ficient for structural analyses. The procedure described in this paper has recently been scaled up further and yields almost 1 g of protein. Although thermostable, the enzyme must be protected against proteolysis, otherwise crystallization is difficult to achieve. The native enzyme is composed of two very similar protomers with molecular weights of 45,000 each. This is consistent with the unit cell symmetry in the enzyme crystals which indicates a moiety of 45,000 daltons as the asymmetric unit (Reid *et al.*, 1973). One equivalent of cysteine can be reacted with Nbs_2 albeit quite slowly at room temperature although the full complement of half-cystine in the protein can be titrated upon denaturation with sodium dodecyl sulfate, showing that the enzyme does not contain disulfide bonds.

The convenient size of this enzyme, its substantial thermal stability, and the high quality of the crystals it yields render it an excellent candidate for the elucidation of the complete three-dimensional structure of an aminoacyl-tRNA synthetase and such studies are now in progress. Since a major source of interest in these enzymes is the structural basis of their bispecificity it is conceivable that this system will prove useful for the preparation and investigation of the complex formed between the enzyme and its cognate tRNA.

Acknowledgments

I thank Dr. B. S. Hartley for his advice and encouragement and Drs. Y. Boulanger and A. Atkinson for help with the purification of the enzyme. This work was done during the tenure of a Travelling Fellowship from the Australian National University.

References

Beicherich, H., von der Haar, F., and Cramer, F. (1972), *Eur. J. Biochem. 26*, 182.
Calender, R., and Berg, P. (1966), *Biochemistry 5*, 1681.
Chirikjian, J. G., Wright, H. T., and Fresco, J. R. (1972), *Proc. Nat. Acad. Sci. U. S. 69*, 1638.
Crestfield, A. M., Moore, S., and Stein, W. H. (1963), *J. Biol. Chem. 238*, 2413.
Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.
Edelhoch, H. (1967), *Biochemistry 6*, 1948.
Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82*, 70.
Hartley, R. W., and Barber, E. A. (1972), *Nature (London), New Biol. 235*, 15.
Hirs, C. H. W. (1956), *J. Biol. Chem. 219*, 611.
Laemmli, U. K. (1970), *Nature (London) 227*, 680.
Reid, B. R., Koch, G. L. E., Boulanger, Y., Hartley, B. S., and Blow, D. M. (1973), *J. Mol. Biol. 80*, 199.
Rymo, L., Lagerkvist, U., and Wonacott, A. (1970), *J. Biol. Chem. 245*, 4308.
Schroeder, W. A. (1972), *Methods Enzymol. 25*, 203.
Stanley, W. M., and Bock, R. M. (1965), *Biochemistry 4*, 1302.
Udenfriend, S., Stein, S., Böhlen, P., Dairman, W., Leimgenber, W., and Weigele, M. (1972), *Science 178*, 871.
Waller, J. P., Risler, J. L., Montheilet, C., and Zelwer, C. (1971), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 16*, 186.

Purification of Rat Urinary Kallikreins and Their Specific Antibody†

Kjell Nustad‡ and Jack V. Pierce*

ABSTRACT: Four biologically active and immunologically identical rat urinary kallikreins (B_1–B_4) have been obtained in highly purified form by pressure dialysis, chromatography on DEAE-Sephadex A-50 and hydroxylapatite columns, and electrofocusing. Molecular weights of 35,300, 33,600, 33,100, and 32,300 were estimated for kallikreins B_1, B_2, B_3, and B_4, respectively, by sodium dodecyl sulfate polyacrylamide disc gel electrophoresis, and of 38,500 for B_3 by Bio-Gel P-200 gel filtration. A sheep was immunized against B_3, and an immune precipitate was prepared from antiserum and a crude urine concentrate. Antigen and antibody were dissociated in 8 M urea and separated on a column of Sephadex G-100. Both antigen and antibody were recovered in highly purified form by this simple procedure.

Mammalian glandular kallikreins are peptidylhydrolases (EC 3.4.4.21) of unique specificity. They are unusual in being able to break two dissimilar peptide bonds, Met–Lys and Arg–Ser, in plasma kininogens to liberate the extremely biologically active decapeptide, kallidin (lysylbradykinin: Lys-Arg-Pro-Pro-Gly-Phe-Ser-Pro-Phe-Arg) (see Pierce, 1968, 1970). The urinary kallikreins are of further interest because of their possible involvement in the regulation of local blood flow (Hilton, 1970), blood pressure and sodium balance (Croxatto and San Martín, 1970; Adetuyibi and Mills, 1972; Margolius *et al.*, 1972; Geller *et al.*, 1972).

To elucidate the origin and physiological function(s) of urinary kallikrein, it was deemed necessary to isolate the pure enzyme and its specific antibody. The present study describes the purification of rat urinary kallikreins, the preparation of specific antiserum, and the simple purification of rat urinary kallikreins and anti-kallikrein from an immune precipitate. Rat urine was found to contain four kallikreins distinguishable by both electrofocusing and polyacrylamide gel electrophoresis.

† From Section on Physiological Chemistry, Experimental Therapeutics Branch, National Heart and Lung Institute, Bethesda, Maryland 20014. *Received November 5, 1973.* A preliminary report of part of this work has appeared elsewhere (Pierce and Nustad, 1972).

‡ Present address: Institute for Surgical Research, Rikshospitalet, Oslo 1, Norway.

Materials and Methods

The following materials were obtained from commercial sources: crystalline ovalbumin, pepsin, trypsin, chymotrypsinogen A, and STI[1] (Worthington Biochemical Corp.); lysozyme and Hammersten casein (Nutritional Biochemicals Corp.); crystallized bovine plasma albumin (Armour); Trasylol (Bayer, Leverkusen, West Germany, identical to Kunitz basic pancreatic inhibitor); *Vibrio cholerae* neuraminidase (Calbiochem); Ultra Pure urea and sucrose, TAME, BAEE, and BAPA (Mann Research Labs); Sephadex G-100, DEAE-Sephadex A-50, and blue dextran (Pharmacia Fine Chemicals, Inc.); ampholines (LKB); Seakem agarose (Bausch and Lomb); SDS (Sigma Chemical Co.). Reagent grade chemicals were used throughout.

