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THE PRESERVATION OF BRADYKININ BY PHENOTHIAZINES IN VITRO

BY

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Chlorpromazine and phenoxybenzamine have been shown to potentiate the actions of bradykinin *in vivo*. To test whether this phenomenon could be due to inhibition of the enzymatic destruction of bradykinin, bradykinin was incubated with either tissue extracts or with carboxypeptidase B. Bradykinin was rapidly destroyed by acetone-dried powders of brain and serum of various animals as well as by purified carboxypeptidase B. The rate of disappearance of bradykinin activity was decreased in the presence of phenothiazine derivatives, phenoxybenzamine and hydroxyzine, but not by compounds of a larger group including other psychotropic drugs, tranquillizers and ganglionic and adrenergic blocking agents. Spectrophotometric studies of the hydrolysis of hippuryl-L-arginine confirmed the presence of a carboxypeptidase B-like activity in brain. The substances that acted as inhibitors of bradykinin destruction were also enzyme inhibitors as measured by this technique. Previous incubation of carboxypeptidase B with phenothiazines and zinc ions greatly reduced the enzymatic inhibition by the phenothiazines, which indicated a possible chelating action by these inhibitors on the metalo-enzyme carboxypeptidase B.

Recent studies (Rocha e Silva, Corrado & Ramos, 1960 ; Lloyd, 1962 ; Kroeger & Krivoy, 1962 ; Krivoy, Lane & Kroeger, 1963) have shown that chlorpromazine and certain sympathetic blocking drugs can potentiate the actions of bradykinin both on the blood pressure and on the nervous system. One possible explanation for these observations is that chlorpromazine retards the enzymatic inactivation of bradykinin. An enzyme in brain capable of destroying bradykinin has been described by Hooper (1962). Erdos & Sloane (1962) have shown that the bradykinin-destroying enzyme(s) present in serum is similar to carboxypeptidase B isolated from hog pancreas. This paper reports an investigation into the presence of an enzyme in brain similar to carboxypeptidase B and capable of destroying bradykinin, and into the drug-induced inhibition of this enzyme.

METHODS

Sources of enzymes

The sources of enzymes in these experiments included acetone-dried powders of brain of dog, cat, rat, pig, rabbit and pigeon ; canine and bovine serum and purified enzymes from commercial sources were also used.

Dogs and cats were anaesthetized with pentobarbitone sodium and were killed either by air embolism or by bleeding followed by saline perfusion. Acetone-dried powders of major brain areas were prepared both from non-exsanguinated as well as from exsanguinated dog and cat brains. Fresh porcine brain was obtained from the abattoir, whereas rat brain was obtained from freshly decapitated laboratory animals.

Acetone-dried powders were prepared by mincing fresh tissue in a blender using four volumes of cold acetone (4° C). The residue was filtered and washed three times with four volumes of cold acetone. The powders were then dried in a vacuum desicator at 4° C for 48 hr and stored at −20° C until ready for use. The acetone-dried powders of rabbit and pigeon brain were obtained from the Nutritional Biochemical Corporation. Chymotrypsin (twice crystallized) and carboxypeptidase B (70 to 110 U/mg, treated with dyflos) were obtained from the Worthington Biochemical Corporation, and the bovine serum was obtained from Hyland Laboratories. Canine serum was prepared from freshly clotted blood of dogs. Carboxypeptidase B activity in acetone-dried powders of dog and pig brain was concentrated by the ammonium sulphate fractionation method described by Folk, Piez, Carroll & Gladner (1960).

Incubation procedures for bioassay

For studies of enzymatic activity of the acetone-dried powder, 25 mg of the material was slowly ground at 4° C with 1 ml. of de Jalon solution, and then centrifuged at 1,200 *g* for 1 hr at 4° C. The supernatant solution was removed and diluted 1:5, 1:10 or 1:20 either with de Jalon solution or with tris(hydroxymethyl)aminomethane buffer. Dilutions of serum, carboxypeptidase B and chymotrypsin were prepared with these same buffers.

Solutions were prepared for incubation by mixing 1 ml. of the enzyme solution (brain extract, serum, carboxypeptidase B or chymotrypsin), 1 ml. of either bradykinin (10^{-7} g/ml.) or oxytocin (0.1 U/ml.) and 1 ml. of either the test drug or de Jalon solution. Except where noted, concentrations of drug represent the concentration before the threefold dilution in the incubation mixtures. All drugs were prepared in de Jalon solution or tris buffer. The mixtures were incubated at 25 or 37° C and at intervals thereafter aliquots were removed, boiled and bioassayed on the rat uterus using bradykinin as a standard.

In certain experiments, to increase the effective concentration of phenothiazines during incubation, both chlorpromazine and bradykinin (final concentration of 3.3×10^{-6} g/ml.) were incubated with the enzyme. Aliquots of these mixtures were diluted 100-times with de Jalon solution before bioassay. In these studies it was necessary to increase the molarity of the tris buffer in the incubate from 0.025 to 0.05 M in order to prevent changes of *p*H.

The incubation mixtures and all the solutions therein contained either de Jalon solution with 0.1 to 0.5 g/l. of sodium bicarbonate, tris buffer (0.025 M) at the desired basic *p*H, or phosphate buffer (0.1 M) for the acidic range. The *p*H was measured before and after incubation. Atropine sulphate and tripelennamine hydrochloride were present in final concentrations of 10^{-7} g/ml.

