Plasma Oxytocinase, Cystine and Leucine Naphthylamidases of Snake, Turtle and Chicken.* (29583)

RUDOLPH H. EHRENSING† (Introduced by H. C. Dessauer)
Department of Biochemistry, Louisiana State University School of Medicine, New Orleans, La.

An oxytocin inactivating enzyme, discovered by Fekete(1) in 1930, appears in plasma of pregnant women after fertilization, reaches maximum activity at parturition and completely disappears from the blood within 4 weeks after cessation of pregnancy(2). Plasma of pregnant women also exhibits increased potential for catalyzing the hydrolysis of the snythetic substrates, L-leucine-β-naphthylamide(3) and L-cystine-di-β-naphthylamide (4). As the L-cystine-di-β-naphthylamidase (CNAase) and oxytocinase activities parallel each other and affect a similar chemical bond, they have been attributed to the same enzyme, CNAase activity being called oxytocinase activity(4,5). Upon starch-gel electrophoresis CNAase separates into 2 bands. These also display L-leucine-β-naphthylamidase (LNAase) activity(6).

Oxytocinase apparently has a limited distribution in plasma of vertebrate animals. Few non-mammalian but many mammalian forms have been studied. The enzyme has been found only in pregnant primates(2,7). Plasma CNAase, assayed in even fewer species, is present in man during obstructive jaundice(8) as well as pregnancy. I have found plasma oxytocinase in a colubrid snake, and LNAase and CNAase in the plasma of a number of non-mammalian forms. This report compares the LNAase, CNAase and oxytocinase activities of a snake, a turtle and a bird with those of man.

Materials and methods. Blood was collected by cardiac puncture from 8 diamond back water snakes (*Natrix rhombifera*) and 8 common snapping turtles (*Chelydra serpentina*), both species from southeast Louisiana (9). Blood was collected from the brachial vein of 8 chickens (*Gallus gallus*), White Leghorn hens and hybrid White Rock × Rhode Island Red roosters. Chickens and turtles were fasted for varying periods and snakes for 3 days prior to blood letting. Heparin was used to inhibit clotting. Plasma was separated from blood cells and assayed immediately or after storage in a deep freeze. No differences in enzyme activities were found between fresh and frozen samples.

CNAase and LNAase were assayed using the colorimetric method of Tuppy and Nesvadba(4). Activities are recorded as μg β-naphthylamide liberated/0.1 ml plasma/hr. Buffer pH was optimal or near optimal for each species. Optimal pH's were determined with the same assay method in succinate, trihydroxyaminomethane (TRIS) maleate, and veronal buffers of varying pH and constant molarity. Incubation temperature was 37°C. Oxytocinase was measured by the method of Golubow and du Vigneaud(10) based on the hypotensive action of oxytocin injected i.v. into chickens(11). Four-tenths ml of a mixture of 0.1 ml plasma, 2 units (6.6 μg) of oxytocin (Syntocinon‡), and 0.0125 M TRIS-maleate buffer pH 6.4 were mixed. An aliquot of 0.1 ml was used to assay zero time activity. After incubating the remaining solution for 6 hours at 37°C, its oxytocin activity was compared to that of the zero time control. Oxytocin incubated in the absence of plasma for 6 hours under the same conditions did not lose activity.

Plasma proteins were fractionated by vertical starch-gel electrophoresis(12). LNAases were located on the gel slabs by a chemical technique(13). Fractions were isolated from gel sections by centrifugation after destruction of gel structure by freeze-thaw(14) or by digestion of the starch with diastase. Eluted fractions were assayed for CNAase and for oxytocinase.

Results. Plasma CNAase and LNAase of

* Supported in part by NSF Grant G-10751 to Drs. H. C. Dessauer and W. Fox.

† NIH Cardiovascular Graduate Research Training Program Fellow

‡ Gift of Sandoz Pharmaceutical Co.

TABLE I. Plasma LNAase, CNAase, and Oxytocinase.

