The Streptokinase–Human Plasminogen Activator Complex

COMPOSITION AND IDENTITY OF A SUBCOMPONENT WITH ACTIVATOR ACTIVITY

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Reactions between purified plasminogen and streptokinase, labelled with ^{131}I and ^{125}I respectively, were investigated by polyacrylamide-gel discontinuous electrophoresis. A molecular complex consisting of both ^{131}I -labelled plasminogen and ^{125}I -labelled streptokinase migrated between plasminogen and streptokinase. This complex contained bovine plasminogen activator activity. The relative quantities of ^{131}I -labelled plasminogen and ^{125}I -labelled streptokinase in this complex were markedly affected by reaction conditions. A fragment that retained 50% or more of the parent activator activity was released from the complex after exposure to mercaptoethanol. This subcomponent had an estimated molecular weight of 70 000, and contained both ^{131}I -labelled plasminogen and ^{125}I -labelled streptokinase.

At least two enzymic activities are demonstrable in mixtures of streptokinase and human plasminogen. Plasmin, the major fibrinolytic protein of the blood is one; activator, an enzyme that rapidly converts bovine plasminogen into plasmin, is the second. Activator is believed to be a complex of human plasminogen or plasmin and streptokinase (DeRenzo, Boggiano, Barg & Buck, 1967b; Summaria, Ling, Groskopf & Robbins, 1968; Ling, Summaria & Robbins, 1965; Hummel, Buck & DeRenzo, 1966; Muramatu, Hayakumo, Onishi, Sato & Fujii, 1969). In the present studies, plasminogen and streptokinase were purified and radioactively labelled before being made to react under controlled conditions of time, temperature and molecular ratio. The results of reaction were monitored by polyacrylamide-gel discontinuous electrophoresis, and the proteins were identified by gel staining, radioactivity and bovine plasminogen activator activity.

In confirmation of the work of others, human plasminogen and streptokinase were found to form a molecular complex with activator activity (DeRenzo *et al.* 1967b; Summaria *et al.* 1968; Ling *et al.* 1965; Hummel *et al.* 1966; Muramatu *et al.* 1969). The composition of the complex, however, varied with the reaction conditions. On exposure of the complex to mercaptoethanol one major fragment was released. This fragment (estimated mol.wt. 70 000) contained activator activity and

components of both plasminogen and streptokinase.

MATERIALS AND METHODS

Purification of plasminogen and streptokinase. Plasminogen was prepared from pseudoglobulin plasminogen (Cutter, Berkeley, Calif., U.S.A.) on CM-cellulose. A glass column (30 cm $\times$ 2.5 cm) was packed by gravity with CM-cellulose (Schleicher and Schuell Co., Keene, N.H., U.S.A.) equilibrated in 7.5 mM-sodium acetate buffer, pH 4.5. Approx. 195 mg of crude plasminogen with 10–15 Remmert & Cohen (1949) caseinolytic units/mg of protein was applied to the column and eluted with a 0.2–0.5 M-NaCl linear gradient (Fig. 1). The eluate was assayed for plasminogen activity in the fibrin clot system described below. Tubes from the plasminogen peak were combined, extensively dialysed against starting buffer, and re-chromatographed with the same gradient. Tubes from the plasminogen peak of the second chromatographic separation contained 23–32 Remmert & Cohen (1949) units/mg of protein including 5–10% of plasmin activity present before activation with streptokinase. About 15 mg of purified plasminogen was recovered. The $E_{1\text{cm}}^{1\%}$ at 280 nm of 16 for plasminogen was used to estimate the plasminogen concentration in the eluate (Robbins, Summaria, Elwyn & Barlow, 1965; Taylor & Botts, 1968). The final preparation showed a single diffuse band by discontinuous electrophoresis at pH 8.5 and a single peak on filtration through Sephadex G-100.

