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PURIFICATION AND PROPERTIES OF A SUBSTANCE P-INACTIVATING ENZYME FROM BOVINE BRAIN*

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Abstract—An enzyme which inactivates Substance P at neutral pH has been extracted and purified from bovine brain. The action of the purified kininase on SP shows a broad pH optimum near 6·4, while proteinase action of the preparation toward hemoglobin is maximal near 4·0. Physical and enzymatic properties of the kininase differ from those of known brain enzymes. The smooth muscle activities of bradykinin and kallidin are also destroyed upon incubation with the enzyme, but eledoisin is unaffected.

The significance of the reported enzyme is discussed in terms of a possible neurohumoral activity of Substance P in the central nervous system.

THE OCCURRENCE of the polypeptide Substance P (SP) in vertebrate neural tissue was reported by von Euler and Gaddum in 1931,[1] and has been confirmed by numerous other workers. Its distribution in the central nervous system of mammals was studied extensively by Pernow[2] as well as by Amin, Crawford and Gaddum,[3] and by Zetler and Schlosser.[4] Highest concentrations were found in phylogenetically older regions of the brain, particularly the hypothalamus.[2] The subcellular localization of SP in bound form in nerve endings separated from brain homogenates has recently been demonstrated.[5, 6]

The observed distribution pattern of SP along with the pharmacologic properties of the partially purified peptide have led some workers to suggest a neurohumoral role in sensory or inhibitory pathways.[7] If such a role is actually played by SP, one might expect to find specific enzymes in brain tissue capable of controlling the concentration of the free peptide within functional limits.

Enzymatic inactivation of SP by neural tissue extract was first reported by Gullbring,[8] who observed that saline extracts of acetone-dried basal ganglia of man and horse rapidly destroyed SP upon incubation. A similar extract from spinal cord of the cow failed to attack the peptide. Umrath,[9] Eber and Lembeck,[10] and Krivoy[11] have confirmed the action of neural tissue extracts on SP preparations. Hooper[12] reported

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the presence of enzyme in dog hypothalamus which inactivated SP. None of these enzymes was isolated, and the degree of specificity for SP was not reported.

In order to study the properties of brain SP-inactivating enzyme (brain kininase), we undertook its purification from bovine tissue. Our investigation was concerned with enzymes active at neutral pH values, since these might be expected to be operative under normal physiological conditions.

MATERIALS AND METHODS

Preparation of Substance *P*

Substance P for use as a substrate was prepared from defatted cattle brain powder (Wilson & Co.) by a modification of a method, essentially that of Pernow,[2] which was developed for extraction of fresh brain.

In a typical bench experiment, a 5·0-g portion of cattle brain powder was suspended in 20 ml of 70% methanol containing 0·2 ml glacial acetic acid and stirred for 1 hr. The extract was recovered by suction filtration and extraction of the residue was repeated. The final residue was washed with 20 ml of 70% methanol and the cake was discarded. The alcoholic filtrates were combined, concentrated *in vacuo* at 50° to a syrup, and then adjusted to 15 ml with water. This fraction was washed three times with 10-ml portions of dichloromethane to remove lipid material, and the aqueous phase was concentrated to 10 ml. Solid ammonium sulfate was added to saturation, and the precipitate which formed within 1 hr was filtered off, washed on the funnel with saturated ammonium sulfate solution, and dissolved in 5 ml water. To precipitate the entrained salts, 4 vol. of 95% ethanol were added and the white granular material which formed was removed by centrifugation. The alcoholic supernatant was evaporated to dryness *in vacuo*, the solids were dissolved in a minimum of water and lyophilized.

This procedure yielded 188 mg of a tan powder which had a potency of 1·9 Euler units/mg when assayed on guinea pig ileum versus a horse intestine preparation of known SP activity.

A large-scale extraction of 50 kg powdered brain residue, by a procedure slightly modified from the method described above, was carried out for us by the Upjohn Laboratories, Kalamazoo, Mich.* The powder was extracted twice with 70% methanol (6·0 and 4·0 l./kg) containing 10 ml glacial acetic acid/l. and filtrates were concentrated *in vacuo* at 50° to 500 ml/kg. Two extractions of the concentrate with dichloromethane (250 ml/kg each) served to remove lipids. After concentration of the aqueous phase to 250 ml/kg and adjustment to pH 4·0 with acetic acid, solid ammonium sulfate was added to near saturation, and the precipitate was allowed to form overnight in the cold before being recovered by centrifugation. The sediment was suspended in saturated salt solution, resedimented and then dissolved in a minimum of water (about 400 ml/kg brain powder). Four volumes of methanol were added with stirring, and the bulky white precipitate was filtered off. The filtrate was taken to dryness *in vacuo*, solids were taken up in water and lyophilized. The product was a tan solid weighing 217·5 g, with a sp. act. of 1·9 units/mg.

