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Research Article

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Partial Purification and Characterization of Contact Activation Cofactor

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ABSTRACT The contact phase of intrinsic clotting involves Factor XI, Factor XII, Fletcher factor, and a fourth activity that we call contact activation cofactor (CAC). All four of these activities are reduced or absent in Dicalite-adsorbed plasma. A modified activated partial thromboplastin time assay for CAC has been defined by using a substrate of Dicalite-adsorbed plasma combined with partially purified sources of Factors XI and XII, and Fletcher factor. The following properties of CAC in plasma have been determined by using the assay: it is stable up to 60 min at 56°C; gradually loses activity at 80°C; is stable between pH 6 and 9; is precipitated by ammonium sulfate between 40% and 50% saturation; is slightly adsorbed by $Al(OH)_3$; and is eluted from DEAE-cellulose after the major protein peaks. A purification procedure has been devised that separates CAC from other known clotting factors. Isolated CAC was less stable than CAC in plasma, but in the presence of dilute human serum albumin it retained full activity for 80 min at 56°C. On gel filtration CAC had an apparent mol wt of 220,000 daltons. These properties are consistent with those described for Fitzgerald factor, which further supports the conclusion that CAC and Fitzgerald factor represent the same activity. Isolated CAC promoted the generation of activated Factor XI (XI_a) in a mixture containing purified Factor XI, Factor XII, and kaolin. The amount of Factor XI_a generated was proportional to the amount of added CAC. No time-consuming reaction between Factor XI or Factor XII and CAC could be demonstrated.

INTRODUCTION

When decalcified plasma is exposed to an activating surface such as glass, intrinsic clotting is initiated through a series of reactions referred to collectively as the contact activation phase of intrinsic clotting. Initially, Factors XI and XII were thought to be the sole reactants, with Factor XII being adsorbed and activated at the surface and then enzymatically activating Factor XI (1-3). However, the picture became more complex with the description of Fletcher factor, which also participated in contact activation (4, 5). Recently, we have demonstrated that an additional plasma activity participates in the interaction of Factors XI and XII in the presence of Fletcher factor and an activating surface (6). We call this activity contact activation cofactor (CAC).[1] CAC, like the other contact activation factors, is readily adsorbed to glasslike surfaces. This property allows one to prepare an artificial assay system for CAC activity. Below we report the details of this assay, some of the properties of CAC activity in plasma, a method for partial purification that separates CAC from other characterized clotting factors, and some properties and reactions of the isolated CAC.

METHODS

All plasmas and plasma fractions were protected from contact with glass surfaces. Plastic equipment was used whenever possible. Essential glass surfaces (cuvettes, columns) were coated with Siliclad (Clay Adams, Div. of Becton, Dickinson & Co., Parsippany, N. J.). Platelet-poor plasmas were prepared from blood taken into either balanced citrate anticoagulant (0.06 M sodium citrate plus 0.04 M citric acid) or acid citrate dextrose anticoagulant by centrifugation at 12,500 *g* for 10 min.

Factor XI-Fletcher factor reagent for CAC assay was the pooled first protein peak from the DEAE-cellulose chromatography of normal plasma or normal plasma adsorbed once with $Al(OH)_3$, prepared as described previously (7). This technique separates Factor XI with Fletcher factor from other known clotting activities. The

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[1] *Abbreviations used in this paper:* CAC, contact activation cofactor, TES, *N*-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid. Activated clotting factors are designated by the subscript "a"; e.g. Factor XI_a, activated Factor XI.

reagent used for most of the experiments below contained about 36% Factor XI and 24% Fletcher factor. It was purified about threefold over starting plasma.

Factor XII reagent for CAC assay. Factor XII was separated from other known clotting factors by DEAE-cellulose chromatography of normal plasma or normal plasma adsorbed once with $Al(OH)_3$ either by the techniques previously described or by a slight modification thereof (7). Saturated ammonium sulfate solution was added to the pooled Factor XII fraction to 25% saturation. The precipitate was discarded, and the solution was brought to 50% saturation. The 50% precipitate, containing the Factor XII, was dissolved in a small amount of buffer and dialyzed to remove ammonium sulfate. This process concentrated Factor XII and largely separated it from anti-XI_a (8). The reagent used for most of these experiments was further rechromatographed on DEAE-cellulose. The final reagent, purified about 3.4-fold over starting plasma, was diluted to about 42% of pooled normal plasma Factor XII activity and stored at −20°C in plastic vials.

Dicalite-adsorbed plasma was prepared by chromatographing normal citrated plasma on a column of the diatomaceous earth powder Dicalite 4200 (Grefco Inc., Dicalite Div., Los Angeles, Calif.). The Dicalite was washed five times with distilled water and five times with *N*-tris (hydroxymethyl) methyl-2-aminoethane sulfonic acid (TES) buffer (0.05 M TES and 0.1 M NaCl, pH 7.35) by suspending the powder in wash solution, allowing the major fraction to settle, and decanting the supernate. The washed powder was then suspended in TES buffer, and the slurry was used to build a column (1–1.1 g Dicalite/ml plasma). Normal citrated plasma was added to the column, and the column was eluted with TES buffer. The major protein peak was pooled (about 1–1.2 times the volume of applied plasma) and incubated at 37°C in glass tubes for up to 2 h until the partial thromboplastin time (9) of the plasma in the presence or absence of kaolin rose to around 150 s. The pH was adjusted to 7.4, and the Dicalite-adsorbed plasma was packed in small samples in plastic vials and frozen at −20°C. The reagent used for most of the experiments reported herein had the following clotting factor activities: Factor II, 63%; factor V, 30%; Factor VIII, 62%; Factor IX, 103%; Factor X, 92%; Factor XI, 4%; Factor XII, 0.6%; Fletcher factor, 0.1%, and fibrinogen, 105 mg/100 ml.