The bioassay was conducted in a 1.5 ml. organ-bath maintained at 30° C. Studies were conducted according to the two-dose bioassay procedure of Burn, Finney & Goodwin (1950). The solutions for assay were added in volumes ranging from 0.02 to 0.08 ml. The amount of fluid in the bath was adjusted so that, after administration of the test solution, the final volume was 1.5 ml. All solutions were adjusted to appropriate bath *p*H before bioassay. The solutions being bioassayed were allowed to remain in contact with the uterus for 1 min at either 3 or 5 min intervals. Only uterine strips sensitive to bradykinin, 10^{-9} g/ml. or less, were used. Analysis of the results was accomplished by measuring the heights of contractions after having previously determined that the curves for latent periods and for areas of contractile response were parallel both for incubated samples and for equivalent standards.

The hydrolysis rates of dipeptides by the forementioned enzyme preparations were studied according to the spectrophotometric method of Folk *et al.* (1960). For studies with drugs, the volume of the substrate solution was 2.8 ml., to which 0.1 ml. each of the drug solution being tested and of the enzyme solution were added. To study the influence of metal ions, mixtures

of the enzyme solution with the drug to be tested and/or the metal ion solution were incubated before the addition of 0.1 ml. of these mixtures to 2.9 ml. of the substrate solution.

Synthetic bradykinin, kindly provided by Dr. R. P. Bircher of the Sandoz Pharmaceutical Company, Hanover, N.J., U.S.A., was used in each of these studies.

RESULTS

Fig. 1 shows the results obtained using standards of bradykinin in which the response (height of contraction) is compared with the log dose of the administered standard. The top curve gives the mean responses, with the standard errors, of thirty-three applications of each of the different standards, to uterine strips from ten different animals ; the lower curve was obtained from four applications of each of the three standards to a single uterine strip.

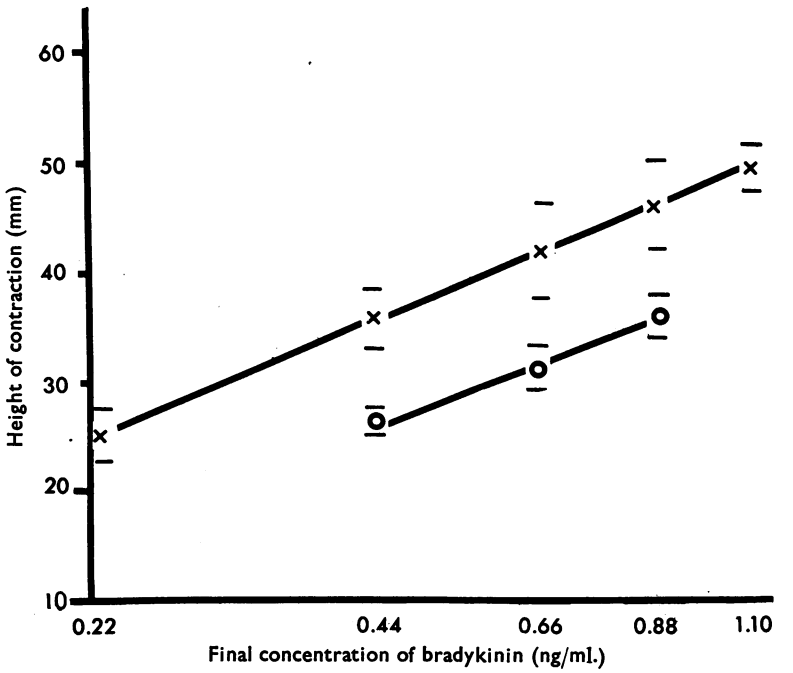

Fig. 1. Calibration curves obtained from bioassays of various concentrations of bradykinin on the rat isolated uterus preparation. Points represent mean heights of contraction, and horizontal lines standard errors; crosses, for a series of five doses of bradykinin; circles, for a series of three doses of bradykinin applied four times to a single uterus preparation.

When bradykinin was incubated with extracts of whole dog brain at *p*H 7.4 and 37° C, there was a progressive disappearance of its biological activity: after 30 min the activity had been reduced to 55% and after 60 min to 25% of the initial value. Boiling the extract before incubation abolished its ability to destroy bradykinin. Full bradykinin activity was observed in solutions tested immediately after mixing, but before incubation. Bradykinin, added after incubation of the brain extract alone, was also fully active. Thus, it seems that the brain extract did not produce

a substance which modified or interfered with the biossay. The rate of disappearance of bradykinin activity was also related to the concentrations of the brain extracts employed (Tables 1 and 2). Qualitatively identical results were obtained with the other extracts, namely acetone powders of exsanguinated dog and cat brain, rat brain and rabbit brain at 1 : 10 dilution.

The bradykinin-destroying activity of the brain extracts was abolished by boiling and depended on *p*H: at *p*H 5.5, about 85% of the activity was retained after an incubation of 60 min. Since nonenzymatic destruction of bradykinin in alkaline media was seen, no studies were performed at a *p*H above 7.8.

