Biochemical Pharmacology, 1963, Vol. 12, pp. 637–646. Pergamon Press Ltd., Printed in Great Britain.

THE ENZYMATIC DEGRADATION OF VARIOUS ANGIOTENSIN II DERIVATIVES BY SERUM, PLASMA OR KIDNEY HOMOGENATE

D. REGOLI, B. RINIKER and H. BRUNNER

Research Laboratories of CIBA Limited, Basle Pharmaceutical Division

(*Received* 26 *January* 1963; *accepted* 27 *February* 1963)

Abstract—Six isomers of angiotensin II, differing from one another only with respect to changes made in the aspartic acid situated at the amino end, were incubated with rat serum, human plasma or rat kidney homogenate. The rate at which the isomers were inactivated was studied by measuring pressor effects in nephrectomized rats. In parallel tests, fragments were demonstrated by means of paper chromatography.

Compounds with an α-L-Asp[1] configuration were inactivated much more rapidly in serum or plasma than α-D-, β-L-, β-D- or desamino-angiotensin II. When the substances were incubated with kidney homogenate, however, the differences were less marked.

Chromatographic studies revealed that, when the α-L-compounds were incubated, not only peptide fragments occurred as a result of aminopeptidasic degradation, but also small quantities (in free form) of all the amino acids contained in the molecule. The α-D-, β-L-, β-D- and desamino-compounds were found to be protected against aminopeptidasic degradation, whereas endopeptidasic degradation proceeded in the same way as in the case of the α-L-compounds. Here, in addition to free amino acids, the isomeric tetrapeptides Asp–Arg–Val–Tyr were also demonstrable. Whereas in the serum of nephrectomized rats approximately 90 per cent of the total degradation process was due to the activity of aminopeptidases and only 10 per cent to that of endopeptidases, the corresponding activity ratio for the enzymes in rat kidney homogenate or in human plasma was approximately 60 per cent : 40 per cent.

THE octapeptide angiotensin II, Asp[1]–Arg[2]–Val[3]–Tyr[4]–Val[5]–His[6]–Pro[7]–Phe[8], is rapidly inactivated both in the organism as well as when incubated *in vitro* with serum, plasma or tissue homogenates. Its inactivation is due to enzymatic degradation,[1–4] a process in which several enzymes are assumed to be involved. The manner in which angiotensin is broken down differs from one type of tissue to another.[1–3] Plentl and Page[5] found that purified carboxypeptidase, pepsin, trypsin and chymotrypsin are capable of destroying angiotensin *in vitro*. Using the synthetic substrate Val[5]-angiotensin II amide, Riniker and Schwyzer[6] succeeded in locating more exactly the sites of attack of these enzymes. Trypsin splits the peptide bond between Arg[2] and Val[3], pepsin between Val[3] and Tyr[4], and chymotrypsin between Tyr[4] and Val[5]. Incubation with leucine–aminopeptidase resulted in stepwise cleavage from the amino end leaving the tripeptide residue His–Pro–Phe. Carboxypeptidase only splits off the terminal Phe[8].

The total activity of the enzymes involved in the degradation of angiotensin is generally referred to collectively as "angiotensinase" activity. Dengler and Reichel,[7] however, concluded from their investigations that it was doubtful whether inactivation

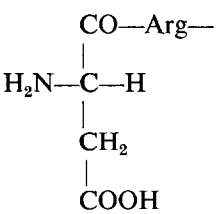
of angiotensin is due to a specific enzyme, or to breakdown by nonspecific peptidases. The studies to be described here were designed to investigate by means of degradation tests on angiotensin II analogues: (1) at what rate angiotensin is broken down in serum, plasma or kidney homogenate, as determined by measuring its pressor activity in biological tests; (2) the metabolic pathways by which angiotensin is broken down, as determined by chromatographic analysis of the cleavage products.

