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PHARMACOLOGICAL SIGNIFICANCES OF PEPTIDASE AND PROTEINASE IN THE BRAIN (REPORT I)— ENZYMATIC INACTIVATION OF BRADYKININ IN RAT BRAIN

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Abstract—The peptidase activity in rat brain was investigated using bradykinin as substrate. The nature and changes during convulsion in activity of the enzymes which inactivates bradykinin (kininase) were examined.

The optimum conditions for the assay of kininase were pH 7·6 and about 45°. The kininase activity in the cerebellum was higher than that in the cerebral cortex or the brain stem.

On subcellular fractionation, the highest kininase activity was observed in the supernatant, with lower activities in the microsomes, mitochondoria and nuclei.

The effects of metal ions (zinc and cobalt ions) and N-ethylmaleimide on the brain kininase activity were different from those on enzyme in the plasma.

The activity of the kininase in the cerebellar region increased during convulsions caused by pentetrazol or picrotoxin, but not by strychnine and, of the subcellular fractions of the cerebellum of a rat after pentetrazol administration, the increase was only found in the nuclear fraction. Increased kininase activity in the cerebellar region induced by pentetrazol, was only observed during convulsions and not in the preconvulsive or intermediate states. This suggests that some alteration in the metabolism of brain protein may occur during convulsion.

IT HAS been reported that bradykinin may play some role in the inflammatory reaction and in pain-producing mechanisms.[1–4] Enzymes which can destroy kinins, that is, so-called kininases, are found in plasma and other biological fluids and in various tissues.[5] In the brain, the existence of enzymes which are capable of inactivating some physiologically active polypeptides has been reported,[6] but the mechanism of inactivation of bradykinin has not been studied in detail. Recently, Ungar *et al.*[7] demonstrated increased protease activity and protein denaturation in rat brain following electrical stimulation of afferent nerves.

We investigated the bradykininase activity in rat brain to obtain information on the possible role of protease in the central nervous system.

METHOD

Bradykinin activity

This was determined on isolated guinea-pig ileum in a 10 ml organ bath. The ileum was bathed in oxygenated Tyrode solution containing atropine sulfate at a concentration of 5 μg/ml at 37°. The time of contact was 90 sec and tests were made every 4 min.

Kininase activity

Kininase activity was determined by adding 5 μg of synthetic bradykinin (from the Institute for Protein Research, Osaka University) in 0·5 ml saline to 0·5 ml of test fluid. The mixture was incubated at 37° and samples of 0·1 ml were removed at intervals and tested on isolated guinea-pig ileum.

Compounds and drugs

The compounds used in experiments on kininase activity were of analytical quality and inorganic cations were used as their chlorides. The stock solutions of these compounds were prepared in 0·05 M Tris-buffer or 0·32 M sucrose solution, at four times the concentration, desired in the incubations. The pH of the solution was 7·4 and each solution was usually preincubated with the enzyme for 20 min.

Pentetrazol (Sankyo Co., Ltd., Tokyo), picrotoxin (Nakarai Chemicals, Ltd., Kyoto) and strychnine nitrate (Konishiseiyaku K. K., Amagasaki) were used as convulsants and were given by subcutaneous injection.

Sources of enzymes

Male rats of the Wistar-strain, weighing about 200 g, were used for all experiments. Rats were killed by decapitation and the brain was rapidly excised and pial blood vessels were removed from the appropriate area before dissection. Particular care was taken to remove as much blood as possible. The brain was separated into the cerebral cortex, the cerebellum and the brain stem. The brain stem included the medulla oblongata, the pons, the mid-brain and diencephalon.

The parts of the brain were homogenized in 9 vol. of 0·32 M sucrose solution at 0° in an all-glass Potter–Elvehjem homogenizer.

