Mechanism of Activation of Human Plasminogen by Streptokinase

PRESENCE OF ACTIVE CENTER IN STREPTOKINASE-PLASMINOGEN COMPLEX*

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SUMMARY

The role of proactivators for the streptokinase activation of human plasminogen was studied. It was found that plasmin, as well as plasminogen, can form activator complex with streptokinase. The relative importance of these two proactivators for the streptokinase activation of human plasminogen is discussed.

The use of the active center-specific reagent, p-nitrophenyl-p-guanidinobenzoate (NPGB) established that an active center appears in the streptokinase-plasminogen complex even before proteolytic activity develops, i.e., before conversion to streptokinase-plasmin. When, in the presence of NPGB, plasminogen is mixed with increasing amounts of streptokinase, equivalent amounts of unactivatable plasminogen are obtained. Physical evidence indicates that the active center develops in the complex before the plasminogen moiety was converted to plasmin, i.e., before cleavage of the single peptide chain of plasminogen. Activator activity, like NPGB-reactivity, is imparted to the complex before proteolytic activity is acquired. It appears that the transitory streptokinase-plasminogen complex plays a dominant role in the streptokinase activation of human plasminogen, as its formation does not depend on the presence of spontaneous plasmin.

The mechanism of activation of the plasma zymogen, plasminogen, by the bacterial protein, streptokinase, has been the subject of numerous investigations. It is well established that the activator of bovine plasminogen is a stoichiometric complex of streptokinase and human plasmin (3-7). Human plasminogen, however, is activated by the addition of streptokinase alone. The streptokinase-induced activation of plasminogen occurs via the hydrolytic cleavage of an arginyl-valyl bond (8); yet, streptokinase itself has no demonstrable activity toward synthetic esters of arginine or lysine (9). These considerations led to the assumption that the activator of human plasminogen must also be the streptokinase-plasmin complex. Blatt et al. in 1964 (10) have demonstrated that a 1:1 molar complex of the two components was indeed an activator for plasminogen of both species. Recently, however, Summaria et al. (11) and Kline and Ts'ao (12), using plasminogen preparations devoid of spontaneous plasmin or containing spontaneous plasmin inhibited with diisopropylphosphorofluoridate, demonstrated activation of human plasminogen by streptokinase, and concluded that streptokinase can activate human plasminogen directly. While we agree that streptokinase can activate human plasminogen in the absence of spontaneous plasmin, evidence presented in this paper shows that (a) streptokinase does not activate human plasminogen by a direct catalytic process; (b) streptokinase-plasminogen complex is the predominant activator species; and (c) spontaneous plasmin, when present, can also serve as a proactivator.

EXPERIMENTAL PROCEDURE

Highly purified preparations of human plasminogen were made by affinity chromatography of human plasma, according to the method of Deutsch and Mertz (13) with minor modifications, as follows: Sepharose 6B (Pharmacia) rather than 4B was used as the matrix. A volume of 500 ml of 2-fold diluted outdated human plasma was passed through a column (2.5 × 20) of coupled Sepharose at room temperature. Adsorbed protein was eluted with 0.2 M L-aminocaproic acid. The eluate was dialyzed in the cold against 0.005 M phosphate buffer, pH 7.4, and the dialysate clarified by centrifugation. The supernatant was freeze-dried and the residue dissolved in 0.05 M Tris-HCl, 0.02 M lysine, 0.1 M NaCl buffer, pH 9.0. A small amount of inactive insoluble material was removed by centrifugation. The preparation obtained exhibited a single, symmetrical boundary in the ultracentrifuge. In acrylamide gel electrophoresis multiple bands were observed also by Deutsch and Mertz (13) and by Brockway and Castellino (14). These authors, as well as Summaria and Robbins (15), using isoelectric focusing, have shown that all bands are activatable, and concluded that plasminogen exhibits charge heterogeneity. The preparation, when examined by gel electrophoresis in sodium dodecyl sulfate and β-mercaptoethanol (16), however, showed a single band, indicating homogeneity with respect to size. Furthermore upon activation with streptokinase there is an appearance of heavy and light chains (17) of plasmin, and complete disappearance of the original plasminogen band as shown by gel electrophoresis under the above conditions. These

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RESULTS

Human Plasmin, as a Proactivator for Streptokinase Activation of Human Plasminogen