METHODS

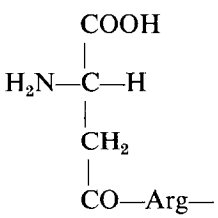
Substances

Except for substance VI, all the compounds studied were analogues of the octapeptide Val[5]-angiotensin II, the only difference between them being the changes made in the amino acid situated at the amino end:[8]

I. α-L-Asp[1]-angiotensin II.
II. α-L-Asp[1](NH_2)-angiotensin II (angiotensin amide).
III. α-D-Asp[1]-angiotensin II.
IV. β-L-Asp[1]-angiotensin II.
V. β-D-Asp[1]-angiotensin II.
VI. Desamino-angiotensin II (with succinic acid in the molecule, instead of aspartic acid).



α-L-form



β-L-form

Immediately prior to the experiment, the compounds were in each instance freshly dissolved in Tyrode's solution[9] (containing no glucose); all glass containers were siliconized in order to prevent adsorption.

Enzyme systems

The breakdown of the above-listed substances was studied using the following systems:

(a) Serum from rats nephrectomized 20 hr prior to the test. For practical reasons blood from nephrectomized animals was employed. Controls with serum from normal animals revealed no differences.

(b) Human plasma rendered incoagulable by the addition of heparin (50 U/ml Liquemin Roche). A few tests were also performed with dried, citrated human plasma (Central Laboratory of the Swiss Red Cross, Berne).

(c) Rat kidney homogenate: 1 g fresh renal tissue was homogenized with 9 ml Tyrode's solution[9] (containing no glucose) in a glass homogenizer (Braun, Melsungen).

(d) Purified leucine-aminopeptidase, from hog kidneys (Worthington, Freehold, N.J.), activated with $MgCl_2$.

The serum, plasma and homogenate were brought to a pH of 7·4 by adding 1 N HCl or 1 N NaOH. To each batch was then added 0·067 M phosphate buffer (pH 7·4)

in an amount equivalent to one-tenth of the original volume. Before addition of the buffer, the kidney homogenate was centrifuged for 10 min at 3000 rev/min, and the supernatant was employed for the reaction. For the blood pressure tests, the kidney homogenate was further diluted with Tyrode's solution to 1:67 ,and to 1:4 for the chromatographic studies.

Blood pressure tests

In order to determine the loss of activity in serum, plasma and kidney homogenate, samples of 0·9 ml were warmed in a water bath to 37°, whereupon 0·4 μg of each of substances I, II and VI was added to the batches in a volume of 0·1 ml. Since the pressor effect of substance III, IV and V is twice as great,[10] these were added in the same volume in amounts of only 0·2 μg. At various times between the second and 120th minute, the reaction proceeding at 37° was interrupted by boiling in a water bath. To obtain a clear supernatant, the samples were brought to a pH of 5·6 by adding 1 N HCl immediately prior to boiling. After boiling they were cooled in ice water and centrifuged; the pressor activity of the supernatant was then tested on the blood pressure of nephrectomized rats.[11] Boiled solutions of the substances under study were used as standards (0·01–0·04 μg per rat) in Tyrode's solution. Boiling did not lead to any loss of pressor activity. The pressor activity recorded after incubation was expressed as a percentage of the original activity.

Samples of the incubation mixture taken immediately before the end of incubation showed the same pressor activity as the supernatant after precipitation. Thus the difference in the loss of activity cannot have been due to a difference in the binding to protein during coagulation.

Of the fragments occurring during the breakdown of angiotensin, only the heptapeptide resulting from detachment of the terminal aspartic acid still displays half the pressor activity of angiotensin II.[12] As this heptapeptide was present only in small quantities, however, the whole pressor activity found following incubatio n was ascribed to angiotensin II.

Chromatographic studies

Paper-chromatographic studies were undertaken in order to determine the fragments occurring during the breakdown of angiotensin. Ten milligrams of the angiotensin derivatives were dissolved in 0·2 ml of a 0·25 M aqueous solution of trihydroxymethylaminomethane (tris buffer), at pH 7·4, and added to 0·8 ml of serum or plasma. Rat kidney homogenate was diluted 1:4 with Tyrode's solution, and 5 mg of each of the angiotensin analogues, dissolved in 0·1 ml tris buffer, was added to 0·4 ml of the homogenate. Following incubation at 37° for 6 or 24 hr in rat serum or human plasma, and for 30 min or 2 hr with kidney homogenate, the reaction was interrupted by boiling for 5 min; the samples were then centrifuged and 10 μl of the supernatant was spotted on Whatman paper No. 1. Descending chromatography was carried out in the following solvent systems:

(1) *sec.*-Butanol–3% NH_3 (100 ml:44 ml).