* We wish to thank Dr. George Cartland and the Upjohn Co., Kalamazoo, Mich., for preparing concentrates.

Purification of crude brain SP

The crude SP product obtained as described above was further purified in our laboratory by solvent extraction and chromatography on Sephadex G-25. The dry powder was extracted twice with glacial acetic acid (7·0 and 4·0 ml/g powder, successively) and the activity was precipitated from the acetic acid solution by the addition of 2 vol. of acetone and 3 vol. of ether. After standing a short time, the precipitate which formed was collected on a filter, washed twice with an acetone–ether mixture (2 : 3, v/v) and finally with ether. The residue was sucked dry and then stored in a vacuum desiccator over calcium chloride. A yield of 0·747 g/g crude SP was achieved, with a specific activity of 2·0 units/mg.

Fractionation by gel filtration was carried out on a column of Sephadex G-25 (ungraded, but with fines removed) which measured 3·6 × 77 cm (850 ml bed, 265 ml void volume). Solvent-purified SP was applied in 10-g portions dissolved in 60·0 ml water, and the column was developed with water at a flow rate of 150 ml/hr.

As development proceeded, the sample separated into two distinct colored bands which corresponded to the main solids bands: a broad brown band representing the unretarded material, and a rather narrow, deep yellow band which moved more slowly. The trailing band, which emerged in approximately one bed volume, had a sp. act. of 10–30 units/mg, while the leading band of material showed less than 1 unit/mg. However, the total activity of the latter fraction was significant, and it was rechromatographed after concentration to 45 ml. A light but distinct yellow band associated with a peak of biological activity separated from the unretarded fraction and was eluted after one bed volume. All chromatographic fractions were lyophilized.

From a total of 68·0 g of solvent-purified material chromatographed on Sephadex G-25, all fractions which exhibited a specific activity of at least 8 units/mg were combined, dissolved in water, filtered and lyophilized to a yellowish-brown grainy solid. This preparation, which weighed 2·475 g and assayed at 12 units/mg, served as an SP substrate of uniform potency for most enzymatic studies described in this report. In some later experiments, a substrate material, which had been purified by an additional pass over a small column of Sephadex G-25 and had a sp. act. of 24 units/mg, was employed.

It is recognized that the SP content of the substrate materials represented less than 0·1 per cent of total solids, since preparations with 100,000 units/mg or more have been prepared by others.[13–15] However, such highly purified materials appear to be very labile in solution, with rapid loss of biological activity.[13] It was felt that the stability of a lower potency substrate justified its use in a study of enzymatic inactivation because of the reproducibility of the experimental system over longer periods of time, and the minimum spontaneous loss of activity during digestion and assay procedures.

Preparation of enzyme extracts from brain tissue

Brains from freshly killed cattle were transported to the laboratory on ice, where they were freed as far as possible from blood and blood clot. They were then wrapped in polyethylene film, stored at — 15°, and utilized as required. Comparison with extracts of fresh brains indicated that the frozen tissue retained full kininase activity for at least 4 months. All extractions were carried out at 5°.

Partially thawed brain tissue (exposed to room temperature for 30–45 min) was macerated in a Waring blendor with 2 ml phosphate buffer (0·002 M, pH 7·0) per g of tissue. The brei was stirred for 20 min and centrifuged at 14,000 *g* for 20 min. The sediment was discarded and the supernatant was either frozen for preservation or immediately subjected to salt fractionation with ammonium sulfate.

A second extract of the sedimented fraction was found to contain about one-third as much enzymatic activity as the initial extract, but was normally omitted.

Determination of enzyme activity

Substance P *inactivation.* Assay of brain extracts and derived fractions for SP kininase activity required digestion with the substrate and subsequent bioassay of remaining SP on isolated guinea pig ileum.

(A). *Digestion.* Each standard digestion mixture contained the following components: substrate, 8·4 units SP; enzyme preparation, 0 to 0·5 ml (0–15 mg protein); buffered saline (0·15 M NaCl, 2 mM phosphate, pH 7·0), 1·5 to 2·0 ml; total volume, 2·0 ml. Tubes were incubated at 37° for 2 hr. Enzyme action was halted by immersing digestion tubes in boiling water for 5 min. Digests were stored in a freezer until bioassays were performed.

