Direct Activation of Human Plasminogen by Streptokinase* (33645)

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The mechanism of activation of human plasminogen by streptokinase has been shown to be due to the cleavage of a single arginyl–valine bond (1, 2). Others suggested that this activation mechanism may be an indirect one involving first the formation of an activator complex, an equimolar complex of human plasmin and streptokinase (3–5). The direct activation involving the cleavage of a peptide bond did not appear to be possible since a synthetic peptide substrate has not been found for streptokinase, whereas all other well-defined activators of human plasminogen, e.g., urokinase, trypsin, and pig-heart activator, have the ability to cleave peptide bonds (6–8). To show whether the direct, indirect, or both mechanisms are operable, would require the removal or inhibition of the plasmin contaminant from the human plasminogen preparation by either physical or chemical methods.

Previous work from our laboratory showed that streptokinase can activate a human plasminogen preparation in which all plasmin activity had been effectively inhibited by prior treatment with DFP (9). Experiments will be described which show that streptokinase can activate human plasminogen directly, rather than through a preexistent active equimolar human plasmin–streptokinase complex.

Materials and Methods. Human plasminogen was prepared from Fraction $III_{2,3}$ by previously described methods (10). Human plasmin was prepared by activating human plasminogen with either streptokinase or urokinase in 25% glycerol (2, 10). The specific activities of the plasminogen and plasmin preparations used in this study were 24–28 casein units/mg of protein (11). Bovine plasminogen was obtained from Parke-Davis and Company. The specific proteolytic activity was 0.4 casein units/mg of solids. A commercial preparation of partially purified streptokinase (Varidase, Lederle Laboratories) containing approximately 5000 units/mg of solids was used.

Human plasminogen was incubated in 10^{-2} *M* diisopropyl phosphorofluoridate (DFP) (Aldrich Chemical Company) for 30 min at 0°, at pH 9.0 (9). Under these conditions, the plasmin contaminant in the plasminogen preparation was completely inhibited (40-fold excess of enzyme assayed). An equimolar human plasmin–streptokinase complex (12) (bovine plasminogen activator) was also incubated in 10^{-2} *M* DFP as described above. Complete inhibition of both proteolytic and bovine plasminogen activator activities was observed (100-fold excess of enzyme assayed) (9). Proteolytic and bovine plasminogen activator activities were determined by procedures previously described (13).

Horizontal starch gel electrophoresis was carried out in the Pherograph (Hormuth) in 8 *M* urea–0.017 *M* sodium formate–0.05 *M* 2-mercaptoethanol, pH 3.2, as previously described (1). Electrophoresis of human plasminogen and plasmin was performed on cellulose acetate membranes at 50 V/cm at room temperature for 30–50 min using the Beckman Microzone system. Protein samples were dissolved in a 0.075 *M* sodium barbital–0.01 *M* lysine buffer at pH 8.6 After electrophoresis, the membranes were stained with 0.2% Ponceau-S fixative dye solution (Beckman). The stained membranes were then rinsed in 5% acetic acid, cleared in a 78% ethanol–22% acetic acid solution, and dried for 15 min at 100°. The dried membranes were scanned with the Beckman model RB Analytrol.

The carboxyl-terminal amino acid residues of the *S*-carboxymethyl derivatives of human plasminogen and plasmin were determined

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from the measurement of the amino acids released by treatment of each preparation with DFP-treated bovine carboxypeptidases A and B (Worthington) at pH 7.0, as previously described (1).

