Nazario, M. (1972), *J. Bacteriol. 112,* 1076.
Nazario, M., and Evans, J. A. (1974), *J. Biol. Chem. 249* (in press).
Okamoto, T., and Kawade, Y. (1967), *Biochim. Biophys. Acta 145,* 613.
Rouget, P., and Chapeville, F. (1971), *Eur. J. Biochem. 23,* 443.
Scatchard, G. (1949), *Ann. N. Y. Acad. Sci. 51,* 660.
Yaniv, M., and Gros, F. (1969), *J. Mol. Biol. 44,* 17.
Yarus, M., and Berg, P. (1967), *J. Mol. Biol. 28,* 479.
Yarus, M., and Berg, P. (1969), *J. Mol. Biol. 42,* 171.
Zubay, G. (1962), *J. Mol. Biol. 4,* 347.

Isolation and Characterization of Porcine Pancreatic Kallikrein†

Martin Zuber and Edgar Sache*

ABSTRACT: Kallikrein was isolated from a defatted extract of partially autolyzed porcine pancreas. A 1300-fold purification was achieved using ammonium sulfate precipitation, calcium phosphate gel adsorption, gel filtration on Sephadex G-100, and DEAE-cellulose chromatography. Repeated DEAE-cellulose chromatography permitted the separation of two major, enzymically active kallikrein components (forms d_1 and d_2) exhibiting distinct mobilities during acrylamide–agarose gel electrophoresis. The latter experiments also revealed the presence of small amounts of an additional, slightly more anionic active kallikrein component (d_3), closely related to form d_2 under the conditions employed for ion-exchange chromatography. By isoelectric focusing, forms d_1 and d_2 could each be resolved into discrete enzymically active peaks (isoelectric points between 3.92 and 4.11). Kallikrein forms d_1 and d_2 both contain a small amount of carbohydrate, 4.6 and 3.2%, respectively. A different hexosamine content was found to be associated with each of these major forms, which may account for some of the observed differences in their elution behavior and electrophoretic mobility. Forms d_1 and d_2 have essentially the same sedimentation coefficient (2.83 S) and closely related amino acid compositions and molecular weights (about 33,000), as determined by gel filtration, sedimentation velocity, and amino acid composition. In both forms, electrophoresis in sodium dodecyl sulfate–polyacrylamide gel in the presence of β-mercaptoethanol separated several components, thus suggesting in each case a disulfide-linked polypeptide chain structure. The enzyme contains no detectable free sulfhydryl groups. Disulfide reducing agents cause loss of activity. Casein or azocoll is not hydrolyzed by kallikrein. In a series of esters, *N*-benzoyl-L-arginine ethyl (or methyl) ester proved to be the best substrate (pH 8.0). *N*-α-Carbobenzoxy-L-lysine benzyl ester is hydrolyzed at nearly a comparable rate. Hydrolysis of *N*-α-carbobenzoxy-L-lysine methyl ester was, however, much slower, that of *N*-α-benzoyllysine methyl ester being nearly negligible. These results show that the specificity is directed primarily toward the esters of arginine and to a lesser extent toward those of lysine. They also indicate that "secondary interaction" implying the nature of the *N*-acyl group of the substrate (position P_2) as well as that of the P'_1 group (according to the nomenclature of Schechter and Berger (1967)), *Biochem. Biophys. Res. Commun. 27,* 157) also contributes to the overall catalytic efficiency of kallikrein. The enzyme also reacts with *p*-nitrophenyl *p'*-guanidinobenzoate giving a burst, followed by a rapid production of *p*-nitrophenol. The enzyme is inhibited by the Kunitz bovine basic proteinase inhibitor. It is also inactivated by diisopropyl phosphorofluoridate, phenylmethanesulfonyl fluoride, diphenylcarbamyl chloride, *p*-aminobenzamidine, and a number of quaternary ammonium compounds. Tosyl-L-lysine or tosyl-L-phenylalanine chloromethyl ketones have no effect on enzyme activity. A slow but definite inhibition was obtained, however, with *N*-α-benzyloxycarbonyl-L-phenylalanine chloromethyl ketone. Following intravenous injection of the enzyme into a dog an immediate and marked fall in arterial blood pressure was observed. The enzyme was identified as porcine pancreatic kallikrein on the basis of its physical and chemical properties and hypotensive action.

It has been proposed that a relationship exists in a number of pathophysiological states between the fibrinolytic, blood coagulation, and vasoactive peptide systems (Eisen, 1964; Back, 1966). Whereas plasmin and thrombin play a key role in fibrinolysis and blood coagulation, respectively, the term kallikrein has been applied to a number of enzymes from various origins (*i.e.*, tissues, body fluids) which rapidly and specifically produce potent physiologically active peptides (kinins) from a plasma α_2-globulin substrate (kininogen). In addition to their strong vasodilatory and hypotensive properties, kinins also increase vascular permeability, produce pain, and modify the migration of leukocytes (Kellermeyer and Graham, 1968). Thus, functionally, a kallikrein is a kininogenase. Such activity, however, does not completely characterize kallikreins since a number of proteins, either proteolytic enzymes (Prado, 1970) or not (Fischer and Udermann, 1970) have also been found to release kinins from a plasma precursor. Kallikreins may thus represent a class of proteolytic enzymes of limited (and still ill defined) specificity for which, at least in the present state of knowledge, a plasma globulin constitutes the best known natural substrate.

In order to understand fully the role of kallikreins in a number of pathophysiological mechanisms, a better knowledge of these enzymes appears desirable particularly with respect to their catalytic activity and specificity. This requires that highly purified enzyme preparations should be readily available.

Porcine pancreas represents a convenient and relatively inexpensive source of glandular kallikrein (EC 3.4.4.21). Although

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the isolation and partial characterization of kallikrein from porcine pancreas have been the subject of a number of studies (Webster, 1970) most of the reports suffer from a lack of details in the procedures employed for the isolation (especially in relation to the early stages of the purification), they do not indicate the yields and/or they appear to involve a great number of laborious steps. Moreover, significant differences in the biological activity of some of the preparations are found (Webster, 1970), which might suggest, at least in some cases, the presence of contaminants or possible degradation during the isolation procedure.

The present communication deals with the isolation and characterization of kallikrein from an extract of partially autolyzed porcine pancreas. The purification of the enzyme (hereafter kallikrein) was monitored by its esterase activity toward Bz-Arg-OEt.[1] Since, besides trypsin and kallikrein porcine pancreas contains other enzymes which also catalyze the hydrolysis of Bz-Arg-OEt (Desnuelle *et al.*, 1969; Voytek and Gjessing, 1971), conclusive identification of the enzyme with kallikrein has resulted from a number of physical, chemical, and biological determinations.

Because of the low amounts of kallikrein present in the starting material and because of some losses observed during enzyme isolation, a pilot-level procedure has been developed and is reported. The procedure was found highly reproducible and is believed to be simpler than existing ones. It has repeatedly led to kallikrein preparations with a high degree of purity as judged by ultracentrifugation and acrylamide gel electrophoresis, and by high kininogenase potency.

Two preliminary accounts of a portion of this work have appeared (Sache and Thély, 1968; Sache *et al.*, 1971).

Experimental Section

Materials. All synthetic substrates were obtained commercially mostly from Sigma Chemical Corp., Mann Research Laboratories, or Cyclo Chemical Corp. Sources of other materials were: ovalbumin, Worthington; bovine serum albumin, myoglobin, casein, domiphen bromide (dodecyldimethyl (2-phenoxyethyl)ammonium bromide), Mann; cytochrome *c*, soybean, lima bean, and ovomucoid trypsin inhibitors, neuraminidase (*Clostridium perfrigens*, Type VI), Sigma; benzamidine hydrochloride, Aldrich Chemical Co.; tetra-*N*-methylammonium iodide, tetra-*N*-butylammonium chloride, K and K Labs; benzethonium chloride (benzyldimethyl[2-[2-(*p*-1,1,3,3-tetramethylbutylphenoxy)ethoxy]ethyl]ammonium chloride), Schwarz; benzalkonium chloride, 50% solution, Touzart-Matignon, Paris; methyl *p*-nitrobenzenesulfonate, diphenylcarbamyl chloride, Eastman Organic Chemicals; phenylmethanesulfonyl fluoride, azocoll, Calbiochem, Lucerne; tosyl-L-leucine-, *N*-α-tosyl-L-lysine- and *N*-tosyl-L-phenylalanine chloromethyl ketones, Cyclo; benzyloxycarbonylphenylalanine chloromethyl ketone, Fox. Porcine trypsin, bovine chymotrypsinogen, α-chymotrypsin, and the Kunitz bovine proteinase inhibitor (Sache *et al.*, 1965) were manufactured by Laboratoire Choay, Paris. Kidney bean (*Phaseolus vulgaris*) trypsin inhibitor was prepared from an alcoholic extract followed by gel filtration on Sephadex G-150 and DEAE-cellulose chromatography. The bovine Kazal-type pancreatic trypsin inhibitor was prepared from pancreas using the chromatographic procedure described by Greene *et al.* (1966) for inhibitor from pancreatic juice. An additional isoelectric focusing separation in a pH gradient between 5 and 8 was employed as the final step in the purification. Two fractions were obtained (p*I* = 5.0 and 6.0) and the major one (p*I* = 6.0), with an amino acid composition in agreement with that published by Greene *et al.* (1966), was used for inhibition experiments. The porcine Kazal-type pancreatic trypsin inhibitor was also prepared from the whole pancreas after Greene *et al.* (1968) but the last step (sulfoethyl-Sephadex) was omitted. DEAE-cellulose (Selectacel type 40; 0.93 mequiv/g) was purchased from Schleicher and Schuell, Keene, N. H. Sephadex and Blue Dextran were obtained from Pharmacia, Uppsala. Urea was recrystallized from 90% ethanol and deionized by passage over an ion-exchange resin (Amberlite MB3) immediately before use. Iodoacetic acid was recrystallized from petroleum ether until colorless (mp 81.5°), iodoacetamide from 50% ethanol, sodium dodecyl sulfate from 95% ethanol, and diphenylcarbamyl chloride from methanol. All other unspecified chemicals were highly purified commercial products.

Protein concentrations were estimated by a modified Lowry procedure, with ovalbumin as standard (Miller, 1959).

Assay Procedures. Proteolytic activity was examined by casein digestion (Hofsten and Reinhammar, 1965). A protein–dye complex (azocoll) was also used as substrate under the conditions indicated by the manufacturer.

Aminopeptidase activity was qualitatively examined on di- and tripeptide substrates by thin-layer chromatography. Aminopeptidase activity against aminoacyl-β-naphthylamides (arylamidase activity) was measured according to Arst *et al.* (1959).

Elastase activity was determined with orcein-impregnated elastin as substrate (Sachar *et al.*, 1955). Exopeptidase activities were determined with hippuryl-L-phenylalanine and hippuryl-L-arginine as substrates for carboxypeptidases A and B, respectively (Folk and Schirmer, 1963; Folk *et al.*, 1960).