		No.	LNAase*	CNAase*	Oxytocinase
Natrix	♀	4	308 ± 34† (264–348)‡	4.28 ± .59 (3.47– 4.85)	Present
	♂	4	206 ± 113 (74–342)	3.49 ± 1.01 (2.34– 4.44)	"
		8	257 ± 95	3.89 ± .88	
Gallus	♀	4	209 ± 20 (181–223)	3.65 ± .37 (3.12– 3.96)	Absent
	♂	4	177 ± 35 (136–222)	3.35 ± .36 (2.84– 3.60)	"
		8	193 ± 31	3.50 ± .37	
Chelydra	♀	3	419 ± 246 (229–697)	8.62 ± 4.52 (5.01–13.70)	Absent
	♂	5	481 ± 133 (257–623)	10.64 ± 3.03 (5.82–13.79)	"
		8	458 ± 171	9.88 ± 3.48	
Homo (from ref. 3, 5, & 10)					
Pregnant			21(3)	5.00(5)	Present(10)
Non-pregnant			12(3)	.20(5)	Absent(10)

* μg β-naphthylamine liberated/0.1 ml plasma/hr. † Mean ± S.D. ‡ Range.

the turtle, snake and chicken were active over a broad range of hydrogen ion concentrations. The enzymes retained over 50% of maximum activity within a region encompassing a 100 to 1000 fold change in hydrogen ion concentration (Fig. 1). Optimal pH's of CNAase and LNAase were approximately equal in the same species. *Gallus* had the most acidic optimal pH and *Natrix* the most alkaline.

LNAase activities in plasma of the snake, chicken and turtle were 16 to 38 times greater than in that of men and non-pregnant women and 10 to 20 times higher than in plasma of pregnant women (Fig. 2, Table I). One-tenth ml of plasma from one turtle, the species with the highest LNAase activity, liberated almost 700 μg of β-naphthylamine in the hour incubation period. CNAase levels in the non-mammalian species were of the same order of magnitude as those of women in late pregnancy. Turtle plasma CNAase was 2.5 to 3 times greater than that of snake and chicken and twice that of pregnant women. CNAase varied in proportion to LNAase in the turtle and snake, having correlation coefficients of +0.86 and +0.75 respectively ($P<.05$). The correlation between the two activities was not clear cut in chickens (correlation coefficient = +0.54; $P>.05$).

Oxytocinase was present only in snake plasma. Oxytocin activity disappeared from snake plasma—oxytocin mixtures after an incubation of 6 hours. Shorter incubations showed that oxytocin inactivation was a function of time. Level of snake oxytocinase was of the same order of magnitude as that of humans in the eighth month of pregnancy. Turtle and chicken plasma-oxytocin mixtures retained 100% of the original oxytocin activity after 6 hrs of incubation. Levels of oxytocinase and CNAase in human plasma as

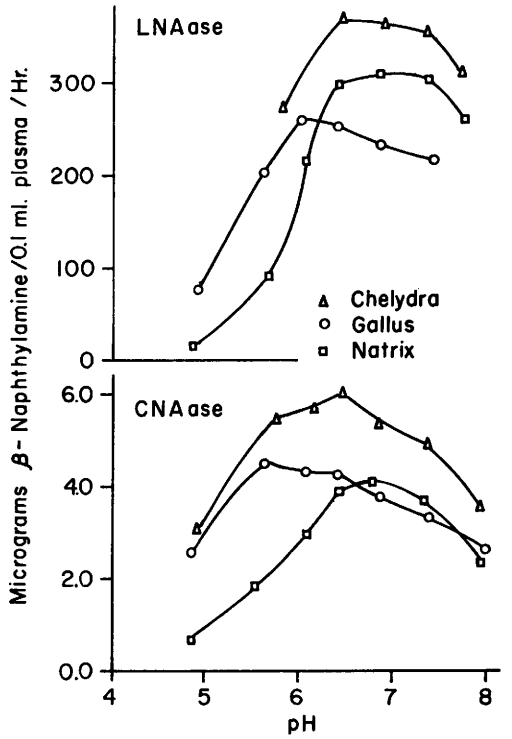

FIG. 1. Naphthylamidase activities of blood plasma of a turtle, *Chelydra*, a snake, *Natrix*, and the chicken, *Gallus*.

