STUDIES ON PHARMACOLOGICAL AND BIOCHEMICAL PROPERTIES OF DEAMINO-DICARBA-[GLY\textsuperscript{7}]-OXYTOCIN (Y-5350)

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Abstract Pharmacological and biochemical properties were investigated on deamino-dicarba-[Gly\textsuperscript{7}]-oxytocin (Y-5350). This is a newly developed compound, in which the disulfide bond and [Pro\textsuperscript{7}] of deamino-oxytocin are substituted by an ethylene linkage and glycine respectively. Bioassayed Y-5350 exhibited 202.6 u/mg of oxytocic activity, 4.6 u/mg of avian depressor activity, 441.2 u/mg of rat milk-ejecting activity and 0.02 u/mg of rat antidiuretic activity, however, a depressor activity was also evident in rats. In particular, the duration of antidiuretic activity was short. Moreover, the oxytocic activity in pregnant rats and rabbits was weak in comparison with oxytocin. Cumulative dose-response studies were carried out on the isolated rat uterus using van Dyke-Hasting solution. The intrinsic activity was the same level as that of oxytocin, and the pD\textsubscript{2} value was 8.62. Oxytocic activity was much enhanced by the existence of 0.5 or 2 mM magnesium ion in vitro. However, the agreement between in vivo and in vitro oxytocic activity was not complete when assay was carried out in the presence of 2 mM magnesium ion. Oxytocin was inactivated by the serum of pregnant women. In the experiment using rats, oxytocin was also destroyed by the extracts of uterus, kidney and liver. In contrast, Y-5350 was not destroyed by any of the enzyme solutions mentioned above.

The role of disulfide linkage in neurohypophysial hormones has been widely investigated (1–7). After investigating the biological activities of crystalline deamino-dicarba-oxytocin (Y-4266), in which the disulfide linkage of deamino-oxytocin was substituted by an ethylene linkage, Yamanaka et al. (8) concluded that the disulfide linkage was not essential for the biological activity of neurohypophysial hormones as reported on an amorphous product of Y-4266 (9–10). Thus, many deamino-dicarba-analogues of neurohypophysial hormones were synthesized and biological activities were investigated. Out of these analogues, deamino-dicarba-[Gly\textsuperscript{7}]-oxytocin (Y-5350) proved to be of great interest, and the pharmacological and biochemical properties were studied in detail.

MATERIALS AND METHODS
Deamino-dicarba-[Gly\textsuperscript{7}]-oxytocin (Y-5350) and deamino-dicarba-oxytocin (Y-4266) have these chemical structures.

\[
\begin{align*}
\text{CH}_2 & \quad - \quad \text{CH}_2 & \quad - \quad \text{CH}_2 & \quad - \quad \text{CH}_2 \\
\text{CH}_2\text{-CO-Tyr-Ile-Gln-Asn-HN-CH-CO-Gly-Leu-Gly-NH}_2 \\
& \quad \quad \text{Y-5350}
\end{align*}
\]
These compounds were synthesized in our laboratory. The following were used, oxytocin (Sankyo, Syntocinon®), posterior pituitary reference standard (The Pharmacopoeia of Japan 8th edition), diethylstilbestrol, urethane, heparin, phenobarbitone sodium and phenoxybenzamine.

The following apparatuses were used, the electric manometer (Nihon Kohden, MP-4T), the carrier amplifier (Nihon Kohden, RP-3), the recorder (Hitachi, QPD-73 or Rikadenki, B-24), the force displacement transducer (Nihon Kohden, SB-1T), the conductivity meter (M&S Instrument Trading Inc.).

Isolated uterus of rat

Virgin Wistar rats weighing 150–200 g were used. Oestrus was induced by diethylstilbestrol (1 mg/kg, s.c.), 24 hr and 48 hr prior to the start of experiments. The animal was stunned by a blow on the head and uterine horn was excised and isolated. Uterine contractions were recorded isotonically on a smoked drum. The weight of load on the uterus was 0.5 g.

