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KIDNEY UROKINASE ACTIVITY FOLLOWING ACUTE EXPOSURE TO LEAD

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Abstract—Urokinase activity was measured in kidney homogenate fractions obtained 2 days after injecting rats with 0.5 mg Pb^{2+}/100 g body weight. The activity was higher in the membrane-containing fractions than in the soluble supernatant fractions and was markedly higher in the preparations derived from the lead-treated rats. The kidney poly(A)$^+$ RNA was obtained from these animals and translated in a rabbit reticulocyte lysate system. In the preparations obtained from the lead-treated rats, there was an increased synthesis of a protein, believed to be urokinase, of pI 8.6 and molecular weight (M_r) 45,000.

The injection of 0.5 to 3.0 mg Pb^{2+}/100 g body weight into mice and rats is associated with changes in the renal proximal tubules. These are changes in the activity of the renal brush border enzymes [1], an increase in mitotic figures, prominence of the nuclei, and the appearance of intranuclear inclusions (reviewed by Choie and Richter in Ref. 2). Subacute exposure to lead salts daily for 1 month results in pathological changes in the renal proximal tubules that include swelling of the lysosomes and changes in the mitochondria and aptical microvilli, as well as persistence of the intranuclear inclusions [3–5].

In mouse kidney following a single injection of lead acetate (0.5 mg Pb^{2+}/100 g body wt), there is an increase in the incorporation of labeled precursors *in vivo* into DNA, RNA, and protein [6, 7]. In rat kidney, the machinery for peptide synthesis exhibits increased activity in homogenates prepared from the Pb^{2+}-treated animals [8]. Our earlier results provided evidence that the charging reaction, as well as the elongation steps and the ribosomes, is more active following lead exposure. In addition, the translatability of poly(A)$^+$ RNA is increased in kidney preparations derived from lead-treated rats [8]. Urokinase mRNA represents about 10% of the kidney poly(A)$^+$ RNA [9], and lead treatment affects the kidney mRNA coding for proteins of the approximate molecular weight of urokinase. The present experiments were carried out to see whether lead treatment would influence urokinase activity and whether the increased translatability of poly(A)$^+$ RNA after lead would be for urokinase-like proteins.

MATERIALS AND METHODS

Animals and chemicals. Male Wistar rats (80–100 g) received food (Purina 5001 Rat Laboratory Chow) and water *ad lib.* Lead acetate [Pb $(CH_3COO)_2 \cdot 3H_2O$], freshly dissolved in distilled water at a concentration of 5 mg/ml, was administered in one intraperitoneal injection of 1 mg/100 g body weight in 0.2 ml (i.e. 0.546 mg Pb^{2+}/100 g body wt). Control rats received the same volume of water (0.2 ml/100 g body wt). The kidneys were obtained 48 hr later; they were pooled either from six control and six treated rats (mRNA isolation) or from two control and two treated rats (homogenate studies). Rabbit reticulocytes were prepared as described previously [10].

L-[U-^{14}C]Leucine (339 Ci/mole) and protosol were obtained from the Amersham Corp., Oakville, Ontario, Canada. Human urinary urokinase (5.5 Sigma units/mg), porcine plasminogen (2 Sigma units/mg), fibrinogen from bovine blood (Type IS), bovine thrombin (587 NIH units/mg), and α-casein from bovine milk were from the Sigma Chemical Co., St. Louis, MO, U.S.A. The other chemicals were obtained as previously described [10].

Kidney homogenate fractions. The kidneys were rapidly removed and chilled in an iced container; all subsequent steps were carried out in the cold. The tissue was homogenized in 3 vol. of buffer 1 containing 0.05 M Tris–HCl (pH 7.5) and 0.1 M NaCl together with 0.25 M sucrose, and the homogenate was filtered through cheesecloth before using it for the assay of urokinase activity. In other experiments, this homogenate was centrifuged at 600 *g* for 10 min to give a crude nuclear fraction (P6). The supernatant fraction (S6) was centrifuged for 15,000 *g* for 15 min, yielding a supernatant fraction (S15) and a mitochondrial-lysosomal fraction (P15).