The homogenates were centrifuged at 700 *g* for 10 min to remove cell debris and any red blood cells still present. Subcellular fractionation was done according to the method of Marks and Lajtha[8] to obtain crude nuclear, mitochondrial and soluble (90,000 *g* supernatant) fractions. Rat plasma was collected by the method of Ferreia and Rocha E. Silva,[9] with some modifications, that is the blood samples were obtained by decapitation with a decapitator. Enzyme protein was determined by the method of Lowry *et al.*[10]

RESULTS

Influence of pH and temperature on kininase activity

Fig. 1 demonstrates the pH optimum of the kininase activity of rat brain. The enzyme had a pH optimum at about 7·6, and its activity diminished above and below this value, little or no activity being present under acidic conditions.

Fig. 2 shows the influence of temperature on the kininase activity. The enzyme was most active at about 45°, and lost its activity at 75°. The plasma kininase showed the same optimum pH and temperature as the enzyme in the brain.

Effect of various compounds on kininase activity

The effects of various compounds on the kininase activity of the brain and plasma are shown in Table 1. Pronounced inhibition is indicated as *. This implies a prolongation of the period required for breakdown of a certain amount of bradykinin from 13 min to 37 min or more. Less pronounced inhibition is indicated as †, and no inhibition as 0.

Metal ions (Zn^{2+}, Co^{2+}, Mg^{2+}, Ca^{2+}) inhibited the kininase activity in the brain, but they had no inhibitory effect on the enzyme in plasma. Chelating agents, such as EDTA blocked both enzyme activities. *N*-Ethylmaleimide, which inhibits enzymes containing sulfhydryl groups, had no detectable inhibitory effect on the kininase

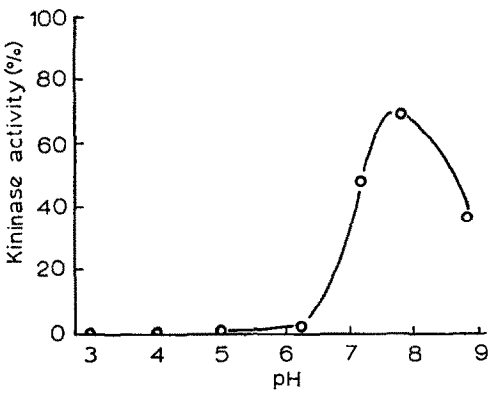

FIG. 1. Effect of pH on kininase activity of rat brain. Synthetic bradykinin was incubated for 9 min at 37° with kininase preparation (supernatant fraction of whole rat brain after centrifugation at 14,500 *g* for 30 min) diluted with buffer. Acetate buffer was used at below pH 7 in a final concentration of 0·1 M. 0·05 M Tris buffer was used above pH 7. Kininase activity was expressed as 100 per cent—residual bradykinin activity (per cent of initial).

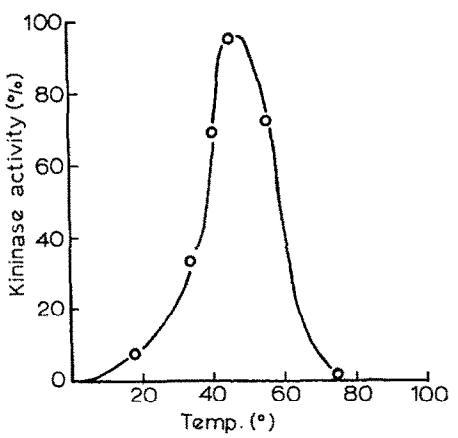

FIG. 2. Effect of temperature on kininase activity of rat brain.
Synthetic bradykinin was incubated with kininase preparation (supernatant fraction of whole rat brain after centrifugation at 14,500 *g* for 30 min) at pH 7·4 for 9 min at various temperatures. Kininase activity was expressed as 100 per cent—residual bradykinin activity (per cent of initial). Determination of kininase is described in the Methods' section.

activity in plasma, whereas it caused marked inhibition of the brain enzyme. The influence of metal ions (Zn^{2+}, Co^{2+}, Mg^{2+}, Ca^{2+}) on the inhibitory effect of EDTA (10^{-4} M) on the kininase activity was examined using the same concentration of ions

(4×10^{-4} M). We found that in plasma Zn^{2+} and Co^{2+} abolished the inhibitory effect of EDTA, while Ca^{2+} and Mg^{2+} had no discernible influence. On the other hand with brain enzyme, metal ions increased the inhibitory effect of EDTA (Fig. 3).