Whenever enzyme activity toward other polypeptides was to be determined, the same procedure was followed but with a 20-min digestion period and with the indicated amounts of substrate per digestion tube: bradykinin, 200 ng; kallidin, 600 ng; or eledoisin, 70 ng.

(B). *Bioassay.* Determination of SP content (smooth muscle-stimulating activity) of digestion mixtures was carried out on isolated strips of guinea pig ileum. Strips were suspended in a 7-ml bath containing oxygenated Tyrode's solution at 37°. Atropine sulfate and chlorpheniramine maleate (0·5 mg/l. each) were included. Contractions were recorded on a smoked drum with a frontal writing lever.

In control tubes, the enzyme fraction was replaced by an equal volume of saline. In some experiments digest tubes containing enzyme preparations were boiled at zero time. Bioassays indicated that none of the enzyme preparations interfered with the estimation of SP activity.

Substance P activity of an unknown solution was determined from the volume which produced the same biological response as a given volume of a standard solution (4·2 units/ml). Bradykinin, kallidin and eledoisin were similarly assayed by comparison with appropriate standard solutions.

(C). *Unit of enzyme activity.* For purposes of comparison, a unit of enzyme activity was arbitrarily defined as that amount of protein per tube which would inactivate 50 per cent of the particular peptide substrate during the specified incubation period under the conditions described above.

Proteinase activity. Proteinase activity of selected enzyme fractions was estimated by incubation with denatured bovine hemoglobin. The fraction soluble in trichloroacetic acid (TCA) was analyzed for its tyrosine content.

(A). *Digestion.* Each digestion tube contained 0·25–0·5 mg enzyme protein and 2·0 mg hemoglobin in 2·0 ml total volume. Enzyme and substrate solutions were adjusted to desired pH values with NaOH or HCl before mixing. Tubes were incubated in a rotary shaker at 37° for 2 hr and each mixture was then brought to 10% TCA by addition of 1·0 ml of a 30% solution (w/v). Precipitates were sedimented by centri-

fuging and the supernatant solutions were analyzed. For autolytic controls, enzyme and substrate were incubated separately and mixed immediately before addition of the TCA solution.

(B). *Analysis for TCA-soluble material.* The TCA-soluble tyrosine released from hemoglobin was determined by the Lowry procedure.[16] TCA was neutralized and aliquots were reacted with the Folin-Ciocalteau reagent. Readings were made at 750 mμ versus a blank containing water in place of digest supernatant. Optical readings were converted to micrograms of tyrosine/ml by reference to a standard curve. Proteinase activity, corrected for autolytic release, was expressed as μg tyrosine released/mg enzyme protein/hr.

Protein determinations

The protein content of enzyme fractions was also determined by the Lowry method. Crystalline bovine serum albumin was used as a standard, and optical readings were made at 750 mμ.

Protein content of chromatographic fractions was estimated routinely from extinction at 280 mμ.

Concentration of protein fractions

Protein fractions were concentrated, where necessary, either by dialyzing against a saturated solution of Carbowax 6000 or in some cases by adding dry Sephadex G-25 dextran gel and recovering the concentrate by suction filtration.

Sources of chemicals

Synthetic polypeptides bradykinin, kallidin, and eledoisin were gifts from Sandoz Pharmaceuticals, Hanover, N.J. Sephadex products were obtained from Pharmacia Fine Chemicals, Inc., New Market, N.J. Ion-exchange celluloses and hydroxylapatite were purchased from Calbiochem Corp., Los Angeles, Calif. Bovine serum albumin and protamine sulfate were obtained from Nutritional Biochemicals Corp., Cleveland, Ohio. Carbowax polyethylene glycol is a product of Union Carbide Corp., South Charleston, W.V. All chemical reagents employed were analytical grade.

EXPERIMENTAL AND RESULTS

Characterization of active component of substrate material

The smooth muscle-stimulating component of substrate materials prepared from bovine brain by the procedures described above exhibited properties characteristic of SP.

The peptide nature of the active material was confirmed by incubation with α-chymotrypsin (Worthington Biochemical Corp.). Solutions of the products purified from cattle brain powder (8·4 units/ml) and fresh cattle brain (8·7 units/ml) were incubated separately with α-chymotrypsin (0·05 mg/ml) for 40 min at 37° (Tyrode solution, pH 7·8). After heat inactivation of the enzyme, bioassay of the digests revealed 93 per cent and essentially 100 per cent inactivation of the brain powder and fresh brain products respectively. The heated enzyme did not influence tissue response to the products.[17]

Biological activity was determined on isolated guinea pig ileum and hen caecum by comparison with a standard SP preparation (75 units/mg) kindly supplied by Dr. B

Pernow. The brain powder product exhibited uniform potency on both tissues, indicating the absence of significant bradykinin activity. Neither atropine nor chlorpheniramine maleate altered the biological response to the preparation.