Kidney mRNA preparation. Kidneys were homogenized as described previously, using autoclaved glassware, in buffer A [0.25 M sucrose, 50 mM Tris–HCl (pH 7.6), 80 mM potassium acetate, 6 mM magnesium acetate] [10]. The homogenate was centrifuged at 15,000 *g* for 20 min at 4° in a Sorvall type SS 34 rotor (r_{av} 5.1 cm). The resultant postmito-

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chondrial supernatant fraction was made 1% (w/v) in sodium deoxycholate and was then centrifuged at 100,000 *g* for 70 min. The pellet was resuspended in buffer B [0.1 M Tris–HCl (pH 9.0), 0.1 M NaCl, 1 mM EDTA] to a final RNA concentration of 20 A_{260} units/ml. SDS* was added at a final concentration of 1.5% (w/v), and the temperature was brought to 20°. To this was added an equal volume of the deproteinizing solution composed of phenol–chloroform–isoamyl alcohol (50:50:1). After shaking it at room temperature for 10 min, the organic phase was separated by centrifugation at 12,000 *g* for 10 min and removed by aspiration. The aqueous phase and interface were re-extracted once more with the same organic phase. Potassium acetate [20% (w/v), pH 5.5] was added to a final concentration of 2%. RNA was precipitated overnight at −20° by the addition of 2 vol. of ethanol. The RNA precipitate was dissolved in water prior to assaying it. This RNA was used to obtain poly(A)$^+$ mRNA by oligo(dT) cellulose chromatography as described previously [10]. The similar yields of poly(A)$^+$ RNA that were obtained from control and treated animals were dissolved in a small amount of distilled water so that 5–20 μg RNA from each preparation could be added to the reticulocyte lysate for translation as described below.

mRNA-dependent translation. The reticulocyte lysate was treated with micrococcal nuclease as described by Pelham and Jackson [11], and the mRNA was assayed as follows. The reaction mixture (50 μl) contained 10 mM Tris–HCl (pH 7.4), 2 mM magnesium acetate, 80 mM potassium acetate, 0.3 mM GTP, 1.3 mM ATP, 5 mM creatine phosphate, 5 μg creatine phosphokinase, 25 μM hemin-Cl, 30 μM of each of nineteen amino acids exclusive of leucine, 0.25 μCi [^{14}C]leucine and 25 μl lysate. Incubation was generally at 26° for 1 hr. The reaction was stopped by applying aliquots of the reaction mixture directly to Whatman 3 MM filter paper. The filters were soaked in ice-cold 10% (w/v) trichloroacetic acid containing 1 mM carrier leucine, heated at 95° for 5 min in 5% (w/v) trichloroacetic acid, and rinsed in cold 5% trichloroacetic acid, ethanol and acetone. The filters were dried, stirred at 30° in 15% (v/v) H_2O_2 for 2 min to decolorize the hemoglobin, rinsed in ethanol and acetone, and dried for counting in scintillation fluid described previously [12] in a Searle model 6892 liquid scintillation counter at 90% efficiency.

Gel electrophoresis and isoelectric focusing. The products of translation contained in 25 μl of the reaction mixture were analyzed by electrophoresis in cyclindrical polyacrylamide gels (5 mm diameter × 100 mm long) containing SDS as described by Laemmli [13]. The discontinuous system consisted of a 10% (w/v) acrylamide gel, pH 8.8, on which a 3% (w/v) polyacrylamide spacer gel, pH 6.8, was layered. Samples and standards were electrophoresed for 4 hr at 22° at a constant current of 2.5 mA/gel [14]. Following fixation in 50% trichloroacetic acid, the gels were sliced (2 mm), solubilized, and counted as described above. In other experiments, isoelectric focusing was carried out similarly in cylindrical gels, after which the gels were placed on polyacrylamide slab gels and subjected to electrophoresis as described by O'Farrell [15]. The gels were stained with Coomassie brilliant blue, destained by diffusion in 7.5% (v/v) acetic acid, dried, and subjected to radioautography using Kodak no screen X-ray film.