Hydroxylapatite was prepared at room temperature by the controlled addition of NaOH and $CaCl_2$ to a saline suspension of $Ca(H_2PO_4)_2$ crystals.[2]

Collection of Rat Urine. Forty female Sprague-Dawley rats (200–300 g) were kept in two stainless steel cages (48 × 58 × 20 cm) and were given water but no food during urine collection. The urine was collected at night onto a stainless steel plate placed at a 45° angle to the bottom of the cage; then into a trough at the lower end of the plate; and finally into bottles kept in ice–water. Solid debris was retained on a 4-mesh stainless steel screen placed just under the cage. Each lot of urine collected overnight (200–300 ml) was filtered through glass wool and stored at −20° until used.

Pressure Dialysis. Concentrating and desalting of protein solutions were done in 1.0-cm flat-width Visking dialysis tubing (Union Carbide Corp.) which had been expanded to about 1.3-cm flat width with water under about 1-kg/cm² nitrogen pressure.[3] The filtration rate was 0.25 ml/cm length of tubing per hr at 3°.

Protein Determination. The absorbance at 280 nm in a 1.0-cm cuvet was used routinely to measure protein concentration of solutions. Dry weights were determined on major fractions by freeze-drying salt-free aqueous solutions.

Phosphate Determination. Fractions from hydroxylapatite chromatography were assayed for P_i by the method of Barton (1948).

Conductivity Determination. The conductivity of fractions from DEAE-Sephadex A-50 chromatography was measured at 25° with a Radiometer conductivity meter Type CDM 2d and conductivity cell Type CDC 114 (0.7-cm path length).

Electrofocusing. A 110-ml electrofocusing column (LKB) was used with a stepwise gradient made according to the manufacturer's directions. The pH of the collected fractions was read at 25° with a Radiometer PHM25 pH meter and then adjusted to about 7.5 with an equal volume of 0.2 M potassium phosphate (pH 7.6).

Electrophoresis in 16.25% Polyacrylamide Disc Gels (Davis, 1964). The separating gel solution was made by dissolving 4.06 g of acrylamide and 27.8 mg of bisacrylamide in 15 ml of water and stirring for 20 min at room temperature; adding 3.1 ml of 3.0 M Tris-HCl (pH 8.9), containing 0.25% (v/v) *N,N,N′,N′*-tetramethylethylenediamine; adding water to 25 ml; and deaerating for 0.5 hr at room temperature with a water pump. After being cooled to about 10° and treated with 18 mg of ammonium persulfate, the solution was added to twelve 0.55-cm i.d. gel tubes to a height of 6.0 cm. After gelling was completed (*ca.* 0.5 hr at room temperature), the stacking gel was prepared by photopolymerizing 0.20 ml of Canalco upper gel solution diluted 1 to 1.25 with water. A mixture of 50 μl of sample (*ca.* 50 μg of protein) and 0.20 ml of undiluted upper gel solution was placed on top of the stacking gel and photopolymerized. A current of 4 mA/gel was applied at room temperature for about 2 hr, when the tracking dye (Bromophenol Blue) had reached the bottom of the separating gels. Gels were stained 1 hr with 0.5% Amido Black in 7% acetic acid and destained in a lateral electrophoretic destainer (Canalco). Gels to be sectioned later were immediately frozen in Dry-Ice–acetone and stored at −20°.

Molecular Weight Determination. The molecular weight of rat urinary kallikrein B_3 was estimated by gel filtration at room temperature on a Bio-Gel P-200 column, using blue dextran, bovine plasma albumin, ovalbumin, and lysozyme as reference compounds (Andrews, 1964). The molecular weights of kallikreins B_1–B_4 were estimated by SDS polyacrylamide disc gel electrophoresis (Shapiro *et al.*, 1967), using chymotrypsinogen A, pepsin, and ovalbumin, and measuring the mobilities relative to that of chymotrypsinogen A (Dunker and Rueckert, 1969). The protein samples were made to 0.5 mg/ml in 1% 2-mercaptoethanol, 4 M urea, and 1% SDS and incubated for 1 hr at 37°, and 5–10 μg of each protein and 3 μl of 0.05% Bromophenol Blue were applied to the top of the gel under electrode buffer (Dunker and Rueckert, 1969). Electrophoresis in 10% polyacrylamide gels was performed according to Weber and Osborn (1969).

Neuraminidase Treatment. Rat urinary kallikreins B_1–B_4 (*ca.* 50 μg) were incubated for 1.0 hr at 37° with 2 units of *Vibrio cholerae* neuraminidase in 0.01 M sodium acetate–0.0015 M $CaCl_2$ (pH 5.6) in a final volume of 60 μl. The incubates with and without neuraminidase were then subjected to polyacrylamide gel electrophoresis as described above.

Immunization with Rat Urinary Kallikrein B_3 (Sussman *et al.*, 1968). Rat urinary kallikrein B_3 (1.2 mg of fraction 4c, Table I) was electrophoresed on twelve 16% polyacrylamide disc gels as described above. Eleven of the gels were frozen in test tubes in Dry Ice–acetone immediately after electrophoresis and stored at −20°. The remaining gel was stained. The B_3 bands (20–30 mg gel weight) from five of the frozen gels were excised by comparison with the stained gel, macerated, suspended in 4 ml of Freund's complete adjuvant (Difco), and injected intramuscularly at multiple sites in a sheep. A second injection was given 30 days later with the remaining six gel sections treated in the same way. A third injection, given on day 43, consisted of 3.3 mg of fraction 5 (Table I) in 2.5 ml of 0.01 M potassium phosphate (pH 6.1) and emulsified with 2.5 ml of Freund's complete adjuvant.

Immunodiffusion in Agarose. Double-diffusion analysis (Ouchterlony, 1949) was done at room temperature for 16 hr in 1.0% agarose in buffered saline (pH 7.5), containing 0.05% NaN_3.

Quantitative Precipitin Reaction. The equivalence point for rat urinary kallikreins in pressure-dialyzed rat urine (fraction 0, Table II) and sheep antiserum to esterase B_3 was determined as described by Kabat and Mayer (1964).

Titrimetric Esterase Assay. The hydrolysis rates of rat urinary kallikrein and esterase A fractions with TAME, BAEE, and ZAME were determined at pH 8.0 by titration with standard 0.01 M NaOH in an Autotitrator TTTlc with Autoburette ABUll (0.25-ml syringe) from Radiometer. The reaction

[1] Abbreviations used are: TAME, α-*N*-tosyl-L-arginine methyl ester; BAEE, α-*N*-benzoyl-L-arginine ethyl ester; ZAME, α-*N*-carbobenzyloxy-L-arginine methyl ester; BAPA, α-*N*-benzoyl-L-arginine *p*-nitroanilide; SDS, sodium dodecyl sulfate; STI, soybean trypsin inhibitor; TCA, trichloroacetic acid; EDTA, ethylenediaminetetraacetate.