Results. Activiation of DFP-treated plasminogen by either streptokinase or a DFP-treated equimolar human plasmin–streptokinase complex in 25% glycerol, in the presence of DFP. Two solutions, each containing 20 mg of the DFP-treated plasminogen preparation in 2.0 ml of 0.05 *M* Tris–0.02 *M* lysine buffer, pH 9.0, were adjusted to a final concentration of 25% glycerol using 99.5% synthetic glycerol (Shell). Each solution was then adjusted with a 1.0 *M* DFP solution to a final concentration of 10^{-2} *M* DFP. To one solution was added 2500 units of streptokinase to give a molar ratio of plasminogen to streptokinase of 415; to the second solution was added 0.3 mg of the DFP-treated equimolar human plasmin–streptokinase complex containing approximately 10,000 units of streptokinase. In the latter mixture, the molar ratio of plasminogen to the streptokinase moiety of the human plasmin–streptokinase complex was 104. To determine the degree of spontaneous activation of plasminogen, a non-DFP-treated plasminogen control preparation in 0.05 *M* Tris–0.02 *M* lysine buffer, pH 9.0, in 25% glycerol was used. To determine enzyme stability, a plasmin control was prepared by activating a non-DFP-treated plasminogen preparation in the 25% glycerol system described above, using a molar ratio of plasminogen to streptokinase of 1360.

All four activation mixtures were then incubated at 25° for a period of 7 days. The proteolytic activity of each mixture was determined every 24 hr, with and without the addition of 500 units of streptokinase/assay tube. The assays carried out without the additional streptokinase on the two activation mixtures containing 10^{-2} *M* DFP showed no proteolytic activity throughout the 7-day period; however, when assayed with the additional streptokinase, both samples showed proteolytic activity. Since the sample was diluted 400-fold during the assay, the DFP

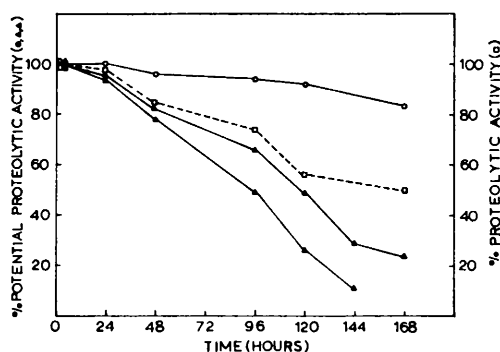


FIG. 1. Rate of activation of human plasminogen in 10^{-2} *M* DFP (see text for details): (○), non-DFP-treated plasminogen control; (□), non-DFP-treated plasmin (streptokinase-activated) control; (△), DFP-treated plasminogen activated with streptokinase in 10^{-2} *M* DFP; and (▲), DFP-treated plasminogen activated with a DFP-treated equimolar human plasmin–streptokinase complex in 10^{-2} *M* DFP.

concentration was reduced to 2.5×10^{-5} *M* where it is noninhibitory. The addition of streptokinase thus provides a means for measuring the plasminogen content of these two mixtures during the activation period. A comparison of the relative decrease with time of the potential plasmin activity (actual plasminogen content) of these two mixtures with the potential plasmin activity of the plasminogen control and the actual plasmin activity of the plasmin control is shown in Fig. 1. A gradual decrease in proteolytic activity occurred in all four activation mixtures; however, 90% of the plasminogen content of the mixture containing the DFP-treated human plasmin–streptokinase complex had disappeared after a period of 144 hr, whereas the plasminogen control during the same period showed only 15% loss in activity. After a period of 168 hr, the mixture containing the DFP-treated plasminogen preparation plus streptokinase lost 75% of its plasminogen content while, for the same period, the plasminogen control lost only 17% of its potential plasmin activity. These differences indicated that plasminogen was converted to plasmin in both activation mixtures containing 10^{-2} *M* DFP. The plasmin produced was subsequently inhibited by the DFP present in the mixture. No plasmin activity could be

detected in either mixture throughout the incubation period.

Spontaneous activation in the plasminogen control, under the conditions described, proceeded to the extent of only 17% over a period of 7 days. Thus, the 75–90% decrease in the plasminogen content in the two activation mixtures containing 10^{-2} *M* DFP was not due to spontaneous plasminogen activation. Plasminogen activation in these two mixtures thus appeared to be due directly to the streptokinase or the streptokinase moiety of the DFP-treated plasmin–streptokinase complex.

