Br. J. Pharmac. (1975), 53, 229-234

SYNTHESIS OF KALLIKREINS BY RAT KIDNEY SLICES

K. NUSTAD[1] & KIRSTEN VAAJE
Institute for Surgical Research and Medical Department B, Rikshospitalet, Oslo 1, Norway

J.V. PIERCE
Section on Physiological Chemistry, Experimental Therapeutics Branch, National Heart and Lung Institute, Bethesda, Md. 20014, USA

1 Four radioactive kallikreins were isolated from rat kidney slices incubated with [^{3}H]-L-leucine.

2 The kallikreins were purified by procedures previously used for the isolation of rat urinary kallikreins (Nustad & Pierce, 1974), and by affinity chromatography on a column of insolubilized anti-rat urinary kallikrein.

3 The kidney kallikreins resembled the urinary kallikreins in their relative amounts, isoelectric points and electrophoretic mobilities on polyacrylamide disc gels.

4 The data indicate that the kidney synthesizes four kallikreins which are released into urine. The kallikreins are not changed by passage through the lower urinary tract.

Introduction

We have recently described the purification of four rat urinary kallikreins which were immunologically and biologically identical, but behaved differently in sodium dodecyl sulphate polyacrylamide disc gel electrophoresis and electrofocusing (Nustad & Pierce, 1974).

These findings and the preparation of specific antibody to the urinary kallikreins made it possible to test in a more definitive manner the hypothesis that urinary kallikrein is derived from the kidney (Werle & Vogel, 1960; Carvalho & Diniz, 1966; Nustad, 1970; Croxatto & Noe, 1972). The present study describes the isolation and characterization of kallikreins synthesized in rat kidney slices incubated with [^{3}H]-L-leucine, and the preparation and utilization of columns of purified anti-rat urinary kallikrein immobilized on agarose.

Methods

Preparation of immunoadsorbent

Purified sheep antibody to rat urinary kallikrein B_3 was obtained as described in Nustad & Pierce (1974). This procedure included immunoprecipitation of concentrated rat urine with the antiserum, dissociation of antigen and antibody by 8 M urea, and separation of antigen from antibody by gel filtration in 8 M urea on a Sephadex G-100 column. The purified antibody (100 A_{280} units of Fraction 1, Table II in Nustad & Pierce, 1974) was covalently bound to Sepharose 4B by the cyanogen bromide method (Axén, Porath & Ernback, 1967), with a 93% yield.

Incubation of kidney tissue with [^{3}H]-L-leucine and preparation of soluble kallikrein

Sixty female Sprague-Dawley rats (200-300 g) were killed by a blow on the neck; the kidneys were removed aseptically and cut into slices (0.1-0.2 mm thick) with a Stadie-Riggs hand microtome (A.H. Thomas Co.). To each gram of tissue (wet weight) was added 10 ml of a medium consisting of: [^{3}H]-L-leucine-4,5 (2 μCi/ml), Eagle Basal Medium (minus L-leucine) with Hanks balanced salt solution (Merchant, Kahn & Murphy, 1964), and 0.05 M Tris HCl, pH 7.4. Before incubation the medium was gassed for 1 min with 95% O_2-5% CO_2. Following incubation in a shaking water bath for 1 h at 37° C, the slices were homogenized by 10 strokes of a Potter-Elvehjem homogenizer (1370 rev/min). An extract was prepared by treating the homogenate with desoxycholate (0.5% w/v), then centrifuging it at 48,000 *g* for 60 min and storing at −20° C. The supernatants from 6 incubates, each derived from the kidneys of 10 rats, were pooled, dialysed against 0.20 M KCl-0.01 M potassium phosphate,

[1] Present address: The Norwegian Radium Hospital, Montebello, Oslo 3, Norway.

pH 7.0, and centrifuged at 48,000 *g* for 4 h to give Fraction 1 (Table 1).

