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MASTER

A Sensitive, Coupled Assay for Plasminogen Activator Using a Thiol Ester Substrate for Plasmin*

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DISCLAIMER

INTRODUCTION

In parallel with the increased interest during the past decade in plasminogen activator involvement in fibrinolysis, tumorigenesis, inflammatory responses, and the expression of hormonal regulation, there has been a rising interest in sensitive and precise methods for the specific assay of plasminogen activator. Several assays for plasminogen activator employ a direct assay method[1-4]. These are remarkably sensitive methods, yet they suffer in comparison to the sensitivity of coupled methods[5-7]. Coupling the assay with plasminogen not only amplifies the sensitivity by the multiplicative effect of plasmin, but insures that only those proteases specific for plasminogen are assayed. The choice of substrate for plasmin is critical. In general, esters are superior to amides in both K_m and k_{cat}, but they suffer a major deficiency in that they are frequently unstable at pH 7-9, the optimum range for plasmin. Green and Shaw[8] synthesized a thiol ester substrate, thiobenzyl benzyloxycarbonyl-L-lysinate (Z-Lys-SBzl), which combines high k_{cat} with alkaline stability relative to the commonly used esters.

In an effort to characterize the plasminogen activator from hepatoma tissue culture (HTC) and its hormonally-controlled inhibitor several of the known direct and coupled methods were found inadequate by reason of either low sensitivity or imprecision. Using Z-Lys-SBzl in a coupled approach we have developed an assay which is superior to the ^{125}I-fibrinolytic assay. It is also extremely sensitive to plasminogen activator (c.2 x 10^{-17} moles of urokinase) and can be used for routine analysis of purification as well as kinetic and binding studies.

EXPERIMENTAL

Methods. Plasminogen was prepared from fresh frozen plasma by the method of Deutsch and Mertz[9]. Purified plasminogen was exhaustively dialyzed against 1 mM HCl, lyophilized, and stored at -20°. Concentration was routinely determined by absorbance at 280 nM; $E^{1\%}_{280nm}$ = 17.0[10]. Plasmin, the gift of Dr. David Aronson, was subsequently exhaustively dialyzed against 1 mM HCl and stored at -20°. Plasmin concentration was determined by active site titrations using p-nitrophenyl-p-guanidino-

benzoate[11]. Urokinase concentration was determined by active site titrations with methylumbelliferyl p-guanidinobenzoate[12,13], using a ratio fluorometer built by Dr. D. Ballou (Dept. of Biological Chemistry).

Fibrinogen was enzymically iodinated[6] using Tris (60 mM)-KCl (300 mM) buffer, pH 7.6. Unbound iodide was separated by chromatography on Dowex 1 (Cl^-). Specific radioactivity was 9×10^{11} cpm/g. Fibrin plates were prepared and fibrinolysis continuous assays were performed as previously described[6]. Discontinuous fibrinolysis assays were also performed, wherein plasminogen and urokinase were incubated in the absence of the fibrin, then added to the ^{125}I-fibrin well.

Plasminogen activator and inhibitor were prepared from HTC cells as previously described[14] except that serum-free conditioned medium additionally contained bovine serum albumin (1 mg/ml) and cell lysates were dialyzed against 100 mM sodium phosphate, pH 7.4, plus 0.1% Triton X-100 to remove free thiols. HeLa cell plasminogen activator was obtained as serum-free conditioned medium[6].

Assay. The plasminogen activator assay is a two-step procedure. In the first step plasminogen is activated to plasmin. The plasmin-catalyzed hydrolysis of Z-Lys-SBzl in the presence of 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) occurs in the second step. All reactions are typically performed in a 24-well tissue culture dish (each 15 mm diameter). This is not necessary, but convenient when handling many assays simultaneously.

The incubation mixture for the first step typically contains: plasminogen, 1.0 µM; glycine, pH 8.50, 120 mM; bovine serum albumin, 0.5 mg/ml; urokinase, 2.7×10^{-12} M. Total volume is 50 µl. The activity equivalent in plasminogen activator from HTC or HeLa cells may be substituted for urokinase. The reaction is started by the addition of plasminogen to the otherwise complete incubation mixture equilibrated at 37°. Normally this incubation is allowed to react for 45 min.

The second step is initiated by dilution of the incubation mixture with 950 µl of the plasmin substrate solution: Z-Lys-SBzl, 200 µM; DTNB 220 µM; Triton X-100 0.01%; sodium phosphate (pH 7.50) 200 mM; sodium chloride, 200 mM. Normally the

second step is quenched after 60 min by the addition of 100 µl of soybean trypsin inhibitor (1 mg/ml in 1 mM HCl). The absorbance at 412 nm may be read immediately or up to 4 hr later. The extinction coefficient of 14,150 cm^{-1}-M^{-1} was assumed for the thiophenolate product of the reaction of benzyl mercaptan with DTNB[15].

Inhibitors were tested by incubation with the first step incubation mixture prior to the addition of plasminogen. Pseudo-first order rate constants, $k_{app}/[I]$, were determined as previously described[16]. Plasmin standard curves were obtained by substituting plasmin for plasminogen and urokinase in step one.