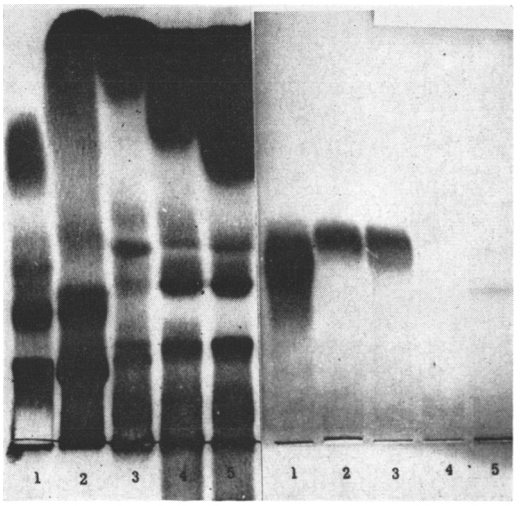

FIG. 2. Plasma leucine naphthylamidases of different species. Picture at left shows starch-gel electropherograms stained with a protein dye of: (1) turtle, *Chelydra*; (2) snake, *Natrix*; (3) chicken, *Gallus*; (4) nonpregnant human female; (5) pregnant human female. Picture on the left is of the other half of the starch gel treated to localize LNAase activities. Anode is to the top of the pictures.

reported by other workers were confirmed (5,10).

The 3 enzyme activities did not vary with sex ($P>.05$) or with stages of estrous cycles in non-mammalian forms. At the time of assay all hens were laying but activities did not differ from those of roosters ($P>.05$). One turtle that was carrying eggs exhibited an LNAase of 331, a CNAase of 7.20. Oxytocinase was absent. The single gravid *Natrix* examined had an LNAase of 312, a CNAase of 4.24 and positive oxytocinase. These levels were not significantly different from those of males ($P>.05$) or nonestrous females ($P>.05$) of the species.

LNAases were located on starch-gel electropherograms of plasma of each species (Fig. 2). LNAase migrated in single bands in *Gallus* and *Natrix* and in two bands in *Chelydra*. No intraspecific differences in the electrophoretic patterns of LNAases were noted. CNAases were localized in the same area of the gel as the LNAases. Attempts to locate the position of oxytocinase in the plasma electrophoretic patterns of the snake and pregnant human were unsuccessful.

Discussion. The presence of CNAase is not a reliable indication of the presence of oxytocinase. Although the plasma of the 3 non-mammalian species had CNAase, only that of the snake exhibited oxytocinase. CNAase and oxytocinase activities may be due to 2 different proteins, as has been recently shown for the related plasma enzymes LNAase and leucine aminopeptidase(15). Berde(7) has compiled several differences between oxytocinase and CNAase.

Plasma oxytocinase in the snake may serve in regulating plasma levels of oxytocic hormone. The neurohypophysis of snakes produces 8-arginine-oxytocin(16). In primates oxytocinase presumably regulates the blood level of oxytocin during pregnancy. Although *Natrix rhombifera* is also a viviparous vertebrate, its oxytocinase activity does not appear to function exclusively in regulation of blood oxytocin levels during pregnancy. Oxytocinase is very active in male as well as female snakes. The chicken also has neurohyposeal oxytocin(17) and is extremely sensitive to circulating oxytocin. However, the chicken has no plasma oxytocinase.

Summary. Plasma of a turtle, a snake and a bird were assayed for leucine naphthylamidase, cystine naphthylamidase and oxytocinase. Levels of both naphthylamidases were very high, did not vary with sex or stages of estrous cycles, and migrated in the same electrophoretic fractions. Oxytocinase was absent from the turtle and bird but was present in both male and female snakes.