Bz-Arg-OEt hydrolysis was followed in a pH-Stat assembly (Radiometer, Copenhagen). The assay was carried out in a total reaction volume of 1 ml containing 20 μmol of Bz-Arg-OEt and 18–20 μmol of $CaCl_2$ in 0.0015 M borate–HCl buffer. The reaction was started by addition of the enzyme solution (4–5 μg). Titrations were performed under a nitrogen stream with 0.01 N NaOH at pH 8.0 in a vessel maintained at 25 ± 0.1° by a water bath. All values were based on the average of at least duplicate assays. Activity was expressed as micromoles of Bz-Arg-OEt hydrolyzed per minute. The values of V and K_m were calculated from initial velocity measurements at various substrate concentrations from Lineweaver–Burk plots where the best-fitting straight line to the data was determined by a least-squares procedure. Esterase activity toward a number of other synthetic esters was also examined under the conditions described above, with slight modifications (*e.g.*, addition of 10% methanol (v/v) to increase solubility), where appropriate.

Total hexose was determined by the anthrone procedure of Roe (1955). Hexosamine was estimated by the procedure of Elson and Morgan (1933) and sialic acid by the thiobarbituric acid method of Warren (1959).

Enzyme Purification. An acetone powder (Pancreatine) obtained from partially autolyzed porcine pancreas was used as the starting material. Isolation of the enzyme was carried out on 10-kg batches of the powder. All steps were performed at 4° unless otherwise noted.

The defatted powder was suspended in 200 l. of 0.005 M phosphate buffer (pH 8.0)–1 M NaCl and stirred for 3 hr. The

[1] Abbreviations used are: OMe, methyl ester; OEt, ethyl ester; Ac, acetyl; Bz, benzoyl; Tos, tosyl; Z, benzyloxycarbonyl; Nbs_2, 5,5′-dithiobis(2-nitrobenzoic acid); DFP, diisopropyl phosphorofluoridate; PMSF, phenylmethanesulfonyl fluoride; TLCK, *N*-α-tosyl-L-lysine chloromethyl ketone; TPCK, *N*-tosyl-L-phenylalanine chloromethyl ketone; ZPCK, *N*-α-benzyloxycarbonyl-L-phenylalanine chloromethyl ketone; *p*-NPGB, *p*-nitrophenyl *p*′-guanidinobenzoate.

TABLE I: Purification of Kallikrein from Porcine Pancreas.[a]

Step	Total Protein (g)	Total Act. (Units)[b]	Sp Act. (Units/mg of Protein)	Recov of Esterase Act. (%)
Starting extract (acetone powder)	5850[c]	650,000	0.1	(100)
1. Ammonium sulfate fractionation (0–60%)	880	306,000	0.3	47
2. Calcium phosphate gel eluate	41.601	149,902	3.6	23
3. Sephadex G-100	8.544	68,607	8.0	11
4. 1st DEAE-cellulose				
Fraction d_1	0.368	19,963	54.2	3
Fraction d_2	0.343	21,760	63.4	3
5. 2nd DEAE-cellulose				
Fraction d_1-I	0.093	10,113	109	1.5
Fraction d_1-II	0.044	4,360	100	0.7
Fraction d_2-I	0.051	5,028	99	0.8
Fraction d_2-II	0.070	9,378	134	1.4

[a] Conditions of purification are given in the text. The data are for 10 kg of starting material. [b] Bz-Arg-OEt units not inhibited by soybean trypsin inhibitor. [c] Protein content in 10 kg of starting material.

suspension was filtered in a frame-pressure filter and solid ammonium sulfate was added to the filtrate to give 60% saturation. The resulting precipitate was dissolved in 0.005 M phosphate buffer (pH 7.6). The solution (20 l.) was dialyzed against two changes of 250 l. of 0.005 M phosphate buffer (pH 7.6) for 30 hr at 4°. Longer dialysis times were avoided as they often resulted in the appearance of precipitate with losses in enzyme recovery. The volume of the diffusate was about 35 l. (25 mg of protein/ml) (step 1, Table I).

Calcium phosphate gel (20 kg) was slowly added with continuous stirring. The suspension was made up to 80 l. with 0.005 M phosphate buffer (pH 7.6), and stirred for 4 hr at room temperature. The gel was collected by filtration and washed twice with 60 l. of the same buffer (pH 7.6), and the washings were discarded. The enzymically active fraction was eluted by suspending the gel in 80 l. of 10% ammonium sulfate and stirring for 2 hr. The suspension was filtered and the filtrate was collected. The eluting process was repeated twice (2 × 40 l.) and the combined eluates were precipitated by the addition of ammonium sulfate to 60% saturation. The precipitate

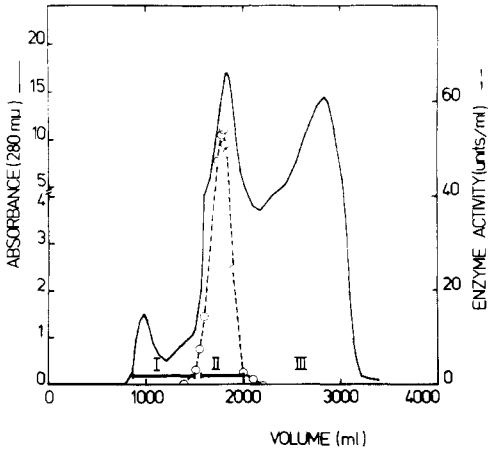

FIGURE 1: Gel filtration on Sephadex G-100 of material eluted from calcium phosphate gel by 10% ammonium sulfate. Eight grams of material from step 2 was dissolved in 60 ml of 0.05 M Tris-HCl (pH 8.0)–0.15 M NaCl buffer and applied to a column (6 × 100 cm) equilibrated and eluted with the same buffer; flow rate, 100 ml/hr; volume of fractions, 15 ml; temperature, 25°: (——) absorbance at 280 mμ; (O - - O) Bz-Arg-OEt hydrolyzing activity in units per ml.

was resuspended in water (4 l.) and dialyzed for 48 hr at 4°. Longer dialysis times were avoided since they also resulted in precipitating material at this step. Any solid material was removed by centrifugation. The dialyzed material was lyophilized (step 2, Table I).

Aliquots (8 g) of this material were applied to a column equilibrated with 0.05 M Tris-HCl buffer (pH 8.0)–0.15 M NaCl, under the conditions stated in the legend to Figure 1. The elution pattern showed three peaks. The Bz-Arg-OEt hydrolyzing activity was confined to peak II (Figure 1) which also possessed other enzymic activities: elastase, a chymotrypsin-like activity (using Ac-Tyr-OEt as substrate), carboxypeptidase A, and carboxypeptidase B. The large molecular weight fraction (peak I) coming through with the void volume contained aminopeptidase and arylamidase activity. Peak III which contained low molecular weight material presumably originating from autolysis was devoid of any activity and was discarded. Peaks I and II were pooled separately, dialyzed at 4° against water (72 hr), and lyophilized. It may be seen that during this step the yield was low (step 3, TABLE I). Since aliquots of the material subjected to gel filtration did not precipitate at room temperature and retained complete activity, losses by precipitation during column operation were unlikely. It was also checked that dialysis at the end of the step did not affect recovery. The reason for this low yield is not known.

The material of high molecular weight (peak I, Figure 1) contains arylamidase activity against leucyl- and alanyl-β-naphthylamide. A number of di- and tripeptides, *e.g.*, glycylglycine, sarcosylglycine, leucylglycine, prolylglycine, arginylvaline, alanylglycine, leucylvaline, glycylglycylglycine, and glycylglycylleucine were also hydrolyzed.

Aliquots (1.5 g) of the enzymically active protein from peak II (Figure 1) were applied to a DEAE-cellulose column previously equilibrated with 0.005 M Tris-acetate buffer (pH 8.0). Bz-Arg-OEt hydrolyzing activity was resolved into two separate peaks which have been constantly observed under the conditions employed (Figure 2). These peaks were eluted at comparatively high NaCl concentration, 0.24 and 0.26 M, respectively. The fractions comprising the peaks, d_1 and d_2 in the order of elution, were pooled, dialyzed against water at 4° (72 hr), and lyophilized (step 4, Table I). When a sample of porcine trypsin was chromatographed under the conditions described in Figure 2, it was immediately eluted with the break-

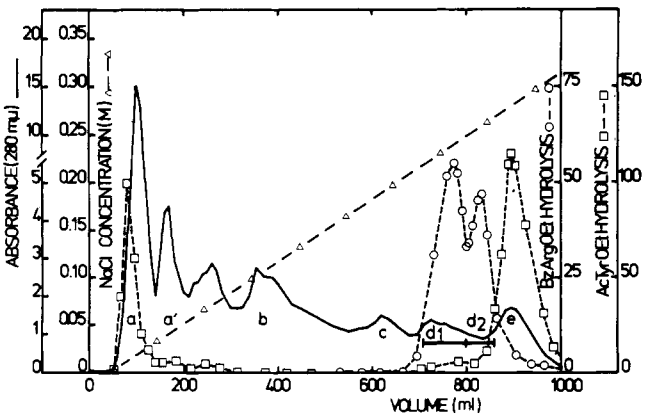

FIGURE 2: Chromatographic separation of porcine kallikrein on DEAE-cellulose. Sample, 1.5 g, of material from step 3 was dissolved in 20 ml of 0.005 M Tris-acetate buffer (pH 8.0) and applied to the column (2 × 28 cm); the column was initially equilibrated with the same buffer and elution was initiated with a linear gradient, with 750 ml of the starting buffer in the mixing chamber and 750 ml of 0.45 M NaCl in 0.005 M Tris-acetate buffer (pH 8.0) in the reservoir; flow rate, 15 ml/hr; volume of fractions, 5 ml; temperature, 25°: (——) absorbance at 280 mμ; (O - - O) Bz-Arg-OEt hydrolyzing activity; (Δ - - Δ) NaCl gradient (NaCl concentration was determined in each fraction by conductivity measurements); other enzymic activities were also tested for. (□ - - □) Ac-Tyr-OEt hydrolyzing activity, peaks a and e (0.28 M NACl); peaks a and a' (122–180 ml) possessed elastase activity; peak b (0.10 M NaCl; 300–455 ml) split hippuryl-L-arginine; peak c (0.20 M NaCl; 550–725 ml) split hippuryl-L-phenylalanine.

through volume, thus suggesting that fractions eluted as d_1 and d_2 were different.

Peaks recovered as fractions d_1 and d_2 were individually rechromatographed on DEAE-cellulose under the same conditions (Figure 3). Each fraction emerged at the same NaCl concentration as in the first DEAE-cellulose column. The descending absorbance limb of fraction d_1 possessed a shoulder, with a corresponding pronounced activity peak (Figure 3A). Fraction d_2 possessed a shoulder on the ascending absorbance limb, with the activity peak nearly symmetrical (Figure 3B). The fractions comprising the peaks were pooled as indicated, dialyzed against water at 4°, and lyophilized. During this step (step 5, Table I) the specific activity of the main fractions (d_1-I; d_2-II) rose to a value twice that obtained in step 4 and rechromatography on DEAE-cellulose was therefore systematically introduced in the purification procedure.

A summary of the purification procedure is given in Table I.