When the diluted Factor XI-Fletcher factor reagent and diluted Factor XII reagent were added to Dicalite-adsorbed plasma in the presence of cephalin, kaolin, and calcium, the clotting times observed were longer than those obtained with diluted congenital Factor V-deficient plasma as a source of Factor XI, Factor XII, and Fletcher factor as well as other clotting activities (see Table I). Factor V-deficient plasma was used to supply missing clotting factor activities to eliminate the possibility that any decrease in clotting time might be due to supplementing the reduced Factor V in the substrate. Since the clotting times in the presence of this plasma were shorter than the clotting times obtained with isolated Factors XI and XII, and Fletcher factor, and Dicalite-adsorbed plasma contained adequate amounts of the other known clotting factors, the diluted Factor V-deficient plasma must be supplying another missing activity to the system. We define this additional activity missing in the Dicalite-adsorbed plasma as CAC.

Absence of CAC in Dicalite-adsorbed plasma was confirmed by failure of Dicalite-adsorbed plasma to promote generation of Factor XI_a in a mixture containing Factor XI-Fletcher factor reagent and Factor XII adsorbed to kaolin. The reaction system was that described previously (6).

TABLE I

Evidence that Dicalite-Adsorbed Plasma Lacks an Activity Other than Factors XI, XII, and Fletcher Factor

Test substance	Clotting time
	s
1. F. XI-Fletcher (9%–6%) + F. XII (10%)	108
2. F. XI-Fletcher (4.5%–3%) + F. XII (5%)	111
3. V-deficient plasma diluted 1/10	86
4. Buffer	113

Clotting mixtures 1 and 2 contained 0.01 ml cephalin 1/10, 0.05 ml Dicalite-adsorbed plasma, 0.05 ml diluted Factor XI-Fletcher factor reagent, 0.05 ml diluted Factor XII reagent, 0.05 ml TES buffer, and 0.01 ml 20 mg/ml kaolin. Clotting mixture 3 contained 0.01 ml cephalin 1/10, 0.05 ml Dicalite-adsorbed plasma, 0.05 ml diluted Factor V-deficient plasma, 0.1 ml TES buffer, and 0.01 ml 20 mg/ml kaolin. Clotting mixture 4 contained 0.01 ml cephalin 1/10, 0.05 ml Dicalite-adsorbed plasma, 0.15 ml TES buffer, and 0.01 ml 20 mg/ml kaolin. After 10 min incubation at 37°C, 0.025 ml 25 mM $CaCl_2$ was added and the clotting times noted. The Factor V deficient plasma contained 75% Factor XI, 84% Factor XII activity and 96% Fletcher factor. Average of two experiments.

CAC assay. The CAC assay was based on the ability of a test substance to correct the abnormal clotting time of a substrate containing Dicalite-adsorbed plasma and partially purified Factors XI, XII, and Fletcher factor in the presence of cephalin, calcium chloride, and kaolin as an activating surface. This substrate contains all known clotting factors except CAC. The reagents were added to a plastic clotting tube in the following order: 0.01 ml cephalin (10), diluted 1/10 in barbital buffer, pH 7.35; 0.05 ml Dicalite-adsorbed plasma; 0.05 ml Factor XI-Fletcher factor reagent; 0.05 ml Factor XII reagent; 0.05 ml test substance; and 0.01 ml kaolin (20 mg/ml) (kaolin, N.F. colloidal, Mallinckrodt Chemical Works, St. Louis, Mo.). A stopwatch was started with the addition of the kaolin, the tube was placed in a heating block at 37°C, and precisely 10 min later 0.025 ml $CaCl_2$ (either 100 mM in early experiments or 25 mM for the later experiments) was added, and the clotting time was noted. A standard curve was prepared by recording the log clotting time of a normal standard plasma diluted in TES buffer against log plasma concentration. Samples for assay were dialyzed against and/or diluted in TES buffer.

Other clotting factor assays. Assays for Factors II, V, VIII, IX, X, XI, XII, and Fletcher factor have been described in detail elsewhere (6). Factor XI_a was detected by adding 0.05 ml test material to a plastic clotting tube containing 0.05 ml cephalin (10) diluted 1/60 in a barbital buffer, pH 7.35, and 0.05 ml hereditary Factor XI-deficient plasma; 0.05 ml of 40 mM $CaCl_2$ was added immediately, and the clotting time was noted. Fibrinogen concentration in Dicalite-absorbed plasma was measured by the method of Blombäck and Blombäck (11). Evidence for small amounts of fibrinogen in purified fractions was obtained by (*a*) double immunodiffusion in 1% agar against rabbit antihuman fibrinogen antiserum (Hyland Div., Travenol

Laboratories, Inc., Costa Mesa, Calif.) and (*b*) by adding 0.025 ml of a thrombin-calcium reagent (5 U/ml bovine topical thrombin adsorbed with $BaSO_4$ containing 0.5 M $CaCl_2$ [Parke, Davis & Company, Detroit, Mich.]) to 0.15 ml test fraction and observing formation of fibrin threads. If no fibrin threads formed within 2 h, an additional 0.025 ml of 10 U/ml thrombin without calcium was added. Test mixtures were observed for 24 h. Thrombin was detected by incubating 0.05 ml test material with 0.1 ml bovine fibrinogen solution, 2 mg/ml (Warner-Chilcott Laboratories, Division Warner Lambert Co., Morris Plains, N. J.) in a plastic tube. If no clot formed within 24 h, the sample was judged free of thrombin. Factor XIII was detected by incubating 0.1 ml congenital factor XIII-deficient plasma (12), 0.1 ml test substance, and 0.1 ml 25 mM $CaCl_2$ containing 0.05 M cysteine at 37°C. After 30 min, 1.5 ml 5 M urea was added, the tubes were capped and placed at room temperature, and the clots were observed over 20 h. Clots from test mixtures lacking Factor XIII dissolved within 10 h.

The amount of clotting factor activity in test samples is expressed as percent of a normal pool plasma standard unless otherwise indicated. In some cases purified reagents appeared to have a trace of another clotting activity, as indicated by a clotting time close to but not as long as the blank. In that case the amount of the contaminant has been shown to be less than the lowest concentration reliably tested.

