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The Plasminogen Activator of Vampire Bat Saliva

By Terence Cartwright

A procedure is described for the production of a highly active plasminogen activator (desmokinase) from the saliva of the vampire bat using gel filtration on Biogel A0.5 m followed by ion-exchange chromatography on DEAE-cellulose. Investigations using specific inhibitors indicated that desmokinase was probably a specific protease similar in some respect to urokinase. Desmokinase was not, however, able to activate plasminogen in solution, and it is postulated that this could be due to a requirement for a fibrin surface for activation to proceed. Desmokinase seems to be more effective than either urokinase or streptokinase in the lysis of preformed blood clots. This property may be explicable in terms of greater affinity of desmokinase for fibrin.

THE MACABRE FEEDING habits of the vampire bats have for some time aroused considerable interest among natural scientists. These animals are unique among the vertebrates in being absolutely dependent on a diet of fresh blood which they obtain by inflicting an apparently painless wound on their victim, which is frequently asleep. It has been noted that these wounds, although superficial, continue to ooze blood for a period of several hours.[1] From this observation it is a relatively short step to the proposal that the vampire bats are somehow able to interfere with normal hemostatis in the victim, and that the mechanism by which they achieve this is carried in their salivary secretions. Accordingly, it has been postulated that vampire bat saliva contains anticoagulants[2,3] or proteolytic enzymes.[1,4] More recently, saliva from the vampire *Desmodus rotundus* has been investigated in the laboratory and has been shown to contain no overt proteolytic activity. Desmodus saliva does, however, contain components which interfere with the hemostatic mechanism of mammalian blood at three distinct levels: an agent which prolongs cephalin time is present, the aggregation of platelets is inhibited, and the saliva contains a potent activator of plasminogen.[5,6]

From the Nuffield Institute of Comparative Medicine, Zoological Society of London, Regent's Park, London, N.W.1. England

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Terence Cartwright, Ph.D.: *Nuffield Institute of Comparative Medicine, Zoological Society of London, Regent's Park, London, N.W.1. England. Present address: Searle Research Laboratories, Lane End Road, High Wycombe, Bucks, England.*

Each of these activities was associated with a distinct protein fraction. The name "desmokinase" was given to the plasminogen activator.[5]

Since thrombolytic therapy involving the use of enzymic activators of plasminogen (for example, streptokinase and urokinase) is becoming more important, it was decided to investigate further the isolation and the possible usefulness of desmokinase. The present paper describes the purification of this material from *Desmodus* saliva and the initial characterization of the active principle.

MATERIALS AND METHODS

Bovine fibrinogen (Armour Pharmaceutical Co.) was 65% clottable with bovine thrombin at 50 NIH U/ml. (Leo Laboratories Ltd.) Human urokinase (reference standard) was also obtained from Leo. Purified human plasminogen was obtained from Kabi Pharmaceuticals Ltd. or prepared by the method of Robbins et al.[7] DEAE-cellulose (DE32) was obtained from Whatman, Sephadex G-200 from Pharmacia, and Biogel A 0.5 m from Calbiochem.*

Assay Procedures

Fibrin plate assay. Plasminogen activator was routinely assayed using the fibrin plate method of Mullertz[8] modified as follows: 350 mg of bovine fibrinogen were dissolved in 100 ml of 0.01 *M* veronal buffer pH 7.35 containing 0.15 *M* NaCl and 25 m*M* $CaCl_2$. Nine milliliters of this solution were clotted with 5 NIH U of thrombin in a standard 10-cm plastic petri dish. No bacteriostat was necessary over the incubation period used (16 hr).

For rapid screening of column effluents, up to ten samples were applied to each plate, and the areas of the lysis zones were measured. For precise determinations, 0.05-ml samples of each test sample were applied in triplicate over a range of five doubling dilutions to the surface of several plates. The log of the resulting lysis areas (measured as the product of two perpendicular diameters) gave a straight line when plotted against log dilution. Over the range of lysis areas used (50–600 sq mm), urokinase and desmokinase gave parallel dose response curves. Each plate included at least one application of a standard preparation of urokinase at 12 CTA U/ml. Preparation potency was calculated from the test sample dilution factor giving the same lysis area as the standard. The standard error obtained using this system was normally below ±6%. Attempts to reduce the error by using larger fibrin–agar plates[9] were not successful since this produced an unacceptable loss of sensitivity. Fibrinolytic activities are quoted throughout as CTA units per milliliter.

