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The angiotensin I converting enzyme activity of subtilisin BPN′ (Nagarse), a crystalline enzyme from *Bacillus subtilis**

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CARLINI and Huggins[1] as well as Walaszek *et al.*[2] reported that an impure preparation of alpha-amylase liberated an oxytocic principle from 5-valine-angiotensin I. Subsequent work by Huggins *et al.*[3] has shown that a bacterial proteinase known as subtilisin BPN′ or Nagarse is the enzyme in crude alpha-amylase which is responsible for the formation of the vasoactive material, substance A. The similarity in action of substance A to angiotensin II, which was shown by Walaszek *et al.*,[2] and the presence of the amino acids of angiotensin II in substance A[4] indicated that Nagarse may indeed possess angiotensin I converting enzyme activity. However, recently Endo *et al.*[5] could not conclusively demonstrate the liberation of angiotensin II activity by incubating angiotensin I with Nagarse. The availability of 5-isoleucine [10-^{14}C(U), leucine] angiotensin I and improved chemical methods†‡[6] of assay for angiotensin I converting enzyme activity enabled us to reinvestigate this problem.

The enzymatic formation of angiotensin II was measured by biological assay on rabbit arotic strips[7] and on isolated rat uteri[1] in an isometric tension apparatus or by the above chemical methods. 5-Isoleucine [10-^{14}C(U), leucine] angiotensin I was synthesized in this laboratory‡ and 5-valine-angiotensin I and II were obtained from CIBA. Subtilisin BPN′ (Nagarse) was obtained from the Enzyme Corp., N.Y. Isometric contractions of the guinea pig ileum were used in the study of the rate of destruction of angiotensin II by Nagarse. All the incubations of angiotensin I with Nagarse were done in 0·05 M sodium phosphate buffer (pH 6·5) at 37°, except in those instances reported otherwise. The radioactivity determinations were made in a Nuclear-Chicago liquid scintillation system, model 720.

Fig. 1 illnstrates the converting enzyme activity of Nagarse, as measured on the rat uterus and the rabbit aortic strip. It can be seen that Nagarse, acting on the substrate angiotensin I(A_1), has the ability to produce a material which stimulates the isolated rat uterus and rabbit aorta.

Studies on the time-course formation and destruction of the oxytocic material were carried out by incubating 2 μg angiotensin I and 5 μg Nagarse contained in 1·5 ml sodium phosphate buffer (0·05 M, pH 6·5). The formation and destruction of the oxytocic principle were assayed on the rat uterus. Peak activity was obtained at 11 min and after 31 min there was almost total destruction of the active principle. The effect of pH on the formation of the active material was determined by using the rabbit aortic strip as the assay system. It was found that the maximal formation of the active material occurred at pH 6 with very little formation below pH 4·5. However, there was considerable formation at pH 8.

A radioactive method† for the assay of the converting enzyme has been developed in our laboratory. This method uses silica gel-impregnated glass-fiber paper (I.T.L.C. type SG-Gelman) to separate angiotensin I, angiotensin II and histidylleucine from an incubation mixture in a solvent system of *n*-butanol–ammonia–water (120:7:7, v/v). When 5-isoleucine [10-^{14}C(U), leucine] angiotensin I is used in the incubation mixture, the histidylleucine liberated by the action of converting enzyme is radioactive, and thus the radioactivity of histidylleucine may be used as a measure of the enzymatic activity. The radioactivity determinations were corroborated in the simultaneous bioassay of angiotensin II on the aortic strip. Table 1 shows the data obtained under these conditions in which we found that the bioassay gave lower values than the chemical method for analysis. This discrepancy was shown to be due to the destruction of the angiotensin II by Nagarse, which was also acting as the converting enzyme. The rate of destruction of angiotensin II by Nagarse was determined on an isolated guinea

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† C. G. Huggins and N. S. Thampi, submitted for publication.

