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Research Article

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Effect of Urokinase Antiserum on Plasminogen Activators: Demonstration of Immunologic Dissimilarity between Plasma Plasminogen Activator and Urokinase

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ABSTRACT Antiserum against purified human urokinase was produced by immunization of Hartley strain guinea pigs. The antiserum was capable of neutralizing the plasminogen activator activity of the antigen and of native urokinase in human urine. The antiserum did not inhibit plasminogen activators of bacterial origin, i.e., streptokinase and staphylokinase; neither did it inhibit urokinase from nonprimate mammals, i.e., dog, pig, rabbit, guinea pig, nor tissue activator or tissue culture supernatants from porcine sources. Partial cross-reactivity against urokinase from primates, i.e., rhesus monkey and baboon, was noted as well as with supernatant from rhesus kidney tissue culture. In vitro studies showed lack of immunologic identity between human urokinase and human milk activator or human tissue activator from adrenal sources but demonstrated immunologic identity between human urokinase and the supernatant from human kidney tissue culture. In vivo studies in man failed to show detectable levels of urokinase activity in peripheral venous or renal venous blood under a variety of clinical states and when stimuli such as exercise, electroshock therapy, or nicotinic acid were used to enhance plasminogen activator activity in the plasma. The results establish that human plasma activator, milk activator, and tissue activator from the adrenals are immunologically distinct from human urokinase.

INTRODUCTION

The ability of urine to lyse a crude fibrin clot was described by Sahli (1) at the turn of the century. Subsequent studies by Sobel, Mohler, Jones, Dowdy, and Guest (2), Williams (3), and Astrup and Sterndorff (4) demonstrated that this property was due to the presence of a plasminogen activator, named urokinase by the former group of investigators (2). Urokinase has been prepared in highly purified form (5, 6), and one group (5) has reported its crystallization. While it is inferred that plasminogen activators appearing in body fluids originate from tissue sources, the original site of urokinase derivation is uncertain.

Since urine from the bladder and from the renal pelvis contains identical urokinase concentrations (7), it is apparent that urokinase is derived either by clearance of plasma activator or directly or indirectly by renal formation; however, direct evidence to support any one of these suppositions is lacking. While limited correlation between plasma fibrinolytic activity and urokinase excretion has been suggested (8), recent reports (9, 10) indicate that the kidney may be the site of urokinase production, and that urokinase excretion into the

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vascular system constitutes a mechanism of physiological significance.

In the present studies, the effect of a specific human urokinase neutralizing antiserum on the biochemical activities of various human, animal, and other plasminogen activators has been determined. The results demonstrate (*a*) that human urokinase is immunologically distinct from both plasma plasminogen activator and human tissue activator prepared from adrenal glands, (*b*) that human renal tissue is capable of urokinase synthesis, but (*c*) that urokinase cannot be detected in the general circulation or renal venous blood of man.

METHODS

Assay of plasminogen activators. Urokinase activity was expressed in Committee on Thrombolytic Agents (CTA) units (11). The activity of other plasminogen activators, i.e. staphylokinase, tissue activators, and activators in tissue culture supernates were expressed in equivalent units (11) as assayed by caseinolytic assay.

Fibrinogen (bovine),[1] 60% clottable by thrombin, was used for the preparation of fibrin plates. Bovine fibrinogen which was 98% clottable[2] by thrombin was used as substrate for ^{125}I iodination.

Bovine thrombin[3] was dissolved in saline at a concentration of 100 U/ml and frozen at −20° in 1-ml aliquots until use.

Human plasminogen was prepared by the method of Kline and Fishman (12) and freeze dried. This material was assayed at 11 Remmert and Cohen casein U/mg of protein when assayed in comparison with a plasmin standard supplied by Michigan State Department of Health and was dissolved in distilled water immediately before use.

Human plasmin was prepared by activation of plasminogen with streptokinase and rendered activator free (13).

Epsilon aminocaproic acid[1] solutions were prepared from crystalline material.

Streptokinase was supplied as Varidase.[4]

Staphylokinase[5] was used in the form of partially purified material which gave an activity of 154 CTA U/mg.

The plasma used was pooled outdated banked plasma stored frozen in small aliquots until use.

Urine was collected fresh and dialyzed in 10-ml aliquots twice against 1 liter of 0.01 M PO_4 buffer, pH 7.6 (2.68 g of Na_2HPO_4, 7 H_2O made up to 1 liter after adjustment of pH) for 18 hr to remove material which gave high spectrophotometric blank values at 280 mμ and to adjust pH to obviate uropepsin activity.

Alpha casein[6] was also used (now available commercially from Worthington Biochemicals, Freehold, N. J.).

Urokinase[6] which assayed at 30,000 CTA U/mg of protein was used for immunization. It was albumin free and lyophilized from 0.1 M PO_4, 0.1 M NaCl solution. The material used in assays was a sterile heat-treated (60°C for 10 hr) preparation with albumin stabilizer, which contained 35,000 CTA U/mg of protein, before the addition of albumin.

Porcine tissue activator[7] contained 15,000 CTA U/mg of protein.

Pig kidney tissue culture concentrate[8] was assayed at 1000 CTA U/mg of protein.

Cytokinase[9] was used in the form of a concentrated solution prepared from serum-free monkey kidney tissue culture supernatant and assayed at 750 CTA U/mg of protein.

Tissue culture supernates were obtained from monolayer RK-13 (rabbit embryo kidney), Hep (carcinoma of larynx), and Hela (carcinoma of cervix) grown from 3 to 5 days on Eagle's growth media containing 2% calf serum. Monkey kidney (MK) tissue culture activator was obtained from supernate of cultures grown on lactalbumin hydrolysate, serum-free media.

Adult human kidney tissue cultures were grown in Eagle's media with 2% horse serum and supernates collected after 5 days growth while the cells were seen to be viable. Material was frozen at −20°C until use.[10]

Crude human milk activator was prepared by centrifuging fresh human milk at 15,000 *g* at 4°C for 20 min and utilizing the supernatant.

Human adrenal tissue activator was prepared by a modification of the methods of Bachmann, Fletcher, Alkjaersig, and Sherry (14, 15). Fresh adrenal glands were collected and stored at −20°C. The frozen adrenals were minced and extracted with 5 M urea at pH 4.0 for 15 min at 4°C, and all subsequent purification steps were performed at this temperature. The urea solution was brought to 25% ammonium sulfate concentration by the addition of salt; the solution was not neutralized. The resulting precipitate was discarded, and the supernatant was brought to 50% ammonium sulfate saturation. After centrifugation the precipitate was dissolved in 5M urea

[1] Nutritional Biochemicals Corporation, Cleveland, Ohio.

[2] Kindly supplied by Dr. Grondahl, Karolinska Institute, Stockholm, Sweden.

[3] Parke, Davis & Co., Detroit, Mich.

[4] Lederle Laboratories, Pearl River, N. Y.

[5] Kindly supplied by Dr. C. Lack, Stanmore Hospital, Stanmore, England.