Plasminogen-free fibrin plates were prepared by heating as described by Lassen.[10] Plasminogen-enriched plates were prepared by adding varying amounts of purified plasminogen to the fibrinogen solution before coagulation.

Whole blood clot lysis. Standard whole blood clots were prepared by clotting 10 ml of freshly drawn human blood with 5 NIH U of thrombin on a Petri dish whose floor was covered with a layer of paraffin wax. The clot was incubated at 37°C until retraction was complete, resulting in a thin disk of clot which did not adhere to the wax surface. 0.5-cm disks were then punched out from the parent clot using a cork-borer and washed in buffered saline until no more red cells were eluted. These clots were then placed in solutions containing various activators in buffered saline and gently stirred during incubation at 37°C. The rate of clot lysis was followed by measuring the increase in absorption at 543 nm caused by the release of red cells as the fibrin mesh was digested.

The Chandler thrombus. Artificial platelet thrombi were produced in rotating plastic tubes as described by Chandler.[11] Clots formed in this way reproduce in part the morphologic features of the in vivo blood clot.

Two-stage assay of activation. Desmokinase was tested for its ability to activate relatively

*Armour Pharmaceutical Co., Maidstone, Kent, England; Calbiochem, London, England; Kabi Pharmaceuticals, London, England; Pharmacia AB, Uppsala, Sweden.

pure plasminogen in solution basically by the method of Alkjaersig et al.[12] In addition to casein, the following were used as indicator substrate: azofibrinogen, fibrinogen, azocollagen, benzoyl arginyl ethyl ester (BAEE), tosyl arginyl methyl ester (TAME), and lysyl ethyl ester (LEE). Plasminogen was prepared from human serum fraction III by the method of Robbins et al.[7]

Estimation of Protein

Protein was determined by the method of Lowry,[13] using as a standard salt-free, crystallized bovine serum albumin (Sigma Chemical Co.). All specific activities are expressed as CTA units per milligram of protein. Protein concentration in column effluents was monitored by measuring the absorption at 280 nm using an LKB Uvicord flow analyzer.

Estimation of Molecular Weight

Molecular weight estimates were made using a calibrated column of Sephadex G-200 basically as described by Andrews.[14] The following highly purified protein preparations were obtained from the Sigma Chemical Co. for use as molecular weight standards: cytochrom c (Type III), soya bean trypsin inhibitor, ovalbumen (Grade V), alcohol dehydrogenase (Yeast, 2X crystallized), globulin (bovine), and fibrinogen. Column runs were performed in 0.15 *M* NaCl, 0.03 *M* tris pH 7.6.

Electrophoresis

Cellulose acetate electrophoresis was performed using citrate buffer pH 6.0 and barbiturate buffer pH 8.6 both at ionic strength 0.1. Polyacrylamide gel electrophoresis was performed according to the method of Ornstein.[16] Enzyme activity was detected in each case by cutting 1-mm segments from the stationary phase and applying these directly to the surface of fibrin plates.

Immunodiffusion

Antisera against crude vampire bat saliva were raised in New Zealand White rabbits by a series of three fortnightly subcutaneous injections of 1 ml of a 50% mixture of crude saliva in Freund's complete adjuvant. Ouchterlony immunodiffusion plates were made from 2% Ionagar No. 2 (Oxoid) in barbiturate buffer (pH 8.6, I = 0.1) and were developed for 48 hr at 4°C.

Handling of Vampire Bats

A colony of 80 bats have been maintained, fed on fresh citrated blood, for about 18 mo. Full details of the management of the colony have been published elsewhere.[16]

For saliva collection, bats were lightly anaesthetized (nitrous oxide 30%, fluothane 2.5% in oxygen for about 2 min), and then placed in perspex stalls which incorporate a collection trough below the animal's head. One drop of 1% pilocarpine nitrate in water was then placed on the buccal mucosa to stimulate salivation. Saliva was then allowed to dribble into the trough until salivation ceased (about 10 min). During this operation, the trough part of the apparatus was cooled to 0°C by packing in ice. The saliva was recovered from the trough and either used immediately or stored frozen at −37°C until required. At this temperature no loss of activity occurred after storage for 6 mo.

RESULTS

Isolation Procedures

All purification procedures were carried out at 4°C. Centrifugations were performed using the MSE High Speed 18 with an angle rotor.

