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## VORWORT

Wir hielten uns für berechtigt, im Rahmen des IV. Internationalen Kongresses für Biochemie ein Symposium über Gerinnungsfaktoren abzuhalten, da die Gerinnungsforschung in den letzten Jahren aus dem Stadium reiner klinischer Empirie herausgewachsen und zur Anwendung exakter biochemischer und biophysikalischer Methoden übergegangen ist. Einige Ergebnisse mögen dies erläutern und die Grenzen, an denen wir angelangt sind, aufzeigen: So ist es gelungen, Fibrinogen herzustellen, das 98% gerinnungsfähiges Eiweiss enthält, und Thrombin so weit zu reinigen, dass es möglich ist, Untersuchungen über das aktive Zentrum des Fermentes durchzuführen. Auch Prothrombin, antihämophiler Faktor und Plasminogen sind weitgehend frei von anderen Gerinnungsfaktoren gewonnen worden. Dank der weitgehenden Reinigung von Fibrinogen und Thrombin ist es gelungen, erfolgreiche Untersuchungen über den Reaktionsablauf und die Kinetik der II. Gerinnungsphase durchzuführen und die Veränderungen, die das Fibrinogenmolekül bei dieser Umwandlung erfährt, zu analysieren. Die Untersuchung der I. Phase mit exakten Methoden dürfte bevorstehen, während bezüglich des Mechanismus der Thrombokinasenbildung einstweilen nur Vermutungen geäussert werden können.

Die biochemische Gerinnungsforschung kann sich aber nicht fruchtbringend weiterentwickeln, wenn sie nicht in engem Kontakt mit der klinischen Forschung steht. Die Klinik bleibt die ständige Quelle, aus der theoretische Forscher seine Anregungen und Probleme schöpft. Sie zeigt ihm in Form der—meist erblich bedingten—hämorrhagischen Diathesen die Folgen des Fehlens des entsprechenden Gerinnungsfaktors in einer Klarheit, die ein Experiment kaum bieten könnte. Die Klinik liefert auch mit dem Blut dieser Patienten dem Forscher jenes Testsystem, an dem er die Wirkung der von ihm isolierten Faktoren am sichersten studieren kann. Es ist bisher nicht gelungen, gleich vollständige Systeme experimentell zu entwickeln. Letzten Endes wird aber die Klinik Nutzniesserin der Ergebnisse der theoretischen Forschungsarbeit sein: Liegt doch die praktische Bedeutung aller dieser Bemühungen darin, durch gereinigte Gerinnungsfaktoren eine spezifische, möglichst ungefährliche und sichere Substitutionstherapie der hämorrhagischen Diathesen zu ermöglichen.

Die Gewebe wurden in die Thematik dieses Symposiums ebenfalls einbezogen. Erst in letzter Zeit ist der Nachweis von Gerinnungsfaktoren in den verschiedensten Geweben gelungen und vor allem einigermassen gewürdigt worden. Das Vorkommen von Gerinnungsfaktoren in den Geweben scheint uns in doppelter Hinsicht von Bedeutung zu sein:

Erstens könnte es möglich werden, Gewebe neben dem nie in ausreichender Menge vorhandenen Plasma als Ausgangsmaterial zur Isolierung gewisser Gerinnungsfaktoren heranzuziehen; zweitens weist diese Tatsache darauf hin, dass die Auslösung von Blutgerinnung und Blutstillung nicht die einzigen Aufgaben sind, die der komplizierte Gerinnungsmechanismus im Organismus zu erfüllen hat: Erst allmählich erkennt man die Bedeutung des Gerinnungsvorganges für die Wundheilung, Bindegewebebildung, Ablauf der Entzündungsvorgänge und Infektabwehr.

Diese Gesichtspunkte waren bei der Auswahl der Themen des vorliegenden Symposiums massgebend. Es sollte vor allem gezeigt werden, wo das Gesicherte aufhört und das Fragliche beginnt, an welchen Stellen die neuesten Ergebnisse erzielt wurden und welche Probleme am dringendsten einer Lösung bedürfen.

Wein, Oktober 1958.

E. DEUTSCH

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# ANTIHEMOPHILIC FACTOR (AHF): SOME ASPECTS OF ITS BIOCHEMISTRY\*

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We use the term antihemophilic factor (AHF) to designate the factor which is lacking in the plasma of patients with severe classical hemophilia (Factor VIII deficiency, hemophilia A). In this paper the following topics are considered: (1) some historical remarks on hemophilia and AHF, (2) methods and difficulties of assay of AHF, (3) plasma fractionation and isolation of AHF, (4) some properties of AHF in plasma and in partially purified preparations, (5) biochemical genetics of AHF, and (6) certain functional and metabolic studies of AHF.