1. Assay of oxytocic activity: The isolated uterus was suspended in de Jalon solution (31–32°C), which was continuously oxygenated. The results were assessed statistically by the four-point assay procedure.

2. Influence of magnesium ion on uterine contractile activity: The isolated uterus was suspended in modified van Dyke-Hasting solution, with (2 mM or 0.5 mM) or without the magnesium ion. A mixture of oxygen and carbon dioxide (95: 5) was bubbled through the bathing solution. The method of cumulative dose-response curve (11) was employed for the analysis of affinity, intrinsic activity and influence of magnesium ion.

Isolated strip of rat mammary gland

The procedure used was generally the same as that of Rydén and Sjöholm (12). Healthy lactating Wistar rats weighing about 300 g were selected on the 3–15 day after parturition. The rats were sacrificed by a blow on the head and the lower mammary glands were isolated. A strip was suspended in Tyrode solution at 38°C. A mixture of oxygen and carbon dioxide (95: 5) was passed through the solution and an initial load of 160 mg was applied. The suspended strips were attached to a force displacement transducer. With this transducer and a carrier amplifier, isometric contractions were measured and recorded. The results were assessed statistically by a four-point assay procedure.

Mammary gland in vivo

Milk-ejection activity was tested according to the modified method of Bisset et al. (13). Healthy lactating Wistar rats weighing about 300 g were used on the 3–15 day after parturition. The rats were anesthetized with urethane (1.5 g/kg, s.c.) within 30–60 min after the separation from their litters. The nipple tip of an abdominal mammary gland was incised and the duct was cannulated with a polyethylene tube connected to an electric manometer, which had been filled with rat serum. By an electric manometer and a carrier am-
plifier, changes of intra-mammary pressure were measured and recorded. The left femoral vein was cannulated with polyethylene tube for intravenous injection. The results were assessed statistically by a four-point assay procedure.

**Blood pressure of chicken**

The avian depressor activities were investigated by the modified method of Stürmer (14). Healthy white Leghorn roosters weighing 1.8–2.3 kg were used. The birds were anesthetized with phenobarbitone sodium (200 mg/kg, i.m.) and the ischiatic artery was cannulated. This cannula was connected with an electric manometer and a carrier amplifier. Changes of arterial blood pressure were recorded. Test solutions were injected in the left wing vein. The results were assessed statistically by a four-point assay procedure.

**Blood pressure of rat**

The assay method established by Dekanski (15) was modified. Male Wistar rats weighing about 300 g were used. At 18 hr prior to the assay the rats were pretreated with phenoxybenzamine (10 mg/kg, s.c.). On the day of assay the rats were anesthetized with urethane (1.75 g/kg, s.c.). The trachea was cannulated to ensure a free airway. The right carotid artery was exposed and cannulated with polyethylene tube, which was connected to an electric manometer. With an electric manometer and a carrier amplifier, changes of mean arterial blood pressure were measured and recorded. Test solutions were injected in the left femoral vein.

**Conductivity of rat urine (Antidiuretic activity)**

The assay method established by Share (16) and Sawyer (17) was modified. Male Wistar rats weighing about 200 g were fasted for about 16 hr, after which an oral administration of 50 ml/kg of 12% ethanol was given. Polyethylene tube was inserted into the jugular vein for the injection of test solutions. The urinary bladder was exposed through a low, midline, abdominal incision. The urethra was ligated, and the bladder catheterized with polyethylene tube provided with an electrode. The electrode consisted of two short stainless steel tubes separated by a short polyethylene tube. The abdomen was then sutured. Next 30 ml/kg of 2.5% ethanol was given via a stomach tube. Urine conductivity was induced by the electrode of the cannula in the bladder, and the changes were amplified by a conductivity meter and recorded. During the assay 2.5% ethanol was given via stomach tube, with the intention to compensate the urinary loss of water in the animals; this procedure also served to maintain the anesthesia throughout the experiment.