TABLE 1

THE INFLUENCE OF NEUROLOGICALLY ACTIVE COMPOUNDS ON THE DESTRUCTION OF SYNTHETIC BRADYKININ WHEN INCUBATED WITH CAT WHOLE BRAIN EXTRACT

Results are from four experiments with two different dilutions of brain extract incubated for 30 min at 37° C and *p*H 7·4, with initial bradykinin concentration $3{\cdot}3 \times 10^{-8}$ g/ml. Controls were run with each bioassay. Values for residual bradykinin activity are percentages of initial concentrations before incubation. Psilocybin is 3-(2-dimethylaminoethyl)indol-4-yl dihydrogen phosphate

Dilution of brain extract	Drug	Drug concentration (g/ml.)	Residual bradykinin activity (%)
1 : 5	None (control)		10–12
	Thioridazine	$3{\cdot}3 \times 10^{-8}$	18–20
	Perphenazine	$3{\cdot}3 \times 10^{-8}$	25–30
	Chlorpromazine	$3{\cdot}3 \times 10^{-8}$	32–37
	Fluopromazine	$3{\cdot}3 \times 10^{-8}$	35–35
1 : 10	None (control)		29–40
	Thioridazine	$3{\cdot}3 \times 10^{-8}$	58–63
	Phenoxybenzamine	$3{\cdot}3 \times 10^{-7}$	75–83
	Hydroxyzine	$3{\cdot}3 \times 10^{-7}$	66–70
	Neostigmine	$3{\cdot}3 \times 10^{-7}$	30–35
	Morphine	$3{\cdot}3 \times 10^{-7}$	28–34
	Reserpine	$3{\cdot}3 \times 10^{-7}$	24–35
	Pentobarbitone	$3{\cdot}3 \times 10^{-7}$	32–40
	Lysergic acid diethylamide	$3{\cdot}3 \times 10^{-7}$	24–31
	Psilocybin	$3{\cdot}3 \times 10^{-7}$	10–26
	Imipramine	$3{\cdot}3 \times 10^{-7}$	31–39
	Chlordiazepoxide	$3{\cdot}3 \times 10^{-7}$	38–45

In view of these findings, it is concluded that extracts of acetone-dried powders of brain contain an enzyme (kininase) capable of destroying bradykinin.

When chlorpromazine was added to the incubation mixture, the rate of destruction of bradykinin was reduced. After 30 min, 65% of the activity was retained in the presence of chlorpromazine (10^{-7}), compared with 55% in the absence of chlorpromazine, and, after 60 min, 50% compared with 24%. Bioassay of incubates containing bradykinin and chlorpromazine (10^{-7}) but no enzyme had full activity, which indicated that this concentration of chlorpromazine did not interfere with the bioassay. Higher concentrations of chlorpromazine interfered with the response of the rat uterus to bradykinin. Bradykinin was fully active when added to previously incubated mixtures of brain extract and chlorpromazine. When the drug and the enzyme solutions were incubated together for 30 min before the addition

of bradykinin, no significant alteration in destruction of bradykinin was seen. The degree of inhibition of the enzyme was proportional to the concentration of chlorpromazine (Fig. 2).

Table 1 lists the other phenothiazine derivatives tested in two experiments. In the presence of chlorpromazine, thioridazine, perphenazine and fluopromazine, there was a reduction of the amounts of bradykinin destroyed after an incubation of 30 min. These results are not sufficient for comparison of the efficacies of the phenothiazines, but they show that each of the derivatives tested inhibited the enzymatic inactivation of bradykinin.

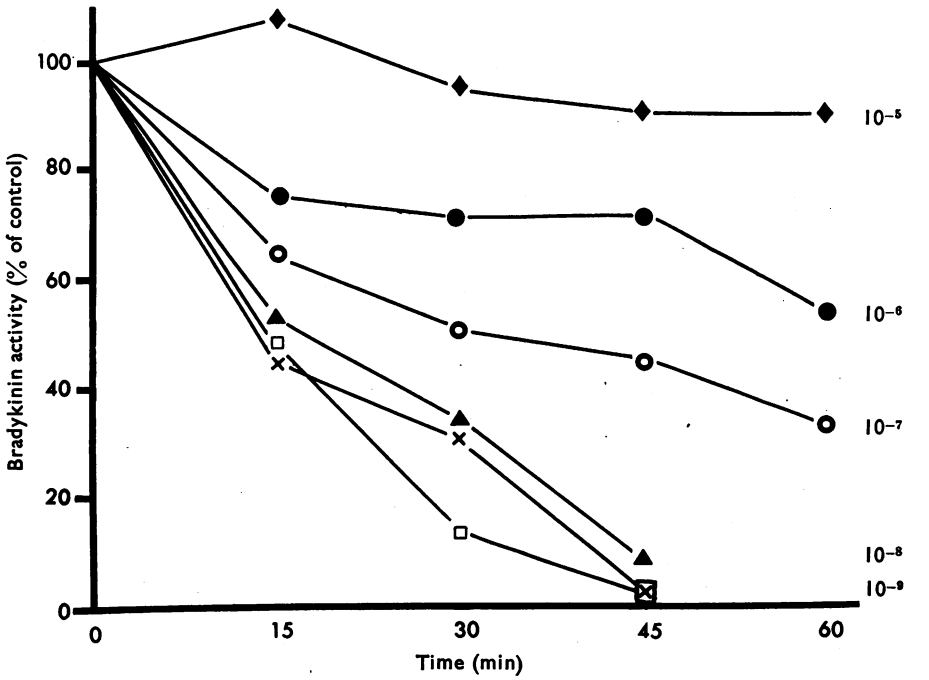

Fig. 2. Inactivation of bradykinin (10^{-5}) incubated with cat brain extract (1 : 10) in the presence of various concentrations of chlorpromazine. Incubation was at 37° C at *p*H 7.6 in tris(hydroxymethyl)aminomethane buffer (0.1 M). Crosses are controls (without chlorpromazine), and the other symbols refer to incubations with chlorpromazine in the concentrations shown.

