A Circulating Inhibitor (Anti-AcG) Specific for the Labile Factor-V of the Blood-Clotting Mechanism

By John H. Ferguson, Charles L. Johnston, Jr., and Doris A. Howell

The recent hematologic literature contains an increasing number of reports of hemorrhagic diathesis related to the presence of some acquired type of circulating inhibitor interfering with the blood-clotting mechanism. Stefanini and Dameshek, on pp. 205–213 of their book, reviewed many of the alleged types and associations with pre-existing conditions, including lupus erythematosus and other systemic diseases, dysproteinemias, pregnancy, transfusions, exposure to ionizing radiations and to a variety of drugs and chemical agents. Special interest is given to those inhibitors which have been shown to be specific for one or other of the protein factors which participate in the normal reactions of coagulation. These may be superimposed upon primary deficiencies of the factor in question, as, for example, anti-AHF or anti-PTC, respectively encountered in occasional cases of hemophilia (AHF-lack) or Christmas disease (PTC-deficiency). There is evidence to suggest that their appearance may at times be related to preceding transfusions, and it is possible that they represent some type of immunosensitization.

An alleged inhibitor specific for AcG (syn. (pro)accelerin, labile factor, factor V, etc.) was described in a case in the German literature (see Discussion). The present investigations establish the temporary presence of a powerful anti-AcG in the blood of an elderly white male who developed hematuria and ecchymoses after a cholecystectomy which presented no hemorrhagic problem at the time of operation. Some of the data which outlined this clotting problem have been presented in previous preliminary communications, but a full analysis has been reserved for the present definitive publication.

Case Report

J.A.Y. is a 78-year-old white male who, on Feb. 21, 1957, following a short history of biliary colic, underwent a cholecystectomy. A small abscess, found between the gall bladder and liver, was drained. No transfusions or foreign protein injections were received, and there was no bleeding complication before, during, or soon after operation. Recovery was satisfactory until Mar. 11, 1957 when hematuria was noted. Cystoscopy, on March 13th, revealed bleeding from both ureters. A Foley catheter was left in place and continued to drain bloody urine. Studies in his local hospital showed: (1) whole blood clotting time (Lee-White): 60 min.; (2) prothrombin time: 45 sec. (control: 12 sec.); (3) hematocrit: 30 per cent; (4) r.b.c. count: 2,730,000; (5) hemoglobin: 11 Gm. 100 ml. There was some question of a low platelet count on two occasions, but clot retraction was satisfactory, and a sternal bone marrow aspirate was reported as...
normal. Treatment included five units of fresh whole blood, adrenosem, vitamin K₁, and 300 mg. cortisone daily for three days prior to referral to Duke Hospital.

**Duke Hospital Report:** On admission (March 19th) the patient’s history provided no significant additional information. Examination showed bloody urine draining through the Foley catheter. There was cracking and easy bleeding of the lips and a few, small, scattered subcutaneous ecchymoses. The cholecystectomy scar was clean and well healed. A urologic consultant believed that the problem was primarily a blood dyscrasia, rather than renal in origin.

Routine clotting studies performed during hospitalization are summarized in table 1. Also the clotting time of recalcified plasma was four times that of the control, and normal blood failed to correct the prolonged clotting and prothrombin times. Liver function studies and other laboratory data, including L.E. tests on several occasions, were essentially negative.

**Progress report:** 100 mg. vitamin K₁ (Mephyton) i.v. had no effect on the clotting or prothrombin times. Two units of fresh blood on the second hospital day raised the hemoglobin from 8.0 to 9.6 Gm. and the hematocrit from 25 to 29 per cent. On the fifth day, the urine appeared clear to gross inspection and another unit of blood was given for the anemia. Following transfusion the hematuria recurred and ACTH gel, 75 units i.m. daily, was started. Within three days the urine was again clear and has since remained free of red cells. The patient was discharged on April 3, 1957, on 20 units ACTH daily.

**Follow-up:** On May 14th, the patient was apparently fully recovered and active. Urine and hematologic examinations were essentially normal, except for mild residual hypoproaccelerinemia (see later). The ACTH therapy was discontinued. On August 22nd, urine and hematologic examinations were now completely normal, with no evidence of recurrence of anti-AcG or bleeding difficulty.

**Family:** There was no known history of abnormal bleeding in the patient’s parents, seven siblings, or his six children. Of the last, four were available for study and are included in the following sections, with negative findings.

## Laboratory Findings

**Routine tests.** Table 1 summarizes the results of the routine clinical and laboratory tests of the patient’s hemostatic and clotting mechanisms. Most of these were performed on admission to the hospital and were followed by the special experimental analyses.
over a two-month period. The significance of the several abnormal test results will emerge as the analysis proceeds, and a critical review of the data will be presented in the discussion.

Special methods. The basic procedure for 1-stage tests follows: 0.1 (or 0.2) ml. of plasma mixture is mixed with 0.2 ml. of Ca-tpln. (equal vols. thromboplastin and 0.02 M CaCl_2) and the clotting timed at 37 C. The 0.1 ml. volume is used for plasmas in the simple prothrombin time test (cf. Quick\textsuperscript{a}). In AcG and anti-AcG assays, the 0.2 ml. represents 0.1 ml. of substrate (aged normal human plasma which has lost most of its AcG, e.g. prothrombin time > 60 sec.) and 0.1 ml. of suitably diluted mixture of agents to be tested. Data on the reagents will be furnished in the appropriate sections. Standard AcG is a BaCO_3-adsorbed beef serum, after the manner of Ware and Seegers\textsuperscript{a}, which proved to be especially useful in these studies. Most tests used an acetone-dried human brain thromboplastin preparation. Normal human plasmas, fresh (N), preserved (N\textsubscript{2}), or aged (A),\textsuperscript{a} were obtained on a number of occasions. Routine preservation of reagents and plasmas was in the frozen state at ~20 C.