Other variations from the standard procedure were tested. Z-Lys-SBzl was substituted by D-Val·Leu·Lys p-nitroanilide. 2,2'-dithiodipyridine and 4,4'-dithiodipyridine, dissolved in dimethylformamide, were substituted on an equimolar basis for DTNB. All absorbance measurements were performed on a Gilford spectrophotometer model 2400-2.

Materials. Fibrinogen (plasminogen-free), urokinase (B grade) and DTNB were the products of Calbiochem-Behring (LaJolla, CA). 2,2'- and 4,4'-dithiodipyridine were obtained from Aldrich Chemical Co. (Milwaukee). Carrier-free ^{125}I (sodium salt) was purchased from Amersham-Searle (Chicago). D-Val·Leu·Lys p-nitroanilide was purchased from Kabi Group (Greenwich, CT). Z-Lys-SBzl was synthesized by Peninsula Laboratories (San Carlos, CA) according to the procedure of Green and Shaw[8]. Methylumbelliferyl p-guanidinobenzoate and soybean trypsin inhibitor (B grade) were obtained from Sigma Chemical Co. (St. Louis). Phe·Pro·ArgCH_2Cl was the gift of Dr. Elliott Shaw. All other chemicals were of reagent grade. 24-well culture plates were purchased from Linbro Co. (Bridgeport, CT).

RESULTS

The sensitivity of the assay depends on the combination of a low "spontaneous" rate of Z-Lys-SBzl hydrolysis with a high enzyme rate. Table 1 presents results of a buffer survey in which sodium phosphate yielded both the highest plasmin-catalyzed rate and the lowest "spontaneous" rate. The effect of Triton X-100 (0.001%-2%) on plasmin activity was determined (data not shown). Optimal activity was broadly

centered on 0.01% Triton X-100, which was 150% of the rate of the detergent-free control. There was no effect on the "spontaneous" rate. Sodium chloride has an inhibitory effect on urokinase and HeLa and HTC plasminogen activators, but below 250 mM no effect was observed on plasmin activity (data not shown). Thus 200 mM NaCl was added to the plasmin assay solution which depressed plasminogen activation 78%. Figure 1 presents the time- and plasmin concentration-dependence of the reaction. The rates are linear at least two hours if the substrate is not depleted. Under these conditions K_m is 19 μM, k_{cat} is 45 s^{-1}.

Several modifications of the standard procedure were attempted in search of increased sensitivity (Table 2). The rate with 4,4'-dithiodipyridine was comparable to that with DTNB but the control (zero plasmin) absorbance was four-fold higher, thus limiting its utility at low enzyme levels. Doubling the substrates produced a 10% sensitivity gain, but with almost double the blank value.

Optimizations of the first step in the reaction (plasminogen activation) were performed by coupling these experiments to optimal plasmin assay conditions. Sodium glycinate gave the highest urokinase activity (Table 1). There was no effect of glycine concentration (20-1600 mM) on urokinase rate nor any effect of Triton X-100 above that attributable to its effect on plasmin (data not shown). Figure 2 presents the time- and urokinase concentration-dependence of the reaction. Urokinase activity is not reproducibly linear beyond 45 min, even in the absence of substrate depletion.

A direct comparison was made to a sensitive ^{125}I-fibrin lysis assay using either a continuous or discontinuous procedure (Figure 3). In the discontinuous assays, whether spectrophotometric or radiometric, the rate is directly proportional to the urokinase concentration, thus upward curvature would be expected on semi-log graph paper. In the continuous assays, both proteases are acting simultaneously (two amplifying mechanisms), thus, based on an analysis of the kinetic equations the response presented on semi-log paper should be linear. It is obvious that the spectrophotometric assay is significantly more sensitive than the radiometric assay.

The standard assay was used to evaluate K_m and k_{cat} for the urokinase-catalyzed activation of plasminogen. K_m and k_{cat} values were 1.7 μM and 44 min^{-1}, respectively. These compare favorably with 1.7 μM and 50 min^{-1} reported by Wohl, et al.[17] for the high molecular weight form of urokinase at pH 7.40, 37°. The K_m of the HeLa plasminogen activator was 1.8 μM; V_{max} was 0.16 ng plasminogen activated/min/ng HeLa protein.

Inhibition of urokinase activity by Phe·Ala·$ArgCH_2Cl$ (Figure 4) and the dexamethasone-induced inhibitor from HTC cells (Figure 5) was also analyzed. The ~~pseudo-first-order rate constants~~ bimolecular constant ($k_{app}/[I]$) for the ~~inhibition~~ inactivation of urokinase by the chloromethyl ketone, whether determined by the coupled plasminogen activation assay or by the direct urokinase-catalyzed hydrolysis of Z-Lys-SBzl (26×10^3 and 24×10^3 min^{-1}-M^{-1}, respectively), are in close agreement. These results differ significantly from those of Kettner and Shaw[16], 2.9×10^3 min^{-1}-M^{-1}, however the pH used here was 1.5 units more basic, which could account for a higher rate. Scatchard analysis of the inhibition of urokinase by the dexamethasone-induced inhibitor from HTC cells yields $K_i \sim 10^{-12}$M, but the stoichiometry cannot be calculated because the concentration of inhibitor is unknown. A K_i in this range has been previously reported by Green[18] for the association of Kunitz pancreatic inhibitor (aprotinin) with bovine trypsin, however most protease-inhibitor complexes have lower affinities.

