Comparison of Susceptibility of Oxytocin and Desamino-Oxytocin to Inactivation by Leucine Aminopeptidase and α-Chymotrypsin.* (27997)

JULIUS GOLUBOW AND VINCENT DU VIGNEAUD
Department of Biochemistry, Cornell University Medical College, New York City

Desamino-oxytocin, an analogue of oxytocin in which the free amino group is replaced by hydrogen, has recently been synthesized in this laboratory(1,2). This analogue possesses higher avian depressor and rat antidiuretic activities than does oxytocin(2,3). Its *in vitro* rat uterine-contracting activity at certain stages of the estrous cycle is higher than that of oxytocin and at other stages approximately the same(4). The milk-ejecting activity in lactating rabbits is approximately the same as that of oxytocin(2,3). It was thought of interest to compare this analogue possessing such striking biological activities with oxytocin in their susceptibility to enzymatic inactivation. As a first step in this direction, 2 highly purified proteolytic enzymes, hog kidney leucine aminopeptidase (LAP) and α-chymotrypsin were used.

Materials and methods. LAP was prepared from freshly obtained hog kidneys by the procedure of Hill and Smith(5). The precipitate resulting from the acetone fractionation step was suspended in 0.005 M Tris buffer at pH 8.0 containing 0.005 M $MgCl_2$ and exhaustively dialyzed against the same solution. The dialyzed solution contained 1.91 mg of protein nitrogen per ml. The proteolytic coefficient C_1, determined against L-leucinamide hydrochloride(6), was found to be 11. The enzyme preparation was refractory to 0.001 M diisopropylfluorophosphate. This solution containing 21 LAP units per ml was used as the source of LAP. The solution was stored in small volumes in the frozen state without diminution in activity, which confirms the observation of Hill and Smith(5). The procedure employed in typical experiments was as follows. LAP (0.40 ml) was first activated by mixture with 0.10 ml of 0.005 M Tris buffer-0.005 M $MgCl_2$, pH 8.0, and 0.50 ml of 0.10 M Tris buffer-0.005 M $MgCl_2$, pH 8.5, and incubation of the solution at 40°C for 1 hour. An aliquot (0.25 ml) of the activated LAP solution was added to a mixture of 0.50 ml of 0.10 M Tris buffer-0.01 M $MgCl_2$, pH 8.5, 0.50 ml of an aqueous solution of oxytocin or desamino-oxytocin containing 1 unit of avian depressor activity/ml, and 1.25 ml of 0.05 M Tris buffer-0.005 M $MgCl_2$, pH 8.5. Immediately after addition of LAP solution, the digestion mixture was mixed rapidly and a 1-ml aliquot was removed, heated in a boiling water bath for 2 minutes, then cooled in an ice bath. The remainder of the digestion solution was allowed to incubate for varying times before the enzyme activity was stopped as described for the zero time sample. Substrate and enzyme controls were performed by using 0.05 M Tris buffer-0.005 M $MgCl_2$, pH 8.0, in place of LAP, and water in place of oxytocin or desamino-oxytocin. In these control experiments aliquots were removed at the same time intervals as were used in the experiments employing the complete reaction mixture. Avian depressor assays were carried out according to the official method for oxytocin by measuring the fall in blood pressure of the chicken as prescribed in the Pharmacopeia(7) with a surgical procedure modified to that of Munsick, Sawyer, and van Dyke (8). In some instances the aliquots were frozen and their biological activity was determined several days later. No difference was observed in the results whether the solutions were assayed immediately or kept frozen for several days.

The typical procedure for the chymotrypsin experiments was as follows. One ml of an aqueous solution of oxytocin or desamino-oxytocin containing 20 units of avian depressor activity/ml was mixed with 0.50 ml of 0.6 M $CaCl_2$, 0.50 ml of 0.2 M Tris buffer, pH 8.0, 1.0 ml of water, and 1.0 ml of 0.1 mg/ml α-chymotrypsin.† Immediately after the addi-

* This work was supported in part by a grant from Nat. Heart Inst., U.S.P.H.S.