Prothrombin time tests. The patient's plasma prothrombin time was prolonged (see table 1). Mixtures of equal (0.05 ml.) volumes of patient's (Y) and normal (N) plasmas also gave lengthened prothrombin times. This at once suggested an inhibitor, since normal plasma might be expected to correct the test, if it were merely a matter of deficiency in one of the factors ordinarily required for these tests. The prothrombin time was shortened, but not to normal, by (a) dilution of Y, (b) addition of AcG, or (c) admixture of Y's plasma and that of either of two daughters (D\textsubscript{1}, D\textsubscript{2}), in tests of April 3, 1957. The D\textsubscript{1}, D\textsubscript{2} test times were perfectly normal. In sharp contrast to the effects of AcG, a beef serum proconvertin (syn. SPCA, stable factor, factor VII, etc.\textsuperscript{a}) did not correct the defect. The above data are illustrated in table 2, for tests made (A) on March 25th and (B) on April 3rd.

Question of "antithromboplastin." The abnormal prothrombin times were demonstrated with a variety of human or animal thromboplastins, including human brain, rabbit brain and "souplastin" (Schieffelin & Co.). Hence, there was no evidence of any species-specific antithromboplastin.

Thromboplastin dilutions. Table 3, tests of March 23d, show results of modifying the "prothrombin time" test by serial dilutions of thromboplastin (rabbit brain). There was an apparent difference between the plasmas of patient (Y) and a fresh normal (N). Besides the longer clotting-time with the 1:1 thromboplastin, Y's series showed a marked lengthening of clotting-times at successive thromboplastin dilutions, becoming too long (+) for accurate measurement beyond 1:8 dil. In tests of May 8th, Y's plasma was old (4 weeks storage at ~20 C.) and was compared with an aged (A) normal plasma in the presence of varying amounts of AcG, added to the plasmas in equal volume. When

<table>
<thead>
<tr>
<th>Table 2.—Prothrombin Time Tests</th>
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<tbody>
<tr>
<td>A. 3-25-57.</td>
</tr>
<tr>
<td>Normal (N) plasma</td>
</tr>
<tr>
<td>Patient (Y) plasma</td>
</tr>
<tr>
<td>Y + N plasma</td>
</tr>
<tr>
<td>Y + AcG</td>
</tr>
<tr>
<td>Y + Proconvertin</td>
</tr>
<tr>
<td>B. 4-3-57.</td>
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<tr>
<td>Daughter (D\textsubscript{1}) plasma</td>
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<tr>
<td>Daughter (D\textsubscript{2}) plasma</td>
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<tr>
<td>Patient (Y) plasma</td>
</tr>
<tr>
<td>Y + saline</td>
</tr>
<tr>
<td>Y + D\textsubscript{1}</td>
</tr>
<tr>
<td>Y + D\textsubscript{2}</td>
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</tbody>
</table>
1:50 AcG was used, the apparent differences between Y and A, in the thromboplastin (human brain) dilution series, were very similar to those in the original tests of March 23d. However, increasing the strength of AcG added to Y diminished the differences, and in the last series (1:1 AcG) the data were essentially comparable to the (A) test series. This argues strongly against any true "antithromboplastin." Rather, the results may be interpreted in terms of differences in AcG content. This raises an important new question concerning the very great significance of the AcG concentration in such test series, which have been used in the past to support claims for the presence of an "antithromboplastin." There has been a gradual recognition of the need to exclude from this designation inhibitors of the plasmatic thromboplastin cofactors.

Preincubation of plasmas with thromboplastin for 1 hour at 37 C. increased the prothrombin time of Y's plasma from 48.4" to 73". The corresponding change in a stored normal (N') plasma (8 per cent AcG) was from 16" to 35". The addition of 10 per cent AcG reduced these latter times, respectively, to 22.8" and 14.4", initially, and to 27" and 15.8", after 10 min. subsequent incubation.

0.05 ml. plasma Y (stored for a month at -20 C.), mixed with 0.1 ml. human brain thromboplastin and subsequently with (a) another 0.05 ml. thromboplastin or (b) 0.05 ml. saline, and then recalcified, gave respective prothrombin times: (a) 49" or (b) 53", initially, and (a) 51" or (b) 55", after 60 min. preincubation at 37 C. These results also failed to substantiate any true "antithromboplastic" action, but again raised the question of AcG content during these tests.

AcG assays. AcG is routinely assayed by comparative tests on the restoration of clotting-times in mixtures with aged normal plasma (see special methods). Current reference standards were dilutions of (1) fresh normal human plasma (N); (2) standard bovine AcG (see above). 1:10 AcG repeatedly assayed very close to 100 per cent and served as a valuable standard in many experiments, especially in assaying for anti-AcG (see below).

