The Purification of Activated Hageman Factor (Activated Factor XII)*

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A method is described for the purification of activated Hageman factor from normal human plasma, utilizing column chromatography with carboxymethylcellulose and diethylaminoethyl cellulose. The product was purified 3000- to 5000-fold or more as measured by a new assay procedure. The purified preparations corrected the clotting defect in the plasma of patients with Hageman trait, accelerated the clotting of normal plasma in silicone tubes, and converted plasma thromboplastin antecedent to an activated form. The preparation was probably contaminated with traces of plasminogen and perhaps Christmas factor as well. The highly purified activated Hageman factor was not inhibited by 10^{-3} M diisopropyl phosphofluoridate.

Hageman factor (Factor XII), a clot-promoting substance found in mammalian plasma, participates in the early stages of blood coagulation (Ratnoff and Colopy, 1955). Its presence is required for the initiation of clotting when blood or plasma comes into contact with glass. Hageman factor may also be concerned with the elaboration of substances in plasma which contract smooth muscle (Margolis, 1957), enhance capillary permeability (Margolis, 1958a), increase blood flow (Webster and Ratnoff, 1961) and accelerate fibrinolysis (Niewiarowski and Prou-Wartelle, 1959; Iatridis and Ferguson, 1961). It is apparently absent from the blood of individuals with Hageman trait, a hereditary disorder in which the clotting time of venous blood is greatly prolonged (Ratnoff and Colopy, 1955). In spite of this, individuals with Hageman trait usually have little or no clinical evidence of a bleeding tendency.

In freshly drawn blood, Hageman factor behaves as if it is in an inactive form. When plasma comes in contact with glass, its Hageman factor is "activated" (Ratnoff and Rosenblum, 1958; Biggs *et al.*, 1958; Lewis *et al.*, 1958; Vroman, 1958; Johnston *et al.*, 1958; Soulier *et al.*, 1959; Waaler, 1959). In turn activated Hageman factor activates plasma thromboplastin antecedent (PTA or Factor XI). In this way, the chain reactions which lead to the formation of a clot *in vitro* may be initiated (Margolis, 1958b; Soulier *et al.*, 1959; Waaler, 1959; Hardisty and Margolis, 1959; Ratnoff *et al.*, 1961).

Several methods for the partial purification of a protein fraction which corrects Hageman factor–deficient plasma have been published. The earliest, from this laboratory (Ratnoff and Rosenblum, 1958), resulted in the isolation of a product later shown to be heavily contaminated with activated PTA. More recent methods, utilizing either normal plasma (Schiffman *et al.*, 1960; Haanen *et al.*, 1960) or plasma deficient in PTA (Ratnoff *et al.*, 1961), either have been unsuitable for large scale purification or have provided only a partially purified product. With one exception (Schiffman *et al.*, 1960) the isolated Hageman factor has been at least partially activated. Problems concerning the method of assay for Hageman factor used in previous studies have made it difficult to assess the degree of purification actually accomplished in earlier attempts.

Extensive purification of a clotting factor that corrects Hageman factor–deficient plasma has now been achieved by column chromatography on carboxymethylcellulose and diethylaminoethyl cellulose. On a protein basis, the product appeared to be purified about 3000–5000 fold or more compared with the plasma from which it was prepared, as measured by a new assay procedure. The yield varied with the degree of purity sought, and was as high as 50% in some preparations. The product was largely or completely in the activated form.

MATERIALS

Citrated normal human plasma was prepared from venous blood drawn from individual donors. Approximately 450 ml of blood was drawn from each donor into silicone-coated bottles containing 50 ml of 0.13 M trisodium citrate dihydrate solution. The blood was centrifuged at 500 × *g* for 15 minutes at room temperature; the plasma was drawn off with silicone-coated syringes and

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The authors wish to dedicate this article to the late Dr. Jerold Rosenblum, our close friend and colleague.

recentrifuged at 2° in silicone-coated bottles again for 15 minutes at 500 *g*.

Platelet-deficient citrated plasma was prepared from normal blood and from the blood of patients with various coagulative abnormalities by a previously described method (Ratnoff *et al.*, 1961). In this technique, the plasma was not allowed to come in contact with glass. This plasma was frozen at −25° until used as the substrate in the assay of various clotting factors.

Aluminum hydroxide gel[1] was diluted to 0.55% by the addition of water.

Diatomaceous earth (Filter Cel) was purchased from Johns-Manville.

Carboxymethylcellulose (CM-cellulose) containing approximately 0.72 meq of acidic groups per g of cellulose was prepared by the procedure of Ellis and Simpson (1956).

Diethylaminoethyl cellulose (DEAE Selectacel, type 40, with a capacity of 1.05 meq per g) was purchased from Brown Company, Berlin, N. H.

Barbital-saline buffer (*p*H 7.5) was composed of 0.025 M barbital and 0.125 M sodium chloride (Ratnoff and Colopy, 1955).

Barbital-saline-citrate buffer was prepared by mixing 85 ml of barbital-saline buffer with 15 ml of 0.13 M trisodium citrate solution.