Increasing Streptokinase or Increasing Streptokinase-Plasmin as Activators—It has been shown earlier that if streptokinase alone is added in increasing amounts to human plasminogen, the amount of plasmin formed in 15 sec reaches a plateau at a certain streptokinase concentration (26). At this plateau only a fraction of the plasminogen present is converted to plasmin in this time interval. Whatever the mechanism responsible for the limitation, it should be overcome, if instead of transferring streptokinase alone to a pool of plasminogen, mixtures of streptokinase and plasminogen were transferred after incubation for 20 min, since such mixtures contain fully formed activator. Under these conditions no limitation in the rate should be observed, and in fact, the activation rate should increase until all plasminogen present is converted to plasmin during the selected 15 sec activation period. (This is the behavior observed, e.g. in the case of activation by urokinase, an enzyme which does not require a cofactor for activating plasminogen.)

Streptokinase was incubated for 20 min with plasminogen in a 0.9:1 molar ratio of streptokinase to plasminogen (50,000 units per mg of plasminogen), and increasing volumes were transferred as activators from this solution to a second solution of plasminogen. The rate of activation in this solution was measured by the amount of plasmin activity developed in 15 sec after activator transfer. In the control experiment the same amounts of streptokinase alone were used as activator. Fig. 1 shows that streptokinase-plasmin is a much more effective activator than streptokinase alone. Maximum activation rate, corresponding to complete conversion of all plasminogen present to plasmin, was achieved in 15 sec when 7,000 units of streptokinase-plasmin complex were added as activator. The same units of streptokinase alone, however, yielded only 56% of complete activity.

Despite considerable evidence (27, 28) that the active center of the plasmin moiety in the streptokinase-plasmin complex is responsible for activator activity, it has been suggested recently (11, 12) that the activator activity may originate in the strepto-
Figure 1. Activation rates of plasminogen following transfer of increasing amounts of streptokinase (SK) alone (Curve a), and of fully activated streptokinase-plasmin complex, containing the same amount of streptokinase (Curve b). Activation rates (ordinate) were measured by the amount of plasmin developed in 15 sec following addition of activator. The plasminogen solution to be activated contained 0.215 mg of protein. The abscissa indicates units of streptokinase transferred per mg of plasminogen contained in the solution to be activated. The plasmin activities transferred with the activator were measured in a control experiment and were subtracted from the plasmin activities obtained; only the plasmin activities arising from the transfer of activator are shown.

Figure 2. PTI inhibition of the azocaseinolytic and activator activities of a stoichiometric streptokinase-plasmin complex, containing 0.05 mg of plasmin protein and 600 units of streptokinase.

Figure 3. Time course of development of azocaseinolytic (Curve a) and activator activities (Curve b) after transfer of streptokinase-plasmin-PTI to plasminogen. For experimental details see text. The ternary complex was prepared by mixing equivalent amounts of streptokinase and plasminogen and a 1.5-fold excess of PTI. Activities are expressed as percentage of those obtained in the absence of added PTI.

The activation of plasminogen by streptokinase is a complex process involving multiple steps, including binding, activation, and catalysis. The availability of PTI, known to combine stoichiometrically with plasmin, permitted us to obtain additional evidence for the role of the plasmin active center in activator activity. In the following experiment a stoichiometric complex of streptokinase and plasmin was titrated with increasing amounts of PTI, and aliquots from the reaction mixture were assayed for proteolytic activity, using azocasein as the substrate, and for activator activity by Lassen's bovine clot lysis method (25). The results shown on Fig. 2 indicate that the two activities are inhibited to the same extent by PTI. Extrapolation of the linear segment cuts the abscissa at 17.4 μmoles of plasmin used. This experiment identifies the active center of the plasmin moiety as the catalytic site essential for the activation of bovine plasminogen.

