The alterations induced by different ions and enzyme inhibitors on the kininase activity of this purified fraction are shown in Table 2. It may be seen that 1 mM concentrations of the ions Cu^{2+} or Zn^{2+} as well as *p*-hydroxymercury benzoate completely inhibited the kininolytic activity; the same effect of these inhibitors was observed with brain neutral proteases.[4]

TABLE 2. EFFECT OF VARIOUS SUBSTANCES ON THE INACTIVATION OF BRADYKININ BY PARTIALLY PURIFIED ENZYME FROM RABBIT BRAIN TISSUE

Compound	Concentration	Inhibition (%)
Iodoacetate	1 mM	0
Ethylenediaminetetraacetate	1 mM	0
8-Hydroxyquinoline	1 mM	0
Diisopropylfluorophosphate	0·3 mM	−100
p-Hydroxymercurybenzoate	1 mM	−100
Bradykinin potentiating factor	0·1 mg/ml	−100
Trasylol	50 μ/ml	−50
Oxytocin	0·2 μ/ml	−50
Cu^{2+}	1 mM	−100
Zn^{2+}	1 mM	−100
Co^{2+}	1 mM	−37
Ca^{2+}	1 mM	−35
Mn^{2+}	1 mM	0
Mg^{2+}	1 mM	0

Complete kininase inhibition was also caused by 0·3 mM diisopropylfluorophosphate, whereas partial inhibition was verified in the presence of Trasylol (50 μml/ml, Bayer) oxytocin (0·2 unit/ml, Syntocinon-Sandoz), 1 mM Co^{2+} and 1 mM Ca^{2+}. In contrast to most of the brain peptidases,[5] EDTA, Mg^{2+} and Mn^{2+} at 1 mM concentrations were ineffective.

BPF (100 μg/ml) completely blocked the bradykininolytic activity of the purified fraction of the rabbit brain homogenate. This result suggests that the BPF potentiating effects of bradykinin on the central nervous system of cats at doses of 20 μg bradykinin and 150 μg BPF may be due to the inhibition of the destruction *in vivo* of the polypeptide by brain kininase.

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Further study on purification of hog pancreatic kallikrein

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RECENTLY many biochemists have focused their attention on the kinin-releasing system and its pharmacological, physiological and pathological effects or roles, and have isolated kallikreins from many sources. It is somewhat difficult to obtain large quantities of pure kallikreins and, since hog pancreatic

kallikrein is the material that has often been employed as the fundamental kinin-releasing enzyme, it seemed necessary to obtain large quantities of the purest hog pancreatic kallikrein in order to pursue studies in this system.

The method employed in this paper was further developed and modified from the method described in our previous papers.[1–10]

Minced hog pancreas was allowed to autolyze at 15–25° for 18–24 hr and was extracted with aqueous solution at pH 6·0. The suspension was heated at 55° for 5 min and, after cooling, was adjusted to pH 4·5, brought to 30% (v/v) in acetone, then filtered in the cold. The supernatant solution