**Uterus and vagina in vivo**

Healthy white rabbits weighing about 3.6 kg (on the 30–31 day of pregnancy) and Wistar rats weighing about 350 g (on the 20–22 day of pregnancy) were used. Laparotomy was performed under urethane (1.5 g/kg, s.c.) anesthesia. A rubber balloon was inserted into one of the uterine horns after careful removal of the one foetus. A polyethylene cannula was connected to the balloon. After closing the incision, the other end of the cannula was led to an electric manometer. With an electric manometer and a carrier amplifier, changes of intra-uterine pressure were measured and recorded. Changes
of intra-vaginal pressure were also recorded on some occasions. The left femoral vein was cannulated with polyethylene tube for intravenous injection.

**Biochemical properties**

(1) **Preparation of enzyme solutions:** Wistar rats weighing about 180 g in the stage of oestrus induced by diethylstilbestrol and Wistar rats weighing about 350 g on the 20–22 day of pregnancy were used. Rats were stunned by a blow on the head and exsanguinated, and three viscera were removed and weighed. A mixture of 1 g of the wet tissue and 0.1 M phosphate buffer (9 ml for kidney or liver, 4 ml for uterus) was homogenated at 0°C and centrifuged at 700 g for 10 min to remove cell debris. The supernatant was separated and used as the enzyme solution. Rat serum was obtained by the centrifugation of arterial blood at 700 × g for 10 min and used as an enzyme solution without dilution. Serum and saliva were obtained from women in the last month of pregnancy and stored in a freezer. On the day of experiment, these were allowed to melt and used as enzyme solution without dilution.

(2) **Preparation of substrate:** Y-5350 or oxytocin was dissolved in (1:400) acetic acid in a concentration of about 8 units per ml. These were diluted 50 times with 0.1 M phosphate buffer solution and used as the substrate.

(3) **pH of phosphate buffer solution:** The pH of solution was 6.5 in the study of the enzymatic action using viscera and saliva, and 7.4 in the study of the enzymatic action using the serum.

(4) **Determination of enzymatic activities:** To 0.6 ml of the enzyme solution, preincubated in 2.6 ml of the 0.1 M phosphate buffer solution, was added 0.8 ml of the substrate solution and incubation at 37°C was carried out. The residual oxytocic activities were determined by analysing the aliquots of reaction mixture. These were tested on the isolated rat uterus according to the method described above.

**RESULTS**

**Oxytocin- and vasopressin-like activities**

The biological activities of Y-5350, Y-4266 and oxytocin are shown in Table 1.

<table>
<thead>
<tr>
<th>Activities</th>
<th>Oxytocin units mg</th>
<th>Deamino-dicarba-[Gly']-oxytocin (Y-4266)</th>
<th>Deamino-dicarba-[Gly']-oxytocin (Y-5350)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxytocin (rat, in vitro)</td>
<td>450</td>
<td>127.4 (118.2–136.6)</td>
<td>202.6 (184.4–222.9)</td>
</tr>
<tr>
<td>Milk ejection (rat, in vivo)</td>
<td>450</td>
<td>143.4 (108.0–219.7)</td>
<td>441.2 (382.6–535.7)</td>
</tr>
<tr>
<td>Milk ejection (rat, in vitro)</td>
<td>450</td>
<td>740.5 (636.4–892.2)</td>
<td>824.8 (647.6–992.2)</td>
</tr>
<tr>
<td>Avian depressor (fowl)</td>
<td>450</td>
<td>37.8 (31.6–45.2)</td>
<td>4.6 (3.9–5.0)</td>
</tr>
<tr>
<td>Pressor (rat)</td>
<td>5</td>
<td>&lt;0.25 depressor</td>
<td></td>
</tr>
<tr>
<td>Antidiuretic (rat)</td>
<td>5</td>
<td>2</td>
<td>~0.02</td>
</tr>
</tbody>
</table>

(J.P. units mg)  * 95% confidence limit
Y-5350 was shown to be more active in respect of oxytocic effect than of the antidiuretic. Moreover Y-5350 had very slight influence on the cardiovascular system as shown in its low activity on the blood pressure of the fowl and rat. Unlike the other compounds, Y-5350 exhibited a temporary depressor action in rats. Typical depressor and antidiuretic actions of Y-5350 are shown in Fig. 1 and Fig. 2, respectively.

