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Some pharmacological properties of a synthetic oxytocin analogue [1-N-carbamoyl-hemicystine-2-O-methyltyrosine]-oxytocin (carbamoyl-methyloxytocin), an antagonist to the neurohypophysial hormones

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Summary

1. A synthetic oxytocin analogue, [1-N-carbamoyl-hemicystine-2-O-methyltyrosine]-oxytocin (carbamoyl-methyloxytocin), has been tested as an antagonist to the actions of oxytocin and vasopressin on the uterus, the mammary gland and blood pressure.

2. The analogue inhibited the response of the isolated rat uterus to both oxytocin and vasopressin without itself stimulating the uterus to contract. The responses to equipotent doses of oxytocin and vasopressin were inhibited equally. There was little or no inhibition of the response to bradykinin, carbachol, angiotensin or 5-hydroxytryptamine with doses of the analogue up to 160 times that required to inhibit the response to oxytocin by 50%. The analogue caused a parallel displacement of the log dose-response curve for oxytocin ; the pA_2 value (2 min contact) varied from 6·4 to 7·1 according to the ionic composition of the solution in the organ bath.

3. The analogue inhibited the response of the rat uterus *in situ* to oxytocin but not to angiotensin or 5-hydroxytryptamine. It did not stimulate the uterus.

4. When, in certain experimental conditions, spontaneous activity occurred in the isolated uterus or the uterus *in situ*, this activity was unaffected by the analogue but the increase in amplitude and frequency of contractions caused by oxytocin was inhibited. The regular rhythm of contractions induced in the

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quiescent uterus by the intravenous infusion of oxytocin was interrupted by intravenous injections of the analogue.

5. The response of the isolated strip of rat mammary gland to the analogue depended on whether or not magnesium was present in the bath solution. In the presence of this ion, the analogue generally caused an increase in tension ; in its absence, it acted as a pure antagonist. As on the isolated uterus, oxytocin and vasopressin were equally inhibited, and the analogue caused a parallel displacement of the log dose-response curve for oxytocin. With 0·9 mM Ca and 1·0 mM Mg, the mean pA_2 value (2 min contact) was $6{\cdot}28 \pm 0{\cdot}08$ (S.E.)

6. In the lactating rat, the analogue inhibited the milk-ejection response to oxytocin and vasopressin but not that to acetylcholine, bradykinin or 5-hydroxytryptamine. A milk-ejection response to the analogue itself was seen occasionally with retrograde arterial but not with intravenous injections.

7. The analogue inhibited the avian depressor response to oxytocin and the rat pressor response to vasopressin.

8. On all assay preparations, the degree of inhibition caused by the analogue was dependent on the dose, and the inhibition could be surmounted by increasing the dose of agonist. Recovery usually occurred within 15 min. These features, together with the parallel displacement of the dose-response curve for oxytocin on the isolated uterus and mammary strip, and the equal inhibition of the responses to oxytocin and vasopressin, suggest that carbamoyl-methyl-oxytocin acts as a specific competitive inhibitor of the neurohypophysial hormones.

9. The structure-activity relationships of analogues of oxytocin having substituents in the terminal amino and phenolic hydroxyl groups, and some practical applications of the carbamoyl-methyl analogue, are discussed.

Introduction

It has been reported that the inhibitory properties of [2-O-methyltyrosine]-oxytocin (methyloxytocin) (Law & du Vigneaud, 1960 ; Beránková, Rychlík, Jošt, Rudinger & Šorm, 1961) can be accentuated by additional substitution of the terminal amino group by a carbamoyl residue (Fig. 1) (Bisset & Smyth, unpublished: quoted by Smyth, 1967b ; Bisset & Clark, 1968 ; Chimiak, Eisler, Jošt & Rudinger, 1968). Whereas methyloxytocin acts as an inhibitor of oxytocin on some assay systems but has an oxytocin-like action on others (for review, see Rudinger & Krejčí, 1968), its N-carbamoyl derivative acts as an antagonist on all the systems which are generally used for the assay of oxytocin. The pharmacological properties of the synthetic oxytocin analogue [1-N-carbamoyl-hemicystine-2-O-methyltyrosine]-oxytocin (carbamoyl-methyloxytocin) are reported in this paper.

Methods

Isolated rat uterus

A uterine horn was removed from a virgin rat, in natural pro-oestrus or early oestrus, and used either on the same day or after being stored overnight at 4° C. The horn was suspended in a 5 or 12·5 ml organ bath at 29°–32° C with van Dyke-Hastings solution (Munsick, 1960) containing (mM) NaCl 114, KCl 6·2, $NaHCO_3$ 30,

NaH_2PO_4 1·0, $CaCl_2$ 0·5–2·5, $MgCl_2$ 0–0·5, glucose 2·8. A mixture of 95% oxygen and 5% carbon dioxide was bubbled continuously through the solution both in the organ bath and the reservoir.

Two different methods were used to test the action of the analogue:

(1) Single doses of the analogue or other drugs were tested and the organ bath was washed out by overflow between doses. When this method was used, tension was recorded isometrically with a Statham universal transducing cell (Model UC3) and micro-scale accessory (Model UL5) coupled with a potentiometric recorder (Goertz, type RE511). An initial tension of 1–2 g was applied. To test for a stimulant action on the uterus, the analogue or other drug was added to the organ bath and washed out as soon as the tension had reached a peak, or, if no contraction occurred, after 2 min. The interval between doses was 4 min. To test for an inhibitory action, the analogue was added to the bath 1 min before oxytocin or other drugs.

(2) A cumulative dose procedure was used (Rudinger & Krejčí, 1962). With this method, contractions were recorded isotonically on a smoked drum. The horn was initially loaded with 2 g and allowed to relax for 30 min; the load was then reduced to 0·5 g. Oxytocin was first tested. When the response to repeated doses had become constant, a cumulative dose-response curve was obtained by doubling the dose when the response to the preceding dose had reached a maximum. The organ bath was not washed out until a maximal response, passing into a contracture, was reached. To test for inhibition the procedure was repeated after adding the analogue to the organ bath 2 min before the first dose of oxytocin, and a pA_2 value was calculated from the displacement of the dose-response curve along the concentration axis (Schild, 1949; van Rossum, 1963).