Streptokinase was prepared from Varidase (Lederle Laboratories, Pearl River, N.Y., U.S.A.) as described by Tomar (1968). Briefly, a 40%-satd. $(\text{NH}_4)_2\text{SO}_4$ precipitate of crude streptokinase was solubilized, dialysed and chromatographed on DEAE-cellulose (Whatman DE 52)

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Fig. 1. Gel-chromatography of plasminogen. Crude plasminogen was eluted by gravity (a) from a column (30 cm $\times$ 2.5 cm) of CM-cellulose in 7.5 mM-sodium acetate buffer, pH 4.5, with a 0.2–0.5 M-NaCl linear gradient formed from 250 ml each of 0.2 and 0.5 M-NaCl. Tubes denoted by the bar were rechromatographed (b) on the same column with the same gradient. Fractions (3 ml) were collected. Samples were adjusted to similar E_{280} and the fibrinolytic activity was estimated by the bovine clot lysis assay. —, E_{280} ; ---, reciprocal of lysis time/6 μ g of protein estimated by using $E_{1\text{cm}}^{1\%}$ at 280 nm = 16.

with an NaCl gradient. Tubes from the streptokinase peak have been shown to contain 85000–120000 units/mg of protein, one major plus one to two faint minor protein bands on discontinuous electrophoresis and a single protein peak on gel filtration (Sephadex G-100). The $E_{1\text{cm}}^{1\%}$ at 280 nm of 10.0 was used for estimating the concentration of streptokinase in the eluates (Taylor & Botts, 1968). Both plasminogen and streptokinase were stored in multiple samples at -60°C until used.

Assays for enzymic activity. Activator activity in the material subjected to electrophoresis on polyacrylamide gels was qualitatively determined by placing it directly in a bovine fibrin–agar plate. The polyacrylamide gels were run in duplicate. One gel was fixed and stained. The second was sliced every 1–2 or 2–3 mm along its horizontal axis and the slices were embedded into fibrin–agar kept at 50 – 60°C . The fibrin–agar plates consisted of 0.25% Nobel agar (Difco Laboratories, Detroit, Mich., U.S.A.), 0.0625% bovine fibrinogen (Cohn fraction I; Armour Laboratories, Chicago, Ill., U.S.A.) and 2 units of bovine thrombin (Parke, Davis, Detroit, Mich., U.S.A.) in a final volume of 10 ml. Bovine plasminogen present in significant quantities in both the fibrinogen and thrombin is converted into the fibrinolytic enzyme plasmin by the activator complex. The diameter of the lytic zone around the slices of the gels was measured after 1–24 h at room temperature (Plate 1). The area of lysis was obtained by subtracting the area of

the gel slices from the total area of lysis. The position of slices that lysed the bovine fibrin was compared with the stained bands in the intact duplicate. To assess the effect of mercaptoethanol on enzymic activity, fibrin–agar plates were also used without embedding gel slices in its matrix (Sgouris, Inman & McCall, 1960). Materials to be assayed were applied directly either to the surface of or into 20 μ l wells cut into the fibrin–agar. Plasmin activity was identified on heated fibrin–agar plates prepared by incubating the plates described above at 85°C for 15–20 min. Serial dilutions of known amounts of plasmin (NIH Standard) and activator complex (NIH Standard plasmin plus streptokinase) were used to establish a standard curve relating area of lysis to quantity of enzyme (Fig. 2).

The caseinolytic assay was modified from that of Norman (1957). Casein (Fisher, King of Prussia, Pa., U.S.A.; 1 ml of a 2% soln.) was incubated for 30 min with 1 ml of sample at 37°C . Then HClO_4 (3 ml, 1 M) was added, and the mixture was kept at 0 – 4°C for 30 min before being filtered through Whatman no. 40 filter paper; the E_{280} was then measured in a Beckman model DB spectrophotometer. Controls of buffer plus casein were also incubated for 30 min at 37°C , processed as above and the resultant extinction was subtracted from that of the experimental samples. These values were converted into units of activity from a standard curve obtained experimentally by using a plasmin standard solubilized in 50% (w/v) glycerol (Division of Biologics Standards, NIH).

The fibrin clot assay was modified from that of Christensen (1945); 0.7 ml of 0.125% bovine fibrinogen and 0.1 ml of the material to be assayed were clotted by 2 units of bovine thrombin (20 units/ml) and then observed at 37°C for complete liquefaction (Tomar, 1968). In estimating plasminogen activity, 100 units of crude streptokinase (Varidase) were added before thrombin.