That the same site is operative also in the activation of human plasminogen was shown by the following experiment. A stoichiometric complex of streptokinase and plasmin was completely inhibited with PTI, and was added to a pool of human plasminogen. At intervals, samples withdrawn from this mixture were analyzed for plasmin development by azocaseinolysis. Curve a in Fig. 3 shows that during the first 20 min following the transfer of the inhibited activator no plasmin activity can be detected. The same amounts of uninhibited complex (not shown) activated 85% of the plasminogen within 5 min. Thus, blocking of the plasmin active site produced inhibition of activation in the human system also. Curve a also shows that after the inactive period the system recovers and is nearly completely activated in 3 hours. It is logical to assume that the increasing rate of plasmin formation seen in Curve a is the result of the liberation of activator from the inhibited complex. To test this hypothesis we again used the bovine clot system which detects the presence of the streptokinase-plasmin activator. Aliquots from the activation mixture were incorporated into clots at intervals and their activator activities determined. Curve b on Fig. 3 shows that following a lag period of about 20 min, activator activity becomes measurable and keeps increasing throughout the rest of the experiment. Thus, the rise in azo-

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5 The decrease in activator activity in this experiment was not due to the displacement of streptokinase by PTI from plasmin, but to the formation of the ternary streptokinase-plasmin-PTI complex which was determined experimentally by titration of the binary complexes with the third component, and will be the subject of a future communication.
Inhibition of bovine plasminogen activator activity of streptokinase-
known concentrations of streptokinase-plasmin as activator.

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plasmin activator, even under conditions of blocking of the
assuming the presence of a trace amount of streptokinase-
trace amounts of free streptokinase-plasmin, under the condi-
the accumulation of streptokinase-plasmin in the system.
inhibited complex resulting in increasing amounts of activator,
increasing rates. The cycle will cease to operate when all
validity of this theory rests on the assumption of the presence of
streptokinase is liberated from the ternary complex. The
the latter will compete for the streptokinase component of the
will catalyze, albeit very slowly, the formation of some plasmin.

The recovery process shown on Fig. 3 can be explained by
known concentrations of streptokinase-plasmin as activator.

*Clot incubated in the absence of added activator and inhibitor.

** Arbitrary units based on a standard curve obtained by using
known concentrations of streptokinase-plasmin as activator.

TABLE I

<table>
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<tr>
<th>Bovine pancreatic</th>
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<td>Controlb</td>
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</table>

* Arbitrary units based on a standard curve obtained by using
known concentrations of streptokinase-plasmin as activator.

** Clot incubated in the absence of added activator and inhibitor.

caseinolytic activity can be regarded as the direct consequence of
the accumulation of streptokinase-plasmin in the system.

The recovery process shown on Fig. 3 can be explained by
assuming the presence of a trace amount of streptokinase-
activator, even under conditions of blocking of the
activator by equivalent amount of PTI. This trace activator
will catalyze, albeit very slowly, the formation of some plasmin.
The latter will compete for the streptokinase component of the
inhibited complex resulting in increasing amounts of activator,
which in turn will catalyze the activation of plasminogen at
increasing rates. The cycle will cease to operate when all
streptokinase is liberated from the ternary complex. The
validity of this theory rests on the assumption of the presence of
trace amounts of free streptokinase-plasmin, under the condi-
tions described. That this is indeed the case was verified by the
PTI-titration of activator already described and shown on Fig. 2.
While excess amounts of PTI appear to suppress completely the
activator activity, the actual data, presented in Table I, show
that even when twice-equivalent PTI had been added, the
amplification provided by the sensitive clot lysis method still
permitted detection of activator activity: the clot lysed in 24
hours, as compared with the control (no activator added) which
was still intact 16 hours after its formation. The presence of
trace amounts of streptokinase-plasmin is indeed to be expected
due to the fact that while inhibition by PTI is stoichiometric,
the affinity of PTI for streptokinase-plasmin is not infinite. In
support of a finite affinity it may be mentioned that Ganrot
(19) observed a slow exchange between radioactive PTI com-
plexed with plasmin and excess unlabeled PTI.

These experiments then demonstrate the essential contribu-
tion made by the plasmin active center to the activator activity
and eliminate all necessity to endow the streptokinase moiety
with any direct catalytic activity.

**Streptokinase Activation of Plasminogen in Presence of In-
hibited Spontaneous Plasmin**—The inhibitor, NPGB, reacts with
plasmin as an active center-specific reagent (29) giving a burst of
p-nitrophenol and inactive, acylated enzyme. Advantage
was taken of this fact in inhibiting the spontaneous plasmin
present in our plasminogen in order to see whether strepto-
kinase could still activate plasminogen. For the following
experiment (Fig. 4) the plasminogen preparation was treated
with NPGB, to cause a rapid and complete inactivation of the
spontaneous plasmin, as determined by the azocasein assay
method. Subsequent addition to the reaction mixture of a 0.1 M
Tris-HCl, 0.1 M lysine buffer, pH 8.5, and incubation for 1 hour
at 25°, destroyed all excess NPGB by nonspecific hydrolysis
(29), as indicated by measurement of p-nitrophenol liberated.