Activation of TLCK-treated plasminogen and SBTI-treated plasminogen with streptokinase. Plasminogen and urokinase-activated plasmin were reacted with 10^{-2} *M* 1-chloro-3-tosylamido-7-amino-2-heptanone (TLCK) in 0.067 *M* phosphate buffer–25% glycerol, pH 7.4 for 1 hr at 25°. Complete inhibition of all plasmin proteolytic activity occurred after 30 min in both preparations (14). The TLCK-treated plasminogen was then activated by streptokinase, using a molar ratio of plasminogen to streptokinase of 1360. Proteolytic activity was measured in the same way as were the DFP-treated activation mixtures since, during the assay, the TLCK was also diluted to a concentration of 2.5×10^{-5} *M* where it was noninhibitory. Plasminogen and TLCK-treated plasminogen were completely activated in 24 hr.

Plasminogen was also incubated with soybean trypsin inhibitor (SBTI) (Pentex) at various molar ratios in 0.02 *M* Tris–0.01 *M* lysine–25% glycerol, pH 8.0, at 25° for 30 min. A molar ratio of SBTI to plasminogen, of 0.5, was sufficient to completely inhibit all plasmin proteolytic activity in the plasminogen preparation. With a ratio of 0.5, it was possible to determine the development of plasmin activity since this amount of SBTI only inhibited approximately 35% of the potential plasmin caseinolytic activity. Ratios greater than 3.0 prevented measurement of caseinolytic activity (15). Plasminogen containing SBTI, with molar ratios of 0.5 (SBTI:plasminogen), were then activated using a molar ratio of plasminogen to streptokinase of 1360. Plasminogen and SBTI-treated plasminogen were completely activated in 24 hr.

Methods used to distinguish human plasmin from human plasminogen. Plasmin formation in the two DFP-containing activation mixtures was determined by: (a) starch gel electrophoresis, (b) cellulose acetate electrophoresis, and (c) carboxyl-terminal amino acid analyses.

(a) Starch gel electrophoresis: Human plasminogen monomer was found to be a single polypeptide chain, whereas human plasmin monomer contains two polypeptide chains connected by a single disulfide bond (1). Starch gel electrophoretic analysis of each reduced activation mixture showed that plasmin was present in each of these mixtures. The electrophoretic behavior of the plasminogen and plasmin controls, the DFP-treated plasminogen preparation activated with streptokinase in 10^{-2} *M* DFP, and the DFP-treated plasminogen preparation activated with the DFP-treated equimolar human plasmin–streptokinase complex in 10^{-2} *M* DFP, in starch gel–8 *M* urea at pH 3.2, after reduction with 1.0 *M* 2-mercaptoethanol for 18 hr, at pH 3.2, at 25°, is shown in Fig. 2. Both DFP-containing activation mixtures consist primarily of the two plasmin chains with some residual plasminogen, with more plasminogen being evident in the mixture activated with streptokinase than with the DFP-treated equimolar plasmin–streptokinase complex. These data confirmed the data obtained by the proteolytic assay.

(b) Cellulose acetate electrophoresis: Human plasminogen can be distinguished from human plasmin by differences in their electrophoretic mobilities on cellulose acetate at pH 8.6. The electrophoretic behavior of plasminogen, urokinase-activated plasmin and streptokinase-activated plasmin is shown in Fig. 3A. The relative mobilities of the three preparations are shown in Fig. 3B. The mobilities of the two plasmin preparations are identical, but the mobility of plasminogen is distinctly different from that of plasmin. The electrophoretic behavior of human plasminogen, urokinase-activated plasmin, the in-

