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Crystalline Deamino-dicarba-oxytocin

Preparation and Some Pharmacological Properties*

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SUMMARY

Deamino-dicarba-oxytocin, a synthetic analogue of oxytocin in which both sulfur atoms are formally replaced by methylene moieties and the terminal amino group by a hydrogen atom, has been obtained as highly pure crystalline material. Upon bioassay this analogue exhibited 160 ± 4.4 units/mg of oxytocic activity, 44 ± 1.7 units/mg of avian vasodepressor activity, 140 ± 7 units/mg of rabbit milk-ejecting activity, 0.10 ± 0.02 unit/mg of rat pressor activity, and 4.7 ± 0.3 units/mg of rat antidiuretic activity.

Cumulative dose-response studies on the isolated rat uterus, mounted in magnesium-free van Dyke–Hastings solution, showed that the maximal attainable contractile response (intrinsic activity) of deamino-dicarba-oxytocin was only 68% of that of the natural oxytocic principle. Deamino-dicarba-oxytocin had a pD_2 value (the negative logarithm of the concentration of the analogue that will evoke a half-maximal effect) of 8.35 ± 0.07; the pD_2 value is a measure of the affinity of the analogue for its uterine receptor.

The presence of 0.5 mM Mg^{++} in the ambient fluid potentiated the contractile capacity of the uterus in response to deamino-dicarba-oxytocin; the intrinsic activity was increased by 35% over its original value.

Solutions in deamino-dicarba-oxytocin were found to be resistant to inactivation during lyophilization from water (pH 6) or aqueous triethylamine (pH ∽ 9).

INTRODUCTION

Several lines of experimental evidence led to the suggestion that the initial step in the "water-conserving" action of neurohypophysial hormones on membrane permeability involves the formation of a mixed hormone-receptor disulfide bond (1, 2). However, subsequent studies revealed that the presence of a disulfide bridge in neurohypophysial hormones is not required for these peptides to evoke this characteristic biological effect (3, 4). Analogues of oxytocin (Fig. 1) in which one or both of the sulfur atoms are substituted by methylene moieties and in which the terminal amino group is replaced by hydrogen (referred to in this paper as "deamino-monocarba-oxytocin" and "deamino-dicarba-oxytocin," compound I) (5) played an important role in arriving at

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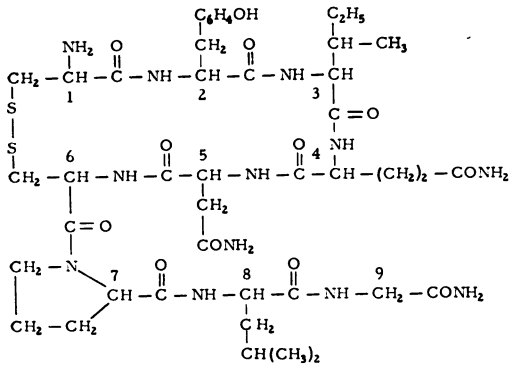

FIG. 1 *Structure of oxytocin*
Numbers indicate the positions of the individual amino acid residues.

the latter conclusion. For the first time one of these hormone analogues has been secured in crystalline form.

This report describes the preparation of *crystalline* deamino-dicarba-oxytocin, some of its chemical properties, and its potencies when assayed for biological activities characteristic of oxytocin. Moreover, the complete dose-response relationship of deamino-dicarba-oxytocin with respect to contractility of the isolated rat uterus has been determined both in the presence and in the absence of magnesium ions. An ancillary feature of these latter experiments was the opportunity to test definitively a recent proposal (6) that the long-known potentiating effect of magnesium ions on the uterotonic response to neurohypophysial hormones may involve coordination of the metal ion with the S—S group of the polypeptides.

MATERIALS AND METHODS

Crystalline deamino-dicarba-oxytocin (I). Crude deamino-dicarba-oxytocin (7) (1.93 g) was charged on a column of silica gel (Merck, 70–325 mesh, 5.0 × 23.0 cm) which had been equilibrated with a mixture of 1-butanol–acetic acid–water (4:1:1 by volume) (S_1). The column was eluted with the same solvent system, and the effluent, monitored continuously for ultraviolet absorption at 280 mμ, was collected in 10-ml fractions (Fig. 2). The fractions in tubes 58–78 were collected and concentrated under reduced pressure, and the resulting residue was dissolved in water (30 ml). The aqueous solution was passed through a column of CM-Sephadex C-25 (H^+ form) (3.5 × 9.0 cm) and then lyophilized; yield, 1.52 g (79% of the crude product). The lyophilized material was found to give a single spot on thin-layer chromatography (silica gel G) with solvent system S_1. Three batches of purified compound I (4.2 g) were combined and dissolved in warm water (45 ml) containing 0.2 ml of acetic acid. Insoluble material was removed by filtration and the filtrate was stored at 4° for 6 days. Long needles appeared in the solution during storage; these crystals were collected by filtration, washed with a small volume of water, and dried over P_2O_5 *in vacuo* at room temperature; yield, 3.6 g (85.7%), $[\alpha]_D^{25}$ −89.1° (*c*, 0.55, in water).