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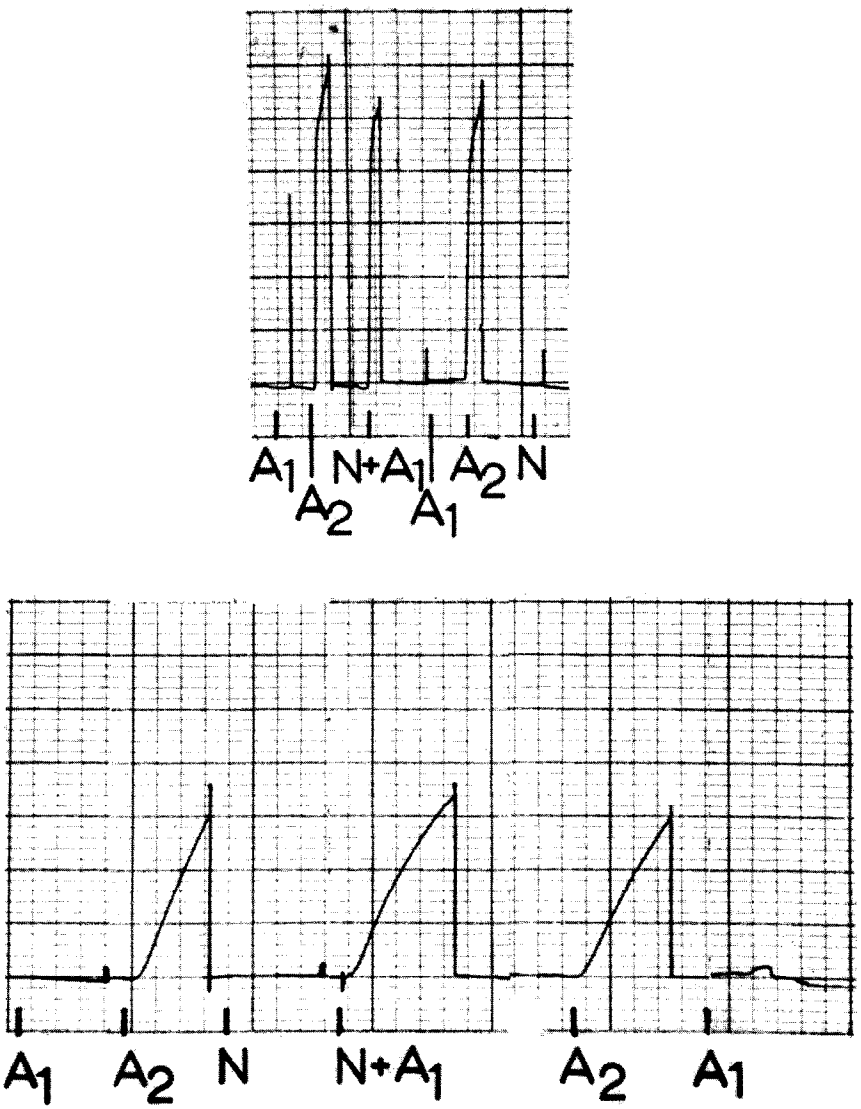

FIG. 1. The formation of angiotensin II as measured on the isolated rat uterus (upper panel) and the isolated rabbit aortic strip (lower panel). A_1 = angiotensin I, A_2 = angiotensin II, N = subtilisin BPN′ (Nagarse). In the upper panel A_1 = 0·1 μg angiotensin I, A_2 = 0·05 μg angiotensin II, N = 0·17 μg Nagarse, N + A_1 = a 0·05-ml aliquot from an incubation mixture of 2 μg A_1 and 5 μg Nagarse/1·5 ml sodium phosphate buffer (pH 6·5, 0·05 M) after 5 min of incubation at 37°. In the lower panel A_1 = 1 μg angiotensin I, A_2 = 0·25 μg angiotensin II, N = 0·25μg Nagarse, N + A_1 = a 0·05-ml aliquot from an incubation mixture of 10 μg A_1 and 5 μg Nagarse/ml sodium phosphate buffer (pH 6·5, 0·05 M) after 5 min of incubation at 37°.

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TABLE 1. USE OF 5-ISOLEUCINE [10-$^{14}C(U)$, LEUCINE] ANGIOTENSIN I IN THE MEASUREMENT OF THE ANGIOTENSIN I CONVERTING ENZYME ACTIVITY OF SUBTILISIN BPN' (NAGARSE)*

Experiment	mμmoles Found (units)†		
	Angiotensin I (radiometric)	Angiotensin II (aortic bioassay)	His. Leu (radiometric)
Control (no enzyme)	16·3	0	0
Exp. 1	9·5	3·5	5
Exp. 2	9·1	3·3	5·5
Exp. 3	11·1		5·8

* 16·3 mμmole 5-Isoleucine [10-^{14}C(U), leucine] angiotensin I were incubated with 10 μg Nagarse at 37° for 5 min.

† One unit of converting enzyme activity is defined as that amount of enzyme required to release 1 mμmole angiotensin II or histidylleucine (His., Leu.) per unit of incubation time.

pig ileum and the data were plotted in Fig. 2. When we corrected for this destruction of angiotensin II, we found fairly good agreement between the chemical and biological methods.

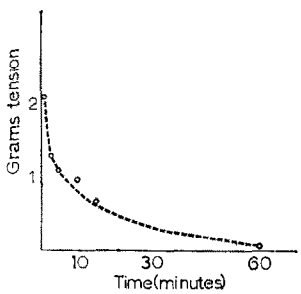

FIG. 2. The enzymatic destruction of angiotensin II by subtilisin BPN' (Nagarse) as a function of incubation time as measured on the isolated guinea pig ileum. Aliquots (0·003 ml) of an incubation mixture of 5 μg angiotensin II and 20 μg Nagarse/ml sodium phosphate buffer (0·05 M, pH 6·5) at 37° were added.

The data presented above show quite clearly that Nagarse does possess angiotensin I converting enzyme activity. However, the activity is manifest only under optimum conditions. The specificity of Nagarse is very broad when compared with renin or the plasma angiotensin I converting enzyme. A pH of 6 was observed to be optimum for Nagarse converting enzyme activity. In addition to the liberation of the oxytocic principle, Nagarse has a destructive effect on the active product itself. This accounts for the low biological activity of the product of incubation in comparison to the higher values observed by the radioactivity measurements.

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