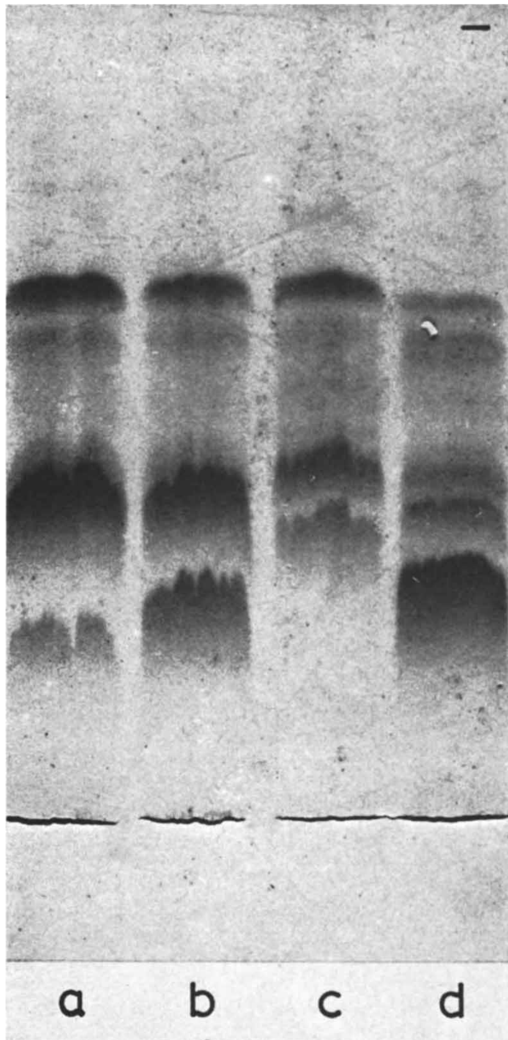
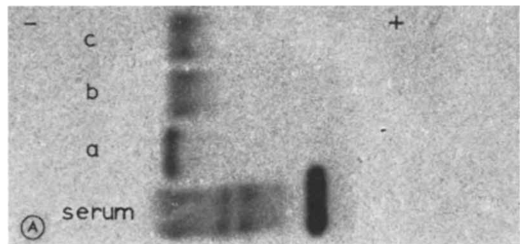


FIG. 2. Horizontal starch gel electrophoretograms of four human plasminogen activation mixtures in 8 *M* urea–0.05 *M* 2-mercaptoethanol–0.017 *M* sodium formate buffer, pH 3.2, after reduction of each preparation with 1.0 *M* 2-mercaptoethanol (see text for details): (a) DFP-treated plasminogen activated with a DFP-treated equimolar human plasmin–streptokinase complex in 10^{-2} *M* DFP; (b) DFP-treated plasminogen activated with streptokinase in 10^{-2} *M* DFP; (c) non-DFP-treated plasmin (streptokinase-activated) control; and (d) non-DFP-treated plasminogen control.

cubated non-DFP-treated plasminogen and plasmin controls, and the DFP-treated plasminogen preparation activated with streptokinase in 10^{-2} *M* DFP is shown in Fig. 4A. The relative mobilities of all five preparations are shown in Fig. 4B. It is apparent from Figs. 3 and 4 that the incubated non-DFP-treated plasminogen control consists of two components, plasminogen and plasmin. The incubated non-DFP-treated plasmin control appears quite skewed, perhaps indicating autolysis. Its mobility is quite different from that of urokinase-activated plasmin which was not incubated. The mobility of the DFP-treated plasminogen preparation activated with streptokinase in 10^{-2} *M* DFP is more similar to the urokinase-activated plasmin control rather than to the incubated non-DFP-treated plasmin control. This was also evident from the starch gel electrophoretic analyses; less autolysis probably occurred due to the rapid inhibition by DFP as the plasmin formed.