1. Fekete, K., *Endokrinologie,* 1930, v7, 364.

2. Page, E. W., *Am. J. Obst. and Gynec.,* 1946, v52, 1014.

3. Green, M. N., Tsou, K. C., Bressler, R., Seligman, M., *Arch. Biochem. & Biophys.,* 1955, v57, 458.

4. Tuppy, H., Nesvadba, H., *Monatsh. Chem.,* 1957, v88, 977.

5. Titus, M. A., Reynolds, D. R., Glendening, M. B., Page, E. W., *Am. J. Obst. and Gynec.,* 1960, v80, 1124.

6. Page, E. W., Titus, M. A., Mohun, G., Glendening, M. B., *ibid.,* 1961, v82, 1090.

7. Berde, B., *Recent Progress in Oxytocin Research,* Charles C Thomas, Springfield, Ill., 1959, p41,

8. Klimek, R., Pietrzycka, M., Gronchowski, J., *Clin. Chim. Acta,* 1962, v7, 398.

9. Schmidt, K. P., *A Checklist of North American*

Amphibians and Reptiles, Univ. of Chicago Press, ed. 6, 1953.

10. Golubow, J., du Vigneaud, V., PROC. SOC. EXP. BIOL. AND MED., 1963, v112, 218.

11. *Pharmacopeia of the United States of America,* 16th Revision, Mack Printing Co., Easton, Pa., 1960 p546.

12. Smithies, O., *Biochem. J.,* 1959, v71, 585.

13. Burstone, M. S., Folk, J. E., *J. Histochem. and Cytochem.,* 1956, v4, 217.

14. Dessauer, H. C., Fox, W., *Proc. XVI Inter. Congr. Zool.,* 1963, v4, 128.

15. Patterson, E. K., Kippel, A., Hsiao, S. H., *J. Histochem. and Cytochem.,* 1961, v9, 609.

16. Heller, H., Pickering, B. T., *J. Physiol.,* 1961, v155, 98.

17. Acher, R., Chauvet, J., Lenci, M. T., *Biochim. Biophys. Acta,* 1960, v38, 344.

Received May 6, 1964. P.S.E.B.M., 1964, v117.

Protection of Functional and Vascular Integrity of the Spleen in Traumatic Shock by Denervation.* (29584)

B. E. M. ZETTERSTROM, C. PALMERIO AND J. FINE

Department of Surgery, Yamins and Kirstein Laboratories, Beth Israel Hospital and Harvard Medical School, Boston, Mass.

Coeliac ganglionectomy prevents the damage to the splanchnic tissues that leads to death in traumatic shock(1). Since the damaged innervated tissues in such a preparation are less sensitive than the denervated tissues to circulating catecholamines, the latter cannot be responsible for the damage. Moreover, whereas the innervated tissues show substantial depletion of their stores of norepinephrine(2,3,4,5), the denervated tissues show no depletion. Hence protection of the latter and survival of the animal may be the result of preventing the release of norepinephrine from its stores in the splanchnic tissues.

This report presents data from studies of the spleen which explore this hypothesis and support its validity.

Method. Under sterile technic and nembutal anesthesia the pedicle of half the spleen in 12 dogs was completely divided transversely, sparing only the large vessels, which were carefully stripped of their nerve supply. Shock was produced, as described below, immediately thereafter in 5 of these dogs, but not until 3 weeks later in the other 7. In 6 additional dogs the pedicle to half the spleen was denervated with xylocaine or nupercain-in-oil injected immediately before inducing shock.

In every experiment the spleen was exteriorized before shock was induced. The flank incision made for this purpose was partly closed around the splenic pedicle. The spleen was covered with a plastic sheet, and kept continuously moistened with saline at 37°C. Care was taken not to induce the shock state until the anesthesia employed to perform the laparotomy had largely worn off.

Hemorrhagic shock was induced by the elevated reservoir technic. The dogs were bled to a mean systolic pressure of 35 mm Hg, and kept at this pressure until they took back 40% of their maximal bleed-out volume, following which normovolemia was restored by transfusion of all the shed blood.

Endotoxic shock was produced by a minimal lethal dose of *E. coli* 0111 B4 endotoxin (1 mg/kg body weight) given intravenously.

Linear contraction of the spleen was measured in millimeters in response to shock, and to intravenous epinephrine or norepinephrine injected before and at various times during shock.

All animals were observed until impending death, at which time they were killed by an overdose of nembutal. Immediately thereafter samples were taken from the 2 halves of the spleen for histological study, for assay

* Aided by a grant from Nat. Inst. Health, Bethesda, Md., and by a contract with Office of Surgeon General, U. S. Army.