**Curve a** in Fig. 4 shows the rate of activation of this inhibited
preparation when activated with 490 units of streptokinase, an
amount less than equivalent to the spontaneous plasmin content,
which was 4500 units. **Curve b** shows the rate of activation of
the uninhibited, control preparation by the same amount of
streptokinase. **Curve c** illustrates the rate of activation of the
inhibited preparation by 5000 units of streptokinase, an
amount in excess of equivalence with the blocked spontaneous plasmin
content. It can be seen that the low amount of streptokinase
activated the inhibited sample extremely slowly, yielding only
5% of the uninhibited control value in 20 min. When the
large amount of streptokinase was used, however, very fast
activation occurred in the inhibited sample. Thus, it is clear
that activation by streptokinase can occur even in the presence
of inhibited spontaneous plasmin, as was observed also by others
before (11, 12) indicating the existence of a mechanism that does
not require plasmin as the cofactor.

If there is indeed such a mechanism, the question may be
asked why did it not function in the above experiment when the
low amount of streptokinase was used?

**Effect of NPGB-inhibition on Streptokinase Affinity of Plasmin**—
The absence of activation when small amounts of streptokinase
are used is due to a preferential affinity of streptokinase to the
spontaneous plasmin when the latter had been inactivated by
prior reaction with NPGB. Due to this preferential affinity of
streptokinase to the inactivated spontaneous plasmin, the strepto-
kinase is trapped and is unavailable to participate in any activa-
tion mechanism. The preferential affinity was demonstrated by the following experiment.

A fully activated sample of human plasmin (0.225 mg) was treated with NPGB to inhibit its proteolytic activity completely. To this preparation was then added active plasmin (0.27 mg) and the mixture titrated with streptokinase. In the control experiment only the untreated active plasmin was titrated. It may be recalled that a stoichiometric complex of streptokinase and human plasmin has decreased proteolytic activity, a property which can be used as a true measure of the specific binding of streptokinase to the plasmin molecule (18). It can be seen in Fig. 5 that in the control experiment (Curve a) there is a sharp decrease in the proteolytic activity of plasmin upon addition of increasing amounts of streptokinase, and streptokinase-plasmin equivalence is reached at 1500 units of streptokinase. When the mixture of NPGB-inactivated plasmin and active plasmin is titrated with streptokinase (Curve b), proteolytic activity decreases with a low slope up to 1000 units of streptokinase. Further addition of streptokinase greatly increases the slope, and equivalence is reached at 2700 units. This compares very well with the equivalence of 2750 units calculated with the assumption that both active and inhibited plasmin combine with streptokinase stoichiometrically. If streptokinase had formed a complex with active plasmin only, the inhibition curve should have been identical with the control, yielding an equivalence of only 1500 units of streptokinase. Since this did not occur, it is evident that streptokinase had reacted with NPGB-inactivated plasmin as well as with active plasmin. Had the affinities been equal, there should have been no change observable in the slope of this curve. Since the initial slope is shallow, it is evident that NPGB-inactivated plasmin has preferential affinity to streptokinase. Thus, the inability of low amounts of streptokinase to activate plasminogen containing NPGB-inhibited spontaneous plasmin is due to the trapping of streptokinase in a complex with the inhibited plasmin. As the amount of streptokinase used exceeds equivalence with that component, the excess free streptokinase becomes available to activate plasminogen by another mechanism to be discussed below. Curve a in Fig. 4 shows, however, that even when streptokinase is below equivalence with the spontaneous plasmin, small amounts of plasmin become discernible after 10 min. The reason for this is that the affinity of streptokinase to NPGB-treated plasmin, although high, is not infinite: small amounts of streptokinase will be dissociated, resulting in the generation of activator activity. It is important to emphasize that the absence of activation by low amounts of streptokinase when the spontaneous plasmin had been inactivated with NPGB should not be interpreted as a demonstration of the essentiality of the presence of spontaneous plasmin for activation with low amounts of streptokinase. It is clear from the data presented above that the reason for the lack of activation was the trapping of streptokinase by the complex. If instead of blocking, the spontaneous plasmin could have been quantitatively removed before the addition of streptokinase, the latter would have been immediately available for activation.

