Mechanisms Involved in Enhancement of Plasma Fibrinolytic Activity by Chloroform

By Leonard A. Moroz and Norbert J. Gilmore

The effects of a single 1-min extraction with chloroform (CHCl₃) on plasma fibrinolytic activity has been examined by ¹²⁵I-fibrin solid phase assay, using normal plasma and plasma depleted of plasminogen (PLG) by lysine-Sepharose affinity chromatography. Fibrinolytic activity of normal plasma is increased (40%-175%), and more than 95% of antiplasmin activity is removed. The increase is demonstrable in PLG-depleted plasma, and is not inhibited by tranexamic acid (0.01 M).

Purified PLG is not activated to plasmin by CHCl₃ treatment. Bio-Gel A 0.5 m fractionation of CHCl₃-extracted, PLG-depleted plasma reveals fractions with the following activities: (1) streptokinase-activatable, PLG-independent fibrinolytic activities; (2) PLG activator activities; and (3) plasmin-stimulated but PLG-independent fibrinolytic activities, which include activities inhibited by hexadimethrine bromide and which cofractionate in part with plasmin-stimulated procoagulant activities. In addition, similar fractionation of nonextracted plasma reveals two non-plasmin fibrinolytic activities (~30,000 and 13,000 daltons) activated by streptokinase and plasmin, respectively. The findings indicate that the enhanced fibrinolytic activity resulting from CHCl₃ treatment is independent of plasmin as the ultimate fibrinolytic enzyme, although activities stimulated by plasmin may contribute, and that such treatment is a useful maneuver for study of PLG-dependent and PLG-independent fibrinolytic mechanisms, and their interactions.

Enhancement of the proteolytic activity of serum and plasma by chloroform treatment has been known since 1903.¹ The generation of increased activity has been confirmed by methods employing casein and gelatin as substrates,²³ and its fibrinolytic potential indicated by studies of clot lysis⁴ and by lysis of fibrinogen.⁶ On the basis of pH and temperature-activity relationships, the identity of this proteolytic activity with plasmin was suggested.⁷ It was subsequently shown, using gelatin as substrate, that chloroform treatment, in addition to decreasing total streptokinase-activated serum protease activity (an effect attributed to enzyme denaturation), also effected removal of more than 90% of the protease inhibitory activity in normal serum.⁸ It was noted, however, that the protease activity generated subsequent to removal of inhibitors by chloroform differed in kinetic properties from the proteolytic activity generated by streptokinase activation of plasminogen to plasmin.⁸ Ogston et al.⁹ demonstrated that euglobulin fractions prepared from normal plasma displayed increased proteolytic activity for both fibrin and casein in the presence of chloroform, although the participation of plasmin was not demonstrated. A role for Hageman factor in the chloroform enhancement phenome-
mon was suggested by the observation that this increased activity was inhibited by addition of hexadimethrine bromide before chloroform treatment, and was not demonstrable in euglobulin fractions prepared from plasma deficient in that factor. Chloroform treatment also resulted in disappearance from serum of the activities of certain complement components (C1, C2, C4, and to a lesser extent, C3), and generated esterase activity in serum which was distinguishable from plasmin by several functional criteria, including temperature and pH activity relationships, resistance to soybean trypsin inhibitor, failure to digest casein, and relative hydrolysis of synthetic substrates. In part, this activity bore functional similarities to C1 esterase, and the chloroform effect was consistent with removal of the corresponding inhibitor (C1NH). However, differential effects on synthetic substrates indicated that more than one esterase activity may have been generated.

We have reexamined the consequences of chloroform treatment of plasma, using a sensitive 125I-fibrin solid phase assay for fibrinolytic activity, both normal plasma and plasma depleted of plasminogen by lysine-Sepharose affinity chromatography, and plasma fractionation on Bio-Gel A 0.5 m. The chloroform effect is shown to be complex, involving the removal of plasmin inhibitors, and the unmasking of plasminogen activators and of plasmin and streptokinase-activated fibrinolytic activities independent of plasmin as the ultimate fibrinolytic enzyme. In addition, evidence is presented for fibrinolytic activities distinct from plasmin which are demonstrable in plasma without prior chloroform treatment.