TABLE 1. EFFECTS OF VARIOUS COMPOUNDS ON KININASE ACTIVITY OF RAT BRAIN AND PLASMA

Compound	Concn. (M)	Relative degree of inhibition	
		Brain‡	Plasma
$ZnCl_2$	2×10^{-5}	*	0
$CoCl_2$	2×10^{-5}	*	0
$CaCl_2$	2×10^{-5}	†	0
$MgCl_2$	2×10^{-5}	†	0
EDTA	2×10^{-5}	*	*
Cysteine	2×10^{-4}	†	†
Glutathione	2×10^{-4}	*	*
NEM	2×10^{-4}	*	0
ATP	2×10^{-4}	*	†
ADP	2×10^{-4}	0	0
Glucose-6-phosphate	2×10^{-4}	0	0
DNP	2×10^{-4}	0	0
KCN	2×10^{-4}	0	0
Iodoacetic acid	2×10^{-4}	0	0

Kininase activity was estimated by mixing 10 μg/ml synthetic bradykinin with enzyme preparation (enzyme protein 100 μg/ml) at 37°, pH 7·4. For preparation of brain enzyme, the supernatant fraction of the cerebral cortex (90,000 *g* supernatant) was used.

* Very effective.

† Less effective, 0: without effect.

‡ Brain: Supernatant fraction of the cerebral cortex.

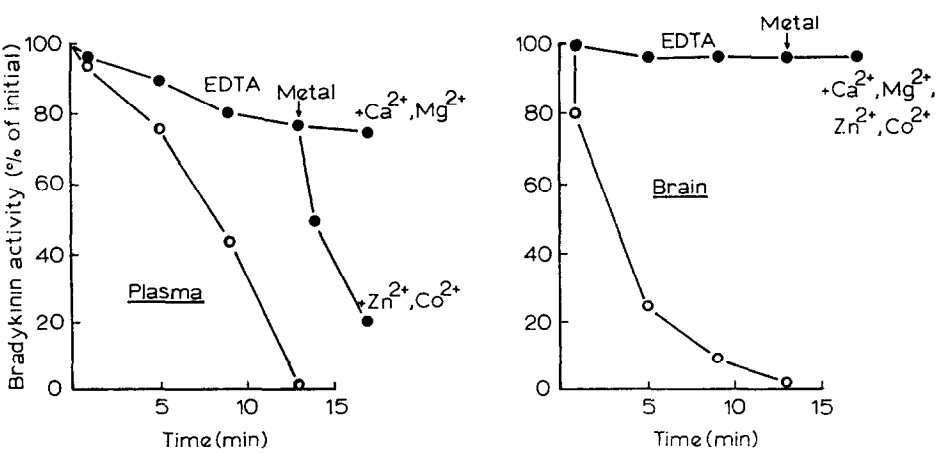

FIG. 3. Effect of metal ions on kininase activity of rat plasma and brain.

Synthetic bradykinin and kininase preparation were incubated, in the presence of 10^{-4} M EDTA (●—●) and without EDTA (○—○). Metal ions (4×10^{-4} M) were added at the point indicated by an arrow. The abscissa represents the time of incubation in min. The conditions of kininase determination were the same as in Table 1, and other details are given in the Methods' section.

The inhibitory effect of glutathione and cysteine on kininase was reduced by adding *N*-ethylmaleimide to plasma, but not to brain (Fig. 4).