Table 1 lists other drugs that were studied. In addition to the phenothiazines, only phenoxybenzamine and hydroxyzine had an inhibitory action.

In further studies the specificity of chlorpromazine for enzymes capable of destroying bradykinin was tested. Table 2 presents the results. With chymotrypsin (5×10^{-6} g/ml.), no difference was detected between samples with and without chlorpromazine. Both canine and bovine serum, diluted 1 : 100, showed a considerable difference between the samples with chlorpromazine and their controls. However, no substantial difference was observed with bovine serum at a dilution of 1 : 10. Chlorpromazine also inhibited carboxypeptidase B (3.5×10^{-5} U/ml.).

TABLE 2

THE INFLUENCE OF CHLORPROMAZINE ON VARIOUS ENZYMATIC PREPARATIONS WHICH DESTROY BRADYKININ

The control mixtures (enzyme and bradykinin alone) were assayed concurrently with the same mixtures containing chlorpromazine. All incubations were at 37° C for 30 min at *p*H 7·4. The initial concentration of bradykinin and chlorpromazine was $3{\cdot}3 \times 10^{-8}$ g/ml.

Enzyme (or enzyme source) and concentration	Residual bradykinin activity (%)
Chymotrypsin (5×10^{-6} g/ml.)	22
Chymotrypsin (5×10^{-6} g/ml.)+chlorpromazine	20
Carboxypeptidase B ($3{\cdot}5 \times 10^{-5}$ U/ml.)	45
Carboxypeptidase B ($3{\cdot}5 \times 10^{-5}$ U/ml.)+chlorpromazine	77
Dog serum (fresh, 1 : 100)	63
Dog serum (fresh, 1 : 100)+chlorpromazine	87
Bovine serum (reconstituted, 1 : 10)	12
Bovine serum (reconstituted, 1 : 10)+chlorpromazine	14
Bovine serum (reconstituted, 1 : 100)	70
Bovine serum (reconstituted, 1 : 100)+chlorpromazine	95
Dog brain (exsanguinated, 1 : 10)	40
Dog brain (exsanguinated, 1 : 10)+chlorpromazine	64
Dog hypothalamus (exsanguinated, 1 : 10)	40
Dog hypothalamus (exsanguinated, 1 : 10)+chlorpromazine	50
Dog cerebellum (exsanguinated, 1 : 10)	20
Dog cerebellum (exsanguinated, 1 : 10)+chlorpromazine	28
Rabbit brain (commerial, 1 : 10)	47
Rabbit brain (commercial, 1 : 10)+chlorpromazine	76

Also listed in Table 2 are the values for similar studies using four different preparations of brain. A consistent pattern was seen, chlorpromazine decreasing the destruction of bradykinin.

When chlorpromazine was added to the incubation mixture containing carboxypeptidase B, the rate of destruction of bradykinin was reduced in direct proportion to the concentration of chlorpromazine (Fig. 3). After 15 min of incubation, the samples containing chlorpromazine (10^{-5} and 10^{-6}) had more than 100% of the activity at zero time (controls). Our results are at present not adequate to explain this phenomenon.

To test the substrate specificity of the enzyme in the acetone-dried powders of dog brain (1 : 10 dilution), oxytocin (0.1 U/ml.) was incubated in place of bradykinin. There was no disappearance of oxytocic activity under these conditions.

The actions of the inhibitors of carboxypeptidase B were further studied using the simplified and rapid spectrophotometric technique of Folk *et al.* (1960). In these experiments 0.1 U of carboxypeptidase B was incubated with 0.001 M-hippuryl-L-arginine. Lowering the *p*H from 7.6 to 6.2 caused a considerable decrease in the hydrolysis rate. At *p*H values of 7.2 and 7.6 0.025 M-tris buffer was used, and at *p*H 6.2 and 6.8 0.1 M-phosphate buffer. The *p*H effect on carboxypeptidase B is very similar to that on serum kininase (Lewis, 1963).

Fig. 4 illustrates the influence of various concentrations of chlorpromazine on this same reaction maintained at a *p*H of 7.6. Increasing the concentration of chlor-