Gel filtration. The molecular weight of the fragment was estimated by gel filtration (Andrews, 1965). The system was calibrated with Blue Dextran, riboflavin and 20 mg each of human immunoglobulin G, bovine albumin, ovalbumin, myoglobin and soya-bean trypsin inhibitor on a column (3.5 cm $\times$ 100 cm) of Sephadex G-200 with 50 mM-tris–0.1 M-KCl buffer, pH 7.5, with 10 mM-mercaptoethanol. The E_{280} was used to identify the protein peaks. The peak tube numbers of these proteins were plotted against their respective mol.wts. Radioactively labelled material was filtered in the same way except that the appearance of radioactivity as well as the E_{280} was used to determine the elution volume. Material used to estimate the molecular weight of the mercaptoethanol-released fragment was obtained in the following manner: radioactively labelled plasminogen and streptokinase were mixed, treated with 10 mM-mercaptoethanol and subjected to electrophoresis on 12 polyacrylamide gels. Of these gels, 11 were sliced and scanned for radioactivity as described in the Materials and Methods section. The twelfth one was fixed, stained and counted for radioactivity. The fragment was identified both by radioactivity and stain. The 11 slices of gel containing the fragment were then incubated overnight at 0 – 4°C in 5 ml of 50 mM-tris–HCl buffer–0.1 M-NaCl, pH 7.6. The eluate lysed an unheated bovine fibrin–agar plate, and migrated in the same position as the initial fragment on re-electrophoresis on polyacrylamide gel. This material was then used to estimate the molecular weight as described above.

EXPLANATION OF PLATE I

Assays for enzymic activity. Polyacrylamide gels were run in duplicate. One gel was fixed and stained. The second was sliced every 1–2 or 2–3 mm along its horizontal axis and the slices were imbedded into fibrin-agar at 50–60°C that was then cooled to room temperature. Lysis could be noted after 1–24 h incubation at room temperature. This photograph was taken after 17 h and shows bovine plasminogen activator activity (clear areas) to be maximum in slices 2 and 3 of this preparation.

Fig. 2. Calibration curve for enzyme activity. Serial dilutions of mixtures of human plasminogen and streptokinase were assayed for activity on heated (a) and unheated (b) fibrin-agar plates. The plasmin preparation was made by incubating 100 μ g of plasminogen with trace quantities of streptokinase overnight at room temperature in 50% glycerol. The activator preparation was made by incubating 100 μ g of plasminogen with 18 μ g of streptokinase in a final volume of 1 ml for 2 min at room temperature before assay. ●, Activator, ○, plasminogen or plasmin.

Radioactively labelled plasminogen and streptokinase. Purified plasminogen and streptokinase were radioactively labelled by the method of McConahey & Dixon (1966) in which 3–5 mg of plasminogen was labelled with 1.0 mCi of ^{131}I (Tracerlab, Waltham, Mass., U.S.A.) (587 mCi/ml) and 1–2 mg of streptokinase was labelled with 0.5 mCi of ^{125}I (Tracerlab) (25.2 mCi/ml). Reaction mixtures (10–12 ml) were dialysed for 48–72 h against a total of 42 litres of 1 mM-HCl (plasminogen) or 0.1 M-phosphate buffer pH 7.4 (streptokinase). No radioactivity could be detected in the final diffusate. This procedure resulted in the combination of 1 g-atom of ^{125}I /250–1200 mol of streptokinase and 1 g-atom of ^{131}I /1400–5500 mol of plasminogen.

All samples were counted in either a Packard Auto-gamma model 3002 spectrometer or a Baird Atomic Well Counter model 810C. A consistent overlap of radioactive spectra required that 24–29% of the ^{131}I radioactivity be subtracted from the ^{125}I count in the final calculation. Materials monitored by discontinuous electrophoresis were either fixed in trichloroacetic acid before staining, destaining, slicing and counting for radioactivity or alternatively sliced and then counted for radioactivity. By counting the radioactivity of the slices directly, all of the radioactivity applied to the gel was recovered. Specific radioactivity (c.p.m./ μ g of protein) was estimated by determining the radioactivity of a known amount of either plasminogen or streptokinase before and from the gel after discontinuous electrophoresis. The difference in radioactivity of duplicate gels was never greater than 20%.