Rat uterus in situ

The method of Bisset, Haldar & Lewin (1966) was used. The intraluminal fluid pressure in the uterine horn of a rat in natural pro-oestrus or oestrus was recorded with a strain-gauge transducer and potentiometric recorder. Uterine activity was measured with a digital integrator. A saphenous artery was cannulated for retrograde arterial injections and a jugular vein for intravenous injections or infusions. To test for inhibition, the analogue was injected 1 or 5 min before oxytocin or other drugs.

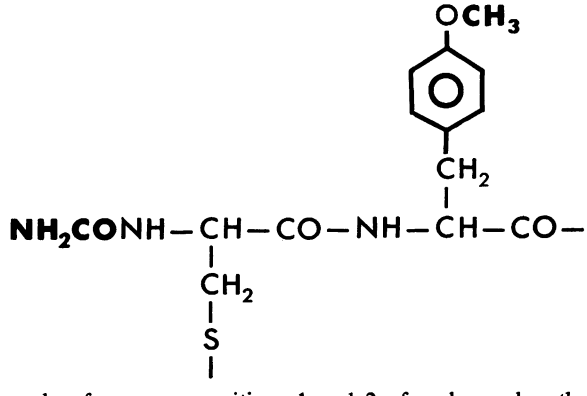

FIG. 1. Partial formula of sequence positions 1 and 2 of carbamoyl-methyloxytocin showing the O-methyl and N-carbamoyl groups (bold-face type).

Isolated strip of rat mammary gland

The method used was based on that of Rydén & Sjöholm (1962). A strip 3 cm × 0·5 cm × 0·5 cm was suspended in a 10 or 12·5 ml organ bath at 38° C containing Tyrode's solution: (mM) NaCl 137, KCl 2·7, $NaHCO_3$ 11·9, NaH_2PO_4 0·42, $CaCl_2$ 0·9–1·8, $MgCl_2$ 0–1·0, glucose 5·6, gassed with a mixture of 95% oxygen and 5% carbon dioxide. Before use, the strip was stored overnight in Tyrode's solution at 4° C. Tension was recorded isometrically by the same method as that used for the isolated uterus or with a Brüel-Kjaer strain-gauge and recorder (Poláček, Krejčí & Rudinger, 1967). An initial tension of 400–500 mg was applied and the tissue allowed to equilibrate for 1–2 h before use. To test for a stimulant action on the strip, the analogue and other drugs were added to the organ bath and washed out by overflow after 2 min. Tests were made every 10–15 min with two changes of the bath fluid in the intervals between doses. In some experiments, a digital integrator was used to estimate tension-time over the 2 min period, as for the uterus *in situ*. To test for inhibition, the analogue was added to the organ bath 1–2 min before oxytocin or other drugs. Values of pA_2 (2 min contact) were calculated from the linear portions of the log dose-response curves.

Mammary gland in situ

Milk-ejection pressure was recorded from a cannulated teat duct in the lactating rat by the method of Bisset, Clark, Haldar, Harris, Lewis & Rocha e Silva (1967). A saphenous artery and jugular vein were cannulated as for the uterus *in situ*. To test for inhibition, the analogue was injected 10–60 s before oxytocin or other substances with milk-ejecting activity.

Chicken blood pressure

Avian depressor activity was tested in anaesthetized roosters (Coon, 1939). After administration of three or four equal doses of standard pituitary (posterior lobe) extract the analogue (60–150 μg; in one experiment 640 μg) was injected, followed by the original dose of extract which was repeated at 5 min intervals.

Rat blood pressure

Pressor activity was assayed in rats, 150–180 g, pithed under ether anaesthesia and maintained on artificial respiration. Arterial blood pressure was recorded from the carotid artery with a strain gauge transducer and potentiometric recorder (1 mm Hg≡1·333 mbar). Drugs were injected, in a volume of 0·2 ml, into a cannulated femoral or external jugular vein. Injections were given as soon as the blood pressure had returned to the base-line after a pressor response. The analogue was injected 1 min before vasopressin or pituitary (posterior lobe) extract.

Materials

The carbamoyl-methyloxytocin was material prepared by total synthesis (Chimiak *et al.*, 1968) and purified by countercurrent distribution. Two batches of the analogue were tested, the first containing 0·5 mg/ml in 0·55% acetic acid and the second, 0·6 mg/ml in 1% acetic acid. All solutions were neutralized with 2 N

Na_2CO_3 before use. Except in tests for milk-ejecting activity by retrograde arterial injection into the mammary gland (see **Results**), no difference was observed between the pharmacological actions of the two batches and no distinction is made in the presentation of results. Other drugs used were synthetic oxytocin (Spofa, Prague & Syntocinon, Sandoz); synthetic arginine vasopressin (Sandoz) and lysine vasopressin (Spofa, Prague); synthetic [1-asparagine, 5-valine]-angiotensin II (Hypertensin, Ciba); synthetic bradykinin (Parke-Davis); 5-hydroxytryptamine creatinine sulphate (May & Baker); acetylcholine chloride (Roche Products) and carbachol (B.P. Savory & Moore). Doses of 5-hydroxytryptamine are expressed in terms of the base. Doses of oxytocin and vasopressin are given in units (oxytocic and pressor units respectively). The weight of peptide corresponding with a given number of units is shown in parentheses. This was calculated on the basis that pure synthetic oxytocin has an oxytocic activity of 500 u./mg (Bodanszky & du Vigneaud, 1959) and pure synthetic arginine vasopresin a pressor activity of 400 u./mg (Boissonnas, Guttmann, Berde & Konzett, 1961).

Results

In general the analogue acted as a specific antagonist to oxytocin and vasopressin and did not itself stimulate the test preparations. The degree of inhibition was dependent on the dose of analogue and in all cases the inhibition could be surmounted by increasing the dose of agonist. With *in situ* preparations, recovery from inhibition was usually complete within 10–20 min and, with isolated organ preparations, after the organ bath had been washed out once or twice at 4 or 10 min intervals.