$C_{45}H_{69}N_{11}O_{12}\cdot 3H_2O$
Calculated: C 53.5, H 7.48, N 15.3
Found: C 53.6, H 7.41, N 15.2

This material gave a ninhydrin-negative and iodine vapor-positive single spot with each of the following chromatographic procedures: thin-layer chromatography on silica gel G, using solvent system S_1, and paper chromatography using the solvent systems 1-butanol–pyridine–water (4:1:1 by volume) and 1-butanol–acetic acid–pyridine–water (15:3:10:6 by volume). A sample hydrolyzed with 6 N HCl in the presence of phenol in a sealed tube for 48 hr gave, upon amino acid analysis, the following ninhydrin-

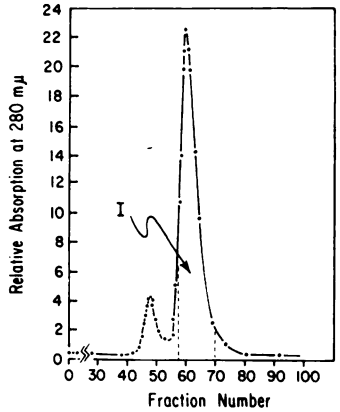

FIG. 2 *Purification of deamino-dicarba-oxytocin (I) by column chromatography*
For experimental details, see the text.

active components, with aspartic acid taken as unity: Tyr, 1.01; Ile, 1.03; Glu, 0.99; Asp, 1.00; Asn, 1.03; Pro, 1.03; Leu, 0.99; Gly, 0.98; ammonia, 3.12.

Stability of deamino-dicarba-oxytocin to lyophilization. Two milligrams of crystalline deamino-dicarba-oxytocin were dissolved in 10 ml of glass-distilled water. Two 0.5-ml aliquots of this solution were transferred to test tubes. To one test tube 1 ml of glass-distilled water was added, and to the other, 1 ml of freshly distilled triethylamine. The solution containing water was at pH 6, and the one containing the aqueous triethylamine was approximately at pH 9. Both test tubes were capped with Parafilm and left at room temperature for 4.5 hr; then the two samples were frozen and lyophilized. The sample originally kept at pH 6 yielded a fluffy white powder, while the other, originally containing triethylamine, yielded a grayish gummy mass. Distilled water (0.5 ml) was added to each lyophilized sample to reconstitute the original concentration. The two samples and the stock solution were diluted identically for avian depressor assay as described by Ferrier *et al.* (8). A four-point procedure was used to determine the activity of the original deamino-dicarba-oxytocin sample (A), the sample lyophilized from water at pH 6 (B), and the sample lyophilized from aqueous triethylamine at approximately pH 9 (C). Three low doses of analogue (one for each sample) were given with three high doses at random. The results showed that deamino-dicarba-oxytocin retained its full biological activity during lyophilization from either water or dilute triethylamine. The activity ratios were: B:A = 1.02, C:A = 1.03, and C:B = 1.04.

Bioassay procedures. Assays for antidiuretic activity were performed on anesthetized, hydrated Sprague-Dawley male rats according to the method of Jeffers *et al.* (9) as modified by Sawyer (10). Rat pressor assays were carried out on atropinized, urethane-anesthetized male rats as described in the United States Pharmacopeia (11). Oxytocic assays were performed on isolated uterine horns from rats in natural estrus according to the method of Holton (12), modified by Munsick (13) with the use of magnesium-free van Dyke–Hastings solution as the bathing fluid. Milk-ejecting activity was determined in anesthetized, lactating rabbits following the procedure of Chan (14). Avian vasodepressor assays were performed on conscious chickens according to the procedure employed by Munsick, Sawyer, and van Dyke (15). The biological activities were measured against the USP Posterior Pituitary Reference Standard as control, and no correction for the 3 molecules of water per molecule of crystalline compound I was made. In all bioassays the four-point design was used, and standard errors were calculated according to the method of Bliss (16).