[2] J. V. Pierce and K. Nustad, manuscript in preparation.

[3] J. V. Pierce, manuscript in preparation.

TABLE I: Purification of Kallikreins from 5.4 l. of Rat Urine.

Fraction	Treatment	Esterase Type	Protein[a] (A_{280} Units)	Sp Act. (EU[b]/A_{280})	Purifcn	Recov (%)
1	Rat urine, pressure dialyzed and freeze-dried (2.40 g)	A + B	2440	0.58		
		B[c]		(0.29)[c]	(1.0)	(100)
2a	DEAE-Sephadex A-50 chromatography of fraction 1	A	800	0.59		
b		B	83	5.9	20	71
3	Hydroxylapatite chromatography of fraction 2b	B	23	15.5	54	52
	Electrofocusing of fraction 3					
4a	p_a 3.50	B_1	1.0	14.5	50	1.9
b	p_a 3.68	B_2	2.8	20.4	70	8.2
c	p_a 3.73	B_3	5.3	22.8	79	17.5
d	p_a 3.80	B_4	3.0	18.8	65	8.2
5	Bio-Gel P-200 gel filtration of fraction 4c	B_3	3.6	25.0	86	13.0

[a] Both fractions 1 and 4c gave an $A_{280}^{1\,cm}$ of 1.0 for a 1.0-mg/ml solution. [b] 1.0 EU (esterase unit) = 1.0 μmol of TAME/min (0.15 M KCl, pH 8.0, 25°). [c] Calculated on the assumption that 51% of the total activity is due to esterase B, as found by DEAE-Sephadex A-50 chromatography.

TABLE II: Purification of Rat Urinary Kallikrein and Its Specific Antibody from an Immune Precipitate.

Fraction		Esterase Type	Protein (A_{280} Units)	Sp Act. (EU[a]/A_{280})	Purifcn	Recov (%)	Immunochemical Identity[c]
0	Rat urine (4.5 l.) pressure dialyzed to 63 ml	B	2900	(0.28)[b]	(1.0)	(100)	Antigen
	Sephadex G-100 gel filtration of immune precipitate with 8 M urea						
1	120–158 ml		135				Antibody
2	158–182 ml	B_{1-4}	10.6	10.4	37	14	Antigen
3	182–225 ml	B_{1-4}	12.5	20.0	72	72	Antigen

[a] 1.0 EU (esterase unit) = 1.0 μmol of TAME/min (0.15 M KCl, pH 8.0, 25°). [b] Calculated on assumption that 51% of total activity is due to esterase B. [c] Determined by Ouchterlony double diffusion.

vessel was kept at 25° and constantly swept with nitrogen. The reaction was started by adding enzyme as the last component after a blank value for spontaneous hydrolysis of ester had been obtained. The final concentrations of the titration mixture were 20 mM substrate and 0.15 M KCl in a volume of 2.5 ml. One esterase unit (EU) is that amount of enzyme which hydrolyzes 1.0 μmol of TAME/min under these conditions.

Colorimetric TAME Esterase Assay. A modification of Roberts' colorimetric method (1958) was routinely used to follow the purification of the TAME esterase activity of rat urine. To tubes containing 0.20 ml of enzyme sample and 0.10 ml of 0.75 M Tris-HCl (pH 8.6) was added 60 μl of 0.13 M TAME-HCl. After incubation at 37° for 1.0 hr, 0.50 ml of freshly prepared 1 M $NH_2OH \cdot HCl$–1.75 M NaOH was added and the solution was kept at 25° for 0.5 hr. After addition of 0.25 ml of 0.37 M TCA–4 M HCl, the solution was treated with 4.0 ml of 0.11 M $FeCl_3$–0.04 HCl and read exactly 5 min later at 525 nm. Standard curves of 0–0.1 EU of human and rat urinary kallikrein standards (determined titrimetrically) were run with each assay.

BAPA Assay. The hydrolysis rate of BAPA was determined at 37° and pH 8.5 in a total volume of 2.2 ml by method II of Erlanger *et al.* (1961).

Proteolytic Activity. The hydrolysis rate of heat-denatured casein was determined by the method of Kunitz (Laskowski, 1955).

Kallikrein Biological Assay. The biological activity of various fractions was determined by incubating 0.10-ml portions in 0.15 M NaCl–1.4 × 10^{-4} M EDTA–0.05 M Tris-HCl (pH 8.5) with 0.1 ml of heated (58°, 30 min) dog plasma diluted 1:4 with Tyrode solution–1.4 × 10^{-4} M EDTA–0.05 M Tris-HCl (pH 8.5) for 4 min at 37°. The kinin released was measured on the isolated guinea pig ileum (Webster and Prado, 1970).

Rat urinary kallikreins were also assayed on the isolated rat uterus (Nustad, 1970), where they caused a contraction without the addition of kininogen; this was found to be the most specific assay for this kallikrein (see Results).

In some cases, bioassay was performed in rats anesthetized with urethane (2.4 g/kg i.p.) by measuring the fall in blood pressure of the carotid artery following injection of sample into the external jugular vein.

Inhibition Studies. A constant amount of enzyme (esterase A or rat urinary kallikrein) and variable amounts of inhibitor (STI or Trasylol) in a final volume of 0.20 ml containing 0.1 ml of 0.75 M Tris-HCl (pH 8.6) were incubated for 0.5 hr at 37°. The incubates were then assayed for esterase activity as described above (Colorimetric TAME Esterase Activity).

The inhibitory effects of sheep antibody to purified rat urinary kallikrein B_3 were studied by incubating each of the four rat urinary kallikreins for 1 min at room temperature with three levels of sheep antiserum or a 2-mg/ml solution of purified antibody (fraction 1, Table II), in a final volume of 0.24