[6] Kindly supplied by Dr. Milton Mozen, Abbott Laboratories, North Chicago, Ill.

[7] Kindly supplied by Dr. Fedor Bachmann, Department of Medicine, Washington University, St. Louis, Mo.

[8] Kindly supplied by Dr. R. L. Hamill, Eli Lilly & Co., Research Laboratories, Indianapolis, Ind.

[9] Kindly supplied by Dr. R. H. Painter, Connaught Medical Research Laboratories, West Willowdale, Ontario.

[10] Kindly supplied by Dr. Maria B. Bernik and Dr. H. C. Kwaan, Northwestern University, Chicago, Ill.

at pH 4.0, refractionated at 33% ammonium sulfate saturation, and the precipitate dissolved in 0.05 M acetic acid. The solution was dialyzed against 0.01 M acetic acid until the resistance exceeded 1000 ohms at room temperature.

The activator was precipitated by raising the pH to 6.5 with sodium hydroxide for 15 min and redissolving in 0.05 M acetic acid; 1 ml of the solution contained 160 CTA U of activator activity.

Urokinase antiserum was produced by injecting the foot pads of Hartley strain guinea pigs with 0.4 mg of the urokinase preparation (in complete Freund's adjuvant) per animal. A booster injection with the same antigen preparation was given after 6 wk and blood collected 2 wk later. Serum was harvested after spontaneous clotting at 37°C for 30 min.

Control guinea pig serum was obtained from the clotted whole blood (37°C for 30 min) of nonimmunized Hartley strain guinea pigs.

Plasminogen activator activity in vitro was determined by the fibrin plate method (16) as modified by Alkjaersig, Fletcher, and Sherry (17), by the plasma clot lysis method (18), and by the euglobulin clot lysis method (19) with the results expressed in fibrinolytic units (20). In certain experiments activator material and antisera were added to the dissolved euglobulin precipitate after its isolation. Epsilon aminocaproic acid (EACA) fibrin plates and antisera and control sera fibrin plates were prepared containing 0.01 M EACA or 0.1% serum, respectively.

Tissue activator and urine from various species were assayed by incubating 2 ml activator containing material with 0.4 ml human plasminogen containing 7.5 Remmert and Cohen casein U, 0.1 ml control or antiserum, and 2.5 ml of 1.4% alpha casein in 0.15 M Tris saline buffer, pH 7.5. After incubation for 30 min at 37°C, casein proteolysis was determined in the trichloroacetic acid filtrate at 275 mμ and the results expressed directly as final optical density. The casein itself incubated under similar circumstances with buffer alone has a blank reading of 0.045.

Plasma plasminogen activator activity was determined by the euglobulin clot lysis method and by ^{125}I-labeled clot lysis (21) with ground glass rod clot carriers and 0.75 Remmert and Cohen casein U of plasminogen per 0.2 ml of plasma clot.

In each instance in which activator activity was studied, a control experiment was done in the presence of 0.01 M EACA; the latter inhibited euglobulin or ^{125}I clot lysis from 86 to 100%, which confirmed that activator activity rather than nonspecific proteolysis or plasmin action was being assayed.

Esterase assays with acetyl-l-lysine methyl ester as substrate were done as previously described (22).

Renin assays[11] were performed on plasma processed by the method of Helmer and Judson (23), and nephrectomized, pentolinium-treated rats were used for assays (24).

[11] Kindly performed by Dr. J. Bourgoignie, Department of Medicine, Washington University, St. Louis, Mo.

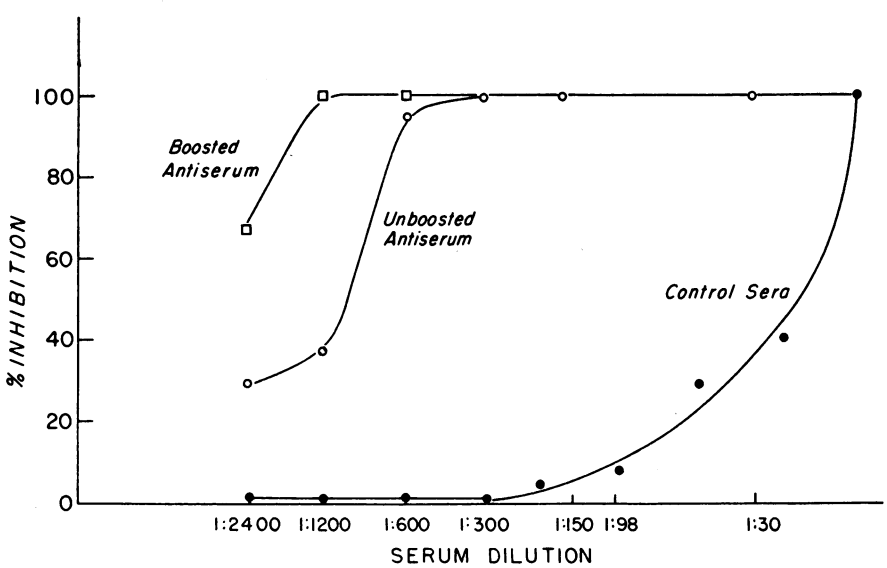

FIGURE 1 Fibrin plate assay. Urokinase, 5 CTA U/ml, final concentration, was treated with either antiserum or control serum in final dilutions ranging from 1:12 to 1:24,000 and the mixtures incubated on standard fibrin plates. Points are the mean of six determinations. □, urokinase (UK) + boosted antiserum; ○, UK + unboosted antiserum; ●, UK + control serum. Per cent of inhibition (ordinate) was calculated on a lysis area basis compared to control experiments with urokinase, 5 CTA U/ml in buffered saline. Serum dilution (abscissa) is plotted on a log scale.

RESULTS

Inhibitory effect of urokinase antisera on human urokinase activity. Fig. 1 demonstrates the inhibitory effect of urokinase antisera on plasminogen activation by purified human urokinase. Human urokinase (5 CTA U/ml of final concentration) and serially diluted antiserum were mixed and urokinase activity assayed by the fibrin plate method. Control serum in a dilution of 1 : 10 completely inhibited urokinase activity, but a dilution of 1 : 100 produced only minimal inhibition. By contrast, unboosted antiserum produced virtually complete inhibition in a dilution of 1 : 600, and boosted antiserum produced complete inhibition at a 1 : 1200 dilution. Thereafter a progressive lessening of inhibition occurred with further dilution of both antisera through 1 : 2400. Boosted antiserum was employed in all subsequent experiments.

Fig. 2 illustrates the action of urokinase antiserum in a plasma clot lysis assay system. Increasing concentrations of urokinase were added to plasma aliquots containing a constant concentration of either antiserum, control serum, or saline. The mixture was immediately clotted with thrombin and the clot lysis time determined. The reciprocal of the lysis time is plotted against urokinase concentrations. The intercept of the plot for inhibition by urokinase antisera with the abscissa indicates that this batch of antiserum (AUKB4) neutralized approximately 50,000 CTA U of urokinase per ml, a finding confirmed by other experiments in which the ^{125}I plasma clot lysis assay was used.