Bovine serum albumin buffers were freshly prepared by dissolving bovine fraction V (Armour) in barbital or Tris buffers at a concentration of 1%.

Tris buffer (0.15 M, *p*H 7.4) was prepared from tris(hydroxymethyl)aminomethane (Sigma 121, Sigma Chemical Company).

A *kaolin-Gliddex* mixture was prepared in the following manner. Gliddex "p," a crude preparation of soybean phosphatides,[2] was suspended at a concentration of 0.1% in 0.15 M sodium chloride solution with a hand glass homogenizer, divided into 5 or 10 ml lots, and stored at −25° until needed. On the day it was to be used, a tube of Gliddex suspension was thawed and kaolin (acid-washed, N.F. Fisher) was added in concentration of 10 mg per ml. The kaolin-Gliddex mixture was homogenized thoroughly in the same way and 0.1 ml of the suspension was pipetted into each of a series of Pyrex tubes. These tubes were kept in an ice bath until used.

Glycerol (U.S.P.) was mixed at a concentration of 60% by weight with Tris buffer.

α-Chymotrypsin (Worthington Chemical Company) was dissolved at a concentration of 20 μg per ml of Tris buffer.

Buffer solutions used for the preparation or storage of activated Hageman factor contained 10^{-4} M *trisodium ethylenediamine tetraacetic acid (versene)*.

[1] Provided through the courtesy of Cutter Laboratories, Berkeley, Calif. The concentration of aluminum hydroxide gel is expressed by the manufacturer as aluminum oxide and the preparation contained 0.6% aluminum oxide.

[2] Provided through the courtesy of the Chemurgy Division, Glidden Paint Company, Chicago.

All glassware was coated with silicone (G. E. Dri-Film SC-87) unless otherwise noted. All assays were performed in Pyrex tubes with an internal diameter of 8 mm. *Centrifugations* were at 2° unless otherwise noted.

Bovine fibrinogen solution containing 300 mg of coagulable protein per 100 ml was purchased from Warner-Chilcott.

Bovine thrombin ("Topical thrombin," Parke Davis Company) was dissolved in barbital-saline buffer at a concentration of 10 N.I.H. units per ml.

Streptokinase (Varidase, American Cyanamid Company) was dissolved in barbital-saline buffer at a concentration of 1500 Christensen units of streptokinase per ml. The preparation used contained 25,000 units of streptococcal desoxyribonuclease for every 100,000 units of streptokinase.

Crude activated Hageman factor was prepared from PTA-deficient plasma, and crude PTA from Hageman factor–deficient plasma, by methods previously reported (Ratnoff *et al.*, 1961).

Diisopropyl phosphofluoridate (DFP)–treated activated Hageman factor was prepared as previously described (Ratnoff *et al.*, 1961; Ratnoff and Davie, 1962).

Methods

An *assay for Hageman factor* was devised by minor modification of the methods of Margolis (1958c) and Rapaport *et al.* (1961). The material to be tested was diluted in buffer to a suitable concentration; in different experiments, the buffer used was barbital-saline, barbital-saline-citrate, or Tris, with or without 1% bovine albumin. One-tenth ml of the solution to be tested was added with a silicone-coated pipet to 0.1 ml of kaolin-Gliddex mixture in an uncoated Pyrex tube. The tube was gently tapped to mix its contents and incubated at 37° for 2 minutes. Then 0.1 ml of Hageman factor–deficient plasma was added with a silicone-coated pipet. The tube was again tapped and incubated at 37° for 8 additional minutes. Finally 0.1 ml of calcium chloride solution was added and a stopwatch started. The calcium chloride solution was 0.025 M if the preparation to be tested was diluted in buffers not containing citrate, and 0.05 M when citrate-containing buffers were used. After 30 seconds, the tube was tilted continually until a clot formed. The end-point was sharp and usually unmistakable. The clotting time was measured from the time calcium chloride solution was added until the clot appeared. Duplicate determinations usually agreed within 3 seconds when the clotting time was less than 100 seconds, and within 6 seconds when the clotting time was less than 200 seconds.

The concentration of Hageman factor in different samples was estimated in the following manner. The most active sample in the group to be tested was diluted serially with the appropriate buffer. Each dilution was then tested and a

calibration curve was drawn. Over a wide range of concentrations the logarithm of the concentration of Hageman factor was inversely proportional to the logarithm of the clotting time. A typical calibration curve when purified activated Hageman factor was used is shown in Figure 1; similar curves were obtained with diluted normal plasma.

One unit of Hageman factor was arbitrarily defined as that amount of activity present in 1 ml of pooled normal human plasma with the assay conditions specified above. The specific activity was taken as the number of units per mg of protein.

This assay did not distinguish between Hageman factor and activated Hageman factor since the sample to be tested was exposed to kaolin, an activator of Hageman factor (Margolis, 1958b). Moreover, the assay was invalidated by the presence of activated PTA, which accelerates the clotting of Hageman factor-deficient plasma (Ratnoff, 1960).