(2) *sec.*-Butanol–*iso*propanol–triethylamine–Veronal–water (100 ml:10 ml:0·8 ml: 1·8 g:60 ml)

(3) *sec.*-Butanol–*iso*propanol–monochloracetic acid–water (70 ml:10 ml:3 g:40 ml).

The chromatograms were developed with Pauly's reagent and with ninhydrin. The degradation products occurring as a result of incubation were identified by comparing their R_f values with those of the amino acids and of peptides produced by synthesis.

In order to obtain quantitative information on the degradation process the identified peptide fragments were chromatographed together with the seven different amino acids of angiotensin II in amounts corresponding to 20 per cent of the amount present in the angiotensin II employed. The intensity of the colour reaction on the paper was compared visually with that for the known test substances. In this way it was possible to make a semiquantitative analysis accurate to approx. $\pm$ 20 per cent.

For the studies undertaken with purified leucine–aminopeptidase, 1 mg angiotensin (substance I) and 1 mg angiotensin amide (substance II) were incubated for 24 hr at 37° in 0·1 ml of 0·1 M tris buffer (pH 8·4) with 0·1 mg leucine–aminopeptidase after adding one drop of toluene. The resultant degradation was investigated by chromatography only, i.e. in the manner already described.

RESULTS

A. Loss of pressor activity

When incubated with rat serum, human plasma or rat kidney homogenate, α-L-angiotensin II amide (substance II) was inactivated more rapidly than the other compounds; α-L-angiotensin II (substance I) proved slightly less sensitive to the inactivating enzymes. The remaining substances (α-D-, β-L-, β-D- and desamino-angiotensin II) were broken down by rat serum or human plasma at a considerably slower rate than substances I and II (Figs. 1, 2 and 3). The rate of inactivation was nearly the same for substances III–VI. When they were incubated with rat kidney homogenate, however, the difference in the speed of degradation as between substances I and II, on the one hand, and substances III–VI, on the other, was not as clear-cut as during incubation with serum (Fig. 2).

The rates of inactivation were the same in fresh, heparinized human plasma and in dried, citrated human plasma. Rat kidney homogenate, with a calculated protein content of 19 per cent of the kidney,[13] was roughly 200–250 times more active than rat serum, whereas human plasma was 2–5 times less active.

B. Chromatographic studies

When α-L-angiotensin or its corresponding amide (substances I and II) were incubated with rat serum, the hepta-, hexa, penta- and tetra-peptides resulting from the splitting off of amino acids from the amino end were identified (Fig. 4). Moreover, after the chromatograms had been developed with ninhydrin, small quantities of all the amino acids present in angiotensin II were also demonstrable in free form. For the sake of greater clarity, Figs. 5, 6 and 7 list only the colour reactions with Pauly's reagent (histidine, tyrosine and their peptides).

When substances III–VI were incubated, no degradation proceeding from the amino end was observed. The molecule did, however, split up into its component amino acids in the same manner as in the case of the α-L-compounds. In addition to the small quantities of free amino acids, the tetrapeptide Asp–Arg–Val–Tyr (α-D-Asp from substance III; β-L-Asp from substance IV; and β-D-Asp from substance V) was also identified (Fig. 4). When the α-L-compounds were incubated, the corresponding tetrapeptide could not be found. This tetrapeptide evidently arose under the influence of

an endopeptidase acting similarly to chymotrypsin. It was apparently comparatively resistant to further degradation, whereas the other half of the molecule was rapidly broken down (e.g. by aminopeptidases, prolidases). The ratio between the four individual amino acids (aspartic acid, arginine, valine and tyrosine) and the intact tetrapeptide Asp–Arg–Val–Tyr was roughly 1:1.