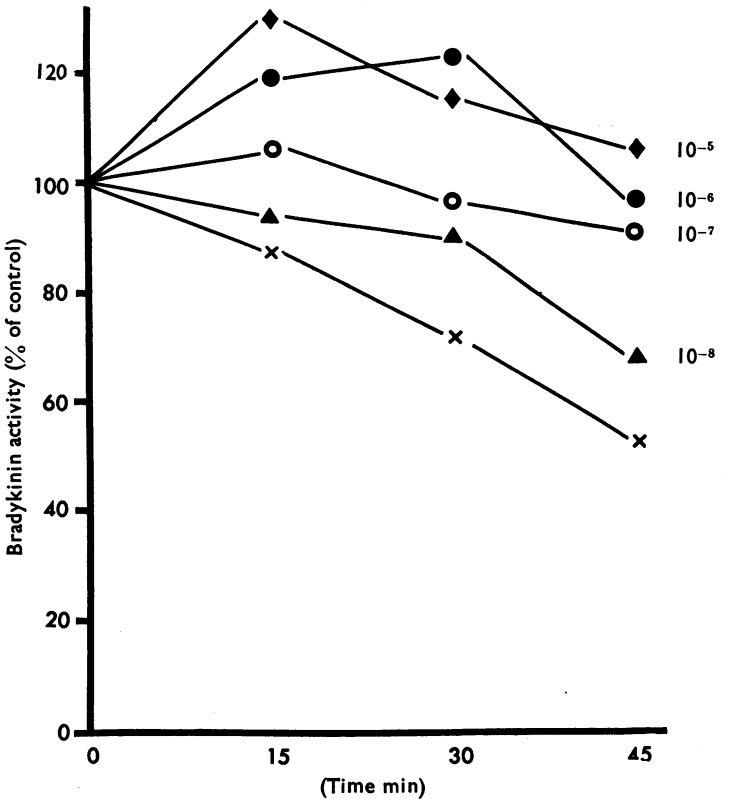

Fig. 3. Inactivation of bradykinin (10^{-5}) incubated with carboxypeptidase B (5×10^{-4} U/ml.) in the presence of various concentrations of chlorpromazine. Incubation was at 37° C at *p*H 7.6 in tris(hydroxymethyl)aminomethane buffer (0.1 M). Crosses represent controls (without chlorpromazine), and the other symbols refer to incubations with chlorpromazine in the concentrations shown.

promazine from 8.3×10^{-7} to 8.3×10^{-5} g/ml. (final concentration) decreased the reaction rate. For the 2 min period, the change in absorbency per minute (ΔA/min) decreased from 0.08 for the control to 0.038 at 4.5×10^{-5} g/ml., and to zero at a concentration of 8.3×10^{-5} g/ml.

As seen in Figs. 5 and 6, many of the same drugs which did not inhibit the destruction of bradykinin when tested by the bioassay procedure again had no effects on the hydrolysis by carboxypeptidase B. Morphine, pentobarbital, tolazoline, hexamethonium, dihydroergotamine, reserpine, lysergic acid diethylamide and neostigmine had no inhibitory activity. Thioridazine, phenoxybenzamine, perphenazine and trifluoperazine all showed activity equal to that of the same concentrations of chlorpromazine.

The two top curves in Fig. 5 show the rates of hydrolysis of the enzyme controls during the experiment; the rates in the presence of morphine, sodium pentobarbitone, neostigmine, lysergic acid diethylamide and reserpine were the same as for the controls.

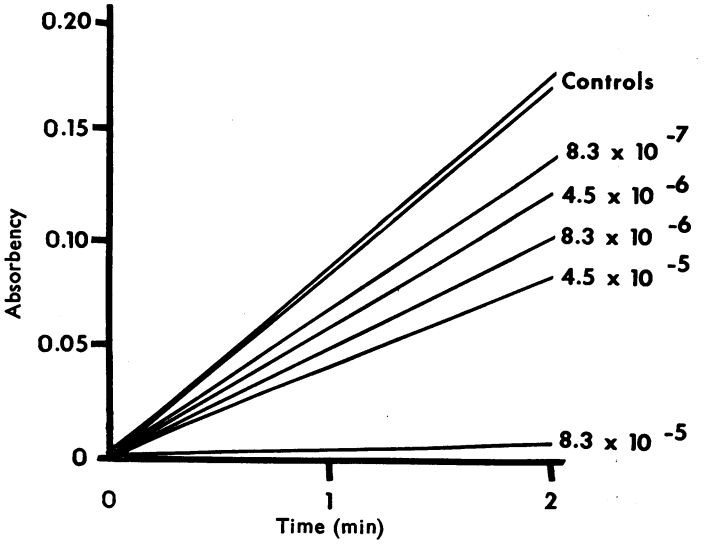

Fig. 4. Effect of various concentrations of chlorpromazine (on right) on the hydrolysis of hippuryl-L arginine by carboxypeptidase B (final concentration, 0.3 U/ml.) at *p*H 7.6 and 25° C. The ordinate gives spectrophotometric absorbency. The drug was added to the reaction mixture just before the addition of the enzyme.