Protein determination and urokinase assay. Protein concentration was determined by the method of Lowry *et al.* [16] using bovine serum albumin as a standard. The urokinase assay was performed by a modification of the method of Highsmith [17]. Kidney homogenate fractions (100 μl), with or without 0.5% (v/v) Triton X-100 [18], were incubated with plasminogen (100 μl buffer 1 containing 20 μg) for 30 min at 37°, at which time α-casein solution (100 μl buffer 1 containing 120 μg) was added for further incubation for 10 min at 37°. The reaction was stopped, and the protein was precipitated by the addition of 1 ml of cold 0.5 M perchloric acid. After 10 min on ice, the mixture was centrifuged at 3000 *g* for 10 min to sediment the protein.

The absorbancy at 275 nm of the resultant supernatant fraction was determined in a Zeiss PM Q II spectrophotometer after subtraction of appropriate blanks. One CTA unit of plasmin activity releases 0.1 μmole tyrosine/min (0.132 is the absorbancy of 0.1 μmole tyrosine/ml at 275 nm). One urokinase unit activates approximately 0.2 CTA units of plasminogen [19].

The fibrinolytic assay was carried out as described by Johnson *et al.* [19] in a buffer containing 0.06 M Tris–HCl (pH 7.5) and 0.09 M NaCl. The reaction mixture for clot formation at 22° contained 0.1 ml thrombin solution (2 μg), 0.2 ml fibrinogen solution (1 mg), and 0.5 ml plasminogen (1.7 mg). Then 0.2 ml kidney homogenate fraction was added with mixing, and the time for the clot to disappear at 37° was measured. Urokinase activity was proportional to the log of the time for lysis [19].

RESULTS

Figure 1 shows the release of tyrosine from α-casein by digestion with plasminogen that had been activated by kidney homogenate (i.e. urokinase activity). There was a linear increase with incubation time from 10 to 60 min using control kidney. With kidney homogenate from the Pb^{2+}-treated rats, the urokinase activity was significantly higher and was linear from 20 to 60 min. The addition of increased amounts of homogenate protein up to about 3 mg from control rats increased α-casein digestion but larger amounts had little further effect. When homogenate protein from Pb^{2+}-treated rats was used and compared to that from controls, a significant increase in α-casein digestion was detected at each point up to 5 mg homogenate protein. When measured in the fibrinolytic assay, the plasminogen activator activity of the homogenates from the Pb^{2+}-treated rats was twice that of the control rats, thus confirming the results of the α-casein digestion assay.

* Abbreviations: SDS, sodium dodecyl sulfate; pI, isoelectric point pH; P6, nuclear fraction; S6, postnuclear supernatant fraction; P15, mitochondrial-lysosomal fraction; and S15, postmitochondrial supernatant fraction.

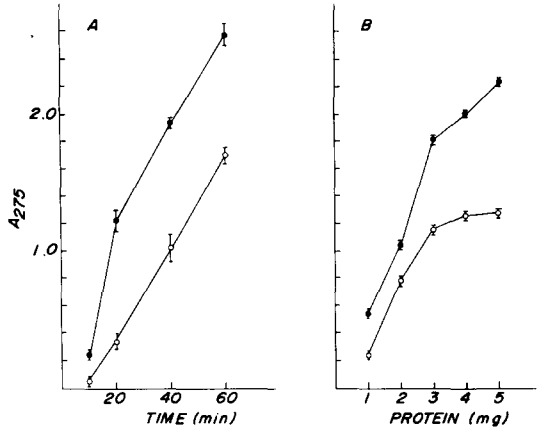

Fig. 1. Urokinase activity of kidney homogenates from control rats and from rats following the injection of lead acetate. (A) Incubation of 2 mg protein with plasminogen for various times prior to the addition of α-casein, as described under Materials and Methods. (B) Incubation for 30 min of various amounts of protein as described under Materials and Methods. Points plotted are mean ± S.E.M. for four preparations. Key: (○) control; and (●) lead.