Assays for PTA, antihemophilic factor (Factor VIII), Christmas factor (Factor IX or plasma thromboplastin component) were performed in the same manner as assays for Hageman factor with two minor exceptions. In each case, the substrate of deficient plasma was added to the mixture of the test substance and the kaolin-Gliddex mixture without the 2-minute delay. In the assay for antihemophilic factor, the concentration of kaolin used was 5 mg per ml of Gliddex (Breckenridge and Ratnoff, 1962).

Assay for fibrinogen, thrombin, prothrombin, proaccelerin (Factor V), pro-SPCA (Factor VII), and Stuart factor (Factor X) were performed by previously described methods (Ratnoff and Davie, 1962).

Assays for plasminogen were performed by incubating a mixture of 0.1 ml of the substance to be tested (diluted with barbital-saline-citrate buffer), 0.1 ml streptokinase (1500 units per ml of barbital-saline buffer), 0.2 ml of bovine fibrinogen solution, and 0.1 ml of bovine thrombin solution (10 N.I.H. units per ml) at 37° in uncoated Pyrex tubes. The clot lysis time was the length of time which elapsed from the addition of thrombin until complete solution of the clot. The *assay for plasmin* was identical except that barbital-saline buffer was substituted for the solution of streptokinase.

Protein was determined by the method of Lowry *et al.* (1951), with a fresh solution of bovine serum albumin used as a standard.

Activated Hageman factor was prepared by adsorption of approximately 1200 ml of pooled citrated normal human plasma with one-tenth volume of aqueous aluminum hydroxide gel for 5 minutes at room temperature and then centrifugation for 10 minutes at 1000 × *g*.

The plasma was then mixed with 7.5 mg of diatomaceous earth per ml, stirred with a magnetic stirrer for 10 minutes, and recentrifuged. The supernatant was incubated in a silicone-coated flask at 37° overnight; a few drops of toluol were added as a preservative.

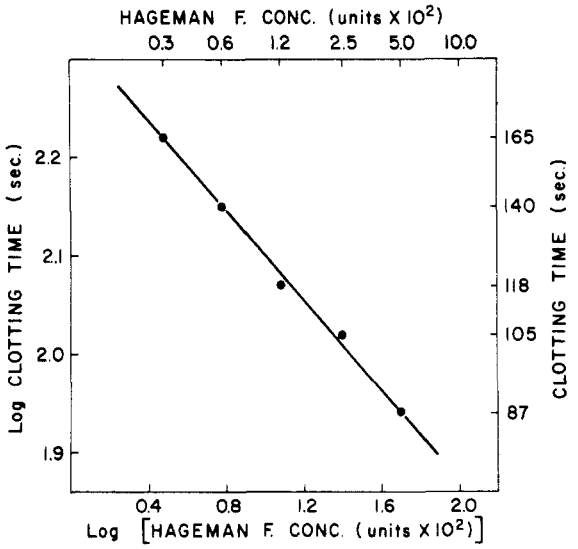

FIG. 1.—The effect of increasing concentrations of activated Hageman factor on the clotting time of Hageman factor-deficient plasma. The logarithm of the clotting time in seconds is plotted against the logarithm of the activated Hageman factor concentration.

The preparation was diluted with an equal volume of 0.15 M sodium acetate buffer (*p*H 5.2) and its *p*H adjusted to 5.2 by the addition of 1 M acetic acid. An equal volume of distilled water was added, bringing the total volume to four times that of the original plasma. Carboxymethylcellulose (18 mg per ml) was added to the acidified diluted plasma and the solution was stirred for 10 minutes at room temperature with the aid of a magnetic stirrer. The plasma was then centrifuged to remove the carboxymethylcellulose. Virtually all of the activated Hageman factor was adsorbed onto the ion exchange resin. The carboxymethylcellulose was washed repeatedly with 0.075 M sodium acetate buffer (*p*H 5.2) containing 10^{-4} M versene, until the washings no longer contained material precipitated by an equal volume of 10% trichloracetic acid. Six washings, each of about one-third the original plasma volume, usually sufficed. The acetate buffer was separated each time by centrifugation.

The washed carboxymethylcellulose was eluted repeatedly with 0.6 M sodium acetate buffer (*p*H 5.2) until the eluting fluid contained only traces of material precipitated by trichloracetic acid. Each elution was with a volume of buffer equivalent to one-third the volume of the original plasma; usually the total volume of eluate was twice that of the original plasma. The combined eluates were recentrifuged to remove traces of carboxymethylcellulose.

Activated Hageman factor was precipitated

TABLE I
A TYPICAL PURIFICATION CHART FOR ACTIVATED HAGEMAN FACTOR

Step	Volume	Total Protein (mg)	Total Units[a]	Specific Activity[b]	Yield (%)
Crude plasma	1,270	89,000	1,270	0.015	100
$Al(OH)_3$	1,300	89,000	1,270	0.015	100
Filter Cel	1,300	89,000	1,270	0.015	100
CM-cellulose eluate	60	540	635	1.2	50
DEAE column	30	69	318	4.6	25
CM-cellulose column	—	2	165	83	13

[a] One unit of activated Hageman factor was defined as that amount of activity present in 1 ml of pooled normal human plasma. [b] The specific activity of activated Hageman factor was taken as the number of units per mg of protein.

from the 0.6 M sodium acetate eluate by the addition of solid ammonium sulfate to 60% saturation. After standing overnight at 4° the protein precipitate was collected by centrifugation at 37,000 × *g* in a Servall Model RC 2 refrigerated centrifuge. The ammonium sulfate precipitate could then be stored at −25° before further purification; no appreciable loss of activity occurred after storage for several months in this state. All the remaining steps in this procedure were performed at 4°.