DISCUSSION

In general base-catalyzed reactions such as the "spontaneous" hydrolysis of Z-Lys-SBzl the choice of buffering agent is often critical if the assay is to be used near its limit of sensitivity. The small list of buffers tested here includes a wide range of "spontaneous" rates and suggests the utility of buffer surveys as a general procedure in assay development.

The standard assay employs 1.0 μM plasminogen which is close to the K_m concentration. An approximately two-fold increase in sensitivity would be realized if a ten-fold higher plasminogen concentration were used, but in most cases the higher sensitivity is not needed, and the use of 10 μM or higher substrate would consume

large amounts of plasminogen.

Since there are no pure inhibitors available which are completely selective for plasminogen activators in the presence of plasmin several other methods were used to depress the activation during the Z-Lys-SBzl step. Primarily, plasminogen activation was inhibited by dilution (20-fold), but the pH shift (1 unit off the optimum) and the addition of chloride yielded substantial additional inhibition (two- and three-fold, respectively). The net activator activity in the second step was less than 1% of its initial value.

The sensitivity with the thiol ester compares favorably with the other substrates tested. As an ester, Z-Lys-SBzl has the advantage of the lowest K_m, and highest k_{cat}. Most of the amide substrates have high K_m and low k_{cat} values. Their chief advantage is their chemical stability. Fluorogenic amide substrates might yield a more sensitive assay based on the greater sensitivity of detection. Were a fluorogenic thiol reagent substituted for DTNB the limit of detection of the hydrolysis of Z-Lys-SBzl might be lowered.

The spectrophotometric assay is considerably more sensitive than the radiometric assay. In the direct comparison (Figure 3) there is no difference between 2.7 and 13.5×10^{-17} moles by the radiometric assay, whereas ΔA_{412} is 0.50 between 5.4 and 13.5×10^{-17} moles. Typically in the Z-Lys-SBzl assay the measured difference between 2.7×10^{-17} moles urokinase and the zero urokinase control is >0.100 A_{412}. The assay is also sensitive to other plasminogen activators. 1 ng of protein from HeLa serum-free conditioned medium yields net $\Delta A_{412} = 0.190$. For 1 µg of protein from HTC whole cell extract the net $\Delta A_{412} = 0.04$. 20 ng of serum-free conditioned medium protein from dexamethasone-induced HTC cells inhibits 90% of the activity present in 7×10^{-16} moles of urokinase.

The wide versatility of the method is demonstrated by the ease of determination of K_m and V_{max} values for plasminogen activation and of inhibition constants for the

interaction of the enzyme with natural or synthetic inhibitors. Since the assay is more rapid and sensitive to low levels of enzyme it is more readily adaptable to monitoring enzyme purification than ^{125}I-fibrinolysis.

In summary, the procedure is a sensitive, precise, and rapid method for the analysis and characterization of plasminogen activators. All reagents and materials are inexpensive and commerically available, the colored products are stable, and the measurement is performed on the standard quality spectrophotometer available in almost all laboratories. It is superior to existing methods both in its simplicity and ease of manipulation and in the quality of the resultant data--kinetic constants rather than arbitray units.

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FIGURE LEGENDS

Figure 1. The time- and plasmin concentration-dependence of Z-Lys-SBzl hydrolysis. A. Reactions were stopped at the time indicated by addition of soybean trypsin inhibitor. Reaction volume was 1.0 ml. [Z-Lys-SBzl] = 200 µM, [DTNB] = 220 µM. Plasmin concentrations were 0.1 (●), 0.3 (○), 0.5 (▣), and 0.8 (□) nM. B. Conditions were as stated in Figure 1A. The reactions were stopped at 20 (●), 40 (○), 60 (▣), 90 (□), and 120 (△) min. The lines were determined by linear regression fit of the data and are drawn to encompass only the linear portion of the data. All data points represent the average of duplicates.

Figure 2. The time- and urokinase concentration-dependence of the plasminogen activation reaction coupled to the hydrolysis of Z-Lys-SBzl by plasmin. The conditions for the thiol ester hydrolysis are described in the Experimental section. A. The reaction was terminated at the indicated time by addition of 950 µl of the plasmin reaction solution as described in the text. Urokinase concentrations were 1.08 (●), 2.7 (○), 5.4 (▣), and 10.8 (□) pM. B. Conditions were as described in Figure 2A. The activations were quenched at 15 (●), 30 (○), and 45 (▣) min. Data at 60 and 75 min are not presented since the response is not a linear function beyond 45 min (see Figure 2A). All times and concentrations relate to the 50 µl activation reaction. The lines represent a linear regression fit of the data and are drawn to encompass only the

linear portion of the data. All data points represent the average of duplicates.