Each labelled protein was compared with unlabelled material from the same preparation by several methods. First, labelled materials always migrated in the same position as the native protein and no pieces split from the radioactive plasminogen and streptokinase by the labelling procedure were detected by either staining or counting the radioactivity of the polyacrylamide-gel slices; i.e., only one peak of radioactivity was observed in each gel. When

a gel was stained and then the radioactivity counted, the peak of radioactivity always coincided with the position of the stained protein, which in turn always corresponded to the position of unlabelled material on a duplicate gel. Trace amounts of the labelled plasminogen and streptokinase were mixed with unlabelled preparations of plasminogen and streptokinase respectively. Radioactivity coincided with the fractions that contained maximum enzymic activity. No change in total activity either of streptokinase or plasminogen after labelling could be detected by the bovine fibrin clot lysis assay, although some plasminogen preparations showed an increase in plasmin accounting for up to 15% of the final activity.

Discontinuous electrophoresis. Discontinuous electrophoresis on polyacrylamide gels was done at pH 8.5 (Davis, 1964). The acrylamide concentration of the lower gel was 7%. In the basic system, both upper and lower gels contained 0.3 M- ϵ -aminocaproic acid (6-amino-hexanoic acid). Samples were run at 4°C at 4 mA/gel on a Canalco model 66 apparatus. Each gel was removed individually from an electrophoresis run when its tracking dye reached the end of the gel. The gels were stained with either Amido Schwarz or Coomassie Blue dye.

Reaction mixtures. Mixtures of human plasminogen and streptokinase in ratios ranging from 1.3/1 to 40/1 by wt. were made to react under several conditions of time and temperature before the mixtures were cooled to 4°C and ϵ -aminocaproic acid was added (final concentration 0.3 M) to inhibit the reaction. With this procedure 18 μ g of ^{125}I -labelled streptokinase was made to react with several different quantities of ^{131}I -labelled plasminogen for 2 min before addition of ϵ -aminocaproic acid. Similarly, 100 μ g of plasminogen was made to react with several different quantities of streptokinase. The effects of time and temperature on the reaction mixture were also studied by incubating 100 μ g of ^{131}I -labelled plasminogen with 18 μ g of ^{125}I -labelled streptokinase. In all these

cases the reaction mixtures were then subjected to discontinuous polyacrylamide-gel electrophoresis.

RESULTS

Streptokinase-plasminogen activator complex

A new band, migrating between streptokinase and plasminogen, was observed when mixtures of purified plasminogen and streptokinase were analysed by discontinuous electrophoresis. This band was shown to contain components of both plasminogen and streptokinase (Fig. 3).

Activator activity was determined by using unheated bovine fibrin-agar plates as described in the Materials and Methods section. Some fibrinolysis occasionally noted with the plasminogen control was presumably caused by spontaneous conversion into and/or contamination by plasmin. No fibrinolysis was noted with the purified streptokinase control. Human plasminogen that had been incubated with streptokinase in 1.3/1-6/1 weight ratios to form activator complex lysed about three times more area on the unheated plates than an equivalent amount of human plasmin (i.e. plas-

Fig. 3. Discontinuous electrophoresis of plasminogen-streptokinase mixtures. Radioactively labelled plasminogen and streptokinase were incubated for 2-5 min at 23°C before 0.3 M- ϵ -aminocaproic acid was added. The mixture was then cooled to 4°C and run in duplicate on polyacrylamide-gel discontinuous electrophoresis. At the completion of the run, one gel was stained and the second was sliced and analysed for radioactivity. (a) 100 μ g of plasminogen; (b) 18 μ g of streptokinase; (c) 50 μ g of plasminogen + 18 μ g of streptokinase; (d) 100 μ g of plasminogen + 18 μ g of streptokinase; (e) 25 μ g of plasminogen + 18 μ g of streptokinase; (f) 100 μ g of plasminogen + 9 μ g of streptokinase. The activator complex migrates between plasminogen and streptokinase and contains radioactive label from both of these proteins. As less plasminogen is used, less plasminogen and less streptokinase appear in the complex while more streptokinase migrates in the native protein (d, c, e). As less streptokinase is used, less appears in the complex (d, f). —, ¹²⁵I label of plasminogen; ---, ¹²⁵I label of streptokinase.

minogen incubated with streptokinase in a 40/1 to 100/1 ratio).