AcG content and clotting tests, with Ca-tpln., on various plasma mixtures. Y's plasma AcG assays were <1 per cent during his hemorrhagic episode. Table 4 summarizes experimental results on testing various mixture of Y plasma, N plasma, and standard AcG. Test (1), Y 1:20 with N 1:2, gave 11.6 sec. as compared with the corresponding control, test (14), N 1:2 alone, 10.4 sec. Test (3), with Y and N both 1:20, gave 37.6 sec., compared with the control, test (16), 15.5 sec. The former represents about 3 per cent AcG, instead of the control's 50 per cent, it being recalled that 1:10 is the routine test plasma dilution which gives the 100 per cent value of 14.2 sec., test (15), in the N reference series. Test (8), Y 1:20 with AcG 1:100, gave 15.6 sec., which was practically the same as 50 per cent N, 15.5 sec. in test (16), but significantly longer than the 14.8 sec. of N 1:20 with AcG 1:100.

Table 3.—Influence of serial dilutions of thromboplastin (tpln) on "prothrombin time" (sec., at 37 C.). Test mixtures: 0.1 ml. plasma (or mixture with equal volume AcG, at strengths stated) + 0.1 ml. tpln. (at dilution stated) + 0.1 ml. 0.02 M CaCl₂. Plasmas: Y = patient; N = fresh normal; A = aged normal.

<table>
<thead>
<tr>
<th>Tpln. dil.</th>
<th>1/1</th>
<th>1/2</th>
<th>1.4</th>
<th>1/8</th>
<th>1/16</th>
<th>1/32</th>
</tr>
</thead>
<tbody>
<tr>
<td>3-23-57: Y</td>
<td>31.4&quot;</td>
<td>52.8&quot;</td>
<td>159.0&quot;</td>
<td>360.0&quot;</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>3-23-57: N</td>
<td>8.9&quot;</td>
<td>11.5&quot;</td>
<td>20.3&quot;</td>
<td>27.0&quot;</td>
<td>35.7&quot;</td>
<td>55.2&quot;</td>
</tr>
<tr>
<td>5-8-57: Y + AcG (1:50)</td>
<td>14.6&quot;</td>
<td>16.0&quot;</td>
<td>19.0&quot;</td>
<td>21.4&quot;</td>
<td>25.6&quot;</td>
<td>33.6&quot;</td>
</tr>
<tr>
<td>5-8-57: Y + AcG (1:50)</td>
<td>30.8&quot;</td>
<td>38.6&quot;</td>
<td>50.0&quot;</td>
<td>64.4&quot;</td>
<td>78.6&quot;</td>
<td>102.0&quot;</td>
</tr>
<tr>
<td>5-8-57: Y + AcG (1:5)</td>
<td>18.7&quot;</td>
<td>22.6&quot;</td>
<td>29.0&quot;</td>
<td>34.4&quot;</td>
<td>43.4&quot;</td>
<td>56.0&quot;</td>
</tr>
<tr>
<td>5-8-57: Y + AcG (1:1)</td>
<td>13.8&quot;</td>
<td>15.6&quot;</td>
<td>20.0&quot;</td>
<td>25.2&quot;</td>
<td>29.0&quot;</td>
<td>36.0&quot;</td>
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</tbody>
</table>
TABLE 4.—AcG assays. Clotting-times (sec., at 37 C.) for 0.1 ml. substrate (aged plasma) + 0.1 ml. test mixture + 0.2 ml. Ca-tpln. Substrate control = 73.0 sec. Dilutions refer to test mixture, before adding substrate, Ca, tpln.

<table>
<thead>
<tr>
<th>Plasma Y</th>
<th>Plasma N</th>
<th>AcG</th>
<th>C.T.</th>
</tr>
</thead>
<tbody>
<tr>
<td>1. 1:20</td>
<td>1:2</td>
<td>—</td>
<td>11.6&quot;</td>
</tr>
<tr>
<td>2. 1:2</td>
<td>1:2</td>
<td>—</td>
<td>29.9&quot;</td>
</tr>
<tr>
<td>3. 1:20</td>
<td>1:20</td>
<td>—</td>
<td>37.6&quot;</td>
</tr>
<tr>
<td>4. 1:2</td>
<td>1:20</td>
<td>—</td>
<td>48.6&quot;</td>
</tr>
<tr>
<td>5. 1:20</td>
<td>—</td>
<td>1:2</td>
<td>10.0&quot;</td>
</tr>
<tr>
<td>6. 1:2</td>
<td>—</td>
<td>1:2</td>
<td>12.9&quot;</td>
</tr>
<tr>
<td>7. 1:20</td>
<td>—</td>
<td>1:50</td>
<td>14.0&quot;</td>
</tr>
<tr>
<td>8. 1:20</td>
<td>—</td>
<td>1:100</td>
<td>15.6&quot;</td>
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<tr>
<td>9. 1:2</td>
<td>—</td>
<td>1:100</td>
<td>26.8&quot;</td>
</tr>
<tr>
<td>10. 1:20</td>
<td>—</td>
<td>—</td>
<td>56.8&quot;</td>
</tr>
<tr>
<td>11. —</td>
<td>1:20</td>
<td>1:2</td>
<td>9.3&quot;</td>
</tr>
<tr>
<td>12. —</td>
<td>1:20</td>
<td>1:50</td>
<td>12.2&quot;</td>
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<tr>
<td>13. —</td>
<td>1:20</td>
<td>1:100</td>
<td>14.8&quot;</td>
</tr>
<tr>
<td>14. —</td>
<td>1:2</td>
<td>—</td>
<td>10.4&quot;</td>
</tr>
<tr>
<td>15. —</td>
<td>1:10</td>
<td>—</td>
<td>14.2&quot;</td>
</tr>
<tr>
<td>16. —</td>
<td>1:20</td>
<td>—</td>
<td>15.5&quot;</td>
</tr>
</tbody>
</table>

test (13). The other test variations confirmed the findings that Y's plasma, 1:2 or 1:20, not only failed to show any assayable AcG, but also contained some factor which was inhibitory to any AcG added either with normal human plasma or in the standard bovine AcG preparation. The inhibition was greater the higher the proportion of Y plasma present.