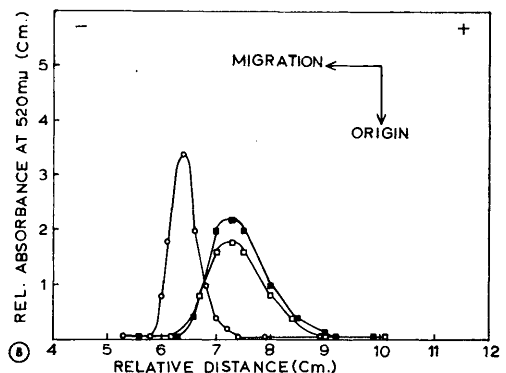


FIG. 3A. Cellulose acetate electrophoresis at pH 8.6 of human plasminogen, urokinase-activated plasmin, and streptokinase-activated plasmin. Electrophoresis was carried out at 50 V/cm for 50 min: (a) human plasminogen; (b) urokinase-activated plasmin; and (c) streptokinase-activated plasmin. (B) The relative mobilities of the samples in (A) obtained by scanning the membrane with the Beckman RB Analytrol: (○), human plasminogen; (■), urokinase-activated plasmin; and (□), streptokinase-activated plasmin.

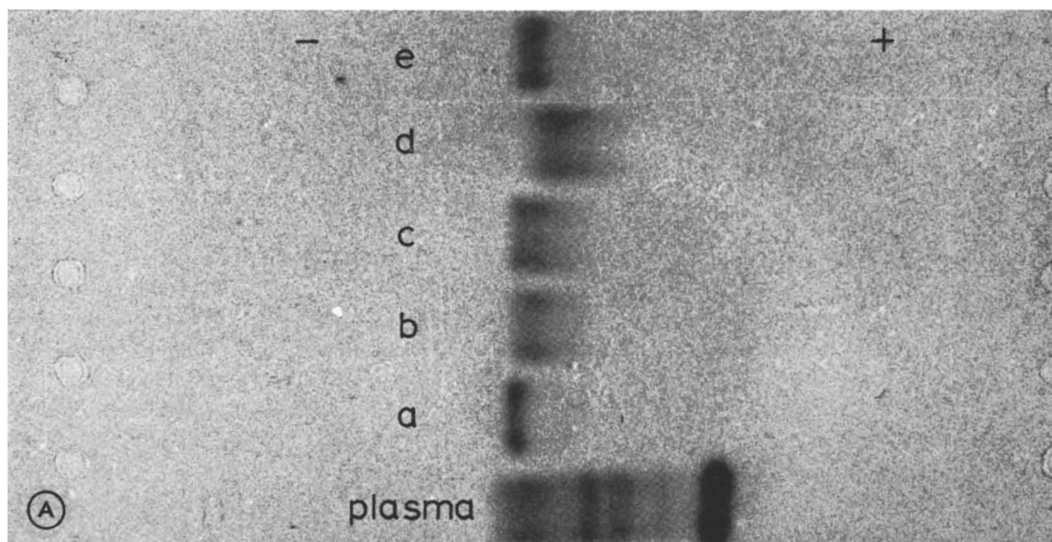
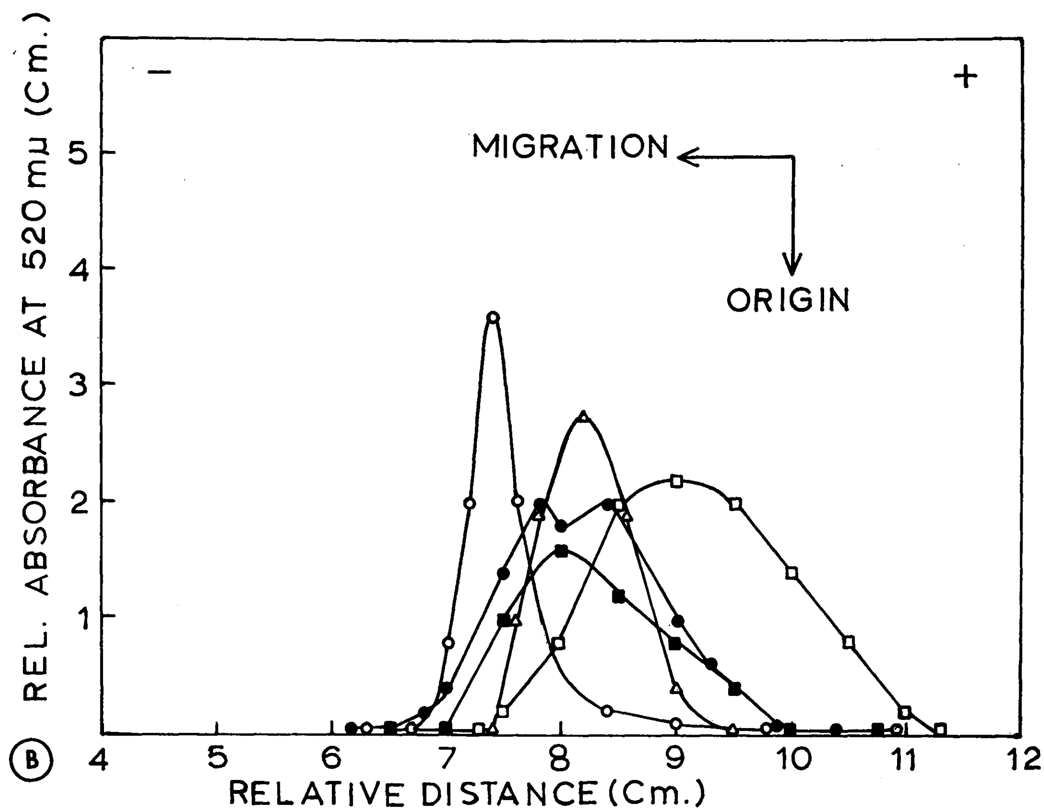