Isolated rat uterus

Concentrations of the analogue up to 6 μg/ml did not cause contraction of the uterus; the threshold for inhibiting the response to oxytocin (0·16–0·64 mu./ml; 0·32–1·28 ng/ml) was 0·03 μg/ml. Figure 2 illustrates a typical experiment using the first of the two procedures described in **Methods**; tension was recorded isometrically and the organ bath washed out between doses. The responses to equipotent concentrations of oxytocin and vasopressin were inhibited by the analogue to the same degree; at a concentration of 0·06 μg/ml the analogue produced 50%, and at 0·12 μg/ml, 75% inhibition. The response to both hormones recovered 4–12 min after the analogue had been washed out of the organ bath. The molar ratio of hormone to analogue for 50% inhibition (the "drug-ratio", Gaddum, Hameed, Hathway & Stephens, 1955) was 1:47 for oxytocin and only 1:2 for vasopressin. In seven other similar experiments, the ratio for oxytocin varied from 62 to 312 with a mean of 166. In six of the eight experiments, the analogue caused no inhibition, and in two only 25% inhibition, of the responses to bradykinin, 5-hydroxytryptamine, carbachol or angiotensin when tested at concentrations up to 160 times those required for 50% inhibition of the response to oxytocin. The lack of an inhibitory effect on the response to bradykinin is illustrated in Fig. 2.

An increase in the calcium concentration of the van Dyke-Hastings solution in the organ bath to 2·5 mM with the addition of magnesium (0·5 mM) caused spontaneous rhythmic contractions of the uterus. Oxytocin (1–10 mu./ml; 2–20 ng/ml) increased the frequency and amplitude of the contractions and, in some cases, the

tonus also. The analogue at concentrations up to 6 μg/ml had no effect on the spontaneous contractions but inhibited the response to oxytocin (Fig. 3).

An experiment in which the cumulative-dose procedure was used is illustrated in Fig. 4. The addition of the analogue to the organ bath at concentrations of 0·3 and 0·6 μg/ml caused a parallel shift of the dose-response curve for oxytocin. From experiments of this type, pA_2 values were determined under various conditions of ionic composition and hormonal state of the uterus. These values (for 2 min contact) ranged from 6·4 to 7·1 (Table 1). Addition of magnesium or an increase in the calcium concentration decreased the pA_2 value, in most cases significantly ($P<0·05$). The values for inhibition of the response to oxytocin and to lysine vasopressin under comparable conditions were equal.

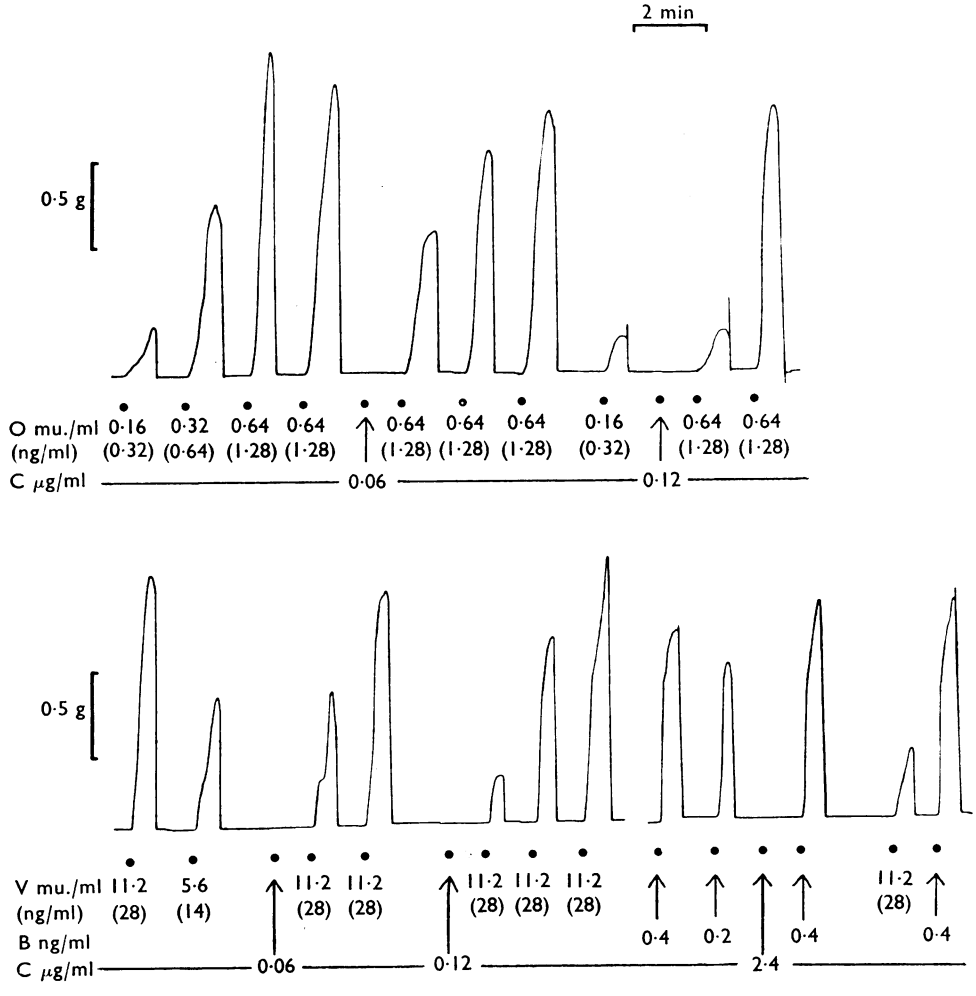

FIG. 2. Isolated rat uterus. Tension recorded isometrically. Van Dyke-Hastings solution with 0·625 mM Ca and no Mg. O, Oxytocin ; V, arginine vasopressin ; B, bradykinin ; C, carbamoylmethyloxytocin. The analogue was added to the organ bath 1 min before oxytocin, vasopressin or bradykinin ; one of these three substances was tested every 4 min. The organ bath was washed out as soon as a contraction reached its peak. The two tracings were obtained from the same uterine horn. In this and subsequent figures, the ordinate scale is linear.