Plasminogen, as Proactivator for Streptokinase Activation

Since spontaneous plasmin-inhibited preparations of human plasminogen could be activated by streptokinase, the possibility that human plasminogen itself could be the proactivator was investigated. To a solution of human plasminogen, the spontaneous plasmin content of which was inhibited by prior treatment with NPGB, was added an equimolar amount of streptokinase (Solution I) and equal volumes of aliquots from this activation mixture were transferred as a function of time to fresh solutions of the same plasminogen (Solution II). Fifteen seconds after the transfer the activation in Solution II was arrested by the addition of azocasein in sodium bicarbonate-ε-amino-caproic acid mixture, shown earlier to arrest activation completely (24). Simultaneously with the transfer of activator, aliquots from Solution I were added to azocasein for the determination of plasmin activity development in the activator preparation. The results in Fig. 6 show that no plasmin is detectable in Solution I up to 30 sec, and maximum plasmin formation requires 5 min (Curve b). However, the activator activity (plasmin developed in 15 sec) and NPGB-plasmin (Curve a). Inasmuch as no plasmin was present in the 15-sec preparation, the activator activity could not have been due to the streptokinase-plasmin complex. It is clear that in the activator preparation (Solution I), initially all of the plasminogen will be present as a complex with streptokinase, and as activation

![Fig. 5. Effect of NPGB-inhibited plasmin on the streptokinase (SK) titration of active plasmin.](http://www.jbc.org/)

![Fig. 6. Development of activator activity in an equimolar mixture of streptokinase and plasminogen.](http://www.jbc.org/)
FIG. 7. a, the conversion of streptokinase (SK)-plasminogen to streptokinase-plasmin. Gel electrophoresis in sodium dodecyl sulfate and β-mercaptoethanol. C, plasminogen control; ′a′, etc., indicate the times at which conversion was arrested by transfer of aliquots to an NPGB-containing solution. Direction of anodic migration: top to bottom.  

b, correlation between development of plasmin activity (azocasein) and cleavage of the plasminogen molecule, as measured by the appearance of the heavy chain. Quantitative determination of the heavy chain was done by determining the fractional weight of the paper under each peak of the scanning record.

Fig. 8. Irreversible inactivation of plasminogen by NPGB in the presence of increasing amounts of streptokinase (SK). Activatable plasminogen was determined after 16 hours of dialysis against 0.001 M HCl by the azocasein method. A 1:1 molar ratio of streptokinase to plasminogen corresponds to 55,000 units of streptokinase per mg of plasminogen, as determined independently on an untreated sample.

As further proof that in the early phase of activation of Solution I plasmin was present only in trace amounts, the following experiment was performed. Solution I was prepared as described above, and aliquots at time intervals after the addition of streptokinase were added (a) to azocasein to determine plasmin activity, as above; and (b) to a solution of NPGB to arrest the activation instantaneously. The relative amounts of plasminogen and plasmin contained in the NPGB-blocked samples were then determined by gel electrophoresis in sodium dodecyl sulfate and β-mercaptoethanol. Fig. 7 shows that plasmin, as indicated by the appearance of heavy and light chains, develops slowly in the activator solution. Figure 7b shows that the appearance of the heavy chain is strictly proportional to the azocaseinolytic activity, strongly supporting the contention that the early activator activity could not have been due to streptokinase-plasmin, as observed earlier by Werkheiser and Markus (26) and elegantly demonstrated by Buck et al. (28).

Reaction of NPGB with Xtreptokinase-Plasminogen Complex—The fact that the streptokinase-plasminogen complex is an activator, i.e. it has the ability to cleave an arginyl-valyl bond in the plasminogen substrate indicates the presence of a catalytic center in the activator complex. That such an active center is indeed present has been demonstrated as follows. To a solution of human plasminogen, a 10-fold excess of NPGB was added, followed by an amount of streptokinase, equivalent to the plasminogen. Fifteen seconds after the addition of streptokinase, the pH of the reaction mixture was adjusted to 3.0 to arrest further activation, and the preparation dialyzed extensively against 0.001 M HCl in the cold to remove excess NPGB. The mixture was then brought to pH 8.5 and immediately assayed for activatable plasminogen by the azocasein method.  