In further work, forms d_1 and d_2 of kallikrein refer to fractions d_1-I and d_2-II from step 5 (Figure 3A,B), respectively.

Isoelectric focusing fractionation was carried out in the LKB 8102 apparatus (LKB-Produkter, Stockholm) with a pH gradient between pH 3 and 5 obtained with a 1% ampholine solution in a column (440-ml capacity) cooled by circulating water (8–10°) and stabilized with a sucrose (0–46%) gradient. The procedure was carried out for 40 hr. At equilibrium the applied potential was 400 V and the current 6 mA. After completion of the experiment, fractions of 2 ml were collected and examined for absorbance at 280 mμ, pH (measured at 25° with a SE-25 pH meter equipped with a GK 2323C microelectrode, Radiometer), and enzyme activity. Selected fractions were pooled and concentrated by vacuum dialysis in collodion bags (Sartorius, Heidelberg) at 4°, first against water, then against 0.005 M Tris-acetate buffer (pH 8.0).

Polyacrylamide gel electrophoresis of the enzyme was carried out in the E.C. Apparatus Corp. Vertical Cell, Philadelphia. The gels were composed of 5% acrylamide–0.7% agarose (Uriel, 1966) in Tris–glycine buffer (pH 8.7). The separation was carried out for 75–150 min at 300 V on a gel slab 17 cm long. Gels were stained with Coomassie Blue and the background was destained electrophoretically.

Acrylamide gel electrophoresis in sodium dodecyl sulfate was performed by the procedure of Weber and Osborn (1969) in 10% acrylamide gels after incubating the samples for 2 hr at 37° in 0.01 M sodium phosphate buffer containing 1% sodium dodecyl sulfate and 1% β-mercaptoethanol. Protein standards used were bovine serum albumin, ovalbumin, pepsin, chymotrypsinogen, cytochrome *c*, and the Kunitz bovine inhibitor (mol wt 6513), with molecular weight values as listed by Weber and Osborn (1969).

Ultracentrifugation Analysis. Sedimentation experiments were performed in a Spinco Model E analytical ultracentrifuge equipped either with a schlieren or an ultraviolet absorption system. Protein concentrations studied were 5 mg/ml with the schlieren optical system and the range 0.28–1.5 mg/ml with the ultraviolet absorption optics. The experiments were carried out in 0.005 M Tris-acetate buffer (pH 8.0)–0.25 M NaCl. The runs were performed in the An-D rotor and a Kel-F analytical cell with 12-mm optical path was employed. Sedimentation coefficients were calculated from a plot of the rate of log of boundary migration *vs.* time. The best-fitting straight line was determined by a computer-performed least-squares procedure which also yielded the standard errors.

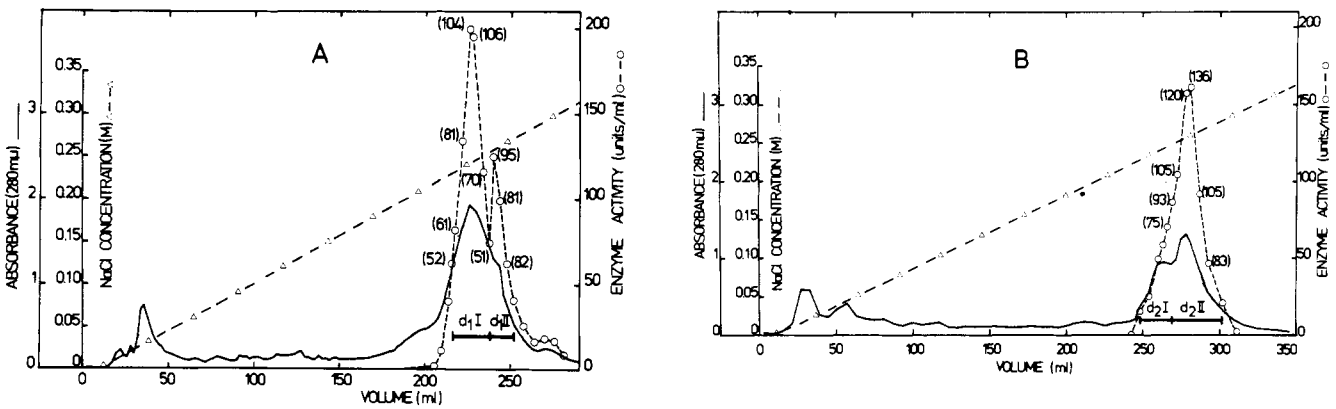

FIGURE 3: Rechromatography of two fractions of partially purified porcine kallikrein on DEAE-cellulose at pH 8.0; 75 mg of kallikrein d_1 (A) and 55 mg of kallikrein d_2 (B) were each dissolved in 5 ml of 0.005 M Tris-acetate buffer (pH 8.0) and applied to a column (1.5 × 22 cm) equilibrated with the same buffer; elution conditions were as in Figure 2, except for the volumes of mixing chamber and reservoir (500 ml); flow rate, 15 ml/hr; volume of fractions, 3 ml; temperature 25°: (——) absorbance at 280 mμ; (O - - O) Bz-Arg-OEt hydrolyzing activity; (Δ - - Δ) NaCl gradient. The number in parentheses are specific activities per unit absorbance. In other experiments, inactive material eluting at low salt concentration was only present as traces.

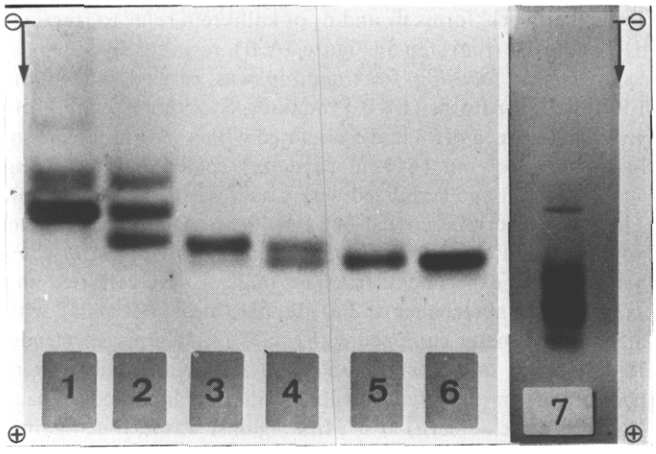

FIGURE 4: Acrylamide-agarose gel electrophoresis (pH 8.7) of porcine kallikrein at different stages of purification. Migration was from top to bottom (anode) under conditions described in the text. Time of migration is 75 min in runs 1–6 and 2 hr in run 7. The sample (1,2: 100 μg; 3–6: 30 μg) was applied in 40% sucrose in a volume of 50 μl or less: 1, calcium phosphate gel (step 2); 2, Sephadex G-100 (step 3); 3–6, DEAE-cellulose (step 5): fractions d_1-I, d_1-II, d_2-I, and d_2-II (Figure 3), respectively. In run 7, 30 μg each of fractions d_1-I and d_2-II were mixed before electrophoresis and run simultaneously for 2 hr (a different enzyme preparation than shown in runs 1–6 was used). Bands not detected in partially purified material (runs 1 and 2) are detected in runs 2–7 through concentration during gel filtration and chromatography.

Amino Acid Analysis. Samples of approximately 3–5 mg were heated in evacuated sealed tubes with 1 ml of twice distilled constant-boiling HCl. Duplicate samples were maintained at 110 ± 2° for 22, 48, and 96 hr and each sample was analyzed according to Spackman *et al.* (1958). Samples of protein were oxidized in duplicate by the method of Moore (1963) and hydrolyzed for 22 hr at 110 ± 2° for determination of cysteine and cystine as cysteic acid and of methionine as the sulfone. Tryptophan was determined by a spectrophotometric method (Bencze and Schmidt, 1957).

Molecular Weight and Related Constants. The molecular weight of the enzyme was estimated by gel filtration experiments (Andrews, 1964). A column of Sephadex G-100 (1 × 60 cm) was equilibrated and eluted with 0.05 M Tris-HCl buffer (pH 7.5)–0.1 M KCl. The void volume (V_0) of the column was taken as the elution volume of Blue Dextran. The column was calibrated with cytochrome *c* (mol wt 13,000), myoglobin (mol wt 17,200), α-chymotrypsin (mol wt 25,000), ovalbumin (mol wt 43,000), and bovine serum albumin (mol wt 68,000) as reference proteins. Since it has been stated that this method correlates with the Stokes radius of a protein rather than with its molecular weight (Siegel and Monty, 1965), the Stokes radius of the enzyme was determined, using the procedure of Ackers (1964). Molecular weight and frictional ratio estimations were performed with the Stokes radius and $s_{20,w}{}^0$ values (Siegel and Monty, 1965). The partial specific volume of the enzyme was calculated from amino acid composition data (Schachman, 1957). Gel filtration also permitted determination of the diffusion coefficient (Siegel and Monty, 1965).

Estimation of Sulfhydryl Groups. The SH content of the enzyme was examined according to Ellman (1959). The Nbs_2 reagent was prepared in 0.1 M Tris-HCl (pH 8.0)–EDTA 10^{-4} M and was used in 18-fold molar excess over the enzyme (0.06 μmol). Determinations were carried out at pH 8.0, either in buffer alone or in the presence of 8 M urea or 0.3% sodium dodecyl sulfate. In other experiments *N*-ethylmaleimide was used for SH groups estimation (Alexander, 1958) in the presence of 8 M urea.

Determination of Free SH Groups Obtained after Reductive Cleavage of the Disulfide Bonds. Reductive cleavage of the disulfide bonds was performed at 0.2% enzyme concentration with 0.15 M $NaBH_4$ in 0.2 M borate buffer–8 M urea (pH 9.1) at 25°. At various times aliquots (0.012 μmol of enzyme) were withdrawn and excess $NaBH_4$ was destroyed by the addition of 50 μl of glacial acetic acid. Foaming of the acidified sample ceased after 2–3 min and the pH was readjusted to 8.0 with 1 N NaOH. The sulfhydryl content was estimated by the Nbs_2 reagent (100-fold molar excess over the enzyme). Titrations were performed in 0.1 M Tris-HCl buffer–8 M urea (pH 8.0) containing 10^{-4} M EDTA, in a final volume of 3 ml.

Results

Enzyme Purification. Starting from an acetone extract from partially autolyzed porcine pancreas, two major forms of kallikrein have been purified by procedures combining ammonium sulfate precipitation, calcium phosphate gel treatment, Sephadex G-100 gel filtration, and repeated DEAE-cellulose chromatography. Results of the individual steps in the purification process are summarized in Table I. A 1300-fold purification was achieved with an overall yield of 4.4%.

Purity. A high degree of purity of each of the main enzyme forms (fractions d_1-I and d_2-II; step 5) was already suggested by the column patterns on DEAE-cellulose, with a fairly constant specific activity for the center of each of the two main peaks (Figure 3A,B).