$Al(OH)_3$-adsorbed plasma was prepared by adding 0.025 ml $Al(OH)_3$ (Alhydrox, Cutter Laboratories, Inc., Wayne, N. J.) to 1 ml test plasma in a plastic tube. The mixture was inverted to mix, incubated for 3 min at 37°C, and centrifuged to remove the $Al(OH)_3$. In some cases the supernate was readsorbed by this method to obtain twice-adsorbed plasma.

Determination of temperature stability. Normal plasma diluted 1/20 or 1/30 was incubated in a covered plastic tube at the desired temperature for variable time intervals and then tested in the CAC assay.

Ammonium sulfate fractionation. Saturated ammonium sulfate solution adjusted to pH 7.4 was added slowly with stirring to normal plasma to bring the mixture to 25% saturation. After 30 min of incubation, the precipitate was separated by centrifugation. Saturated ammonium sulfate solution was added to the supernate slowly with stirring to increase the concentration of ammonium sulfate in the mixture to 33%. The mixture was incubated for 30 min and centrifuged. The fractions precipitating at 40% and 50% saturation were similarly obtained from the 33% and 40% saturation supernates, respectively. All procedures were carried out at 4°C with chilled reagents. Precipitates were dissolved or suspended in small volumes of TES buffer and dialyzed exhaustively against TES buffer. They were diluted to one fifth or one tenth original plasma volume for assay.

Determination of pH stability. 1 ml of normal plasma was dialyzed against 100 ml of buffer overnight at 4°C. The following buffer solutions were used: pH 5 and 6, 0.05 M sodium acetate plus 0.1 M sodium chloride adjusted to desired pH; pH 7, 7.35, and 8, 0.05 M TES plus 0.1 M sodium chloride adjusted to desired pH; pH 9 and 10, 0.05 M sodium carbonate plus 0.1 M sodium chloride adjusted to desired pH. The dialyzed plasmas were diluted 1/20 and 1/40 in TES buffer, pH 7.35, and assayed for CAC activity.

Estimation of molecular size by gel filtration. Samples were chromatographed on a column of Sephadex G-200, 2.5 × 90 cm, with 1 cm of Sephadex G-25 layered on the top. The column was equilibrated and eluted with 1.5 M NaCl-0.025 M Tris, pH 7.2, containing partially purified human serum albumin to produce an $A_{280} = 0.1$. Fractions of 3.5 ml were collected automatically. The column was standardized with human gamma globulin (Pentex Biochemical, Kankakee, Ill.), which gel filters as a molecule of 205,000 daltons (13); aldolase (Pharmacia Laboratories, Inc., Piscataway, N. J.) 158,000 daltons; bovine serum albumin (Worthington Biochemical Corp., Freehold, N. J.) 70,000 daltons; and chymotrypsinogen A (Pharmacia) 25,000 daltons. Void volume was defined by blue dextran (Pharmacia). A standard curve was prepared by plotting log molecular weight against elution volume/void volume (13).

Partially purified human serum albumin used in these systems was prepared from the Calbiochem (San Diego, Calif.) crystalline B-grade albumin by binding to DEAE-cellulose at pH 7.2, 0.02 M barbital or Tris, and 0.03 M NaCl. After washing the column with six column volumes of buffer or until protein effluent fell to background, the albumin was eluted with 0.8 M NaCl in buffer and stored in concentrated solution at −20°C. At concentrations used for experiments below it had no detectable Factor II, V, VIII, IX, X, XI, XII, Fletcher factor, or CAC activity.

Purified Factor XI was prepared from normal plasma by a slight modification of a previously described technique (6). Plasma was chromatographed on DEAE-cellulose; the isolated Factor XI was adsorbed to and eluted from $BaSO_4$, then chromatographed on SP-Sephadex at pH 5.2 with a linear gradient between 0.05 M sodium acetate-0.0001 M EDTA containing 0.05 M NaCl and the same buffer containing 0.5 M NaCl; the resultant Factor XI fraction was chromatographed on Dicalite 4200 in the presence of human serum albumin (Calbiochem, crystalline, B-grade) and concentrated by ultracentrifugation. The purified Factor XI fraction had a specific activity of about 5,600 (% Factor XI/A_{280}), which represented a purification of about 3,500-fold over normal plasma. A diluted sample that clotted in the kaolin-activated Factor XI assay in 75 s produced a clotting time of 235 s in the absence of an activating surface. At the time of use, this fraction contained about 300% Factor XI activity. When diluted to about 20% Factor XI activity, it had no detectable Factor II, VIII, IX, or X activity; factor V, about 0.1%; Fletcher factor, about 0.1%; Factor XII, less than 0.1%. At 8% Factor XI, it had much less than 0.1% CAC. The fraction was diluted to desired concentrations in solutions containing purified human serum albumin at a concentration producing $A_{280} = 0.1$.

Purified Factor XII. Normal plasma, dialyzed against a pH 7.2 buffer containing 0.02 M Tris, 0.03 M NaCl, and 50 μg/ml Polybrene (hexadimethrine bromide, Aldrich Chemical Co., Inc., Milwaukee, Wis.) was chromatographed on DEAE-cellulose equilibrated with this buffer. The Factor XII fractions, which eluted after the first protein peak, were pooled and adsorbed to DEAE-Sephadex A-50 equilibrated with pH 8.1 buffer containing 0.01 M Na_2HPO_4, 0.005 M EDTA, and 50 μg/ml Polybrene. Factor XII was eluted with a straight line gradient to pH 4.6 buffer containing 0.15 M NaH_2PO_4, 0.25 M NaCl, and 0.001 M EDTA according to the method of Revak, et al. (14). The pooled Factor XII fraction, which eluted following the initial protein peak, had a specific activity of 595 (% Factor XII/A_{280}). This represented a purification of about 370-fold over normal plasma. At a concentration producing a clotting time of 63 s in a kaolin-activated Factor XII

assay, this fraction failed to clot Factor XII-deficient plasma in a Factor XII assay in the absence of an activating surface in 360 s. The fraction at time of use contained 84% Factor XII and no detectable Factor II, V, VIII, IX, or X activity. When diluted to 10% Factor XII activity, it had 0.1% Fletcher factor, much less than 0.1% CAC, and no detectable Factor XI.