The rate of this modification seems to be proportional to the rate of plasmin formation, as observed also by Buck et al. (28).
method. No activatable plasminogen was found, indicating that all of the plasminogen had reacted with NPGB during the 15 sec before the adjustment to pH 3.0. That the NPGB-reactive species was indeed plasminogen and not plasmin is evident from the data shown in Fig. 6 which indicate that in an equivalent complex no plasmin is formed up to 30 sec. (This point will be further documented below.) If NPGB can indeed react with the active center of streptokinase-plasminogen, then addition of increasing amounts of streptokinase to plasminogen should result in the formation of increasing amounts of inactive complex with an equivalent decrease in activatable plasminogen. The data in Fig. 8 show a linear correlation between the amount of inactivated plasminogen and the streptokinase added to the reaction mixture: again, all of the plasminogen is inactivated by NPGB when a stoichiometric amount of streptokinase is added.

Now, if the streptokinase-plasminogen complex is indeed the activator species responsible for activation of human plasminogen, presence of NPGB in the activation mixture should result in an instantaneous arrest of activation. Thus, when streptokinase is added to plasminogen in the presence of NPGB only two species should be found in the reaction mixture even after long periods of incubation at pH 8.5, namely, activatable plasminogen and inactivated streptokinase-plasminogen complex. When an equimolar amount of streptokinase is used, all plasminogen should be in the inactive complex form. That this is indeed the case is shown by the experiment illustrated on Fig. 9. To plasminogen solutions in 0.1 M Tris-HCl, 0.1 M lysine, pH 8.5, and excess NPGB, increasing amounts of streptokinase were added. The solutions were incubated for 1 hour at 25°, conditions sufficient to hydrolyze all excess NPGB. Samples were then analyzed for activatable plasminogen. The results definitely establish that the amount of unactivatable plasminogen is proportional to the amount of streptokinase used; activation must have been arrested, since in no case was the unactivatable plasminogen found to be in excess of the amount of streptokinase used, i.e. no catalytic formation of plasmin has occurred.

An unequivocal demonstration that the NPGB-reactive species is indeed streptokinase-plasminogen complex was made possible by polyacrylamide gel electrophoresis, and was based on the fact that plasminogen is made up of a single polypeptide chain, whereas plasmin consists of two chains held together by disulfide bonds (17). To plasminogen in the presence of excess NPGB was added an equimolar amount of streptokinase, and the reaction mixture incubated for 30 min at 25°. It was then made 0.14 M with β-mercaptoethanol and 0.1% sodium dodecyl sulfate. The gels were analyzed by autoradiography, indicating the presence of increasing amounts of streptokinase-plasminogen complex. The direction of anodic migration is from top to bottom. In Gel 3 the slow and fast migrating bands correspond to the heavy and light chains of plasmin, respectively. In Gel 4, the band migrating between the former two is modified streptokinase.

It is evident, therefore, that due to complex formation with streptokinase, plasminogen has acquired the ability to react with NPGB, indicating that an active center on the plasminogen molecule has become available.

McClintock and Bell (30) have independently and simultaneously come to the same conclusions regarding the streptokinase-plasminogen complex. Their studies also showed that NPGB reacts with streptokinase-plasminogen, indicating the appearance of an active center in the complex before cleavage of the plasminogen chain had occurred. Their kinetic studies suggested a two-step mechanism whereby streptokinase and plasminogen act in sequence.

The preliminary reports of both the present work (2) and that of McClintock and Bell (31) were presented at the same session of the FASEB meetings, on April 14, 1971 in Chicago, Illinois.
plasminogen react rapidly to form a complex, followed by the slower process of active center development.