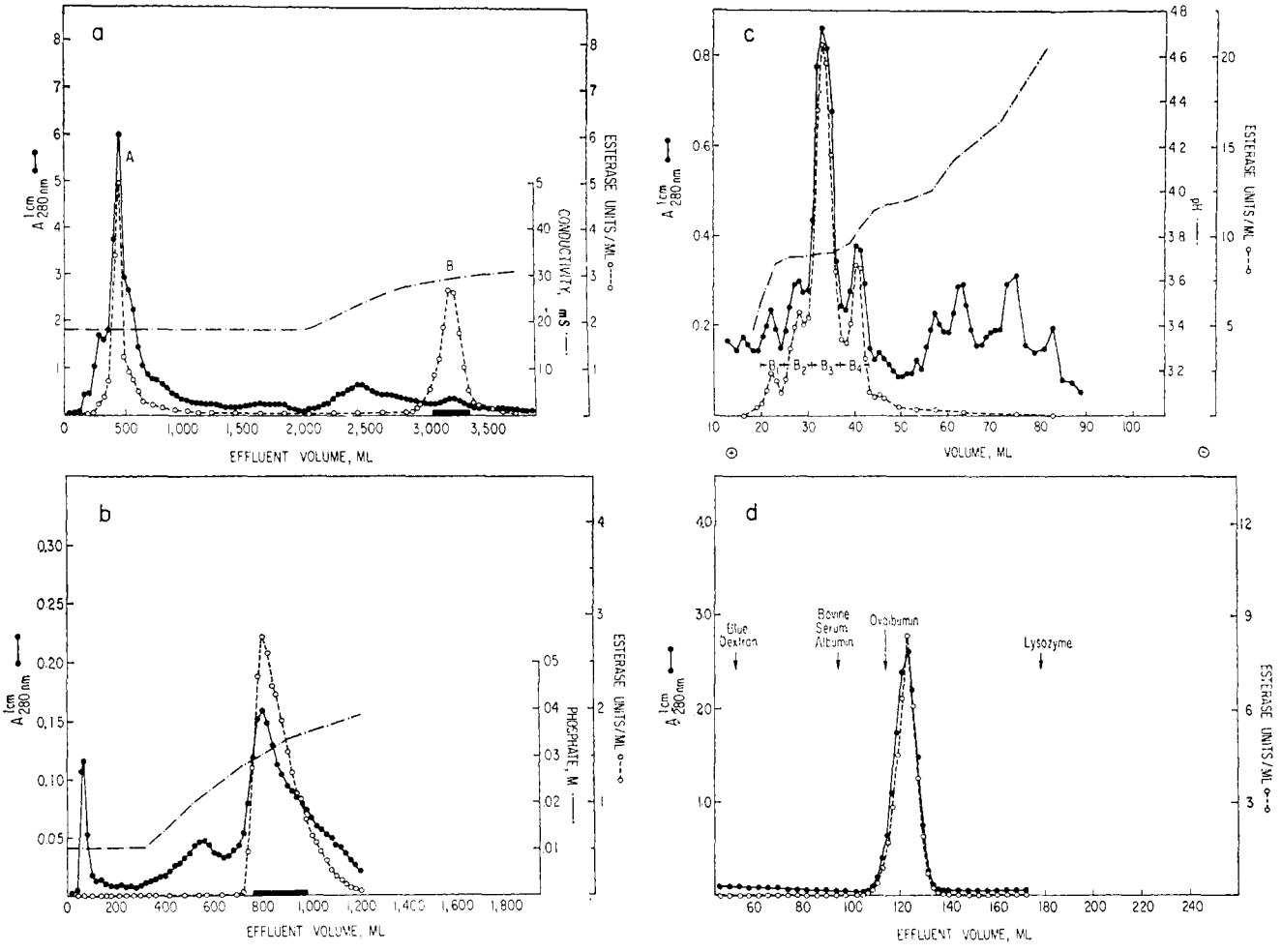

FIGURE 1: Purification of rat TAME esterases at 4° (fraction numbers refer to Table I). (a) DEAE-Sephadex A-50 chromatographic separation of esterases A and B: column, 2.5 × 89 cm; starting sample, 2.5 g of fraction 1 in 30 ml; eluents, 1.6 l. of 0.20 M KCl–0.01 M potassium phosphate (pH 7.0); 4.0 l. of a linear gradient of 0.20 M KCl–0.01 M potassium phosphate (pH 7.0) to 0.50 M KCl–0.01 M potassium phosphate (pH 7.0); fraction volume, 20 ml; flow rate, 40 ml/hr. (b) Hydroxylapatite chromatography of rat urinary kallikrein: column, 1.25 × 37 cm; starting sample, 83 A_{280} units of fraction 2b in 27 ml; eluents, 200 ml of 3 M NaCl–0.01 M sodium phosphate (pH 6.0); 1500 ml of a linear gradient of 3 M NaCl–0.01 M sodium phosphate (pH 6.0) to 3 M NaCl–0.05 M sodium phosphate (pH 6.0); fraction volume, 10 ml; flow rate, 7.3 ml/hr. (c) Electrofocusing of 11.5 A_{280} units of fraction 3 in a 110-ml column containing 2.14 ml of 40% Ampholine, pH 3–5; 400–1000 V/6.7 hr; 1000 V/12.4 hr; fraction volume, 1.0 ml; flow rate, 120 ml/hr. (d) Bio-Gel P-200 gel filtration of fraction 4c in 1 M NaCl–0.01 M sodium phosphate (pH 7.0): column, 1.56 × 103 cm; starting sample, 5.3 A_{280} units in 2.5 ml; fraction volume, 2.0 ml; flow rate, 2.0 ml/hr.

ml, and measuring the biological and TAME esterase activities of the incubates. Normal sheep serum and a γ-globulin fraction from the same serum served as controls.

Results

Purification of Rat Urinary Kallikreins B_1–B_4. The following steps in the purification of rat urinary kallikreins were carried out at 4°, and are summarized in Table I and Figure 1.

STEP 1. PRESSURE DIALYSIS. Rat urine (5.4 l.) was thawed in 1-l. portions, centrifuged if necessary, and added to four 80-cm lengths of pressure-expanded dialysis tubing and to the connecting 1.0-l. reservoir. About 1.5 l. of urine could be processed in 24 hr. The dialysand was collected in about 1% of the starting volume and a clay-like precipitate was removed by centrifugation. The urine concentrate was further pressure dialyzed against 3 M NaCl–0.01 M sodium phosphate (pH 7.0) to release a considerable amount of amber-colored material into the dialysate, then equilibrated against distilled water while under pressure and freeze-dried to give fraction 1 (Table I).

STEP 2. DEAE-SEPHADEX A-50 CHROMATOGRAPHY. Fraction 1 was dissolved in 0.20 M KCl–0.01 M potassium phosphate (pH 7.0) and adsorbed to a DEAE-Sephadex A-50 column equilibrated with the same buffer. The column was developed as described in Figure 1a. Under these conditions, 49% of the TAME esterase activity was not adsorbed (peak A), whereas 51% was eluted as one peak (peak B; identified by direct action on isolated rat uterus as rat urinary kallikrein) as a conductivity of 29 mS, corresponding to about 0.32 M KCl–0.01 M potassium phosphate (pH 7.0). The most active fractions from peak B were combined and concentrated by pressure dialysis against 3 M NaCl–0.01 M sodium phosphate (pH 6.0) to give fraction 2b.