Saliva preparation. When stored saliva was thawed or when fresh saliva stood at 0°C for a while, stringly filaments of mucous were precipiated. These were removed by centrifugation (5000 *g*, 10 min, 4°C) before the saliva was subjected to column chromatography. Plasminogen activator activity was virtually

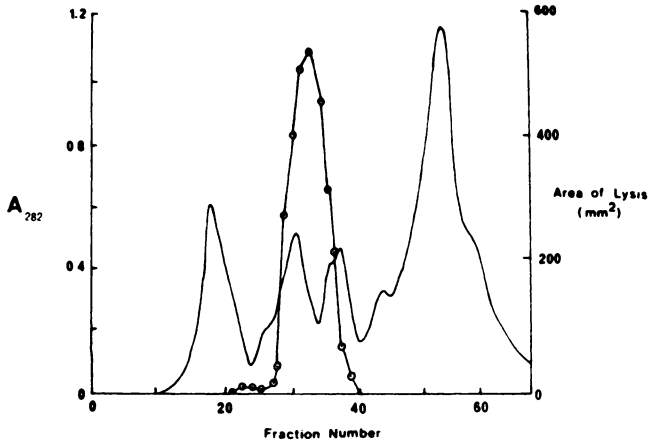

Fig. 1. Initial separation of crude desmodus saliva on a Biogel A 0.5-m column. Area of lysis on fibrin plates was used as a preliminary measure of activity when scanning column effluents.

all recovered in the supernatant fluid. The amount of mucus observed was very variable and initial activity measurements were usually made after this step. Dialysis before proceeding produced no activity loss but did not improve subsequent separation and was therefore abandoned after a few early purification runs.

Gel filtration on Biogel A 0.5 m. Up to 3 ml of the centrifuged saliva was applied directly to a 1.2 × 50-cm column of Biogel A 0.5 m equilibrated with a citrate buffer pH 6.45, I = 0.1. Flow rate was maintained at 30 ml/hr using an LKB Perpex peristaltic pump. Two-four-milliliter fractions were collected and stored at 4°C until assayed. All fractions were tested for activity by a single application to fibrin plates, and those showing activity were reassayed in the quantitative system. Desmokinase was eluted from the Biogel with the relatively early protein fractions (Fig. 1). Purification at this stage was about eightfold with very good yield. The large, low-molecular-weight peak is accounted for in part by residual pilocarpine and is considerably reduced after dialysis.

Ion-exchange chromatography on DEAE-cellulose. Considerable difficulty was encountered in the evolution of a further purification step for desmokinase since irreversible binding to the resin occurred in several ion-exchange systems. A reasonable separation was achieved when the pool of active material from the Biogel column was chromatographed on a 1.5 × 25-cm column of DEAE-cellulose equilibrated with 0.03 *M* Tris pH 7.6. In the first runs the activator was eluted from the column by use of a linear NaCl gradient in the same buffer, but stepwise elution was finally adopted (Fig. 2). Samples were again kept at 4°C awaiting assay and then frozen at −37°C. The most highly purified material was about 80-fold purified over the starting material, and showed a specific activity of about 29,000 CTA U/mg of protein. Details of the yields and purifications obtained at each step are summarized in Table 1.

Physicochemical Properties

Characterization studies were performed using purified desmokinase preparations with a specific activity of 7–18000 CTA U/mg of Lowry protein.

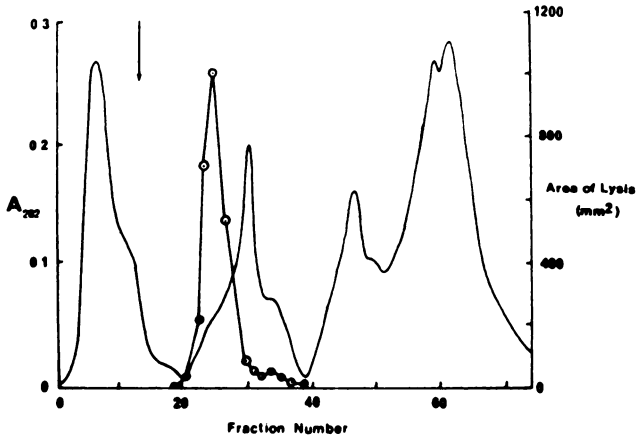

Fig. 2. Ion-exchange chromatography of partially purified desmokinase on DEAE-cellulose equilibrated with 0.03 M tris buffer pH 7.6. Desmokinase was eluted by passing the same buffer containing 0.15 M NaCl applied at the point arrowed. The final large protein peak was eluted at 1.0 M NaCl.