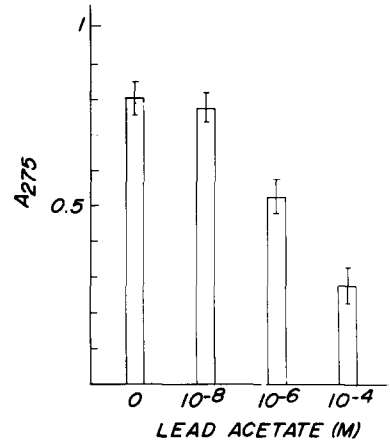

Fig. 2. Urokinase activity of kidney homogenates from control rats following the addition of lead acetate *in vitro*. Kidney homogenate (2 mg) was incubated for 30 min as described under Materials and Methods. Lead acetate was added to the homogenate in the molar concentrations indicated. Bars are means ± S.E.M. for four preparations.

The distribution of urokinase activity was studied in homogenate fractions of kidneys from control and Pb^{2+}-treated rats (Table 1). The activity was increased significantly in the kidney fractions from the lead-treated rats when the pellets from the 600 *g* step (P6) and from the 15,000 *g* step (P15) were studied. The former contains cell membranes, nuclei and cell debris while the latter contains mitochondria and lysosomes. While the supernatant fraction from the 600 *g* step (S6) showed a small but statistically significant increase in homogenates from the lead-treated rats compared to control rats, there was no such change in the supernatant fraction from the 15,000 *g* step (S15). The greatest urokinase activity was seen in P15, the sediment containing mitochondria and lysosomes, both in fractions from the control rats and from the lead-treated rats. The addition of a membrane-solubilizing agent such as Triton X-100 to the P6 and P15 pellets resulted in about twice as much urokinase activity both in the control fractions and in the fractions obtained from the lead-treated rats. This increased proteolytic acitivity after detergents has been described previously [18, 20].

The kidney weight and the protein concentration of the kidney homogenate fractions of the lead-treated rats were not different statistically from those of the control rats. The total urokinase activity, calculated per 100 mg wet wt was greatest for the pellet from the 15,000 *g* step, i.e. the fraction containing the mitochondria and lysosomes, both for control and lead-treated preparations, in agreement with previous reports [18, 21].

Since the urokinase activity of the mitochondrial-lysosomal fraction (P15) was higher in kidney preparations from Pb^{2+}-treated rats than from control rats, just as in kidney preparations treated with a detergent, it seemed possible that Pb^{2+} in the kidney might act directly on membranes to release urokinase activity. With this in mind, Pb^{2+} was added directly to kidney homogenates obtained from control rats (Fig. 2). The addition of 10^{-8} M Pb^{2+} did not affect urokinase activity. However

Table 1. Urokinase activity of kidney homogenate fractions from control rats and from rats following the injection of lead acetate*

	Enzyme activity (A_{275}/mg protein)					
	No Triton			Triton X-100		
Fraction	Control	Pb^{2+}	% Increase	Control	Pb^{2-}	% Increase
S6	0.55 ± 0.015	0.66 ± 0.009†	20	0.52 ± 0.025	0.64 ± 0.020†	23
P6	0.68 ± 0.040	1.12 ± 0.017†	65	1.61 ± 0.160	2.12 ± 0.040†	32
S15	0.76 ± 0.042	0.77 ± 0.035	Nil	0.88 ± 0.046	0.77 ± 0.030	Nil
P15	1.42 ± 0.046	1.97 ± 0.046†	39	2.36 ± 0.150	3.74 ± 0.040†	58

* Various homogenate fractions were prepared and incubated for 30 min as described under Materials and Methods. Abbreviations: S6, supernatant fraction from 600 *g* sedimentation step; P6, crude nuclear and cell membrane fraction; S15, postmitochondrial supernatant fraction; and P15, mitochondrial and lysosomal fraction. Values are expressed as mean ± S.E. for three preparations.