MATERIALS AND METHODS

Reagents and materials were obtained from the following sources: tris (hydroxymethyl) aminomethane (Tris) and L-lysine hydrochloride, Sigma Chemical Co., St. Louis, Mo.; chloroform (reagent grade) and disodium ethylenediaminetetraacetic acid (EDTA), Fisher Scientific Co., Montreal, Canada; Sepharose 4B, Pharmacia, Uppsala, Sweden; Bio-Gel A 0.5 m, Bio-Rad Laboratories, Richmond, Calif.; trans-4-amino-methylcyclohexanecarboxylic acid (AMCHA, trannexamic acid) and hexadimethrine bromide (Polybrene), Aldrich Chemical Co., Milwaukee, Wis.; Varidase (streptokinase-streptodornase, for intramuscular use), Lederle Products Department, Cyanamid of Canada, Ltd., Montreal, Canada; human fibrinogen, Connaught Medical Research Laboratories, Toronto, Canada; disposable polystyrene culture tubes (Lab-Tek No. 4411, 12 × 75 mm, Lab-Tek Products, Miles Laboratories, Westmont, Ill.); monospecific antisera to human α1-macroglobulin and C1 esterase inhibitor, Behringwerke AG, Marburg-Lahn, West Germany; α1-antitrypsin radial immunodiffusion kits, Kallestad Laboratories, Inc., Chaska, Minn. Urokinase (Abbott 38790) was a generous gift of Dr. J. Donahoe, Abbott Laboratories, North Chicago, Ill.

Plasma samples. Venous blood was collected from normal individuals with the use of a tourniquet, without glass contact where indicated, and anticoagulated with EDTA (final concentration, 0.005 M). Plasma was separated by an initial centrifugation at 700 g for 10 min, followed by further centrifugation of the supernatant plasma at 10,000 g for 10 min. Although similar effects of chloroform on fibrinolytic activity were observed with freshly prepared and frozen plasma subsequently rethawed, the studies reported here were all performed with fresh preparations.

Assay of fibrinolytic activity. The 125I-fibrin solid phase assay method, together with methods for isolation, purification, and quantitation of electrophoretically homogeneous plasminogen have been described previously. Briefly, the assay was based on release of radioactive degradation products from polystyrene assay tubes coated with 125I-fibrin (human), originally adsorbed to the assay tubes as plasminogen-free 125I-fibrinogen, and subsequently converted to 125I-fibrin by treatment with plasminogen-free thrombin. The absence of con-
taminating plasminogen has been demonstrated by appropriate studies with urokinase and streptokinase, and the assay yields radioactive fibrin degradation products comparable to those obtained with a nonradioactive fibrin clot prepared conventionally by thrombin treatment of plasminogen-free fibrinogen. Assay tubes were coated with approximately 1.5 µg 125I-fibrin per tube (specific activity, 35,000-50,000 counts/min/µg fibrin).

**Spontaneously activated plasmin.** The plasmin used in these studies was prepared from a normal plasma by affinity chromatography on lysine-Sepharose, with EACA gradient elution as described by Brockway and Castellino, followed by chromatography on Bio-Gel A 0.5 m. This gradient method resolved plasminogen into two fractions, designated F-1 and F-2, distinguishable by different mobilities on polyacrylamide disc gel electrophoresis at acid pH. Fraction F-1 from this particular plasma was 75% activated to plasmin, and fraction F-2, the preparation used here, was maximally activated, as judged by failure to generate further fibrinolytic activity on incubation with urokinase or streptokinase at concentrations up to 100 units/ml, far in excess of the lowest concentrations of these activators producing detectable activation of plasminogen in the 125I-fibrin assay. Similar spontaneously activated preparations have been described by others. On SDS polyacrylamide disc gel electrophoresis, this plasmin preparation yielded two protein bands after reduction (0.05 M 2-mercaptoethanol, 12 hr, 37°C), both of greater mobility than the single protein band observed with a similarly treated preparation of unactivated plasminogen. There was no protein band in the plasmin preparation which corresponded to the single band observed with reduced, nonactivated plasminogen, providing structural confirmation of the maximal activation determined functionally.