Chromatography on DEAE-cellulose. The ammonium sulfate precipitate was dissolved in 60 ml of 0.025 M phosphate buffer, *p*H 6.8, containing 1×10^{-4} M versene and dialyzed against 2 liters of the same solution for 4 hours. The dialysis was then continued for an additional 4 hours against 2 liters of 0.025 M barbital buffer, *p*H 7.45 containing 1×10^{-4} M versene. The dialyzed solution was centrifuged for 10 minutes at 10,000 × *g* and the precipitate discarded. In this step, most of the plasminogen was precipitated while the bulk of the activated Hageman factor remained in solution. The clear supernatant, which contained approximately 10 mg protein per ml, was divided in half, and each portion placed on a 2 × 22 cm DEAE-cellulose column previously equilibrated with 0.025 M barbital buffer, *p*H 7.45. The protein was eluted from the column by a buffer gradient established by placing 175 ml of 0.025 M barbital buffer (*p*H 7.45) in the mixing chamber and 175 ml of a mixture of 0.025 M barbital buffer (*p*H 7.45) in 0.25 M NaCl in the adjacent reservior. In the procedure employed the volumes in the mixing chamber and resevoir were variable but proportional (Long, 1961). Ten ml fractions were collected in plastic tubes at a rate of 25 ml per hour. The fractions containing the activated Hageman factor obtained from both columns were combined and dialyzed overnight against 4 liters of 1×10^{-4} M versene and lyophilized.

Chromatography on CM-cellulose. The lyophilized protein from the DEAE-cellulose columns was dissolved in 30 ml of 0.025 M sodium acetate buffer (*p*H 5.2) and placed on a 2 × 22 cm CM-cellulose column previously equilibrated with 0.025 M sodium acetate (*p*H 5.2). The protein was eluted from the column by a buffer gradient established by placing 150 ml of 0.025 M sodium acetate buffer (*p*H 5.2) in the mixing chamber and 150 ml of 0.6 M sodium acetate buffer (*p*H 5.2) in the adjacent reservior. Chromatography was continued with the higher ionic strength buffer to complete the elution of activated Hageman factor. Approximately 10-ml fractions were collected in plastic tubes at a rate of 25 ml per hour. The most active fractions were combined, dialyzed overnight against 4 liters of 1×10^{-4} M versene, and lyophilized. The final product contained 2–20% protein as measured by the Lowry method and was stable for at least 6 months in the lyophilized state at 4° or −25°.

RESULTS

Purification.—A summary of a typical fractionation of activated Hageman factor from pooled normal human plasma is shown in Table I. The aluminum hydroxide gel, which removed prothrombin, pro-SPCA, Christmas factor, Stuart factor, and a portion of the PTA in plasma, adsorbed little or no activated Hageman factor. The adsorption with diatomaceous earth (Filter Cel) freed the plasma of any remaining PTA. The chromatographic fractionation of activated Hageman factor on DEAE-cellulose and CM-cellulose columns is shown in Figures 2 and 3. The yield ranged from 10 to 50%, with an over-all purification around 5000-fold relative to the original plasma.

Clotting Properties of Activated Hageman Factor.—Activated Hageman factor, prepared by the method described, corrected the defect in Hageman factor–deficient plasma (Table II). It also accelerated the coagulation of normal plasma in silicone tubes, an effect previously observed with preparations purified 100- to 200-fold (Ratnoff *et al.*, 1961). Moreover, the clot-promoting activity of activated Hageman factor was enhanced by preincubation with PTA, its normal substrate (Table III). The details of the activation of PTA by less pure preparations of activated Hageman factor have been described previously (Ratnoff *et al.*, 1961).

TABLE II

CLOT-PROMOTING ACTIVITY OF PURIFIED ACTIVATED HAGEMAN FACTOR

Assay Plasma	Final Concentration of Hageman Factor (μg/ml)	Clotting Time
Hageman factor–deficient	5	55 sec.[a]
	1.25	72 sec.
	0.31	85 sec.
	Buffer	>240 sec.
Normal	2	10 min.[b]
	0.5	13.5 min.
	0.125	16.0 min.
	Buffer	17.5 min.

[a] Lyophilized activated Hageman factor was dissolved in barbital-saline buffer containing 1% bovine serum albumin and diluted serially in Tris buffer. The samples were then assayed for Hageman factor activity by the method described. [b] Clotting time (in duplicate) of a mixture of 0.1 ml of activated Hageman factor dissolved in barbital-saline-citrate buffer containing bovine serum albumin *or* buffer, 0.1 ml of normal platelet-deficient citrated plasma, and 0.1 ml of 0.05 M $CaCl_2$ in silicone-coated tubes at 37°.