The effectiveness of various individual isolation steps was examined by electrophoresis in acrylamide–agarose gel at pH 8.7. As shown in Figure 4, enzyme forms d_1 (run 3) and d_2 (run 6) migrated as nearly homogeneous bands. In other preparations, however, an additional component showing greater mobility toward the anode was observed. This component appeared in tubes with maximum esterase activity (Figure 3B), being mostly associated with tubes in the second half of the descending limb of the activity peak, and present as a single electrophoretic band in only one or two tubes (about 298–300 ml, Figure 3B). Since tubes in the descending limb of the elution profile of Figure 3B were completely devoid of any measurable chymotrypsin-like activity but possessed high specific activity toward Bz-Arg-OEt, this component should represent another form of the enzyme (form d_3). When pooled and lyophilized kallikrein forms d_1 and d_2 (step 5, Table I) were subjected to electrophoresis as a mixture, the resulting pattern (run 7, Figure 4) showed that the distinct mobilities of forms d_1 and d_2 were retained. The pattern also shows that when present, form d_3, as compared with the two other forms, occurs in much lower amounts (the thin slower moving band observed in this particular preparation was occasionally found in other preparations and may represent slight protein contamination; densitometry tracings, however, indicated that this material represents only approximately 2.5% of total stainable material). It was also observed that after incubation with neuraminidase (1:1 by weight) in 0.1 M acetate buffer (pH 5.0) for 26 hr at 37°, the electrophoretic pattern of run 7 (Figure 4) remained unchanged.

Forms d_1 and d_2 were examined in the analytical centrifuge at pH 8.0. The schlieren pattern obtained when each enzyme form (5 mg/ml) was subjected to high-speed centrifugation showed only one symmetrical peak. Both forms behaved similarly. Linear extrapolation to zero protein concentration of the sedimentation coefficient values was performed from determinations carried out at five concentrations of enzyme (0.28–1.5 mg/ml). The $s_{20,w}{}^0$ value (form d_1) was 2.83 S (SE ±0.056). The slope value (−0.019) indicated small concentration depen-

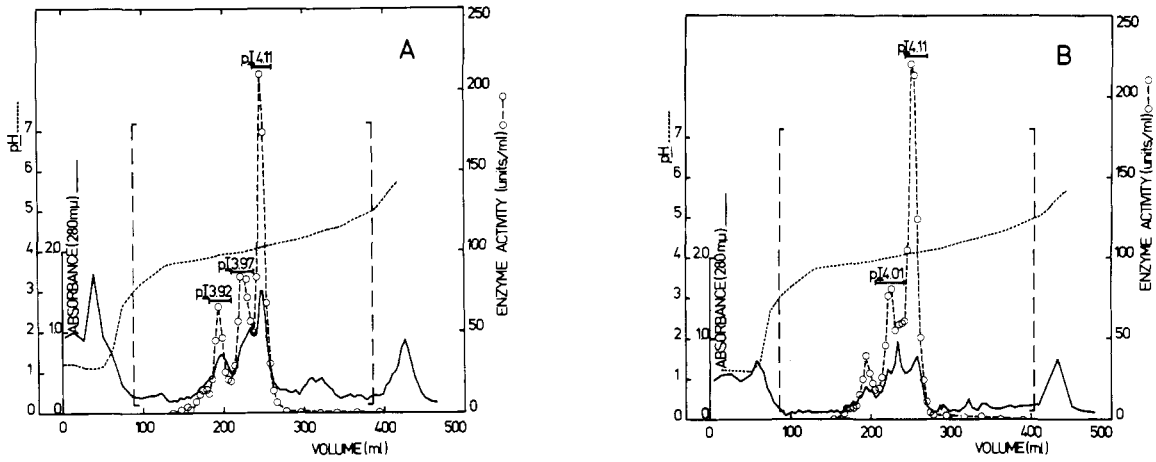

FIGURE 5: Isoelectric focusing of porcine kallikrein in the pH range 3-5. (A) Isoelectric focusing of form d_1 and (B) of form d_2; both forms are already purified fractions from step 5 (see text): (——) absorbance at 280 mμ; (- - -) pH (measured at 25°); (O - -O) Bz-Arg-OEt hydrolyzing activity. Brackets in the diagram indicate the area of electrofocusing. The areas outside the brackets represent the electrolytes of the anode and cathode in which no Bz-Arg-OEt hydrolyzing activity could be detected. Experimental conditions are given in the text.

dence of the sedimentation coefficient. The same s value, within limits of experimental error, may be assigned for form d_2.

In order to assess their homogeneity further, forms d_1 and d_2 were subjected to isoelectric focusing separately in the range between pH 3 and 5. A typical pattern obtained with each form is shown in Figure 5. Form d_1 (Figure 5A) was resolved into three major esterase activity peaks, pI = 3.92, 3.97 and 4.11 and form d_2 (Figure 5B), into essentially two major active peaks, pI = 4.01 and 4.11 (isoelectric point values, ±0.05 pH unit, are average from four to five separate determinations). Both forms also contained additional minor activity peaks, pI = 3.75 and 3.82 (d_1) and pI = 3.93 (d_2). Thus, the two kallikrein forms show patterns which do not differ significantly, except for the active material of lowest isoelectric point (*i.e.*, pI = 3.92-3.93 and below) which appears to be more largely associated with enzyme form d_1. Peak pI = 3.92 (Figure 5A) usually had a lower specific activity (about 80-100 units/mg of protein), that of peak 4.11 in Figure 5B being the highest (about 163 units/mg of protein). Thus, the specific Bz-Arg-OEt esterase activities of the peaks resolved by isoelectric focusing were not found to substantially differ from those of the starting fractions (109-134 units/mg of protein, Table I). A low Ac-Tyr-OEt splitting activity could also be detected (pI = 4.08-4.20) which was not inhibited by tosyl-L-leucine chloromethyl ketone, a chymotrypsin C inhibitor (Tobita and Folk, 1967).

Stability. Each form of purified kallikrein (500 μg/ml) could be stored at 4° in 0.005 M Tris-acetate buffer (pH 8.0) for at least 12 weeks, without any measurable change in esterase activity. Salt-free lyophilized preparations were tested after 1 year, also without appreciable loss of activity. At pH values above 4.5 (1-hr incubation, 25°) each of the kallikreins retained its activity almost completely when assayed at pH 8.0. Incubation for 1 hr at and below pH 4.0 resulted in considerable loss of activity. This instability may presumably account for the lower specific activity associated with the fractions of lowest isoelectric point obtained in isoelectric focusing experiments.

Heat inactivation was measured by heating each enzyme form at 25-120° for 10 min in sealed ampoules. When the temperature was increased above 58 ± 1°, a rapid loss in enzyme residual activity first occurred until 66-70°, followed by a slight regain in activity, that has been constantly observed. At 100° appreciable residual activity could still be observed and complete inactivation was obtained only after the temperature had reached 120-130°. Both forms of kallikrein showed comparable thermal properties.

In 8 M guanidine hydrochloride both forms of kallikrein were completely inactivated after 5 min at 37°.

Amino Acid Analysis. The results of the amino acid analysis of both forms of kallikrein, as well as data for porcine trypsin (Travis and Liener, 1965) and the data of Fritz *et al.* (1967) for porcine pancreas kallikrein, are shown in Table II. Both forms of kallikrein are very similar in composition. In order to obtain a more precise comparison of the amino acid compositions, the deviation function of Harris *et al.* (1969) was calculated: $D = [\Sigma(X_{1,i} - X_{2,i})^2]^{1/2}$ (where $X_{1,i}$ is the mole fraction for amino acid i in protein 1 and $X_{2,i}$ the mole fraction of the same amino acid in the protein being compared). The D value, which is a measure of the divergence of amino acid compositions, increases with increasing dissimilarity in the amino acid compositions being compared. The D value calculated from the data in the analyses (including all residues) was 0.012. This low value is interpreted as indicating that the two forms may be of identical amino acid composition (the D value for unrelated proteins would be near 0.100).

Molecular Weight and Related Constants. From gel filtration experiments molecular weights of 34,500 and 31,000 (average of three separate determinations) were determined for forms d_1 and d_2 of kallikrein, respectively. Since the standards used were globular proteins, these values are based upon the assumption that kallikrein is a globular protein. This assumption was found valid since frictional ratios, f/f_0, of 1.34 and 1.28 were obtained for forms d_1 and d_2 of the enzyme, which suggest an approximately spherical shape for both molecules (Table III). From the pore radius of the gel determined by gel filtration of proteins of known Stokes radius, the Stokes radii of d_1 and d_2 were determined as 2.83 and 2.65 mμ, respectively. Combining these values with the $s_{20,w}^0$ obtained from the ultracentrifuge (2.83 S) and the partial specific volume ($\bar{v}$ = 0.723 cm^3/g) calculated from amino acid composition data for each enzyme fraction (Table II), molecular weights of 32,725 and 30,643 were obtained for forms d_1 and d_2, respectively.

The molecular weight of the enzyme was also estimated from its amino acid composition by the method of Delaage (1968). The data were computed on an IBM 1130 machine. The application of this method to the amino acid analyses of kallikrein (Table II) yielded molecular weights of 32,566 ± 1000 and 33,280 ± 1200 for forms d_1 and d_2, respectively.

TABLE II: Amino Acid Composition of Kallikrein, Forms d_1 and d_2.

Amino Acid	Residues per Molecule[a]					
	Form d_1		Form d_2			
	Found[b]	Nearest Integer	Found[b]	Nearest Integer	Trypsin[f]	Kallikrein[g]
Lys	12.2	12	11.6	12	10	13
His	8.9	9	8.9	9	4	10
Arg	5.5	5–6	4.6	5	4	4
$^1/_2$-Cys[c]	11.0	12	12.3	12	12	10
Asp	33.3	33	33.5	33–34	18	33
Thr[d]	18.8	19	18.0	18	11	18
Ser[d]	17.9	18	17.6	18	24	17
Glu	28.9	29	29.0	29	17	28
Pro	19.7	20	20.9	21	10	20
Gly	26.8	27	28.0	28	26	27
Ala	16.7	17	16.2	16	16	16
Val	13.0	13	13.3	13	16	13
Met[c]	4.9	5	4.9	5	2	5
Ile	13.9	14	13.7	14	15	14
Leu	22.7	23	24.1	24	16	24
Tyr	9.9	10	8.7	9	8	9
Phe	13.3	13	12.0	12	4	13
Trp[e]	9.3	9	9.5	9	6	9

[a] Based on a molecular weight of 33,000. [b] Average values from the 22-, 48-, and 96-hr hydrolysates, in duplicate. [c] Determined after 22-hr hydrolysis of performic acid oxidized protein (Moore, 1963). Values for cysteic acid are corrected assuming a recovery of 94%. [d] Corrected values calculated by extrapolation to zero hydrolysis time. [e] Determined spectrophotometrically (Bencze and Schmidt, 1957). [f] Travis and Liener (1965). [g] Fritz *et al.* (1967).