**Chloroform extraction of plasma.** Plasma (1 volume) was extracted with chloroform (2 volumes) for 1 min at room temperature by gentle inversion in glass or plastic centrifuge tubes (as indicated), and immediately centrifuged (2000 rpm, 20 min, 4°C, International PR-6 centrifuge). The supernatant plasma layer was carefully aspirated, and residual chloroform gently evaporated with a stream of nitrogen. Buffer and plasma controls were carried out, as indicated, for the chloroform and nitrogen treatments, respectively.

**Preparation of plasminogen-free plasma.** Plasma (5-7 ml) was passed through a column (1.0-1.25 ml bed volume in a 3/4 inch Pasteur pipette, or in a plastic syringe barrel) of lysine-Sepharose 4B prepared by the method of Deutsch and Mertz, and equilibrated with Tris-NaCl buffer (0.015 M Tris, 0.15 M NaCl, pH 7.4). Effluent plasma obtained in this manner did not yield a precipitin line by double diffusion with a monospecific rabbit antiplasminogen antiserum, did not generate additional fibrinolytic activity on incubation with urokinase under conditions capable of detecting 0.2 µg plasmin generated/ml, and was free of plasminogen by a solid phase inhibition radioimmunoassay (sensitivity, 10 ng plasminogen/ml) employing polystyrene tubes coated with specific rabbit antiplasminogen antiserum, and 125I-labeled pure human plasminogen.

**Fractionation of plasma by gel filtration.** Plasma (1 ml) was applied to a column (0.9 x 87 cm) of Bio-Gel A 0.5 m equilibrated with Tris-NaCl buffer. Fractions (1.8 ml) were collected at room temperature. The column was calibrated, as described previously, with markers of the following molecular weights: blue dextran 2000 for void volume, human fibrinogen (341,000), aldolase (158,000), bovine serum albumin (65,000), horseradish peroxidase (40,000), chymotrypsin (23,000), myoglobin (17,000), and cytochrome c (13,000). The absorbancy of the column effluent was monitored at 280 nm.

**Assay of chromatographic fractions.** Aliquots of fractions (0.1 ml) were assayed for fibrinolytic activity by the 125I-fibrin method, either alone, with pure plasmin, or with pure plasmin and hexadimethrine bromide. Aliquots were incubated (37°C, 30 min) with 0.1 ml of Tris-NaCl buffer, or the same volume of buffer containing plasmin (final concentration, 1 µg/ml). The final concentration of hexadimethrine bromide was 100 µg/ml. Activities of fractions plus plasmin, or of fractions plus plasmin and hexadimethrine, are expressed (after subtraction of activities of fractions alone) as a percentage of the activity of plasmin alone.

To assay for streptokinase-activatable fibrinolytic activities in fractions of plasminogen-depleted plasma, aliquots (0.1 ml) of each fraction were incubated (37°C, 30 min) in 125I-fibrin assay tubes with 0.1 ml of Tris-NaCl buffer containing streptokinase (final concentration, 0.05 units/ml). Activities obtained with fractions alone were subtracted from corresponding activities with streptokinase to obtain streptokinase-activated fibrinolytic activity.
Similarly, fractions were assayed for plasminogen activator activity by incubating aliquots (0.1 ml) for 30 min at 37°C with 0.1 ml of Tris-NaCl buffer containing purified human plasminogen (final concentration, 5 µg/ml). The plasminogen preparation used had no fibrinolytic activity in the 125I-fibrin assay in the absence of urokinase or streptokinase. Activator activity in fractions was calculated by subtracting activity of fractions alone from activity of fractions together with plasminogen.

Procoagulant activity of chromatographic fractions was monitored by determining their effect, in the presence and absence of plasmin, on the clotting time of whole blood. Aliquots of fractions (0.05 ml) were incubated (37°C, 30 min), with and without plasmin (final concentration, 0.2 µg/ml), in combination with 0.4 ml of normal human venous blood, collected with the use of a tourniquet, but without anticoagulant, and chilled on ice prior to start of the assay. The reciprocals of the clotting time (min⁻¹) were calculated, and results expressed as a percentage of the control values obtained with blood and buffer, or with blood, buffer, and plasmin.

