. That this may have been due to thrombin contaminants is suggested since globulin fractions similarly derived from normal plasma showed spontaneous coagulation.

The only clear cut evidence of dissociation of fibrinogen and AHF, prior to this study, is that reported by Graham, et al.8'9 in canine hemophilia. Most of their observations are consistent with ours. In discord, however, are their results with canine plasma defibrinated by heat or thrombin10 where antihemophilic activity was unimpaired. These discrepancies are probably referable to technical differences.*

The disappearance of antihemophilic activity early in coagulation explains the low antihemophilic activity of serum contrasted with plasma. Whereas in canine blood, AHF disappears in parallel with prothrombin,10 in man substantial amounts are consumed before any measurable loss of prothrombin. This suggests

* Graham, et al.10 heated the canine plasma to 52 C. for 10 minutes in contrast to our conditions of 56 C. for 2'1/2 minutes. Also, they used 0.4 to 1.5 units of thrombin per ml. of plasma whereas we added 5.
that disappearance of AHF is not directly related to prothrombin consumption. Nevertheless, the data in both species indicate that anything which retards prothrombin conversion retards AHF consumption. Interestingly enough, in the coagulation of canine thrombocytopenic blood, AHF disappearance is delayed, but once it begins, it proceeds at normal velocity.¹⁰

Also worthy of comment are other "coagulation" changes which occur in afibrinogenemic blood. The disappearance of Ac-globulin cannot be attributed to fibrin deposition. It probably results from the effect of evolved thrombin which then converts the plasma Ac-globulin into the active serum type; the latter, being far less stable, disappears very rapidly. The postulate of Carter and Warner⁴⁰ that the plasma form is less active by virtue of its being bound, or otherwise rendered impotent, by plasma fibrinogen, and that during coagulation it is converted to the active (serum) form when freed of fibrinogen, is disproved by finding normal Ac-globulin activity in afibrinogenemic plasma.

The velocity of both Ac-globulin and prothrombin disappearance seems greater in the coagulation of afibrinogenemic blood than in normal. Since thrombin labilities Ac-globulin as well as platelets, these findings suggest that normally fibrinogen-fibrin acts as an antithrombin by rapid adsorption and/or mechanical occlusion of thrombin, thus reducing its action on other clotting constituents. In afibrinogenemia, thrombin elaboration and the effects consequent to its formation may thus proceed faster than normal.

It is still not clear whether fibrinogen-fibrin has additional antithrombic function, e.g. heparin co-factor activity, as has recently been implied.⁴⁸ A difficulty inherent in this question is the fact that orthodox tests for thrombin are predicated on its ability to convert fibrinogen to fibrin.* Accordingly, residual thrombin (or thrombin inactivation) is measured in systems which contain fibrinogen. Thus, the extent to which the heparin per se may affect the thrombin fibrinogen interaction and the degree of thrombin adsorption is difficult to evaluate.

This becomes strikingly evident when the data are closely scrutinized. It would appear (fig. 8) that with added heparin, afibrinogenemic plasma could not inactivate more thrombin than in the absence of heparin. This would suggest that the co-factor was identical with fibrinogen. This interpretation is not supported, however, by those experiments where fibrinogen was incorporated in the presence vs. absence of the plasma in question (table 8). Under these circumstances, the clotting ability of thrombin was far more impaired when both heparin and plasma were present than in the presence of either alone. There can be little question, therefore, that plasma contains a moiety which is not fibrinogen and which with heparin interferes with the action of thrombin on fibrinogen.†

The above studies permit portrayal of the coagulation sequence, exclusive of the fibrinogen-fibrin mechanism, as follows: Within minutes after blood is exposed to a foreign surface, the platelets begin to undergo morphologic changes,

* Recently Sherry and Troll reported on the enzymic splitting by thrombin of a synthetic substrate.⁴⁸ The possible application of their work to this problem is intriguing.
† A minor note of caution is indicated. Waugh,⁴⁴ Ferry,⁴⁵ and others have shown that thrombin converts fibrinogen to a visible clot via certain intermediary steps, the final one being a deposition of insoluble fibrin from soluble fibrin. The precise point where heparin plus co-factor acts remains obscure.
agglutinate, and lyse. In close parallel, perhaps simultaneously, AHF disappears rapidly, Ac-globulin becomes activated and is rapidly consumed, while SPCA is elaborated from its precursor. Although these changes are well along before a measurable amount of prothrombin has been converted, they are most likely caused by small amounts of thrombin which are evolved early since they can be duplicated by the direct addition of thrombin to plasma. Unfortunately, close examination of the time relationships does not allow more precise delineation of the sequential order of these changes.