Polyacrylamide gel electrophoresis in the presence of sodium dodecyl sulfate was also employed for further estimate of the molecular weight of forms d_1 and d_2 of kallikrein. The standard incubation conditions of Weber and Osborn (1969) were modified in order to ensure as complete dissociation as possible of the proteins. The patterns obtained for forms d_1 and d_2 after incubation for 1–10 min at 100° in the presence of 10 M urea and 1% β-mercaptoethanol are shown in Figure 6. Both forms

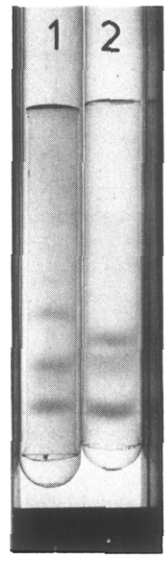

FIGURE 6: Sodium dodecyl sulfate–polyacrylamide gel electrophoresis of kallikrein. Direction of migration is from top to bottom (anode). *Sample load, 20 μg. All incubations are in 1% sodium dodecyl sulfate–10 M urea buffer containing 1% β-mercaptoethanol for 10 min at 100°:* 1, kallikrein form d_1; 2, kallikrein form d_2; gel length, 6 cm.

exhibit essentially three major faster moving bands, of molecular weight (±500) 17,000, 11,000 (diffuse band) and about 7000 in form d_1, and 13,500, 11,000 (diffuse band), and about 7000 in form d_2. Additional trace bands were also observed in each form, consistent with molecular weights of 43,000, 30,000, and 24,000. It is not known whether the latter components represent incompletely dissociated enzyme or result from possible aggregation phenomena in spite of the relatively stringent conditions employed. These data strongly suggest that kallikreins d_1 and d_2 both have disulfide-linked polypeptide chain compositions, possibly as the result of internal cleavage points occurring during activation such as found in α-chymotrypsin (Desnuelle, 1960) or trypsin (Schroeder and Shaw, 1968). Table III summarizes some physical properties of kallikreins d_1 and d_2.

Determination of Sugar Content. Both enzyme forms contain hexose(s) and hexosamine(s) but only a negligible amount of sialic acid was found in either of these forms (Table IV). While the amount of total neutral hexose is nearly identical in kallikreins d_1 and d_2, form d_1 has a higher hexosamine content, as determined from three separate enzyme preparations.

TABLE III: Some Physical Properties of Kallikrein.

Parameter	Method	Kallikrein d_1	Kallikrein d_2
Sedimentation coefficient, $s_{20,w}{}^0$	Sedimentation velocity	2.83	2.83
Molecular weight	Gel filtration	34,500	31,000
	Amino acid composition[a]	32,566	33,280
	Obtained from $s_{20,w}{}^0$, $\bar{v}$, and a[b]	32,725	30,643
	SDS[c]–polyacrylamide gel electrophoresis	17,000, 11,000 7000	13,500, 11,000 7000
	Light scattering[d]	33,500	33,500
Partial specific volume ($\bar{v}$), cm^3/g	Amino acid composition	0.723	0.723
Stokes radius (a), mμ	Gel filtration	2.83	2.65
Frictional ratio (f/f_0)	Calculated[b]	1.34	1.28
Diffusion coefficient (D), $cm^2\ sec^{-1}$	Calculated[b]	7.6×10^{-7}	8.1×10^{-7}
Isoelectric point (pI)	Isoelectric focusing	3.92, 3.97, 4.11	4.01, 4.11

[a] Delaage (1968). [b] Siegel and Monty (1965). [c] SDS = sodium dodecyl sulfate. [d] Cattan (personal communication).

TABLE IV: Carbohydrate Composition of Kallikrein.[a]

	Hexose[b]		Hexosamine[c]		Sialic Acid	
Form	g/ 100 g	Calcd Residues/ 33,000 g	g/ 100 g	Calcd Residues/ 33,000 g	g/ 100 g	Calcd Residues/ 33,000 g
d_1	1.8	3.3	2.8	4.3	0.04	0.04
d_2	1.55	2.8	1.6	2.4	0.015	0.016

[a] The anthrone reaction for total hexose was applied to sample either without prior hydrolysis of after treatment with 2 N H_2SO_4 (3 ml/4 mg) at 100° for 4 hr, passage of hydrolysate on a column of Dowex 1-X8, elution by water, and concentration to dryness at 25°. Hexosamine content was estimated after hydrolysis (2 ml/4 mg) with 4–6 N HCl at 100° for 4–8 hr. The hydrolysate was transferred to a column of Dowex 50-X4 (H^+ form, 100–200 mesh) which was washed with distilled water; hexosamine was eluted with 2 N HCl and the eluate was evaporated to dryness at 50°. For sialic acid estimation the sample was hydrolyzed with 0.1 N H_2SO_4 (2 ml/5 mg) at 80° for 1 hr and taken to dryness. [b] Estimated as β-D-glucose. [c] Estimated as α-D-glucosamine.

Effect of Divalent Metal Ions and Metal Chelators. Each of the kallikreins was incubated at pH 8.0 for 1 hr and 25° with a number of divalent metal ions as their chlorides, at the indicated concentration. An aliquot (20 μl) was removed and assayed for activity in a final volume of 1 ml. Hg^{2+} (0.001 M), Cu^{2+} (0.01 M), and Zn^{2+} (0.01 M) completely inhibited enzyme activity while some other cations (*i.e.*, Ni^{2+}, Co^{2+}, Fe^{2+}, Cd^{2+}) also exerted significant inhibition. Mn^{2+} (0.05 M), however, had no effect on activity, in contrast with the results of Fiedler and Werle (1968). Ca^{2+} (0.01 M) was also without marked effect but at higher concentration (1 M) enzyme activity was increased by 15–20%. This is in contrast with results reporting some inhibition of kallikrein by Ca^{2+} (Takami, 1969a).

Neither of the chelating agents, EDTA (0.1 M, 1 hr, 25°) or *o*-phenanthroline (0.01 M, 2 hr, 25°) had any effect on enzyme activity. The complete inhibition obtained after 1-hr incubation in 0.001 M $HgCl_2$ could be reversed and enzyme activity fully restored after 3 hr by the addition of EDTA (60-fold molar excess).

Effect of Sulfhydryl Reagents and Disulfide Reducing Reagents. Incubation at 25° and pH 8.0 of both forms of the enzyme with *p*-chloromercuribenzoate, iodoacetate or iodoacetamide (40°, pH 8.5), *N*-ethylmaleimide, or Nbs_2 did not influence esterase activity, therefore indicating that the enzyme contains no free SH capable of participating in catalysis. In contrast, 0.1 M β-mercaptoethanol or 0.01 M dithiothreitol (in 8 M urea) markedly decreased enzyme activity of each of the kallikreins, indicating that intact disulfide bridge(s) appear estial for catalysis.

Determination of Free Sulfhydryl Groups Obtained after Reductive Cleavage of Disulfide Bonds. The time course of formation of SH groups after reduction by 0.15 M sodium borohydride in the presence of 8 M urea is shown in Figure 7. A rapid appearance of SH groups was observed, with a maximum yield after 1 hr: 5.4, SE ±0.21 (7 experiments) and 6.8, SE ±0.17 (4 experiments) for forms d_1 and d_2, respectively. Longer reaction times did not result in further reduction. Instead, a decrease in the number of titratable free SH groups was found, suggesting that some reoxidation occurred. Figure 7 also shows

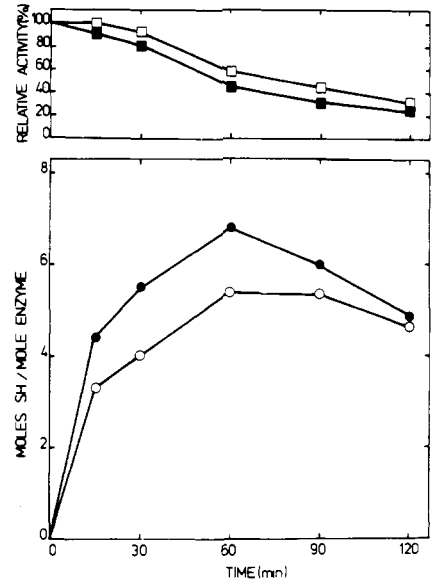

FIGURE 7: The reduction of kallikrein with sodium borohydride. Enzyme (2.4 mg) was dissolved in 1.1 ml of 0.2 M borate buffer–8 M urea (pH 9.1) and reduction was started by the addition of 0.12 ml of 1.5 M sodium borohydride (in 0.2 M borate buffer–8 M urea). Reduction proceeded at 25°, at the pH of the solution (pH 9.9). Circles, SH content estimated by the method of Ellman (see text): (○) form d_1; (●) form d_2. Squares (top), enzyme activity determined immediately after removal of aliquots, at indicated times. Activity expressed as percentage of control without sodium borohydride: (□) form d_1; (■) form d_2.

that upon sodium borohydride reduction most of the catalytic activity disappears. It should be noted, however, that in both enzyme forms two disulfide bridges can be reduced without loss of activity. Since, under the conditions used for reduction, 8 M urea alone had only negligible effect on enzyme activity, the observed loss is unlikely to be a consequence of denaturation by urea. From Figure 7 it is also seen that reoxidation of SH groups is not associated with a regain in activity, possibly suggesting some impairment in the refolding of the protein. The possibility, however, of inactivation by reductive cleavage of other bonds, *e.g.*, peptide bonds, by sodium borohydride should not be excluded.

Effect of pH. The effect of pH on the rate of hydrolysis of Bz-Arg-OEt (25°) was measured over the range pH 5.0–9.5. At pH values higher than 9.5, spontaneous hydrolysis of Bz-Arg-OEt was observed, thus making reproducible data difficult to obtain. The hydrolysis of Bz-Arg-OEt by kallikrein showed a sigmoid pH-velocity profile over the pH range 5.0–9.5, with maximum activity at pH 8.0. The data could be fitted with a calculated titration curve for a group with a pK' of 6.35. The plot of log v *vs.* pH (Dixon, 1953) indicated a pK' of 6.5. These results suggest that protonation of a group of pK' = 6.5 or so in the enzyme–substrate complex, most likely the imidazole group of histidine, leads to inactivation of the enzyme.