Plasminogen and streptokinase were identified and quantitated on gels by their respective radioactive labels. On the gels, plasminogen-streptokinase complexes of 2/1-22/1 by wt. (approx. 1/1-13/1 molar ratio) were demonstrated. Because of this variation of combining ratios of the components in the activator complex, the effects of various plasminogen/streptokinase ratios in the initial incubation mixture and of time and temperature were examined.

Constant streptokinase concentration, varying plasminogen concentration. The amount of plasminogen and streptokinase in the complex was a direct function of the amount of plasminogen used in the incubation mixture. With the concentration of streptokinase constant, approx. $4.6\mu\text{g}$ of plasminogen was associated with about $0.54\mu\text{g}$ of streptokinase for each $10\mu\text{g}$ of plasminogen used in

the reaction system (Fig. 4). This represents a weight ratio of 8.5/1 or an approx. ratio of 5/1.

Constant plasminogen, varying streptokinase. A direct relationship existed between the amount of streptokinase used and that associated with plasminogen. Approx. $2.4\mu\text{g}$ of streptokinase associated with plasminogen for every $10\mu\text{g}$ of streptokinase used. Although the amount of plasminogen associated with streptokinase also increased as the amount of streptokinase in the reaction mixture increased, a linear relationship was not apparent (Fig. 5).

Streptokinase in excess. As the amount of streptokinase relative to plasminogen was increased, more streptokinase was observed to migrate as native protein. Simultaneously, one to three rapidly migrating bands were visible on the polyacrylamide gels and were found to contain only the

Fig. 4. Effect of varying the plasminogen concentration. In five experiments (●, △, ○, ■) with two different preparations of streptokinase and plasminogen $18\mu\text{g}$ of streptokinase was made to react with various quantities of plasminogen. All incubations were at room temperature for 2 min then ϵ -aminocaproic acid was added before electrophoresis in the cold. (a) Amount of streptokinase incorporated into the complex as related to μg of plasminogen used ($0.45\mu\text{g}$ of streptokinase incorporated for every $10\mu\text{g}$ of plasminogen used). (b) Amount of plasminogen incorporated into complex as related to μg of plasminogen used ($4.6\mu\text{g}$ of plasminogen incorporated for every $10\mu\text{g}$ of plasminogen used).

Fig. 5. Effect of varying the streptokinase concentration. In four experiments (●, △, ○, ■) with three different preparations of streptokinase and plasminogen $100\mu\text{g}$ of plasminogen was made to react with various quantities of streptokinase. All incubations were at room temperature for 2 min, then ϵ -aminocaproic acid was added before electrophoresis in the cold. (a) Amount of streptokinase incorporated into the complex as related to μg of streptokinase used ($0.24\mu\text{g}$ of streptokinase incorporated for every $10\mu\text{g}$ of streptokinase used). (b) Amount of plasminogen incorporated into the complex as related to μg of streptokinase used. A linear relationship was not evident.

^{125}I -labelled streptokinase. These bands were thought to represent digestion products from streptokinase resulting from its cleavage by plasmin and/or activator.

Plasminogen in excess. Plasminogen was converted into plasmin with small amounts of radio-labelled streptokinase (40/1–100/1 weight ratio). This streptokinase consistently migrated with plasminogen in the same position as activator complex. With ^{131}I -labelled plasminogen and unlabelled streptokinase, one or more smaller peaks containing radioactivity from ^{131}I -labelled plasminogen were seen. These were thought to represent pieces split from plasminogen during its conversion into plasmin or from plasmin by autolysis.

Effect of time. The longer streptokinase and plasminogen were incubated before addition of ϵ -aminocaproic acid, the more plasminogen was present in the complex. At all temperatures tested, more streptokinase appeared as split products as the time of the incubation increased. This increase of split products derived from streptokinase was usually associated with the decrease in the amount of streptokinase in the complex. This was most clearly shown at 37°C . (Table 1).