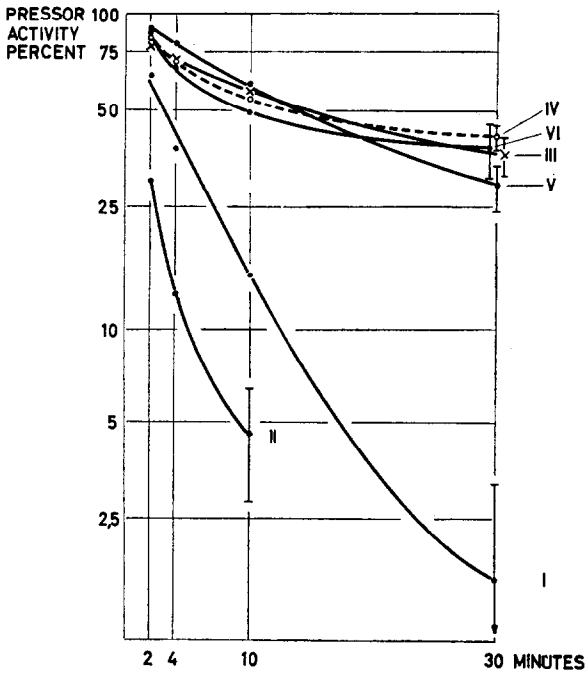


FIG. 1. Inactivation of angiotensin isomers after varying periods of incubation with rat serum at 37° and pH 7·4.

I = α-L-Asp^1-angiotensin II.
II = α-L-Asp^1(NH_2)-angiotensin II (angiotensin amide).
III = α-D-Asp^1-angiotensin II.
IV = β-L-Asp^1-angiotensin II.
V = β-D-Asp^1-angiotensin II.
VI = Desamino-angiotensin II.

The pressor activity recorded after incubation was expressed as a percentage of the activity originally displayed by the angiotensin derivative in question. Each point represents the mean value from three to five determinations. In order to indicate the variability, $\bar{x} \pm s\bar{x}$ has in each instance been included at the end-point. In the case of the other points, however, the variability has been omitted for the sake of simplicity.

A comparison between the values of breakdown via the various pathways reveals that approximately 90 per cent of the total inactivation of the two α-L-compounds was accounted for by aminopeptidasic degradation and only approximately 10 per cent by the action of the chymotrypsin-like enzyme followed by further degradation (Fig. 5).

With rat kidney homogenate, qualitatively the same degradation processes and the same difference in the breakdown of the various angiotensin isomers were observed

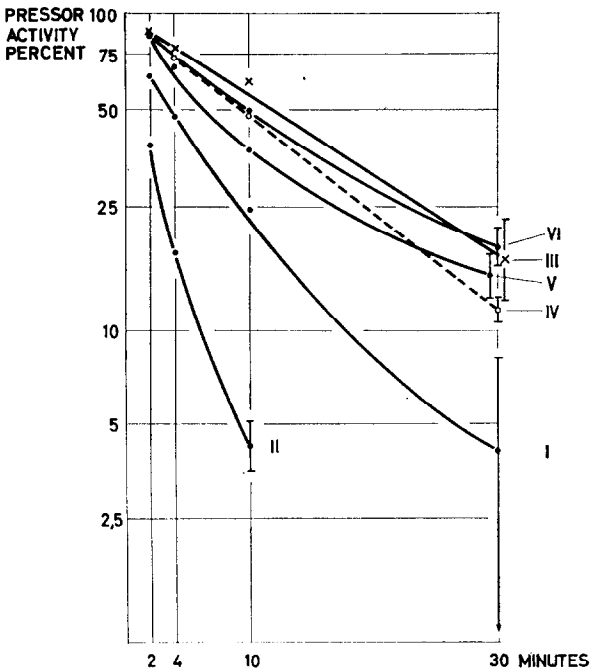


FIG. 2. Inactivation of angiotensin isomers after varying periods of incubation with rat kidney homogenate at 37° and pH 7·4. For further details, see Fig. 1.