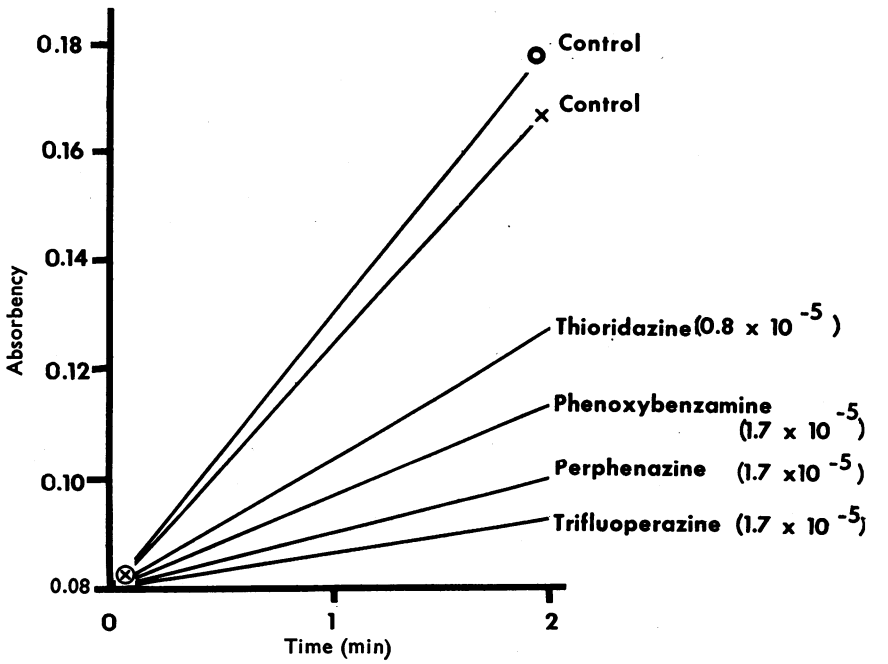

Fig. 5. Influence of certain psychotropic drugs on the hydrolysis of hippuryl-L-arginine by carboxypeptidase B (0.3 U/ml.) at *p*H 7.6. The ordinate gives spectrophotometric absorbency. The two reaction rates at the top (circles and crosses) are for the control runs. Drugs (on right) were added just before the addition of the enzyme to the reaction mixture. Final concentrations of the drugs (g/ml.) in the reaction mixture are given.

Fig. 6 shows minor inhibitory effects of a group of ganglionic and adrenergic blocking agents. Only hydroxyzine, tolazoline and hexamethonium caused a significant inhibition. However, the concentrations required were more than ten-times that for chlorpromazine. Mecamylamine and dihydroergotamine were without noticeable effect.

Having determined that purified pancreatic carboxypeptidase B was sensitive both to *p*H and to drugs in a manner similar to the extracts of acetone-dried powders, the activities of brain extracts were also spectrophotometrically assayed. The increase in carboxypeptidase B-like activity from one exsanguinated dog brain during the initial stages of purification was determined. The initial extract of an acetone-dried powder had an activity of 2.32 U/ml. This extract represents the activity from

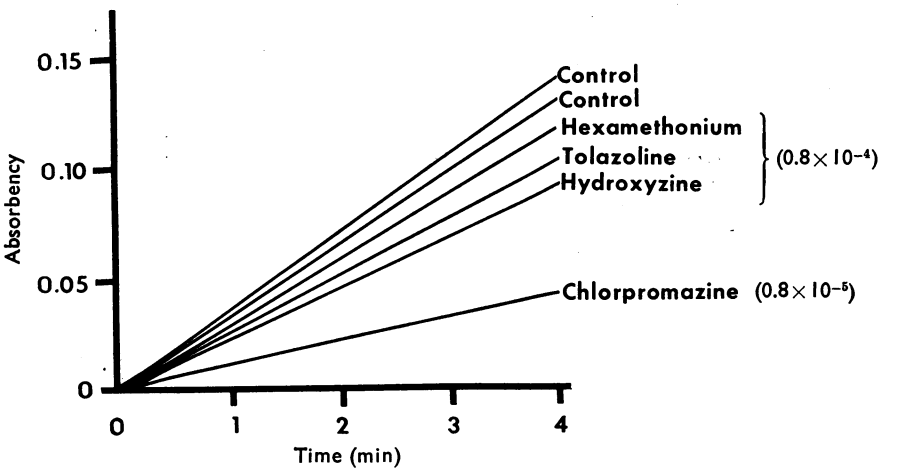

Fig. 6. Influence of certain ganglionic and adrenergic blocking agents on the hydrolysis of hippuryl-L-arginine by carboxypeptidase B (0.08 U/ml.) at *p*H 7.6. Chlorpromazine (0.8×10^{-5} g/ml.) was run as a control. Drugs (on right) were added just before the addition of the enzyme to the reaction mixture. Final concentrations of the drugs in the reaction mixture are given.

20 mg of dried powder in 1 ml. of extraction fluid. Samples obtained from the first stage of purification, namely ammonium sulphate precipitation followed by dialysis, had an activity of 4.6 U/ml. Further concentration by batch adsorption and elution from diethylaminoethylcellulose increased the activity to 6.96 U/ml.

To test the possibility that chlorpromazine was interfering with the enzyme by chelating the zinc ion of the metallo-enzyme, the influence of previous incubation of carboxypeptidase B with chlorpromazine and equimolar concentrations of zinc ion was studied. The change in absorbency for the enzyme-substrate control was 0.22 absorbency units/4 min (ΔA/4 min). The addition of chlorpromazine (2.5×10^{-5} and 2.5×10^{-6} M) to the enzyme-substrate mixture completely inhibited the hydrolysis of hippuryl-L-arginine. Previous incubation of zinc sulphate (2.5×10^{-6}M) with chlorpromazine (2.5×10^{-6}M) completely prevented the inhibition of the enzyme reaction, whereas zinc sulphate (2.5×10^{-5} M) was only partially

effective in overcoming the inhibition of the reaction by 2.5×10^{-5} M-chlorpromazine (0.14 absorbency units). Thus, chlorpromazine was greatly, if not totally, prevented from inhibiting carboxypeptidase B in the presence of zinc ions. However, if the enzyme, substrate, zinc sulphate and chlorpromazine were incubated simultaneously without prior incubation, the action of chlorpromazine was not modified. These results indicate that the presence of the substrate, hippuryl-L-arginine, prevents zinc ions from antagonizing, reversing or preventing the inhibition by chlorpromazine, a phenomenon which had been observed for carboxypeptidase A using its corresponding substrate (Vallee, 1961).