† $P < 0.05$, by Student's *t*-test.

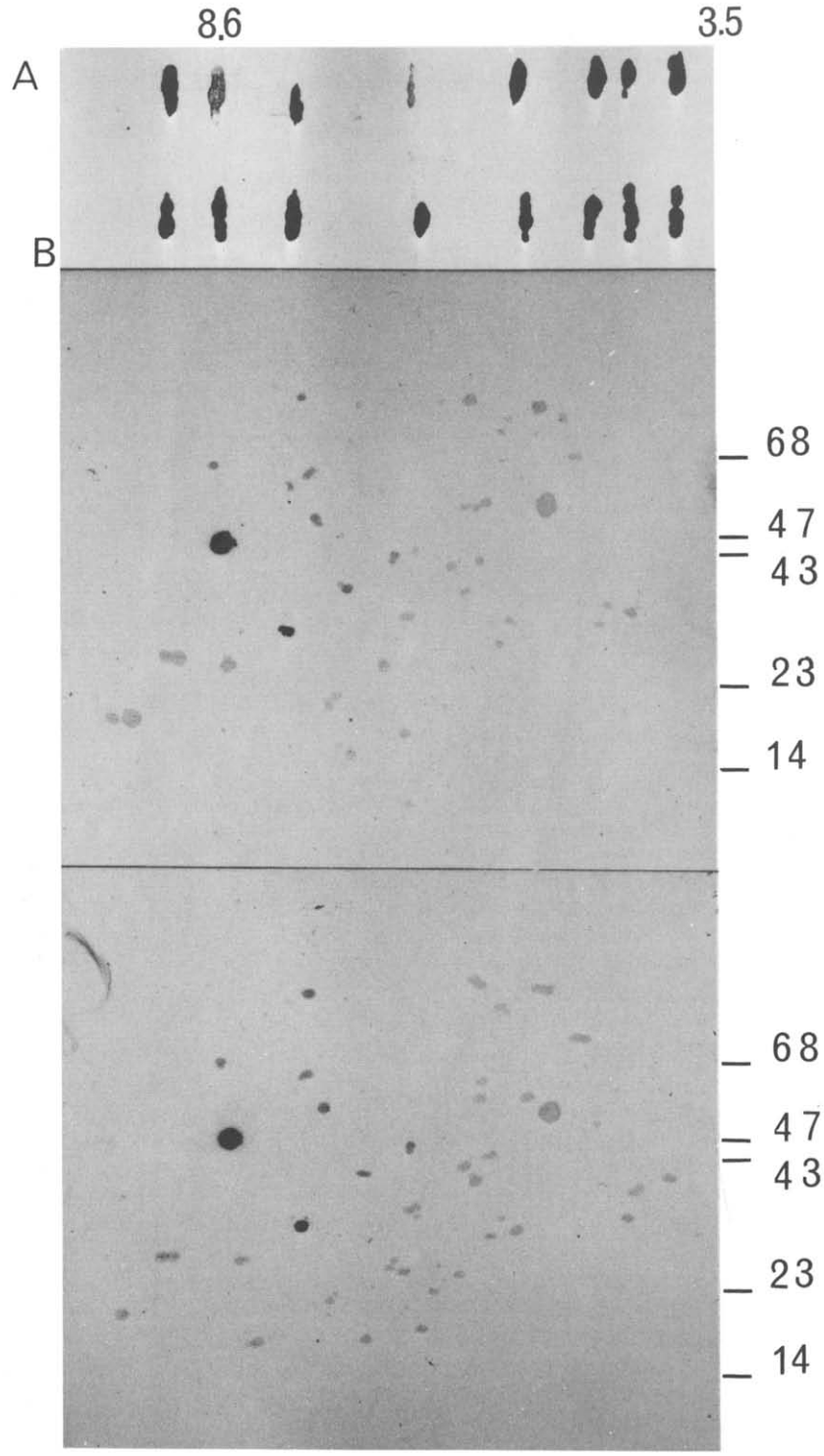

Fig. 3. Isoelectric focusing and SDS gel electrophoresis followed by radioautography of proteins synthesized from [^{14}C]leucine in mRNA-dependent reticulocyte lysates in response to kidney poly(A)$^+$ RNA. Poly(A)$^+$ RNA (5 μg) from control or treated animals was incubated in 25 μl lysate containing [^{14}C]leucine as described in Materials and Methods and the lysate subjected to (A) isoelectric focusing or (B) isoelectric focusing followed by SDS gel electrophoresis. Control lysate (upper gel A and upper gel B) contained 10,000 dpm. Lysate from lead-treated animals (lower gel A and lower gel B) contained 18,000 dpm. The pH and radioactivity of the gel slices following isoelectric focusing was determined on similar gels after soaking in distilled water as described previously [8]. The molecular weights (M_r) following SDS gel electophoresis in the second dimension were determined using marker proteins; bovine serum albumin (68,000), human urokinase (47,000), ovalbumin (43,500), trypsin (23,300) and lysozyme (14,300). Exposure time at $-70°$ was 6 hr (A) and 14 hr (B).

higher concentrations (10^{-6} M Pb^{2+} and 10^{-4} M Pb^{2+}) caused statistically significant ($P < 0.05$) decreases in urokinase activity. Thus, the increased activities seen in homogenates from animals treated with Pb^{2+} *in vivo* are unlikely to arise from Pb^{2+} acting directly on the enzyme, in view of the opposite direction of the effect when Pb^{2+} was added *in vitro*.

Poly(A)$^+$ mRNA was obtained from the kidneys of control rats and of rats injected with lead and translated in a rabbit reticulocyte lysate using [^{14}C]leucine as a precursor of proteins. The newly labeled peptide products of the translation assay were separated first by isoelectric focusing gels (Fig. 3A) and then by SDS polyacrylamide slab gels (Fig. 3B). In the former, it can be seen that there were eight major groups of labeled proteins by radioautography, the most basic proteins being of isoelectric points of approximately pH 9.0, 8.6, and 8.0.