Partially purified CAC. 100 ml of normal human plasma, adsorbed once with $Al(OH)_3$, was dialyzed against Tris buffer, pH 7.2 (0.02 M Tris plus 0.03 M NaCl) and applied to a 2.5 × 100 cm DEAE-cellulose column (DE-52, Whatman) equilibrated with the same buffer. The column was washed with the starting buffer until the first protein peak eluted. Then an exponential gradient was connected consisting of 1,300 ml of high salt buffer containing 0.673 M NaCl and 0.02 M Tris, pH 7.2, in the reservoir and 1,950 ml of the starting Tris buffer in the mixer flask. Fractions of about 12 ml were collected automatically. CAC eluted beyond the major protein peaks, well separated from Factors XI and XII, at about 0.27–0.3 M salt. Fletcher factor eluted with the Factor XI peak. A typical elution pattern is shown in Fig. 1. The pooled CAC fractions, which also contained Factor V and small amounts of Factor VIII activity, were brought to 50% saturation with ammonium sulfate. The precipitate was dissolved in a minimum volume of 0.025 M Tris buffer, pH 7.0, containing 1.5 M NaCl and applied to a 2.5 × 100 cm agarose column, Bio-Gel A-1.5m 60–100 mesh (Bio-Rad Laboratories, Richmond, Calif.). The column was equilibrated and eluted with the same high ionic strength buffer. 3-ml fractions were collected automatically. This step separated CAC from the majority of the Factor V, as shown in Fig. 2, as well as from Factor VIII. The CAC obtained by this procedure was purified about 40-fold over normal plasma. Purification data for a typical preparation are shown in Table II. The purified CAC fraction prepared by this method, used for the mechanism experiments below, contained the following activities relative to a standard pooled normal plasma: CAC, 40%; Factor XII, 0.3%; Factor VIII, 1.2%; Factor V, 1.4%; less than 0.1% Fletcher factor; and no detectable Factor II, VII, IX, X, XI, or thrombin. No clottable fibrinogen was found, but the pres-

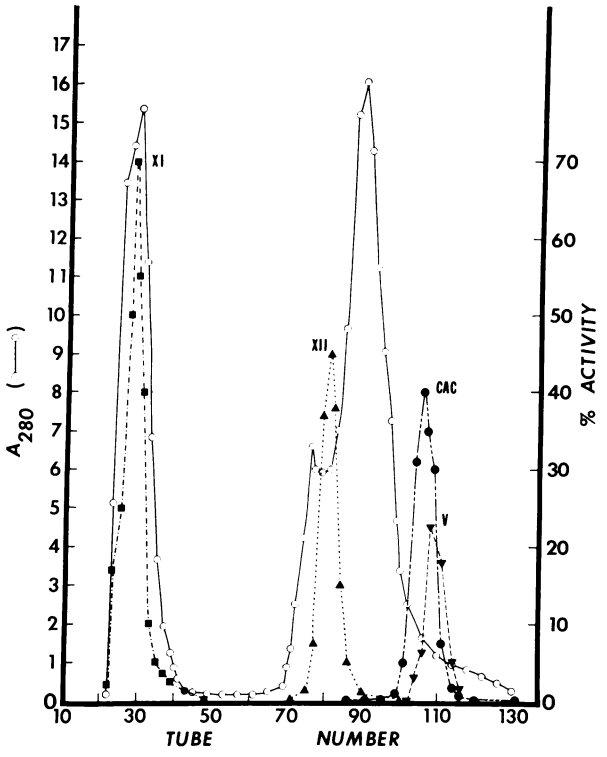

FIGURE 1 Elution pattern on DEAE-cellulose chromatography of $Al(OH)_3$-adsorbed normal human plasma. Symbols: A_{280}, O——O; Factor XI, ■ -·-·- ■; Factor XII, ▲---▲; CAC, ●— - - —●; Factor V, ▼- - - -▼. 100 ml of adsorbed plasma was applied in 0.05 M NaCl-Tris buffer pH 7.2. After elution of the first protein peak, an exponential gradient was applied consisting of 1,300 ml of 0.693 M NaCl-Tris buffer, pH 7.2, in the reservoir and 1,950 ml of 0.05 M starting buffer in the mixer flask. Fractions were 12 ml each.

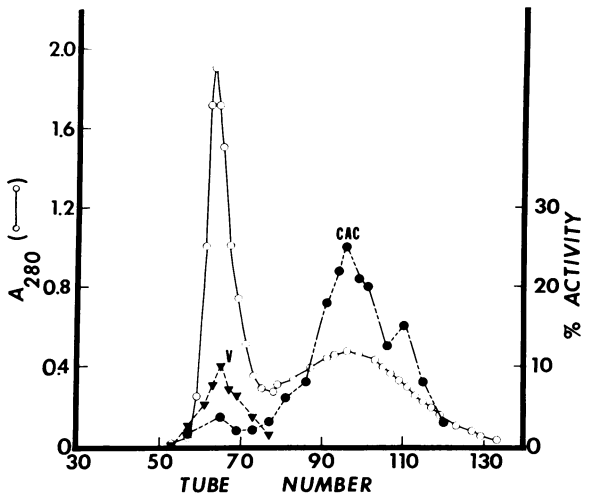

FIGURE 2 Elution pattern on agarose Bio-Gel A-1.5 m of CAC fraction from DEAE. Symbols are the same as in Fig. 1. The column was equilibrated and eluted with 0.025 M Tris, pH 7.0, containing 1.5 M NaCl. Fractions 101 through 116 of column shown in Fig. 1 were pooled, precipitated by 50% ammonium sulfate, dissolved in column buffer, and applied directly to the column. Fractions were 3 ml each.