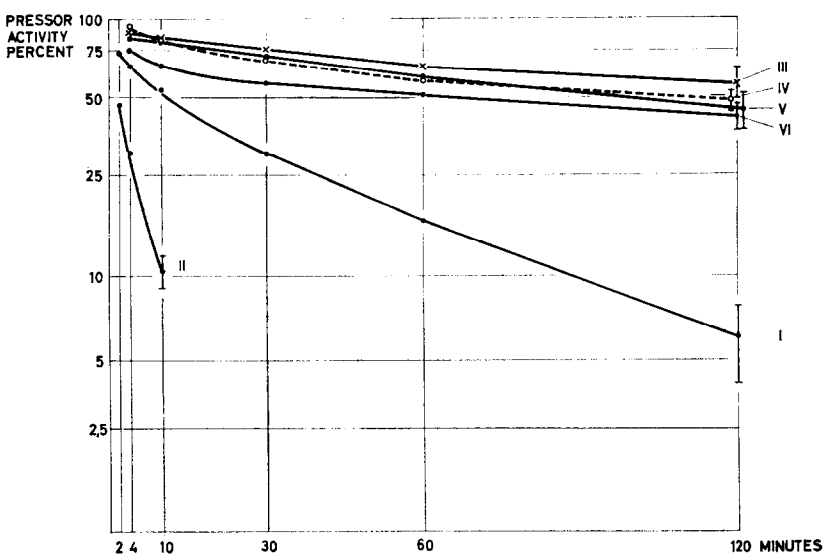


FIG. 3. Inactivation of angiotensin isomers after varying periods of incubation with human plasma (+heparin 50 U/ml) at 37° and pH 7·4. For further details, see Fig. 1.

as with rat serum. The rate of breakdown was consistently greater with kidney homogenate than with rat serum.

When substances III–VI were incubated with kidney homogenate, the isomeric tetrapeptides Asp–Arg–Val–Tyr were found in larger quantities than following incubation with rat serum; on the one hand, in the case of incubation with rat kidney

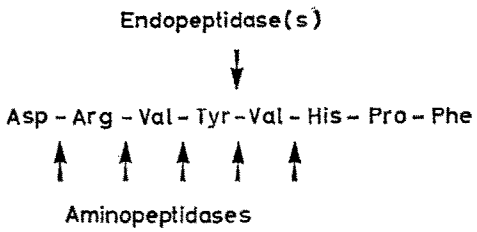


FIG. 4. Diagram illustrating the breakdown of α-L-angiotensin II.

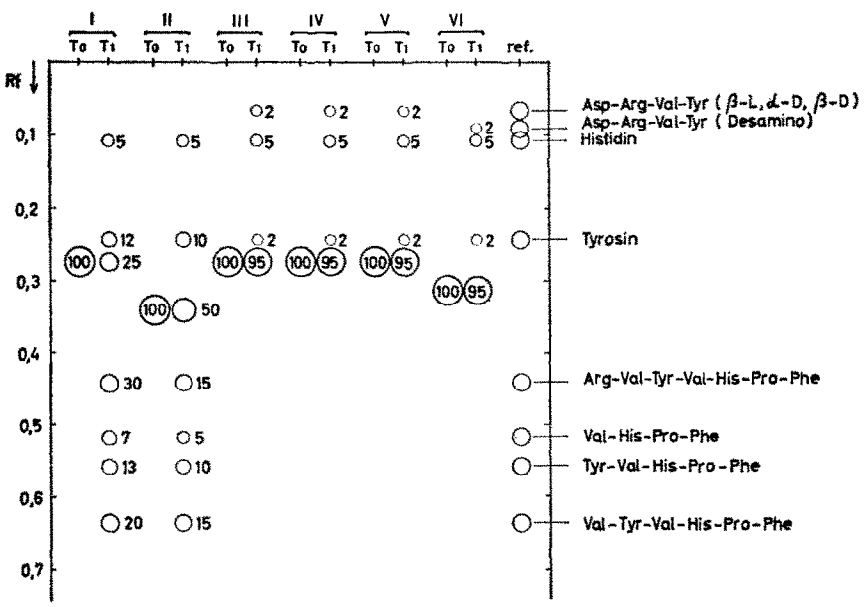


FIG. 5. Diagram showing degradation products demonstrable by chromatography after incubating angiotensin II isomers with rat serum for 6 hr at 37° and pH 7·4. Same substances as in Fig. 1. Paper chromatography, using as solvent system *sec*-butanol: 3% NH_3 (100:44); developed with Pauly's reagent. The figures listed represent percentages (calculated on a molar basis) of the angiotensin II originally present and are mean values obtained from three tests. Ref., reference substances; T_0, chromatography without incubation; T_1, incubation for 6 hr.