Rat uterus in situ

Experiments were carried out on nine rats in pro-oestrus in which there was no spontaneous uterine activity. The threshold dose of oxytocin for causing an increase in uterine tension was 0·5–1·0 mu. 1–2 ng) by intravenous and 0·1 mu. (0·2 ng) by retrograde arterial injection. In every experiment the analogue inhibited the response to oxytocin without itself stimulating the uterus to contract. It was tested in doses up to 120 μg ; the dose required for 50% inhibition was 12–15 μg. A typical experiment is illustrated in Fig. 5. The injection of 24 μg analogue abolished the response

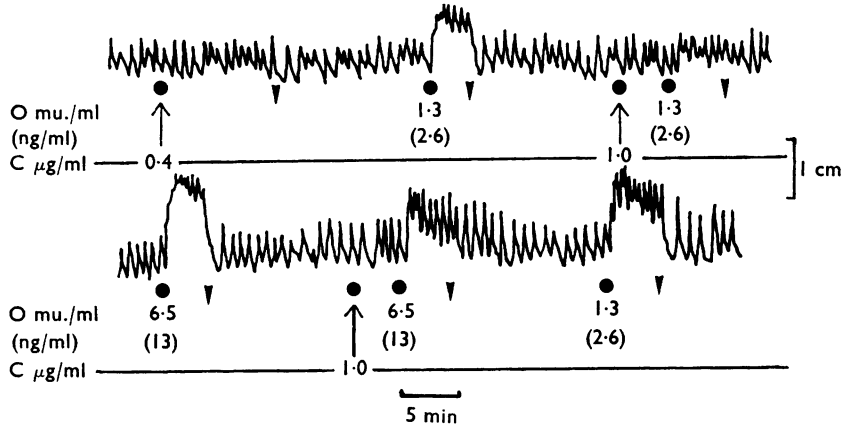

FIG. 3. Isolated rat uterus. Effect of carbamoyl-methyloxytocin on the spontaneously contracting uterus and its response to oxytocin. Isotonic contractions. Van Dyke-Hastings medium with 2·5 mM Ca and 0·5 mM Mg. O, Oxytocin ; C, carbamoyl-methyloxytocin. Downward arrows denote washing out.

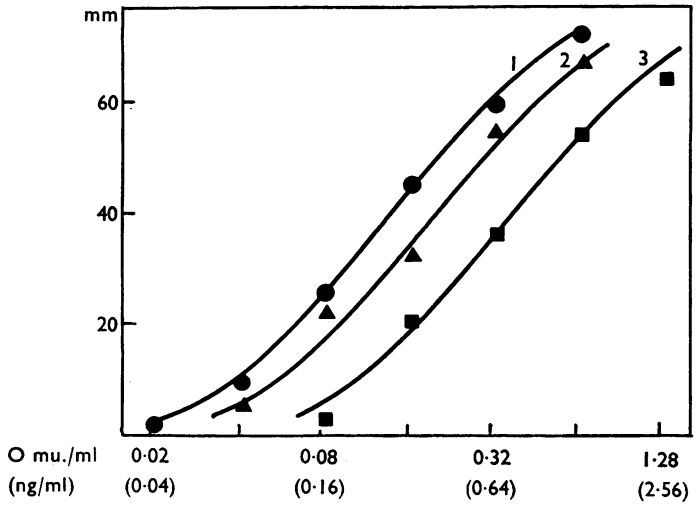

FIG. 4. Isolated rat uterus. Contractions recorded isotonically. Cumulative dose-response curves for oxytocin alone (1) and in the presence of 0·3 (2) and 0·6 (3) μg/ml carbamoyl-methyloxytocin added to the organ bath 2 min before the first dose of oxytocin. Van Dyke-Hastings medium with 1·0 mM Ca and no Mg. Ordinate, Height of contraction in mm of record ; abscissa, concentration of oxytocin (O).

TABLE 1. *Inhibition of the response of the isolated uterus to oxytocin and lysine vasopressin by carbamoyl-methyloxytocin under various experimental conditions*

Agonist	Concentration of Ca (mM)	Concentration of Mg (mM)	No of experiments	pA_2† (mean±S.E.)	Differences significant at $P<0.05$
Oxytocin	0·5	0	20	6·90±0·05	a
Oxytocin	0·5	0·5	21	6·61±0·06	b
Oxytocin	1·0	0	18	6·70±0·12	c
Oxytocin	1·0	0·5	10	6·40±0·08	d
Oxytocin	0·2*	0	4*	7·09±0·16	
Lysine vasopressin	0·5	0	15	6·96±0·06	

* Uteri from rats spayed 14–21 days before the experiment washed with calcium-free medium until the response to oxytocin disappeared and restored to van Dyke-Hastings medium with 0·2 mM Ca. As indicated in the last column of the table, the differences between a and c, b and d and c and d are significant at $P<0.05$.
† 2 min contact.