Figure 3. A comparison of spectrophotometric and radiometric coupled assays for urokinase. The spectrophotometric assay (⊗) was performed according to the standard procedures outlined in the text. The volume of continuous radiometric assay is 1.0 ml in 100 mM sodium glycinate, pH 8.50 with 50 nM plasminogen. Total CPM released at 60 (□) and 105 (▨) min of incubation are presented. The latter time is the total time required by the discontinuous assays. In the discontinuous radiometric assay (○) plasminogen (1.0 μM) was activated by urokinase in the glycine buffer for 45 min then a 50 μl aliquot was added to the ^{125}I-fibrin-coated well containing 950 μl of 200 mM sodium phosphate and 200 mM NaCl. Total CPM released after 60 min in the presence of fibrin are presented. All reactions were performed at 37°. All radiometric points are averages of triplicates. All spectrophotometric points are averages of duplicates.

Figure 4. The inactivation of urokinase by Phe·Ala·ArgCH_2Cl. Prior to activity determination by the standard assay, urokinase (1.08×10^{-10} M) was incubated with the indicated concentration of the tripeptidyl inhibitor in 80 mM sodium glycinate, pH 8.50 with 1 mg/ml bovine serum albumin, 25°. At the indicated times 5 μl of the reaction solution were diluted into 85 μl of the same glycine buffer at 0°. At the completion of the inactivation reaction (8 min) all activation reactions were initiated simultaneously by addition of 10 μl of 10 μM plasminogen and removal to a 37° incubator. 900 μl rather than 950 μl of plasmin reaction solution was used. $k_{app}/[I] = 260 \times 10^3$ min^{-1}-M^{-1}.

Figure 5. Scatchard analysis of the inhibition of urokinase by the plasminogen activator inhibitor from hepatoma tissue culture cells. The inhibitor test solution was the serum-free conditioned medium from dexamethasone-induced (10^{-7} M, 16 hr) cells. 1-5 μl of test solution were incubated with urokinase (2.7×10^{-16} moles) for 15 min at 25° in 45 μl total volume. The activation reaction was started by the addition of 5 μl of 10 μM plasminogen and shift to 37°. E is the concentration of active urokinase; r is the concentration of inactive urokinase.

Table 1

Effects of Various Buffers on Plasmin and Urokinase Activity[a]

	Relative Rates of Hydrolysis (% of Rate in Sodium Phosphate)		
	Plasmin		Urokinase
Buffer	Enzymic	Non-enzymic	Enzymic
Phosphate (Na)	100	100	100
MOPS[b]	92	100	118
Barbital[b]	93	250	-
Tris[b]	76	420	102
Glycine[b]	79	220	143
Triethanolamine[b]	87	290	100
Tricine[b]	86	290	104
Pyrophosphate[b]	75	120	69
Phosphate (K)[c]	82	-	100
HEPES[c]	84	-	126
TES[c]	92	-	116

[a] All assays were performed at 37°. Plasmin activity was determined by continuous recording of A_{412}. Urokinase activity was determined by the standard plasminogen activation assay described herein. In each case the pH profile (6.5 - 10) for sodium phosphate was obtained for comparison. The pH optima were: plasmin, 7.5; urokinase, 8.5 - 9.0. There was no variation in the blank rate in the urokinase assay. All buffers are 100 mM. pH was measured before and after incubation, and these values agreed ± 0.05.

[b] pH 8.50

[c] pH 7.50

Table 2

Variations on the Standard Plasmin-catalyzed Hydrolysis of Z-Lys-SBzl Coupled with DTNB[a]

Substrate/Chromogen	Sensitivity (A/nM)
Z-Lys-SBzl/DTNB	2000
Z-Lys-SBzl/DTNB (2X)[b]	2260
Z-Lys-SBzl/2,2'-dithiodipyridine[c]	700
Z-Lys-SBzl/4,4'-dithiodipyridine[d]	2030
D-Val·Leu·Lys p-nitroanilide[e]	600