Effect of temperature. Temperature variation produced no noticeable effect on the quantity of plasminogen complexed. Less incorporation of

streptokinase occurred at 37°C than at the other temperatures tested (Table 1). In general, more split products originating from streptokinase were seen at 23°C and 37°C than at 4°C . Fibrin plate assays for activator activity and casein assays for plasmin activity were done on these six samples. In spite of the appearance of split products there was little change in activator activity as a function of time. Plasmin activity showed only a slight decrease, perhaps caused by autolysis.

Formation of complex by reaction of plasmin with streptokinase. Plasmin, made by preincubating ^{131}I -labelled plasminogen with either streptokinase or urokinase (made available by courtesy of Dr Alan Johnson, New York Blood Bank), formed activator complex with radioactively labelled streptokinase. A new band migrating between plasminogen and streptokinase was visible on polyacrylamide gel electrophoresis. This new band consisted of label from both streptokinase and plasmin and was found to have activator activity when tested on unheated bovine fibrin-agar plates.

Studies on a subcomponent identified after treatment with mercaptoethanol

The activator complex was formed as previously described by incubation of $100\mu\text{g}$ of plasminogen with $18\mu\text{g}$ of streptokinase at room temperature

Table 1. *Distribution of protein after reactions between ^{131}I -labelled plasminogen and ^{125}I -labelled streptokinase*

A $100\mu\text{g}$ sample of ^{131}I -labelled plasminogen was made to react with $18\mu\text{g}$ of ^{125}I -labelled streptokinase under six conditions of time and temperature before the addition of 0.3M ϵ -aminocaproic acid. The products were subjected to discontinuous electrophoresis on polyacrylamide gel. Radioactivity and amounts of protein were determined as described in the Materials and Methods section. Radioactive label was found in four places on the gels after reaction: A, aggregated, protein remaining in the stacking gel; N, native, protein corresponding to a major band of unaltered protein; C, complexed, protein associated with another protein in a new major band on gel; SP, split products, protein corresponding to faint bands migrating ahead of either the native or complexed material. No less than 92% of the starting material was recovered. With time, the amounts of plasminogen in the complex and streptokinase as split products increased leading to a greater plasminogen/streptokinase complex ratio.

Treatment	Type of protein ... Component	Protein (μg)				Total protein (μg)	Plasminogen/ streptokinase weight ratio in complex
		A	N	C	SP		
4°C, 2 min	Plasminogen	14.0	50.0	29.0	3.0	96.0	3.6/1
	Streptokinase		14.0	8.0	2.0	24.0	
4°C, 60 min	Plasminogen	4.0	48.0	48.0	5.0	105.0	6/1
	Streptokinase		4.0	8.0	8.0	20.0	
23°C, 2 min	Plasminogen	6.0	51.0	37.0	2.0	96.0	5/1
	Streptokinase			7.2	12.0	19.2	
23°C, 60 min	Plasminogen	4.0	33.5	55.0	6.8	99.3	14/1
	Streptokinase			4.0	14.0	18.0	
37°C, 2 min	Plasminogen	6.0	50.0	35.0	7.0	98.0	7/1
	Streptokinase		2.7	5.4	9.2	17.3	
37°C, 60 min	Plasminogen	8.0	28.0	48.0	8.0	92.0	16/1
	Streptokinase		1.3	2.7	13.0	17.0	

for 5 min. The ϵ -aminocaproic acid was added, followed by mercaptoethanol to give a final concentration of 10 mM. The samples remained at room temperature for 5–15 min at pH 7.0–7.5. Under these conditions little or no activator activity was lost before electrophoresis (Fig. 6). The samples were then analysed by polyacrylamide-gel discontinuous electrophoresis in the presence of 10 mM-mercaptoethanol.

In this way, a new band was seen in the polyacrylamide gel. This band, presumably a fragment released from the reduced complex, migrated somewhat behind streptokinase and well ahead of plasminogen or complex. The mercaptoethanol-released fragment was not degraded in 10 mM-mercaptoethanol by further incubation for 72 h at 0–4°C or for 24 h at room temperature. However, when the concentration of mercaptoethanol was increased to 0.1 M the fragment was split into multiple pieces within 15 min at room temperature and all activator activity was destroyed, as determined with bovine fibrin-agar plates.