Urokinase antiserum produced comparable inhibitory effects on purified urokinase and native human urokinase in urine, and these data are shown in Fig. 3. The figure displays three sets of urokinase assays performed by the caseinolytic method (results expressed in OD). The solid top line shows assay values obtained with a dilution series of buffered urokinase and the bottom line the values obtained when urokinase antiserum was added to each solution to a concentration of 0.02 %. These lines represent the reference assay curves in the presence and absence of antisera.

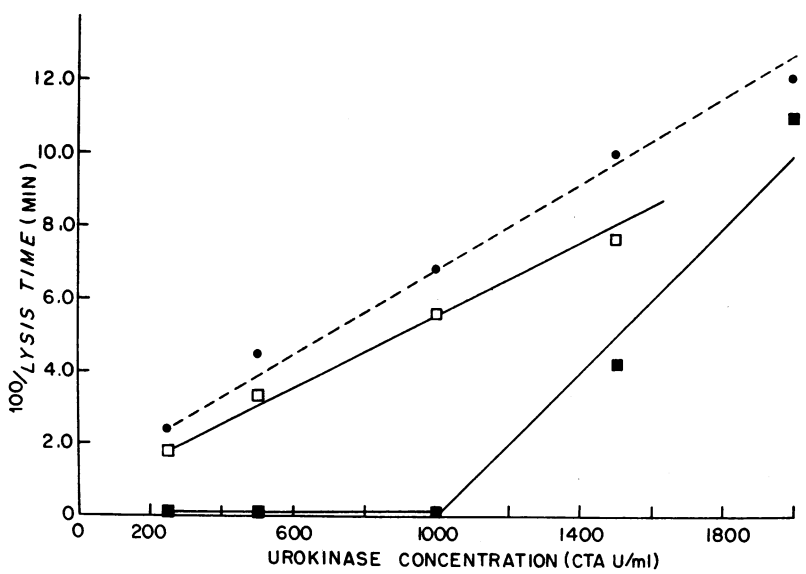

FIGURE 2 Specific inhibition of urokinase by urokinase antisera shown with whole plasma clot lysis method. Urokinase (200–2000 CTA U/ml) was added to bank plasma, the mixture clotted immediately with thrombin, and clot lysis determined (expressed as 100 lysis time on the ordinate). Urokinase concentration is shown on the abscissa. ●---●, urokinase in buffered saline, pH 7.6; ■——■, urokinase plus urokinase antiserum, 1%; □——□, urokinase plus control serum, 5%. The intercept of the antiserum plot (■——■) with the abscissa, corrected for antiserum dilution, indicates that this antiserum neutralized approximately 50,000 CTA urokinase U/ml.

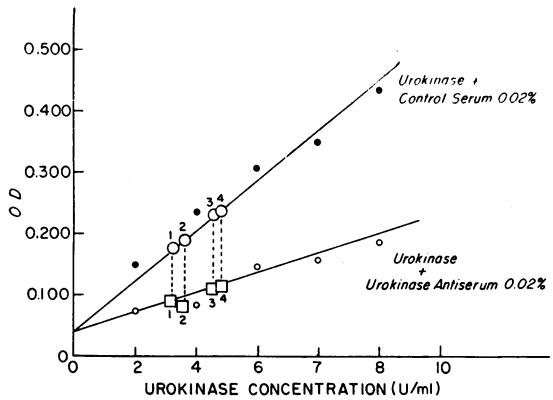

FIGURE 3 Caseinolytic assay. Equivalent inhibition of urokinase antiserum against purified and native human urokinase (urine). Ordinate, OD by casein assay; abscissa, urokinase concentration. ●——●, purified urokinase plus 0.02% control serum; ○——○, purified urokinase plus 0.02% antiserum; ○ (1–4), individual human urine assay values; □, 1–4), urine assays with 0.02% antiserum. Antiserum produced identical inhibition against human urines and purified urokinase.

The four paired assay values (marked 1–4, ○- - - -□) are assay values with four individual urine specimens performed in the absence and presence of antiserum. They superimpose perfectly on the purified urokinase reference lines and demonstrate that the antiserum displayed an identical neutralizing titer against both native urokinase in human urine and purified urokinase. Higher concentrations of antiserum produced complete inhibition of urokinase activity in urine.

When similar experiments were performed with the acetyl-1-lysine methyl (ALMe) esterase assay rather than the caseinolytic assay, inhibition of urokinase by its antiserum was not demonstrable.

The kinetics of urokinase inhibition by antiserum was studied with the casein assay to quantify the velocity of plasminogen activation. The ordinate of Fig. 4 shows 1/velocity (1/OD, casein proteolysis) and the abscissa 1/substrate (1/plasminogen concentration). The Lineweaver-Burk plot demonstrates inhibition of the competitive type, a common pattern observed when antibodies inhibit the biochemical actions of enzymes.

Specificity of urokinase antiserum. The influence of urokinase antiserum on a variety of plasminogen activators and animal urines is shown in Fig. 5 and in Table I. Fig. 5 demonstrates that the antiserum failed to specifically neutralize either streptokinase (15 U/ml) when combined with a trace of human plasmin as a proactivator source, as shown by solid lines, or porcine tissue activator, shown by dashed lines, in a fibrin plate assay system. Significant differences between assay values when these activators were treated with control serum (open symbols) or urokinase antiserum (closed symbols) were not detected. The antiserum also failed to inhibit the activity of another plasminogen activator of bacterial origin, staphylokinase, when tested by the euglobulin clot lysis method.

Fig. 6 illustrates the assay principle used in determining the per cent of inhibition by urokinase antiserum against the plasminogen activators listed in Table I. The casein assay method was used. Since assay mixtures containing urokinase and control serum and those containing urokinase and antiserum each yield linear increases of OD as a function of urokinase concentration, 100 % inhibition by antiserum was defined, at any activity level, as the difference between these readings. For the experiment shown in Fig. 6, serial dilutions of 0.5, 1.0, and 1.5 ml of dialyzed baboon urine were assayed in the presence of 0.02 % control serum and 0.02% antiserum. The values with control serum are plotted on the upper reference assay line and show a virtual linear increase in activity with urine volume. Addition of antiserum also produced a linear reduction in baboon urine assay values, but this was proportionately less than that with purified human urokinase; the Fig. 6 data indicate that the inhibition averaged 77 % of that observed with human urokinase. Similar studies were performed with other activators, and Fig. 7 summarizes findings with baboon urine, rhesus kidney tissue culture supernate (48 % inhibition), cytokinase (37 % inhibition) rhesus urine (50 % inhibition, and human kidney tissue culture supernate (98 % inhibition).