RESULTS AND DISCUSSION

Pharmacological studies have shown that the replacement of the terminal amino group by hydrogen in oxytocin (Fig. 1) does not have a detrimental effect on the biological potency of this neurohypophysial hormone; in fact, crystalline deamino-oxytocin has been found to be more potent than oxytocin in a number of bioassay systems (8). Two laboratories have described the preparation by independent routes of an analogue of deamino-oxytocin in which both sulfur atoms were substituted by methylene moieties (deamino-dicarba-oxytocin) (5, 7).

Although the amorphous preparation of Kobayashi *et al.* (7) proved to have a higher degree of biological activity than observed in earlier studies,[1] thin-layer chromatography [unlike paper electrophoresis and paper chromatography (7)] revealed the presence of a minor impurity on develop-

[1] Since Kobayashi *et al.* (7) reported their preliminary biological activities, extensive bioassays have been carried out in our laboratories on this amorphous material and the following potency values were found: 134 ± 6.4 units/mg of rat oxytocic activity, 33.2 ± 1.4 units/mg of avian vasodepressor activity, 0.10 ± 0.04 unit/mg of rat pressor activity, and 6.8 ± 0.6 units/mg of rat antidiuretic activity. For amorphous deamino-dicarba-oxytocin prepared in another laboratory (5), the following values were reported: 9 IU/mg of rat oxytocic activity, 2.5 IU/mg of avian vasodepressor activity, 0.7 IU/mg of antidiuretic activity, and no rat pressor response to doses up to 20 μg.

TABLE 1

Crystalline deamino-dicarba-oxytocin and deamino-oxytocin: comparison of biological potencies[a]

Compound	Depressor (fowl)	Oxytocic (rat)	Milk-ejecting (rabbit)	Pressor (rat)	Antidiuretic (rat)
Deamino-dicarba-oxytocin	44 ± 1.7	160 ± 4.4	140 ± 7	0.10 ± 0.02	4.7 ± 0.3
Deamino-oxytocin[b]	975 ± 24	803 ± 36	541 ± 13	1.44 ± 0.06	19.0 ± 1

[a] Expressed in USP units/mg.

[b] Values reported by Ferrier *et al.* (8).

ment with iodine vapor. It was hoped that this minor component would be removed if the crystallization of deamino-dicarba-oxytocin could be achieved. Unfortunately, while the dicarba analogue did crystallize from dilute acetic acid [a solvent from which deamino-oxytocin had previously been crystallized (8)], the impurity did not diminish appreciably even after repeated recrystallizations. Hence, the deamino-dicarba-oxytocin preparation was subjected to chromatography on a silica gel column; upon elution with the solvent system 1-butanol–acetic acid–water (4:1:1 by volume), chromatographically pure deamino-dicarba-oxytocin was obtained from the slower moving, major peak (Fig. 2). Subsequently, crystallization of material associated with this peak from dilute acetic acid gave compound I as fine needles containing 3 molecules of water per molecule of peptide.

The biological activities of crystalline deamino-dicarba-oxytocin in several assay systems are compared in Table 1 with those of crystalline deamino-oxytocin. It can be seen that compound I exhibits 5–20% of the activity of deamino-oxytocin. The crystalline deamino-dicarba-oxytocin displays a considerably higher degree of biological activity than was reported by some workers for amorphous preparations. Moreover, these findings confirm earlier studies (3–5, 7, 17–19) on amorphous preparations of the deamino-monocarba and deamino-dicarba analogues, in which it was concluded that the disulfide bridge is not essential for the biological activity of neurohypophysial hormones.

It has been established that the responsiveness of the isolated rat uterus to naturally occurring neurohypophysial peptides and synthetic analogues can be affected by changes in the magnesium ion concentration in the assay solution (for a recent summary, see ref. 20); similar observations have been described for vascular and intestinal smooth muscle and mammary gland myoepithelium (21–23). Pharmacological studies involving the response *in vitro* of the rat uterus to cumulative increases in the dosage of neurohypophysial peptides suggested that a concentration of 0.5 mM Mg^{++} in the ambient fluid increases the affinity of the peptides for their uterine receptor (21, 24–28); however, magnesium ions can also alter the intrinsic activity of these agents; i.e., the ability of the peptide-receptor complex to provide an effective stimulus-response coupling (24, 27, 28). The finding of an increased intrinsic activity in the presence of 0.5 mM Mg^{++} for 8-lysine-vasopressin, as well as for oxytocin (27, 28), emphasizes the fact that small alterations in intrinsic activity can readily escape attention.