TABLE III

THE EFFECT OF ACTIVATED HAGEMAN FACTOR UPON PTA

Test Mixture	Clotting Time[a] (min.)
PTA, 0.1 ml, + buffer, 0.1 ml	44
Activated Hageman factor, 0.1 ml, + buffer, 0.1 ml	48
PTA, 0.1 ml, + activated Hageman factor, 0.1 ml, incubated separately and then mixed	38
PTA, 0.1 ml, + activated Hageman factor, 0.1 ml, incubated together	28
Buffer, 0.2 ml	48

[a] Clotting time (in duplicate) of a mixture of 0.2 ml of the test mixture, 0.1 ml of PTA-deficient plasma and 0.1 ml 0.05 M calcium chloride in silicone-coated tubes at 37°. The test mixture was composed of purified activated Hageman factor (0.7 μg per ml of bovine albumin solution in barbital-saline-citrate buffer), PTA (at a concentration equal to that of the plasma from which it was prepared) in barbital-saline buffer, *or* the two respective buffers. These substances were incubated for 30 minutes either together or separately, and then combined prior to testing.

The Effect of Diisopropyl Phosphofluoridate upon Activated Hageman Factor.—Previously, it was shown that preparations of activated Hageman factor purified 100- to 200-fold from PTA-deficient plasma were substantially inactivated by treatment with diisopropyl phosphofluoridate (Becker, 1960; Ratnoff *et al.*, 1961). This effect was confirmed in the present studies (Table IV). However, with highly purified preparations of activated Hageman factor little or no inhibition occurred by pretreatment at 25° for 60 minutes with 10^{-3} M diisopropyl phosphofluoridate (Table IV). Similar results were obtained after diisopropyl phosphofluoridate treatment in the presence of kaolin, a substance which converts Hageman factor to an active form (Mallett, Davie, and Ratnoff, unpublished observations).

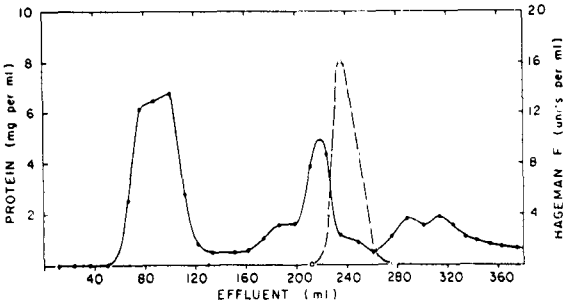

FIG. 2.—The chromatography of activated Hageman factor on a 2 × 22 cm DEAE column. Protein was eluted from the column by a buffer gradient between 0.025 M barbital buffer, *p*H 7.45, and 0.025 M barbital buffer–0.25 M NaCl, *p*H 7.45; ●—● refers to protein; O– O refers to Hageman factor activity. See text for details.

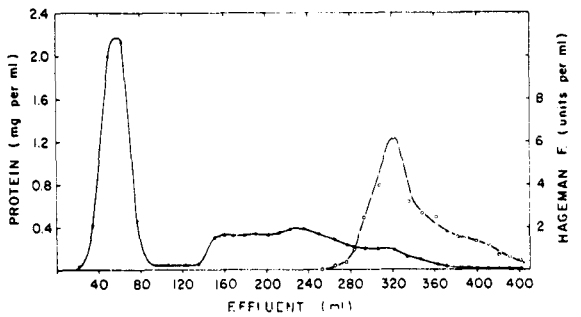

FIG. 3.—The chromatography of activated Hageman factor on a 2 × 22 cm CM-cellulose column. Protein was eluted from the column by a buffer gradient between 0.025 M NaAc buffer, *p*H 5.2, and 0.6 M NaAc buffer, *p*H 5.2; ●···● refers to protein; O—O refers to Hageman factor activity. See text for details.

Stability of Activated Hageman Factor.—When activated Hageman factor was dissolved in barbital-saline or Tris buffer, it deteriorated rapidly when incubated at 37°, appreciable activity being lost within 30 minutes. It remained stable for 96 hours at −25° in either of these buffers, but when it was thawed it deteriorated quickly even at 0°. The preparation could not be stabilized by storage in 50% glycerol or in 0.15 M sodium acetate buffer (*p*H 5.2). Activated Hageman factor was stabilized, however, by dissolving it in a 1% solution of bovine albumin in Tris or barbital buffers. Under these conditions it was stable for at least a month at −25°, and upon thawing was stable at 37° for 30 minutes (Table V). Slight deterioration occurred after three hours at this temperature. No such stabili-

TABLE IV
EFFECT OF DIISOPROPYL PHOSPHOFLUORIDATE (DFP) ON ACTIVATED HAGEMAN FACTOR

Test Solution[a]	Clotting Time[b] (sec.)
Purified activated Hageman factor, untreated	121
Purified activated Hageman factor, DFP-treated	122
Crude activated Hageman factor, untreated	76
Crude activated Hageman factor, DFP-treated	117

[a] Purified activated Hageman factor, 2 mg per ml, *or* crude activated Hageman factor (one-tenth original plasma concentration), untreated or treated with 10^{-3} M DFP. [b] Hageman factor activity, tested by the method described.