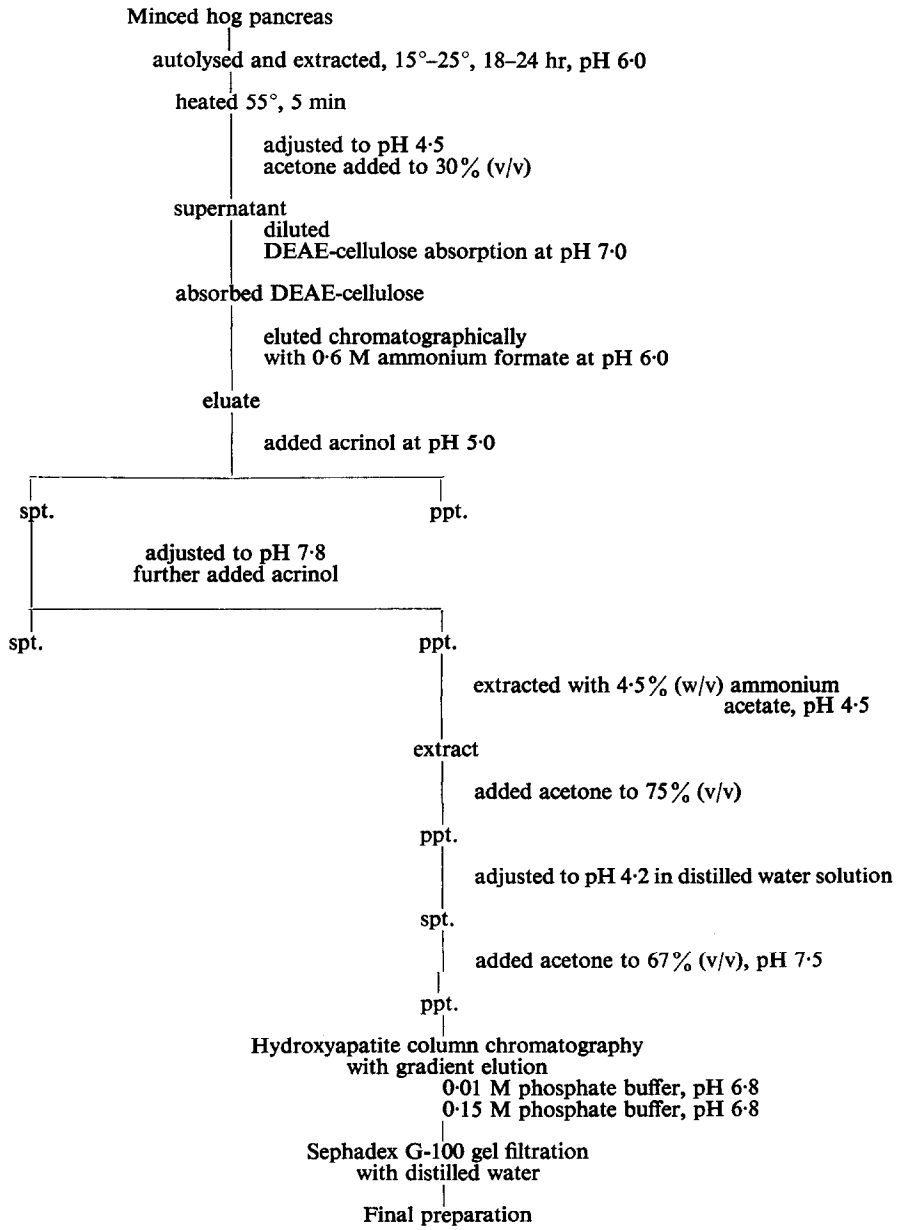

FIG. 1. Purification of hog pancreatic kallikrein.