STEP 3. HYDROXYLAPATITE CHROMATOGRAPHY. Fraction 2b was adsorbed to a hydroxylapatite column which was then developed as described in Figure 1b. All of the TAME esterase activity was eluted as one peak at a phosphate concentration of 0.03 M. The most active fractions were pooled, pressure dialyzed, and equilibrated against 0.01 M sodium phosphate (pH 6.0) to give fraction 3 (10 ml).

STEP 4. ELECTROFOCUSING. Fraction 3 was divided into two equal parts and electrofocused in two separate runs. To each 5 ml of starting sample was added 2.15 g of sucrose and 0.15 ml of 40% Ampholine (pH 3–5) and made to 8.8 ml with water.

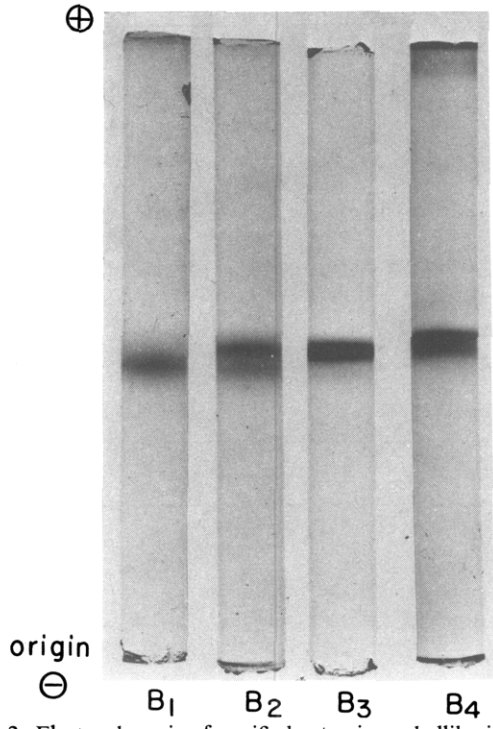

FIGURE 2: Electrophoresis of purified rat urinary kallikreins (fractions 4a–d, Table I) in 16.25% polyacrylamide gels.

This solution replaced those fractions in the stepwise gradient with which it was isopycnic (near the middle of the gradient). Four TAME esterases peaks were obtained with p*I*'s of 3.50, 3.68, 3.73, and 3.80, and were named B_1, B_2, B_3, and B_4, respectively (Figure 1c; Table I). The most active fractions of B_3 (the major peak) and all of B_1, B_2, and B_4 were pooled separately, dialyzed against 3 M KCl–0.01 M potassium phosphate (pH 6.0), concentrated by pressure dialysis, and equilibrated against 0.01 M sodium phosphate (pH 7.0) to give fractions 4a–4d.

STEP 5. BIO-GEL P-200 GEL FILTRATION. Fraction 4c was filtered through a Bio-Gel P-200 column. The protein and TAME esterase activity were eluted as a single symmetrical peak at 2.34 times the void volume (Figure 1d). The molecular weight of esterase B_3 was estimated to be 38,500 (Andrews, 1964). The active fractions were combined, pressure dialyzed, equilibrated against distilled water, and freeze-dried to yield fraction 5 (Table I).

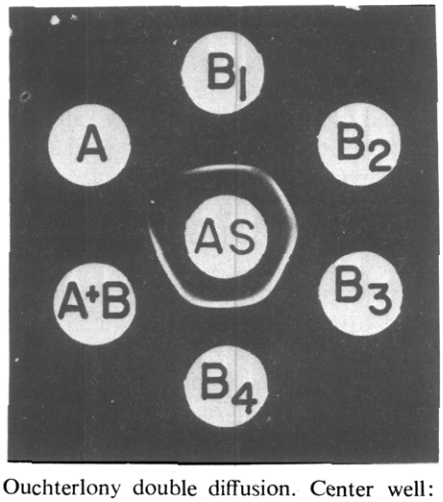

FIGURE 3: Ouchterlony double diffusion. Center well: undiluted sheep antiserum to rat urinary kallikrein B_3; peripheral wells: B_1–B_4, purified rat urinary kallikreins B_1–B_4; A + B, pressure-dialyzed rat urine (fraction 1, Table I); A, esterase A (fraction 2a, Table I). See text under Results for further details.

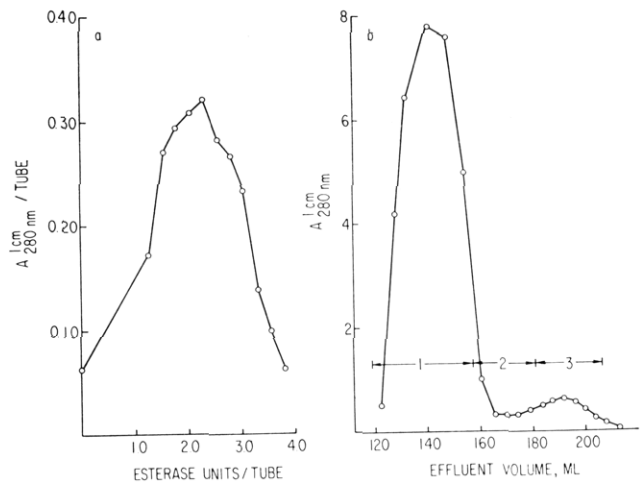

FIGURE 4: Purification of rat urinary kallikreins and their antibody by immunoprecipitation and gel filtration. (a) Quantitative precipitin curve of crude rat urinary kallikrein and sheep antiserum to rat urinary kallikrein B_3. (b) Sephadex G-100 gel filtration of the immune precipitate with 8 M urea–0.20 M NaCl–0.01 M sodium phosphate (pH 6.0); column, 2.5 × 90 cm. starting sample, 254 A_{280} units of immune precipitate in 12 ml of buffered 8 M urea; fraction volume, 5.0 ml; flow rate, 11 ml/hr.