## HISTORICAL ASPECTS

AHF has been an elusive clotting factor, and a word of caution may be in order at the beginning. Much uncertainty surrounds the older literature. This is pointed up by the discovery in 1952 of a new bleeding diathesis, a sex-linked recessive disorder called PTC deficiency or Christmas disease. With many of its clinical and laboratory findings identical with true hemophilia, it became obvious that studies in the past had not discriminated between AHF and PTC. Only in those older studies in which the patients used for reference were still living or where affected members of the same pedigree were available can one be certain whether AHF or PTC or both were being considered. In our own work for the past 12 years, we have depended on a single strain of hemophilic dogs for definition of AHF. This strain has a clotting defect indistinguishable from true human hemophilia.

With the understanding that history cannot be precise, it is interesting to trace the development of the main ideas current today regarding hemophilia and AHF. The slow coagulation of hemophilic blood was recognized first. Sahli<sup>1</sup> showed that there was no lack of fibrinogen. Addis<sup>2</sup> showed that thrombin formed slowly, and after reliable methods

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\* Studies by the authors, cited in this paper, were aided by grants from the National Institutes of Health.

for prothrombin became available, Brinkhous<sup>3</sup> showed that prothrombin disappeared very slowly in clotting hemophilic blood. There gradually emerged the idea that the delayed clotting in hemophilia is due to absence of a specific plasma factor, genetically determined. Attempts to concentrate and purify this factor were begun. Addis<sup>2</sup> appears to have had some success, but he thought he was working with prothrombin. After it was demonstrated that small amounts of normal plasma had a corrective effect on the clotting of hemophilic blood (Govaerts and Gratia<sup>4</sup>, Patek and Stetson<sup>5</sup>, and others), Pohle and Taylor<sup>6</sup>, Bendien and van Creveld<sup>7,8</sup>, and others prepared crude globulin fractions of plasma which had the same effect. We use the term, antihemophilic factor, for this activity advisedly, since it has not been adequately characterized to be certain that it is in fact a globulin.

Another idea regarding the plasma abnormality in hemophilia has many advocates. It has been proposed that the procoagulant factors are present in normal amounts, but that there is an excess of an inhibitor, anticephalin, which is genetically determined and which results in hemophilia (Tocantins *et al.*,<sup>9-12</sup> Seegers, Johnson *et al.*,<sup>13</sup> Feissly<sup>14</sup>, Verstraete<sup>15</sup>, and others). An analysis of much of the data which appears to support this view has been made in our laboratory by Graham and Barrow<sup>16,17</sup>. They conclude that it is not necessary to invoke the inhibitor hypothesis to explain any of this body of data. Regardless of the hypotheses as to the basic causation of the hemophilic clotting defect, workers generally agree that there is a factor in plasma which will temporarily correct, at least partially, the clotting and hemostatic defect in hemophilia.

### ASSAY OF AHF

The problems encountered in devising an accurate assay procedure for AHF are many. Correction of the basic clotting defect in hemophilic blood as well as correction of the hemostatic defect in hemophilic subjects appear to be necessary as minimal criteria for recognition of AHF. It is difficult to test AHF-containing preparations in this way regularly. It appears desirable to standardize *in vitro* assay procedures with preparations known to be effective when administered *in vivo* to hemophilic subjects.

*In vitro* tests are of many types, and all appear to present problems of specificity, sensitivity, and accuracy. *Specificity*: A number of coagulant factors are able to shorten the prolonged clotting time of hemophilic blood without altering the fundamental defect—the block in prothrombin utilization. This “corrective” action has been shown by Graham *et al.*<sup>18,19</sup> for plasma and serum eluates (from BaSO<sub>4</sub>), and by Deutsch and Schaden<sup>20</sup> for certain serum fractions. Graham and Barrow<sup>17</sup> demonstrated that asbestos treatment of hemophilic plasma seemingly caused unmasking of