Colorimetric assay for α-N-carbobenzyloxy-L-arginine methyl ester (ZAME) hydrolysis

The purification of rat kidney esterases was followed using Roberts' (1958, 1960) method with a minor modification. The reaction between ZAME and 2 M alkaline hydroxylamine (NH_2OH . HCl) was very rapid, maximum colour developed after 30-60 s incubation and then diminished. In contrast, the reactions of α-*N*-benzoyl-L-arginine ethyl ester (BAEE) and α-*N*-tosyl-L-arginine methyl ester (TAME) with alkaline hydroxylamine were complete in 1 and 12 min, respectively; the colour yield was not reduced after long incubation periods. To each test tube containing 0.20 ml of enzyme and 0.10 ml of 0.75 M Tris HCl, pH 8.5, was added 75 µl of 0.10 M ZAME. After incubation for 1.0 h at 37°C, the tubes were treated with 0.50 ml of freshly prepared 2.0 M NH_2OH . HCl-3.5 M NaOH and mixed. After 30 s, 0.25 ml of 0.37 M TCA-8.0 M HCl and then 4.0 ml of 0.11 M $FeCl_3$-0.04 M HCl, were added. The absorbance at 525 nm was determined 5 min later in a 1.0-cm cuvette. The amount of enzyme which hydrolyses one µmole of ZAME per min under these conditions was defined as one esterase unit (EU). A standard curve with ZAME was used to calculate the amount of ester hydrolysed by the enzyme preparations.

Conductivity determination

The conductivity of fractions from the DEAE-Sephadex column was determined in milliSiemens (mS) at 25°C, using a conductivity cell CDC 314 and conductivity meter CDM 3 (Radiometer).

Liquid scintillation counting

Disc gel sections of 10 to 60 mg (wet weight) were weighed in glass counting vials and dissolved by incubation with 0.1 ml of 30% hydrogen peroxide for 20 h at 50°C (Young & Fulhorst, 1965). The vials were cooled to room temperature, left without caps for 1 h, treated with 0.5 ml of Soluene, and incubated for 2 h at 50°C. The liquid scintillation fluid (10 ml per vial) contained: 400 g of naphthalene, 25 g of PPO, 0.25 g of POPOP, 1925 ml of dioxane, 1925 ml of xylol, and 1150 ml of absolute alcohol. Counting efficiency for tritium was 43%, using a Mark II Liquid Scintillation Counter (Nuclear Chicago).

Protein determination

This was measured by the adsorbance at 280 nm in a 1-cm cuvette, and in main fractions, also by the method of Lowry, Rosebrough, Farr & Randall (1951). Pure urinary kallikrein B_3 (Fraction 4c, Table 1 in Nustad & Pierce, 1974) was used as a protein standard.

The biological assay of kallikrein, phosphate determination, electrofocusing, and electrophoresis in 16.25% polyacrylamide disc gels are described in Nustad & Pierce (1974). The pressure dialysis of protein solutions and preparation of hydroxyapatite will be reported elsewhere.

Materials

The following were used in addition to the materials listed in Nustad & Pierce (1974), we obtained [^{3}H]-L-leucine-4,5 (15 Ci/mmole TRK 75) from Amersham/Searle; α-*N*-carbobenzyloxy-L-arginine methyl ester (ZAME) from Cyclo Chemical; sodium desoxycholate and bovine serum albumin (Fraction V) from Sigma Chemical Co.; Sepharose 4B from Pharmacia; cyanogen bromide from Fluka; ultrapure sucrose from Mann; 2,5-diphenyloxazole (PPO) and 1,4-di(2-(5-phenyloxazolyl))-benzene (POPOP) from Koch-Light Lab., Ltd; Soluene 100 from Packard Instrument Co. and Aprotinin from Bayer, Leverkusen.

Results

Rat urinary kallikrein hydrolysed ZAME much more rapidly than the more commonly used substrates, TAME and BAEE (Nustad & Pierce, 1974). Consequently, hydrolysis of ZAME was used to follow the purification of kallikrein from kidney tissue, where the concentration of kallikrein is low and a sensitive assay is required (Nustad, 1970). The purest preparation of rat urinary kallikrein B_3 (23 TAME EU/mg) had a specific activity of 430 ZAME EU/mg; the purity of kallikrein preparations was therefore calculated assuming that pure enzyme should have a specific activity of 430 ZAME EU/mg. Kallikrein activity was also determined on the isolated rat uterus without addition of kininogen (Nustad & Pierce, 1974).