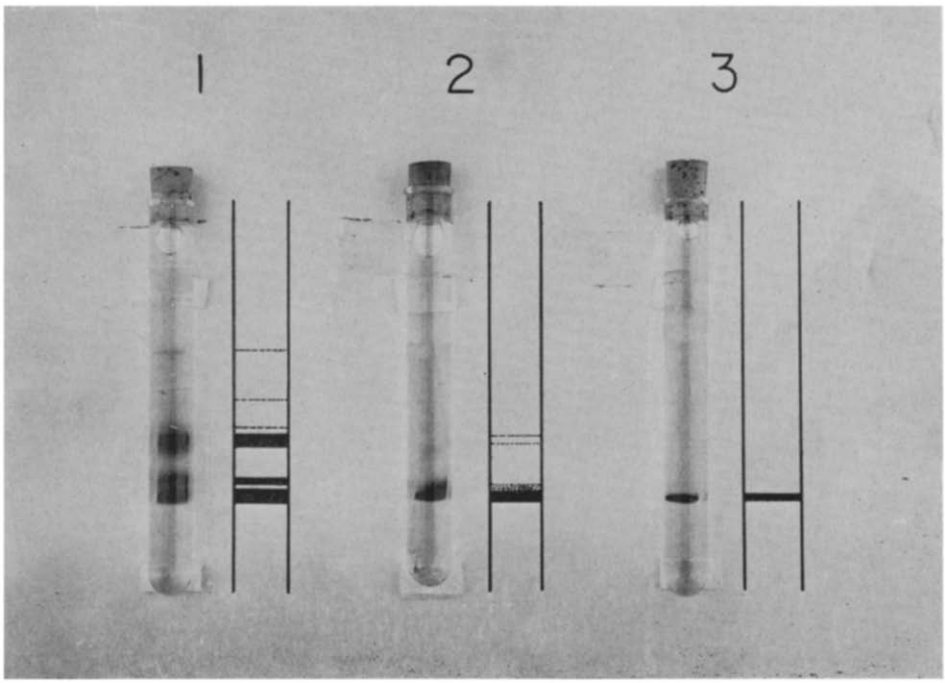

FIG. 2. Disc electrophoresis of hog pancreatic kallikrein. 1 = crude preparation, 20 FU/E_{280}; 2 = fraction after hydroxyapatite chromatography, 950 FU/E_{280}; 3 = the final purest preparation, 1200 FU/E_{280}. Current = 2·5 mA per gel, 150–200 V, 90 min. Stained with 1% (w/v) amido black 10 B.

B.P.—*facing page 550*

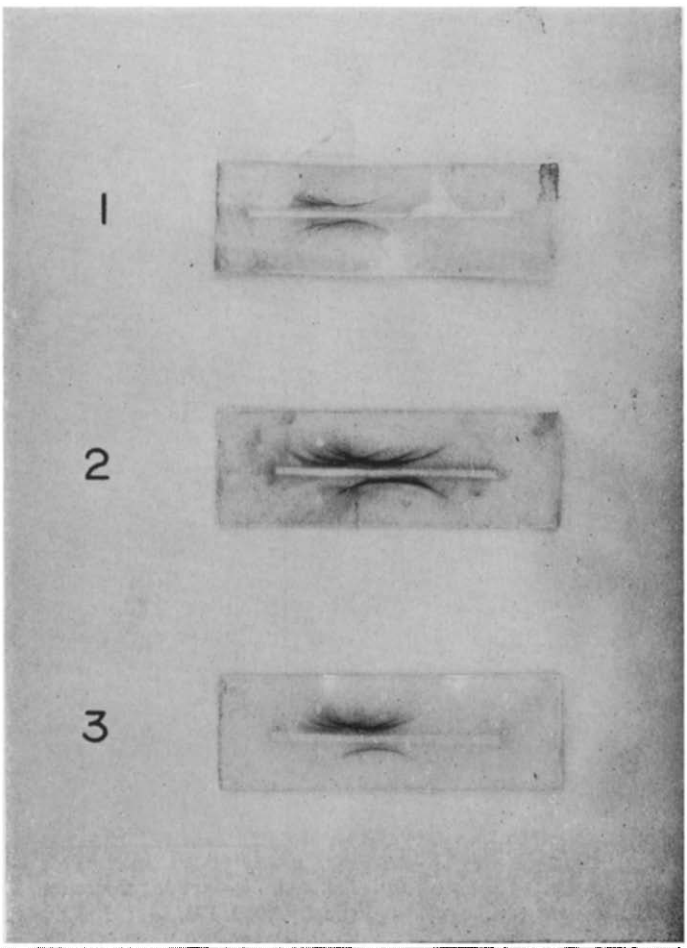

FIG. 3. Immunoelectrophoresis of several preparations of hog pancreatic kallikrein. 1 (upper and lower) = fraction just before hydroxyapatite chromatography, 155 FU/E_{280}; 2 (upper) = crude preparation, 20 FU/E_{280}; (lower) = fraction after hydroxyapatite chromatography, 950 FU/E_{280}; 3 (upper) = crude preparation, 20 FU/E_{280}; (lower) = final purest preparation, 1200 FU/E_{280}. Electrophoresis: 7·5 mA, 100–160 V, 90 min. Diffusion: 36 hr at 4° on slide glass. Medium: 1·5% (w/v) agar gel with Tris-HCl buffer, pH 8·5, μ = 0·05. Antiserum was prepared in rabbits injected with crude hog pancreatic kallikrein (20 FU/E_{280}), total 1500–2000 FU with Freund's complete adjuvant, then given booster injections a few times for 3 months.