Chromatography on carboxymethyl-Sephadex C-25 or Amberlite IRC-50 resulted in the adsorption of more than 90 per cent of the activity, although large losses (up to 80 per cent) were normally experienced in attempts to recover the activity by elution with 1 M ammonium acetate, pH 5·5,[15] or 0·1 N HCl.

According to Zetler,[18] 18 per cent of the smooth muscle activity of crude SP from cattle brain is due to related peptides separable on acid alumina. Passage of our preparations over acid alumina (California Biochemical Corp.) in 60% ethanol showed less than 10 per cent of the activity to be unabsorbed. Recovery of SP from the column by elution with water gave variable yields with routinely large losses (50–80 per cent). Elution with basic solutions failed to improve recovery. Low and variable recoveries of SP during chromatography, especially on alumina, have been noted by others.[19]

Since no more than 5 per cent of the substrate activity has been found to resist inactivation by the bovine brain enzyme preparation, any active components other than SP must also be susceptible to the enzyme fraction.

Purification of SP *kininase activity from brain extract*

Aqueous extracts of bovine brain prepared as described above demonstrated enzymatic inactivation of SP in the standard assay system. Such extracts were subjected to certain precipitation and chromatographic procedures in order to separate the desired enzyme activity from other cell constituents.

Step 1: Salt fractionation. Preliminary experiments indicated that neutral kininase activity was precipitable between 30 and 65 per cent saturation with ammonium sulfate. Brain extracts were brought to 30 per cent saturation by addition of the solid salt (216 g/l.) at 5° with constant stirring. The solution pH was maintained at 7·0 (glass electrode) with 3 N NaOH. The precipitate which formed was collected after 15 min by centrifuging at 9500 rev/min for 15 min. The sedimented fraction contained little activity and was discarded, while the supernatant solution was brought to 65 per cent saturation by further addition of salt (252 g/l. extract) with pH adjustment to 7·0. This precipitate, which possessed the bulk of the activity, was taken up in buffer and dialyzed against three changes of 2 mM phosphate buffer, pH 7·0. Assay of the protein fraction obtained by this procedure showed a purification of about 1·5 to 2.

When the product from step 1 was subjected to fractional precipitation at 30, 40, 50, and 65 per cent saturation, appreciable kininase activity was found in all but the last fraction. In several experiments the highest activity was located in the 40–50 per cent fraction, but this result was not obtained consistently.

Step 2: CM-Sephadex adsorption. Treatment of the product from step 1 with CM-Sephadex (Na^+ form, pH 7·0) removed some inactive material, including any hemoglobin present.

The protein solution (approximately 20 mg/ml) was either passed over a bed of CM-Sephadex C-25 or, in later experiments, was stirrred with three successive portions of gel (60 mg dry wt./g protein) and separated by centrifugation. This procedure removed 15–40 per cent inactive protein, giving a purification of about 1·4.

Step 3: Protamine sulfate fractionation. The active product obtained in step 2 was further purified by precipitating inactive material with protamine sulfate. To achieve maximum precipitation of inactive material, protamine sulfate was added as a clarified neutral (pH 7), (w/v) solution at a ratio of 0·1 mg/mg brain protein. After sedimentation of the precipitate, SP kininase activity was precipitated by 65% saturated ammonium sulfate. The salted-out fraction contained the initial activity, with a 1·5 to 2-fold increase in sp. act.

The described purification steps yielded a product which, on the basis of protein content, represented a purification of kininase activity from the initial homogenate of 40-fold, with overall yield of 40 per cent. The data from a typical experiment are summarized in Table 1.

TABLE 1. PURIFICATION OF KININASE FROM BOVINE BRAIN

Preparation	Protein (g)	Sp. act.*	Total units	Yield (%)
Homogenate	23·03	0·07	1600	100
Aq. extract	2·505	0·9	2200	140
Step 1				
30–65% Salt fraction	1·086	1·8	1500	95
Step 2				
CM-Sephadex fraction	0·777	2·5	1900	120
Step 3				
Protamine sulfate fraction	0·214	2·9	620	40

* Activity with Substance P as substrate. Expressed in arbitrary units per mg protein as defined in text. Details of assay are described in text.

Other fractionation methods tested. Several precipitation and adsorption techniques were investigated in further attempts to purify enzyme activity toward SP. These were found to be of little value under the conditions employed.