Other analytic methods. α₁-antitrypsin was quantitated with commercial radial immunodiffusion kits, while α₂-macroglobulin and Cl esterase inhibitor were determined semiquantitatively by double immunodiffusion analysis of serial dilutions of plasma with monospecific commercial antisera.

RESULTS

Effects of Chloroform Extraction on Fibrinolytic Activity of Plasma

A single 1-min extraction of plasma with two volumes of chloroform resulted in increased fibrinolytic activity. For example, lysis of 125I-fibrin by 0.1-ml aliquots of an untreated plasma (30-min incubation, 37°C) was 47.3 ng ± 2.6 SEM (five replicate determinations), while activity of the same plasma after chloroform extraction was 65.1 ng ± 2.4 SEM, an increase of 40% (p < 0.01). In another normal plasma, corresponding activities were 36.1 ng ± 2.7 SEM (untreated) and 100.9 ng ± 5.8 SEM (chloroform extracted).

Relative Independence From Plasminogen of the Chloroform Effect

Increased fibrinolytic activity in plasma following chloroform extraction is independent of plasminogen (Table 1). The increase in activity in untreated plasma (+158%) is of the same order as the increase produced in plasma depleted of plasminogen by lysine-Sepharose affinity chromatography (+147%) or in plasma passed through an unsubstituted Sepharose 4B column (+140%). In addition, the increased fibrinolytic activity in chloroform-extracted plasma is

<table>
<thead>
<tr>
<th>Plasma Treatment</th>
<th>125I-Fibrin Lysed (ng)</th>
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<tbody>
<tr>
<td></td>
<td>Before Extraction</td>
</tr>
<tr>
<td>None</td>
<td>60.6 ± 1.7</td>
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<tr>
<td>Sepharose 4B column</td>
<td>67.4 ± 1.7</td>
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<td>Lysine-Sepharose 4B column</td>
<td>65.9 ± 2.4</td>
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Plasma (0.005 M EDTA), collected and processed without glass contact, was passed through a lysine-Sepharose 4B column, or a column of unsubstituted Sepharose 4B, equilibrated with Tris-NaCl buffer. An aliquot of starting plasma was diluted with Tris-NaCl buffer (0.2 volumes) to correct for plasma dilution during passage through the columns. Portions of all three plasma samples were extracted with chloroform as described in Materials and Methods, and aliquots of extracted and unextracted plasma (0.1 ml) were incubated (37°C, 60 min) in 125I-fibrin assay tubes coated with 1.5 µg 125I-fibrin (35,000 counts/min/µg). Results are expressed as mean ± SEM (five replicate determinations).
Fig. 1. Effect of chloroform extraction on fibrinolytic and anti-plasmin activity of normal human plasma. Serial dilutions of plasma in Tris-NaCl buffer (0.1 ml) were incubated (37°C, 30 min) with and without plasmin (0.1 ml, final concentration, 10 μg/ml), in assay tubes coated with 125I-fibrin (80,400 counts/min/tube, 53,600 counts/min/μg fibrin). Left, plasminogen-rich plasma. Right, plasma depleted of plasminogen by lysine-Sepharose affinity chromatography. Open circles, plasma alone. Open triangles, chloroform-extracted plasma alone. Closed circles, plasma incubated with plasmin. Closed triangles, chloroform-extracted plasma incubated with plasmin. Horizontal interrupted line, lysis produced by plasmin alone.

not inhibited by tranexamic acid (AMCHA) at a concentration of this agent (0.01 M) which effectively inhibits fibrin digestion by plasmin.13

Removal of Antiplasmin Activity and Appearance of New Fibrinolytic Activities After Chloroform Extraction

The low fibrinolytic activity of whole plasma is attributed in part to the effects of normal plasma inhibitors of plasmin (antiplasmins).18,19 The effects of chloroform extraction on the plasmin inhibitory capacity of normal plasma are illustrated in Fig. 1. Using a fixed dose of electrophoretically homogeneous, maximally activated human plasmin, untreated normal plasma inhibits plasmin lysis of 125I-fibrin, with 50% inhibition at a plasma dilution of approximately 1/100, and complete dilution of inhibitory activity at 1/300 (Fig. 1, left, closed circles). Net fibrinolysis at plasma dilutions beyond 1/300 does not exceed levels attributable to the added plasmin alone.