Polyacrylamide-gel slices containing the fragment were active in the direct bovine fibrin-agar assay (Fig. 7). The fragment retained much of the fibrinolytic activity (50–100%) present in the parent complex. Eluates of these slices from the gel with the mercaptoethanol-released fragment were also active on bovine fibrin-agar plates. Radioactive label from both ^{125}I -labelled strepto-

kinase and ^{131}I -labelled plasminogen was found in the fragment. In Table 2 a typical set of results is shown from an experiment in which 100 μg of plasminogen was made to react with 18 μg of streptokinase. In all experiments, the combined radioactivity of the fragment released by mercaptoethanol and remaining complex of the reduced sample was close to the amount of radioactivity in the untreated sample.

Molecular weight estimates. The molecular weight, as determined by Sephadex G-200 gel filtration, was estimated to be 70 000 (Fig. 8). After filtration the fragment again contained activator activity and migrated in the same position on polyacrylamide-gel discontinuous electrophoresis.

DISCUSSION

The present studies confirm that human plasminogen or plasmin combines with streptokinase to form an effective activator of bovine plasminogen. By using radioactively labelled purified streptokinase and plasminogen, we have been able to estimate the amount of each of these proteins in the activator complex. Our results show that the composition of the complex depends on at least four factors, i.e. the time and temperature of the reaction, and the quantities of streptokinase and plasminogen in the mixture. Complexes with plasminogen/streptokinase ratios of 2/1 to 22/1 by weight (approx. 1/1 to 13/1 molar ratio) could be formed by altering these factors (Table 1).

Evidence for an equimolar complex is threefold. Activator activity has been noted to be maximum when the plasminogen/streptokinase molar ratio approaches unity (Kline & Fishman, 1961). Assays for comparative activity of the complexes were not done in the present investigation. In studies with an analytical ultracentrifuge, plasminogen and streptokinase were found to form a complex in ϵ -aminocaproic acid with a sedimentation velocity of 5.0–5.2 S and an estimated molecular weight of 150 000; that is, about the same values as those expected for an equimolar complex. However, the investigators who used this method to demonstrate complex-formation noted that when lysine was used instead of ϵ -aminocaproic acid, the sedimentation velocities and molecular weights varied from 5 to 11 S and from 138 000 to 308 000 respectively (Davies, Englert & DeRenzo, 1964; Ling, Summari & Robbins, 1967). Davies and his colleagues also noted that the increase in the sedimentation velocity of the complex was concentration-dependent (Davies *et al.* 1964).

On starch-gel electrophoresis a band migrating between streptokinase and plasminogen could be seen with mixtures of these materials (DeRenzo *et al.* 1967b; Summari *et al.* 1968). When

Fig. 6. Effect of mercaptoethanol on complex. Equal quantities of a human plasminogen-streptokinase complex and the same human plasminogen-streptokinase complex after treatment with 10 mM-mercaptoethanol at pH 7.0–7.5 were serially diluted and assayed for activator activity on unheated bovine fibrin-agar plates. ●, Activator; Δ, mercaptoethanol-treated complex.

Fig. 7. Polyacrylamide-gel electrophoresis of the fragment. Areas of lysis in bovine fibrin-agar plates were plotted against the polyacrylamide gels to relate activator activity to protein bands. (a) Control plasminogen (100 µg) with some activity caused either by contamination with or spontaneous conversion into plasmin; (b) control streptokinase (18 µg) with no activity; (c) complex (100 µg of plasminogen and 18 µg of streptokinase) with activator activity correlating well with appearance of band in gel; (d) fragment (100 µg of plasminogen and 18 µg of streptokinase treated with 10 mM-mercaptoethanol) with activity present in bands of both fragment and residual complex.

streptokinase was used in greater than equimolar quantities with plasminogen, uncomplexed streptokinase could be seen. When plasminogen was used in greater than equimolar quantities, excess of plasminogen could not be identified (DeRenzo *et al.* 1967b). In the present investigation, plasminogen and streptokinase were identified in two ways: by their position on polyacrylamide gels and by their specific radioactive label. With labelled components, semiquantitative analysis of the complex and the split products arising from the reaction mixture were possible. The present results also suggest that DeRenzo and his colleagues were not able to identify plasminogen because it was primarily complexed with streptokinase.