Fractional precipitation with acetone or acid (pH 5·3) resulted in variable and sometimes complete loss of activity. That the time of exposure may be critical in acid fractionation was suggested by the recovery of all activity when the acidified solution was immediately neutralized without separation of precipitated solids.

The enzyme could be adsorbed onto calcium phosphate gel or hydroxylapatite, and eluted between 0·15 and 0·20 M phosphate buffer (pH 6·5). Recovery of activity ranged from 15–40 per cent, with little increase in specific activity. Likewise, the enzyme was adsorbed onto DEAE-Sephadex or DEAE-cellulose at pH 6·5 and an active peak could be eluted by 0·3 M NaCl, but recovery was usually about 15 per cent with decreased sp. act.

The causes for the failure of these commonly employed enzyme fractionation methods are not apparent. Activity was not restored by admixture of various DEAE-Sephadex fractions, nor by chromatography in the presence of a divalent metal (Mg^{2+}).

Properties of the partially purified enzyme

Rate of enzymatic inactivation of substance P. The rate of SP inactivation by brain kininase was studied for digestion periods from 5–120 min. Identical digestion mixtures containing 0·2 ml enzyme fraction (2·82 mg protein) per tube were incubated at 37°, and at specified intervals tubes were transferred to a boiling water bath to

terminate enzyme action. The zero-time mixture (boiled enzyme control) was heated immediately after addition of enzyme. Substrate controls containing no enzyme were incubated for the full 2-hr period to detect any spontaneous loss of SP activity under the experimental conditions employed. The extent of SP inactivation was determined by bioassay for remaining activity.

The data presented in Fig. 1 indicate that the inactivation rate under these conditions is approximately linear up to about 40 per cent destruction, but shows a declining rate

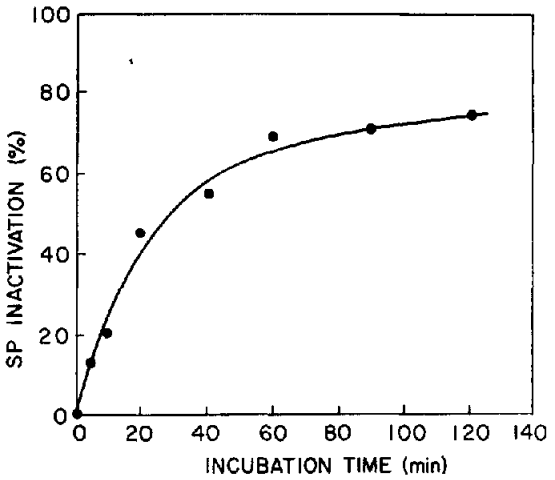

FIG. 1. Inactivation of SP by brain kininase.

with continued incubation. Both the boiled enzyme control and the substrate control retained full SP activity, demonstrating that SP destruction proceeded only in the presence of the enzyme.

At higher enzyme levels or longer digestion times, about 5–10 per cent of the smooth muscle activity was usually still detectable. These results may indicate the presence of components which bind SP or otherwise protect it from enzymatic destruction, or the presence of low levels of smooth muscle stimulants resistant to kininase action.

Since, for enzyme assays, it was desirable to limit the quantity of enzyme protein included in each digest tube, the 2-hr period was selected as a standard incubation time, and appropriate dilutions of enzyme fractions were tested in the standard system. The 50 per cent inactivation equivalent for each preparation was determined by interpolation.

pH optimum for Substance P *inactivation.* The effect of pH on enzymatic inactivation of Substance P was determined for the range 3·5–8·5. Enzyme and substrate solutions were adjusted separately to the desired values and digestion was carried out as previously described. Acidic tubes were neutralized with NaOH before the terminal heating step.

The pH optimum for this reaction lies near 6·4, with little or no inactivation occurring below pH 5·0 (Fig. 2). These data confirm that the enzyme studied is active at normal physiological pH values, and therefore may participate in the metabolism of SP *in vivo*.

