also appeared in this case that a novel reaction took place accompanied by the ring expansion from thiazole to 1,4-thiazine ring.

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103. Yukio Ishida, Hideki Moritoki, and Michiko Onishi : Effects of Two Kinds of Oxytocin Antagonists on the Isolated Rat Uterus.

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On the way of our studies with drug receptors of acetylcholine (ACh), barium chloride and oxytocin on the isolated rat uterus, we found that the action of oxytocin was inhibited competitively by hydrogen ion[1] and simple phenolic compounds.[2] And then we found that carbobenzyloxylated peptides containing tyrosine inhibited competitively the action of oxytocin. Among these small peptides, carbobenzyloxy-L-tyrosyl-L-tyrosine ethylester (Cbz-Tyr-TyrOEt) showed the most active inhibition.[3] This compound showed competitive antagonism to oxytocin, but non-competitive to ACh and barium chloride from the shift of dose-responsecurve.

In this report, relative inhibitory activities of derivatives of tyrosyltyrosine were tested. These compounds are Cbz-Tyr-TyrOEt, Tyr-TyrOEt which is decarbobenzyloxylated, and Cbz-Tyr-TyrOH which has a carboxylic acid. Among these derivatives, original Cbz-Tyr-TyrOEt was the most active antagonist. Then, estrogens were also tested. They were estradiol and diethylstilbestrol. All these compounds showed competitive antagonism to oxytocin from the shift of dose-response curve. Furthermore, we have found that cystine diethylester (CySDE) had the competitive action to oxytocin. It is interesting that this is a new type of antagonist which has never been reported. This compound was not so strong inhibitor, but more active than thioglycollate which was reported to have the inhibitory action to oxytocin by Martin and Schild.[4] Parameters for these competitive antagonists were obtained by the method introduced by Schild.[5]

From their chemical structures, they may be divided into two types of antagonists : one of which has phenol group such as peptides containing tyrosine and estrogens and so on, and the other, CySDE and thioglycollate which may be concerned with -S-S- group of oxytocin. The compounds of two types showed the same competitive inhibition to oxytocin by means of their shifts of dose-response curve, but it is suggested that they may have different mechanisms of inhibition considering their chemical structures. So, we examined synergistic antagonism of both groups in combination.

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*1 Sho-machi, Tokushima (石田行雄, 守時英喜, 大西美智子).

1) Y. Ishida : Yakugaku Zasshi, **81**, 1717 (1961).
2) *Idem* : *Ibid.*, **81**, 1722 (1961).
3) Y. Ishida, K. Hara : This Bulletin, **12**, 972 (1964).
4) P. J. Martin, H. O. Schild : Nature, **196**, 382 (1962).
5) H. O. Schild : Pharmacol. Rev., **9**, 242 (1957).

Experimental Method

The Isolated Uterus of Diestrus Rats——The uterus of healthy vargin rats from 5 to 15 days after overiectomized was used. Body weights of rats were 200±20 g.

Modified Lock-Ringer's Solution[6]——NaCl 8.8 g., KCl 0.40 g., $CaCl_2$ 0.04 g., $MgCl_2$ 0.018 g., $NaHCO_3$ 0.40 g., KH_2PO_4 0.02 g., Na_2HPO_4 0.08 g. and glucose 0.50 g. in a litre of distilled water. The bath temperature was maintained at 28°.

Some authors recommended that the uterus from estrus rat should be used to assay of oxytocin. In our experiments, the estrus uterus often showed spontaneous movement and all or none response, but the diestrus uterus of our preparation showed hardly such spontaneous movement and exerted a graded response, if one used this modified Lock-Ringer's solution. This solution contains reduced amounts of calcium and magnesium ions. A small amounts of magnesium ions increased sensitibity of oxytocin.