Affinity constant and intrinsic activity

The cumulative dose-response curves of Y-5350 and oxytocin are shown in Fig. 3, in which the figures represent the average value of three different rat uteri. The affinity constants of Y-5350 and oxytocin, in the absence of magnesium ion, were 8.62 and 9.13, which were expressed in terms of the pD₂ value. The intrinsic activity of Y-5350 was almost...
the same level as that of oxytocin. However, the slope of the cumulative dose-response curve of Y-5350, in the dose range from $1 \times 10^{-9}$ to $1 \times 10^{-8}$ M, was slightly steeper than that of oxytocin in the dose range from $3 \times 10^{-10}$ to $3 \times 10^{-9}$ M.

**Influence of magnesium ion on oxytocic activity**

Cumulative dose-response curves of Y-5350 and oxytocin are shown in Fig. 4. In these experiments, modified van Dyke-Hasting solution was used, with or without the addition of 0.5 mM magnesium ion. Activities of both Y-5350 and oxytocin were strongly potentiated by 0.5 mM magnesium ion in terms of their oxytocic action. Moreover, they were assayed against the J.P. posterior pituitary reference standard with or without

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**Table 2. Influence of magnesium ion on oxytocic activity of oxytocin, deamino-oxytocin, deamino-dicarba-oxytocin and deamino-dicarba-[Gly']-oxytocin in van Dyke-Hasting solution.**

<table>
<thead>
<tr>
<th>Polypeptides</th>
<th>Without Mg**</th>
<th>2 mM Mg**</th>
<th>R Mg**</th>
</tr>
</thead>
<tbody>
<tr>
<td>Oxytocin*</td>
<td>546 ± 18</td>
<td>420 ± 10</td>
<td>0.77</td>
</tr>
<tr>
<td>Deamino-oxytocin*</td>
<td>551 ± 17</td>
<td>476 ± 15</td>
<td>0.86</td>
</tr>
<tr>
<td>Deamino-dicarba-oxytocin (Y-4266)</td>
<td>129 ± 8</td>
<td>279 ± 16</td>
<td>2.16</td>
</tr>
<tr>
<td>Deamino-dicarba-[Gly']-oxytocin (Y-5350)</td>
<td>218 ± 9</td>
<td>648 ± 13</td>
<td>2.97</td>
</tr>
</tbody>
</table>

(J.P. units mg, Mean ± S.E.)

*: Values reported by Chan and Kelley (1967)
(U.S.P. units mg, Mean ± S.E.)

R Mg** = $\frac{2 \text{ mM Mg}^+}{\text{Without Mg}^+}$

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**Fig. 4.** Cumulative dose-response curves of oxytocin (a) and deamino-dicarba-[Gly']-oxytocin (b) in van Dyke-Hasting solution (modified by Munsick) without addition of magnesium ion (○—○) and with 0.5 mM added magnesium ion (○—○). Each curve represents an average of four experiments involving four different rats.
the addition of 2 mM magnesium ion. The same potentiation of Y-5350 was obtained in 2 mM magnesium ion as shown in Table 2.

**Oxytocic activities in rats and rabbits in vivo**

Oxytocic activities of Y-5350 in vivo were compared with those of oxytocin in pregnant animals. Typical data are shown in Fig. 5 and Fig. 6. In both figures, the dose injected in all cases was equal to that obtained in the oxytocic assay in vitro. In the pregnant rats and rabbits, uterine contracting responses were weak with a single dose of Y-5350. In this study, intravaginal pressure changes induced by a single injection of Y-5350 and oxytocin were also recorded. Y-5350 and oxytocin showed no obvious effect on vaginal movement, when injected at a dose range from 5 to 50 mu/kg (Fig. 6).