Table I lists the results of experiments designed to determine the percent of inhibition by urokinase antiserum of a variety of plasminogen activators classified with respect to primate, human, or other mammalian origin. The tabulated data are the average of duplicate experiments, and studies were confirmed by assaying 2–4 individual activator specimens. Neutralization studies were initially performed at 0.02 % antiserum or control serum concentration and confirmed at 0.1 % serum con-

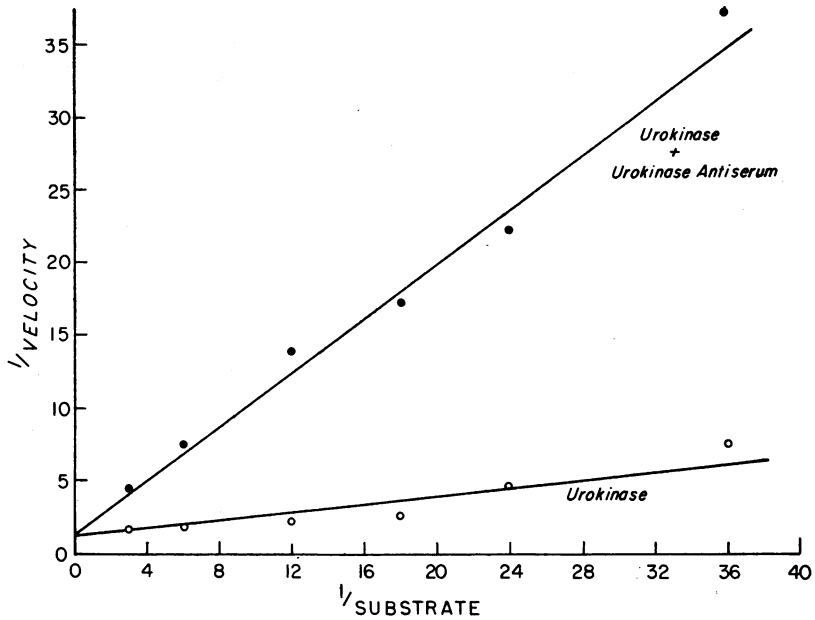

FIGURE 4 Inhibition kinetics of urokinase by antiserum, Lineweaver-Burk plot. Ordinate is $1/A_{275}$, caseinolytic assay. Abscissa is 1/ substrate (S); S is human plasminogen. The concentration of the stock solution is 75 Remmert and Cohen U/ml. Incubation time is 30 min. Urokinase concentration is 10 CTA U/assay tube. Antiserum concentration, 0.04%. ●——●, urokinase plus 0.04% antiserum; ○——○, urokinase plus buffered saline, pH 7.0. The results indicate inhibition of the competitive type.

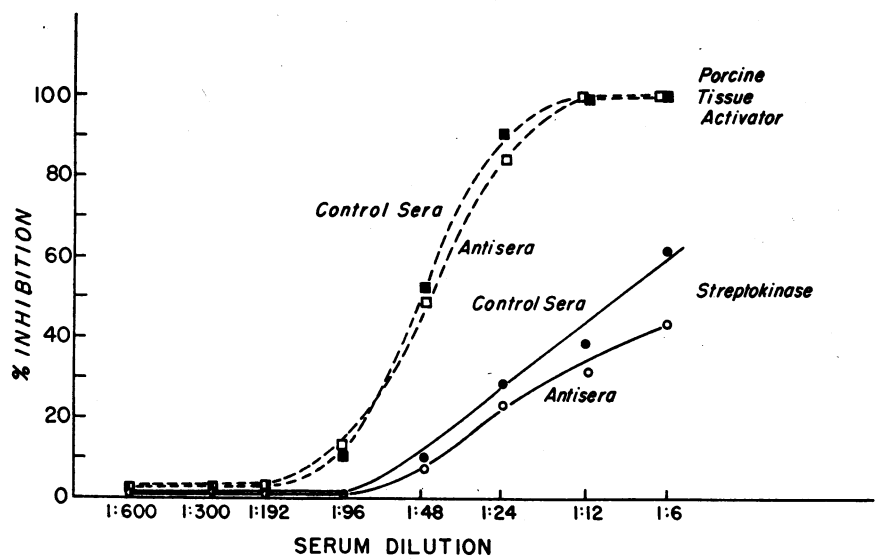

FIGURE 5 Fibrin plate assays of streptokinase and porcine tissue activator demonstrating lack of specific inhibition when treated with urokinase antiserum compared to control sera.

■---■, control sera
□---□, urokinase antisera } plus porcine tissue activator, 2.0 CTA U/ml;

●——●, control sera
○——○, urokinase antisera } plus streptokinase, 2.0 U/ml.

centration. In instances in which activator activity was suboptimal for the caseinolytic method or excessive blank values were encountered, the euglobulin lysis or fibrin plate method was used as noted.

In a few tissue culture media samples, the rabbit kidney tissue culture, and human Hep and Hela cell line tissue cultures, no plasminogen activator activity was detected even by the most sensitive methods, such as euglobulin clot lysis. A low degree of activity could have been masked by inhibitors in the 2 % calf serum necessary for cell growth.

The data indicate that antiserum to human urokinase has a negligible effect against the urokinase, tissue activator, and tissue culture supernates from nonprimate sources, i.e., dog, pig, rabbit, and guinea pig. Per cent of inhibition varied from 0–4 %. In contrast antiserum has a significant effect against activators from primate urinary tract sources. The per cent of inhibition against rhesus urine, rhesus kidney tissue culture supernate, and cytokinase (partially purified rhesus kidney culture supernate) is in the 37–50 % range. Baboon urine cross-reacted to a greater degree and showed 77 % inhibition.

Plasminogen activators from human sources treated with urokinase antiserum fall into two categories: first those which were completely inhibited, including all batches of purified human urokinase, human urine, and human kidney tissue culture supernate; and secondly, those with zero

TABLE I

Per Cent Inhibition of Plasminogen Activator by Urokinase Antiserum: Caseinolytic Assay (Results in OD)

Mammalian (nonprimate) sources	Antiserum 0.1 %	Inhibition
		%
Guinea pig, Swine, Canine, Rabbit urine		0–4
Porcine kidney tissue culture media		0–4
Porcine heart tissue activator		0–4
Rabbit kidney tissue culture media (RK-13)	No activity detected	

Primate sources	Saline	Control serum 0.02 %	Antiserum 0.02 %	Inhibition
				%
Rhesus urine	0.211	0.214	0.150	50
Rhesus kidney tissue culture media	0.181	0.179	0.133	48
Cytokinase	0.206	0.198	0.151	37
Baboon urine	0.184	0.168	0.110	77
Human sources				
Human urokinase	0.181	0.179	0.075	100
Human urine	0.190	0.180	0.076	100
Human kidney tissue culture media	0.245	0.248	0.116	98
Hep, Hela cell line tissue culture media	No activity detected			

	Saline	Control serum 0.1 %	Antiserum 0.1 %	Inhibition
				%
Human tissue activator,* adrenal source	396	400	377	4.5
Human milk activator*	383	392	380	3.0
Plasmin, activator free*	490	462	466	0

* Fibrin plate assay. Results expressed as lysis area in mm^2.