Polyacrylamide Disc Gel Electrophoresis of Rat Urinary Kallikreins B_1–B_4. Purified rat urinary kallikrein B_3 (fraction 4c, Table I) moved with the tracking dye front when the standard 7% polyacrylamide gel was used, but only about one-half as fast in 16.25% gels. Figure 2 shows the results of electrophoresis of kallikreins B_1–B_4 in such gels. Rat urinary kallikrein B_3 showed one major band and faintly visible bands on either side which may correspond to B_1 + B_2 and B_4. An unstained gel (stored at −20°) of B_3 was sectioned, homogeneized in saline–0.01 M sodium phosphate (pH 7.0), and assayed for TAME esterase activity. The activity, recovered in 66% yield, was confined to the B_3 band. Incubation of each of the four kallikreins with neuraminidase did not change their mobilities in polyacrylamide gel electrophoresis.

Each of the four kallikreins gave a single band when run in 10% polyacrylamide gels containing SDS. Measurement of the mobilities of these bands at the sharp front and of those of pepsin and ovalbumin relative to that of chymotrypsinogen A gave molecular weights of 35,500, 33,600, 33,100, and 32,300 for kallikreins B_1–B_4, respectively.

Immunology. When rat urinary kallikrein B_3 was injected into a sheep, no precipitating antibody was found on day 30. After the first booster injection on day 30, however, precipitating antibody was found on day 43. A second booster injection on day 43 gave an appreciable increase in antibody titer on day 52 which did not perceptibly change thereafter to day 124. The antiserum used in this study was provided by a large bleeding on day 52.

As shown in Figure 3, this antiserum gave a single line of identity with kallikreins B_1–B_4 (all *ca.* 20 EU/ml) and pressure-dialyzed rat urine (38 EU/ml, 50% of which is due to esterase A), but no discernible line with a high concentration (130 EU/ml applied three times) of esterase A (fraction 2a, Table I). Thus, rat urinary kallikreins B_1–B_4 are immunologically identical and the antiserum contains detectable precipitating antibody only to them. No precipitin line was observed with human urinary kallikrein (*ca.* 20 EU/ml).

TABLE III: Substrate Specificity of Rat Urinary Kallikrein and Esterase A.

Substrate	Concn (mM)	pH	Temp (°)	Sp Act. (μmol/min per A_{280})	
				Kallikrein	Esterase A
α-*N*-Carbobenzyloxy-L-arginine methyl ester	20	8.0	25	346	1.65
α-*N*-Benzoyl-L-arginine ethyl ester	20	8.0	25	193	3.22
α-*N*-Tosyl-L-arginine methyl ester	20	8.0	25	25	1.51
α-*N*-Benzoyl-L-arginine *p*-nitroanilide	2.4	8.5	37	0.5	0.07
α-*N*-Benzoyl-DL-methionine methyl ester	10	8.0	25	0	
Casein	0.5%	8.0	37	0	0

Purification of Rat Urinary Kallikreins B_1–B_4 and Their Specific Antibody from an Immune Precipitate. Rat urine concentrate (63 ml; fraction 0, Table II) and sheep antiserum (70 ml) were mixed at the equivalence point found for these same solutions by quantitative precipitin analysis (Figure 4a), made to 700 ml with 0.15 M NaCl–0.01 M sodium phosphate (pH 7.5), stirred for 1.0 hr at 37° and for 24 hr at 0°, and centrifuged at 0°. The white precipitate was evenly suspended seven times with fresh 20-ml portions of ice-cold saline–phosphate buffer, and centrifuged each time at 0°. The final wash had an adsorbance of 0.065 at 280 nm. The precipitate was dissolved in buffered 8 M urea and filtered through a Sephadex G-100 column (Figure 4b; Table II). Three pools were made, as shown in Figure 4b, and pressure dialyzed against saline–phosphate (pH 6.0). Fraction 1 (204 A_{280} units before dialysis) contained no TAME esterase activity, but rather behaved like antibody to esterase B on immunodiffusion plates and like a γ-globulin in polyacrylamide disc gels (Figure 5). Fraction 3 (13.4 A_{280} units before dialysis) behaved like a mixture of kallikreins B_1–B_4 on polyacrylamide disc gel electrophoresis: the band corresponding to B_3 was the most prominent one, as previously found in the original purification of the kallikreins. The specific activity was about that calculated for a mixture of B_1–B_4 (fractions 4a–d, Table I), *viz.*, 20 EU/A_{280}. Fraction 3 was dialyzed against distilled water and freeze-dried to give 12.5 mg. Fraction 2 (12.8 A_{280} units before dialysis) contained TAME esterase activity but the specific activity was only 10 EU/A_{280}: this fraction behaved like the immunogen on Ouchterlony plates, and gave polyacrylamide disc gel bands corresponding to kallikreins B_1–B_4, as well as a band with mobility between the γ-globulin band of fraction 1 and the B_1–B_4 bands of fraction 3. TAME esterase activity was found associated only with the rat urinary kallikrein bands.

Assuming that all of the protein of fraction 1 before dialysis is antibody to rat urinary kallikrein, that the molecular weights of antigen and antibody are 33,000 and 160,000, respectively, and that their $A^{1\%}_{1\text{ cm}}$'s are 10 and 14, respectively, then one can calculate that the immune precipitate contained 1.6 mol of antibody/mol of antigen and that the antibody titer of the antiserum is about 2 mg/ml.

Substrate Specificity. Of the typical synthetic L-arginine substrates of trypsin listed in Table III, ZAME was by far the best substrate for rat urinary kallikreins. No hydrolysis of casein was observed when 30 μg of pure rat urinary kallikrein (fraction 3, Table II) was incubated with 0.5% casein for 30 min at 37° and pH 8.0, whereas 0.3–0.5 μg of bovine trypsin gave appreciable hydrolysis under the same conditions. No discernible hydrolysis of *N*-benzoyl-DL-methionine methyl ester was found for pure rat urinary kallikreins.

Biological Activity. Direct Action on the Rat Uterus. Rat urinary kallikreins B_1–B_4 without added substrate caused equal contraction of the rat uterus when equal amounts of TAME esterase activity were added to the organ bath, whereas no such activity was observed on the guinea pig ileum. The contraction caused by the kallikreins was slow like that of bradykinin, but came after a much longer lag period. A dose of 200 ng of each kallikrein was equivalent to 30–40 ng of bradykinin. Esterase A had no such activity on the isolated rat uterus.

Preincubation of rat urinary kallikreins (200 ng) in Tyrode solution for 3 min at 37° with Trasylol (14 μg) or purified antibody (200 μg) abolished the oxytocic effect, whereas carboxypeptidase B (75 μg/ml) had no effect. However, carboxypeptidase B added to the organ bath at a lower concentration (25 μg/ml) 15 sec before the kallikreins (200 ng) significantly reduced their oxytocic effect.