Proteinase activity. The activity of the purified enzyme toward protein was determined with denatured hemoglobin as substrate. Maximum proteinase activity was demonstrated near 3·5–4·0 (Fig. 2) as previously reported by other workers.[20] A low level of proteinase activity was also observed near pH 8·0, as reported by Marks and Lajtha for rat brain homogenate.[20]

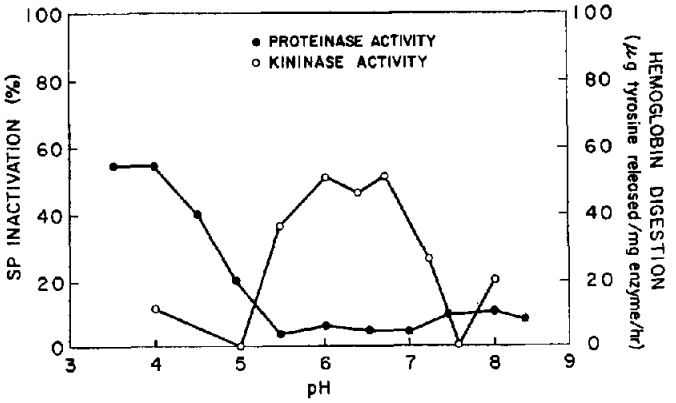

FIG. 2. Effect of pH on kininase and proteinase activities of partially purified brain enzyme.

Behavior on Sephadex columns. Chromatography of salt-fractionated enzyme preparations on Sephadex gels was carried out in 2 mM phosphate buffer at pH 7·0. Kininase activity was found to be completely excluded from Sephadex G-50 and G-75. Upon chromatographing on a Sephadex G-200 column, the enzyme was found to be somewhat retarded behind the elution front, but emerged in less than one void volume, indicating a probable molecular weight of 100,000–150,000.

Marks and Lajtha have reported from gel filtration data a molecular weight of about 60,000 for acid proteinase from rat brain.[21] We analyzed the G-200 effluent for proteinase activity at pH 3·9. and found that bovine neutral kininase and acid proteinase activities were not separated on our column.

Metal ion requirements. The activities of many recognized peptidases depend on the presence of divalent metal ions, especially Mg^{2+}, Mn^{2+}, Co^{2+}, and Zn^{2+}.[22] Uzman, van den Noort and Rumley[23] found that human and mouse brain dipeptidase lost 80 per cent of its activity toward glycylleucine in the presence of EDTA, but could be reactivated by Zn^{2+}. Tripeptidase activity toward glycylglycylglycine was not inhibited by EDTA. Dialysis resulted in total and ireversible loss of both dipeptidase and tripeptidase activities.

In the present investigation, bovine brain SP kininase did not appear to be inactivated to any measurable extent by dialysis or by desalting on a Sephadex G-25 column implying the absence of a dissociable metal activator. The addition of EDTA (10^{-4} M) to the digestion mixture did not noticeably reduce the rate of SP destruction. The addition of Mg^{2+}, Mn^{2+}, Co^{2+}, or Zn^{2+} at a concentration of 10^{-4} M did not alter the rate of SP inactivation.

Sulfhydryl requirement. The possible role of sulfhydryl groups in the activity of the brain enzyme was investigated for inhibition by sulfhydryl blocking agents and for activation by reducing agents.

(a). Reducing compounds. Although no decline in activity due to air oxidation was normally observed during the described purification sequence, certain digestions were carried out in the presence of reducing compounds (glutathione, mercaptoethanol) to determine their effects on the reaction rate. However, the presence of these substances interfered with the bioassay for SP, even after air oxidation or exposure to hydrogen peroxide. No conclusions could be made as to the possible effect of reducing compounds on the enzyme activity.

(b). Inhibitors. Iodoacetic acid (10^{-3} M) and hydrogen peroxide (10^{-2} M) were tested for the ability to inhibit SP destruction by blocking or oxidizing essential sulfhydryl groups on the enzyme molecule. Enzyme preparations were preincubated 30 min at 25° with the test compound before carrying out the standard digestion of SP. Hydrogen peroxide had no influence on the rate of peptide destruction, while iodoacetic acid reduced the inactivation rate by about one-third.

Stability of the purified enzyme. SP-kininase preparations could be stored at — 15° for several months at all stages of purification without detectable loss of activity. Lyophilization of the purified material yielded a product which was completely soluble upon reconstitution and which retained essentially all of the initial activity.

Activity toward other polypeptides. The susceptibilities of some other naturally occurring polypeptides, with similar pharmacologic properties, to the partially purified enzyme were determined. Synthetic bradykin, kallidin, and eledoisin were employed as substrates at several stages of purification of the enzyme.

Bradykinin and kallidin (*N*-lysyl bradykinin) were inactivated by all of these enzyme fractions, whereas eledoisin was not inactivated by any of the fractions tested (Table 2).

TABLE 2. RELATIVE ACTIVITY OF BOVINE BRAIN KININASE FRACTIONS TOWARD BIOLOGICALLY ACTIVE POLYPEPTIDES

	Kininase activity*			
Enzyme preparation	SP	Bradykinin	Kallidin	Eledoisin
Buffer extract supernatant	0·8	15	3½	0
Salt fraction (30–65%)	1·2	30	3½	0
CM-Sephadex fraction	1·6	40	4	0

* Expressed in arbitrary units per mg protein as defined in text.