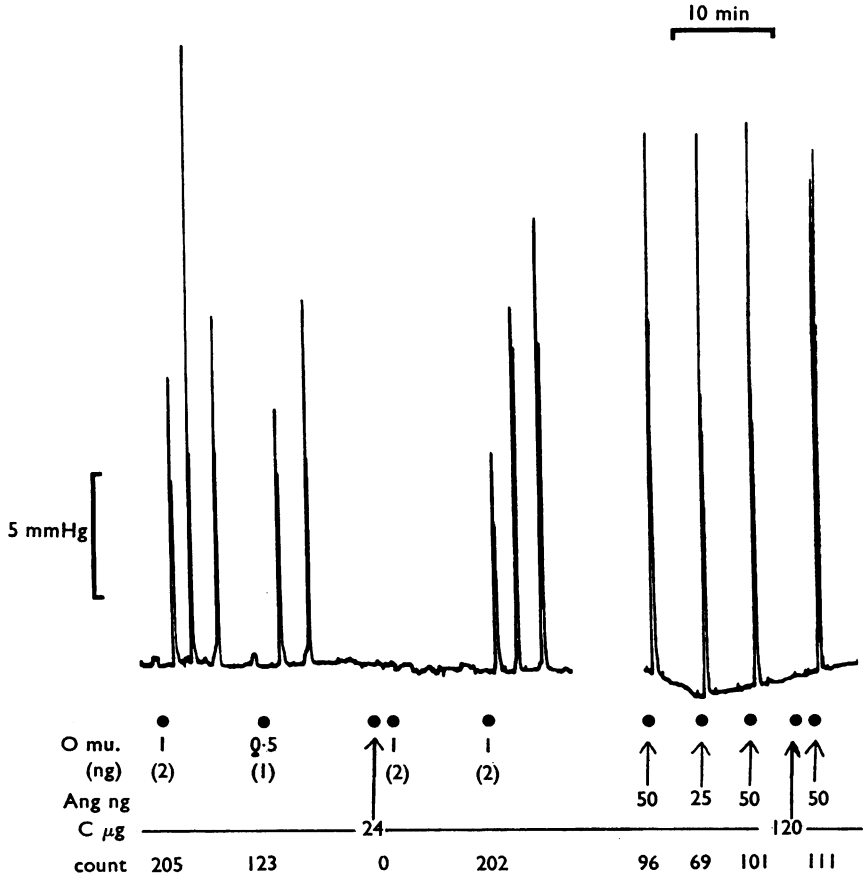

FIG. 5. Rat uterus *in situ*. Recording of intraluminal fluid pressure in uterine horn: O, Oxytocin; Ang, angiotensin; C, carbamoyl-methyloxytocin. Intravenous injections. The analogue was injected 1 min before oxytocin or angiotensin; one or other of these two drugs was injected every 10 min. An integrator was used to provide 2 min (angiotensin) or 10 min (oxytocin) counts, proportional to tension-time (see **Methods**); these counts are shown in the bottom row of the figure.

to an intravenous injection of 1 mu. (2 ng) oxytocin ; recovery was complete within 10 min (section A). Section B of Fig. 5 shows the effect of the analogue on the response to angiotensin. This peptide has been shown to produce a short-lasting contraction of the rat uterus *in situ* (Bisset, Haldar & Lewin, 1966). The response to 50 ng angiotensin was not reduced by 120 μg analogue, that is 5 times the amount required for complete inhibition of the response to oxytocin. In a similar experiment in which 12 μg analogue caused >75% inhibition of the response to an intravenous injection of 1 mu. (2 ng) oxytocin, 60 μg did not inhibit the response to an intravenous injection of 4 μg 5-hydroxytryptamine.

The effect of the analogue on the response to a continuous intravenous infusion of oxytocin is illustrated in Fig. 6. There was no spontaneous uterine activity in the preparation. Section A shows the responses to intravenous injections of oxytocin which produced bursts of contractions lasting for 5 or 10 min. The continuous intravenous infusion of oxytocin at the rate of 0·9 mu. (1·8 ng)/min during the part of the experiment shown in section B induced a regular rhythm of uterine contractions which tended to increase gradually in amplitude. There was no increase in the basal intrauterine pressure. The intravenous injection of 30 μg analogue caused an abrupt cessation of uterine contractions followed by a slow recovery. Similar but larger inhibitory responses were obtained with 60 and 120 μg. As shown by the integrated counts of tension-time, the uterine activity remained below the initial control level for a period of at least 25 min after each injection. The decrease in uterine activity caused by stopping the infusion of oxytocin was small compared with the inhibitory effect of the analogue.

In two experiments rats in oestrus were used. The uteri showed a regular rhythm of spontaneous contractions. Oxytocin (8 mu.; 16 ng) increased the frequency and amplitude of contractions. The analogue, 60 μg, produced only a small (12%) or no reduction in the integrated count of spontaneous uterine activity, but it inhibited the response to oxytocin.

Isolated strip of rat mammary gland

The results depended on whether or not Mg was present in the bath fluid. In its presence, the analogue generally stimulated the strip ; in its absence, it acted as a pure antagonist.

Fifteen experiments were carried out with Mg-free solution containing 1·8 mM Ca. The analogue (0·24–3·2 μg/ml) did not cause contraction of the strip in any experiment whereas threshold responses to oxytocin were obtained with 0·04–0·32 mu./ml (0·08–0·64 ng/ml). In nineteen experiments the solution contained 1·0 mM Mg and 0·9 mM Ca. In thirteen of these the analogue caused contraction in concentrations of 0·15–3 μg/ml. The time course of the response did not differ from that seen with oxytocin. The contractions were generally weak even with high doses, and only occasionally, with low doses, was the response found to vary with the dose. On two preparations, a 2+2 dose assay was possible, giving a potency of 0·007–0·009 u./mg. In the other six experiments, with concentrations of 0·4–6·4 μg/ml, contraction was not observed.

In all experiments the analogue inhibited the response to oxytocin regardless of whether or not it had itself caused contraction. The lowest effective concentration for causing 50% inhibition was 0·08 μg/ml. The mean pA_2 (2 min contact), calcu-