Lack of species specificity of the inhibitor. Besides the above comparable inhibitions of human and bovine AcG, a canine factor V preparation, obtained through the courtesy of Dr. R. H. Wagner, UNC Dept. of Pathology, was similarly inhibited.

Pre-incubation with AcG. Since some types of inhibitor23 need a time period for maximal effect, and since a similar finding had been claimed for the alleged anti-AcG in the earlier case reported,13 this question was also studied with the present patient's plasma. In three parallel series, run simultaneously, 1 ml. of "standard" AcG was pre-incubated at 37 C. over 1 hour with (a) saline, control; (b) 1:20 Y plasma, stored 7 weeks at −20 C.; (c) 1:20 aged normal plasma, A. The AcG assays showed no significant progressive loss of AcG in any of the test series. There was only the initial loss of about 60 per cent AcG in (b) due to the inhibitor in Y's plasma. Similar results were also obtained at room temperature, 22 C., and with several dilutions of Y's plasma or serum. The "immediate" character of the anti-AcG effect was rather surprising. However, it did permit a simple procedure for quantitative assay of the inhibitor, as follows.

Anti-AcG assays. The inhibitor titer was determined from the reduction in AcG test activity on adding to standard 1:10 AcG (100 per cent) equal volumes of serial dilutions of Y's serum, plasma, or certain fractions.

Table 5 shows the actual test clotting-times in corresponding dilution series for Y's plasma and serum. The AcG-saline control gave 15.6 sec. The very
close point-to-point comparison at corresponding dilutions clearly indicated that all the plasma anti-AcG survived the clotting process and could be quantitatively assayed in the serum.

When normal serum was substituted for saline, in another control, the same 15.6 sec. clotting-time was obtained. This must mean that the normal human serum contained no detectible AcG nor anti-AcG.

Table 6 shows data for some of the tested titers (dilutions) of Y's plasma and certain fractions. These values are given as percentage inhibition, computed by difference between the found (residual) AcG value and the original 100 per cent. The 1:20 dilutions show the comparisons particularly well, the percentage inhibition here being about 69 (64–72) with (a) the original plasma, oxalated or citrated, (b) BaSO₄-adsorbed oxalated plasma, (c) the supernatant (sup.) after 25 per cent saturation with (NH₄)₂SO₄, and none in the sediment (sed.), (d) practically all in the sediments after 33 per cent or 50 per cent saturation with (NH₄)₂SO₄. The fractions were made from oxalated Y plasma and the sediments, removed by centrifugation, were redissolved in imidazole-buffered saline, pH:7.3, to the original plasma volume, in each instance. In all tests, the inhibitor effect became minimal (<15 per cent) at a titer of 1:160 to 1:320. These preliminary data suggested that the anti-AcG accompanied certain of the plasma globulin fractions and was not adsorbed by BaSO₄.

Nature of the inhibitor.

Stability data. (a) Temperature. The plasma anti-AcG proved to be remarkably stable at −20 C. (frozen), 4 C., 22 C., and 37 C. At room (1) or refrigerator temperatures, its potency was unaltered in several weeks, and

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**Table 5.—Inhibition of Bovine AcG**

<table>
<thead>
<tr>
<th>Additives</th>
<th>Clotting-times (sec.) AcG Assays</th>
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<tbody>
<tr>
<td></td>
<td>Plasma</td>
</tr>
<tr>
<td>Y 1:0.2</td>
<td>42.0&quot;</td>
</tr>
<tr>
<td>Y 1:2</td>
<td>26.0&quot;</td>
</tr>
<tr>
<td>Y 1:8</td>
<td>22.0&quot;</td>
</tr>
<tr>
<td>Y 1:64</td>
<td>17.0&quot;</td>
</tr>
<tr>
<td>Y 1:256</td>
<td>16.6&quot;</td>
</tr>
</tbody>
</table>

AcG-saline 15.6"

**Table 6.—Titer of Anti-AcG: Per Cent Inhibition**

<table>
<thead>
<tr>
<th>Y's Plasma and Fractions</th>
<th>1:20</th>
<th>1:160</th>
<th>1:320</th>
</tr>
</thead>
<tbody>
<tr>
<td>Citr. plasma</td>
<td>64</td>
<td>22</td>
<td>10</td>
</tr>
<tr>
<td>Oxal. plasma</td>
<td>69</td>
<td>23</td>
<td>14</td>
</tr>
<tr>
<td>BaSO₄ plasma</td>
<td>69</td>
<td>25</td>
<td>14</td>
</tr>
<tr>
<td>(NH₄)₂SO₄:</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>25% sat., sup.</td>
<td>69</td>
<td>21</td>
<td>—</td>
</tr>
<tr>
<td>25% sat., sed.</td>
<td>0</td>
<td>5</td>
<td>—</td>
</tr>
<tr>
<td>33% sat., sed.</td>
<td>68</td>
<td>3</td>
<td>—</td>
</tr>
<tr>
<td>50% sat., sed.</td>
<td>72</td>
<td>14</td>
<td>14</td>
</tr>
</tbody>
</table>
at 37°C (2) there was no change in 48 hours. At 60°C (3) there were only
minor fluctuations in test values over 1 hour. At 70°C (4) a progressive fall
in titer occurred, with practically complete inactivation in 30 minutes. At
boiling temperature (5), the anti-AcG was entirely destroyed within 5 min-
utes. Figure 1 shows the anti-AcG test values, as percentage inhibition, with
a 1:1 titer in the plasma assay. Comparable data were also obtained in as-
says at 1:20 titer.