Kinetic Determinations. Lineweaver–Burk plots of initial rates of hydrolysis of Bz-Arg-OEt by both forms of the enzyme are shown in Figure 8A. The nonlinearity of these plots indicates that the reaction fails to follow simple Michaelis–Menten kinetics. At substrate concentration higher than 3×10^{-2} M a decrease in reaction velocity was observed suggesting substrate inhibition. Extrapolation of low substrate concentration data gave identical K_m values of 5×10^{-4} M for both forms of the enzyme; k_{cat} values were calculated from V values obtained from extrapolation of the curves at low substrate concentration and the concentration of the enzyme, assuming a molecular weight of 33,000 for both forms (k_{cat} = 74 and 100 sec^{-1} for d_1

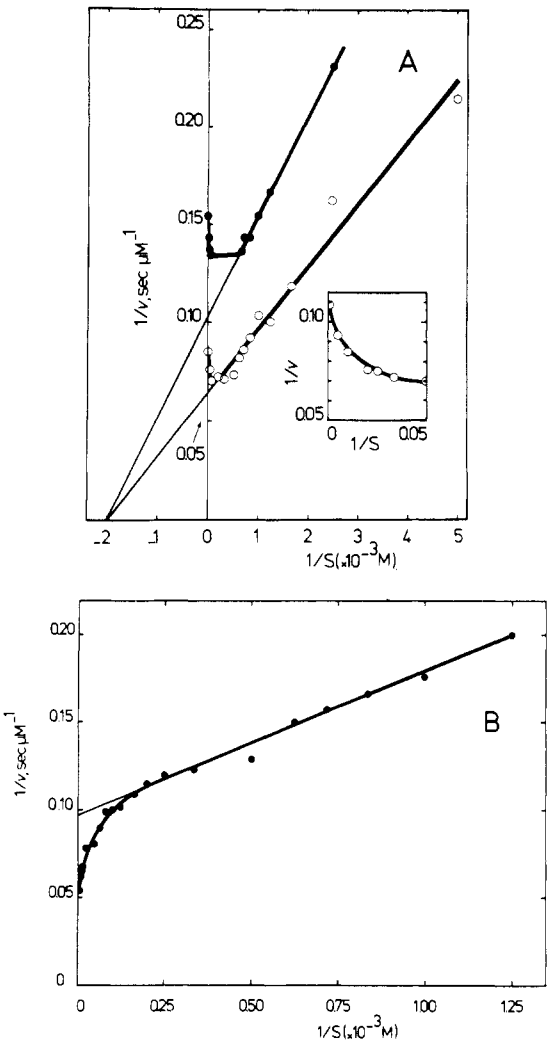

FIGURE 8: Effect of substrate concentration on the rate of hydrolysis by kallikrein of Bz-Arg-OEt (A) (○) form d_1 (enzyme concentration 0.21 μM); (●) form d_2 (enzyme concentration 0.097 μM); 25 ± 0.1°, pH 8.0. Inset, detail of decrease in reaction velocity of kallikrein (form d_1) at high substrate concentration. (B) Hydrolysis of Tos-Arg-OMe (enzyme concentration 3.03 μM, form d_2).

and d_2, respectively). With Tos-Arg-OMe at high substrate concentration, an increase in reaction velocity was found suggesting substrate activation in this case (Figure 8B). The constants (kallikrein d_2), obtained from graphical extrapolation of the curve for low substrate concentration, were K_m = 8.1×10^{-4} M and k_{cat} = 3.4 sec^{-1}. Deviation from linearity in Michaelis–Menten kinetics has been explained by the effect of secondary site binding upon catalysis (Trowbridge *et al.*, 1963; Béchet and Yon, 1964). It was also recently shown (Kallen-Trummer *et al.*, 1970) that efficient acceleration of trypsin-catalyzed ester hydrolysis only takes place if the substrate (or effector) contain a positive charge and an aromatic sulfonamide group at a critical distance of each other. Similar considerations may also apply to kallikrein. A pH-dependent Bz-Arg-OEt inhibition and Tos-Arg-OMe activation of kallikrein were also observed by Fiedler and Werle (1968).

The effect of temperature on kallikrein activity was measured at 20–55° with Bz-Arg-OEt as substrate. From the slope of an Arrhenius plot, the activation energy was determined to be 10,703 and 8200 cal per mol for forms d_1 and d_2, respectively.

Substrate Specificity. The enzyme readily hydrolyzes Bz-Arg-OEt, Bz-Arg-OMe, and α-*N*-Z-L-lysine benzyl ester, while Tos-Arg-OMe is hydrolyzed at a much slower rate and the hydrolysis of Ac-Tyr-OEt is very low (Table V). *N*-Benzoyl-DL-arginine-β-naphthylamide and *N*-benzoyl-DL-arginine-*p*-nitroanilide were hydrolyzed at barely discernible rates. The extremely low activity or absence of activity toward two common substrates of the proteolytic enzymes, casein and azocoll, is also shown. In some preparations, a small caseinolytic activity (amounting to less than 10% that of trypsin) was associated with the enzyme obtained from steps 4 or 5. After isoelectric focusing, however, and under the conditions employed (250 μg of enzyme in the assay, *i.e.*, 25-fold excess by weight over standard conditions when using trypsin, a maximum value, possibly an overestimate, corresponding to 0.5% of the activity of trypsin, was found (peak p*I* = 4.11 in Figure 5A). This negligible hydrolysis of casein is in contrast with the results of Takami (1969b).

Since the specificity of kallikrein toward simple esters shows a number of similarities with trypsin, its reaction with *p*-NPGB (Chase and Shaw, 1969) was also examined. The "burst" behavior was observed. In contrast with trypsin it was followed by a rapid postburst *p*-nitrophenol production (k_{pb} = 0.72 min^{-1}; 0.1 M Veronal buffer (pH 8.3), 25°; enzyme, form d_1, 2×10^{-6} M; *p*-NPGB, 5×10^{-5} M; ϵ_{410} of *p*-nitrophenol measured as 16,414). A similar behavior was found for form d_2. This reactivity of kallikrein with *p*-NPGB suggests an acyl-enzyme mechanism. The rapid postburst release of *p*-nitrophenol, however, makes *p*-NPGB less satisfactory as a titrant for kallikrein than for trypsin.

Poly(L-arginine), poly(L-lysine), and salmin, although not poly(L-ornithine) are hydrolyzed by kallikrein (Sache *et al.*, 1971). The enzyme also exerts proteolytic activity toward bonds involving the carbonyl group of arginine in a series of peptides that were specifically synthesized (Lefrancier and Sache, in preparation) to help define enzyme specificity.

Effects of Inhibitors. As expected, among the naturally occurring trypsin inhibitors examined, only the Kunitz bovine inhibitor was found effective (for a review of extensive previous work, see Vogel and Werle, 1970). This inhibitory effect of the Kunitz inhibitor is significantly weaker for kallikrein than for trypsin, since in a 1:1 inhibitor to enzyme molar ratio, kallikrein still exhibits 25% remaining activity. In the presence of a 2.5-fold molar excess of inhibitor (Table VI), the remaining activity of kallikrein is 13%. The hydrolysis of poly(L-arginine), poly(L-lysine), and salmin by kallikrein is also inhibited by the Kunitz inhibitor, whereas the Kazal-type (bovine) and ovomucoid trypsin inhibitors are without effect.

Incubation of the enzyme with the quaternary ammonium compound benzalkonium chloride (a mixture of C_8–C_{18} alkyldimethylbenzylammonium chlorides) also resulted in an inhibition, that was found more effective at pH 5.0 than at pH 8.0 (Table VI). This inhibition could not be reversed or decreased by the addition of a polyanion, either poly(L-glutamic acid) (mol wt 61,000; 1.2 molar excess) or heparin (about 10-fold molar excess). Kallikrein is competitively inhibited by *p*-aminobenzamidine with a K_i of 1.0×10^{-3} M (enzyme form d_1, 25°, pH 8.0). The enzyme is also inactivated by diphenylcarbamyl chloride (Table VI), a chymotrypsin inhibitor also capable of inactivating trypsin (Erlanger and Cohen, 1963). The sensitivity of the enzyme to organofluorides was tested using PMSF and DFP (Table VII). The trypsin inhibitor TLCK in 200-fold molar excess over the enzyme showed no effect. TPCK in 1000-fold molar excess (up to 120-hr exposure) and methyl *p*-nitrobenzenesulfonate in 100- to 1000-fold molar excess (1 hr) were also not inhibitory. In contrast to TPCK,

TABLE V: Hydrolysis of Various Substrates by Kallikrein.

Substrate	Concn (mM)	Rel Enzyme Concn	Rel Act. (%)	
			d_1	d_2
N-Benzoyl-L-arginine ethyl ester[a]	20	1	100	100
N-Benzoyl-L-arginine methyl ester	20	0.6	114	100
p-Toluenesulfonyl-L-arginine methyl ester, HCl	20	10	9.8	8.5
p-Toluenesulfonyl-L-lysine methyl ester, HCl	20	10	1.3	Trace
p-Toluenesulfonyl-L-ornithine methyl ester, HCl	20	10	2.0	1.3
N-Benzoyl-L-histidine methyl ester[b]	20	20	3.0	3.6
N-Benzoyl-L-leucine ethyl ester[b]	20	100	Trace	Trace
N-α-Benzoyl-L-lysine methyl ester	20	10	8.2	7.2
N-Benzoyl-L-methionine methyl ester[b]	20	100	Trace	Trace
N-Benzoyl-DL-arginine-β-naphthylamide, HCl[c]	0.45	200	Trace	Trace
N-Benzoyl-DL-arginine-*p*-nitroanilide[d]	1.5	200	Trace	Trace
N-α-Carbobenzoxy-L-lysine benzyl ester, *p*-tosylate	20	1	86	
N-α-Carbobenzoxy-L-lysine methyl ester, HCl	20	2	39	
N-Acetyl-L-lysine methyl ester, HCl[e]	20	10	5.6	4.4
N-Acetylglycyl-L-lysine methyl ester, acetate[f]	20	5	26.5	22.3
N-Acetyl-L-tryptophan ethyl ester[b]	20	20	Trace	Trace
N-Acetyl-L-phenylalanine ethyl ester	20	10	2.3	1.7
N-Acetyl-L-tyrosine ethyl ester[b,g]	20	10	2.4	3.4
N-Acetyl-L-glycine methyl ester	20	10	Trace	
N-Acetyl-L-glycine ethyl ester	20	10	Trace	Trace
N-Acetyl-L-alanyl-L-alanyl-L-alanine methyl ester	20	10	Trace	Trace
p-Nitrophenyl acetate[h]	1.6	16	0	0
Casein	0.5%	50	Trace	Trace
Azocoll	0.5%	100	0	0

[a] Conditions for hydrolysis of ester substrates were as described for assay of Bz-Arg-OEt (0.02 M ester substrate in 0.0015 M borate-HCl buffer containing 0.018 M $CaCl_2$; pH 8.0; 25 ± 0.1°; final volume, 1 ml). Relative enzyme concentration is kallikrein concentration relative to concentration used in experiment with Bz-Arg-OEt (5 μg). Relative activities are expressed as per cent of Bz-Arg-OEt activity (rates of Bz-Arg-OEt hydrolysis were 109, SE ±4.3 (12 preparations) and 134, SE ±6.9 (11 preparations) μmoles/min per mg of protein for forms d_1 and d_2, respectively). Relative activity denoted as "trace" implies less than 1% hydrolysis. [b] Methanol concentration in assay is 10%. [c] According to Blackwood and Mandl (1961). [d] According to Ganrot (1966). [e] A K_m of 5.8×10^{-3} M was determined. [f] A K_m of 6.9×10^{-4} M was determined, with enzyme activation at high substrate concentration. [g] Values of 1.63 and 1.66% were found for major peaks from isoelectric focusing, p*I* = 4.11 in Figure 5A,B. [h] According to Krisch (1966).

ZPCK (1000-fold molar excess) caused a slow decay in enzyme activity, obeying apparent first-order kinetics, $t_{1/2}$ = 133 hr.