TABLE II
Summary of Purification Steps

Test material	Volume	A_{280}	CAC	Specific activity	Recovery
	ml		%	*% CAC/ A_{280}*	*% origina CAC*
Original plasma	100	55.38	90	1.6	—
$Al(OH)_3$-adsorbed plasma	110	43.92	80	1.8	98
DEAE-CAC	187	1.60	23	14.4	48
50% $(NH_4)_2SO_4$ ppt.	7.9	12.32	520	42.2	46
Agarose-CAC	24	0.55	40	73	11

CAC activity is expressed as percent of pooled normal plasma activity.

ence of a trace of fibrinogen was indicated by a faint line that formed on double immunodiffusion against rabbit anti-human fibrinogen antiserum. The isolated CAC lacked plasminogen-plasmin by the Hyland EnzoDiffusion fibrin plate test that detected plasminogen-plasmin in normal plasma diluted 1/100. Part of this CAC reagent was concentrated by dialysis against dry Sephadex G-200 beads before storage at −20°C. Unless noted otherwise, this reagent was diluted to desired concentration in solutions containing the partially purified human serum albumin described above at a concentration producing $A_{280} = 0.1$.

Partially purified Factor V was a pooled first peak from the agarose chromatography step in the purification of CAC. The fraction used in experiments below contained the following activities: Factor V, 22%; Factor VIII, 2%; CAC, 0.8%; Fletcher factor, less than 0.1%; and no detectable Factor II, IX, X, XI, XII, thrombin, fibrinogen, or plasminogen.

Trypsin (Sigma Chemical Co., St. Louis, Mo.) Type III, twice recrystallized from bovine pancreas, was made up in 0.001 N HCl at 6 mg/ml, diluted to 400 μg/ml in citrate-saline, and stored at −20°C. At the time of use it was further diluted to 40 μg/ml.

Protein was estimated as absorbancy at 280 nm.

RESULTS

CAC assay. The effects on the CAC assay of different kaolin concentrations, calcium ion concentrations, and incubation times were studied. Changes in kaolin concentration between 10 and 25 mg/ml caused little change in clotting time (Table III). 20 mg/ml kaolin was selected as the standard reagent. Varying the calcium concentration caused marked changes in clotting time (Table IV). In some early experiments, assays were carried out with 100 mM $CaCl_2$. The curves were reproducible, and the results were consistent. However, the clotting times were quite long, and the results could easily be affected by factors influencing available calcium ions. Therefore, 25 mM $CaCl_2$ was selected for standard use.

The incubation time required to produce a minimum clotting time in this assay was quite long, around 15 min for a test plasma diluted 1/10, around 25 min for a 1/50 dilution of test plasma, and up to 30 min when buffer was substituted for test substance. It was impractical to use a 30-min incubation routinely, so 10 min was selected as the standard interval. Results have been reproducible with this incubation time. A dilution curve obtained with a standard frozen normal plasma with 20 mg/ml kaolin, 25 mM $CaCl_2$, and a 10-min incubation time is shown in Fig. 3.

TABLE III
Effect of Kaolin on CAC Assay

Kaolin	Clotting time
mg/ml	*s*
40	54
25	51
20	48
10	49
0	120

Clotting mixtures contained 0.01 ml cephalin 1/10, 0.05 ml Dicalite-adsorbed plasma, 0.05 ml Factor XI-Fletcher factor reagent, 0.05 ml Factor XII reagent, 0.05 ml normal plasma diluted 1/20, and 0.01 ml kaolin. After 5 min incubation 0.025 ml of 25 mM $CaCl_2$ was added and the clotting time noted.

TABLE IV
Effect of $CaCl_2$ Concentration on CAC Assay

$CaCl_2$	Clotting time
mM	*s*
100	72
80	62
60	59
40	51
30	48
25	47
20	49
15	65

Clotting mixtures contained 0.01 ml cephalin 1/10, 0.05 ml Dicalite-adsorbed plasma, 0.05 ml Factor XI-Fletcher factor reagent, 0.05 ml Factor XII reagent, 0.05 ml normal plasma 1/20, 0.01 ml kaolin 20 mg/ml. After 5 min incubation 0.025 ml of $CaCl_2$ was added and the clotting time noted.

Since Factor V in the Dicalite-adsorbed substrate was somewhat reduced, the possibility existed that Factor V added in a test substance might reflect as apparent CAC activity. To test this possibility we assayed a partially purified Factor V preparation prepared as a by-product of CAC purification (see above). A fraction with 22% Factor V activity had only 0.8% apparent CAC activity. This small activity represented the maximum possible effect of Factor V in this assay; the effect may be even less, since the Factor V fraction may have contained some CAC. Therefore, an effect of factor V did not appear to be a significant problem in this assay unless a test material contained very high concentrations of Factor V. For greatest accuracy, fractions to be assayed for CAC activity should be di-

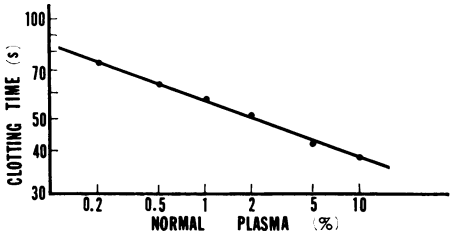

FIGURE 3 Dilution curve in CAC assay.

TABLE V

Effect of Temperature on CAC Activity in Plasma

Temperature	Incubation time			
	0	15 min	30 min	60 min
	% CAC			
Room temperature	98	—	95	—
37°C	98	95	92	102
56°C	101	92	92	89

Plasmas were diluted 1/30 before heating. Results are expressed as percent of original untreated CAC activity remaining after heating. Average of two experiments.

luted to a Factor V level of about 10% or less. Our standard curves in the CAC assay began at 1/10 dilution of the normal plasma.