Chloroform-extracted plasma, on the other hand, exhibits two effects. Firstly, there is a marked decrease in antiplasmin activity (closed triangles), with 50% inhibition at a plasma dilution of only 1/3 to 1/4, representing an approximate 96%-97% decrease in antiplasmin activity. In addition, the net fibrinolytic activity on further dilution of the treated plasma reached a level
53% greater than the activity exhibited by plasmin alone (horizontal interrupted line). This increment cannot be accounted for by the activity of chloroform-treated plasma alone, which is minimal (open triangles), and is also not attributable directly to an effect of the extraction procedure on plasmin. The preparation of plasmin is already maximally activated (no further generation of fibrinolytic activity on incubation with urokinase), and incubation of plasmin with chloroform-extracted buffer does not result in enhanced activity. Lysis on incubation (30 min, 37°C) of 0.1 ml Tris-NaCl buffer with 0.1 ml plasmin (final concentration, 10 µg/ml) is 547 ng ¹²⁵I-fibrin ± 34 SEM (five replicate determinations), while corresponding activity for plasmin and chloroform-extracted buffer is 449 ng ± 10 SEM (0.01 < p < 0.02 Student’s t test), a decrease of 22%. These findings indicate that chloroform extraction, in addition to removing most of the antiplasmin activity, also permits the generation from treated plasma, in the presence of plasmin, of additional fibrinolytic activity.

Since plasmin itself is capable of catalyzing the conversion of plasminogen to plasmin, further studies were performed with plasma depleted of plasminogen by lysine-Sepharose chromatography, and subsequently extracted with chloroform (Fig. 1, right). The completeness of removal of plasminogen by this treatment was indicated by lysis of 65.4 ng ¹²⁵I-fibrin by plasma (0.1 ml) incubated with urokinase (40 CTA units/ml, 37°C, 30 min), as compared to lysis of 66.1 ng by plasma (without urokinase) prior to passage through the lysine-Sepharose column. As shown in Fig. 1, right, incubation with plasmin of serial dilutions of plasminogen-free, chloroform-extracted plasma resulted in a similar increment in maximal fibrinolysis to that observed with plasminogen-rich plasma (Fig. 1, left), indicating that the additional fibrinolytic activity generated by plasmin was independent of plasminogen. Activation of purified plasminogen to plasmin by chloroform extraction was not observed.

Analysis of the Chloroform Effect by Gel Filtration Fractionation of Plasma

When plasminogen-free plasma is fractionated on Bio-Gel A 0.5 m, and fibrinolytic activity of fractions is assayed in the presence of plasmin (Fig. 2, panel A), almost all fractions exhibit significant antiplasmin activity. The exception is lower molecular weight material which exhibits increased fibrinolytic activity in the presence of plasmin, with peak activity (tube 31) eluting slightly ahead of cytochrome c (MW 13,000). Although the activity of this fraction (68% greater at tube 31 than plasmin alone, which lysed 6,600 counts/min of ¹²⁵I-fibrin) is clearly increased, it has negligible fibrinolytic activity in the absence of plasmin (710 counts/min ¹²⁵I-fibrin lysed by 0.1 ml of tube 31, after subtraction of background control, 785 counts/min). Similar activity is present in both chloroform-extracted and untreated plasminogen-free plasma (see below).

Further resolution of the antiplasmin activity (Fig. 2, panel A) is obtained by assaying fractions at a 1/10 dilution (Fig. 2, panel B). Three inhibitory peaks are now apparent, one in the void region, and two additional peaks at tube 22 (MW 90,000) and tube 25 (MW 45,000). At this dilution, the low molecular weight peak of plasmin-generated fibrinolytic activity is no longer apparent.