**Enzymatic inactivation in vitro**

The results of the enzymatic inactivation of Y-5350 and oxytocin in vitro are shown in Fig. 7 and Fig. 8. Oxytocin destroying activities were observed in the serum of pregnant women. However, oxytocin was stable in serum of rats, and in saliva of pregnant women. In both pregnant and non-pregnant rats, extracts of uterus, kidney and liver showed oxytocin destroying activities. On the contrary, the activity of Y-5350 was maintained in the serum of pregnant women, and also in the above three tissue extracts of rats under the same condition.
Fig. 7. Inactivation of deamino-dicarba-[Gly']-oxytocin (○—○) and oxytocin (●—●) by serum (1), liver (2), uterus (3) and kidney (4) of pregnant rats in vitro.

Fig. 8. Inactivation of deamino-dicarba-[Gly']-oxytocin (○—○) and oxytocin (●—●) by serum (1) and saliva (2) of pregnant women in vitro.
DISCUSSION

Deamino-oxytocin, in which terminal amino-group of oxytocin is replaced by hydrogen, showed more potent biological activities than oxytocin (18). Further interest was directed to an analogue of deamino-oxytocin, which has an ethylene linkage in place of the disulfide bond. Thus, crystalline Y-4266 was synthesized and biological activities were investigated in various bioassay systems. It was apparent that Y-4266 exhibits 5–20% of the activity of deamino-oxytocin, and it was concluded that the disulfide linkage is not essential for the activity of neurohypophysial hormones. In consequence of the foregoing, many deamino-dicarba-analogues of neurohypophysial hormones were synthesized and their biological activities were investigated (9–10). Out of these analogues, Y-5350 attracted interest on account of its low vasopressin-like activity as compared to its high oxytocin-like activity. In particular, Y-5350 exhibits weak effects on blood pressure in rats and chicken and on antidiuresis in rats.

Pharmacological studies involving the response in vitro of the rat uterus to cumulative increases in the dosage of Y-5350 and oxytocin were carried out. Yamanaka et al. (8) reported that intrinsic activity of Y-4266 was only 68% of that of oxytocin. In the present study, intrinsic activity of Y-5350 was revealed to be the same as that of oxytocin. However, the cumulative dose-response curve of Y-5350 was steeper than that of oxytocin. In the above cumulative experiment, the influence of 0.5 mM magnesium ion was also investigated on the oxytocic activities of Y-5350. Many investigators using various organs reported the enhancement of biological activities of oxytocin by magnesium ion (12, 19–22). The affinity of Y-5350 to isolated rat uterus was much enhanced by the addition of 0.5 mM magnesium ion to the bathing fluid, a phenomenon similar to that of oxytocin.

Furthermore, with respect to the role of magnesium ion on the oxytocic activity of neurohypophysial hormones in vitro and in vivo, Chan and Kelley (23) reported the complete agreement between in vivo and in vitro activities, which was assayed in the presence of 2 mM magnesium ion being equal to the normal serum level. In Y-5350, however, such agreement between in vivo and in vitro was not observed. Consequently, further studies were done to elucidate the difference between Y-5350 and oxytocin in terms of the biological activities in vitro. It has been reported that the oxytocin destroying enzyme, oxytocinase, is present in pregnant women and in pregnant rhesus monkey (24). However, no oxytocin destroying activity was detected in the plasma of other nonpregnant or pregnant animals of the mammalian. Oxytocin was destroyed by the extracts of uterus (25), kidney (26) and liver (25). In contrast with oxytocin, Y-5350 was not destroyed by the serum of pregnant women and by the extracts of rat’s uterus, kidney and liver under the same conditions. In a recent study (27), the half-life time of Y-5350 in the blood was about 2–3 min in rabbits. This value was nearly the same as that of oxytocin reported by Chaudhure and Walker (28).

Finally, the lack of agreement between in vivo and in vitro oxytocic activity of Y-5350 remains to be investigated. It is a most interesting feature that Y-5350 has specific uteroconstrictive activity (oxytocin-like) with little effect on blood pressure (vasopres-
sin-like).

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