**DISCUSSION**

The Nature of Proactivator—The experiments presented in this study show that all proactivator activity can be accounted for by either plasmin or plasminogen. The fact, first established by Blatt et al. (10), and confirmed here, that maximum activator activity toward human plasminogen is obtained when streptokinase and plasminogen are present in a 1:1 molar ratio, limits the choice of proactivator to either plasminogen or plasmin. Clearly, if any other proactivator such as e.g. the one recently isolated by Takada et al. (32) from human plasma had been responsible for the mechanism of activation, the streptokinase-plasminogen ratio at maximum activator activity would have been less than 1.0. Inasmuch as the streptokinase-plasmin complex is an activator of human plasminogen, it was logical to assume that spontaneous plasmin when present in plasminogen preparations can form streptokinase-spontaneous plasmin complex and participate in the activation mechanism. However, attempts to demonstrate such a role for spontaneous plasmin in the present study were not successful for the following reasons. The practically instantaneous formation of a streptokinase-plasminogen complex possessing activator activity precludes the possibility of estimating the contribution of streptokinase-spontaneous plasmin to the rate of activation. This could be demonstrated only if the specific activator activities of streptokinase-plasminogen and of streptokinase-plasmin were very different. Fig. 6 shows that this is unlikely to be the case. It is obvious, therefore, that no correlation can be expected between the level of spontaneous plasmin present in any given plasminogen preparation and the rate at which streptokinase can activate plasminogen. Data presented in this paper show that when the spontaneous plasmin is inhibited with NPGB, addition of low levels of streptokinase results in activation only after a long lag period. At first this was interpreted to mean that the presence of spontaneous plasmin was essential for activation by low levels of streptokinase. However, since it was later demonstrated that streptokinase had a higher affinity for NPGB-inactivated plasmin than for active plasmin, it became obvious that the reason for lack of activation was that streptokinase was trapped in the complex with inactivated plasmin and was therefore unavailable for the formation of the streptokinase-plasminogen complex. Inasmuch as complex formation between streptokinase and plasmin, and streptokinase and plasminogen are both extremely rapid, and the specific activator activities of the two complexes appear to be comparable, there seems to be no reason to doubt that spontaneous plasmin, when present, will make a contribution to the activation process, to an extent not exceeding its fractional content in the preparation. Kline and Ts'ao (19) recently claimed that spontaneous plasmin makes no contribution to the activation process, since they found no difference in the activation rates, at varying streptokinase levels of two plasminogen preparations containing 2.8, and less than 0.1% spontaneous plasmin, respectively. It seems to us that this is exactly the result one would expect if both plasmin and plasminogen could serve as proactivators, and were of comparable efficiency.

**Streptokinase-Plasminogen Complex as Activator**—That human plasminogen itself is a cofactor for the streptokinase-activation of bovine plasminogen was suggested by the work of Werkheiser and Markus (26) and by Buck et al. (28). They showed that upon the addition of streptokinase to human plasminogen there is a rapid development of bovine plasminogen activator activity in the reaction mixture, the rate of its appearance greatly exceeding the development of plasmin activity. These results suggested that fully formed plasmin is not essential for activator activity, and that the complex of streptokinase with plasminogen itself could serve as an activator. In the present paper we have extended these observations to the activation of human plasminogen also (Fig. 6). The data presented in this paper as well as the independent study of McClintock and Bell (30) provide experimental evidence that an active center is formed in the zymogen molecule upon interaction with streptokinase. Plasminogen, which does not react with NPGB, an active center-directed, irreversible inhibitor of plasmin, acquires this ability upon complex formation with streptokinase. The fact that NPGB reacts with plasminogen in the complex before the critical peptide bond is broken clearly indicates that the exposure of the active center in the zymogen molecule is due to a conformational change induced by streptokinase. A mechanism for the streptokinase activation of human plasminogen can be proposed as follows. In this scheme streptokinase-plasminogen stands for the complex possessing the exposed active center.

$$\text{streptokinase} + \text{plasminogen} \rightarrow \text{streptokinase-plasminogen} \quad (1)$$

$$\text{Plasminogen} \rightarrow \text{Plasmin} \quad (2)$$

$$\text{streptokinase-plasminogen} \rightarrow \text{streptokinase-plasminogen + plasmin} \quad (3)$$

It should be mentioned that recently other views concerning the mechanism of streptokinase-activation of human plasminogen have been expressed. Thus, Summaria et al. (11), as well as Kline and Ts'ao (19) proposed that streptokinase is capable of activating human plasminogen directly. Inasmuch as there is by now sufficient evidence to show that when streptokinase is added to human plasminogen, it immediately forms stable, stoichiometric complexes with plasmin and plasminogen both of which can function as activators, leaving little, if any, streptokinase in the free state, there is no necessity to ascribe to streptokinase an independent enzymatic role. Recently the claim has been made (33, 34) that streptokinase, upon prior interaction with human plasmin or plasminogen, becomes modified and when separated can directly activate bovine plasminogen. A critical examination of these results will only be possible after publication of the detailed data.

**REFERENCES**

Mechanism of Activation of Human Plasminogen by Streptokinase: PRESENCE OF ACTIVE CENTER IN STREPTOKINASE-PLASMINOGEN COMPLEX
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