Time and temperature studies showed that the plasmin-streptokinase system has some similarities to an enzyme-substrate system in which streptokinase serves as substrate for plasmin and/or activator. The complex may be comparable with an

enzyme-substrate intermediate, which is thought to occur, at least transiently, in most proteolytic reactions. An increase in the amount of streptokinase-split products appeared simultaneously with a decrease in streptokinase in the complex, suggesting that at least some of these digested pieces originated from the complex. At 4°C, the streptokinase contribution to the complex appeared to be stabilized and the complex itself was more readily apparent than at 37°C. This is in some ways similar to the results of Tsao & Kline (1969), who found less activator activity when they increased the temperature of a mixture of plasminogen and streptokinase from 22° to 37°C. Thus, it seems that streptokinase rapidly combines with and activates plasminogen but then itself acts as a substrate for either plasmin and/or activator. The second step, at least, would then appear to be dependent upon time and temperature. Therefore streptokinase might be an example of an activator substrate

Table 2. *Distribution of radioactivity of ^{131}I -labelled plasminogen and ^{125}I -labelled streptokinase after treatment with mercaptoethanol*

A duplicate set of samples containing 100 μg of ^{131}I -labelled plasminogen and 18 μg of ^{125}I -labelled streptokinase were kept for 5 min at 23°C. One sample was then treated with 10 mM-mercaptoethanol for 15 min at 23°C. Both samples were subjected to electrophoresis on polyacrylamide gel and the radioactivity was determined as previously described. A new major band was identified after treatment with mercaptoethanol. This band contained both ^{131}I -labelled plasminogen and ^{125}I -labelled streptokinase. Abbreviations are as for Table 1; F, protein fragment.

Treatment	Component	Protein (μg)					Total protein (μg)
	Type of protein ...	A	N	C	SP	F	
No mercaptoethanol	Plasminogen	5	56	33	9	—	103
	Streptokinase	—	4	5	7.5	—	16.5
Plus mercaptoethanol	Plasminogen	—	44	11	14	36	105
	Streptokinase	—	4	2	3	9	18

Fig. 8. Estimation of molecular weight of fragment. A 20 mg sample of each marker protein was filtered through a column (3.5 cm $\times$ 100 cm) of Sephadex G-200 with 0.5 M-tris-0.1 M-KCl-10 mM-mercaptoethanol buffer, pH 7.5. The flow rate was 20 ml/h, and 3 ml fractions were collected. The position of the fragment was determined by measurement of radioactivity and E_{280} , as well as activator activity. The marker proteins were γ -globulin (A), bovine albumin (C), ovalbumin (D), soybean trypsin inhibitor (E) and myoglobin (F). The fragment was eluted at B.

(Dixon & Webb, 1964). At all temperatures, as the time of reaction was prolonged more plasminogen was incorporated into the complex. Thus, as the duration of the incubation was increased, more plasminogen was associated with less streptokinase in an increasingly plasminogen-rich complex.

A fragment released from the plasminogen-streptokinase complex by treatment with mercaptoethanol contained 50–100% of the activator activity present in the original complex. Components of both ^{125}I -labelled streptokinase and ^{131}I -labelled plasminogen were identified in this complex, the molecular weight of which was estimated to be 70 000.

Since streptokinase has no disulphide bonds (Taylor & Botts, 1968; DeRenzo, Siteri, Hutchings & Bell, 1967a), the break in the complex to form the fragment should have occurred at one or more of the 22 disulphide bonds of plasminogen (Robbins *et al.* 1965).

Direct determination by radioactivity would suggest that five-sixths of the complex is plasminogen, and one-sixth is streptokinase. However, interpretation may be inaccurate because the molecules may not be uniformly labelled. Thus the specific radioactive label of that portion of the molecule found in the fragment is not known.

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