Electrophoretic and immunodiffusion analysis. Desmokinase of the highest specific activity was apparently homogenous when subjected to cellulose acetate electrophoresis at pH 8.6 or 6.0. At pH 6.0 the activator migrated very slightly towards the cathode, while at pH 8.6 it moved anodically with a mobility of 1.3 sq cm/V/sec $\times 10^5$. On polyacrylamide gel electrophoreis, even the purest preparations showed several closely spaced bands. It was not possible to divide the gel into short enough segments to determine whether one or all of these were associated with activator activity.

DEAE-cellulose-treated preparations of desmokinase showed only a single precipitin line when diffused against crude saliva, which itself usually showed at least five discernible lines. The presence of neutralizing antibodies could not, however, be convincingly demonstrated.

Molecular weight determinations. Using a Sephadex G-200 column of 42.5 cm length, a linear relationship was obtained between log molecular weight and elution volume. Desmokinase was eluted as a single symmetrical peak slightly in advance of alcohol dehydrogenase (mol wt 126,000) in a position corresponding to a molecular weight of about 150,000.

Stability of desmokinase. Desmokinase is a relatively robust enzyme, stable for at least 6 mo when kept frozen at −37°C and for several days at 4°C at neutral pH. It is relatively stable to elevated temperatures at acid or neutral

Table 1. Activator Purification

Fraction	Specific Activity (CTA U/mg protein)	% Yield	Purification Factor
Crude, centrifuged saliva	360	100	1.0
Biogel filtered	2700	95	7.5
DEAE-cellulose treated:			
1. Pooled	17700	72	49
2. Best fraction	29160	40	72

Table 2. Stability of Desmokinase to Extremes of Temperature and pH

pH	0°C	37°C	56°C	80°C
3.0	100	90	71	0
5.0	100	97	92	0
7.0	100	100	96	10
11.0	80	62	25	0

Desmokinase was exposed to various temperatures and pH values for a period of 10 min and then returned to neutral pH at 25°C. Figures show percentage activity remaining after this treatment.

pH, but activity rapidly declines above pH 9.5 (Table 2). Desmokinase may be freeze-dried without serious loss of activity.

Reactivity of desmokinase. Desmokinase caused rapid lysis of both plasminogen-rich blood clots and fibrin plates. The material was, however, completely inactive when applied to fibrin plates in which the plasminogen had been destroyed by heating. Addition of further plasminogen to unheated plates resulted in enhanced lysis initially proportional to the amount of plasminogen added but finally reaching a plateau at a level which varied with different fibrinogen batches. No proteolytic or esterolytic activity was ever demonstrable in the purified preparations using several different substrates, although crude saliva did show a strong esterase activity (K_m with BAEE $5.5 \times 10^{-4} M$ at pH optimum 8.7 at 37°C) which was purified out at the gel-filtration stage.

In addition to its reaction with fibrin plates, desmokinase was able to cause rapid lysis of the "Chandler thrombus," and to digest whole blood clots (prepared as described earlier) at a rate faster than could streptokinase or urokinase of the same unitage (Fig. 3). Reactivity towards plasminogen solutions in the two-stage plasminogen assays was, however, much lower than predicted from the fibrin plate assays. The possibility that this was due to a requirement for some cofactor present in the fibrinogen preparations used was tested by performing the activation reaction in the presence of fibrinogen solution. No increased activity was observed. If, however, the fibrinogen was then clotted, rapid lysis occurred.

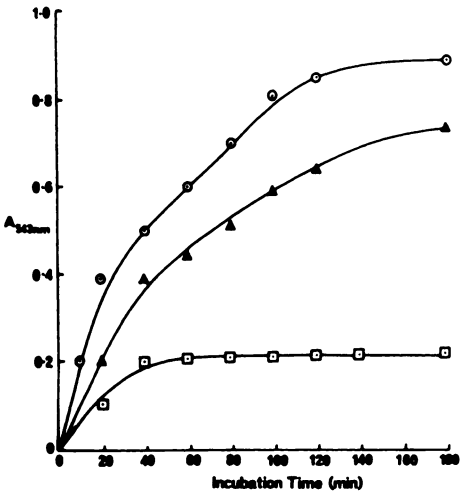

Fig. 3. The relative rates of whole blood clot lysis by urokinase at 2800 CTA units/ml (▲ ——— ▲) and desmokinase at 960 CTA units/ml (⊙ ——— ⊙). Clot lysis is assessed by measuring absorption at 543 nm caused by release of red cells from the clot. A control clot (⊡ ——— ⊡) shows the background cell release caused by mechanical disruption. Desmokinase has completely lysed the clot after 3 hr, at this time a refactory "core" remains in the urokinase tube and will persist for at least 8 hr more.