Enzymatic activity toward bradykinin was purified to approximately the same extent as activity toward SP while specific activity for kallidin remained nearly unchanged throughout the fractionation steps. According to Boissonnas *et al.*,[14] SP possesses the *N*-terminal sequence Arg-Pro-Pro found in bradykinin. Since kallidin differs from bradykinin only by the additional *N*-terminal lysyl residue, the data in Table 2 indicate that the specificity of the bovine brain kininase may rest at least partly on the amino-terminal peptide structure of the substrate.

DISCUSSION

Enzymes involved in the metabolism of peptides and proteins of neural tissue have been detected but, for the most part, have not been extensively purified or characterized.

Ansell and Richter first reported in 1954 the detection of neutral proteinase activity in human brain homogenates.[24] The enzymatic activity toward endogenous substrates

ceased within 1–1½ hr after death. Extracts of fresh brain or brain acetone powder failed to digest denatured hemoglobin.

Lajtha[25] extracted from rat brain a proteinase with a pH optimum of 7·6, which required an energy source for maximum activity. This enzyme was purified from rat and calf brains and separated from acid proteinase by Marks and Lajtha,[21] who reported a molecular weight greater than 100,000 and an association with the lighter cellular particles and fragments.

Guroff[26] purified a neutral calcium-activated proteinase from the soluble fraction of rat brain (pH optimum 7·1). The enzyme was subject to irreversible inactivation in the absence of sulfhydryl compounds and was quite labile in the presence of calcium ions.

Polypeptidase activity of brain tissue has been reported by several workers. Ansell and Richter[24] detected a release of amino acids from peptone by rat brain homogenate, but the action ceased within 1 hr. According to Gullbring,[8] basal ganglia of man and horse destroyed SP at pH 6·5, while SP inactivation by an extract of guinea pig brain was reported by Krivoy[11] to exhibit a pH maximum between 6 and 7. Hooper[12] believed that the enzyme in dog hypothalamus which destroyed SP (a broad pH optimum near 6) was distinct from one which destroyed vasopressin (pH optimum about 7·0). Bradykinin, however, was destroyed most rapidly at pH 7·1. None of these polypeptidases was purified or further characterized.

Uzman *et al.*[23] studied the action of human and mouse brain extracts on a number of glycine peptides, and detected dipeptidase and tripeptidase activities. Both enzymes were irreversibly inactivated by dialysis, and the dipeptidase was inhibited by EDTA. The tripeptidase exhibited only aminopeptidase activity.

The bovine brain neutral kininase activity which we are reporting is stable in frozen tissue or partially purified forms without added reducing agents, is not inactivated by dialysis or EDTA, and requires no calcium or energy source for its action on basic polypeptides. On the basis of available evidence, it is unlikely that this enzyme is identical with previously recognized neutral brain proteinases or peptidases. In the absence of further characterization of neural polypeptidases, no comparison is possible with the bovine product.

The mechanism by which SP activity is destroyed upon incubation with the brain enzyme is unknown. The nature of the reaction products has not been investigated and the molecular structure of SP has not been described. Trypsin and chymotrypsin are known to inactivate SP by splitting it into several peptides, apparently without releasing free amino acids.[14]

Only with high purity SP can one hope to elucidate the chemical transformation which results in loss of biological activity of this peptide. Bradykinin and kallidin, however, offer the possibility of determining the types of peptide bonds which are labile to the action of brain kininase, and investigations with these and other peptides are in progress. Preliminary studies indicate that products of bradykinin digestion include arginine, proline, phenylalanine, and at least two unidentified ninhydrin-positive components.[27]

The relatively slow rate of destruction of SP in comparison with bradykinin and kallidin may reflect the presence of inhibitory factors in the substrate preparation, rather than a preference for the synthetic substrates. Likewise, one must accept with some reservations comparisons of pH optima for SP destruction which have been

carried out on substrate material containing less than 1% active polypeptide. The difficulties encountered in attempting to determine a precise pH optimum for the bovine enzyme may be due in large measure to impurities.

The susceptibility of SP to enzymatic alteration in intact brain tissue may be modified by its state of binding *in vivo*. Recent investigations by several laboratories[5, 6, 28–30] indicate that brain SP normally occurs largely in bound form.