TABLE V
TEMPERATURE STABILITY OF ACTIVATED HAGEMAN FACTOR[a]

Preincubation Time (min.)	Preincubation Temperature (°C)	Clotting Time[b] (sec.)
30	0	80
30	37	76
30	56	86
15	60	111
30	60	126
5	80	>300

Lyophilized activated Hageman factor (0.2 mg/ml) was dissolved in barbital-saline-citrate containing bovine serum albumin and incubated for the indicated time in silicone-coated tubes. [b] Hageman factor activity was tested by the method described in the text.

zation was provided by dissolving activated Hageman factor in solutions of gelatin.

In bovine serum albumin solution, activated Hageman factor was relatively resistant to heating at higher temperatures (Table V). No more than 25% of activity was lost after incubation at 56° for 30 minutes, and 75 to 80% was lost at 60° for the same period. However, less than 1% activity remained when activated Hageman factor was incubated at 80° for 5 minutes.

The purification of activated Hageman factor from normal plasma was carried out in the presence of 10^{-4} M versene. This substance slowed the deterioration of crude Hageman factor during its storage at −25°. There was no evidence that it had a stabilizing effect during brief intervals at 0° or 37°. Lyophilized activated Hageman factor, however, still contained large amounts of versene which were present during dialysis prior to freeze-drying.

The ability of activated Hageman factor to correct the defect of Hageman factor–deficient plasma was decreased by incubation with small amounts of chymotrypsin (Table VI); this reaction suggests that the activated Hageman factor is a protein. The presence or absence of carbohydrate in the final product has not been established. Likewise, physical studies have been difficult owing to the small amounts of protein available, its limited solubility, and the instability of the highly purified preparations in the absence of bovine serum albumin.

TABLE VI
THE EFFECT OF CHYMOTRYPSIN ON ACTIVATED HAGEMAN FACTOR[a]

Mixture	Clotting Time (sec.)
Activated Hageman factor + buffer	120
Activated Hageman factor + chymotrypsin	190

[a] One tenth ml of activated Hageman factor (0.2 mg per ml of bovine albumin in barbital-saline-citrate buffer) was incubated with 0.1 ml of Tris buffer *or* 0.1 ml of chymotrypsin (20 μg per ml of Tris buffer) for 30 minutes at 37° in silicone-coated tubes. The contents of each tube were then diluted 10-fold with barbital-saline-citrate buffer and the concentration of Hageman factor determined by measuring its effect upon the clotting time of Hageman factor–deficient plasma by the method described.

Tests for Contaminating Proteins in the Purified Activated Hageman Factor.—Purified activated Hageman factor was prepared by techniques designed to minimize contamination with other clotting factors. In fact, no PTA, antihemophilic factor, pro-SPCA, proaccelerin, Stuart factor, prothrombin, thrombin, or fibrinogen could be detected. Traces of Christmas factor–like activity, comparable to less than 1% of the amount of plasma of equivalent activated Hageman factor activity, were present. Whether this represented contamination with Christmas factor or an effect of activated Hageman factor upon the assay for Christmas factor was not clear (Table VII).

TABLE VII
CHRISTMAS FACTOR ACTIVITY OF PURIFIED ACTIVATED HAGEMAN FACTOR

Fraction Tested	Clotting Time[a] (sec.)
Normal plasma, diluted 1:640	180
Activated Hageman factor, 6 μg/ml	190
Buffer	195

[a] Clotting time of a mixture of 0.1 ml Hageman factor or plasma, diluted with bovine albumin solution in barbital-saline-citrate buffer, 0.1 ml Christmas factor-deficient plasma, 0.1 ml kaolin-Gliddex mixture, and 0.1 ml 0.04 M calcium chloride solution, tested by the method described. The amount of activated Hageman factor used was approximately equivalent to normal plasma, diluted 1:5.

TABLE VIII
FIBRINOLYTIC ACTIVITY OF PURIFIED ACTIVATED HAGEMAN FACTOR

Test Solution	Clot Lysis Time[a] (min.)
Plasma, diluted 1:160, + streptokinase	6
Plasma, diluted 1:640, + streptokinase	11
Activated Hageman factor, 3 μg./ml, + streptokinase	7.5
Activated Hageman factor, 3 μg./ml, + buffer	>120
Buffer + streptokinase	>120

[a] Clot lysis time, in duplicate, at 37° in Pyrex tubes of 0.1 ml plasma (diluted with barbital-saline-citrate buffer) *or* lyophilized activated Hageman factor (dissolved at a concentration equivalent in Hageman factor activity to $^4/_{10}$ that of the plasma from which it was prepared in barbital-saline-citrate buffer), 0.1 ml streptokinase (1500 units per ml) in barbital-saline buffer *or* barbital-saline buffer, 0.2 ml bovine fibrinogen and 0.1 ml bovine thrombin (10 units per ml).