Testing of the immunoadsorbent

(a) *Rat urinary kallikrein* The capacity of the immunoadsorbent to bind rat urinary kallikrein was determined by passing a sample of pressure-dialysed and freeze-dried rat urine (233 mg of protein, calculated to contain 4 mg of pure kallikrein), in 6 ml of 0.15 M NaCl-0.01 M sodium phosphate, pH 7.4, over a 1.0 x 10-cm column of Sepharose 4B containing 36 mg of purified anti-

body. The column was washed with 130 ml of 0.15 M NaCl-0.01 M sodium phosphate, pH 7.4; 10 ml of 3 M NaCl-0.20 M sodium phosphate, pH 6.0; and 40 ml of 0.15 M NaCl-0.01 M sodium phosphate, pH 6.0. About one-half of the kallikrein (2 mg) in the starting sample was adsorbed on to the column. The molar ratio of antibody/bound antigen was calculated to be 3.7, whereas a ratio of 1.6 had been calculated for the immune precipitate from which the purified antibody had been obtained (Nustad & Pierce, 1974). The binding capacity of purified antibody on the column would then be 43% of that found for antibody in the antiserum.

The bound kallikrein was eluted with 8 M urea-0.15 M NaCl-0.01 M sodium phosphate, pH 6.0. After removal of urea by dialysis against 0.15 M NaCl-0.01 M sodium phosphate, pH 6.0, and concentration by pressure dialysis, 51% of the kallikrein activity which had been bound to the column was recovered. This compares well with a recovery of 45% from the immunoprecipitate (Table II in Nustad & Pierce, 1974), where kallikreins were recovered in two fractions after gel filtration in 8 M urea with purities calculated to be 42 and 82%, that is an average of 62%. The kallikreins recovered from the immunoadsorbent were 55% pure. Electrophoresis in 16.25% polyacrylamide gels showed one major band in the middle of the gel with faint bands on either side—a pattern indistinguishable from that found for the kallikreins isolated from the immunoprecipitate (Fraction 3 of Figure 5 in Nustad & Pierce, 1974).

(b) *Rat kidney kallikrein* A 50-ml extract (0.15 M NaCl-0.01 M sodium phosphate, pH 7.4) of kidney cortex from 10 rats (Fraction 1, Table 1) was analysed as described above with a similar immunoadsorbent column. As in the case of urinary kallikrein, 50% of the ZAME esterase activity was recovered after the removal of urea, but this preparation was less than 1% pure. Polyacrylamide disc gel electrophoresis showed many bands staining with Amido Black, none of which could be identified with kallikrein. It was therefore decided that the large-scale purification of kallikrein from incubates of rat kidney slices and [^{3}H]-L-leucine should follow the same procedure previously used to purify the urinary enzymes (Nustad & Pierce, 1974). The immunoadsorbent was reserved for the final purification step.

Purification of rat kidney kallikrein from tissue incubated with [^{3}H]-L-leucine

The purification of kidney kallikrein (Table 1, Figure 1) was carried out at room temperature (20-22° C) except for the electrofocusing step (4° C).

Step 1 Desoxycholate-solubilized kallikrein from the incubated kidney slices of 60 rats was prepared as described in methods. This extract (Fraction 1, Table 1) contained 4200 ct/min per mg of protein.

Step 2 Fraction 1 in 1.5 l of 0.20 M KCl-0.01 M potassium phosphate, pH 7.0, was adsorbed on to a DEAE-Sephadex A-50 column equilibrated with the same buffer. The column was developed as described in Figure 1a. All of the applied esterase activity was eluted as one peak at 40 mS, corresponding to 0.32 M KCl-0.01 M potassium phosphate, pH 7.0 (Figure 1a). The most active fractions were pooled, concentrated by pressure dialysis, and equilibrated against 3.0 M NaCl-0.01 M sodium phosphate, pH 6.0, to give Fraction 2.