Location of kininase

The activities of kininase were examined in three regions of rat brain: the cerebral cortex, the cerebellum and the brain stem, and were measured in the supernatants

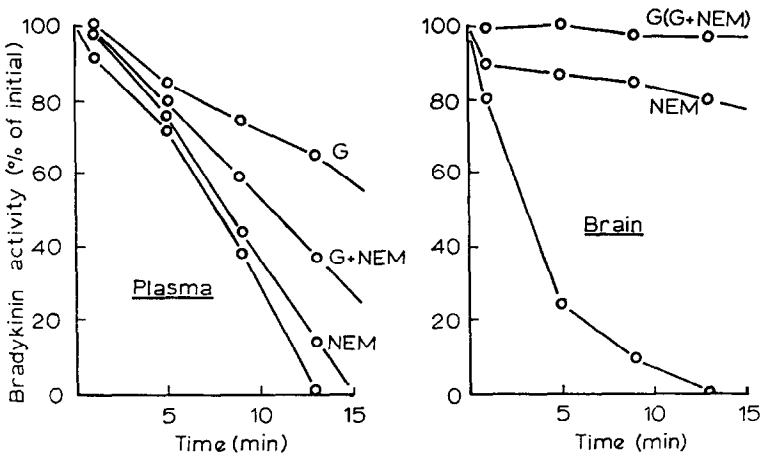

FIG. 4. Effect of glutathione and/or NEM on kininase activity of rat plasma and brain. G: Glutathione (2×10^{-4} M), NEM: *N*-Ethylmaleimide (2×10^{-4} M). The conditions of kininase determination were the same as in Table 1. For kininase determination, see Methods.

which were obtained by centrifugation at 700 *g* for 10 min and diluted 1:160 with 0·32 M sucrose solution. The kininase activity in the cerebellar region was almost twice those in the brain stem and in the cerebal cortex regions on a protein basis. In addition, the activities of kininase were almost the same in these latter two regions.

Distribution of kininase activity in subcellular fractions

Fig. 5 demonstrates the kininase activity in subcellular fractions of rat brain. Experiment in the brain stem, gave the same result and activities as in the cerebral cortex region. In the three regions of rat brain, the highest activity was found in the supernatant, with lower activities in the microsomal, mitochondrial and nucleal fractions. This order of activity was confirmed with preparations containing more enzyme protein.

Effect of convulsants on kininase activity

It has been reported that excitants and convulsants caused increased —SH and protease levels in "resting" brain.[11] In these experiments, inactivation of bradykinin by brain tissue was used as a measure of peptidase activity and the effects of pentetrazol, picrotoxin and strychnine on kininase activity were examined. Drugs were administered by subcutaneous injection in a dose of LD_{100} to obtain typical effects of convulsants on kininase activity. Immediately after the arrest of heart beat, the enzyme preparations were obtained as quickly as possible by the method described

above, Fig. 6 shows the effect of pentetrazol (200 mg/kg) on kininase activity of rat brain, and, the change of its activity in the brain stem region was almost the same as that in the cerebral cortex. With the picrotoxin treatment (20 mg/kg), the result was very similar to that found with pentetrazol administration.

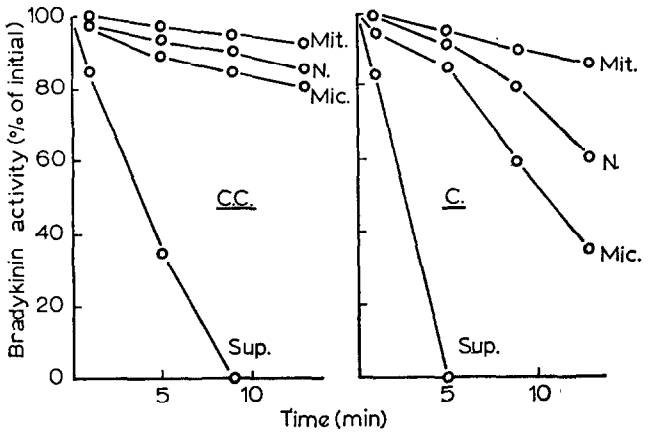

FIG. 5. Distribution of kininase activity in subcellular fractions of rat brain.
Synthetic bradykinin (10 μg/ml) was incubated with each fraction (enzyme protein 100 μg/ml) at 37°. N.: Nuclear fraction, Mit.: Mitochondrial fraction, Mic.: Microsomal fraction, Sup.: Supernatant fraction. Other details are given in Methods. [C. C.]: Cerebral cortex, [C.]: Cerebellum.