None of the preparations of purified activated Hageman factor tested had fibrinolytic activity. Each of four such preparations, however, had plasminogen-like activity approximating 1% that of an amount of plasma with equivalent Hageman factor activity (Table VIII). This was measured by estimating the time required for the lysis of bovine fibrin in the presence of streptokinase. It is unclear whether this activity was due to contamination with plasminogen or represented an effect of activated Hageman factor itself (Niewiarowski and Prou-Wartelle, 1959; Iatridis and Ferguson, 1961).

DISCUSSION

To judge the virtues of a purification process, an accurate assay of the substance under study must be available. Previous assays for Hageman factor have provided only crude estimates of its concentration. To determine the concentration of Hageman factor accurately, it must be activated completely prior to its measurement. This can be done by bringing the preparation into contact with glass, kaolin, or some other effective agent. Only in its activated form will Hageman factor correct the defect in plasma deficient in Hageman factor or accelerate the clotting of normal plasma. Moreover, the only specific way to measure the concentration of Hageman factor is to test its effect upon a substrate of plasma obtained from a patient with Hageman trait. Such plasma, however, inhibits the *activation* of Hageman factor by surfaces (Ratnoff and Rosenblum, 1958). It is therefore essential that the activation of Hageman factor be complete before the addition of the substrate of Hageman factor–deficient plasma. Neither the assays we have used in earlier studies nor those that have been published by others have allowed sufficient time for the complete surface activation of Hageman factor. The result of this omission is to underestimate the Hageman factor activity in whole plasma or serum, and therefore to overestimate the degree of purification achieved in the preparation of this substance.

The assay used in the present study avoids this difficulty by allowing the preparation to be tested to be in contact with the activating surface, kaolin, for 2 minutes before the addition of Hageman factor–deficient plasma; a longer period was unnecessary. The assay did not avoid an equally serious problem concerning the specificity of the assay. Once a clotting factor is activated, it will correct any deficient plasma, providing the added activated factor participates in clotting at the same stage or later than the factor lacking in the deficient plasma. Thus, activated PTA rapidly shortens the clotting time of Hageman factor–deficient plasma.

Our failure to appreciate this difficulty led us to assume that an earlier preparation, rich in PTA and activated PTA, contained Hageman factor 500 or more times purified (Ratnoff and Rosenblum, 1958). Whether the highly purified activated clotting factor described in the present paper is identical to the factor lacking in patients with Hageman trait could not be ascertained, since it also might participate after Hageman factor in the clotting scheme. The factor does, however, activate PTA and does not correct the defect in PTA-deficient plasma. Moreover, it induces permeability activity in normal or Hageman factor-deficient plasma (Ratnoff and Miles, 1962, unpublished). Similar activity can be induced by exposing diluted normal plasma to glass surfaces, but glass does not induce permeability properties in plasma deficient in Hageman factor (Margolis, 1958; Ratnoff and Miles, 1962, unpublished observations).

In the present report, a method is described for the preparation of activated Hageman factor in which several steps were take to insure its separation from other known clotting factors, particularly PTA. In the course of its preparation, the Hageman factor was either largely or totally activated, presumably by contact with carboxymethylcellulose, an agent which, like glass, accelerates clotting (Ratnoff *et al.*, 1961). PTA was removed partly by adsorption onto aluminum gel and partly by treatment with diatomaceous earth. In this step, suggested by Soulier and Prou-Wartelle (1960), the plasma was exposed to a small amount of diatomaceous earth; Soulier postulated that this step activated a small amount of Hageman factor which then activated the PTA in the plasma. The activated PTA was then inactivated by the inhibitors in plasma. This explanation may not be entirely adequate, since we have been unable to deplete plasma of all its PTA by the addition of purified activated Hageman factor.

The final product had high Hageman factor activity relative to its content of protein; the activated Hageman factor appeared to be purified 3000–5000 fold. This calculation assumes that the color yield of bovine serum albumin and activated Hageman factor are identical in the protein determination by the method of Lowry *et al.* (1951). The yield varied from preparation to preparation, in part depending upon the degree of purification achieved; yields as high as 50% of the activity in the original plasma were accomplished. The estimates of purity and yield are subject to the errors in the determination of Hageman factor activity which have already been discussed. Presumably most or all of the purified Hageman factor was activated, for it both accelerated the clotting time of normal plasma and converted PTA to an activated form in silicone-coated tubes. The method of assay presumably fully activated the Hageman factor in the test plasma, but proof of this is by no means certain.

The preparation of activated Hageman factor was contaminated with salts, principally versene, used as a stabilizing agent. Total removal of salts was impractical, since this left so little solid after lyophilization that it was difficult to recover the material remaining in the flask.

The preparation was devoid of other known clotting factors, with the possible exception of Christmas factor. In high concentration, it accelerated the coagulation of plasma deficient in Christmas factor minimally. Since the Christmas factor–deficient plasma itself clotted upon the addition of calcium and "cephalin," it is likely that it was not totally devoid of Christmas factor. Under these circumstances, activated Hageman factor may have accelerated its coagulation nonspecifically, through its influence upon earlier steps of the clotting process.