(b) pH. The influence of pH on the inhibitor was studied by cautiously
adding N/10 HC1 or N/10 NaOH to Y's plasma, determining the pH with
the glass electrode, rechecking after 2 to 2½ hrs., and then restoring to the
original pH (8.25) before testing at (I) 1:1 and (II) 1:20 titers, accurate
as to volumes. Figure 2 shows the excellent stability over the wide range
between pH 5 and 10. The inhibitor potency was slightly weakened at pH
4.0 and at 11.5. At pH 2.5, about half the activity was lost in 2 hours.

(c) Fat solvents. Five-minute shaking with an equal volume of either
pure ether or benzene (C₆H₆) did not alter the inhibitor test values, whether
determined immediately or when retested 24 hours later. These results differ
from previous reports on an allegedly similar (anti-AcG) inhibitor¹³ and on
one possibly of another type.²⁷

![Graph showing temperature stability of anti-AcG](image)

**Fig. 1.**—Temperature stability of anti-AcG.
Nondialysability. 1:5 dilutions of plasma Y (April 3, 1957) remained in the refrigerator, at 4 C., from May 2nd, after tests of pH stability (see (b) above). Two of them, which on that date had been exposed for 2½ hrs. to pH 5.6 and 10.0, respectively, and then restored to pH 8.25, were retested 21 days later and found to have the same inhibitor potency (anti-AcG assays) as originally. They were, therefore, pooled and retested before and after dialysis for 24 hours against saline at 22 C., room temperature. The inhibitory titers remained unchanged. Besides affording additional evidence of the stability of the inhibitor, these tests showed that the active factor in Y’s plasma was nondialysable.

Serum Proteins. No cryoglobulins were demonstrable in Y’s plasma or serum, kept under observation at 4 C. The “distilled water screening test” for macroglobulins was negative and no atypical proteins were demonstrable by electrophoresis. The usual five serum protein peaks were identified electrophoretically by their typical mobilities as albumin and α, α₂, β, and γ globulins.

Paper electrophoresis, was performed at Duke Hospital on Mar. 29, 1957 on fresh serum (Y), through the courtesy of Dr. W. R. Rundles. Of the total protein (5.9 Gm./100 ml.), the following “percentages” were computed: albumin 56, globulins 44, including α₁ 9, α₂ 14, β 8, and γ 13.

Moving boundary electrophoresis (“Spinco”) was carried out at UNC, with the cooperation of Drs. J. L. Irvin (Biochemistry) and R. H. Wagner (Pathology), on two serum (Y) samples in barbiturate buffer, pH 8.6, ionic strength 0.1. Figure 3: (I) data of April 3, 1957 (when anti-AcG was present in the sample tested): albumin 45.8, globulins 54.2, including α₁ 8.55, α₂ 18.47, β 11.12, and γ 16.12, are of similar type to the paper chromatography results; (II) data of May 15, 1957 (when inhibitor had disappeared): albumin 56, globulins 44, including α₁ 7.4, α₂ 10.25, β 15.1, and γ 11.4. The above data, obtained by planimetry, were compared with accepted normal values namely: albumin 56 ± 3, globulins 44 ± 3, including α₁ 7.2 ± 1.2, α₂ 8.7 ± 1.5, β 12.8 ± 2.3 and γ 14.4 ± 2.4 (percentages of total protein, for which current data state the normal range to be 6.3 to 7.7 Gm./100 ml., with an A/G ratio 1.3 to 2.2).
Interpretation: These electrophoretic data show that Y's serum had an abnormal globulin pattern, quantitatively, during the period (March and April) when high-titer anti-AcG was demonstrable in this patient's blood. The nature of the anomaly was a considerable increase in $a_2$, and some increase in $a_1$, possibly at the expense of the $\beta$ globulins. The $\gamma$ globulin remained normal. The A/G ratio was reversed (0.85) on April 3, 1957, at the time of the greatest increase in the $a_2$ globulin. Such a pattern is commonly encountered in acute infections. Hence it is merely an assumption that the anti-AcG may be related to the altered globulin pattern. The protein pattern had returned to normal by May 15th, at which time the inhibitor was no longer demonstrable. Unfortunately for these studies, the return to normal cancelled plans for attempting chromatographic separation of the inhibitor.

Partial thromboplastin time tests (P.T.T.). This one-stage test is a modified plasma recalcification clotting-time determination in which a brain cephalin suspension is substituted for the tissue thromboplastin of the usual (e.g. Quick's) prothrombin time test. In the routine, now being followed in a joint clinical research program of the UNC Physiology and Pathology Departments, under the supervision of Dr. J. B. Graham of the latter Department, a patient's (e.g. Y's) plasma P.T.T. is compared with (1) fresh normal (N) plasma, (2) (equivolume) mixtures of patient and normal, (3) similar mixtures of each with various "deficient" plasmas, such as hemophilic (AHF-), Christmas disease (PTC-), Stuart factor deficiency (Stuart-), aged (A) normal plasma (AcG-), etc.