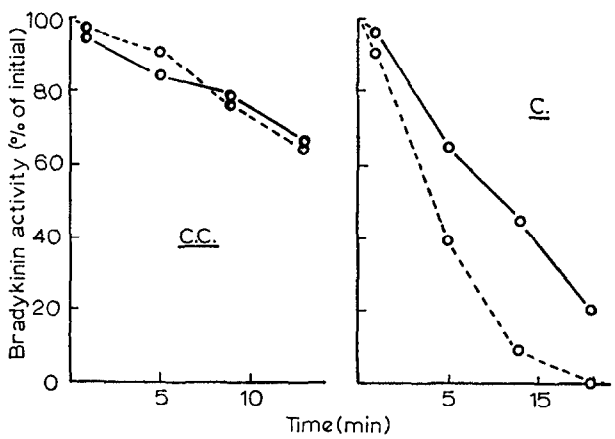

FIG. 6. Effect of pentetrazol on kininase activity of rat brain.
Pentetrazol was given subcutaneously in a dose of 200 mg/kg (○ - - - ○). After convulsive death, the enzyme preparations were obtained as described in the Methods' section. As a control, saline was administered (○—○). Synthetic bradykinin (10 μg/ml) was incubated with enzyme preparation (supernatant fraction after centrifugation of each region at 700 g for 10 min, enzyme protein 0·6 mg/ml) at 37°. For kininase determination, see Methods. [C. C.]: Cerebral cortex, [C.]: Cerebellum.

On the other hand, strychnine did not affect the kininase activities in any of the three regions of rat brain: the cerebral cortex, the cerebellum or the brain stem. The effect of strychnine in the cerebral cortex region is shown in Fig. 7.

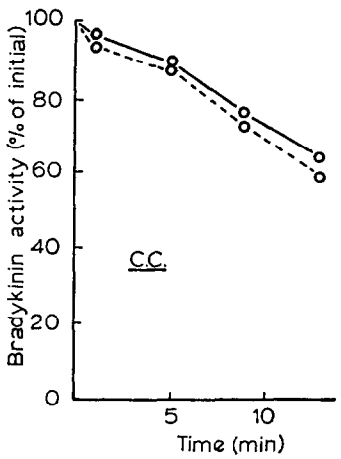

FIG. 7. Effect of strychnine on kininase activity of rat brain.
Strychnine was given by subcutaneous injection in a dose of 4 mg/kg. The conditions of kininase determination were the same as in Fig. 7. ○——○: Saline treated, ○ - - - ○: Strychnine nitrate 4 mg/kg. [C. C].: Cerebral cortex.

Effect of pentetrazol on the kininase activity in subcellular fractions

Since pentetrazol and picrotoxin enhanced the kininase activity in the cerebellar region, the changes of enzyme activity in subcellular fractions of this region were investigated.