Step 3 Fraction 2 in 25 ml of 3.0 M NaCl-0.01 M

Table 1 Purification of kallikreins synthesized in kidney slices from 60 rats

Fraction	*Treatment*	*Protein (mg)*	*Specific activity (EU*/mg)*	*Purification*	*Recovery (%)*
1	Rat kidney extract	3600	0.05	(1)	(100)
2	DEAE-Sephadex A-50 chromatography of Fraction 1	414	0.34	7	82
3	Hydroxyapatite chromatography of Fraction 2	37	2.2	46	47
4	Electrofocusing of Fraction 3	17	3.8	80	38
5	Affinity chromatography of Fraction 4	0.75	33.0	700	15

* 1.0 EU (esterase unit) = 1.0 μmole of ZAME/min (pH 8.5, 37° C).

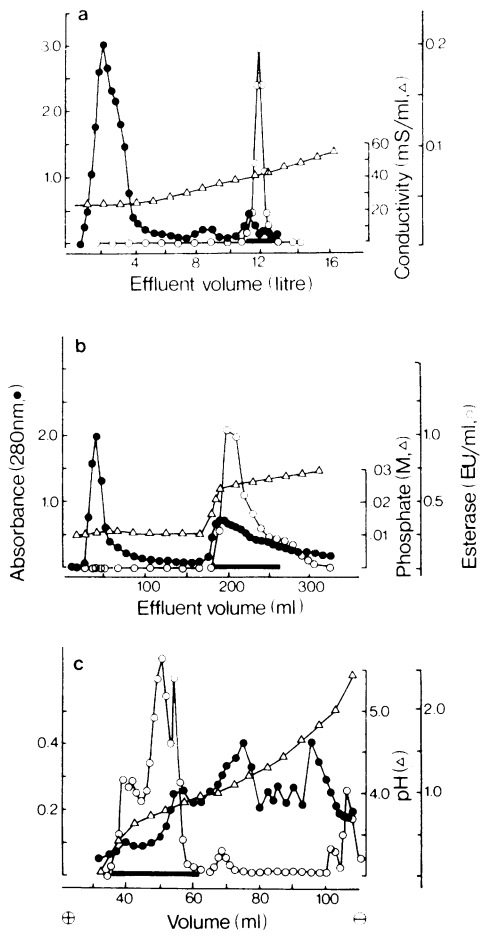

Figure 1 (a) DEAE-Sephadex A-50 chromatography. Column: 5 x 74 cm. Starting sample: 3600 mg of Fraction 1 (Table 1) in 1.5 l of the equilibration buffer (0.20 M KCl-0.01 M potassium phosphate, pH 7.0). Eluents: 4.0 l of the equilibration buffer; 10.0 l of a linear gradient from the equilibration buffer to 0.50 M KCl-0.01 M potassium phosphate pH 7.0. Fraction volume: 20 ml. Flow rate: 40 ml/hour. Inside right ordinate: conductivity (mS/ml, △). (b) Hydroxyapatite chromatography. Column: 1.25 x 29 cm. Starting sample: 414 mg of Fraction 2 (Table 1) in 25 ml of the equilibration buffer (3 M NaCl-0.01 M sodium phosphate, pH 6.0). Eluents: 110 ml of the equilibration buffer; 800 ml of a gradient from the equilibration buffer to 3 M NaCl-0.05 M sodium phosphate, pH 6.0. Fraction volume: 4.0 ml. Flow rate: 3.5 ml/hour. Inside right ordinate: phosphate (M, △). (c) Electrofocusing. Column: 110 ml containing 0.8% Ampholine, pH 3-5. Starting sample: 37 mg of Fraction 3 (Table 1) in 3.3 ml of 0.01 M sodium phosphate, pH 7.0. Operation: 400 → 1000 V/5 h, 1000 V/13 hour. Fraction volume: 1 ml. Elution flow rate: 120 ml/hour. Inside right ordinate: pH (△).

sodium phosphate, pH 6.0, was adsorbed to a hydroxyapatite column and was developed as described in Figure 1b. All of the esterase activity was eluted as one peak at a phosphate concentration of 0.024 M. The most active fractions were pooled, concentrated by pressure dialysis, and equilibrated against 0.01 M sodium phosphate, pH 7.0, to give Fraction 3.