Biological Effects. The decrease of the arterial blood pressure of the dog after intravenous injection of the enzyme *in vivo* was used as physiological evidence of its kallikrein nature. Injection of 0.25 μg/kg body weight of animal (pentothal anesthetized) produced an average drop in blood pressure of about 17% (dog 1; 24 kg) to 23% (dog 2; 14 kg). Forms d_1 and d_2 of the enzyme could not be distinguished in their biological effects. With both forms the drop in blood pressure occurred about 10 sec after the time of injection and was only obtained for a few seconds. This drop in blood pressure was no longer observed when the enzyme was incubated with the Kunitz bovine inhibitor prior to injection. Since a lyophilized commercial sample of kallikrein (Kalleone retard, Bayer) containing 40 biological units (for definition of KU units, see Webster, 1970) was found to hydrolyze 6.3 μmol of Bz-Arg-OEt under the standard conditions described in the Experimental Section, the relationship of 1 Bz-Arg-OEt esterase unit to 6.3 biological units was determined. Thus, administration of 0.25 μg/kg of the enzyme preparations used in the above experiments (assaying about 122 units/mg) are equivalent to about 0.19 KU/kg. The extent of the blood pressure decrease such as obtained in these experiments is in agreement with the known effects of kallikrein on the cardiovascular system (Brecher and Brobmann, 1970).

The enzyme had no accelerating effect on blood coagulation either *in vitro* (Raby, personal communication), in the presence of recalcified citrated blood, or *in vivo* (Hureau and Vairel, personal communication).

Discussion

The purification of the enzyme was monitored by its esterase activity toward Bz-Arg-OEt. The enzyme was characterized as an anionic protein at pH 8.0, differing from porcine anionic trypsin (Voytek and Gjessing, 1971) essentially in substrate specificity and lack of susceptibility to the soybean trypsin inhibitor. Evidence of the enzyme's identity with porcine kallikrein was provided by its kininogenase potency.

Kallikrein, like a number of other pancreatic enzymes, is probably synthesized as an inactive zymogen although this view has been questioned (Hilton and Jones, 1968). Two different prekallikreins have been characterized in porcine gland homogenates (Fiedler and Werle, 1967) while partial prekallikrein purification from the juice has also been reported (Greene *et al.*, 1968). However, under the conditions used in the present study, the enzyme was obtained from the glands in

TABLE VI: Effects of Some Enzyme Inhibitors on the Activity of Kallikrein.[a]

Inhibitor	Concn (M or %)	Time of Incubn (min)	Enzyme Act. (% of Control)
Kunitz bovine inhibitor	1.5×10^{-5}	5	13
Kazal-type (bovine) inhibitor	8.0×10^{-4}	5	98
Kazal-type (porcine) inhibitor	8.0×10^{-4}	5	98
Soybean trypsin inhibitor	0.5%	5	100
Lima bean trypsin inhibitor	0.5%	5	100
Kidney bean inhibitor	0.5%	5	100
Ovomucoid	0.5%	5	100
Potato inhibitor I	0.25%	5	98
Potato inhibitor II	0.25%	5	100
ϵ-Aminocaproic acid (EACA)	1	60	100
Diphenylcarbamyl chloride[b,c]	3.8×10^{-4}	60	75
Benzamidine	1.0×10^{-2}	30	97
Tetra-*N*-methylammonium iodide	1.6×10^{-1}	60	94
Tetra-*N*-butylammonium chloride	1.1×10^{-1}	60	91
Domiphen bromide	2.4×10^{-3}	30	49
Benzethonium chloride	2.4×10^{-3}	30	52.5
Benzalkonium chloride[d]	2.4×10^{-3}	30	25
Benzalkonium chloride[e]	2.4×10^{-3}	30	6
Acetylsalicylic acid	1.5×10^{-2}	5	100

[a] The purified enzyme (about 6–7 μM) was incubated at 25° in 0.005 M Tris-acetate buffer (pH 8.0)–0.25 M NaCl with the inhibitor in the concentration shown and for the time indicated. An aliquot (20 μl) was added to the titration vessel containing the substrate solution (substrate, 0.02 M Bz-Arg-OEt, in 0.0015 M borate-HCl buffer containing 0.018 M $CaCl_2$; pH 8.0; 25 ± 0.1°; final volume, 1 ml). Since essentially identical results were obtained with both forms of kallikrein only data concerning form d_1 are presented. [b] Inhibition by diphenylcarbamyl chloride was performed at pH 8.0 by mixing equal volumes of enzyme (7.5 μM) and inhibitor (in 40% methanol). An appropriate control was incubated in 20% methanol. [c] Inactivation obeyed apparent first-order kinetics ($t_{1/2}$ 152 min). Division of the pseudo-first-order rate constant by inhibitor concentration gave a calculated second-order rate constant of 12.2 M^{-1} min^{-1}. [d] Incubation in 0.1 M Tris buffer (pH 8.0). [e] Incubation in 0.1 M acetate buffer (pH 5.0).

TABLE VII: Effect of Organofluorides on the Activity of Kallikrein.[a]

Inhibitor	Final Concn (M)	Time of Incubn (min)	pH of Incubn	Temp of Incubn (°C)	Enzyme Act. (% of Control)
PMSF[b]	1.0×10^{-2}	30	7.0	37	23
PMSF	5.0×10^{-3}	60	7.0	25	39
DFP	5.0×10^{-3}	30	7.4	25	68
DFP	5.0×10^{-3}	60	7.4	25	48

[a] Equal volumes of enzyme solution (form d_1) in 0.005 M Tris-acetate buffer (pH 8.0) and organofluoride in 50% isopropyl alcohol (PMSF) or 10% isopropyl alcohol (DFP) were mixed and incubated, as indicated. Enzyme final concentration, 7.5 μM. Appropriate controls were incubated in each experiment in 25 or 5% isopropyl alcohol. [b] Inactivation of kallikrein at pH 8.0 and 25° by PMSF at various concentrations ($I \gg E$) obeyed pseudo-first-order kinetics. A saturation effect in the kinetics of enzyme inactivation was determined (Kitz and Wilson, 1962), with a limiting velocity of inactivation k_2 of 0.03 min^{-1}; $K_i = 4.6 \times 10^{-3}$ M; $t_{1/2}$ at saturation is 28 min.

active form. Upon trypsin incubation, no further increase in esterase activity was obtained at various stages of the preparation. Trypsin incubation could also not bring about the transformation of one form of kallikrein into another. The purification presented provides a highly reproducible procedure for the isolation in relatively few steps of two distinct major forms of kallikrein from dried extracts of porcine pancreas, with a 1300-fold increase in specific activity. Examination by the two criteria of sedimentation velocity and polyacrylamide gel electrophoresis indicated a high degree of purity for both forms of the enzyme.

The presence in porcine pancreas of two distinct major forms of kallikrein is in agreement with the findings of other laboratories (Habermann, 1962a; Fritz *et al.*, 1967; Takami, 1969b). In addition to these two major forms, evidence for a third faster migrating form (d_3), present in much smaller amounts was repeatedly obtained by electrophoresis in acrylamide–agarose gels. Although a new active form (kallikrein B′) was recently isolated by Fiedler *et al.* (1970), kallikreins B′ and d_3 are probably not related, since the major component of kallikrein B′ has an isoelectric point of 4.32 whereas no Bz-Arg-OEt esterase activity was found at this pH in the isoelectric focusing experiments reported here. Spontaneously activated kallikrein B′ also exhibits a lower esterase activity (depending, however, on the assay conditions; Fiedler *et al.*, 1970) as compared with the other forms of kallikrein. In contrast, the specific esterase activity found in this study for kallikrein d_3 was comparable with that of the two other forms, d_1 and d_2.

The present report of multiple kallikreins is not unusual in view of the well-known polymorphic appearance of a number of exocrine proteins in porcine pancreas. The actual origin of the differences between the two major forms of kallikrein is not known but could be related, at least in part, to their different hexosamine content (Table IV). Fritz *et al.* (1967) have shown their kallikrein preparation to contain multiple forms that could be attributed to a varying content of sialic acid. After removal of sialic acid by neuraminidase treatment, only two forms of kallikrein (A and B)[2] could be characterized by electrophoresis. Our data, however, show that even after neuraminidase treatment there is evidence for at least three electrophoretically distinguishable components.

From the data in Table III, a molecular weight of 33,000 has been assumed for both forms of kallikrein. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis in the presence of a reducing agent clearly indicates that kallikreins d_1 and d_2 each consist of disulfide-linked chains (the sums of the molecular weights of the chain components also being consistent with a molecular weight of about 33,000 for both forms of the unreduced enzyme).

[2] On account of their chromatographic behavior on DEAE-cellulose and in spite of differences in the elution conditions, kallikreins d_1 and d_2 were tentatively identified with kallikreins B and A, respectively (Fiedler and Werle, 1967). This view is largely supported by the fact that upon isoelectric focusing the isoelectric points of 4.05 obtained for the major peaks each in forms B and A (Fiedler *et al.*, 1970) are very similar to those reported here (p*I* = 4.11, Figure 5A,B).

Kallikreins d_1 and d_2 possess comparable amino acid compositions, each of which in good agreement with that given by Fritz *et al.* (1967) for an unresolved mixture of two forms of the enzyme. These authors, however, found 10 residues of half-cystine/mol instead of 12 in this work. Our results are in contrast with the composition reported by Moriya *et al.* (1969), in which methionine and isoleucine could not be detected. An interesting property of the two kallikreins is their high content of proline, glycine (7.0 and 9.5 residues %, respectively), and disulfide bonds. This may account, at least in part, for the high stability of the enzyme above pH 4.0.

Concerning the catalytic and active-site characteristics of the two major forms of kallikrein described here, it should be emphasized that both forms share very similar properties.

Since enzyme activity is not affected by SH blocking reagents and less than 0.1 residue free SH group is present per mol of protein, the involvement of an SH group is ruled out for activity. Thus, in each enzyme form the twelve half-cystine residues must be present as six disulfide bridges. As judged by the effect of a number of reducing agents, disulfide bridge(s) are essential for activity. Although high concentrations of DFP and PMSF were necessary to achieve a relatively small degree of inhibition, these experiments suggest that serine may be involved in the active site (Habermann, 1962b; Fiedler *et al.*, 1969). The interaction with *p*-NPGB, also recently reported by Fiedler *et al.* (1972), shows that an acyl-enzyme is a probable intermediate during catalysis. Inhibition by diphenylcarbamyl chloride may also be indicative of the presence of an active serine, as well, possibly, of an aromatic binding site in the substrate binding region. From Table V it may be seen that when a benzyloxycarbonyl group replaces a benzoyl group in the *N*-acyl moiety of L-lysine methyl ester, the relative activity is increased by a factor of about 5. While both groups represent hydrophobic substituents at P_2 (using the nomenclature of Schechter and Berger, 1967), the increased distance of about 2.6 Å between the phenyl ring and the carbonyl group of the susceptible ester bond in the benzyloxycarbonyl derivative, as compared to the benzoyl derivative (Kortt and Liu, 1973), probably exerts a favorable influence upon the catalytic process. As the activity of kallikrein toward the benzyl ester of α-*N*-Z-L-lysine is more than twice that found toward the methyl ester derivative, an aromatic site may also be implied at P'_1. The occurrence of one or more aromatic binding sites in the substrate binding region is also inferred from the ability of the enzyme to hydrolyze *N*-Z-L-phenylalanine-*p*-nitrophenyl ester (unpublished data). These results suggest that, although the esterase activity of kallikrein is directed primarily toward the esters of arginine, and to a lesser extent toward those of lysine, "secondary" binding sites must play an important part in the overall catalytic efficiency of the enzyme.