homogenate, only about 60 per cent of the breakdown of the two α-L-angiotensins was caused by aminopeptidasic degradation, whereas approximately 40 per cent was due to the activity of the chymotrypsin-like enzyme (Fig. 6); the corresponding ratio in rat serum worked out at 90:10. On the other hand, when the compounds were incubated with kidney extract, only a quarter of the tetrapeptides were broken down further

into amino acids as compared with half during incubation with rat serum (Fig. 6).

In human plasma (Fig. 7), enzymatic degradation proceeded at a slower rate than in rat serum. The qualitative differences in the breakdown of the angiotensin isomers were the same for incubation with human plasma as for incubation with rat serum or rat

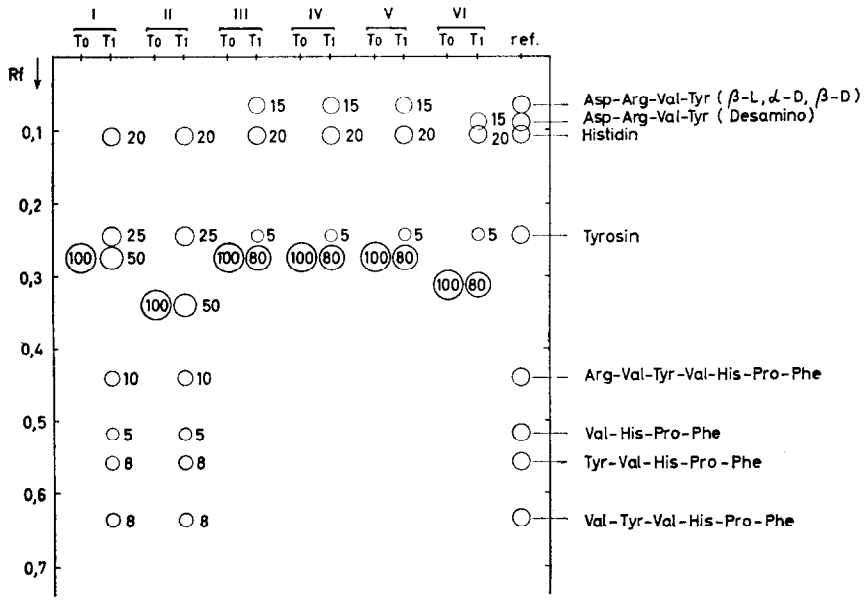


FIG. 6. Diagram showing degradation products demonstrable by chromatography after incubating angiotensin II isomers with rat kidney homogenate for 30 min at 37° and pH 7·4, For further details, see Fig. 5; T_1 in these experiments, 30 min.