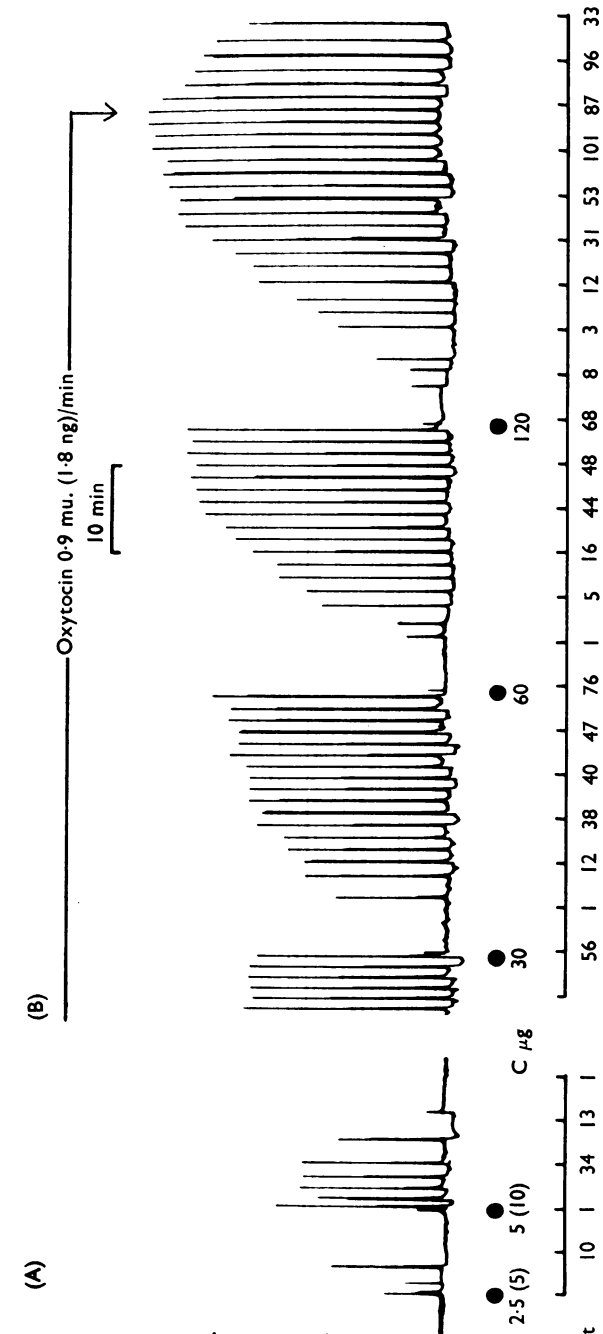

FIG. 6. Rat uterus *in situ*. Recording as in Fig. 5. O, Oxytocin; C, carbamoyl-methyloxytocin. (A), Responses to intravenous injections of oxytocin. Note absence of spontaneous uterine activity. (B), A sustained rhythm of uterine contractions was produced by the continuous intravenous infusion of oxytocin at the rate of 0·9 mu. (1·8 ng)/min. During the infusion the analogue was injected intravenously. At the arrow the infusion was stopped. The numbers in the bottom row of the figure give 5 min integrator counts (see legend to Fig. 5). Each injection of the analogue was made at the end of a 5 min control period, as soon as the intraluminal fluid pressure had returned to the baseline.

lated from fifteen experiments with 0·9 mM Ca and 1·0 mM Mg, was 6·28 ± 0·08 (S.E.).

The analogue acted as an antagonist not only to oxytocin but to vasopressin also. An experiment in which its effects on the response to these two substances were compared is illustrated in Fig. 7. The analogue did not itself cause an increase in tension of the strip. At a concentration of 0·06 μg/ml it caused 50% and at 0·12 μg/ml 75% inhibition of the response to 0·32 mu. (0·64 ng)/ml oxytocin (section A). The response to oxytocin recovered 10 min after the analogue had been washed out of the organ bath. The responses to 0·32 mu. (0·64 ng)/ml, 0·16 mu. (0·32 ng)/ml and 0·08 mu. (0·16 ng)/ml oxytocin were closely matched by 11·2 mu. (28 ng)/ml, 5·6 mu. (14 ng)/ml, and 2·8 mu. (7 ng)/ml vasopressin (section B). The concentrations of analogue (0·06 and 0·12 μg/ml) which had caused 50 and 75% inhibition of the response to 0·32 mu. (0·64 ng)/ml oxytocin caused practically the

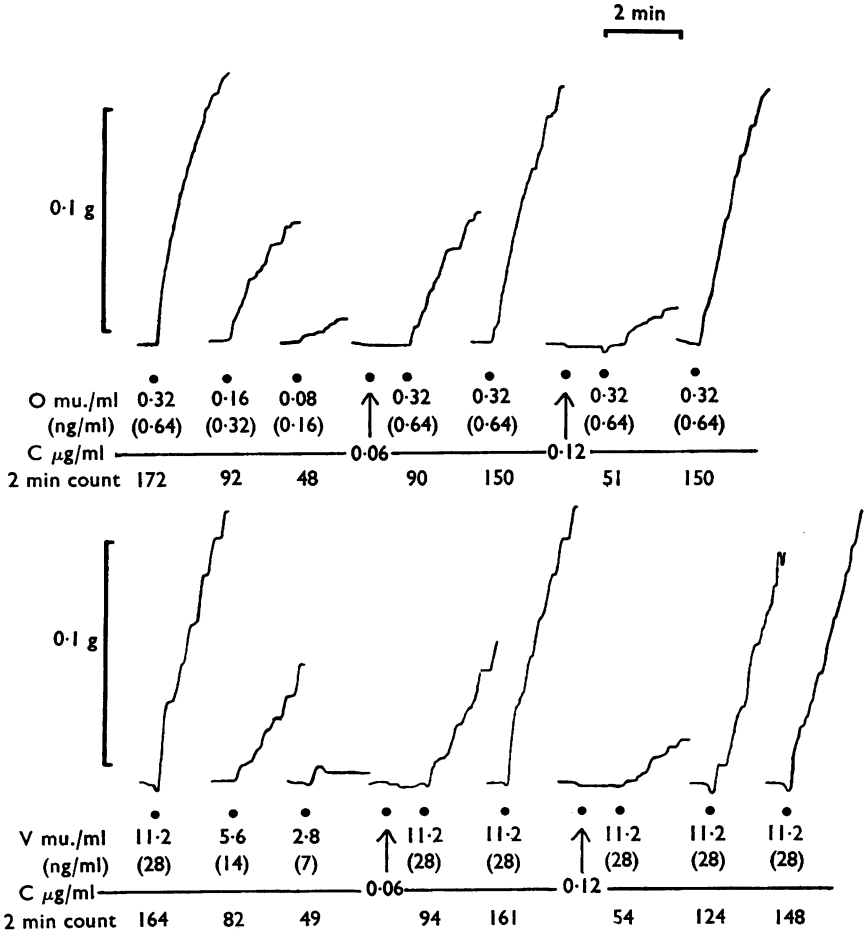

FIG. 7. Isolated strip of rat mammary gland; 1·8 mM Ca, no Mg. Tension recorded isometrically. O, Oxytocin; V, arginine vasopressin; C, carbamoyl-methyloxytocin. The analogue was added to the organ bath 1 min before oxytocin or vasopressin. The bath was washed out 2 min after each addition of oxytocin and vasopressin; one or other of these two drugs was tested every 10 min. The numbers in the bottom row of the figure give 2 min integrator counts (see legend to Fig. 5).

same percentage inhibition of the response to 11·2 mu. (28 ng)/ml vasopressin. The molar ratio of hormone to analogue for 50% inhibition (the " drug ratio ") was 1 : 170 for oxytocin and only 1 : 4·3 for vasopressin. Smith (1961) showed that the potency of arginine vasopressin, in assays on the isolated strip of rat mammary gland, was only $3{\cdot}2 \pm 0{\cdot}24\%$ of the potency of oxytocin. Our result is consistent with this estimate.