FIG. 4A. Cellulose acetate electrophoresis at pH 8.6 of: (a) human plasminogen, (b) urokinase-activated plasmin; (c) incubated non-DFP-treated plasminogen control; (d) incubated non-DFP-treated plasmin (streptokinase-activated) control; and (e) DFP-treated plasminogen activated with streptokinase in 10^{-2} *M* DFP. Electrophoresis was carried out at 50 V/cm for 30 min. (B) The relative mobilities of the samples in (A) obtained by scanning the membrane with the Beckman RB Analytrol: (○), human plasminogen; (■), urokinase-activated plasmin; (●), non-DFP-treated plasminogen control; (□), non-DFP-treated plasmin (streptokinase-activated control); and (△), DFP-treated plasminogen activated with streptokinase in 10^{-2} *M* DFP.



(c) Carboxyl-terminal amino acid analyses: Carboxypeptidases A and B treatment of the *S*-carboxymethyl derivatives of the DFP-treated plasminogen preparation activated with streptokinase and the DFP-treated plasminogen preparation activated with the DFP-treated human plasmin–streptokinase complex, in 10^{-2} *M* DFP, showed that these preparations contained in addition to one residue of asparagine, 0.61 and 0.79 residues of arginine, respectively. These values correspond fairly well with the estimation of plasmin formation (75 and 90%) obtained by the proteolytic assay.