That many of the above reactions take place early after exposure to a “foreign” surface, deserves special comment. Occurring in blood which may manifest no physical gelation, they constitute evidence of “chemical coagulation.”

It is evident that in order to minimize alterations, the coagulation sequence must be prevented or stopped within a few minutes after blood withdrawal. Fluidity alone is no assurance of the efficacy of procedures designed to obtain blood and its components in an unaltered state. A better criterion is the absence of SPCA (in contrast to pro-SPCA) in the plasma.

Although hemorrhage is a prominent feature in afibrinogenemia, it is also evident that coagulable blood is not essential for at least some degree of hemostasis. Despite incoagulable blood, our subjects experienced long intervals of freedom from hemorrhage. This has been noted also by Macfarlane and Lawson.

The bleeding time in afibrinogenemia as in hemophilia, is frequently normal, whereas in parahemophilia and thrombocytopenia it is elevated. Although the significance for hemostasis of a normal vascular tree must not be minimized, of great importance is the agglutination of platelets, their adhesion to endothelial surfaces, and their lysis. In afibrinogenemia these functions appear unimpaired, whereas, in parahemophilia they are retarded and in thrombocytopenia they are inadequate. On the other hand, the normal bleeding time of hemophilia despite the retarded platelet changes, is explicable by the fact that the abnormality is corrected by tissue thromboplastin available at the site of trauma.

The relationship between fibrinogen concentration and the prothrombin time is of both practical and theoretic interest. If the prothrombin time of a given plasma sample is normal, its fibrinogen level must be in excess of 100 mg. per cent. Below this, the prothrombin time progressively rises in conformity with the curve correlating the two. Indeed, such a curve permits a rough calculation of the fibrinogen concentration from the prothrombin time if the prothrombin and prothrombin-conversion factors are normal and if the fibrinogen level is sufficiently low to give a considerably elevated prothrombin time. At such levels, orthodox methods for plasma fibrinogen determination may be unreliable.

SUMMARY

In a study of three subjects with incoagulable blood due to congenital afibrinogenemia, information is presented regarding some basic aspects of coagulation independent of the fibrinogen-fibrin conversion mechanism. The following facts have been established: (1) Shortly after the blood is exposed to glass, the earliest detectable changes are morphologic alteration in the platelets and their progressive agglutination and lysis. (2) Almost in parallel, plasma antihemophilic activity, originally normal, declines rapidly and practically disappears. (3) Also
within minutes, SPCA evolves from its precursor in a normal manner. (4) Ac-globulin is rapidly consumed. (5) These changes are well along before detectable amounts of prothrombin have disappeared. (6) Prothrombin consumption proceeds at normal velocity, or slightly faster than normal. (7) Thrombin addition to afibrinogenemic plasma induces platelet agglutination, but this does not occur when thrombin is mixed with platelets alone. (8) The "natural" antithrombic activity of afibrinogenemic plasma is normal. (9) The heparin co-factor of antithrombic activity was demonstrable. (10) Quantitative data were obtained relating the one-stage prothrombin time with the fibrinogen concentration.

The theoretic and practical implications of the observations are discussed.

**Summario in Interlingua**

Super le base de un studio de tres patientes cuje sanguine esseva non-coagulabile in consequentia de lor afibrinogenemia congenite, observationes es presentate relative a certe aspectos fundamental de coagulation independente del mechanismo de conversion del fibrinogeno in fibrina. Le sequente factos esseva establite: (1) Brevemente post que le sanguine esseva exponite a un superficie de vitro, le prime detegibile cambiamentos esseva le alteration morphologic del plachettas e lor progredente agglutination e lyse. (2) Plus o minus parallelmente, le activitate hemophilic del plasma—que comenciava per esser normal—se deteriorava rapidemente e dispareva quasi integreme. (3) Etiam intra pauc minutas, le accelerator del conversion de prothrombina seral (ACPS) se disveloppava cormalmente cx su antecedente. (4) Globulina Ac esseva consumite rapidemente. (5) Iste alterationes esseva ben avaintiate ante que quanti-tates detegibile de prothrombina habeva disparite. (6) Le consumption de prothrombina procedeva a un velocitate normal o levemente supernormal. (7) Le addition de thrombina al plasma afibrinogenemic induceva le agglutination del plachettas, sed isto non occurreva quando thrombina esseva miscite con plachettas in isolation. (8) Le activitate antithrombic natural de plasma afibrinogenemic esseva normal. (9) Le co-factor de heparina esseva demonstrabile in le activitate antithrombic. (10) Datos esseva obtenite que establi un relation inter le tempore prothrombinic a phase unice e le concentration del fibrinogeno.—Le implicationes practic e theoric del observationes es discutite.

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Congenital Afibrinogenemia: A Study of Some Basic Aspects of Coagulation

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