(a) Mar. 26, 1957: The above P.T.T. tests, with Y's plasma, gave the results shown in table 7. They indicated: (1) marked abnormality of Y itself, (2) also Y + N, and (3) Y + any of the "deficient" plasmas mentioned (each of which was normalized by N plasma). These findings indicated the presence

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Normal (N)</th>
<th>Patient (Y)</th>
</tr>
</thead>
<tbody>
<tr>
<td>-</td>
<td>93&quot;</td>
<td>272&quot;</td>
</tr>
<tr>
<td>N</td>
<td>93&quot;</td>
<td>241&quot;</td>
</tr>
<tr>
<td>AHF-</td>
<td>80&quot;</td>
<td>172&quot;</td>
</tr>
<tr>
<td>PTC-</td>
<td>76&quot;</td>
<td>156&quot;</td>
</tr>
<tr>
<td>Stuart-</td>
<td>81&quot;</td>
<td>233&quot;</td>
</tr>
<tr>
<td>AcG-</td>
<td>81&quot;</td>
<td>233&quot;</td>
</tr>
</tbody>
</table>
of an inhibitor, the nature of which was defined by the other studies in this paper.

(b) May 15, 1957: P.T.T. tests on Y were normal on this occasion, viz.
(1) Y = 100", (2) Y + N = 99", (3) N = 106".

(c) Aug. 22, 1957: Similar normality was noted on this occasion.

_A Circulating Inhibitor (Anti-AcG)_

A CIRCULATING INHIBITOR (ANTI-AcG) of an inhibitor, the nature of which was defined by the other studies in this paper.

(b) May 15, 1957: P.T.T. tests on Y were normal on this occasion, viz.
(1) Y = 100", (2) Y + N = 99", (3) N = 106".

(c) Aug. 22, 1957: Similar normality was noted on this occasion.

_Thromboplastin generation tests (T.G.)_ The original Biggs-Douglas\textsuperscript{1} technic, III of figure 4, and the Bell-Alton\textsuperscript{2} modification, I and II of figure 4, (substituting brain cephalin for the platelet suspension) were both used on occasion.

(a) Mar. 26, 1957: I of figure 4. Compared with the control (0), N plasma + N serum; Y plasma + Y serum, gave markedly abnormal results (1). These showed also with (2), Y plasma + N serum; (3), N plasma + Y serum; (4), equal volumes Y + N plasmas and N serum; and (5), N plasma and equal volumes Y + N sera. This is typical of an inhibitor\textsuperscript{16}.

(b) July 25, 1957: This series, II of figure 4, was designed to show the effects of Y's inhibitor on the conversion of prothrombin to thrombin, in the presence of fully formed plasma thromboplastin. Tests were performed on fresh, normal, Y citrated plasma of April 1st, and aged normal citrated plasma (actually Mr. Y's daughter D\textsubscript{1}) of April 5th. Hence, the patient's plasma (1) was used as _substrate_ in the final step of the T.G. test, comparison being made with a fresh normal plasma substrate (2) and the above aged normal plasma substrate (3). The last two behaved in a typically normal fashion. The patient test, however, was grossly abnormal. This is a clear indication that the inhibitor interferes with some factor (AcG) essential for the conversion of prothrombin (see Discussion.).

(c) May 15, 1957: Fresh materials from the patient Y of this date (when

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![Fig. 4.—Thromboplastin generation tests (see text).](image-url)
the inhibitor was not detectable by other technics) were tested. The results are shown in series III of figure 4: control curve (0), normal platelets, serum, and plasma; (1) Y plasma + N serum; (2) N plasma + Y serum; and (3) N plasma, N serum, and Y platelets. All tests were perfectly normal (see Follow-up Tests).

Prothrombin utilization rate (P.U.R.). Prothrombin consumption was tested by measuring the residual prothrombin, at 15 minute intervals, in recalcified plasma (or mixtures) held at 37 C. The most significant determination is usually after 1 hour.

(a) March 28, 1957: Y’s plasma utilized only 33 per cent (instead of the normal’s >90 per cent prothrombin in 60 min., according to assays by the “improved” 2-stage technic.

(b) April 3, 1957: Y’s P.U.R., measured by the specific 1-stage method, was essentially normal in 45-60 min. It was, however, much accelerated in the 15 to 30 min. period, resembling the normal control, when AcG was added. In the absence of added AcG, the 15 min. 1-stage test gave a 150 per cent “prothrombin” figure. This is a frequent finding in many normal plasmas, being usually interpreted as due to the activation of proconvertin to convertin (“serum accelerator”), although Quick regards it as evidence for a “prothrombinogen.”

(c) May 14, 1957: Y’s plasma again gave normal P.U.R. tests by the 1-stage method.

(d) Aug. 22, 1957: A 1-hr. prothrombin consumption of 94 per cent (2-stage method) was perfectly normal.

Other possible inhibitors. There was no evidence for (1) abnormal “anti-thrombin,” (2) “heparin-like” inhibitor, or (3) inhibitors of plasmatic thromboplastin cofactors (AHF, PTC, etc.), according to the following tests:

1. Plasma thrombin clotting-times. Y’s and N’s plasmas gave essentially similar test times with a series of thrombin dilutions (30-1 thrombin units) per 0.3 ml. test mixture, at 37 C.

Y’s serum inactivated added thrombin progressively, comparable to normal serum.

2. Protamine titration test. Y’s plasma (Mar. 8, 1957) was recalcified at 37 C. in the presence of serial dilutions of protamine sulfate. The saline control gave a 21½ min. clotting-time, which is a considerable prolongation (normal: 1½ to 6 min.). Below its own inhibitory concentrations, protamine improved this, but it was most effective, C.T.: 13½ min., at minimal concentrations (0.1 to 0.2 µg./ml.). This, therefore, was similar to many normal plasmas, except for the longer clotting-times throughout, and could not be regarded as evidence for any “heparin-like” factor. Lack of AcG could explain the long clotting-times.