Discussion. A controversial point that has not been satisfactorily resolved concerning the activation of human plasminogen by streptokinase is whether streptokinase can convert plasminogen to plasmin directly or whether a human plasmin–streptokinase complex must first form, which then activates plasminogen. Both mechanisms appear to be possible (16, 17). The activation of plasminogen by a human plasmin–streptokinase complex was suggested to be a two-step first order kinetic reaction dependent upon the streptokinase concentration (4, 18). In these studies we have shown that the plasmin contaminant in our plasminogen preparations is not essential for streptokinase activation of the proenzyme; thus streptokinase acts directly on human plasminogen. However in the absence of DFP, the predominant mechanism of activation is through the human plasmin–streptokinase complex. Contaminating plasmin activity in the plasminogen preparation was completely inhibited by prior treatment of the plasminogen with either DFP, TLCK, or SBTI. We had previously shown that both DFP and TLCK react stoichiometrically with the serine and histidine residues, respectively, in the active center of plasmin (14). Activation was then carried out using very low molar ratios of streptokinase to plasminogen. Plasminogen treated with TLCK or SBTI activated as quickly as untreated plasminogen; plasminogen treated with 10^{-2} *M* DFP, then dialyzed free of the DFP, also activated as quickly as untreated plasminogen. However, plasminogen treated with DFP and then activated in 10^{-2} *M* DFP activated at a much slower rate. Even with a ratio of streptokinase to plasminogen three times as great as that in a control activation mixture, only 75% of the plasminogen was converted to plasmin after a period of 7 days in 10^{-2} *M* DFP, whereas complete activation occurred in 24 hr in the absence of DFP. Activation occurred, but was also considerably slower when DFP-treated plasminogen was activated in 10^{-2} *M* DFP with a DFP-treated equimolar plasmin–streptokinase complex. Plasmin formation in these systems was determined by proteolytic activity, starch gel and cellulose acetate electrophoresis, and carboxyl-terminal amino acid analyses.

In the absence of inhibitors, plasminogen mixed with streptokinase may be activated by either streptokinase itself, the plasmin contaminant in the plasminogen, the plasmin–streptokinase complex, the streptokinase moiety of the complex, the plasmin moiety of the complex, or the dissociated products of the complex. Treatment of plasminogen with SBTI inhibited the plasmin contaminant (15), thus eliminating the activation effect due to that component; however, it did not prevent formation of the plasminogen–streptokinase complex nor subsequent events, and consequently, did not noticeably affect the rate of plasminogen activation. TLCK and DFP also effectively inhibited the contaminating plasmin; however, due to the instability of TLCK (19), no subsequent products are inhibited and the treated plasminogen was completely activated. However, DFP at a concentration of 10^{-2} *M* in the glycerol–buffer used in these studies, was shown to inhibit the proteolytic activity of the plasmin that formed, but did not prevent plasminogen activation. Since 10^{-2} *M* DFP would inhibit any potential plasmin–streptokinase complex that may form, the rate of plasminogen activation was considerably decreased because activation occurred primarily through the direct action of streptokinase on plasminogen. Activation of plasminogen by the DFP-treated equimolar plasmin–streptokinase complex and by streptokinase was near-

ly complete in 7 days in 10^{-2} *M* DFP. It appears that activation of plasminogen by the complex was probably due to the streptokinase moiety. It has been postulated that the human plasmin–streptokinase complex is in equilibrium with plasmin and streptokinase, and that dissociation of the complex can occur at 37° in the presence of low levels of streptokinase, whereas high levels of streptokinase stabilize the complex (20). The exact mechanism of human plasminogen activation by streptokinase still remains obscure; however, this study indicates that, although the human plasmin–streptokinase complex activates human plasminogen, it is not essential, and that the activation of human plasminogen by streptokinase will occur in the absence of any preexistent plasmin.

Summary. Human plasminogen was treated with either DFP, TLCK, or SBTI under conditions which completely inhibited the plasmin contaminant in our preparation. Each treated plasminogen preparation could be completely activated with low molar ratios of streptokinase to plasminogen in 24 hr, in 25% glycerol. Activation of DFP-treated plasminogen with either streptokinase or a DFP-treated equimolar human plasmin–streptokinase complex could also be carried out in 10^{-2} *M* DFP, in 25% glycerol; however, much longer periods of time were required to obtain nearly complete activation. Under these conditions for activation, the plasmin contaminant in our human plasminogen preparation is therefore not essential for streptokinase-activation of the proenzyme. These data indicate that streptokinase, alone or in the complex, acts directly on human plasminogen. A new electrophoretic method for differentiating human plasminogen from human plasmin is described.

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