Step 4 Ultrapure sucrose (2.15 g) and 40% Ampholine pH 3-5 (0.15 ml) were added to ice-cold Fraction 3 in 3.3 ml of 0.01 M sodium phosphate, pH 7.0, and made to 8.8 ml with distilled water. This fraction replaced those tubes in the stepwise gradient with which it was isodense. Five esterase peaks with kallikrein activity were obtained with isoelectric points of 3.52, 3.65, 3.74, 3.80, and 4.0 (Figure 1c). The four kallikrein peaks with isoelectric points of 3.52 to 3.80 were pooled; dialysed against 3.0 M NaCl-0.01 M sodium phosphate, pH 6.0; and concentrated by pressure dialysis while equilibrating against 0.15 M NaCl-0.01 M sodium phosphate, pH 7.4, to give Fraction 4. The fifth kallikrein with pI 4.0 was not used since preliminary experiments indicated that it probably arose from the other four kallikreins by minor bacterial action.

Step 5 Fraction 4 in 50 ml of 0.15 M NaCl-0.01 M sodium phosphate, pH 7.4, was applied to a 1.0 x 4.0-cm column of immobilized antibody to rat urinary kallikrein. The column was then washed with 115 ml of 0.15 M NaCl-0.01 M sodium phosphate, pH 7.4; 16 ml of 3.0 M NaCl-0.20 M sodium phosphate, pH 6.0; and 50 ml of 0.15 M NaCl-0.01 M sodium phosphate, pH 6.0. No esterase activity appeared in the effluent, the last 20 ml of which had an $A_{280\,nm}$ of less than 0.015 and no radioactivity. The column was then eluted with 50 ml of 8.0 M urea-0.15 M NaCl-0.01 M sodium phosphate, pH 6.0. A small protein peak with total $A_{280\,nm}$ of 0.34 was collected in 3 ml, dialysed against 0.15 M NaCl-0.01 M sodium phosphate, pH 6.0, and concentrated by pressure dialysis to 1.7 ml (Fraction 5). Electrofocusing of 0.6 ml of this fraction in a pH 3-5 gradient showed an enzyme profile like that of Fraction 4.

Disc gel electrophoresis of purified [^{3}H]-rat kidney kallikreins

Samples of 180 μl of Fraction 5 were electrophoresed in 16.25% polyacrylamide disc gels. A gel stained with Amido Black showed impurities at the cathodic end of the gel, a major band with a mobility of 25% of that of the dye front, and a minor band with faint bands on either side with a

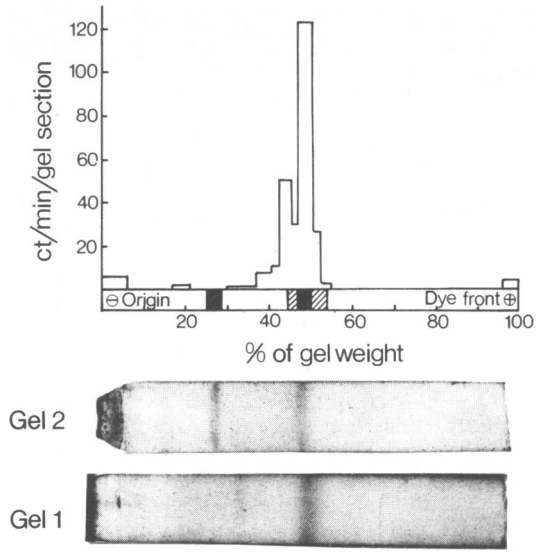

Figure 2 Electrophoresis of purified rat urinary and kidney kallikreins in 16.25% polyacrylamide disc gels at 20° C with 4 mA/tube for 2 hours. Gel 1. 1.4 EU (3.3 μg protein) of rat urinary kallikrein B_3. (Fraction 4c, Table I in Nustad & Pierce, 1974.) Gel 2. 2.3 EU (67 μg protein) of rat kidney kallikreins (Fraction 5, Table 1). The figure above shows Gel 2 sectioned, dissolved in 30% hydrogen peroxide and counted for tritium.

mobility of 50% of that of the dye front (Figure 2).