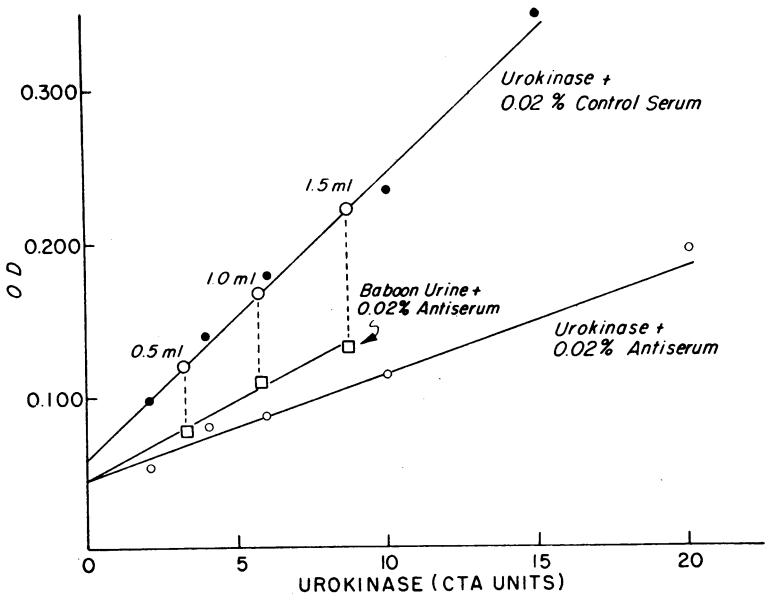

FIGURE 6 Caseinolytic assay. Inhibition of baboon urine by antiserum to human urokinase. Assay system and symbols are same as in Fig. 3; three concentrations of baboon urine are being tested. At each baboon urine concentration, urokinase antiserum inhibited activity proportionately less than against purified human urokinase, and the per cent of inhibition was calculated on this basis. (Human urokinase inhibited by antiserum equals 100%.) The values shown in Table I were determined in this manner.

or negligible cross-reaction including human tissue activator, extracted from adrenal glands, and milk activator.

Control experiments performed by either eliminating plasminogen (in the caseinolytic procedure) or by testing in the presence of 10^{-2} M EACA reduced assay values to 0–14 % of initial values, and confirm that results refer to plasminogen activator concentrations uninfluenced by significant contamination with proteolytic enzymes.

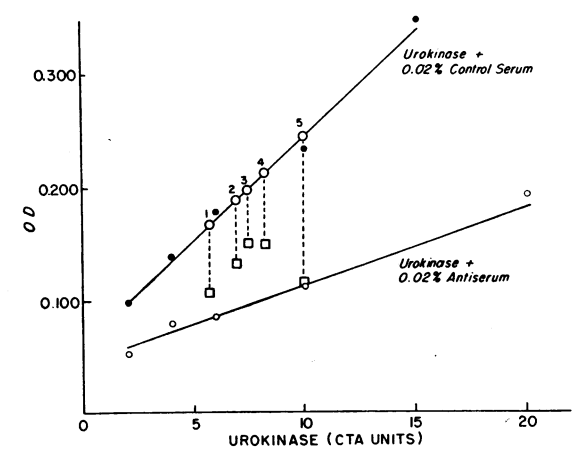

FIGURE 7 Per cent of inhibition of several primate and human source plasminogen activators by human urokinase antiserum. The caseinolytic method is used. 1, baboon urine (dialyzed), 77% inhibition; 2, rhesus kidney tissue culture media, 48% inhibition; 3, cytokinase (partially purified activator from No. 2), 37% inhibition; 4, rhesus urine, 50% inhibition; 5, human kidney tissue culture media, 98% inhibition. Reference urokinase assay lines are designated as in Fig. 3 (●——● and ○——○). Test activators assayed in 0.02% control serum (○ 1–5) are plotted on the upper reference curve and connected by dashed lines to assays in 0.02% antiserum (□ 1–5).

Immunodiffusion. Ouchterlony plates were used for immunodiffusion studies of the urokinase preparation used for immunization and a more highly purified urokinase preparation [12] (156,000 CTA U/mg of protein); these two preparations showed a weak line of identity. An additional stronger precipitin line was present between the antiserum well and that containing the less pure urokinase preparation.

Studies on in vivo plasma plasminogen activator. The possibility that urokinase might be formed in the kidney, diffuse into plasma, and there appear as plasma activator has been suggested by recent studies (10, 25). Our in vivo studies have been designed to test this hypothesis, i.e., whether under any of a variety of experimental circumstances urokinase could be identified in either renal venous blood or in the general circulation.

Five volunteer subjects, three normal and two with Laennec's cirrhosis proved by biopsy, were studied after exercise, rapid infusion of 100 mg of nicotinic acid, or therapeutic electroshock. Blood was sampled from peripheral veins 10 min after

[12] Kindly supplied by Dr. W. White, Abbott Laboratories, North Chicago, Ill.