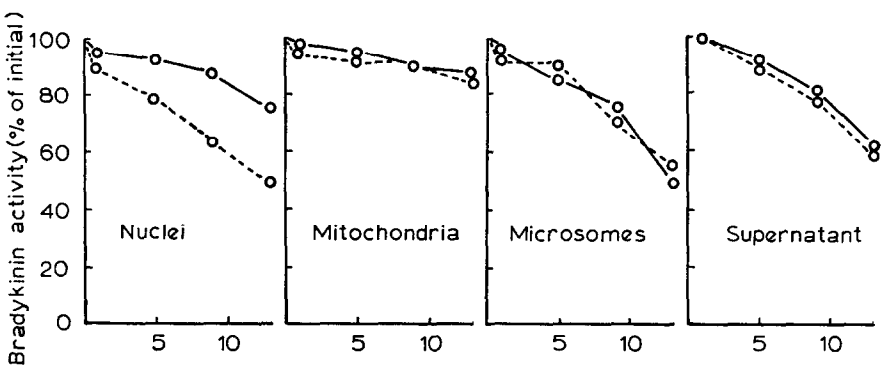

FIG. 8. Effect of pentetrazol on the distribution of kininase activity in the subcellular fractions of rat cerebellum.
Pentetrazol was administered by subcutaneous injection in a dose of 200 mg/kg (○ - - - ○). After convulsive death, the cerebellum was removed and subcellular fractionations were obtained by the method of Marks and Lajtha[8] as crude nuclear, mitochondrial, microsomal and soluble (90,000 *g* supernatant) fractions. As a control, saline was injected (○—○). Synthetic bradykinin (10 μg/ml) was incubated with enzyme preparations containing 0·6 mg/ml of particulate protein, or supernatant fraction containing 0·15 mg/ml of protein.

A great difference in enzyme activity between control and pentetrazol treated rats, was found in the nuclear fraction but no difference in the other fractions. Pentetrazol, at a concentration of 10^{-5} M, was added *in vitro* to each fraction of the cerebellar

region of saline treated rats. At this concentration it had no effect on kininase activity but at higher concentrations (10^{-4}—10^{-3} M) it decreased enzyme activity.

Changes in kininase activities during pentetrazol-induced seizure

After subcutaneous injection of 200 mg/kg of pentetrazol, the kininase activities in the three regions of rat brain were estimated in relation to behavior. The behavioral stages were classified as follows. After injection of pentetrazol, rats first exhibited loss of spontaneous motility (preconvulsive state). Then they became tremorous and clonic convulsions followed abruptly. After clonus, excessive loss of spontaneous motility was observed (intermediate state). Then tonic seizure increased leading to death (convulsive state and death). In the state of death, material was removed as quickly as possible after confirming that the heart had stopped.

There were no differences in kininase activity between saline and pentetrazol treated animals in either the cerebral cortex or the brain stem in these four states, but only remarkable changes were observed in the cerebellar region during the convulsive state and death (Fig. 9).

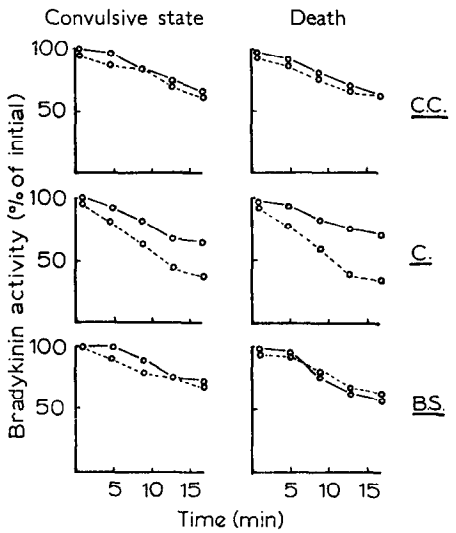

FIG. 9. Changes in kininase activities during pentetrazol-induced seizures.
After subcutaneous injection of pentetrazol, 200 mg/kg, kininase activities of the three regions of rat brain were measured in relation to behavior (○ - - - ○). The classification of behavioral stages is described in the text. As a control, saline was administered (○——○). Synthetic bradykinin (10 μg/ml) was incubated with enzyme preparation (supernatant fraction of each brain region at 700 *g* for 10 min obtained by centrifugation, enzyme protein 0·6 mg/ml, in [C] enzyme protein 0·3 mg/ml) at 37°. [C. C.]: Cerebral cortex, [C.]: Cerebellum, [B.S.]: Brain stem.