When plasminogen-free plasma is extracted with chloroform prior to frac-
Although the peaks of inhibitory activity shown in Fig. 2, panel B (void Fig. 2. Analysis of chloroform effect by fractionation of plasma on Bio-gel A 0.5 m. Removal of antiplasmin activity and appearance of plasmin-stimulated, plasminogen-independent fibrinolytic activity. Plasminogen-free plasma, with and without chloroform extraction, was fractionated as described in Materials and Methods, and aliquots of fractions tested for fibrinolytic activity with and without plasmin (final concentration, 1 μg/ml). Activity of fractions plus plasmin (after subtraction of activity of fractions alone) is expressed as percentage of activity of plasmin alone (6600 counts/min 125I, fibrin lysed, 42,000 counts/min/μg fibrin). Hexadimethrine bromide was used at a final concentration of 100 μg/ml. Plasminogen (PLG) eluted at tube 23, as determined by 280-nm absorbancy profile of electrophoretically homogeneous PLG preparation, confirmed by fibrinolytic activity of fractions assayed with streptokinase (20 units/ml).

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Fig. 3. Procoagulant activity of nonextracted and chloroform-extracted plasma fractions. Plasminogen-free normal plasma, with and without prior chloroform extraction, was fractionated on Bio-Gel A 0.5 m as in Fig. 2. Fractions were assayed for effect on clotting time of whole blood, with and without addition of plasmin, as described in Materials and Methods. (A) Plasminogen-free plasma. (B) Plasminogen-free chloroform-extracted plasma. (C) Plasminogen-free plasma, fractions assayed with plasmin. (D) Plasminogen-free, chloroform-extracted plasma, fractions assayed with plasmin. Reciprocals of clotting times with fractions are expressed as percentages of reciprocals of clotting times with buffer alone (A and B), or with buffer and plasmin (C and D).

Effect of Plasma Fractions on Coagulation

Hageman factor and its fragments have procoagulant activity, and plasmin can activate Hageman factor. Since hexadimethrine bromide, an inhibitor of Hageman factor activation and function, alters the profile of plasmin-stimulated fibrinolytic activity (Fig. 2), plasma fractions have been tested for their effects on the clotting time of whole blood (Fig. 3). Plasminogen-free plasma volume, tube 22, tube 25) would correspond to three known inhibitors of plasmin function (α2-macroglobulin; MW 900,000; Cl esterase inhibitor; MW, 90,000 α1-antitrypsin; MW 45,000), there was no significant change in the plasma concentrations of these three proteins, determined immunochemically, following chloroform extraction, suggesting that the effect of chloroform was to produce functional inactivation without physical removal, or alternatively, that other as yet uncharacterized inhibitors were involved.

The effects of hexadimethrine bromide, an inhibitor of Hageman factor activation, on plasmin-stimulated fibrinolytic activity are shown in Fig. 2, panel D. This agent produces striking changes in the fibrinolytic profile. Stimulatory effects are abolished in tube 21 (MW 110,000), and in tubes 24–26 (MW 35,000–60,000). In addition, the low molecular weight activity (tubes 30–33) is no longer apparent. Remaining peaks of activity are at tubes 18 (MW 230,000), tube 20 (MW 150,000), tubes 22 and 23 (MW 75,000–90,000), and tubes 27–29 (MW 18,000–30,000).
EFFECT OF CHCl₃ ON FIBRINOLYSIS

(panel A) exhibits inhibitory (anticoagulant) activity in many fractions corresponding to a molecular weight range of 60,000–250,000. After chloroform extraction (panel B), this anticoagulant effect is no longer evident, and peaks of procoagulant activity are present at tube 15 (void) and tube 21 (MW, 110,000). In both chloroform-extracted (panel A) and nonextracted plasma (panel B), comparable procoagulant activity is present in later eluting fractions (tubes 27–34).

Fractions were similarly assayed with the addition of pure plasmin (final concentration, 0.2 µg/ml) (panels C and D). With nonextracted plasminogen-free plasma, addition of plasmin resulted in procoagulant activity in fractions (panel C) which were anticoagulant in the absence of plasmin (panel A). Addition of plasmin to fractions of chloroform-extracted plasma resulted in a slight increase in the uniformly distributed procoagulant activity from tubes 15–24. In both untreated and chloroform-extracted plasma, addition of plasmin to fractions produced comparable increases in the low molecular weight procoagulant activity. In the chloroform-extracted material, there was definite accentuation of the increased activity at tubes 30 and 31, corresponding to the elution volume of the plasmin-stimulated fibrinolytic activity in this region (Fig. 2, panels A and C).