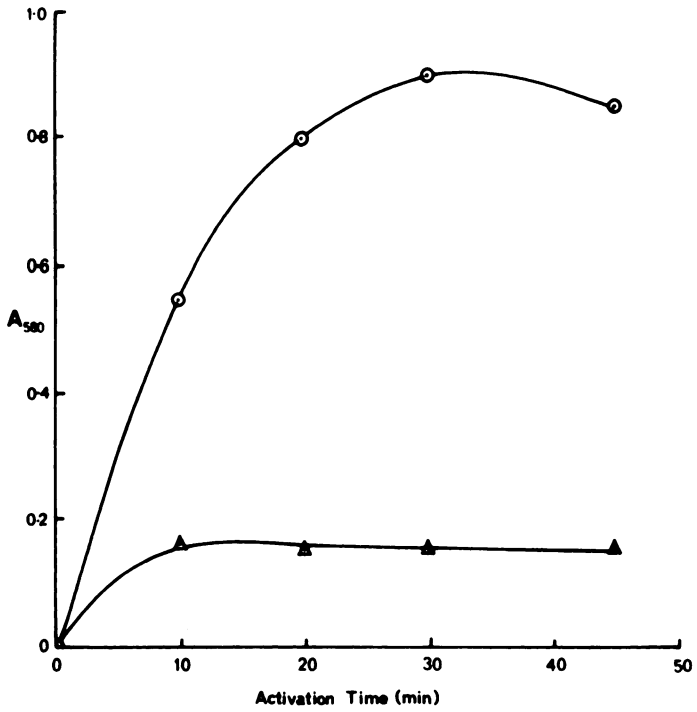

Fig. 4. Desmokinase and urokinase in a two-stage assay with purified plasminogen. The indicator substrate is TAME whose hydrolysis was assayed by methanol release using the colorimetric method of Siegelman et al.[20] ⊙ ——— ⊙ Urokinase, 120 CTA U/ml; ▲ ——— ▲ desmokinase, 1200 CTA U/ml.

It was also considered possible that the desmokinase preparations could contain an inhibitor of activation which, if it were of different molecular size, might be separated from the activator by differential diffusion in the fibrin plate situation. Precedents for this type of situation have been reported.[17,18] This was, however, shown to be unlikely since when desmokinase was added to a system where purified plasminogen was being activated by urokinase, no change in either the rate or the extent of the activation was produced. Desmokinase preparations were also shown to have no effect on the proteolysis of various proteins by plasmin.

The possibility was also tested that the particular form of plasmin produced by desmokinase might be incapable of attacking the casein normally used as indicator substrate in the two-stage assay. When other indicator substrates were employed the same results were obtained, and only very slight activity was observed with azofibrinogen, azocollagen, and various esters of arginine and lysine. A typical two-stage assay, with urokinase used as a positive control, is shown in Fig. 4.

The effect of inhibitors. In an attempt to relate the reactivity of desmokinase to specific functional groups, desmokinase was pretreated with several specific protein reagents. Treatment with diisopropylfluorophosphate (DFP) followed by removal of excess reagent resulted in a complete and irreversible inactivation of desmokinase. The sulphydryl reagents iodoacetamide and p-chloro-

mercuribenzoate, however, had no effect on desmokinase at 10^{-1} *M*. Activity was reduced by about one-third when desmokinase was treated with 10^{-2} *M* mercaptoethanol followed by 10^{-2} *M* iodoacetamide.

Reversible inhibition of desmokinase activity was investigated by adding inhibitors to the fibrin plates before coagulation. Results were considered to represent inhibition of activation if the effect produced when desmokinase was added to the plates was more marked than when the activation step was completely eliminated by adding plasmin direct to the plate. Generally the activation step (in the case of streptokinase and urokinase as well as of desmokinase) was much more sensitive to inhibition than was the lysis of fibrin by plasmin.

In this system, desmokinase was completely inhibited by the classic anti-activator epsilon aminocaprioc acid at 5×10^{-3} *M*. Arginine and BAEE at 10^{-3} *M* were both shown to have a marked inhibitory effect on desmokinase, although plasmin was also affected before their concentration could be raised high enough to achieve complete inhibition of desmokinase.