Properties of CAC in plasma. CAC activity in normal plasma was very stable. Plasma stored 1 wk at 4°C in a plastic vial retained 75-80% original CAC activity. The effect of short-term incubation at room temperature, 37°C, or 56°C is shown in Table V. About 90% of initial CAC activity survived exposure to 56°C for up to 60 min. At 80°C, CAC activity decreased in diluted plasma. After 30 min, the clotting time of a typical diluted sample rose from 45 to 50 s. This represented an average loss of 48% of starting activity (three experiments).

CAC activity was relatively stable between pH 6 and 9 (see Table VI). Exposure to pH 10 overnight resulted in significant loss of activity.

CAC activity precipitated from normal plasma between 40 and 50% saturation with ammonium sulfate (Table VII). During this fractionation and subsequent dialysis, CAC activity was consistently lost. CAC was slightly adsorbed by $Al(OH)_3$ (Table VIII).

Some properties of isolated CAC. The effect of temperature on partially purified CAC is shown in Table IX.

TABLE VI

Effect of pH on CAC Activity in Plasma

pH	% CAC
5	74
6	90
7	88
7.35	92
8	79
9	87
10	66

Results are expressed as percent of CAC activity remaining in the treated fractions compared to the CAC activity of original untreated plasmas retained at 4°C as controls. Average of two experiments.

TABLE VII

Effect of Ammonium Sulfate Fractionation on CAC Activity in Plasma

Test material	% CAC
$(NH_4)_2SO_4$ precipitates	
25%	2.1
33%	2.2
40%	2.2
50%	23
50% supernate	2

Results are expressed as percent of original plasma CAC activity in each fraction at original plasma volume. Average of three experiments.

CAC was relatively stable over short periods of time at room temperature. Incubation for 20 min at 37 or 56°C produced a significant drop in CAC activity, but this effect could be prevented by the addition of dilute albumin. Incubation of the CAC at 78°C resulted in loss of activity in the presence or absence of albumin; however, decay was more gradual in the mixture containing albumin. On gel filtration on Sephadex G-200, CAC eluted as a molecule with an approximate mol wt of 220,000 (data not shown).

Reactions of CAC. CAC was first identified as an activity required to facilitate the generation of activated Factor XI in a mixture containing Factors XI and XII, and Fletcher factor (6). Further studies of this reaction are shown in Table X. No time-consuming reaction occurred over 20 min between purified Factors XI and XII incubated together in the presence of kaolin (line 1). Addition of either diluted normal plasma or congenital Fletcher factor-deficient plasma to the reaction mixture caused prompt generation of procoagulant activity in the reaction mixture (lines 2 and 3). These results demonstrated that added Fletcher factor was not required for the reaction and suggested that CAC alone was sufficient to promote generation of Factor XI_a in the mixture.

Further experiments with the partially purified CAC are presented in Table XI. For these experiments lower kaolin concentrations were used than in the experiments

TABLE VIII

The Effect of $Al(OH)_3$ Adsorption on CAC Activity in Plasma

Test material	% CAC
Plasma	90
Once-adsorbed plasma	80
Twice-adsorbed plasma	73

Results are expressed as percent CAC in the test sample relative to a normal pooled plasma. Average of three experiments.

TABLE IX

Effect of Temperature on Partially Purified CAC

Temperature	Buffer 0	Buffer 20 min	Buffer 40 min	Buffer 80 min	Buffer-albumin 0	Buffer-albumin 20 min	Buffer-albumin 40 min	Buffer-albumin 80 min
	% CAC							
Room temperature	94	82	66	74	92	96	96	98
37°C	94	61	48	—	92	84	89	92
56°C	94	46	36	27	92	88	88	88
78°C	94	54	35	20	92	78	70	61

A CAC fraction, prepared as described in the text and concentrated by dialysis against powdered Sephadex G-200 (Pharmacia), contained about 100% CAC in 1.5 M NaCl. It was diluted 1/10 either in distilled water or distilled water containing purified human serum albumin to produce an A_{280} = 0.1. The mixtures were further diluted 1/4 in TES buffer (0.05 M TES, 0.1 M NaCl, pH 7.35) or buffer containing purified human serum albumin to produce an A_{280} = 0.1, and heated as shown. Samples were assayed without further dilution. Results are expressed as percent of CAC relative to the amount in pooled normal plasma. Average of two experiments.

shown in Table X, because the higher kaolin concentration inhibited the reaction with purified CAC. Isolated CAC effectively promoted generation of activated Factor XI as shown in line 2. No preliminary time-consuming reaction could be demonstrated between CAC and Factor XI alone; rather activity decreased on incubation (line 3). Incubation of CAC with Factor XII produced no significant increase in apparent Factor XII activity, as shown in line 4.

The effects of varying the concentration of CAC on the kinetics of generation of activated Factor XI in a reaction mixture containing Factors XI, XII, and CAC in the presence of kaolin are shown in Fig. 4. Reducing the concentration of CAC in the reaction mixture produced a proportional drop in the amount of activated Factor XI formed. These results suggest that CAC may be consumed during the generation of activated Factor XI.

The effect of soluble trypsin on CAC was tested by incubating isolated CAC with trypsin at a concentration of 1.5-2.7 μg/ml in the reaction mixture. This concentration of trypsin rapidly activated the purified Factor XI, as we have demonstrated previously (6). No increase in procoagulant activity occurred over 20 min, as detected in the CAC assay, in the absence of an activating surface.

TABLE X

Generation of Factor XI_a Activity in Mixtures Containing Purified Factors XI, XII, and Diluted Plasmas

Test mixture	Incubation time 15 s	5 min	10 min	15 min	20 min
	clotting time in s				
XI (15%) + XII (20%) + Buffer	161	221	221	226	231
XI (7.5%) + XII (10%) + Normal pl. 1/10 (av. 2)	166	104	98	96	94
XI (7.5%) + XII (10%) + Fletcher def. pl. 1/10 (av. 3)	167	132	116	112	106

At zero time, 0.01 ml of 20 mg/ml kaolin was added to a plastic tube containing 0.1 ml of each test reagent, and the sample was mixed well on a vortex mixer. At the times indicated, the sample was mixed again, and a 0.05-ml portion was removed and tested in the Factor XI_a assay system as described in the text. The normal plasma contained 140% Factor XII, 125% Factor XI, 100% Fletcher factor, and 100% CAC. The Fletcher factor-deficient plasma contained 73% Factor XII, 87% Factor XI, and 78% CAC.