An unstained gel (stored at −20° C) was sectioned, homogenized in 0.15 M NaCl-0.01 M sodium phosphate, pH 7.3, and assayed for esterase and kallikrein activities. Both activities were found in the same fractions with 78% and 64% recoveries, respectively, and corresponded exactly to the bands in the middle of the stained gel. The same correspondence was found for rat urinary kallikreins treated in the same way.

The stained gel of Fraction 5 was sectioned, dissolved in hydrogen peroxide and counted. The radioactivity was recovered in 77% yield, in those sections from the middle of the gel where the kallikrein activity was found (Figure 2).

Bioassay of purified [^{3}H]-rat kidney kallikrein

The five esterases with pI's 3.50-4.0 gave contractions of the isolated rat uterus proportional to their esterase activities. The ability to contract the isolated rat uterus without addition of kininogen was previously found to be typical for rat urinary kallikreins (Nustad & Pierce, 1974). The oxytocic activity of each kallikrein was inhibited by antiserum to rat urinary kallikrein B_3 and by aprotinin, but not by soybean trypsin inhibitor.

Discussion

The newly synthesized kallikreins isolated from kidney slices incubated with [^{3}H]-L-leucine were indistinguishable from kallikreins isolated from the urine of rats of the same strain. The isoelectric points of the kidney enzymes–3.52, 3.65, 3.74 and 3.80–agree closely with those found earlier for the urinary enzymes–3.50, 3.68, 3.73 and 3.80. Furthermore, their relative amounts were similar.

The relative amount of enzyme activity under each peak of urinary kallikreins B_1-B_4 (Figure 1c in Nustad & Pierce, 1974) were: 5, 12, 66 and 19%, whereas the corresponding figures for kidney kallikreins (Figure 1c) were: 11, 15, 52 and 22%, respectively. The slight differences in the distribution of enzyme activities were probably due to the difference in the slopes of the pH gradient. Finally, the mobilities of the kidney and urinary enzymes in polyacrylamide gel electrophoresis were the same, and the active bands from the kidney contained almost all of the radioactivity of the applied sample. Besides indicating the identity of kidney and urinary kallikreins, these results show that kallikreins synthesized and released by the kidney are not changed during their passage through the lower urinary tract.

Kidney kallikreins presumably act on kininogen to produce kallidin (lysyl-bradykinin) in the kidney. Although the kininogen could come from the blood, there is some evidence that the kidney itself contains kininogen (Sardesai, 1968; Werle & Zach, 1970). Since kallikreins are synthesized in the kidney and free kinins are found in urine, the kallikrein-kinin system may participate in the local regulation of renal blood flow. This could be due to direct effects on vascular smooth muscles by kinins formed in the intercellular space. However, an indirect action of kinins on renal haemodynamics could be mediated through release of prostaglandins in the medulla by kinins formed in tubular urine (McGiff, Terragno, Malik & Lonigro, 1972; Stoner, Manganiello & Vaughan, 1973; McGiff, Crowshaw & Itskovitz, 1974). A role of kinins in the regulation of sodium excretion was suggested by Adetuyibi & Mills (1972) and Marin-Grez, Cottone & Carretero (1972). A role in the general regulation of blood pressure should also be considered, in view of several reports of decreased urinary kallikrein excretion in patients with essential hypertension (Elliot & Nuzum, 1934; Werle & Korsten, 1938; Margolius, Geller, Pisano & Sjoerdsma, 1971; Chernova &

Nekrasova, 1971; Margolius, Geller, de Jong, Pisano & Sjoerdsma, 1972).

The present study, demonstrating the synthesis of kallikreins in the kidney and their apparent identity with the urinary enzymes, contributes to the growing evidence for a physiological role of the kinin system.

References

ADETUYIBI, A. & MILLS, I.H. (1972). Relation between urinary kallikrein and renal function, hypertension, and excretion of sodium and water in man. *Lancet*, **2**, 203-207.

AXÉN, R., PORATH, J. & ERNBACK, S. (1967). Chemical coupling of peptides and proteins to polysaccharides by means of cyanogen halides. *Nature, Lond.*, **214**, 1302-1304.

CARVALHO, I.F. & DINIZ, C.R. (1966). Kinin-forming enzyme (kininogenin) in homogenates of rat kidney. *Biochim. biophys. Acta*, **128**, 136-148.