AHF activity, since the clotting time became normal; however, such "corrected plasmas" did not result in normalization of prothrombin consumption, either *in vitro* or *in vivo*. Thrombin in trace amounts will greatly shorten the partial thromboplastin time of hemophilic plasma.<sup>21</sup> Other examples of non-AHF coagulants affecting the assay procedures could be cited. It was shown in 1951 that AHF is not adsorbed onto  $\text{BaSO}_4$ <sup>22</sup> and only partially adsorbed onto  $\text{Al}(\text{OH})_3$ .<sup>23</sup> Advantage can be taken of this fact by preliminary adsorption of plasmas or their fractions to remove interfering coagulant factors before the assay procedures are carried out. The possible effect of Hageman factor on AHF assays has not been resolved. Most assays rely upon hemophilic blood or plasma as substrate. Included in this group are the recalcified clotting time, the partial thromboplastin time, the two-stage prothrombin utilization test, and the thromboplastin generation test (TGT). In general, tests based on changes in the clotting time of whole blood or recalcified plasma appear least specific. Substrates made artificially low in AHF are also used, as in the assay for thromboplastic plasma component<sup>24</sup> and the AHF assay of Wolf<sup>25</sup> in which the first supernatant of the ether fractionation process is used. Seitz filtration also removes AHF.

*Sensitivity.* This is secondary to problems of specificity. In general, most of the tests are sensitive to small changes in AHF within a limited range of concentrations. Comparative studies are limited. Recently Rodman and Graham<sup>26</sup> showed that the partial thromboplastin procedure was more sensitive over a wider range of AHF concentrations than the TGT.

*Accuracy.* At best, the results of assay for AHF have an error of plus or minus 10%.

#### PURIFICATION

Two requirements for successful purification of AHF are (1) a specific and reproducible assay procedure, as outlined above, and (2) a satisfactory source of AHF. For starting material, only plasma has been used successfully. The AHF content of various plasmas thus becomes important. If human plasma is considered as having unit activity per ml, then sheep plasma has about 2.5 units, dog plasma 5 units, cow plasma 2-3 units, and pig plasma 5-10 units. These values for relative potency of different plasmas vary somewhat according to the assay procedure used. Unfortunately, human plasma is one of the weakest sources of AHF. On the basis of the best purification data, in terms of activity and protein N, it would appear that plasma AHF does not exceed 3-5 mg%.<sup>27</sup>

The apparent lability of AHF in plasma must be recognized in any program of purification. Both the presence of platelets and a source of thrombin can contribute to AHF lability. It has been shown that with reduced platelet levels, AHF disappears from native plasma more slowly

than with a full complement of platelets.<sup>28</sup> Penick and associates<sup>22,54</sup> have demonstrated that AHF is remarkably stable if prothrombin and related coagulants are first adsorbed from plasma. Bidwell used  $\text{Al}(\text{OH})_3$  adsorption in her purification procedure.<sup>29</sup> Surgenor<sup>30</sup> has stressed that the AHF activity of a fraction is improved if it is promptly separated from the bulk of Fraction I. Fantl<sup>31</sup> has reported that AHF is stable if the pH of the plasma is maintained at 6.4.

*Methods of purification.* (a) *Dilution and acidification* of plasma was one of the earliest procedures used, but has largely been abandoned (Patek and Stetson<sup>5</sup>, Bendien and van Creveld<sup>8</sup>, Alexander<sup>32</sup>, and others). (b) *Ethanol precipitation.* Fraction I is the best known of the preparations used. As prepared by Method 6 of the Cohn group,<sup>33</sup> it contains little AHF activity. As methods of assays and conditions for stabilization of AHF have come to be appreciated, improved preparations have been obtained. Combined with heating and acid precipitation, bovine Fraction I has been purified approximately 34 times.<sup>34</sup> The contributions of the Blombäcks<sup>35,36</sup> have been outstanding. They have selectively extracted the crude Fraction I with glycine-ethanol-buffer mixtures to obtain a human product 100 times purified, with a low fibrinogen content. Our best ethanol preparation, obtained by selective extraction of an ethanol precipitate obtained from fibrinogen-free canine plasma has been about 200 times purified.<sup>27,33</sup> (c) *Ether precipitation.* Kekwick, Wolf and associates<sup>38,39</sup> have prepared a potent AHF concentrate from human plasma, using the low temperature, ether precipitation technic. This product contains fibrinogen. It appears to be about 10-30 times purified. Van Creveld<sup>40</sup> and coworkers have obtained from this material a fibrinogen-free preparation about 20 times concentrated. (d) *Salting-out procedures.* These methods have been used mainly by Bidwell<sup>29</sup> and ourselves (1952). Bidwell, using adsorbed bovine plasma, salted out AHF with phosphate buffer at pH 6.8 and reprecipitated with strong citrate solutions. Potent products, with a purification of 100-400 times or more, were obtained. We have confirmed this work. Using porcine plasma, and by absorbing impurities with tricalcium phosphate as a final step, we have obtained an AHF preparation 800 times purified, with good yields. Our best preparations have been obtained by multiple precipitation of dog plasma. Preparations 2000 times purified with 30% yields have been obtained. These preparations are 10,000 times as active, per mg. protein, as human plasma. However, solubility is limited, and characterization of these preparations is just beginning. (e) *Adsorption and elution procedures.* These procedures have been used by a number of investigators, including van Creveld and associates (1937), Lorand and Laki<sup>41</sup>, and Johnson, Seegers and associates.<sup>42,43,44</sup> We have repeated the experiments of Lorand and Laki, and found that if the starting material is first adsorbed by  $\text{BaSO}_4$ , the final eluates from kaolin were active in