In two experiments, the response to 5–10 μg acetylcholine was not inhibited by 4–20 times the dose of analogue producing 50% inhibition of the response to oxytocin.

Mammary gland in situ

The first batch of analogue was tested by retrograde arterial injection only. In five rats, doses of 2·5–10 μg at first produced milk-ejection responses which were matched by retrograde arterial injections of 10–40 μu. (0·02–0·08 ng) oxytocin, but repeated injections induced tachyphylaxis.

The second batch did not elicit a milk-ejection response when a dose of 30 μg was tested by retrograde arterial injection in a rat responding to 10 μu. (0·02 ng) oxytocin or a dose of 60 μg in a rat responding to 2 μu. (0·004 ng). It was tested both by retrograde arterial and intravenous injection for an inhibitory effect on the response to oxytocin. The retrograde arterial injection of 6 μg 15 s before a retrograde arterial injection of 40 μu. (0·08 ng) oxytocin caused 50% inhibition of the response to oxytocin. However, the milk-ejection response to acetylcholine injected 15 s after the analogue was also inhibited and the injection of 0·9% NaCl solution 10, 15 or 20 s before oxytocin caused an inhibition similar to that observed with the analogue. Possibly the inhibitory effect produced in these conditions was caused by a change of temperature or vasoconstriction in the mammary gland. Whatever the cause of the inhibition, the retrograde arterial route of injection is evidently unsuitable for the analogue, at least if the interval between the injections of analogue and oxytocin is short. When the analogue was given by intravenous injection it inhibited the response to oxytocin specifically, and the degree of inhibition was approximately the same with intervals of 10, 20, 30 and 60 s between the injections of analogue and oxytocin. Therefore, the procedure adopted in all subsequent experiments was to give the analogue by intravenous injection 1 min before an intravenous or retrograde arterial injection of oxytocin or other substances with milk-ejecting activity.

Intravenous injections of the analogue were tested in twelve rats. In every one, the analogue inhibited the response to oxytocin without itself eliciting an increase in milk-ejection pressure. The largest dose of analogue tested was 240 μg. On a weight basis the amount of analogue injected was at least 27,000 and in one experiment 500,000 times the threshold dose of oxytocin. Expressed in another way, the milk-ejecting activity of the analogue was $<0{\cdot}001$ u./mg.

The degree of inhibition produced by the analogue depended on the dose (see Fig. 8 (section A)). Doses of 12–60 μg were required to produce 50% inhibition of the response to 20 or 40 μu. (0·04–0·08 ng) oxytocin by retrograde arterial or 200–1,000 μu. (0·4–2 ng) by intravenous injection. The lowest molar ratio of oxytocin to analogue for 50% inhibition (the " drug ratio ") by the same route of injection was 1 : 5,500. The inhibitory effect of the analogue was of short duration;

even after the response to oxytocin had been abolished, it usually recovered completely within 15–20 min.

The action of arginine vasopressin on the mammary gland was also inhibited by the analogue. The milk-ejecting activity of this hormone in the lactating rat is equivalent to about 14% of its antidiuretic activity (Bisset *et al.*, 1967). In one experiment, identical milk-ejection responses were obtained with intravenous injections of 200 μu. (0·4 ng) oxytocin and 1,400 μu. (3·5 ng) vasopressin. The response to oxytocin was inhibited 50% by 12 μg of the analogue, 75% by 24 μg and 87·5% by 48 μg. The response to vasopressin was inhibited 87·5% by only 12 μg. In this experiment, the molar ratio of hormone to analogue for a given degree of inhibition (the " drug ratio ") was 30 times higher for vasopressin than for oxytocin.

The analogue acted as a specific antagonist to oxytocin and vasopressin. The experiment shown in Fig. 8 illustrates how it may be used in conjunction with 2-bromolysergic acid diethylamide (BOL), a specific antagonist to 5-hydroxytryptamine (5-HT), to distinguish between this substance and oxytocin. Section A shows graded inhibition of the response to oxytocin. The analogue did not itself cause an increase in milk-ejection pressure. After each dose of analogue the response to oxytocin recovered within 15 min. Later in the experiment (section B), the sensitivity to oxytocin increased. The responses to 200 and 400 μu. (0·4 and 0·8 ng) were then matched by intravenous injections of 2 and 4 μg 5-HT. The analogue did not reduce the milk-ejection response to 4 μg 5-HT, although the dose tested, 96 μg, was 8 times the threshold for inhibiting oxytocin. In contrast, the response to 5-HT was partially inhibited by 20 μg and completely by 40 μg BOL and this did not reduce the response to 400 μu. (0·8 ng) oxytocin. In five other experiments, the milk-ejection responses to retrograde arterial injections of 2 μg bradykinin or 10–40 ng acetylcholine were not reduced by doses of the analogue up to 5 times that required to produce 50% inhibition of the response to oxytocin in the same rat.

The effect of the analogue in inhibiting the action of oxytocin given by continuous intravenous infusion in the lactating rat is shown in Fig. 9. Section A shows milk-ejection responses to intravenous injections of oxytocin. The infusion of oxytocin at the rate of 1·3 mu. (2·6 ng)/min during the part of the experiment shown in section B produced a sustained increase in milk-ejection pressure accompanied by small rhythmic oscillations. The response was similar to the " plateau " or " tonic " type of reaction to the infusion of oxytocin described in the rabbit by Cross (1958) and by Berde & Cerletti (1960). The intravenous injection of 30, 60 or 120 μg of the analogue caused an abrupt fall in milk-ejection pressure. The oscillations were at first abolished for 1 or 2 min and then restored with increased amplitude. The milk-ejection pressure and the amplitude of the oscillations returned to their control levels 15–30 min after the injections of the analogue. The absolute fall in pressure produced by the injections was similar to that observed when the infusion of oxytocin was stopped.