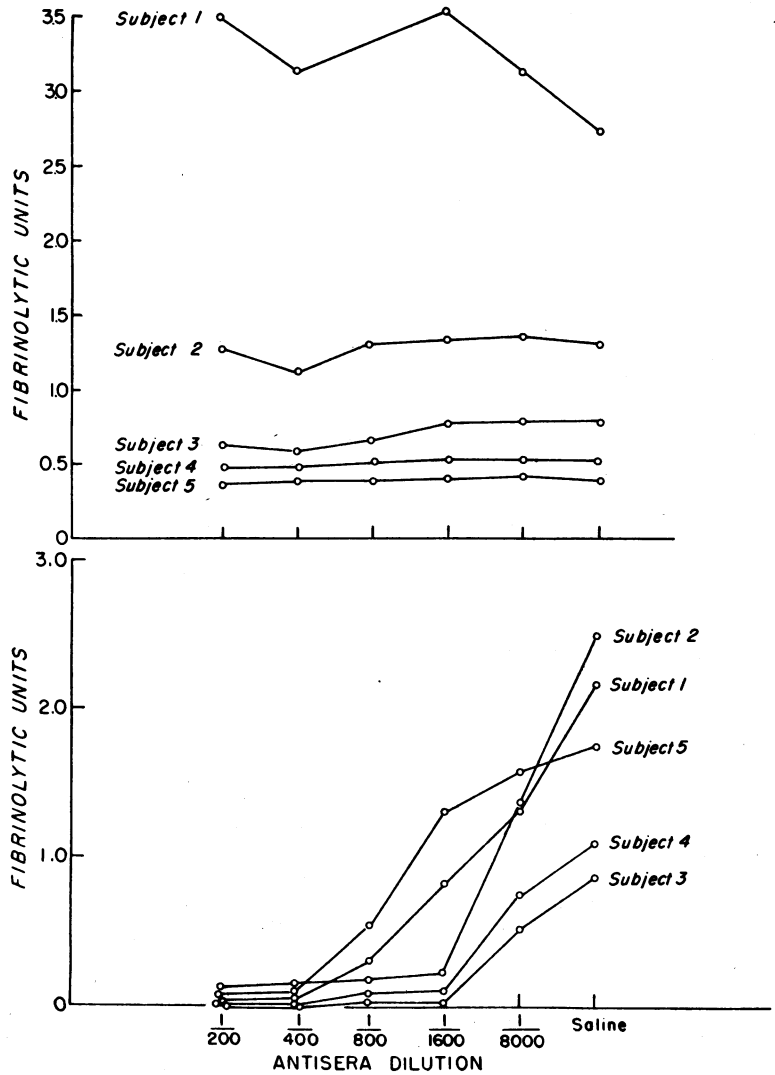

FIGURE 8 (Top section). Lack of inhibition by urokinase antisera against plasma plasminogen activator in human plasmas. Plasmas were drawn 10 min after drug injection or physical stimulus. Euglobulin lysis assays performed with 0.5–0.01% urokinase antisera. Paired assays (not shown) performed in the presence of control sera gave identical results. Subject 1, 58 yr white female, Laennec's cirrhosis, infused with 100 mg of nicotinic acid, i.v.; subject 2, 22 yr white male volunteer stimulated by exercise; subject 3, 39 yr white female 10 min posttherapeutic electroshock; subject 4, 44 yr white male 10 min posttherapeutic electroshock; subject 5, 56 yr Negro male, Laennec's cirrhosis, infused with 100 mg of nicotinic acid, i.v.

(Bottom section). Marked inhibition is produced by urokinase antisera against urokinase-enriched, zero-time plasmas of subjects depicted in upper section. Euglobulin lysis assays were performed in the presence of 0.5–0.01% urokinase antisera. Sufficient urokinase was added to zero-time subject plasmas to approximate fibrinolytic activity noted after the procedures performed above. Paired studies with control sera (not shown) gave minimal nonspecific inhibition ranging from 5–22% with 0.5% control serum.

the procedure and activator concentration studied by the euglobulin clot lysis method. The redissolved euglobulin precipitates of these plasmas were divided into aliquots and treated respectively with serial dilutions of either urokinase antiserum or control serum. The mixtures were clotted with thrombin and the lysis time determined. The results shown in Fig. 8 (upper section) failed to demonstrate specific inhibition of plasma plasminogen activator when assayed in the presence of urokinase antiserum ranging from 0.5 % to 0.01 %, final concentration.

The paired studies with serial dilution of control guinea pig serum gave virtually identical results with those depicted in Fig. 8 with a mean difference of $+1.75\% \pm 7.2$ between the use of control sera and urokinase antisera in the assay, a value within the limits of assay error.

In contrast, experiments shown in Fig. 8 (lower section), performed by adding sufficient urokinase to zero-time plasma samples to simulate euglobulin lysis activity after stress, demonstrated highly significant differences between the actions of control serum and antiserum on urokinase-enriched plasma. Treatment of urokinase-enriched plasma with urokinase antiserum produced virtually complete inhibition in all experiments with antiserum concentrations of 0.25 % or higher and substantial inhibition in concentrations as low as 0.01 %. Control guinea pig serum had a minor nonspecific

inhibitory effect on euglobulin lysis activity of urokinase-enriched plasma ranging from 5.7 to 22 % inhibition by the 0.5% concentration.

Five additional subjects, three normal and two patients with cirrhosis, were similarly studied with the ^{125}I-labeled clot lysis assay. In this instance, antisera and control sera were added directly to subject plasma after the experimental procedures and to the zero-time plasma samples which had been enriched with urokinase. Because the experimental subjects exhibited variable enhancement of plasma plasminogen activator levels after stress, the results in Fig. 9 are expressed as per cent of inhibition produced by the sera compared to assays performed with saline and are displayed as the mean values for all five subjects.

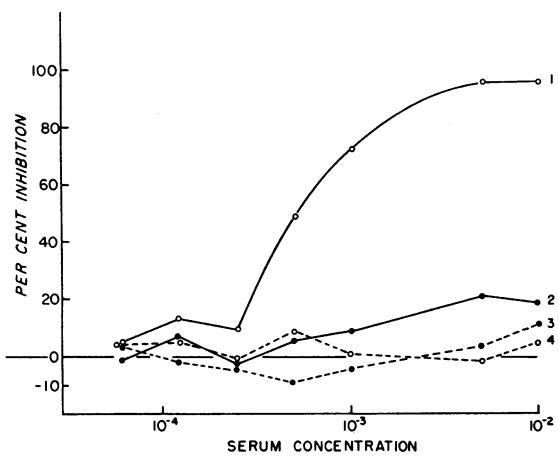

FIGURE 9 Failure of urokinase antiserum to inhibit plasma plasminogen activator activity in human plasma. Assays by the ^{125}I clot lysis technique of human plasma samples drawn 10 min after stimulus. Five individual subjects were studied and each value plotted is the mean of the five separate determinations. Per cent of inhibition produced by the antiserum or control serum (ordinate) is plotted against serum concentration (abscissa). Whereas zero-time patient plasmas to which urokinase had been added were inhibited by antiserum (line 1), plasmas from patients with enhanced levels of plasminogen activator were not (line 2). Line 1, urokinase-enriched, zero-time plasma plus urokinase antiserum; line 2, subject plasma drawn 10 min poststimulation (containing plasma activator) plus urokinase antiserum; line 3, subject plasma drawn 10 min poststimulation (containing plasma activator) plus control serum; line 4, urokinase-enriched, zero-time subject plasma plus control serum. Subjects studied were 25 yr female with exercise, 25 yr and 20 yr males with exercise, 58 yr female cirrhotic with 100 mg of nicotinic acid, i.v., and 38 yr female after therapeutic electroshock.

Though high concentration of antiserum produced apparently minor inhibitions of activity in the plasma samples drawn after stress, the differences between treatment with antiserum (line 2) and control serum (line 3) were not statistically significant ($P > 0.1$). In contrast, the effect of control serum (line 4) and antiserum (line 1) on urokinase-enriched plasma was markedly different. Control serum failed to produce significant inhibition in any concentration, whereas antiserum produced virtually complete inhibition in concentrations above 10^{-3}, and definite inhibition was noted in concentrations as low as $10^{-3.4}$.

Renal venous blood studies. The following studies were performed to determine whether urokinase could be detected in either renal venous or inferior vena cava plasma samples, particularly under conditions in which high levels of plasma plasminogen activator were present. Sample collection was performed by catheterization under fluoroscopic control or direct aspiration at surgery, and sampling was done simultaneously or with a very short interval required for catheter flushing. The six subjects studied were being investigated for suspected renal vascular hypertension or for cardiac abnormalities, and the clinical data and results of euglobulin clot lysis, ^{125}I-labeled clot lysis, and plasma renin assays are shown in Table II. All subjects except No. 3 had normal renal function by chemical and radiographic criteria. In subjects 2–5, no specific stimulus other than that of the investigational catheterization or surgery was used to enhance plasma plasminogen activator concentration. In subject 6, 100 mg of nicotinic acid was injected via catheter into the renal vein. In subject 1, contrast media was injected into the left renal vein before the collection of this final sample.