DISCUSSION

Evidence for an additional activity that participated with Factors XI and XII in the contact phase of intrinsic clotting was first presented by us in 1960 (7). It was observed that the combination of partially purified plasma fractions containing Factor XI or XII was insufficient to correct the defect in Celite-adsorbed normal plasma, though adequate amounts of all other known

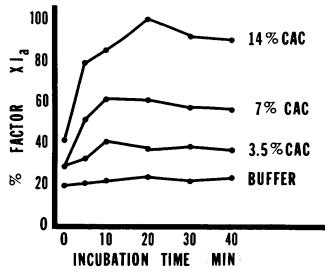

FIGURE 4 Effect of varying the concentration of CAC on the generation of Factor XI_a in mixtures containing purified Factors XI, XII, and CAC. At zero time, 0.01 ml of 2 mg/ml kaolin was added to a mixture containing the equivalent of 0.1 ml of Factor XI (15%), 0.1 ml Factor XII (20%), and 0.1 ml CAC at one of the concentrations shown in the figure. The sample was well mixed on a vortex mixer, and at the times shown 0.05 ml samples were removed and tested for Factor XI_a activity. A standard curve was made by testing dilutions of a mixture containing 14% CAC sampled between 20 and 30 min incubation. The undiluted mixture was arbitrarily designated 100%. A plot of the log of the concentrations of the mixture versus the log of the clotting times formed a straight line, from which the other clotting times could be converted to percent.

TABLE XI

Interaction of Isolated Factors XI, XII, and CAC

Test mixture	Incubation time					
	15 s	5 min	10 min	15 min	20 min	30 min
	clotting time in s					
XI (7.5%) + XII (10%) + buffer*	244	—	237	—	232	221
XI (7.5%) + XII (10%) + CAC (7%)‡	216	167	148	145	143	—
XI (7.5%) + buffer + CAC (7%)§	273	—	298	—	328	331
Buffer + XII (10%) + CAC (7%)§	202	—	218	—	196	193

At zero time, 0.01 ml of 2 mg/ml kaolin was added to a plastic tube containing 0.1 ml of each test reagent, and the sample was mixed well on a vortex mixer. At the times indicated, the sample was mixed again, and a 0.05 ml portion was removed and added to a plastic clotting tube containing 0.05 ml cephalin 1/60 and 0.05 ml deficient plasma; 0.05 ml 40 mM $CaCl_2$ was added, and the clotting time was noted. Mixtures of lines 1, 2, and 3 were tested with Factor XI-deficient plasma. The mixture shown in line 4 was tested with Factor XII-deficient plasma.

* Average of 4.

‡ Average of 5.

§ Average of 3.

clotting activities were present. In 1965 a new clotting abnormality was described, Fletcher factor deficiency, in which there was a defect in the contact activation mechanism unrelated to Factors XI and XII (4). It was suggested that Fletcher factor might be the activity missing in our earlier experiments (5). However, when the properties of Fletcher factor were characterized and Fletcher factor was shown to be the same as prekallikrein (15-18), it became clear that our crude Factor XI preparation would have contained Fletcher factor. Assays of Fletcher factor activity in Factor XI reagents prepared later confirmed this conclusion. Therefore, our results could not be explained by an absence of Fletcher factor. Moreover, we have demonstrated that in a mixture containing Factor XI, Fletcher factor, and Factor XII adsorbed to kaolin, a fourth activity present in dilute Fletcher factor-deficient plasma was required to generate activated factor XI (6). This fourth activity involved in contact activation we call CAC. The experiments presented herein were undertaken to define more clearly the nature of CAC and its role in the contact phase of intrinsic clotting.

To measure CAC activity we have devised a one-stage activated partial thromboplastin time assay with Dicalite-adsorbed plasma combined with sources of Factors XI, XII, and Fletcher factor. CAC was initially detected in a two-stage system that measured generation of Factor XI_a. The new assay has the advantages that it does not require a deficiency plasma, all reagents can be prepared relatively easily from normal plasma, and all reactions occur in the single clotting tube. This assay can be used over a wide range of activity, 0.2-10% of normal plasma activity, and when the data are plotted as log clotting time versus log activity concentration, a linear relationship is found that makes it easy to evaluate intermediate levels of CAC.

The first goal of this study was to determine whether CAC activity represented a unique new clotting activity or whether it was a newly recognized function of an established clotting factor. The properties of CAC in plasma appeared to differentiate it from the other known clotting factors. Unlike Factors II, VII, IX, and X, the majority of plasma CAC activity remained in Al-$(OH)_3$- adsorbed citrated plasma. CAC was strongly adsorbed onto Dicalite along with Factors XI, XII, and Fletcher factor, but it could be separated from these activities by DEAE-cellulose chromatography. CAC was much more stable to high temperature than Factors V, VIII, or fibrinogen.

Confirmation of the unique nature of CAC was obtained by fractionation of normal human plasma according to the partial purification scheme outlined above. CAC activity separated from all the other known clotting activities. Furthermore, conditions of purification were relatively mild, and the other clotting activities could be recovered in fractions removed from the CAC fractions. Therefore, CAC activity was associated with a molecule clearly distinguishable from all other characterized clotting factors.