When the isoelectric focusing gels were sliced and counted, the preparations from the lead-treated rats were 80% more radioactive for protein of pI 8.6 (i.e. 1050 dpm for control and 1810 dpm for treated) and 60% more radioactive for protein of pI 8.0. The other areas were less than 40% more radioactive on preparations following lead treatment.

Isoelectric focusing gels similar to these were subjected to SDS gel electrophoresis in the second dimension and radioautographed. Many labeled peptides from the isoelectric focusing gels were removed during SDS gel electrophoresis because of small size. Since fluorography and long exposure times were not used, the labeled products (approximately 50) of only the most abundant poly(A)$^+$ mRNA species were detected. The poly(A)$^+$ mRNA directed relatively major amounts of incorporation of [^{14}C]leucine into two proteins of isoelectric point and mol. wt of 8.6 and 45,000, respectively, and of 8.0 and 32,000, respectively. These two proteins showed increased density by radioautography in samples derived from Pb^{2+}-treated rats and possibly are the major two forms of urokinase such as described in other animal species [22, 23]. Because the amount of protein produced in these experiments is extremely low, it is not possible to perform a direct urokinase assay on the gels [24, 25]. When marker proteins were subjected to the same electrophoretric procedures, human urokinase had a pI of 8.6 and a mol. wt (M_r) of approximately 47,000 with a second band that exhibited a slightly lower M_r. When the SDS gel electrophoresis was carried out in tube gels and the 45,000 dalton band was eluted and used as a source of protein for isoelectric focusing gels, the dpm per slice for proteins of pI 8.6–9.0 was 1000 dpm using preparations from Pb^{2+}-treated rats compared to 500 dpm using preparations from control rats.