DISCUSSION

The observation that desmokinase is inactive on plasminogen-free fibrin plates is in agreement with the observation of Hawkey,[5] and supports her view that desmokinase is a specific plasminogen activator. This view is strengthened by the experiments described here showing that enrichment with purified plasminogen enhances the lytic effect of desmokinase. Studies on the species specificity of desmokinase (Hawkey and Cartwright, unpublished results) again indicate a specificity of mechanism of attack, since although clots from all mammals studied are lysed by desmokinase those from birds are not. In this connection it is of interest that the saliva of the bird-attacking vampire *Diaemus youngii* will cause lysis of plasminogen-rich avian clots but not mammalian ones (Cartwright and Hawkey).[21] Large numbers of plasminogen activators of varying potency are now known to be present in the digestive secretions of hematophagous animals.[18] Among these desmokinase ranks very high in terms of potency and exhibits some properties which are potentially highly desirable from the point of view of causing lysis of an established thrombus.

In the present study it has proved difficult to study the way in which the vampire bat plasminogen activator acts because of its lack of reactivity in the two-stage assay systems. This has meant that it was not generally possible to consider the reaction of desmokinase separate from that of the newly created plasmin and therefore that it was not possible to investigate fully the kinetics of plasminogen activation. Immediately this implies that there is a significant difference between the way in which desmokinase reacts and the mechanism by which urokinase (which served as a positive control in all two-stage assays) is able to activate plasminogen.

Considerable evidence now exists that the plasminogen activation process is a proteolytic event catalysed by the activator. This process may also proceed slowly by a spontaneous mechanism, or it may be achieved by nonspecific proteolysis, for example by trypsin. Urokinase has been shown to have a specific proteolytic activity towards plasminogen, and also to be capable of hydrolyzing the synthetic esters BAEE and LEE. From this it has been inferred

that the peptide bonds broken when plasminogen is converted to plasmin are adjacent to arginine or lysine residues.[19] Desmokinase would appear to operate by a similar mechanism. Inhibition by diisopropylfluorophosphate is a characteristic feature of the group of proteases and esterases with an "active serine" residue involved in the active center. Furthermore, although desmokinase seems unable to hydrolyze BAEE or LEE itself, the activation of plasminogen is inhibited by both BAEE and by arginine. This might imply that, as is the case with several other "active serine" proteases, this activator attacks its substrate at a point adjacent to an arginine residue. This view would be strengthened if it were possible to demonstrate that the inhibition of activation was of a competitive nature.

The lack of inhibition by sulfhydryl reagents indicates that no sulfhydryl groups in desmokinase are essential for activity. Treatment with mercaptoethanol was, however, inhibitory, and it is possible that desmokinase contains some functionally important disulfide bonds.

The failure of desmokinase to react with pure plasminogen in solution is a finding which is difficult to explain and is unique among the plasminogen activators so far described. The results presented here indicate that this phenomenon is not a function of the loss of a cofactor occurring in the fibrinogen samples, nor is it due to the presence of antiactivators in the desmokinase preparations, since the latter had no effect on the normal reaction of urokinase with plasminogen. Antiplasmins may also be ruled out since desmokinase did not affect the reaction of plasmin with any of the indicator substrates listed. The use of several indicator substrates also showed that it was very unlikely that desmokinase produced a plasmin molecule with an unusual specificity which made it unreactive towards a particular substrate.

Since desmokinase was extremely reactive towards the plasminogen contained in fibrin plates, in the Chandler thrombus, and in the whole blood clots (in all of which situations solid clots were present) it seems possible that there might be some stereochemical requirement for the presence of a fibrin surface as well as plasminogen if desmokinase is to cause plasminogen activation. If this were the case, it might mean that an added specificity is conferred on desmokinase to ensure that plasmin production is rigidly controlled to occur only in the presence of its correct substrate.

From the point of view of therapeutic clot lysis it is of particular interest that, in vitro, desmokinase is able to dissolve whole blood clots and Chandler thrombi more efficiently than can either urokinase or streptokinase. Indeed, streptokinase is virtually completely inactive towards the Chandler thrombus, due, apparently, to its inability to penetrate the platelet-fibrin thrombus by diffusion. The success of the much larger desmokinase molecule may be related to a special affinity for fibrin which is reflected in the possible requirement for fibrin in the activation reaction as mentioned above.

Although some of the properties of desmokinase recommend it as a therapeutic agent, and very highly active preparations result from an uninvolved purification procedure, the unavailability of the source animal, and the fact that rabies virus is probably endemic in the vampire bat population are serious obstacles to the use of desmokinase in thrombolytic therapy.

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