Effects of Chloroform Treatment on Plasminogen Activator Activity, and on Streptokinase-activatable, Plasminogen-independent Fibrinolytic Activity of Plasma

To determine if chloroform extraction influenced other fibrinolytic mechanisms, plasminogen-free plasma fractions were also assayed for fibrinolytic activity alone, in the presence of streptokinase, and in the presence of purified, fibrinolytically inactive plasminogen (Fig. 4). In fractions of plasminogen-free plasma alone (upper panel, left), intrinsic fibrinolytic activity was found throughout the elution profile, indicating the presence of significant non-plasmin-mediated activities. After chloroform extraction (upper panel, right), in addition to enhanced activity at tubes 14 and 15 (void), suggesting removal of some inhibitors of fibrinolytic activities there, there were moderate decreases in activity at tubes 24, 25, 28, 29, and 31, perhaps reflecting enzyme inactivation.

On incubation with streptokinase (middle panel, left), at concentrations which do not activate plasminogen significantly (Fig. 5), unextracted plasminogen-free plasma gives peaks of activity at tubes 14 and 15 (void), tubes 18–20 (MW 145,000–235,000), and tube 29 (MW 18,000), indicating that nonextracted plasma contains streptokinase-activatable fibrinolytic activity which is in excess of that attributable to the fractions alone, and which is not due to plasminogen-plasmin, which would be expected to yield fibrinolytic activity at tube 23 (MW 80,000). After chloroform extraction (middle panel, right), there is decreased activity at the void volume, and at tubes 16, 17, 19, 20, and 29. In addition, however, there is the striking appearance of increased activity (eight- to nine-fold increase) in tube 21 (MW 120,000), consistent with the removal by chloroform of an inhibitor of streptokinase activation of a non-plasminogen-derived fibrinolytic activity.

On addition of completely inactive plasminogen, there are net increases in fibrinolytic activity at the void volume, at tubes 19 and 20 (MW 145,000–
Fig. 4. Analysis of chloroform effect by plasma fractionation. Effects on streptokinase-activatable, plasminogen-independent fibrinolytic activity of plasma, and on plasminogen activator activity of plasma. Plasminogen-free plasma, with and without chloroform extraction, was fractionated on Bio-Gel A 0.5 m as described. Left, plasminogen-free plasma. Right, plasminogen-free plasma extracted with chloroform. Fibrinolytic activity (upper panels), streptokinase-activatable fibrinolytic activity (middle panels) and plasminogen activator activity (bottom panels) were assayed as described in Materials and Methods. Assay tubes were coated with $^{125}$I-fibrin (36,000 counts/min/μg, 1.5 μg/tube).

185,000), at tube 22 (MW 90,000) and at tube 28 (MW 24,000), in fractions from nonextracted, plasminogen-free plasma (bottom panel, left). After prior chloroform extraction (bottom panel, right), the activity in the void region is virtually unchanged, and plasminogen activator activity at tube 20 (MW 145,000) is decreased by 70%. There is a new activator activity now apparent at tube 18 (MW 235,000), and at tubes 28 and 29 (MW 18,000–24,000), suggesting the possible removal in these regions of inhibitors of either plasminogen activation, or of plasmin-mediated fibrinolysis, or of chloroform activation of plasminogen activator.
**Other Observations: Fibrinolytic Activity of Fractions of Normal Plasma**

Although the studies described thus far were designed to exclude the participation of endogenous plasminogen in plasma, fibrinolytic activity of fractions of normal plasma, obtained without glass contact, were also examined (Fig. 5). Fractions of unextracted plasma (upper left) exhibited low grade activity across the profile, with a prominent peak at tubes 18 and 19 (MW 185,000-235,000). After chloroform extraction (upper right), this peak was absent, and there was a general increase in activity across the profile (approximately 60% greater total activity), indicating that the chloroform-induced increment was heterogeneously distributed in terms of molecular weight. In both upper profiles, there was no obvious peak at tube 23, where plasminogen eluted both physically and functionally, indicating the absence of significant plasmin-mediated fibrinolytic activity in normal plasma, and the absence (upper right) of significant activation of plasminogen to plasmin, confirming the failure to detect such activation with chloroform extraction of purified plasminogen.