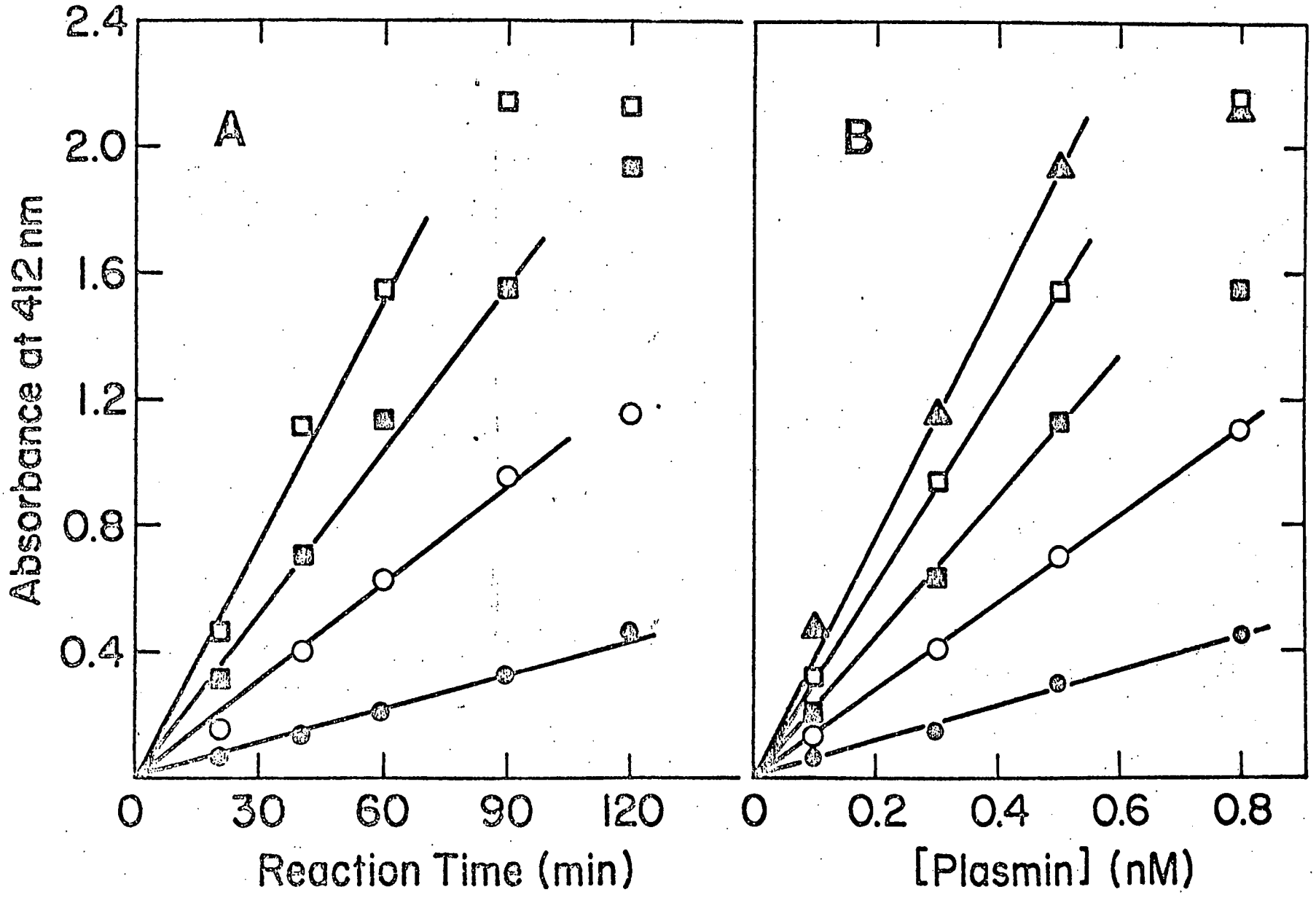
[a] Plasmin was varied 0-1 nM in 60 min assays which were terminated by addition of 100 µl soybean trypsin inhibitor as detailed in the Experimental section. DTNB reactions were measured at 412 nm.