DISCUSSION

It has been mentioned that bradykinin plays some role in the signs and pathological responses seen in inflammation, and the enzyme which inactivates bradykinin may participate both locally and in the general circulation in controlling the plasma kinin concentration.[3] It was also suggested that the protease-polypeptide system was involved in local vasomotor control within the central nervous system and, when in excess, the components of the system might be connected with disease.[12] Recently, Ramu and

Bergmann[13] reported the role of the cerebellum in blood pressure regulation. In our present investigation, the enzyme which inactivates bradykinin in the rat brain was found to have the same temperature optimum as the enzyme in plasma. It was most active at about 45°. This shows that the enzyme inactivating bradykinin in the brain, may act in maintaining the normal status of brain function as it does in the circulation, when taken into consideration that in a pathological condition such as thermal injury, kinin level in the lymph shows slight elevation, while plasma kinin forming enzyme concentration was markedly increased.[3] The effect of metal ions and —SH reagent indicates that the enzyme which inactivates bradykinin in the brain is different from that in the plasma. Metal ions and metal chelating agents, such as EDTA, blocked the enzymatic activity in the brain but not the plasma. This suggests that different enzymes may be present in plasma and brain and the fundamental differences in their properties might be found on further purification. Hooper[14] described the intracellular distribution of hypothalamic enzymes which inactivate oxytocin in dogs and observed that the nuclear and microsomal fractions generally did not contain any detectable amounts of enzyme which destroys oxytocin whereas the mitochondrial and supernatant fractions did. However, in our present investigation, the enzyme which inactivates bradykinin had highest activity in the supernatant, and lower activity in the microsomal, mitochondrial and nuclear fractions. On the other hand, Lajtha[15] has shown that in the rat or sheep brain, the mitochondrial preparations in media buffered at pH 3·8 and 7·6 had the highest endogenous proteolytic activity, with lower activities in the nuclear, microsomal and supernatant fractions. The fact that the enzymes which inactivate these physiologically active polypeptides and the enzymes which hydrolyse haemoglobin or casein are present in different cell fractions, might imply that these enzymes are responsible for some physiological function or dynamic state of protein turnover in the central nervous system.

The cerebellar region showed higher kininase activity than either the cerebral cortex or the brain stem. However, Hooper[16] found that in dog brain the cerebellum and the cortex contained almost equal quantities of the enzyme and had the same specific activity. These different results might be due to the difference in the experimental animal used.

In 1959, Chapman *et al.*[12] demonstrated that human cerebrospinal fluid from patients with disorders of the central nervous system, such as vascular headache of migraine type or chronic schizophrenia, formed a vasodilator polypeptide on incubation with plasma globulin. On the other hand, Ungar *et al.*[7] described changes in the configuration of proteins in the cerebral cortex after stimulation of afferent nerves, and also in isolated nerves stimulated *in vitro*. They also reported that prolonged stimulation, in addition to causing structural changes, caused breakdown of protein and the appearance of proteolytic activity. In our present experiments, inactivation of bradykinin by brain tissue was used as a measure of peptidase activity, and this activity was measured in the brain of rats after subcutaneous injection of convulsants. Pentetrazol or picrotoxin markedly enhanced the activity of kininase in the cerebellar region but caused no change in the cerebral cortex or brain stem, whereas strychnine had no effect on brain kininase. After administration of these convulsants to rats, the changes in brain proteinase activities were also investigated, using denatured haemoglobin as substrate,[17] and results similar to those described here were obtained.

From these results, some changes in the metabolism of brain protein might be

expected during convulsion, but these drugs may have different sites of action. The increased kininase activities in the cerebellar region which were induced by pentetrazol and picrotoxin, might not be the direct effects of either drug, for in *in vitro* experiments pentetrazol, picrotoxin and strychnine, when added to enzyme preparations from rats treated with saline, had no effects on the kininase activity in the cerebral cortex and reduced the enzyme activities in the cerebellar and the brain stem regions.[17] Changes in kininase activities during pentetrazol-induced seizure were observed in the cerebellar region during the convulsive state and death. This result suggests that the changes in kininase activities are manifestations or results of increased functional activities. In subcellular fractions of the cerebellum of a rat after pentetrazol administration, the changes of enzyme activity were only found in the nuclear fraction. On the form of existence of this enzyme, it is remained further to be investigated. We are now investigating the relationships between the actions of convulsants and protease activity using other substrates, and results will be published elsewhere.

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