Subject 1 showed comparable levels of plasminogen activator in the right renal and inferior vena cava plasma samples. The left renal plasma was assayed at considerably higher levels in the sample taken after contrast media injection, probably because of the vasoactive effect of this agent in high concentration. The remainder of the subjects showed comparable titers of plasma plasminogen activator in renal and inferior vena cava (IVC) plasma. Subject 3 produced fibrinolytic assay values of a similar magnitude to the other subjects in spite of impaired renal function. Renin

TABLE II

Fibrinolytic Activity and Renin Levels in Venous Plasma

No.	Subject	Sampling method	Clinical status	Stimulus	Assay method	Right kidney	Left kidney	Inferior vena cava
1	35 yr white female	Catheter	Teflon graft in right renal artery inserted for hypertension secondary to stenosis. Normotensive, normal IVP and normal clearance studies at time of sampling for follow up renin levels.	None	Euglobulin ^{125}I Renin	0.198 U 2.27 % 341 U	0.860 U 10.31 % 295 U	0.100 U 2.70 % 374 U
2	33 yr white female	Catheter	Renal venous catheterization for intermittent hypertension. Normal IVP and renal clearances.	None	Euglobulin ^{125}I Renin	0.159 U 2.89 % 267 U	0.210 U 2.89 % 302 U	0.150 U 2.75 % 330 U
3	52 yr white male	Catheter	Renal venous catheterization for hypertension and bilateral renal artery stenosis, more severe on the left by angiography. IVP normal; BUN, 35 mg/100 ml, serum creatinines 1.35 mg/100 ml.	None	Euglobulin Renin	0.134 U 697 U	0.221 U 1313 U	0.157 U 604 U
4	39 yr Negro female	Surgery	Exploratory surgery for renal cyst in lower pole of left kidney. IVP, otherwise normal; BUN, 10 mg/100 ml. Renal blood aspirated at surgery.	None	Euglobulin ^{125}I		0.70 U* 4.76 %	1.05 U 5.5 %
5	22 yr Negro male	Catheter	Cardiac catheterization for mild pulmonary vascular stenosis. BUN, normal.	None	Euglobulin ^{124}I		0.63 U 5.01 %	
6	23 yr white male	Catheter	Cardiac catheterization for pulmonary valvular stenosis. BUN, normal.	100 mg nicotinic acid i.v. into renal vein.	Euglobulin ^{125}I	0.49 U 5.8 %		0.51 U 6.4 %

IVP, inferior vena cava plasma; BUN, blood urea nitrogen.
* Peripheral venous sampling.

levels bore no relationship to fibrinolytic activity in these studies.

Table III shows the effect on euglobulin lysis and ^{125}I-labeled clot lysis assay values of treating the renal and IVC plasma samples with two concentrations of antiserum and control serum, and, as previously, experiments with urokinase-enriched plasma are included for comparison. Results are expressed as the mean and range of per cent of inhibition produced by the sera.

These data show that in both assay systems, control serum produced a small degree of inhibition in renal and IVC plasma. Treatment with urokinase antiserum as seen in Table III is highly inhibitory against urokinase-enriched plasma. By contrast, plasma plasminogen activator in either renal venous or IVC plasma was not specifically inhibited by urokinase antiserum.

DISCUSSION

Plasminogen activators are widely distributed in various organs and body fluids; they are named with reference to the site of occurrence. Thus the term tissue activator designates activators extracted from specific tissues; plasma and milk activators are those activators present in plasma and milk, respectively, and urokinase, the activator present in urine. Except in the case of human urokinase which has been highly purified (5, 6) and porcine heart activator which has been substantially purified (15), little is known of the properties, precise origins, and interrelationships of the individual activators. Of particular significance are the interrelationships and identities of tissue activators, plasma activator, and urokinase.

The finding of concomitant increases in the plasma fibrinolytic activity and urokinase excre-

TABLE III

Per Cent Inhibition Produced by Antiserum or Control Serum of Plasma Plasminogen Activator in Renal Venous Plasmas and Inferior Vena Cava Plasma

Euglobulin lysis method

Activator source	n	Antisera 0.5 %	Control sera 0.5 %	P*	Antisera 0.12 %	Control sera 0.025 %	P*
		%	%		%	%	
		19	18		13	12	
Renal plasma	9	3–35	0–39	>0.1	0–29	0–29	>0.1
		22	21		10	12	
Inferior vena cava plasma	5	21–23	21–23	>0.1	5–15	4–16	>0.1
		93	13		89	9	
Urokinase-enriched plasma	5	90–100	6–23	<0.001	85–92	3–23	<0.001

^{125}I clot lysis method

Activator source	n	Antisera 0.25 %	Control sera 0.25 %	P*	Antisera 0.025 %	Control sera 0.025 %	P*
		%	%		%	%	
		16	12		17	17	
Renal plasma	7	4–30	0–28	>0.1	4–35	8–27	>0.1
		16	17		12	16	
Inferior vena cava plasma	4	11–23	9–32	>0.1	0–36	11–22	>0.1
		97	7		77	8	
Urokinase-enriched plasma	6	96–98	0–10	<0.001	65–88	0–21	<0.001

n, number of subjects.

* Significance of difference between paired antiserum and control serum studies.

tion after exercise by Celander and Guest (26) and during cardiac bypass procedures by von Kaulla and Riggenbach (27) suggested that plasma activator may be excreted by the kidney as urokinase. The data of Smyrniotis, Fletcher, Alkjaersig, and Sherry (8) on urokinase excretion in a variety of disease states were also generally consistent with this hypothesis.

In contrast, early reports by MacFarlane and Pilling (28), Williams (3), and recent experimental studies by Holemans, McConnell, and Johnson (29), Boongaard, Vreeken, Bleyenberg, and Deggeller (30), and Dudok de Wit (31) using vasoactive drugs to produce transient enhancement of plasma activator levels failed to demonstrate increased urokinase excretion. The latter author reported that plasma activator was not additive in neutralizing urokinase inhibitors in plasma. Urokinase has also been reported as possessing greater thermostability than plasma activator at low pH (32). The relationship of urokinase and tissue activator is similarly undefined except for reports that they have similar thermostability (33). Urokinase and porcine tissue activator have been reported as having different ratios of affinity to plasminogen in solution (as in plasma) vs. plasminogen in the gel phase (as in a fibrin clot) (15).

Recent evidence, indicating (*a*) that activator concentration is higher in renal venous than arterial blood in both man and animals (34) and (*b*) that plasma activator concentration fell during mercuric chloride intoxication (9, 25) and clamping of the renal vessels (10) suggests that the kidney may serve as a significant source of plasma activator. A clearing function for plasma activator by the liver is suggested by studies which detected increased levels of this activator in animals in which the hepatic vessels had been clamped (10), in humans with cirrhosis (35), and in animals in which liver transplantation was being performed (36). Bachmann has shown clearance of both plasma activator and exogenously injected urokinase into bile by the liver (37). However, defini-

tive identification of urokinase in systemic blood or renal venous plasma has not been accomplished, and the evidence for its presence in these fluids has been inferential.