DISCUSSION

Of the numerous questions posed by the results here presented, those which require discussion pertain to the presence of a kininase or kininases in brain, capable of inactivating the plasma kinin, bradykinin, the possible identity of such an enzyme, the ability of certain chemical structures to inhibit the enzyme(s) and the possible role of this inhibition *in vivo*.

The presence of peptidases in the brain is currently much investigated. The work of Uzman, Van den Noort & Rumley (1962), Hooper (1962), Pope (1959) and others indicates that extracts of brain can split or inactivate various peptide structures. Our results show the presence of an enzyme in acetone powders of exsanguinated brain of a number of species, capable of inactivating bradykinin. According to our findings the disappearance of bradykinin during incubation is a consequence of interaction with a constituent of the tissue extract, in contrast to the production of interfering substances. This interaction is likely to be enzymatic since it depends on *p*H in a manner similar to an enzymatic reaction. More important, boiling of the tissue extract or of the purified carboxypeptidase B prevents subsequent reaction with bradykinin.

The inhibition of enzymatic activity of the brain extracts studied is probably due to a direct action of the inhibitor tested on the enzyme, and not the consequence of drug interference with the bioassay, or of drug-induced synthesis of interfering substances. This conclusion is supported by the fact that incubation of tissue extract with an inhibitor produced no alteration in the response to bradykinin added after incubation.

Failure to observe substantial inhibition of enzymatic activity of high concentrations of brain extract or of serum may be accounted for by the inactivation and/or binding of the phenothiazine by protein material. However, since similar results were seen with high concentrations of purified carboxypeptidase B, a simpler explanation may be the fact that there is a necessary relationship between the concentration of enzyme and the effective concentrations of the inhibitor.

Since the enzyme appears in exsanguinated brain, it appears to be a constituent of brain. However, because this enzymatic activity is present in serum, it is presumed that tissue fluids (lymph) also possess it. It has not yet been ascertained if the enzyme systems in the various tissues studied are identical, but in view of their inhibition by the phenothiazine tranquillizers, hydroxyzine and phenoxybenzamine, some similarity is suggested.

Crude brain extracts were subjected to purification procedures similar to those used by Folk *et al.* (1960) for pancreatic carboxypeptidase B ; preparations were produced with increased abilities of splitting the dipeptide, hippuryl-L-arginine. The results obtained, using these semi-purified extracts which were studied spectrophotometrically, substantiate the fact that brain possesses enzymatic activity capable of inactivating bradykinin.

Although the concentration of the peptidase is substantially less in brain than in pancreas, it could account for destruction of considerable quantities of bradykinin in the brain. A unit of carboxypeptidase B is defined as that amount of enzyme capable of splitting 1 μmole of hippuryl-L-arginine in 1 min at 25° C, or approximately 328 μg of the substrate. Using the bioassay technique, 3.5×10^{-5} U/ml. of carboxypeptidase B was initially capable of splitting approximately 2.5 ng/min of bradykinin. Relating these two reactions and assuming equal sensitivity of the substrates to the enzyme, 1 U of enzyme would hydrolyse about 75 μg/min of bradykinin, or close to 0.1 μmole/min. The bioassay technique can distinguish the results of an enzyme concentration of 10^{-5} U/ml., whereas the spectrophotometric assay is limited at about 0.01 U/ml. of activity. Thus, the bioassay requires roughly 1,000-times less enzyme than spectrophotometry. The difficulty in assaying brain tissues spectrophotometrically could be explained by the fact that a fifteen- to twentyfold concentration of the enzyme by the purification procedure of Folk *et al.* (1960) was still not sufficient for an assay by the spectrophotometric method. This same explanation could account for our inability, as well as that of Erdos & Sloane (1962), to assay the serum kininase by the spectrophotometric method.

The similarity of the kininase in brain tissues to the carboxypeptidase B-like activity of brain and pancreas is further confirmed by the considerable inhibitory activity of the phenothiazines on the enzymatic hydrolysis of both synthetic bradykinin and dipeptides. That the same drugs inhibit at the same concentration lends support to the idea that the enzymes are similar.

Results from the spectrophotometric assays indicate that, on a molar basis, the phenothiazines and phenoxybenzamine are among the most potent inhibitors of the kininase, carboxypeptidase B. A final concentration of 2.5×10^{-5}M-chlorpromazine caused a 50% inhibition without previous incubation, whereas 2.5×10^{-6}M-chlorpromazine caused complete inhibition after 15 min of previous incubation. These values represent inhibitory activities some 100 to 1,000-fold greater than for the most potent compounds previously studied, such as 1,2-phenanthroline (Erdos & Sloane, 1962). The increase in inhibition after previous incubation of the enzyme and the phenothiazines suggests that the mechanism of inhibition involves a rate process or some reaction in which a prior action of phenothiazine is important.