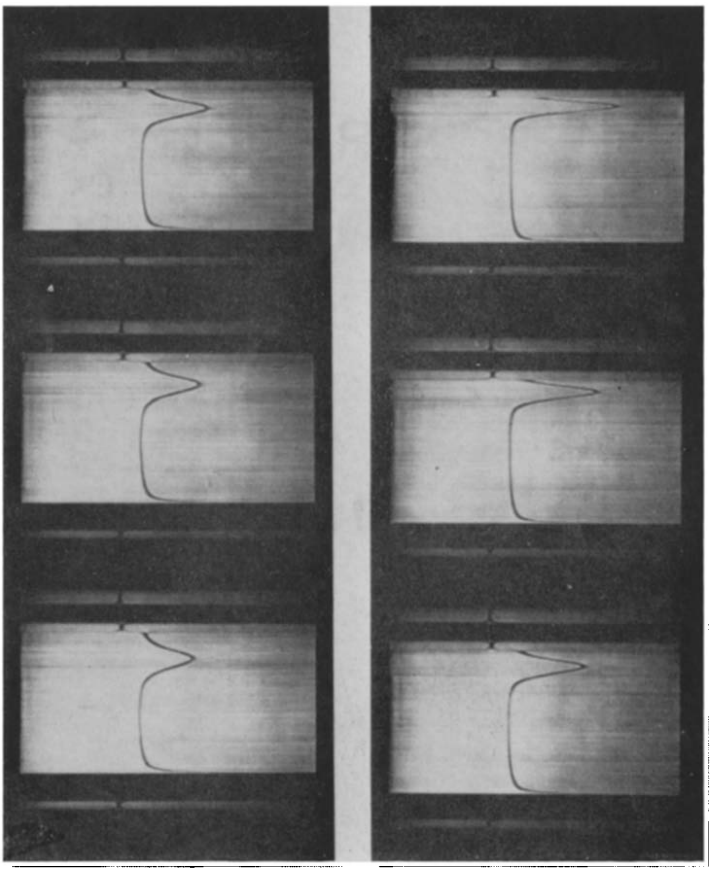

FIG. 4. Ultracentrifugal analysis of the purest hog pancreatic kallikrein. Protein concentration = 7·2 E_{280}/ml in 0·1 M phosphate buffer, pH 6·8; 55,430 rpm; temperature 88 ± 0·4°. Photographs were taken 41 min after the start and at 16-min intervals thereafter.

was subjected to the following steps: DEAE-cellulose treatment, acrinol (2-ethoxy-6,9-diamino-acridinium lactate) precipitation, extraction with ammonium acetate, acidic pH treatment and acetone precipitation, methods which are similar to those described in our most recent paper,[10] except for the modification of pH in the acrinol precipitation illustrated in Fig. 1.

The material obtained after acetone 67% (v/v) precipitation (120–160 FU/F_{280}) was further purified by chromatography on a hydroxyapatite column (1·6 × 25 cm) equilibrated with 0·01 M phosphate buffer, pH 6·8. Elution was carried out with an increasing buffer gradient of 0·01–0·15 M phosphate, pH 6·8 (flow rate 6·0 ml/hr). Active material appeared in one peak eluted with 0·08 to 0·1 M buffer and its purity was about 1000 FU/E_{280}, although it still had trace impurities (see Fig. 2). This fraction was pooled and filtered through a Sephadex G-100 gel column equilibrated with freshly distilled water. Material with a purity of 1200 FU/E_{280} was obtained (yield 6–9 per cent, 2280-fold purification, calculated from the starting extraction material). As shown in Figs. 2–4, this material was clearly found to be homogeneous.