In 1965 Lesuk, Terminiello, and Traver (5) described a method for the preparation of crystalline human urokinase; this crystalline material fulfilled several biophysical criteria for homogeneity and had a molecular weight of 54,000. Subsequently White, Barlow, and Mozen (6), who, by alternative methods prepared urokinase of comparable specific activity to that of Lesuk et al., described preparation inhomogeneity and the presence of at least three molecular species (S_1, mol wt 31,500 and S_2, mol wt 54,700, the main types). Type S_1 urokinase had a specific activity approximately twice that of S_2, but antisera prepared against S_1 showed reaction of identity with S_2 material. However, recent studies by Lesuk, Terminiello, Traver, and Groff (38) substantiate their original claims that native urokinase is probably a single molecular species of 54,000, mol wt and suggest that White's S_1 fraction may have been subjected to artifactual proteolytic cleavage during purification.

The urokinase preparation used for immunization purposes in the present study was predominantly in the high molecular weight (54,000, mol wt) form, and the antiserum neutralized, on a biochemical activity basis, equal quantities of both the purified urokinase preparation used as antigen and native urokinase in human urine. This antiserum permits the detection of urokinase in biological fluids.

Antiserum specificity for human urokinase was established by demonstrating (*a*) lack of reactivity against the biochemical action of nonhuman and nonprimate plasminogen activators, including streptokinase, staphylokinase, and animal urines, tissue kinases, and supernates from animal cell culture lines and (*b*) lack of reactivity against human plasminogen activator extracted from adrenal glands and human milk activator. These experiments demonstrate immunologic dissimilarity between human urokinase and mammalian (nonprimate) urokinases. The plasminogen activator in rhesus monkey urine, baboon urine, and in unpurified and partially purified (cytokinase) supernates from rhesus monkey kidney cell tissue culture could be completely inhibited by urokinase antiserum. However, antiserum inhibitory titer against these activators was less than that against human urokinase, indicating partial cross-reactivity against these materials. These findings indicate partial immunologic identity and suggest a common phylogenetic derivation of the urokinase molecule in the two primates and in man. The similar inhibitory titers (37–50 %) against rhesus urine and supernate activator material from rhesus renal cell culture suggest that urokinase is produced in vitro by these cell lines. In the parallel situation with human material, identical inhibition was produced by urokinase antiserum against human urine, purified human urokinase, and supernate from human kidney cell culture (98–100 %), a finding which suggests that the latter cell lines produce human urokinase in vitro.

It is generally accepted that inhibition of enzymes by their antibodies involves steric hindrance to the access to the catalytic site (39, 40), and frequently the kinetics of the inhibition are those of competitive inhibition, as in our studies. The steric hindrance hypothesis would provide an explanation for the strong inhibition seen with high molecular weight substrate (plasminogen), with no inhibition when the complex was tested on low molecular weight substrate (amino acid ester). Other explanations which might be invoked are (*a*) the difference of molar concentration of the respective substrates or (*b*) the difference in K_m between the respective enzyme substrate reactions. However, in view of certain recent studies by Shapira and Arnon (41) on the mechanism of inhibition of papain by its specific antibodies, studies in which they too described inhibition of enzymatic action when large molecular weight substrate (casein), but not when low molecular weight substrate (amino acid ester) was used, steric hindrance would seem the most likely explanation for our findings.

Whether or not the urokinase antibodies prepared for this study were wholly of the precipitating variety remains uncertain, but at least on Ouchterlony plates a precipitin line, which was almost certainly attributable to urokinase precipitation, was seen. Shapira and Arnon also indicate that aggregation state of antigen-antibody complexes is of only minor importance with regard to complex biologic activity.

The chief purpose of these studies was to deter-

mine whether urokinase could be definitely identified in the plasma of man, and if so to determine the extent to which this specific enzymatic moiety, urokinase, contributed to total plasma activator activity. However, all attempts to demonstrate the presence of urokinase in human plasma were unsuccessful, although for technical reasons studies were performed in patients exhibiting enhanced rather than resting levels of plasminogen activator.

Our data were obtained in normal volunteers in which enhanced levels of plasma plasminogen activator were induced by exercise, nicotinic acid administration, and therapeutic electroshock; in some subjects undergoing anesthesia, surgery, or inferior vena cava catheterization, high levels of plasma activator occurred spontaneously, and stimuli were not employed.

Assay accuracy was confirmed by the use of two methods, the ^{125}I clot lysis assay and the euglobulin lysis time, which gave concordant results. Assay specificity was assured through the demonstration, first, that the antiserum did not inhibit plasmin and second, by the use of control studies in which 0.01 M EACA (an inhibitor of plasminogen activation) was added to the plasma samples. EACA treatment of subject plasma samples or urokinase-enriched plasma reduced activity to below 0.1 U in the euglobulin assay and to 0–14 % of initial activity with the ^{125}I-labeled clot assay.

The peripheral venous samples, tested by the euglobulin clot lysis method, were wholly negative for the presence of urokinase and the results of additional studies with the ^{125}I technique on these samples were similarly interpreted. However, as shown in Fig. 9, anomalous but not statistically significant discrepancies were observed with high antiserum and control serum concentrations used in the ^{125}I-labeled clot lysis experiment. Though these anomalous results were almost certainly due to technical artifact (see Table II in which discrepancies were absent), the ^{125}I technique experiments were insufficient to rigorously exclude the presence of trace amounts of urokinase in plasma. However, both sets of experiments together would indicate the absence of urokinase from the plasma samples.

Additional studies were performed using blood drawn centrally from the distal inferior vena cava below the renal veins and from the renal veins, the latter site being the one at which urokinase concentration, if present, would presumably be the highest. The results, (Table III) with both ^{125}I-labeled clot and euglobulin lysis methods, demonstrate that in none of the six subjects was urokinase detected in renal vein or inferior vena cava plasma. It was also noted that fibrinolytic levels of plasma drawn from renal veins were not significantly different from those in the inferior vena cava, with the single exception of assays from the left renal vein of subject 1 in which elevated values were believed to result from the vasoactive effects of contrast media injected into the vessel immediately before sampling.

Furthermore, since it is known that the 50% plasma clearance rate of plasminogen activator averages 13 ± 5 min (37), it would be expected, were the kidney the principal site of activator production, that the activities between the distal IVC and renal vein samples would differ. However consistent differences between the fibrinolytic activity of the IVC and renal venous samples were not observed.

Our findings indicate that urokinase is immunologically distinct from human plasma activator, and that urokinase could not be detected in peripheral or renal venous plasma. Urokinase is also immunologically distinct from human milk activator and the tissue activator extracted from human adrenal glands. It is inferred that plasma activator is probably derived from tissue activator, especially that found in relation to blood vessels (42–44).

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