our partial thromboplastin assay procedure, but inactive in the two-stage prothrombin utilization test, which is more specific for AHF under these conditions. Bidwell<sup>29</sup> obtained similar results, identifying the procoagulant activity as Christmas factor. (f) Many other purification procedures have been used, but with indifferent success. These include heavy metal precipitation<sup>45</sup> and cryoconcentration.<sup>46</sup>

*Separation of AHF from fibrinogen.* This separation has been one of the most troublesome in purification of AHF. Heat denaturation of fibrinogen, careful defibrination with thrombin, fuller's earth adsorption of fibrinogen, dextran sulfate precipitation of fibrinogen (Surgenor and Steele<sup>47</sup>, using the method of Walton and Pennell<sup>48</sup>), and selective precipitation of AHF<sup>29,36</sup> have all been used. It was demonstrated in 1951 by Graham and associates<sup>22</sup> that cautious heat treatment of plasma would precipitate the fibrinogen, leaving AHF in solution. This principle has been used by van Creveld and associates<sup>40</sup> and others. It was demonstrated by Wagner and associates<sup>23,49</sup> that fuller's earth would selectively adsorb fibrinogen from canine plasma. This preliminary fractionation procedure has proved to be most important in obtaining the potent AHF fractions referred to above.

#### PROPERTIES OF AHF

*Heat denaturation.* The greater heat stability of AHF in comparison to fibrinogen has been emphasized.<sup>22</sup> However, conditions have not yet been found whereby all fibrinogen could be removed and all AHF activity maintained. Bidwell<sup>29</sup> has the most complete data on heat stability of AHF, using a bovine plasma fraction. She reported that the AHF activity was stable for 5 min at 48–49° C at pH 6.8. Wagner and Thelin<sup>37</sup> have extended this work, determining loss of AHF activity with periods of heating up to 3 hr. Without exception, unusual rather than typical denaturation curves were obtained. The expected curve is a straight line, when the log AHF activity is plotted against time. The curve instead flattens out, with no further loss of activity after 30–90 min. Two possibilities for this behavior have occurred to us: (a) There are two types of AHF in our preparation, one being more stable than the other; this may be related to protein-protein interaction. (b) The instability is not inherent lability of AHF, but rather is caused by the presence of a relatively heat labile enzyme that can inactivate AHF.

*Ultraviolet absorption of AHF preparations.* Our best porcine and canine AHF preparations have shown a typical protein curve, with a peak at 280 $\mu$ . The ratio of the reading at 280 over 260 was 1.6. Further, no anomalies in the visible range were observed.<sup>37</sup>

*Dialysis.* Plasmas adsorbed with BaSO<sub>4</sub> or Al(OH)<sub>3</sub> can be thoroughly dialyzed in the cold without loss of AHF activity. As might be expected,

the range of conditions for dialysis is more limited with purified preparations.

*Electrophoresis of AHF.* Paper electrophoretic studies of AHF have been reported from two laboratories. With a bovine preparation, only  $\beta$ -globulin was found.<sup>34</sup> Van Creveld and coworkers<sup>40</sup> migrated a human preparation on paper, eluted the strips, and used the TGT to locate the activity. They also found that AHF activity was concentrated in the  $\beta_2$ -globulins. With highly purified preparations, we have found irreversible adsorption on paper, demonstrated both by change in rate of migration with concentration, and by the test of Monty *et al.*<sup>50</sup> With free electrophoresis of barium sulfate-treated canine plasma, followed by careful sampling, activity was found in all regions, even in the albumin fraction of the ascending limb, thus indicating a considerable amount of protein-protein interaction. Work on continuous curtain electrophoresis of plasma<sup>51</sup> demonstrated a separate globulin peak, which contained AHF activity. This procedure, of course, surmounts largely the irreversible adsorption troubles which one encounters with ordinary paper electrophoresis. In discussing electrophoresis, one should perhaps recall the report by Bernfeld and Stefanini<sup>52</sup> that hemophilic plasma is different electrophoretically from normal plasma. We have been unable to confirm this in a number of studies on hemophilic dogs, and other workers have been unable to confirm this in hemophilic human patients.