With the foregoing considerations as background we compared the response of the isolated rat uterus to deamino-dicarba-oxytocin and to oxytocin in Mg^{++}-free solution and subsequently in a solution containing 0.5 mM Mg^{++}. Qualitative differences in the uterotonic responses to deamino-dicarba-oxytocin and oxytocin were noted in the cumulative dose-response experiments when the latter were carried out in the absence of Mg^{++}. For example, in the case of deamino-dicarba-oxytocin, but not in the case of oxytocin, the onset of the response appeared to be delayed, and partial relaxation of the uterus occurred promptly after the attainment of maximal contraction (compare curves *A* and *C*, Fig. 3). Nevertheless, deamino-dicarba-oxytocin was more difficult to "wash out" after completion of a dose-re-

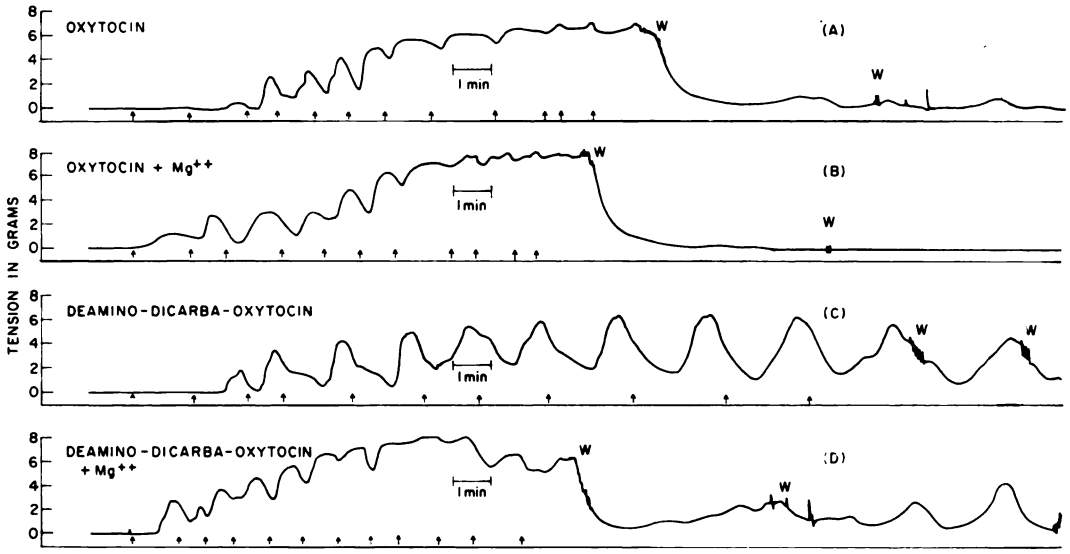

FIG. 3 *A typical response pattern of isolated rat uterus (in natural estrus) to increasing (cumulative) doses of oxytocin and deamino-dicarba-oxytocin*

Oxytocin and deamino-dicarba-oxytocin (curves *A* and *C*, respectively) in magnesium-free van Dyke-Hastings solution. Oxytocin and deamino-dicarba-oxytocin (curves *B* and *D*, respectively) with 0.5 mM Mg^{++} added to the bathing fluid. As detailed previously (27), the contractions were recorded isometrically and the doses of agonist were increased geometrically according to the $\frac{1}{3}$ $\log_{10}$ procedure until a maximum response was reached. In the experiments shown, the starting concentration of oxytocin was 0.1 mμM and that of deamino-dicarba-oxytocin was 0.52 mμM. An arrow indicates application of agonist (milliunits); *W* indicates washout.

sponse study than was oxytocin, as indicated by a prolonged, albeit partial, contractile response (compare curves *A* and *C*, Fig. 3). When 0.5 mM Mg^{++} was added to the bath, the general pattern of the uterine response to deamino-dicarba-oxytocin more closely resembled the response to oxytocin. At the higher dose levels the contractions remained at their maximal levels for longer periods of time, and there was little evidence of relaxation between successive contractions.