The molecular weight of the purest kallikrein was estimated to be 25,200 by sedimentation equilibrium, although the previous result was 33,000 with our 570 FU/mg preparation.[8] Qualitatively the amino acid and sugar composition was similar to that previously reported by us.[6, 7] As shown in Table 1, the enzyme activities of the purest kallikrein were measured and compared with vasodilator

TABLE 1. COMPARISON OF BIOLOGICAL, ESTEROLYTIC AND PROTEOLYTIC ACTIVITY WITH VARIOUS PURITIES OF HOG PANCREATIC KALLIKREIN

	Vasodilator activity*	Esterolytic activity†	Proteolytic activity†
67% acetone precipitate	65 188	27 90	176 73
Eluate, hydroxyapatite column chromatography	702 1035	217 221	1·7 1·5
Sephadex G-100 gel filtrate	1126 1250	232 260	< 0·01 < 0·01

* Dog assay was done for vasodilator activity.[10]
† Substrates for esterolytic and protolytic activities were TAMe and casein respectively.

activity. From these results, the purest hog pancreatic kallikrein can be said not to have caseinolytic activity. The purest material was found to be unstable under conditions of complete dryness and, therefore, all studies were performed based on E_{280} without dryness. Quantitative determination of protein with this material was also carried out by the method of Lowry *et al.*,[11] with bovine serum albumin as the protein standard. Since 1 E_{280} of the purest kallikrein corresponded to about 0·5 mg of the dried protein, the specific activity, indicated as FU/mg, would be assumed to be much more than 1200.

Recently, properties of hog pancreatic kallikrein were reported by several investigators[12–15] and some discrepancies concerning molecular weight, specific activity and the possibility of artifact during treatment were discussed. Our purified material was also somewhat different from those previously described. It was reported by Werle *et al.*[14, 15] and by Habermann[12] that more than one kallikrein is present in hog pancreas. However, the present results do not allow a selection of which of these kallikreins corresponds to our purest kallikrein. Our studies on purification are being continued in order that large quantities of the purest material may be produced, so that the above questions may be answered.

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Oxidation of styrene in liver microsomes

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DIHYDRODIOLS are metabolic products of oxidation of aromatic hydrocarbons such as naphthalene in mammalian liver microsomes.[1, 2] Oxidation of the nonaromatic double bond of some cyclic compounds to glycols has also been demonstrated. Thus, dihydronaphthalene[3] and indene[4] give rise to glycols in mixed-function oxidations in liver microsomes.

El Masri *et al.*[5] showed that rabbits fed styrene (phenylethylene) excrete the glucuronide of phenylethylene glycol in the urine. We have found that the dihydroxylation of this noncyclic olefin occurs in the microsomes of mammalian liver.[6] This communication describes some of the characteristics of glycol formation from styrene.

The enzyme preparation was the reconsituted lyophilized 9000 *g* supernatant fraction, prepared as previously described,[4, 7] from homogenates of livers of male Holtzman rats or male New Zealand White rabbits that had been injected i.p. with sodium phenobarbital, 75 mg/kg/day (rats) or 15 mg/kg/day (rabbits), for 4 days. The last injection occurred 20 hr before the animals were killed. Incubation, deproteinization and extraction of glycol with ethyl acetate were carried out by methods similar to those employed for the study of indene metabolism.[4] The substrate solution containined 50 μmole styrene in 0·5 ml dimethylformamide. The recovery of added phenylethylene glycol in the extraction procedure was essentially complete.

Gas chromatograms of the extracts in two different systems are shown in Fig. 1. In each case, peaks were observed in the chromatograms of experimental extracts which were identical in retention time to those given by authentic phenylethylene glycol. All other peaks in the experimental chromatograms were matched by peaks in those of blank samples prepared as above, but with the styrene and dimethylformamide added at the end of the incubation period, immediately before deproteinization. In each system, two peaks were observed for phenylethylene glycol reference samples and corresponding peaks were obtained from experimental extracts. This phenomenon was investigated with the OV-1/OV-17 column. One peak varied in height over a 10-fold range proportionally to the amount of glycol injected, whereas the other was not proportional to sample size, but was relatively constant in height. This is also shown in tracings E and F of Fig. 1, in which one peak was higher in the chromatogram of an extract made after 60 min of incubation than in that after 10 min, whereas