The Antagonistic Action——The isolated uterus at first was immersed in the above Ringer's solution which contained a standard dose of the inhibitory drug. After 10 minutes, the response was made in a solution that contained the same standard dose of it and each concentration of oxytocin.*2

The experiments of synergism of two antagonists were followed by the method of Takagi and Takayanagi.[7,8]

Experimental Results

(1) Competitive Antagonism of CySDE and Affinities of Antagonists to Oxytocin Receptor——After the dose-response curve of oxytocin had been estimated on the isolated rat uterus, the dose-response curves were obtained in the presence of CySDE $2.0\times10^{-4}M$, $4.0\times10^{-4}M$ and $8.0\times10^{-4}M$. The shape of the movement of oxytocin dose-response curves showed that CySDE is a competitive antagonist of oxytocin. The PA_2 value of CySDE was calculated by equation (i) from the shifts of these curves and it became 4.08.

The PA_2 value is a parameter, as introduced by Schild[9] for a competitive antagonist. Under ideal circumstances the PA_2 value is equal to the negative logarithm of the affinity of the competitive antagonist. It causes a parallel shift of the dose-response curve of an agonist. The degree of shifting is a measure of the affinity :

$$PA_2 = -\log [B]_x + \log (x-1) \qquad (i)$$

where $[B]_x$ is the dose of the antagonist which causes a shift of a factor x.[4]

The PA_2 values of other antagonists were as follows : Cbz-Tyr-TyrOEt 5.38, Tyr-TyrOEt 3.96, Cbz-Tyr-TyrOH 3.64, diethylstilbestrol 5.68, estradiol 4.55, *p*-nitrophenol 3.89 and thioglycollate 2.00.

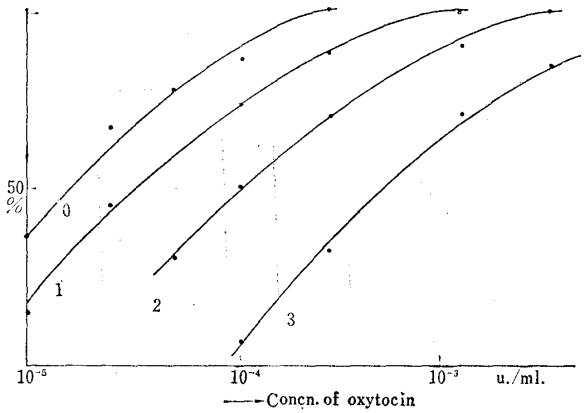

Fig. 1. Competitive Antagonism of Cystine Diethylester to Oxytoxin

0 : dose-response curve of oxytocin alone
1 : with CySDE $2\times10^{-4}M$
2 : with CySDE $4\times10^{-4}M$
3 : with CySDE $8\times10^{-4}M$

(2) Synergism of Two Antagonists to Oxytocin Receptor——The equation (ii)[7] shows additive effect of two antagonists B and C to an agonist A. In this case, A is concentration of oxytocin, B and C are definite concentrations of two antagonists. K_A is the dissociation constant of oxytocin that was estimated as the concentration of oxytocin

$$y/y' = A/K_A/(1+A/K_A+B/K_B+C/K_C) \qquad (ii)$$

*2 Oxytocin is Atonin sold by Teikoku-Zoki Co. (in Japan).

6) H. Kumagai, S. Ebashi, F. Takeda : Jap. J. Pharmacol., **2**, 65 (1952).
7) K. Takagi, I. Takayanagi : Jap. J. Pharmacol., **14**, 458 (1964).
8) E. J. Ariens, A. M. Simonis : J. Pharm. Pharmacol., **16**, 289 (1964).
9) H.O. Schild : Brit. J. Pharmacol., **2**, 251 (1947); **4**, 277 (1949).

to 50% of the maximum response. K_B and K_C were obtained respectively from the experiment of antagonism to oxytocin. K_B and K_C were dissociation constants of antagonists B and C that were obtained respectively from the experiment of antagonism to oxytocin. y' is maximum response and y is response of oxytocin in the presence of B and C. When y/y' is 1/2, A can be calculated from equation (ii), this value of A is theoretical one. If experimental result A' is equal to theoretical A, the combined effect of B and C may be additive, whereas, if A' is larger than A, it is suggested that the combined effect of B and C may be supper-additive or potentiative.