3. Assays for AHF, PTC, and their inhibitors. These two plasma co-factors for thromboplastin generation (see above) were assayed by the prothrombin consumption test method, specially modified in the present studies, by the addition of AcG (BaCO₃-adsorbed beef serum) to overcome the anti-AcG effect. Without this, the assays failed. Known hemophiliac (AHF-) and PTC-deficient plasmas were used as substrates, being mixed with AcG 1:10 and Y plasma 1:5 before recalcification at 37 C. The P.U.R. was
followed over 1 hr. by either 2-stage or 1-stage prothrombin assays. In these systems, the substrate defect was fully corrected by Y's plasma. By comparison with fresh normal plasma dilutions, AHF in Y's plasma was quantitatively assayed at 70 per cent. There was insufficient PTC substrate available, however, for exact quantitation of that factor, but enough was saved for inhibitor tests. In these, mixtures of Y (1:1) and N (1:5) plasmas, again in AcG-supplemented systems, showed no difference from N (alone) in correcting both deficiencies. Anti-AHF and anti-PTC were thus excluded.

Normal test findings. As noted in table 1, a number of other possibilities failed in confirmation because of normal test findings. Platelets were normal (Duke Hospital tests) and the tourniquet test negative. That clot retraction was slow could be attributed to the “buffing”—we use this term in the historical sense, when, in the old days of “blood-letting,” it denoted a combination of slow clotting and rapid r.b.c. sedimentation, which permitted formation of a fibriniferous supernatant layer. This well describes the clotting (March 20, 1957) in Y's case. There was no fibrinolysis. Prothrombin assay normal at 122 per cent or 126 per cent, by specific 1-stage or 2-stage methods, respectively, and proconvertin was a very satisfactory 80 per cent, with no evidence of any antiproconvertin (methods24).

Follow-up Tests
May 14–15, 1957: Prothrombin times were normal (11" to 12") for patient (Y), daughter (D3, pregnant 6 m.o.), son (S1), and control (N), and also for equivolume mixtures of any pairs of these plasmas. Prothrombin assay (110 per cent) and P.U.R. (95 per cent prothrombin consumption in 1 hr.), by specific 1-stage method, were normal.

AcG assays: Y’s plasma AcG level was restored to 45 per cent on May 14th and to 63 per cent on May 15th. D3 plasma was 82 per cent AcG on the latter date. Our “low normals” seldom reach 65 to 60 per cent.

Anti-AcG was no longer demonstrable, even in undiluted Y plasma or serum. In fact, the AcG now present significantly increased the AcG test values when plasma was mixed with the 1:10 standard AcG in a fashion similar to the earlier studies. Sera obtained on May 15th, after two days in the refrigerator (4 C.) were mixed with (a) 1:10 AcG or (b) 1:20 AcG and gave the following clotting-times (sec.) in the AcG testing: (I) Saline controls: (a) 15.8", (b) 19.5"; (II) Y serum: (a) 15.7", (b) 18.9"; (III) N serum: (a) 15.6", (b) 19.0".

P.U.R., P.T.T. and T.G. tests (see previously) were now completely normal.

August 22, 1957: All studies were normal, including clotting-time (10 min.), clot-retraction, prothrombin time (10.8 sec.), prothrombin assay (1-stage: 100 per cent; 2-stage 118 per cent), proconvertin (100 per cent). AcG (100 per cent). No inhibitor was demonstrable, and the P.T.T. test (Y: 82.5 sec.; N: 82.5 sec.) and P.U.R. (94 per cent prothrombin consumption (2-stage) in 1 hr.) were perfectly normal. T.G. tests were also normal and very similar to those of May 15th (see series III, fig. 4).

Discussion
In the light of the foregoing experimental analysis, the abnormalities in
certain of the routine tests (table 1) and in the special test systems described, may all be interpreted as the result of a severe AcG deficiency in the blood of patient Y, and this can be accounted for by the presence of a powerful inhibitor, specific for the labile factor (factor V). The diagnosis of hypoproaccelerinemia due to a specific inhibitor (anti-AcG) seems clearly established.

Clinical evaluation. There are an increasing number of reports of bleeding disturbances associated with one or another type of circulating anticoagulant. The true etiology is often obscure, and the case of Mr. Y is no exception. It is interesting that his clinical course seemed to be worse (as to hematuria) after transfusions on two occasions and to be progressively improved, with disappearance of the inhibitor, on continued ACTH-cortisone therapy. The evidence for an immunosensitization is not conclusive, however, especially as the hematuria was first noted prior to any transfusions. The absence of gamma-globulin increase prevents any conclusion which the converse might suggest as to an immune type "antibody." The other distinct possibility is a dysproteinemia, perhaps associated with the stress of his gall-bladder infection. The question of "collagen disease" has been considered in our case. The absence of a positive L.E. test or corroborative clinical findings render this possibility highly unlikely. There is little to implicate the liver beyond the suspicion that it is the chief organ associated with the maintenance of plasma proteins. Whether or not the anomalous globulin (electrophoretic) pattern, especially the $\alpha_2$ increase, had any true correlation with the inhibitor, other than coincidence in time, was not fully determined. It would appear that the anti-AcG was a globulin protein, nondialysable, and with a rather remarkable stability to temperature, pH, and fat solvents. This unique case has features not described in any other that we have been able to find in the hematologic literature.