CHERNOVA, N.A. & NEKRASOVA, A.A. (1971). Urinary kallikrein in hypertensive vascular disease and reno-vascular hypertension. *Kardiologiia*, **11**, 84-89.

CROXATTO, H.R. & NOE, G.E. (1972). Kallikrein-like enzyme in purified rat renal extracts containing renin. *Commentarii, Pontificia Acad. Sci.*, **11**, 1-24.

ELLIOT, A.H. & NUZUM, F.R. (1934). The urinary excretion of a depressor substance (kallikrein of Frey and Kraut) in arterial hypertension. *Endocrinology*, **18**, 462-474.

LOWRY, O.H., ROSEBROUGH, N.J., FARR, A.L. & RANDALL, R.J. (1951). Protein measurement with the folin phenol reagent. *J. biol. Chem.*, **193**, 265-275.

MARGOLIUS, H.S., GELLER, R., PISANO, J.J. & SJOERDSMA, A. (1971). Altered urinary kallikrein excretion in human hypertension. *Lancet*, **2**, 1063-1065.

MARGOLIUS, H.S., GELLER, R.G., DE JONG, W., PISANO, J.J. & SJOERDSMA, A. (1972). Urinary kallikrein excretion in hypertension. *Circulation Res.*, **30-31** *(suppl. II)*, 125-131.

MARIN-GREZ, M., COTTONE, P. & CARRETERO, O.A. (1972). Evidence for an involvement of kinins in regulation of sodium excretion. *Am. J. Physiol.*, **223**, 794-796.

McGIFF, J.C., TERRAGNO, N.A., MALIK, K.U. & LONIGRO, A.J. (1972). Release of a prostaglandin E-like substance from canine kidney by bradykinin. *Circulation Res.*, **31**, 36-43.

McGIFF, J.C., CROWSHAW, K. & ITSKOVITZ, H.D. (1974). Prostaglandins and renal function. *Fedn Proc.*, **33**, 39-47.

MERCHANT, D.J., KAHN, R.H. & MURPHY, W.H., JR. (1964). *Handbook of Cell and Organ Culture.* Minneapolis: Burgess Publishing Co.

NUSTAD, K. (1970). The relationship between kidney and urinary kininogenase. *Br. J. Pharmac.*, **39**, 73-86.

NUSTAD, K. & PIERCE, J.V. (1974). Purification of rat urinary kallikreins and their specific antibody. *Biochemistry*, **13**, 2312-2319.

ROBERTS, P.S. (1958). Measurement of the rate of plasmin action on synthetic substrates. *J. biol. Chem.*, **232**, 285-291.

ROBERTS, P.S. (1960). The esterase activities of human plasmin during purification and subsequent activation by streptokinase or glycerol. *J. biol. Chem.*, **235**, 2262-2266.

SARDESAI, V.M. (1968). Determination of bradykinin in blood and bradykininogen in tissues. *Can. J. Physiol. Pharmacol.*, **46**, 77-79.

STONER, J., MANGANIELLO, V.C. & VAUGHAN, M. (1973). Effects of bradykinin and indomethacin on cyclic GMP and cyclic AMP in lung slices. *Proc. natn. Acad. Sci.*, **70**, 3830-3833.

WERLE, E. & KORSTEN, H. (1938). Der Kallikreingehalt des Harns, des Speichels und des Blutes bei Gesunden und Kranken. *Z. ges. exp. Med.*, **103**, 153-162.

WERLE, E. & VOGEL, R. (1960). Über die Kallikreinausscheidung im Harn nach experimenteller Nierenschädigung. *Arch. int. Pharmacodyn. Thér.*, **126**, 171-186.

WERLE, E. & ZACH, P. (1970). Verteilung von Kininogen in Serum und Geweben bei Ratten und anderen Säugetieren. *Z. klin. Chem. klin. Biochem.*, **8**, 186-189.

YOUNG, R.W. & FULHORST, H.W. (1965). Recovery of S^{35} radioactivity from protein-bearing polyacrylamide gel. *Analyt. Biochem.*, **11**, 389-391.

(Received April 15, 1974)