The subcellular localization of brain kininase is of importance to any proposed metabolic role, but the present study did not investigate this area. No attempt was made to prevent osmotic disruption of subcellular structures, although one experiment in which 0·25 M sucrose solution was employed for the initial extraction revealed no reduction in kininase activity extracted.

The fact that Stern[7] failed to observe any pharmacological effects after intraventricular administration of highly purified SP does not exclude a normal function for this polypeptide in the central nervous system. Enzymatic destruction or simply failure to penetrate to normal receptor sites could explain the observed results.

While the mechanism of action of many psychotropic drugs remains obscure, there is good evidence to indicate that at least some drugs directly inhibit enzymes (e.g. monoamine oxidases) which inactivate endogenous pharmacologically active molecular species. The possibility that other classes of psychotropic agents may exert their effects by altering the rate of enzymatic destruction of SP is now subject to investigation *in vitro* with the purified brain kininase. Significant acceleration or inhibition of kininase action by recognized psychotropic agents would be extremely interesting and should merit investigation.

REFERENCES

1. U. S. VON EULER and J. H. GADDUM, *J. Physiol., Lond.* **72**, 74 (1931).
2. B. PERNOW, *Acta physiol. scand.* **29**, *suppl.* 105, (1953).
3. A. H. AMIN, T. B. B. CRAWFORD and J. H. GADDUM, *J. Physiol., Lond.* **126**, 596 (1954).
4. G. ZETLER and L. SCHLOSSER, *Arch. exp. Path. Pharmak.* **224**, 159 (1955).
5. J. CLEUGH, J. H. GADDUM, A. A. MITCHELL, M. W. SMITH and V. P. WHITTAKER, *J. Physiol., Lond.* **170**, 69 (1964).
6. R. W. RYALL, *Nature, Lond.* **196**, 680 (1962).
7. P. STERN, *Ann. N.Y. Acad. Sci.* **104**, 403 (1963).
8. B. GULLBRING, *Acta physiol. scand.* **6**, 246 (1943).
9. K. UMRATH, *Pflügers. Arch. ges. Physiol.* **258**, 230 (1953).
10. O. EBER and F. LEMBECK, *Arch. exp. Path. Pharmak.* **229**, 139 (1956).
11. W. A. KRIVOY, *Br. J. Pharmac. Chemother.* **12**, 361 (1957).
12. K. C. HOOPER, *Biochem. J.* **83**, 511 (1962).
13. K. VOGLER, W. HAEFELY, A. HÜRLIMANN, R. O. STUDER, W. LERGIER, T. STRÄSSLE and K. H. BERNEIS, *Ann. N.Y. Acad. Sci.* **104**, 378 (1963).
14. R. A. BOISSONNAS, J. FRANZ and E. STÜRMER, *Ann. N.Y. Acad. Sci.* **104**, 376 (1963).
15. H. ZUBER, *Ann. N.Y. Acad. Sci.* **104**, 391 (1963).
16. O. H. LOWRY, N. S. ROSEBROUGH, A. L. FARR and R. J. RANDALL, *J. biol. Chem.* **193**, 265 (1951).
17. H. EDERY, *Br. J. Pharmac. Chemother.* **22**, 371 (1964).
18. G. ZETLER, *Ann. N.Y. Acad. Sci.* **104**, 416 (1963).
19. B. PERNOW, personal communication.
20. N. MARKS and A. LAJTHA, *Biochem. J.* **438**, (1963).
21. N. MARKS and A. LAJTHA, *Biochem. J.* **97**, 74 (1965).
22. E. L. SMITH, in *The Enzymes* (Eds. P. D. BOYER, H. LARDY and K. MYRBACK), vol. 4, p. 6. Academic Press, New York (1960).
23. L. L. UZMAN, S. VAN DEN NOORT and M. K. RUMLEY, *J. Neurochem.* **9**, 241 (1962).

24. G. B. ANSELL and D. RICHTER, *Biochim. biophys. Acta* **13**, 92 (1954).
25. A. LAJTHA, in *Regional Neurochemistry* (Eds. S. S. KETY and J. ELKES), p. 25. Pergamon Press, Oxford (1961).
26. G. GUROFF, *J. biol. Chem.* **239**, 149 (1964).
27. D. L. CLAYBROOK and H. W. HOUSE, JR., unpublished observations.
28. A. INOUYE and K. KATAOKA, *Nature, Lond.* **193**, 585 (1962).
29. R. W. RYALL, *Biochem. Pharmac.* **11**, 1234 (1962).
30. E. DE ROBERTIS, G. ARNAIZ, L. SALGANICOFF and A. PELLEGRINO DE IRALDI, *Nature, Lond.* **194** 794 (1962).