Comparison with data in literature. The considerable literature on circulating inhibitors of blood coagulation was searched diligently. Only one series of reports13,14 was found in which the existence of "a specific inhibitor of Factor V" was claimed. A critical review of these data raises questions as to (1) adequacy of controls, (2) the small degree of "inhibition" (cf. natural instability of AcG), (3) alleged absence of inhibitor in patient's serum, (4) alleged inactivation on extracting plasma with ether or petroleum ether, (5) alleged demonstration also in (a) five out of six siblings, whose factor V (AcG) assays were low (23 to 46 per cent), and (b) in normal aged oxalate plasma. It is suggested that the German case was undoubtedly a congenital hypoproaccelerinemia22 (Owren's25 "parahemophilia"), but the evidence for an inhibitor complication was unconvincing.

Significance of AcG in blood clotting. The present case offered an exceptional opportunity to learn more about the role of AcG at several stages of the blood-clotting process. Hörder and Sokal15 had used a case of "parahemophilia" (since no inhibitor was claimed, it was probably not the patient of their preceding publications) to study thromboplastin generation and to conclude that (a) AcG (Factor V) is necessary for blood thromboplastin generation, but (b) is not necessary in the subsequent prothrombin
A CIRCULATING INHIBITOR (ANTI-ACG)

conversion. The earlier work of Biggs, Douglas, and Macfarlane\(^4\) on factors concerned with thromboplastin generation has been extended by several recent workers. In particular, Hougie\(^{17}\) has recently published a study, including observations on systems containing reagents from our\(^{22}\) earlier case of severe congenital hypoproaccelerinemia (Owren's disease). Hougie's data afford good support for the idea that AcG is needed for thromboplastin generation, but at a later phase than the interaction of platelet (lipid) and plasmatic co-factors (AHF, PTC, Stuart factor, etc.). Using the AcG-deficient patient's plasma as substrate, no differences were observed, compared with normal plasma substrate, in testing the thromboplastin generated from normal platelets, plasma, and serum. The last finding, common to the works cited\(^{15,17}\) must remain inconclusive because of the carry-over of AcG from the thromboplastin generating system into the prothrombin conversion stage (testing on substrate plasma). Our case, having a strong inhibitor able to neutralize any AcG present, made possible a new evaluation of the role of AcG in the later clotting phases, i.e. after generation of thromboplastin. Thus, in the data of series II, figure 4, Mr. Y's plasma was used as substrate in testing thromboplastin generation from a normal system (lipid, plasma, serum). The grossly abnormal clotting-times are clear evidence that AcG (which has acted normally in generating the thromboplastin) has a significant additional role in some later phase connected with the conversion of prothrombin to thrombin. This confirms the essentiality of AcG in the latter process, which we\(^{21}\) had previously noted in artificial clotting systems deprived of AcG by boiling treatment, including the tissue thromboplastin.

**In summary**, the patient’s abnormal test findings were:

(1) lack of AcG, together with the presence of high-titer anti-AcG.

(2) prolonged clotting-times of whole blood or recalcified plasma, which could be due to lack of AcG.

(3) prolonged “prothrombin time.”

(4) prolonged P.T.T., including failure to correct aged normal and various “deficient” plasmas (table 7), all explicable on the assumption that the inhibitor renders ineffective any AcG which may have been present initially.

(5) abnormal thromboplastin generation (T.G.), with the characteristic test pattern of an inhibitor (fig. 4).

(6) deficient P.U.R., on at least one occasion. We\(^{22}\) had previously noted prothrombin consumption defect in severe AcG-deficiency (congenital).

Besides disclosing an apparently unique case of clotting disorder with hemorrhagic tendency, our studies have presented some new evidence concerning the role of AcG in clotting and in a variety of special tests for the coagulative function of blood. All test systems which need AcG will fail of their special purpose if this factor is not present in sufficient amounts. Its lack may, indeed, be imposed on such systems when the presence of a powerful anti-AcG, as in Mr. Y's plasma or serum, inhibits such AcG as is knowingly supplied. Only by adding enough AcG were we able to perform AHF and PTC assays, rule out the “antithromboplastin” possibility, etc. Y's plasma failed to correct various “deficient” plasmas (see above). This
did not mean that the patient had all these deficiencies, but merely one of
them, namely, AcG lack. It was his anti-AcG which nullified the other tests,
because AcG, while not the specific factor being tested, was nevertheless
an essential component of the special test systems.

SUMMARY

Study of the present hemorrhagic disorder establishes, without doubt, the
presence of a powerful specific anti-AcG. The deprivation of AcG due to
this inhibitor confirms the view that the labile factor plays a key role in
the blood clotting system in at least two phases, namely, (1) during thrombo-
plastin generation, and (2) later, during the conversion of prothrombin
to thrombin. AcG is an essential component of many special clotting-test
systems.

SUMMARIO IN INTERLINGUA

Es presentate un caso de disordine hemorrhagic. Le studio de illo establi
sin dubita le presentia de un potente inhibitor specific anti AcG. Le depriva-
tion de AcG como effecto de iste inhibitor confirma le conception que le factor
labile ha un rolo cardinal in le systema del coagulation sanguinee in al minus
duo phases, i.e. (1) durante le generation de thromboplastina e (2) plus
tarde, durante le conversion de prothrombina in thrombina. AcG es un com-
ponente essential de multe systemas special de essayage coagulatori.

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A CIRCULATING INHIBITOR (ANTI-ACG)

A Circulating Inhibitor (Anti-AcG) Specific for the Labile Factor-V of the Blood-Clotting Mechanism

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