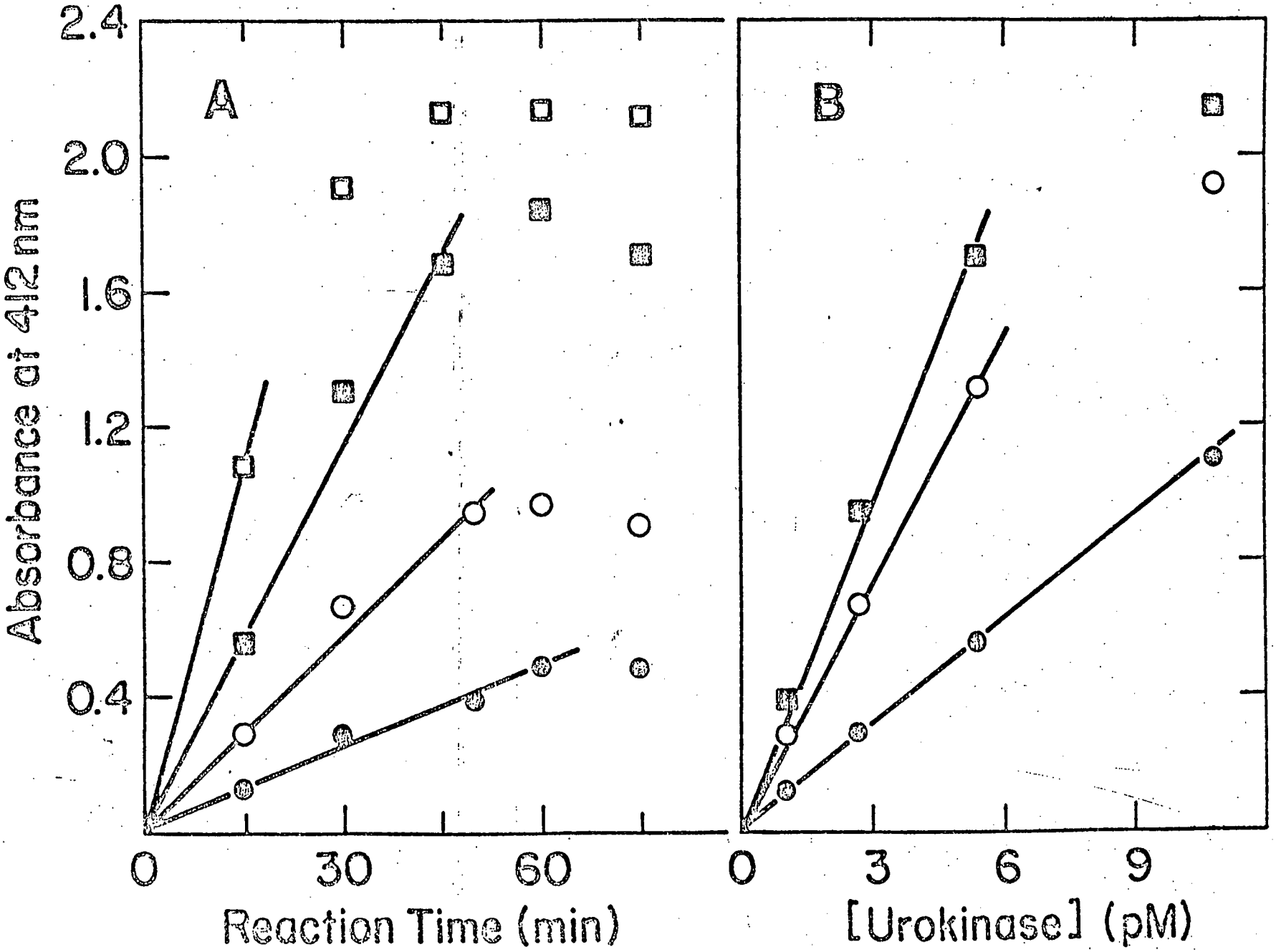
[b] Both reagents were at twice the standard concentrations.

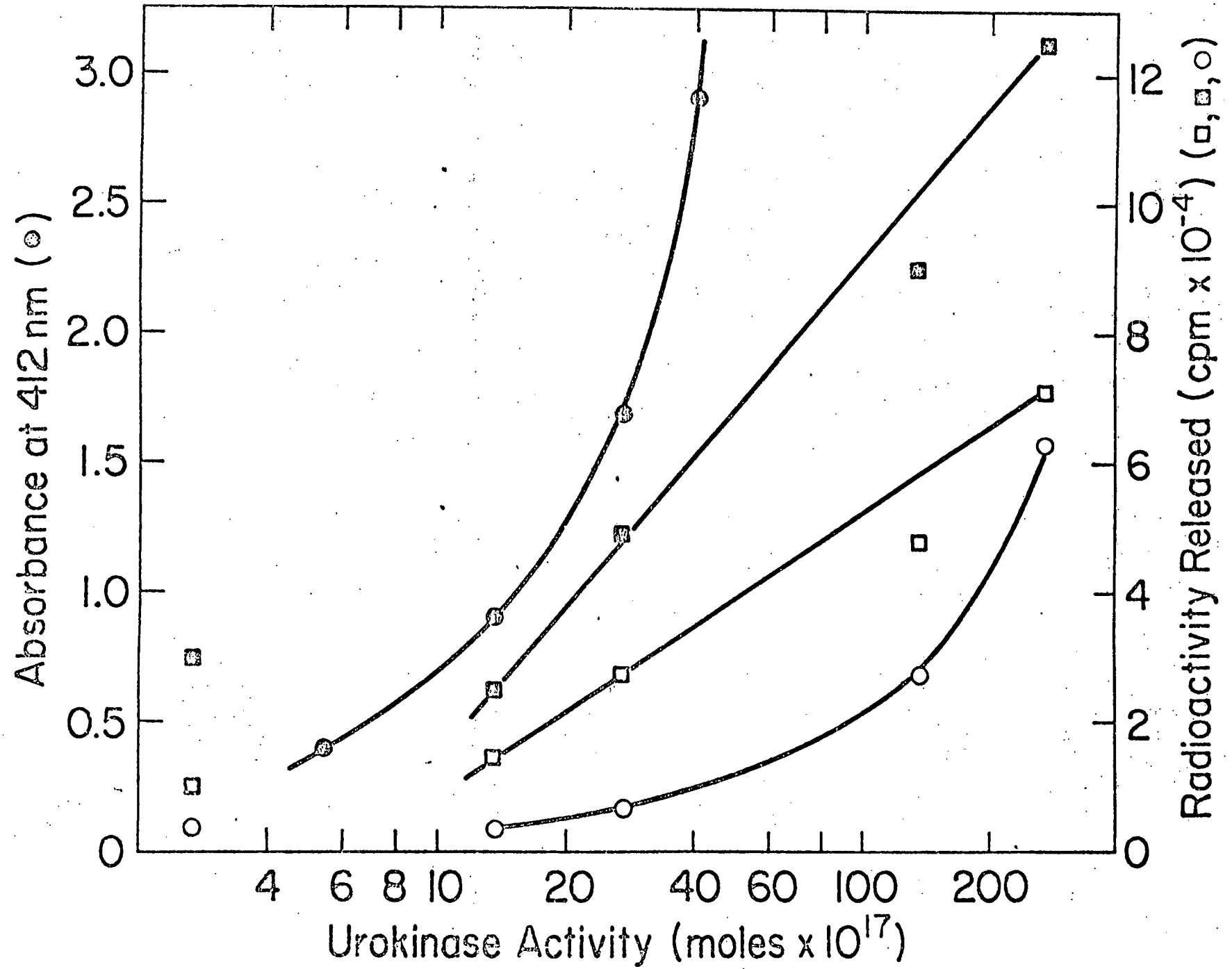
[c] Measured at 270 nm, the wavelength of maximum ΔA.