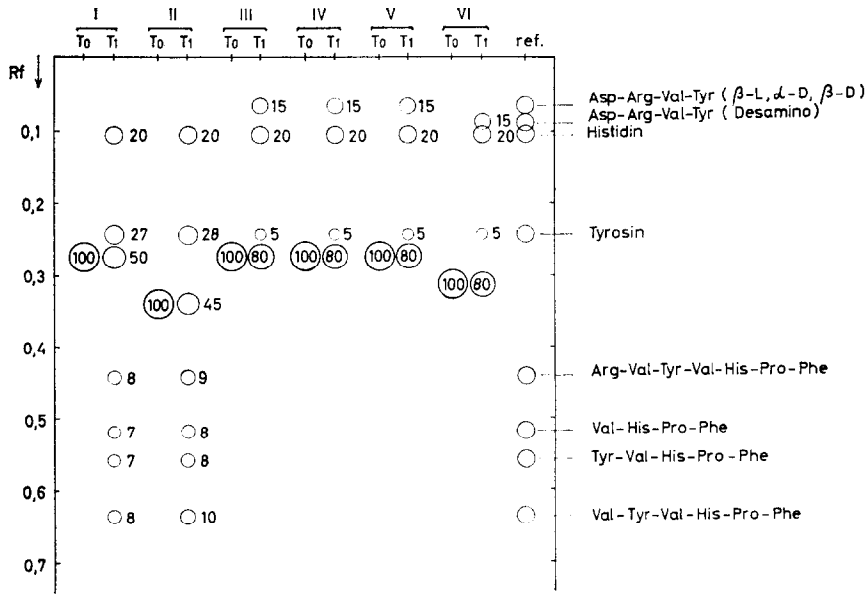


FIG. 7. Diagram showing degradation products demonstrable by chromatography after incubating angiotensin II isomers with human plasma (+heparin 50 U/ml) for 24 hr at 37° and pH 7·4. For further details, see Fig. 5; T_1 in these experiments, 24 hr.

kidney homogenate. The ratio of aminopeptidasic to endopeptidasic degradation resembled the ratio obtained with kidney homogenate, and once again a fairly large quantity of Asp–Arg–Val–Tyr tetrapeptides was also demonstrable. Thus, human plasma behaves similarly to rat kidney homogenate and differs from rat serum.

In order to check the values estimated on the basis of the chromatographic findings, one batch was also subjected to biological analysis. There was found to be a close correlation between the loss of pressor activity and the degree of degradation as estimated from the chromatogram. Longer incubation times (24 hr in rat serum, and 2 hr in kidney homogenate) resulted in almost complete destruction of the angiotensins.

In the tests for the demonstration of fragments by means of chromatography, the method employed necessitated using for incubation concentrations of angiotensin analogues 25,000 times higher than in the biological investigations. When the degradation rates under these two sets of conditions were compared, it was found that with the higher concentrations the rate of degradation was slower, i.e. that the enzyme had become saturated. Besides these differences in the degradation rate, incubation with rat serum was also associated with a shift in the relative degradation rate of α-L-angiotensin II as compared with α-L-angiotensin amide: whereas at a low concentration α-L-angiotensin II amide was in each of five experiments invariably broken down more quickly than α-L-angiotensin II, the opposite was found to be the case in three chromatographic tests in which the pressor activity was also recorded. No explanation can be offered for the fact that in rat serum angiotensin II was more rapidly broken down at a high concentration (Fig. 5), but not when it was incubated with human plasma or with kidney homogenate.

The degradation of substances I and II in response to incubation with purified leucine–aminopeptidase was investigated only by means of chromatography. After incubation for 24 hr, angiotensin II amide was destroyed to the extent of 90 per cent, whereas only traces of α-L-angiotensin had been broken down.

DISCUSSION

Angiotensin II derivatives in which L-aspartic acid at the amino end was linked to arginine, the second amino acid, in the α-position were inactivated and split more rapidly than compounds combining with D-aspartic acid or D- or L- aspartic acid linked in the β-position. The difference can be explained by the assumption that the overall degradation is mainly due to aminopeptidases, which are capable of attacking only the α-L-configuration.

Leucine-aminopeptidase cannot be the sole enzyme responsible for the process in question, since α-L-angiotensin II was broken down by this enzyme at a far slower rate than α-L-angiotensin II amide. This difference might explain why in biological tests angiotensin amide is inactivated more rapidly than any of the other compounds. Angiotensin II must, however, be attacked by one or more other aminopeptidases. Glenner *et al.*[14] have demonstrated the existence in rat kidneys of an enzyme—referred to as aminopeptidase A—which specifically splits off L-aspartic or L-glutamic acid bound in the α-position at the amino end. It is conceivable that such an enzyme might play a role in the destruction of α-L-angiotensin II by splitting off the aspartic acid. The resultant heptapeptide, which displays only half the pressor activity of the octapeptide, may in turn be attacked by leucine-aminopeptidase and rapidly broken down to the tripeptide His–Pro–Phe.[5] This tripeptide was not found in any of the

samples studied—a fact which indicates that it, too, must be quickly destroyed. The peptidases (prolidasis) participating in this cleavage are not known.