The purified activated Hageman factor was probably contaminated with traces of plasminogen. The addition of streptokinase induced fibrinolytic activity, as tested upon a substrate of bovine fibrin. The alternative possibility, that activated Hageman factor potentiated the action of streptokinase upon the plasminogen which contaminated the fibrin substrate, could not be ruled out (Niewiarowski and Prou-Wartelle, 1959; Iatridis and Ferguson, 1961).

In an earlier study, we reported that crude Hageman factor, prepared from PTA-deficient plasma, was partially inhibited by diisopropyl phosphofluoridate. Although we have been able to confirm this observation with crude preparations of Hageman factor, the highly purified preparation was unaffected by 10^{-3} M diisopropyl phosphofluoridate. A possible explanation is that diisopropyl phosphofluoridate inactivated some undescribed enzyme present in the crude preparation which participates in clotting prior to PTA. Exploration of the reasons for the discrepancy in behavior of diisopropyl phosphofluoridate toward crude and purified preparations of activated Hageman factor should be of great interest.

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Aminomalonic Decarboxylase*

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A study has been made of the aminomalonic decarboxylase of rat liver, and evidence has been obtained for the role of pyridoxal phosphate as a co-factor. Under the test conditions employed, the enzyme appears to be specific for aminomalonic acid, and is inhibited by several structural analogues (*e.g.* α-methylaminomalonic acid, L-serine) which are not substrates. The nonenzymic decarboxylation of aminomalonic acid and α-methylaminomalonic acid at *p*H 6 is promoted by pyridoxal phosphate or pyridoxal (10^{-4} M and higher), and diethyl aminomalonate is decomposed at this *p*H in the presence of 10^{-5} M pyridoxal phosphate.

The existence of an enzyme that catalyzes the decarboxylation of aminomalonic acid was reported in a brief note by Shimura *et al.* (1956), who found it in the silk-gland tissue of silkworms and in rat-liver homogenates. In their studies of the silk-gland enzyme, these authors demonstrated that glycine and CO_2 were the products of the reaction, that the *p*H optimum was near *p*H 6, and that the enzymic activity was inhibited by cyanide and hydroxylamine. A careful search of the biochemical literature has failed to reveal any subsequent paper on this subject by the above authors. A later note from their laboratory (Nagayama *et al.*, 1958) reported the finding, in the silk-gland of silkworms and in rat-liver homogenates, of a transaminase that catalyzes the amination of ketomalonic acid (mesoxalic acid) by L-alanine and several other amino acids. The possibility was raised that a route of glycine synthesis in the silk-gland may be: hydroxymalonic acid (tartronic acid) to ketomalonic acid to aminomalonic acid to glycine. The question of the role of aminomalonic acid as a glycine precursor had been discussed several times previously in the biochemical literature (Knoop, 1914; Haas, 1916; Knoop and Oesterlin, 1927); in particular, Shemin (1946) excluded this possibility in the conversion of L-serine to glycine in the intact rat because the COOH-C^{13}/N^{15} ratio in the glycine (isolated as urinary hippuric acid) produced was the same as in the administered labeled serine. In his well-known note, Ogston (1948) commented on this conclusion by calling attention to the possibility that aminomalonic acid, although a symmetrical compound, may be handled asymmetrically by an enzyme so as to cause the selective loss, by decarboxylation, of only one of the apparently equivalent carboxyl groups. Later work on the mechanism of the serine-glycine interconversion has led to the recognition of the role of the tetrahydrofolic acid compounds as co-factors (for a review, see Huennekens and Osborn, 1959), and the possible intermediate role of aminomalonic acid in amino acid metabolism has received little attention recently.

Because of our interest in the possibility that aminomalonic acid may be a naturally occurring amino acid, it appeared desirable to examine more closely the enzyme-catalyzed decarboxylation reported by Shimura *et al.* (1956). In the present communication we describe experiments with a preparation from rat liver, and offer evidence in favor of a pyridoxal phosphate-dependent enzymic process. Observations are also reported on the catalysis of the nonenzymic decarboxylation of aminomalonic acid by pyridoxal phosphate. During the course of these studies with model systems, we noted that Neuberger (1961) had already observed the pyridoxal phosphate-promoted decarboxylation of aminomalonic acid.

A number of syntheses of aminomalonic acid have been described since its first preparation by Baeyer (1864) by the reduction of oximinomalonic acid (obtained from isonitrosobarbituric acid) with sodium amalgam. Later methods based on this approach have involved the preparation of diethyl isonitrosomalonate by the treatment of diethylmalonate with $NaNO_2$ and acetic acid (Cerchez, 1930; Zambito and Howe, 1960), followed by reduction with aluminum amalgam (Piloty and Neresheimer, 1906; Putochin, 1923; Cerchez, 1930), with hydrogen sulfide (Johnson and Nicolet, 1914), with Pt or Pd and H_2 (Putochin, 1923; Schneider, 1937; Snyder and Smith,

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