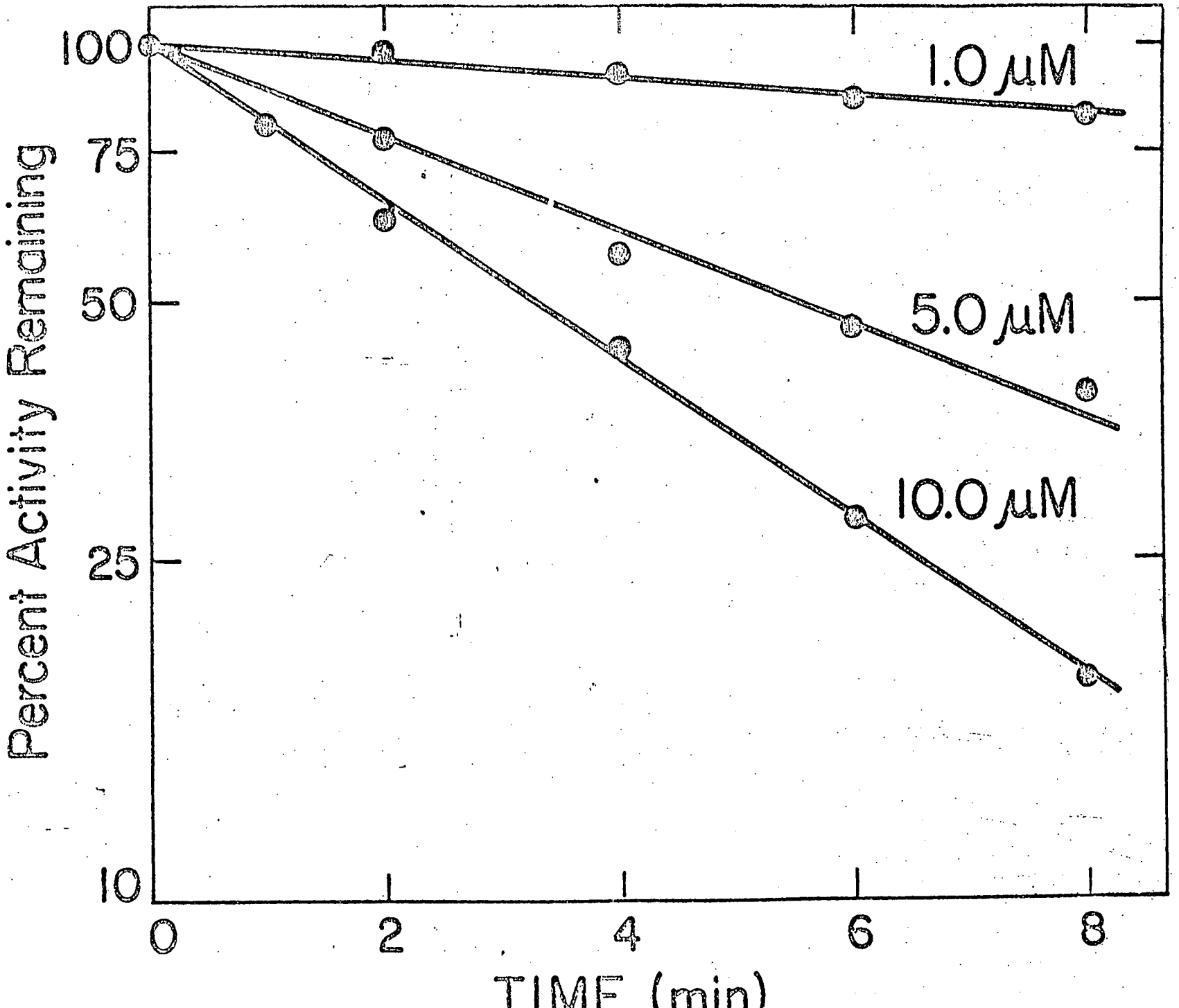
[d] Measured at 324 nm, the wavelength of maximum ΔA.