While the alkylammonium derivatives had no effect, the other three quaternary ammonium compounds examined in this work (Table VI), which all possessed one or more benzene rings, inactivated the enzyme to some extent. Such compounds are known to affect a number of enzyme-catalyzed reactions (Mäkinen, 1968; Mäkinen and Mäkinen, 1970; Feldbau and Schwabe, 1971). In addition to electrostatic effects, evidence for the specific interaction of benzalkonium chloride with serine proteases at low pH values has been proposed (Feldbau and Schwabe, 1971). Thus, the reaction of kallikrein with benzalkonium chloride may serve as further evidence that the enzyme is a serine esterase although some inactivation also occurred at pH 8.0, which is higher than that reported for other serine proteases (Feldbau and Schwabe, 1971). Domiphen bromide, which has no effect on cationic trypsin (Mäkinen and Mäkinen, 1970), also inactivates kallikrein, possibly because the two molecules are oppositely charged. This inhibition, however, is more probably specific since it was not affected by the addition of a polyanion (heparin), either at pH 5.0 or 8.0.

Although the pH–velocity experiments suggest that histidine may be involved at the active site, failure of TLCK to react with the enzyme (Mares-Guia and Diniz, 1967) was confirmed in this study. The lack of interaction could result from a restricted specificity for lysine as compared to arginine, from the nature of the amino-terminal blocking group (*e.g.*, the *p*-toluenesulfonyl group; Tos-Arg-OEt is a poor substrate as compared to the benzoyl derivative), or both. Hence, the interaction with arginyl alkylating agents (Shaw and Glover, 1970) or replacement of the *p*-toluenesulfonyl group by another group such as a benzyloxycarbonyl group in the chloromethyl ketone inhibitor (particularly in view of the inhibition achieved by ZPCK in contrast to TPCK) should be of interest. It may also be that the active histidine in kallikrein is more distant from the specificity pocket than in trypsin.

The recent finding that methyl *p*-nitrobenzenesulfonate inactivates α-chymotrypsin by methylation of an essential histidine (Nakagawa and Bender, 1969) prompted the use of this reagent in the present study. However, as with trypsin, no modification in kallikrein activity was found.

While trypsin and kallikrein have in common the ability to hydrolyze esters of arginine and lysine, they not only show considerable differences in a number of physical and chemical properties, but also in their biological action. While trypsin accelerates human blood coagulation (Yin, 1964), kallikrein, either *in vitro* or *in vivo*, has no effect on blood clotting. Either trypsin or kallikrein will produce hypotension in the dog. Yet, to obtain comparable hypotension, considerably higher amounts of trypsin (Hureau and Vairel, personal communication) were necessary.

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References

Ackers, G. K. (1964), *Biochemistry 3*, 723.
Alexander, N. M. (1958), *Anal. Chem. 30*, 1292.
Andrews, P. (1964), *Biochem. J. 91*, 222.
Arst, H. E., Manning, R. T., and Delp, M. (1959), *Amer. J. Med. Sci. 238*, 598.
Back, N. (1966), *Fed. Proc., Fed. Amer. Soc. Exp. Biol. 25*, 77.
Béchet, J.-J., and Yon, J. (1964), *Biochim. Biophys. Acta 89*, 117.
Bencze, W. L., and Schmidt, K. (1957), *Anal. Chem. 29*, 1193.
Blackwood, C., and Mandl, I. (1961), *Anal. Biochem. 2*, 370.
Brecher, G. A., and Brobmann, G. F. (1970), *in* Handbook of Experimental Pharmacology: Bradykinin, Kallidin and Kallikrein, Vol. XXV, Erdös, E. G., Ed., Berlin, Springer-Verlag, p 351.

Chase, Jr., T., and Shaw, E. (1969), *Biochemistry 8,* 2212.
Delaage, M. (1968), *Biochim. Biophys. Acta 168,* 573.
Desnuelle, P. (1960), *Enzymes 4,* 93.
Desnuelle, P., Gratecos, D., Charles, M., Peanasky, R., Baratti, J., and Rovery, M. (1969), *in* Structure-Function Relationships of Proteolytic Enzymes, Desnuelle, P., Neurath, H., and Ottesen, M., Ed., Munksgaard, Copenhagen, p 21.
Dixon, M. (1953), *Biochem. J. 55,* 161.
Eisen, V. (1964), *Brit. M. Bull. 20,* 205.
Ellman, G. L. (1959), *Arch. Biochem. Biophys. 82,* 70.
Elson, L. A., and Morgan, W. T. J. (1933), *Biochem. J. 27,* 1824.
Erlanger, B. F., and Cohen, W. (1963), *J. Amer. Chem. Soc. 85,* 348.
Feldbau, E., and Schwabe, C. (1971), *Biochemistry 10,* 2131.
Fiedler, F., Hirschauer, C., and Werle, E. (1970), *Hoppe-Seyler's Z. Physiol. Chem. 351,* 225.
Fiedler, F., Müller, B., and Werle, E. (1969), *Eur. J. Biochem. 10,* 419.
Fiedler, F., Müller, B., and Werle, E. (1972), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 24,* 41.
Fiedler, F., and Werle, E. (1967), *Hoppe-Seyler's Z. Physiol. Chem. 348,* 1087.
Fiedler, F., and Werle, E. (1968), *Eur. J. Biochem. 7,* 27.
Fischer, G., and Udermann, H. (1970), *Arzneim.-Forsch. 20,* 580.
Folk, J. E., Piez, K. A., Carroll, W. R., and Gladner, J. A. (1960), *J. Biol. Chem. 235,* 2272.
Folk, J. E., and Schirmer, E. W. (1963), *J. Biol. Chem. 238,* 3884.
Fritz, H., Eckert, I., and Werle, E. (1967), *Hoppe Seyler's Z. Physiol. Chem. 348,* 1120.
Ganrot, P. O. (1966), *Acta Chem. Scand. 20,* 175.
Greene, L. J., Di Carlo, J. J., Sussman, A. J., and Bartelt, D. C. (1968), *J. Biol. Chem. 243,* 1804.
Greene, L. J., Rigbi, M., and Fackre, D. S. (1966), *J. Biol. Chem. 241,* 5610.
Habermann, E. (1962a), *Hoppe Seyler's Z. Physiol. Chem. 328,* 15.
Habermann, E. (1962b), *Hoppe Seyler's Z. Physiol. Chem. 328,* 24.
Harris, C. E., Kobes, R. D., Teller, D. C., and Rutter, W. J. (1969), *Biochemistry 8,* 2442.
Hilton, S. M., and Jones, M. (1968), *J. Physiol. 195,* 521.
Hofsten, B. V., and Reinhammar, B. (1965), *Biochim. Biophys. Acta 110,* 599.
Kallen-Trummer, V., Hofmann, W., and Rottenberg, M. (1970), *Biochemistry 9,* 3580.
Kellermeyer, R. W., and Graham, R. C. (1968), *N. Eng. J. Med. 279,* 754.
Kitz, R., and Wilson, I. B. (1962), *J. Biol. Chem. 237,* 3245.
Kortt, A. A., and Liu, T. Y. (1973), *Biochemistry 12,* 328.
Krisch, K. (1966), *Biochim. Biophys. Acta 122,* 265.
Mäkinen, K. (1968), *FEBS (Fed. Eur. Biochem. Soc.) Lett. 2,* 101.
Mäkinen, K. K., and Mäkinen, P. L. (1970), *Biochim. Biophys. Acta 206,* 143.
Mares-Guia, M., and Diniz, C. R. (1967), *Arch. Biochem. Biophys. 121,* 750.
Miller, G. M. (1959), *Anal. Chem. 31,* 964.
Moore, S. (1963), *J. Biol. Chem. 238,* 235.
Moriya, H., Kato, A., and Fukushima, H. (1969), *Biochem. Pharmacol. 18,* 549.
Nakagawa, Y., and Bender, M. L. (1969), *J. Amer. Chem. Soc. 91,* 1566.
Prado, J. L. (1970), *in* Handbook of Experimental Pharmacology: Bradykinin, Kallidin and Kallikrein, Vol. XXV, Erdös, E. G., Ed., Berlin, Springer-Verlag, p 156.
Roe, J. H. (1955), *J. Biol. Chem. 212,* 335.
Sachar, L. A., Winter, K. K., Sicher, N., and Frankel, S. (1955), *Proc. Soc. Exp. Biol. Med. 90,* 323.
Sache, E., Maillard, M., Toubiana, R., and Choay, J. (1971), *C. R. Acad. Sci., Ser. A 272,* 647.
Sache, E., and Thély, M. (1968), *C. R. Acad. Sci., Ser. A 226,* 1200.
Sache, E., Thely, M., and Choay, J. (1965), *C. R. Acad. Sci., Ser. A 260,* 3491.
Schachman, H. K. (1957), *Methods Enzymol. 4,* 32.
Schechter, I., and Berger, A. (1967), *Biochem. Biophys. Res. Commun. 27,* 157.
Schroeder, D. D., and Shaw, E. (1968), *J. Biol. Chem. 243,* 2943.
Shaw, E., and Glover, G. (1970), *Arch. Biochem. Biophys. 139,* 298.
Siegel, L. M., and Monty, K. J. (1965), *Biochem. Biophys. Res. Commun. 19,* 494.
Spackman, D. H., Stein, W. H., and Moore, S. (1958), *Anal. Chem. 30,* 1190.
Takami, T. (1969a), *J. Biochem. (Tokyo) 66,* 651.
Takami, T. (1969b), *Seikagaku 41,* 777.
Tobita, T., and Folk, J. E. (1967), *Biochim. Biophys. Acta 147,* 15.
Travis, J., and Liener, I. E. (1965), *J. Biol. Chem. 240,* 1962.
Trowbridge, C. G., Krehbiel, A., and Laskowski, M., Jr. (1963), *Biochemistry 2,* 843.
Uriel, J. (1966), *Bull. Soc Chim. Biol. 48,* 969.
Vogel, R., and Werle, E. (1970), *in* Handbook of Experimental Pharmacology: Bradykinin, Kallidin and Kallikrein, Vol. XXV, Erdös, E. G., Ed., Berlin, Springer-Verlag, p 213.
Voytek, P., and Gjessing, E. C. (1971), *J. Biol. Chem. 246,* 508.
Warren, L. (1959), *J. Biol. Chem. 234,* 1971.
Weber, K., and Osborn, M. (1969), *J. Biol. Chem. 244,* 4406.
Webster, M. E. (1970), *in* Handbook of Experimental Pharmacology: Bradykinin, Kallidin and Kallikrein, Vol. XXV, Erdös, E. G., Ed., Berlin, Springer-Verlag, p 131.
Yin, E. T. (1964), *Thromb. Diath. Haemorrh. 12,* 307.