(a) Synergistic Antagonism of Cbz-Tyr-TyrOEt and CySDE to Oxytocin——In Fig. 2, curve a is the dose-response curve of oxytocin alone. ED_{50}*3 of oxytocin (K_A) became 3.5×10^{-5} u./ml. from curve a. Curve b is the curve of oxytocin in the presence of CySDE $4.0\times10^{-4}M$, and curve c is that in the presence of Cbz-Tyr-TyrOEt $2.0\times10^{-5}M$. From these curves, K_B and K_C became $10^{-3.70}$ (that is, PA_2 of CySDE=3.70) and $10^{-5.60}$ (that is, PA_2 of Cbz-Tyr-TyrOEt=5.60). Curve b+c is that in the presence of both antagonists with above doses. Therefore, experimental result A' was 6.5×10^{-4} u./ml. And then, theoretical value A calculated from the equation (ii) became 2.45×10^{-4} u./ml. As experimental result A' was larger than calculated value A, this synergistic antagonism may be potentiative. This represents the different mechanism between Cbz-Tyr-TyrOEt and CySDE.

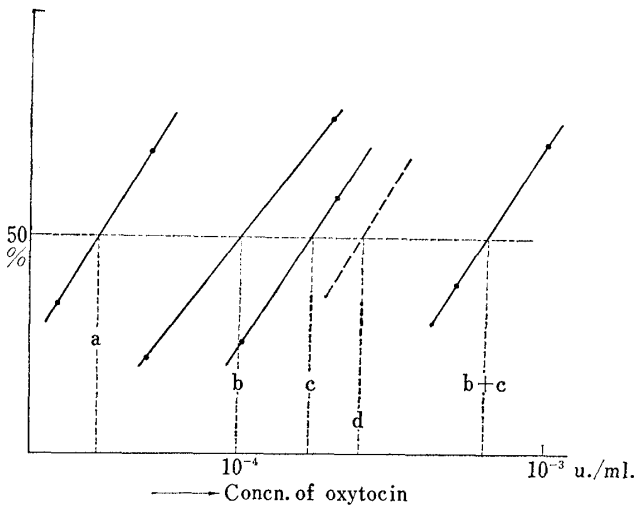

Fig. 2. Potentiative Antagonism of Carbobenzyloxy-L-tyrosyl-L-tyrosine and Cystine Diethylester to Oxytocin

a : oxytocin alone
b : with CySDE $4\times10^{-4}M$
c : with Cbz-Tyr-TyrOEt $2\times10^{-5}M$
b+c : with above both antagonists (experimental result A')
d : calculated value A

(b) Synergistic Antagonism of Cbz-Tyr-TyrOEt and Estradiol to Oxytocin——Concentrations of two antagonists were used $10^{-5}M$ for Cbz-Tyr-TyrOEt, and $6.0\times10^{-5}M$ for estradiol. In this experiment, experimental result A' was 1.9×10^{-4} u./ml. and calculated value A was 2.0×10^{-4} u./ml. A was almost equal to A', so this synergism may be additive. It shows the same antagonistic mechanism between Cbz-Tyr-TyrOEt and estradiol (Fig. 3).

(c) Synergistic Antagonism of CySDE and Estradiol to Oxytocin——The concentration of CySDE was $4.0\times10^{-4}M$, and estradiol was $6.0\times10^{-5}M$. Experimental result was 1.6×10^{-3} u./ml. and calculated value became 1.7×10^{-4} u./ml. As A' is about 10 times as large as A, this synergistic antagonism represents potentiation with two antagonists (Fig. 4).

Discussion

A number of antagonists towards oxytocin and vasopressin have been found by other workers recently; Cash, *et al.*[10] found acetylation products of oxytocin and

*3 PD_2 of oxytocin became to 10.15, as calculated from 500 u./mg. of it and its molecular weight of about 1000.

10) W. D. Cash, B. L. Smith : J. Biol. Chem., **238**, 994 (1963).