Repeated addition of rat urinary kallikrein to the rat uterus at 8-min intervals gave a slow reduction of the response, which was completely abolished after 8–10 hr. Also, storage of the rat uterus in de Jalon solution for 4 days at 4° absolished the direct contraction by the kallikreins. No change in the response to bradykinin was observed by these preparations. It was calculated that contractions equivalent to 14–20 μg of bradykinin were obtained from such a rat uterus.

Kinin Formation. Purified rat urinary kallikreins released kinin from dog plasma equivalent to 20–32 μg of bradykinin/

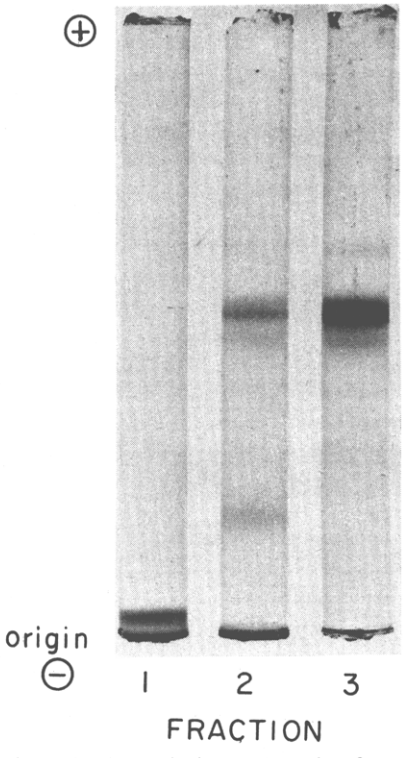

FIGURE 5: Polyacrylamide gel electrophoresis of rat urinary kallikrein and antibody fractions from 8 M urea Sephadex G-100 gel filtration of the immune precipitate.

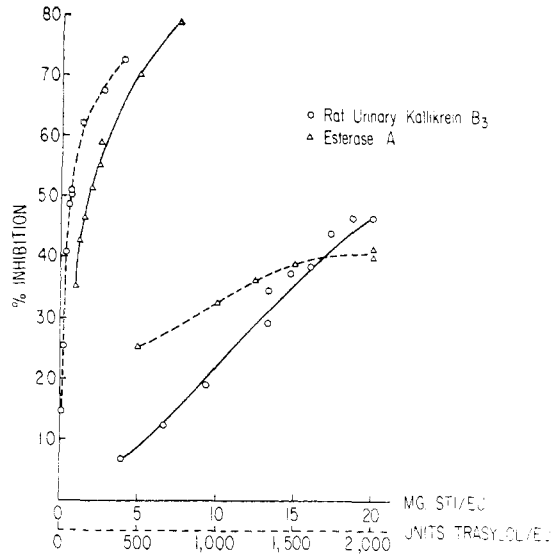

FIGURE 6: Inhibition of TAME activity of rat urinary kallikrein B_3 and esterase A by STI and Trasylol (contains *ca.* 0.14 μg of protein/inhibitor unit according to Schultz *et al.*, 1963).

min per mg, and lowered the blood pressure of anesthetized rats. The fall in blood pressure came after a lag period typical of kallikreins, but not observed when bradykinin was injected. Esterase A, with a TAME esterase activity 30 times greater than that which gave a good response with the kallikreins, had no effect on the rat blood pressure. However, incubation of esterase A with dog kininogen gave kinin equivalent to 0.12 μg/min per A_{250}.

Inhibition Studies with Esterase A and Rat Urinary Kallikrein. As shown in Figure 6, the TAME esterase activity of rat urinary kallikrein B_3 is strongly inhibited by Trasylol but only weakly by STI, whereas just the converse is true for esterase A.

Preincubation for 1 min of about 3 μg of each of the purified rat urinary kallikreins with 10, 50, and 100 μl of either sheep antiserum to rat urinary kallikrein B_3 or purified antibody solution (2.0 mg/ml of fraction 1, Table II) gave 0, 50, and nearly 100% inhibition, respectively, of the biological activity. It can be calculated on the basis of assumptions made above that about 5 mol of antibody are required for 50% inhibition of 1 mol of rat urinary kallikrein. No inhibition of their TAME esterase activity was observed. Neither normal sheep serum nor a γ-globulin fraction from the same serum had any effect on the kininogenase activity of the four kallikreins.

Discussion

The four rat urinary TAME esterases B_1–B_4 are kallikreins since they release kinins very rapidly from native kininogens, lower the blood pressure in the rat in a manner typical of kallikreins, directly contract the isolated rat uterus, and have no detectable caseinolytic activity. They are indistinguishable biologically and immunochemically. The small differences in isoelectric points and electrophoretic mobilities cannot be ascribed to varying numbers of sialic acid residues, since no mobility changes were observed after neuraminidase treatment. The four kallikrein forms observed may be derived from a single proenzyme by a complex activation mechanism.

Previous preparations of kallikreins from human (Moriya *et al.*, 1963), horse (Prado *et al.*, 1962, 1963), hog (Werle and Trautschold, 1963), and rat (Mares-Guia *et al.*, 1970; Porcelli and Croxatto, 1971) urines had not shown such heterogeneity. However, some other glandular kallikreins did exhibit heterogeneity. For example, four forms of rat submandibular kallikrein have been purified (Ekfors *et al.*, 1967), and hog pancreatic kallikrein has been resolved into two forms (Fiedler and Werle, 1967). In addition, we have found that human urinary kallikrein can be separated into multiple isoelectric and molecular weight forms (Pierce and Nustad, 1972).

Some of the purification methods used for rat urinary kallikreins could be successfully applied to the human enzymes. The efficiency of these methods recommends their use in attempts to purify other glandular kallikreins. However, DEAE-Sephadex chromatography, which separated esterase A and rat urinary kallikrein and produced a sixfold increase in specific activity of the latter, was an inefficient step in the purification of human urinary kallikreins. On the other hand, hydroxylapatite chromatography failed to separate esterase A and rat urinary kallikrein, but was able to resolve human urinary kallikreins A and B (Pierce and Nustad, 1972)[2].

Rat TAME esterase A failed to release kinin from rat plasma. However, with heterologous plasma, a smooth muscle contracting substance was formed. The much greater lability of esterase A than rat urinary kallikrein may explain why others (Margolius *et al.*, 1972; Geller *et al.*, 1972) found that rat urine collected under a variety of experimental conditions gave essentially the same amounts of kallikrein activity whether it was measured by hydrolysis of [^{3}H]TAME (Beaven *et al.*, 1971) or by bioassay.