A classification of the carboxypeptidases A and B as metallo-enzymes is well established (Folk *et al.*, 1960 ; Vallee, 1961). Chlorpromazine possesses a strong affinity for certain metal ions (Borg, 1961). Although zinc ions will protect the enzyme if added concurrently with the chlorpromazine, the failure of zinc ions to restore enzymatic action after chlorpromazine suggests that the total inhibitory action may not be solely that of chelation with the zinc from the enzyme, carboxypeptidase

B. Subsequent denaturation of the apoenzyme, enzyme minus its metal, is a possibility. Another possibility is that the substrate or one of its products may cause steric hindrance to the metal replacement. The work of Erdos & Sloane (1962), Ferreira & Rocha e Silva (1962) and Aarsen & Kemp (1962) all points to the role of metal ions in the action of serum kininase; however, their conclusions conflict as to whether this enzyme is a metallo-enzyme or a metal-activated enzyme.

These results suggest that a potentiation of the vascular and neurogenic actions of bradykinin induced by chlorpromazine or phenoxybenzamine (Rocha e Silva *et al.*, 1960; Rocha e Silva, 1960; Kroeger & Krivoy, 1962; Weatherred, Kroeger & Smith, 1963) could arise from kininase inactivation. This view conflicts with the currently held concept that potentiation of bradykinin *in vivo* only involves the adrenergic blocking action of these drugs. The fact that intravenous injections of carboxypeptidase B greatly diminished the depressor response to bradykinin (Erdos, Renfrew, Sloane & Wohler, 1963) indicates the rapid and potent actions of the peptidases, either exogenous or endogenous. It is recognized, however, that other mechanisms may be responsible for the potentiation, since hexamethonium and dihydroergotamine, although potentiating bradykinin in rats (Lloyd, 1962), cause very slight inhibition of carboxypeptidase B *in vitro*.

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REFERENCES

AARSEN, P. N. & KEMP, A. (1962). Influence of metals on the activity of bradykinin-destroying enzyme kininase. *Brit. J. Pharmacol.*, **19**, 442–450.

BORG, D. C. (1961). Chemical and physiological specificity in metal-binding: a model metal-phenothiazine system involving a free-radical component. *Fed. Proc.*, **20**, Suppl. 10, 104–109.

BURN, J. H., FINNEY, D. J. & GOODWIN, L. G. (1950). *Biological Standardization*, pp. 185–186. London: Oxford University Press.

ERDOS, E. G., RENFREW, A. G., SLOANE, E. M. & WOHLER, J. R. (1963). Enzymatic studies on bradykinin and similar peptides. *Ann. N.Y. Acad. Sci.*, **104**, 222–234.

ERDOS, E. G. & SLOANE, E. M. (1962). An enzyme in human blood plasma that inactivates bradykinin and kallidins. *Biochem. Pharmacol.*, **11**, 585–592.

FERREIRA, S. H. & ROCHA E SILVA, M. (1962). Potentiation of bradykinin by dimercaptopropanol (BAL) and other inhibitors of its destroying enzyme in plasma. *Biochem. Pharmacol.*, **11**, 1123–1128.

FOLK, J. E., PIEZ, K. A., CARROLL, W. R. & GLADNER, J. A. (1960). Carboxy-peptidase B. IV. purification and characterization of the porcine enzyme. *J. biol. Chem.*, **235**, 2272–2276.

HOOPER, K. C. (1962). The catabolism of some physiologically active polypeptides by homogenate of dog hypothalamus. *Biochem. J.*, **83**, 511–517.

KRIVOY, W. A., LANE, M. & KROEGER, D. C. (1963). The action of certain polypeptides on synaptic transmission. *Ann. N.Y. Acad. Sci.*, **104**, 312–329.

KROEGER, D. C. & KRIVOY, W. A. (1962). The interaction between chlorpromazine and bradykinin. *Pharmacologist*, **4**, Abstract 174.

LEWIS, G. P. (1963). Pharmacological actions of bradykinin and its role in physiological and pathological reactions. *Ann. N.Y. Acad. Sci.*, **104**, 236–247.

LLOYD, S. (1962). Vascular responses of the rat to bradykinin. *Brit. J. Pharmacol.*, **19**, 503–507.

POPE, A. (1959). The intralaminar distribution of dipeptidase activity in human frontal isocortex. *J. Neurochem.*, **4**, 31–41.

ROCHA E SILVA, M. (1960). Biochemistry and pharmacology of bradykinin. In *Polypeptides which Affect Smooth Muscles and Blood Vessels*, ed. SCHACHTER, M., p. 210. London: Pergamon Press.

ROCHA E SILVA, M., CORRADO, A. P. & RAMOS, A. O. (1960). Potentiation of duration of the vasodilator effect of bradykinin by sympatholytic drugs and by reserpine. *J. Pharmacol. exp. Ther.*, **128**, 217–226.

UZMAN, L. L., VAN DEN NOORT, S. & RUMLEY, M. K. (1962). Properties and classification of some brain peptidases. *J. Neurochem.*, **9**, 241–252.

VALLEE, B. L. (1961). The "active catalytic site," an approach through metalloenzymes. *Fed. Proc.*, **20**, Suppl. 10, 71–80.

WEATHERRED, J. G., KROEGER, D. C. & SMITH, E. L. (1963). Pressure response in the dental pulp chamber to inferior alveolar nerve stimulation. *Fed. Proc.*, **22**, 287.