A second metabolic pathway was found to consist of a cleavage process brought about by a chymotrypsin-like enzyme. The fraction of angiotensins metabolized by this means varied in the different systems studied. The degradation produced by this enzyme invariably proceeded at a slower rate than that produced by aminopeptidases. The action of the chymotrypsin-like enzyme gave rise to two different types of tetrapeptides, of which, however, it was possible to demonstrate only Asp–Arg–Val–Tyr, the isomeric peptides protected against aminopeptidases and occurring during the breakdown α-D-, β-L-, β-D- and desamino-angiotensin. The other tetrapeptide, Val–His–Pro–Phe, was split up so rapidly under the influence of other enzymes that only free amino acids could be traced.

There was no evidence of destruction by carboxypeptidases. The latter, according to Riniker and Schwyzer,[6] split off only the phenylalanine at the acid end. No heptapeptide having the appropriate configuration could be found after incubation either with plasma or with kidney homogenate.

The rate at which naturally occurring α-L-angiotensin II was inactivated by human plasma, which in our experiments was mixed with phosphate buffer in a ratio of 10:1, coincided with the values obtained by Khairallah *et al.*[3] although these investigators employed human plasma diluted with phosphate buffer in a ratio of 1:10. As the inactivation rate does not increase proportionally to the angiotensin II concentration, any difference might be attributable to variations in the concentration employed for incubation.

With the exception of desamino-angiotensin II, which has a weaker effect than angiotensin II,[15] the pressor activity of the compounds resistant to aminopeptidases, as determined in nephrectomized rats, was approximately 50 per cent greater than that of the α-L-analogues. A comparison between equipressor doses has shown that the duration of action of the compounds III–VI is from two to three times longer than that of the α-L-compounds.[10] This finding would appear to suggest that the sum of the activity of all the enzymes in the organism of the rat participating in the breakdown of angiotensin is similar to that of the enzymes in kidney homogenate or human plasma, but not to that in the serum of nephrectomized rats.

REFERENCES

1. E. BRAUN-MENENDEZ, J. C. FASCIOLO, L. F. LELOIR, J. M. MUNOZ and A. C. TAQUINI, *Renal Hypertension* p. 166. Charles C. Thomas, Springfield, Ill. (1946).
2. E. KEMP and I. RUBIN, *Arch. Biochem.* **96**, 56 (1962).
3. P. A. KHAIRALLAH, I. H. PAGE, F. M. BUMPUS and R. R. SMEBY, *Science* **138**, 523 (1962).
4. D. KLAUS, *Klin. Wschr.* **40**, 701 (1962).
5. A. A. PLENTL and I. H. PAGE, *J. exp. Med.* **79**, 205 (1944).
6. B. RINIKER and R. SCHWYZER, *Helv. chim. acta* **44**, 658 (1961).
7. H. DENGLER and G. REICHEL, *Experientia* **16**, 36 (1960).
8. B. RINIKER, H. BRUNNER and R. SCHWYZER, *Angew. Chem.* **74**, 469 (1962).
9. A. VON MURALT, *Einführung in die praktische Physiologie* p. 75. Springer, Berlin–Heidelberg (1948).
10. H. BRUNNER and D. REGOLI, *Experientia* **18**, 504 (1962).
11. F. GROSS and F. SULSER, *Arch. exp. Path. Pharm.* **229**, 374 (1956).
12. R. SCHWYZER and H. TURRIAN, *Vitamins & Hormones* **18**, 237 (1960).
13. H. D. CREMER, *Biochem. Taschenbuch* p. 830. Springer, Berlin–Göttingen–Heidelberg (1956).
14. G. G. GLENNER, P. J. MCMILLAN and J. E. FOLK, *Nature, Lond.* **194**, 867 (1962).
15. K. ARAKAWA, R. R. SMEBY and F. M. BUMPUS, *J. Amer. Chem. Soc.* **84**, 1424 (1962).