Rat urinary kallikreins cause a direct contraction of the isolated rat uterus. The direct action of kallikreins on certain smooth muscles was first noted by Werle *et al.* (1937), and a similar effect of trypsin was observed by Rocha e Silva (1939). Also, Beraldo *et al.* (1966) have noted that rat urinary kallikrein contracts the rat uterus but has no effect on other smooth muscle preparations. The present study confirms and extends the earlier results, inasmuch as the direct action of the enzyme is inhibited both by Trasylol and by specific antibody to rat urinary kallikrein.

The direct action on rat uterus is probably due to kinin liberation from kininogen indigenous to the uterus, since prior addition of carboxypeptidase B to the organ bath reduced the effect, and storage of the uterus for four days or repeated treatment with kallikrein abolished the response to the enzyme, but not to bradykinin.

The TAME esterase activity of the kallikreins was extraordinarily stable, thus allowing the use of 8 M urea for dissociation and gel filtration of the immunoprecipitate. However, the biological activity was somewhat labile: the ratio of biological to TAME esterase activity in the purified kallikreins B_1–B_4 was only 36% of that of the starting pressure-dialyzed urine (fraction 1, Table I). This may be attributed partly to our having determined the TAME activities first and the biological activities much later, when the fractions had been frozen and thawed several times. Such losses of biological activity with retention of esterase activity have also been observed for thrombin (Seegers *et al.*, 1967) and human plasma kallikrein (Pierce, unpublished data).

While several reports indicate the kidney origin of urinary kallikrein (Werle and Vogel, 1960; Carvalho and Diniz, 1966; Nustad, 1970), we wanted to test this hypothesis more decisively than was previously possible. The isolation, from incubates of L-[^{3}H]leucine with rat kidney slices, of tritiated kallikreins closely similar to the urinary enzymes will be described elsewhere.[4]

K. Nustad, J. V. Pierce, and K. Vaaje, manuscript in preparation.

References

Adetuyibi, A., and Mills, H. (1972), *Lancet 2*, 203.

Andrews, P. (1964), *Biochem. J. 91*, 222.

Barton, C. J. (1948), *Anal. Chem. 20*, 1068.

Beaven, V. H., Pierce, J. V., and Pisano, J. J. (1971), *Clin. Chim. Acta 32*, 67.

Beraldo, W. T., Araujo, R. L., and Mares-Guia, M. (1966), *Amer. J. Physiol. 211*, 975.

Carvalho, I. F., and Diniz, C. R. (1966), *Biochim. Biophys. Acta 128*, 136.

Croxatto, H. R., and San Martín, M. (1970), *Experientia 26*, 1216.

Davis, B. J. (1964), *Ann. N. Y. Acad. Sci. 121*, 404.

Dunker, A. K., and Rueckert, R. R. (1969), *J. Biol. Chem. 244*, 5074.

Ekfors, T. O., Riekkinen, P. J., Malmiharju, T., and Hopsu-Havu, V. K. (1967), *Hoppe-Seyler's Z. Physiol. Chem. 348*, 111.

Erlanger, B. F., Kokowsky, N., and Cohen, W. (1961), *Arch. Biochem. Biophys. 95*, 271.

Fiedler, F., and Werle, E. (1967), *Hoppe-Seyler's Z. Physiol. Chem. 348*, 1087.

Geller, R. G., Margolius, H. S., Pisano, J. J., and Keiser, H. R. (1972), *Circ. Res. 31*, 857.

Hilton, S. M. (1970), *in* Handbook of Experimental Pharmacology, Vol. 25 (Bradykinin, Kallidin, and Kallikrein), Erdös, E., Ed., New York, N. Y., Springer-Verlag, p 389.

Kabat, E. A., and Mayer, M. M. (1964), *in* Experimental Immunochemistry, 2nd ed, Springfield, Ill., C. C Thomas, p 22.

Laskowski, M. (1955), *Methods Enzymol. 2*, 26.

Mares-Guia, M., Silva, E., and Diniz, C. R. (1970), *Advan. Exp. Med. Biol. 8*, 65.

Margolius, H. S., Geller, R. G., de Jong, W., Pisano, J. J., and Sjoerdsma, A. (1972), *Circ. Res. 31, Suppl. II*, 125.

Moriya, H., Pierce, J. V., and Webster, M. E. (1963), *Ann. N. Y. Acad. Sci. 104*, 172.

Nustad, K. (1970), *Brit. J. Pharmacol. 39*, 73.

Ouchterlony, Ö. (1949), *Acta Pathol. Microbiol. Scand. 26*, 507.

Pierce, J. V. (1968), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 27*, 52.

Pierce, J. V. (1970), *in* Handbook of Experimental Pharmacology, Vol. 25 (Bradykinin, Kallidin, and Kallikrein), Erdös, E., Ed., New York, N. Y., Springer-Verlag, p 21.

Pierce, J. V., and Nustad, K. (1972), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 31*, 623 Abstr.

Porcelli, G., and Croxatto, H. (1971), *Ital. J. Biochem. 20*, 66.

Prado, E. S., Prado, J. L., and Brandi, C. M. W. (1962), *Arch. Int. Pharmacodyn. Ther. 137*, 358.

Prado, J. L., Prado, E. S., Brandi, C. M. W., and Katchburian, A. V. (1963), *Ann. N. Y. Acad. Sci. 104*, 186.

Roberts, P. S. (1958), *J. Biol. Chem. 232*, 285.

Rocha e Silva, M. (1939), *Arch. Inst. Biol. Sao Paulo 10*, 93.

Schultz, F., Kraut, H., and Bhargava, N. (1963), *Naturwissenschaften 50*, 375.

Seegers, W. H., Schroer, H., and Marciniak, E. (1967), *in* Blood Clotting Enzymology, Seegers, W. H., Ed., New York, N. Y., Academic, p 103.

Shapiro, A. L., Viñuela, E., and Maizel, J. V., Jr. (1967), *Biochem. Biophys. Res. Commun. 28*, 815.

Sussman, H. H., Small, P. A., Jr., and Cotlove, E. (1968), *J. Biol. Chem. 243*, 160.

Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244*, 4406.

Webster, M. E., and Prado, E. S. (1970), *Methods Enzymol. 19*, 681.

Werle, E., Götze, W., and Keppler, A. (1937), *Biochem. Z. 289*, 217.

Werle, E., and Trautschold, I. (1963), *Ann. N. Y. Acad. Sci. 104*, 117.

Werle, E., and Vogel, R. (1960), *Arch. Int. Pharmacodyn. Ther. 126*, 171.