The affinity constant (expressed in terms of the pD_2 value, which is the negative logarithm of the concentration of agonist that will evoke a half-maximal effect; see ref. 29) of deamino-dicarbo-oxytocin in the absence of Mg^{++} was found to be 8.35 ± 0.07, and the intrinsic activity (measured as the maximal attainable contractile response) of deamino-dicarba-oxytocin was only 68% of the intrinsic activity of oxytocin (Fig. 4). However, in the presence of 0.5 mM Mg^{++} there was a striking increase in the maximal contractile response to deamino-dicarba-oxytocin, which then amounted to 92% of that of oxytocin, corresponding to an increase in intrinsic activity of $[(0.92 - 0.68)/0.68] \times 100 = 35\%$. The Mg^{++}-induced increase in intrinsic activity of the analogue proved to be highly significant statistically ($p < 0.001$). The addition of Mg^{++} to the assay solution also enhanced the affinity of the analogue for the uterine receptor, the pD_2 value increasing to 8.55 ± 0.08.

The above data show that oxytocin and deamino-dicarba-oxytocin differ not only in their affinity for the uterine receptor, but also in their capacity to effect maximal stimulation of the contractile elements of this tissue. Deamino-dicarba-oxytocin is strongly potentiated by 0.5 mM Mg^{++}, a finding which militates against the suggestion that potentiation by Mg^{++} depends on Mg^{++} activation of a hormone-receptor thiol-disulfide interchange (25), and is incompatible with the hypothesis that the S—S

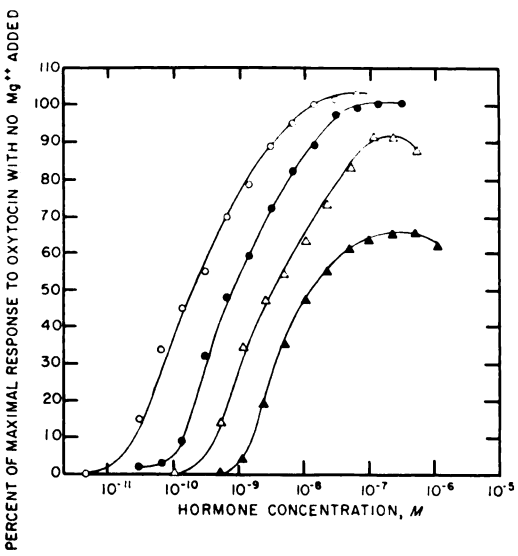

FIG. 4 *Cumulative dose-response curves of oxytocin and deamino-dicarba-oxytocin in Munsick's fluid with and without 0.5 mM added Mg^{++}*

●——●, oxytocin without added Mg^{++}; ○——○, oxytocin with added Mg^{++}; ▲——▲, deamino-dicarba-oxytocin without added Mg^{++}; △——△, deamino-dicarba-oxytocin with added Mg^{++}. The experimental procedure followed for obtaining dose-response curves has been described previously (27). Each curve represents an average of at least nine experiments involving five different rats.

group of neurohypophysial peptides is actively involved in the formation of a peptide-metal-receptor complex (6). Moreover, on the basis of data obtained in the present study and in previous studies of the potentiation by Mg^{++} of 8-alanine-oxytocin, 8-alanine-oxypressin, and their deamino analogues (27), the terminal amino group appears not to be the critical moiety for a physiologically significant coordination involving magnesium. The latter contention is also supported by the finding that the contractile effect of deamino-oxytocin on avian smooth muscle is potentiated by Mg^{++} (30).

Natural and synthetic neurohypophysial peptides have repeatedly exhibited a loss of biological activity in the course of certain procedures commonly employed for peptide isolation, such as concentration and lyophilization (31, 32). A most striking example of this phenomenon was encountered in the case of diseleno-oxytocin (an analogue of oxytocin in which the disulfide bridge is substituted by a diseleno bridge), which lost 87% of its avian vasodepressor activity during a single lyophilization from water at pH 6.0 (33). In the same study analogous experiments with deamino-oxytocin, deamino-1-seleno-oxytocin, and deamino-6-seleno-oxytocin indicated that the inactivation occurs at the lyophilization step. Evidence is accumulating which implicates disulfide interchange as the critical step in this denaturation (34–36). In this context, it was of interest to investigate whether deamino-dicarba-oxytocin, in which the disulfide bridge is replaced by two methylene moieties, would retain its biological activity during lyophilization. Hence we subjected one sample of the crystalline analogue to lyophilization from water, and another sample to lyophilization from aqueous triethylamine, the analogue having been kept for 4.5 hr at room temperature with base concentrations as described by Yamashiro *et al.* (36). The recovery of fully active peptide from both experiments strongly supports the contention that disulfide bond interchange is the critical step in the denaturation of neurohypophysial hormones during lyophilization.

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