Extensive studies were not made of fibrinolytic activity in fractions of normal plasma after maximal activation, since the very high activities generated by addition of optimal concentrations of urokinase or streptokinase (increases up to 100-fold) tended to obscure the fibrinolytic activities described in this study. However, fractions were assayed in the presence of streptokinase at concentrations which did not appreciably activate plasminogen (Fig. 5, lower). The notable finding, with or without prior chloroform extraction, was the presence...
of a peak of fibrinolytic activity at tube 27 (MW 30,000). This activity was distinct from plasminogen-plasmin, which eluted at tube 23.

DISCUSSION

This study confirms earlier reports that chloroform treatment increases fibrinolytic activity of plasma4,5,9 and indicates that this enhancement occurs in the absence of plasminogen, and without the participation of plasmin as the ultimate fibrinolytic enzyme. It extends previous demonstrations that chloroform removes most antiproteinase activity in serum8 by clearly showing that the inhibitors removed are active against plasmin. It also provides evidence for additional mechanisms that may contribute to chloroform-mediated enhancement of fibrinolysis, and which may have physiological significance. In addition to unmasking of plasminogen activator activities, chloroform extraction permits the detection of plasmin- and streptokinase-activated fibrinolytic activities in plasma, which are distinguishable from plasminogen-plasmin by their molecular weight or streptokinase sensitivity. It remains to be determined whether these activities result from a direct activating effect of chloroform (although plasminogen itself does not appear to be activated by this treatment), or are a consequence of antiplasmin activity removal. In favor of the latter possibility are the observations that plasma inhibitors of plasmin are active against other proteinases in plasma,20,22 the evidence suggesting that chloroform treatment abolishes C1 esterase inhibitor (C1INH) activity,12 and the removal, in the present study, of antiplasmin activity in fractions with molecular weights corresponding to those of C1INH and α1-anti-trypsin (90,000 and 45,000 daltons, respectively).

The present studies demonstrate several fibrinolytic activities distinct from plasmin. Although plasmin is considered to be the major streptokinase-activatable fibrinolytic enzyme in plasma,18 the present findings indicate that other fibrinolytic activities may be influenced by this activator. These observations also raise the question of the relative contribution to normal, spontaneous fibrinolytic activity in plasma of processes culminating in plasmin generation from plasminogen, as distinguished from activities generated by plasmin, or activities which, although activated by the same factors (e.g., streptokinase), are independent of the plasminogen–plasmin system. Since fibrinolytic activity in normal plasma is not appreciably influenced by plasminogen depletion (Table 1 and Fig. 5), these additional activities may account for a significant portion of normal fibrinolytic activity. Further evidence for this view is presented in another paper.28

The activities described here may relate to factors previously described by others. The unmasking of these fibrinolytic activities by chloroform extraction and their partial inhibition by hexadimethrine suggest a link with the observations of Ogston et al.,9 who found a requirement for Hageman factor in the chloroform effect, although the participation of plasminogen-plasmin, as distinguished from proteolytic activity in general, has not been conclusively shown. Hageman factor and its fragments have procoagulant activity,24 Hageman factor can be activated by plasmin,25 and hexadimethrine bromide, an inhibitor of Hageman factor activation and function,26,27 alters the profile of plasmin-stimulated fibrinolytic activity (Fig. 2). The finding (Fig. 3) that this activity corresponds
in elution profile to plasmin-stimulated procoagulant activity suggests a possible relationship to the MW-12,000 fragment of Hageman factor which has been described.24 Although it has not been directly demonstrable by the less sensitive fibrin plate method,29 proteolytic activity is generally assumed to underly the activation of other enzymatic processes by Hageman factor and its fragments.29

Operationally, normal inhibitors in plasma have posed significant obstacles to the biochemical dissection of fibrinolytic mechanisms. The methodology described here provides a useful approach to assay of antiplasmins and demonstrates the usefulness of chloroform extraction as a maneuver for identifying and characterizing fibrinolytic activities other than plasmin and for examining their interactions with that enzyme.

ACKNOWLEDGMENT

The authors wish to thank E. Hervas, M. Kim, and K. H. Man for their expert technical assistance.

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Mechanisms involved in enhancement of plasma fibrinolytic activity by chloroform

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