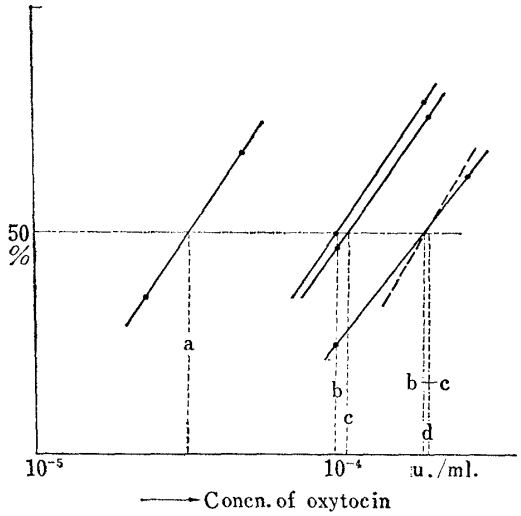

Fig. 3. Additive Antagonism of Carbobenzyloxy-L-tyrosyl-L-tyrosine and Estradiol to Oxytocin

a : oxytocin alone
b : with Cbz-Tyr-TyrOEt $10^{-5}M$
c : with estradiol $6.0\times10^{-5}M$
b+c : with above both antagonists (experimental result *A'*)
d : calculated value *A*

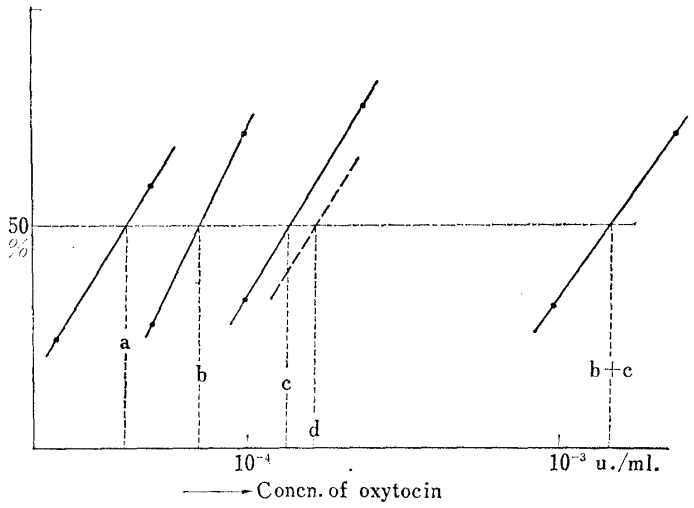

Fig. 4. Potentiative Antagonism of Cystine Diethylester and Estradiol

a : oxytocin alone
b : with CySDE $10^{-4}M$
c : with estradiol $6.0\times10^{-5}M$
b+c : with above both antagonists (experimental result *A'*)
d : calculated value *A*

vasopressin, Bisset, *et al.*[11] 1-N-carbamyl-oxytocin, Guttmann, *et al.*[12] homotyrosyl$^{2:3}$-oxytocin and Martin and Schild[4] thioglycollate and α-thioglycerol. In addition to the above antagonists, we have found Cbz-Tyr-TyrOEt, estrogens, *p*-nitrophenol and cystine diethylester (CySDE) to exhibit the competitive inhibition.

It is generally suggested that an essential active center of oxytocin may be -S-S- bond from the physiological nature of a large number of analogous peptides of oxytocin which were synthesized mainly by V. du Vigneaud and B. Berde and *et al.* and especially considering that 2-phenylalaninyl-oxytocin which does not contain tyrosine molecule has weak oxytocic activities. We have suggested from our pharmacological experiments that there are two active centers of oxytocin on the contractile effect of uterus. One of them, which is an active center of oxytocic action, may be a -S-S- bond in the oxytocin molecule, and the other, which is a binding site promoting and characterizing oxytocic activities, may be the tyrosine residue of oxytocin. Thus, it would be reasonably expected that these phenolic compounds can antagonize oxytocin without concerning the -S-S- bond.

The authors are indebted to Professor Keijiro Takagi of the University of Tokyo for his advices and encouragement.

Summary

It has been found that cystine diethylester (CySDE) is a new antagonist to oxytocin on the isolated rat uterus. It was not so strong inhibitor, but more active than thioglycollate reported by P.J. Martin and H.O. Schild.

It was suggested from synergistic antagonisms of following both groups that there are two types of antagonists to oxytocin : one of which has phenol group such as

11) G.W. Bisset, A.M. Poisner, D.G. Smyth : J. Physiol., **170**, 12p (1964).
12) St. Guttmann, P.-A. Jaquenoud, R.A. Boissonnas, H. Konzett, B. Berde : Naturwissenschaften, **44**, 632 (1957).