† Lot No. 6004-5, Worthington Biochemical Corp., Freehold, N. J. A 1 mg/ml solution was prepared in 0.001 *N* HCl. One ml of this solution was mixed with 9 ml of 0.12 M $CaCl_2$ to give a 0.1 mg/ml solution.

tion of enzyme, 2 ml of the reaction solution was removed, heated in a boiling water bath for 2 minutes, and then kept frozen until assayed for biological activity. The remainder of the reaction mixture was incubated at 37°C for 3½ hours and then inactivated and removed for assay in the same manner as was the zero time sample.

Results and discussion. Incubation of oxytocin with LAP for 24 hours at 40°C at a substrate concentration of 0.2 unit of avian depressor activity per ml and an enzyme concentration of 0.84 LAP unit per ml resulted in destruction of approximately 50% of the activity. On the other hand, when desamino-oxytocin represented the substrate none of the activity was destroyed under these conditions. An increase of the incubation time to 48 hours did not alter the results with either compound. In the control experiments oxytocin and desamino-oxytocin were both found to be stable to incubation for 48 hours in the absence of enzyme and LAP itself was found to have no demonstrable avian depressor activity.

The fact that LAP was without any effect on desamino-oxytocin is in agreement with the reported specificity of this enzyme(6). Our results on the effect of LAP on the biological activity of oxytocin complement those of Hill and Smith on the hydrolysis of oxytocin by this enzyme. On the basis of ninhydrin determinations, they showed that oxytocin is attacked very slowly by highly purified LAP (5). Each amino acid present in oxytocin was found in dialysates of the reaction mixture, although less than stoichiometric quantities of tyrosine and isoleucine were present. They noted that peptides lacking a disulfide bridge are hydrolyzed much more rapidly than oxytocin by LAP.

Incubation of desamino-oxytocin with α-chymotrypsin for 3½ hours at 37°C at a substrate concentration of 5 units of avian depressor activity per ml and an enzyme concentration of 0.025 mg per ml resulted in complete loss of activity, whereas 70% of the activity disappeared when oxytocin was used in place of desamino-oxytocin. When the destruction of these 2 compounds was measured after 90 minutes, it was found that all of the activity of the desamino-oxytocin had disappeared while approximately 50% of the activity of oxytocin was still present. These data indicate that desamino-oxytocin is more susceptible to the action of α-chymotrypsin under these conditions than is oxytocin. The inactivation of oxytocin by chymotrypsin confirms the early work of Croxatto *et al.*(9,10).

Summary. The susceptibility of oxytocin and desamino-oxytocin to attack by hog kidney leucine aminopeptidase and α-chymotrypsin has been investigated by measurement of their avian depressor activity before and after incubation with the enzymes. On this basis desamino-oxytocin appeared to be resistant to the action of leucine aminopeptidase whereas 50% of the activity of oxytocin was lost after treatment with the enzyme. On the other hand, desamino-oxytocin was found to be completely inactivated by α-chymotrypsin under conditions in which some of the activity of oxytocin still remained.

The authors wish to express their appreciation to Miss Shirley R. Pomeroy, Miss Lenore McAteer, and Mr. Hans Holzhauser for carrying out the bioassays.

1. du Vigneaud, V., Winestock, G., Murti, V. V. S., Hope, D. B., Kimbrough, R. D., Jr., *J. Biol. Chem.*, 1960, v235, PC64.

2. Hope, D. B., Murti, V. V. S., du Vigneaud, V., *ibid.*, 1962, v237, 1563.

3. Chan, W., du Vigneaud, V., *Endocrinology*, in press.

4. Chan, W., O'Connell, M., Pomeroy, S. R., *ibid.*, in press.

5. Hill, R. L., Smith, E. L., *J. Biol. Chem.*, 1957, v228, 577.

6. Smith, E. L., in *Methods in Enzymology*, Vol. II, Colowick, S. P., Kaplan, N. O., Eds., Academic Press Inc., New York 1955, p88.

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

8. Munsick, R. A., Sawyer, W. H., van Dyke, H. B., *Endocrinology*, 1960, v66, 860.

9. Croxatto, H., Alliende, J., Croxatto, R., *Rev. med. y aliment Santiago, Chile*, 1942-1943, v5, 230.

10. Croxatto, H., Croxatto, R., Illanes, G., Salvestrini, H., *ibid.*, 1942-1943, v5, 300.

Received October 5, 1962. P.S.E.B.M., 1963, v112.