*Ultracentrifuge studies of AHF.* We have studied the behavior of AHF in the ultracentrifuge in whole plasma by using the sampling technique of Hogeboom and coworkers and a swinging bucket head.<sup>37</sup> At the end of a  $2\frac{1}{2}$  hr sedimentation run at 35,500 r.p.m. (average  $g$ :120,000), samples were removed and assayed for fibrinogen and for AHF.

The entire top centimeter of the tube was almost cleared of AHF, while fibrinogen had sedimented only slightly. The total recovery of AHF was 94% in this experiment, and no inhibitory activity was observed in any of the fractions. On the basis of these experiments, one would conclude that AHF activity sediments much more rapidly in plasma than does fibrinogen. Whether this rapid sedimentation is a reflection of a molecular weight greater than that of fibrinogen, or of an AHF-protein complex, cannot be determined. Preliminary work with Mr. Thelin with purified preparations has not shown this rapid sedimentation.

*Inactivation of AHF by enzymes.* Two enzyme preparations, fibrinolysin and thrombin, have been shown to destroy AHF. Fibrinolysin in relatively low concentrations (0.8 Loomis units per ml) was found to inactivate a partially purified antihemophilic preparation. In one experiment, 70% of the AHF was lost within 1 min after adding fibrinolysin.<sup>49</sup> Thrombin inactivation of AHF has been studied in our laboratory, especially by Penick<sup>53,54</sup>. As little as 0.5 Iowa units of highly purified thrombin per ml

of plasma caused nearly complete inactivation of AHF within 5 min. This reaction was markedly accelerated when calcium was available. The presence of another substrate, fibrinogen, did not alter this inactivation phenomenon, suggesting that thrombin has a higher affinity for AHF than for fibrinogen. Recent work, indicating that thrombin acts on an arginyl-glycyl bond in the substrate fibrinogen,<sup>55</sup> suggests that a similar linkage occurs in the AHF molecule.

The inactivation of antihemophilic factor by thrombin and fibrinolysin may partially explain the instability of AHF which has been repeatedly observed in fractions. Traces of these contaminants can ruin a preparation. Penick and associates<sup>56</sup> have demonstrated that, if blood is carefully collected, the AHF is relatively stable even after storage for as long as 3 or more weeks. It has been suggested that incipient thrombin formation is the cause of the instability so often seen. The recent findings of Penick<sup>54</sup> also shed light on the old observation of Graham and associates<sup>22</sup> that AHF is stabilized in adsorbed plasma. If plasma is first adsorbed by BaSO<sub>4</sub> or other adsorbents which remove prothrombin and related pro-coagulants, it is stable. Even after adding Ca<sup>++</sup>, and cephalin or other phospholipid, the AHF remains unaltered. Whether other enzymes inactivate AHF is at present undetermined.

*Comparison with platelet cofactor I.* In connection with AHF, the findings of Seegers and associates<sup>57</sup> regarding platelet cofactor I are pertinent. This factor is effective, along with platelet factor 3 and Ca<sup>++</sup>, in the conversion of isolated prothrombin. It has been obtained not only from normal plasma, but also from plasmas and sera (ether-treated hemophilic plasma, and normal and ether-treated serum) ordinarily believed to be devoid of AHF activity. This cofactor has been obtained in highly purified form. It is a protein with an isoelectric point of 6.4. Compared to AHF, it appears to be more heat labile. The activity of this factor can be simulated by a number of substitutes, such as Benadryl or platelet cofactor II (PTC), particularly if small amounts of thrombin are present. If these differences from AHF are fundamental, the platelet cofactor I may be concerned with an alternative pathway in the activation of prothrombin. Many examples of alternative pathways in the coagulation process have been demonstrated by Seegers in the past few years.

### BIOCHEMICAL GENETICS

The sex-linked recessive inheritance of classical hemophilia appears firmly established. Thus fabrication of AHF appears related to the H gene on the X-chromosome. The varying AHF levels in pedigrees of patients with milder and subclinical forms of hemophilia have given rise to much speculation. All the data available indicate that in these forms the defective