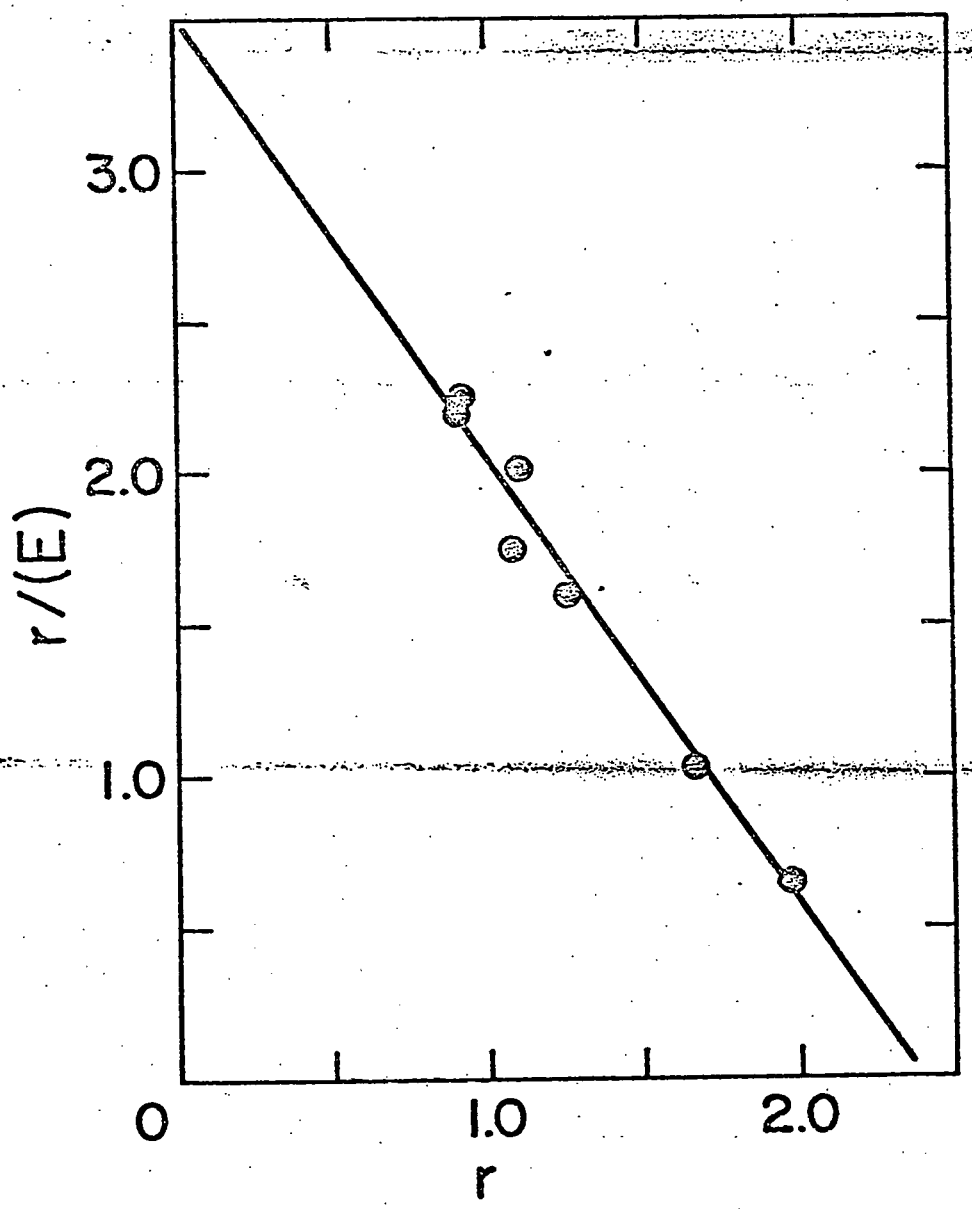
[e] Measured at 383 nm, the wavelength of maximum ΔA.


A
B
Absorbance at 412 nm
2.4
2.0
1.6
1.2
0.8
0.4
0
30
60
90
120
Reaction Time (min)
0
0.2
0.4
0.6
0.8
[Plasmin] (nM)


A
B
Absorbance at 412 nm
2.4
2.0
1.6
1.2
0.8
0.4
0
30
60
Reaction Time (min)
0
3
6
9
[Urokinase] (pM)


Absorbance at 412 nm (●)
Radioactivity Released (cpm x 10⁻⁴) (□,○)
Urokinase Activity (moles x 10¹⁷)
0
0.5
1.0
1.5
2.0
2.5
3.0
0
2
4
6
8
10
12
4
6
8
10
20
40
60
100
200


100
75
50
25
10
Percent Activity Remaing
0
2
4
6
8
TIME (min)
1.0 μM
5.0 μM
10.0 μM


3.0
2.0
1.0
0
1.0
2.0
r/(E)
r