CAC was also differentiated from two components of the fibrinolysis system: plasminogen and Factor XII-cofactor (plasminogen proactivator). Factor XII-cofactor is a plasma activity through which Factor XII can initiate fibrinolysis. It elutes from anionic exchange resins with the Factor XI fraction (19, 20). In contrast, CAC is bound to the resin at low ionic strength

and elutes far beyond Factor XI in the presence of much higher salt concentrations, as shown above. Therefore, these two activities, which have rather similar names and both of which react with Factor XII, are quite different physically. The absence of detectable plasminogen-plasmin in partially purified CAC was important because plasmin can activate Factor XII (21). Contamination of a CAC reagent with this activity could lead to spurious results in reaction mixtures containing Factor XII.

For each previously defined clotting factor, rare patients have been identified who have a congenital deficiency of that activity. Therefore, one might expect to find a CAC-deficient patient. Recently, a new clotting abnormality has been described known as Fitzgerald trait (22). Mr. Fitzgerald's plasma had a defect in the contact phase of intrinsic clotting that could not be explained by the absence of a known contact factor—Factor XI, Factor XII, or Fletcher factor. The activity lacking in this plasma was called Fitzgerald factor. We have tested this plasma in our assay system and found that it lacks CAC and that it is corrected by partially purified CAC (23). Therefore, Mr. Fitzgerald appears to be the first recognized CAC-deficient patient.

Added evidence for the identity of CAC and Fitzgerald factor is found in a comparison of some of the properties recently described for Fitzgerald factor (24) and those of CAC reported above. Both activities are slightly adsorbed by $Al(OH)_3$, adsorbed to glasslike surfaces, unusually stable at elevated temperatures, and have simliar elution patterns on anionic exchange resins. The temperature stability of this molecule is particularly noteworthy; it appears to be the most stable clotting factor yet characterized.

At first glance the size of CAC (reported mol wt 220,000) appears to be quite different than the size of Fitzgerald factor (reported mol wt about 160,000). However, this difference is more apparent than real. In both studies size was estimated by comparison of the elution pattern of the activity with the elution patterns of known standards on gel filtration. In both studies CAC or Fitzgerald factor eluted very close to a gamma globulin standard. Therefore, the actual sizes of CAC and Fitzgerald factor are similar. We find CAC to be slightly larger than gamma globulin. The large discrepancy in reported molecular weights arises from the assignment of different molecular weights to the gamma globulin standard. In the Fitzgerald study, gamma globulin was assigned a mol wt of 160,000, which is its size determined by methods other than gel filtration. We have used a mol wt of 205,000 for gamma globulin, which is its apparent size on gel filtration (13). These definitions necessarily will lead to different molecular weights for similar results. A more definitive determination of the molecular weight of CAC-Fitzgerald factor must await preparation of a highly purified CAC-Fitzgerald factor reagent suitable for physical analysis of the protein.

The identification of CAC as Fitzgerald factor raises the possibility that CAC may be not only a clotting activity but also a participant in the kinin-generating system, because there is suggestive evidence that Fitzgerald factor (CAC) may be high molecular weight kininogen (24). A close relationship between clotting and kinin formation has been established previously by the demonstration that two other contact phase clotting factors, Factor XII and Fletcher factor, play central roles in the reactions leading to liberation of kinin (15, 16, 18, 21, 25). CAC may represent a third link between these two interrelated systems.

CAC shares another important characteristic with Factor XII and Fletcher factor. Patients lacking these activities have no known hemostatic abnormality (3, 4, 22). Hence, none of these activities seems to be required for normal clotting in vivo. The clotting reactions of Factor XII, Fletcher factor, and CAC that are studied in vitro may occur in vivo, but presumably alternate physiologic pathways also exist that can lead to the activation of Factor XI.

The experiments described above provide some insights into the role of CAC in the generation of Factor XI_a. We have shown previously that CAC activity promotes activation of Factor XI in mixtures containing Factors XI, XII, and Fletcher factor (6). Herein we demonstrate that CAC alone is able to promote generation of Factor XI_a in mixtures containing purified Factors XI and XII in the presence of kaolin. Both isolated CAC and the CAC activity in diluted Fletcher factor-deficient plasma were effective. The purified Factor XI reagent used in these experiments at 10% activity contained about 0.05% Fletcher factor activity, and the purified Factor XII reagent at 10% activity contained 0.1% Fletcher factor. Therefore, it is not possible to exclude an effect of traces of Fletcher factor. However, the results strongly suggest that CAC is a primary cofactor for this reaction.

Kinetic studies of the mechanism of interaction of CAC with Factors XI and XII in the presence of kaolin indicate that the amount of product formed is proportional to the amount of CAC present. Therefore, one might expect that CAC forms a complex with one of the other reactants that limits the amount of product formed. However, we could demonstrate no time-consuming preliminary reaction between either Factor XI or Factor XII and CAC in the presence of kaolin with Factor XI- or Factor XII-deficient plasma as substrate (Table XI). In one experiment, mixtures containing CAC and either Factor XI or factor XII were tested with Fitzgerald-deficient

plasma as substrate. Both reaction mixtures showed a decrease in activity over 20 min, indicating again that no detectable time-consuming reaction occurred in these mixtures. One plausible explanation of these results is that CAC and either Factor XI or Factor XII combined very rapidly in a reaction that is not demonstrated under these circumstances and that the product of the reaction is the active species. In fact, two other reactions in the clotting sequence have been described which occur almost instantaneously: the interaction of Factors IX_a and VIII and the interaction of Factors X_a and V (*26, 27*). Interestingly, the interaction of Factors IX_a and VIII reportedly can occur at glass surfaces (*26*). No physiochemical evidence has been reported for a molecule that would have the properties of a complex of CAC combined with Factor XI or Factor XII, but it seems quite possible that such a complex might exist only at the glass surface and that during isolation the complex would be dissociated. Further experiments will be required to test this hypothesis.

This study has demonstrated that CAC is a unique clotting activity. Strong evidence indicates that it is the same as the recently described Fitzgerald factor. CAC promotes generation of Factor XI_a in mixtures containing purified Factors XI and XII and kaolin. It appears to play an important role by limiting the amount of Factor XI_a that can be generated in the mixture.

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