DISCUSSION

Plasmin formed by plasminogen activators is believed to play a role in a number of processes such as fibrinolysis, cell migration and neoplasia. The fibrinolytic activity of human urine was shown to be due to the prescence of a plasminogen activator called urokinase [26, 27] and recently the purified enzyme has been isolated from human plasma [24]. Plasminogen activators from tissues other than kidney appear to be distinct from the urokinase, and their RNA has been prepared from human melanoma cells [28]. Cultured renal tubular cells from porcine kidney produce large amounts of plasminogen activator, especially under the influence of the hormone, calcitonin (25); human kidney cell cultures, human urine and rabbit kidney have also provided sources of plasminogen activator activity [18, 29]. We are not aware of reports for rodent kidney, although rodent brain apparently has plasminogen activator activity [30]. Moreover, human kidney and cultured human embryonic kidney have provided poly(A)$^+$ mRNA which directs the synthesis of urokinase [9].

The activity of the plasminogen activator in kidney homogenates was measured by α-casein digestion and by fibrinolysis. Preparations from control rats and rats that had received one i.p. injection of Pb^{2+} (0.5 mg Pb^{2+}/100 g body wt) 48 hr prior to the experiment were used, and there was a marked increase in the activity in the Pb^{2+}-treated animals. Several observations suggest that this proteolytic activity was not due to an increase in non-specific proteolytic enzymes but rather was due specifically to an increase in the plasminogen activator. For example, the assays used were dependent upon the presence of plasminogen. Moreover, the poly(A)$^+$ mRNA that was obtained from the Pb^{2+}-treated animals was more active in overall translation [8], and, specifically, in directing the incorporation of [^{14}C]leucine into a protein of an approximate molecular weight of 50,000 and a pI of 8.6. Increased incorporation is also found with crude kidney homogenate preparations [8], and this would not be the case if increased amounts of various proteolytic enzymes were present in these preparations, since newly synthesized peptides thus would be hydrolyzed rapidly. The location of the greatest plasminogen activator activity in the lysosomal-mitochondrial fraction resembled that reported by Maciag *et al.* [21] for rabbit kidney and cultured pig kidney [18, 21].

The molecular weight and the pI of the rat kidney proteins, whose mRNA is more translatable following Pb^{2+}, resemble those reported for human urokinase [9, 24]. Similarly, rabbit kidney plasminogen activator resembles human urokinase [31]. Thus, it appears unlikely that the rat kidney plasminogen activator is of the tissue type. Neither the urinary urokinase nor the tissue plasminogen activators of the rat have been characterized, to our knowledge. The low quantity available in rat kidney makes this difficult. Nevertheless, the molecular weight and pI of the protein coded by a major proportion of kidney poly(A)$^+$ in the rat suggest that the protein is similar to human urokinase.

The presence of a cytosolic Pb^{2+}-binding protein of mol. wt 63,000 [32] and the presence of lead-induced intranuclear inclusion proteins (mol. wt 32,000 and pI 6.3) [5] appear unrelated to the protein described here of mol. wt 45,000 and pI 8.6. The events resulting from a single exposure to a low dose of Pb^{2+} could be triggered, e.g. by Pb^{2+} directly activating a serine protease such as plasminogen activator. This seems not to occur since Pb^{2+} added directly to tubes decreased plasminogen activity (Fig. 2). Similarly, Pb^{2+} might directly activate mRNA

but, if so, it would be expected that the mRNA would yield a number of products rather than one major protein. If, for example, Pb^{2+} stimulated the release of calcitonin through its interaction with Ca^{2+} and phosphate, then this might possibly explain the increased plasminogen activator activity [25]. In any case, not only are some brush border enzymes reduced in their activities but some enzymes, such as alkaline phosphatase of brush border [1] and plasminogen activator of membrane fractions, are increased in activity in the kidneys of rats that have received one injection of Pb^{2+} (0.5 mg/100 g body wt).

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