Chicken blood pressure

In one of four animals, injection of the analogue (60 μg) caused a slight decrease in the blood pressure ; in the remaining experiments there was no significant depressor response. The response to pituitary (posterior lobe) extract given after

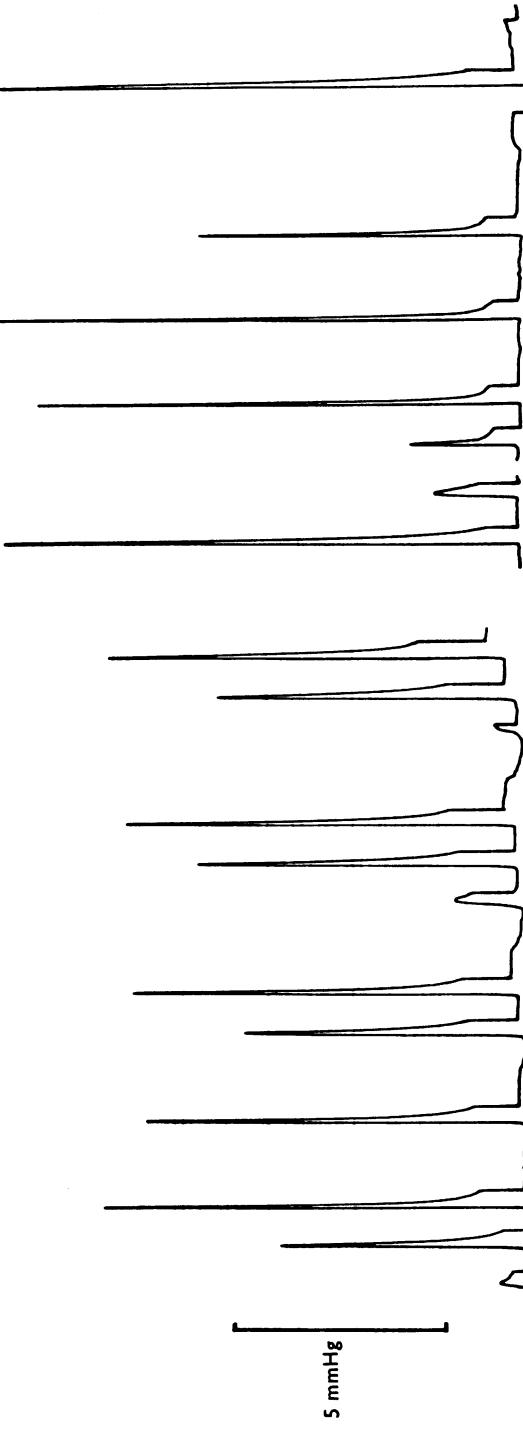

FIG. 8. Milk-ejection pressure in lactating rat. O, Oxytocin; 5-HT, 5-hydroxytryptamine; C, carbamoyl-methyloxytocin; BOL, 2-bromolysergic acid diethylamide. Intravenous injections. The analogue was injected 1 min before oxytocin or 5-HT, and BOL 1 min before 5-HT. The recorder was switched off within 30 s after the injection of oxytocin or 5-HT; one or other of these two substances was injected every 5 minutes.

the analogue was at first completely inhibited and then gradually recovered (Fig. 10). After doses of 60–150 μg in three experiments the original responses to the extract were restored after 20–30 min ; in the remaining experiment recovery after 540 μg of the analogue took about 45 minutes.

Rat blood pressure

In six experiments in which doses up to 600 μg were tested, the analogue did not cause a rise of blood pressure. The pressor response to vasopressin or pituitary extract was inhibited by doses of 60 μg and above. Figure 11 shows graded inhibition of the response to vasopressin. It was calculated from the dose-response curve for vasopressin that 80 μg analogue caused 38% and 160 μg 60% inhibition of the response to 8 mu. (20 ng) vasopressin with full recovery within 5–10 minutes. In this experiment, the analogue itself caused a fall of blood pressure which was not reproduced by a control injection of saline. It is possible that the analogue produced this fall by inhibiting a small amount of residual or endogenous vasopressin in the circulation. Inhibition of the response to vasopressin following the injection

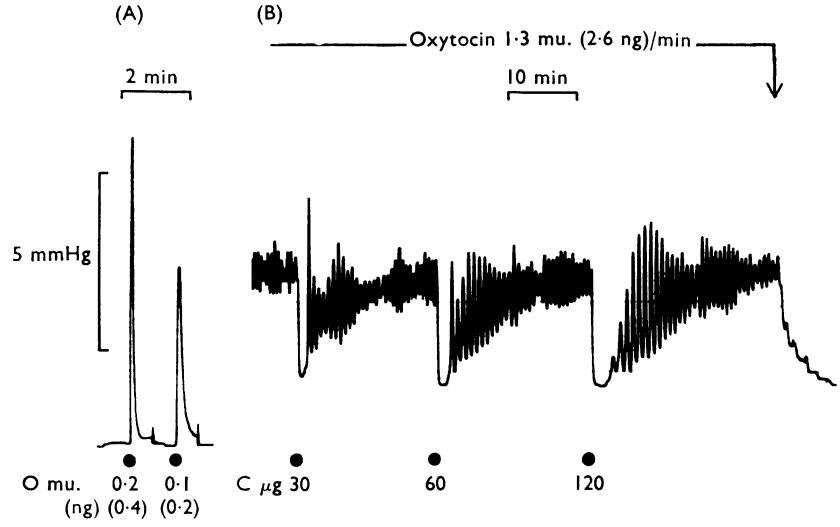

FIG. 9. Milk-ejection pressure in lactating rat. O, Oxytocin ; C, carbamoyl-methyloxytocin. (A), Milk-ejection responses to intravenous injections of oxytocin (recorder switched off 30 s after injections). (B), A sustained increase in milk-ejection pressure was produced by the continuous intravenous infusion of oxytocin at the rate of 1·3 mu. (2·6 ng)/min. During the infusion, the analogue was injected intravenously. At the arrow the infusion was stopped.

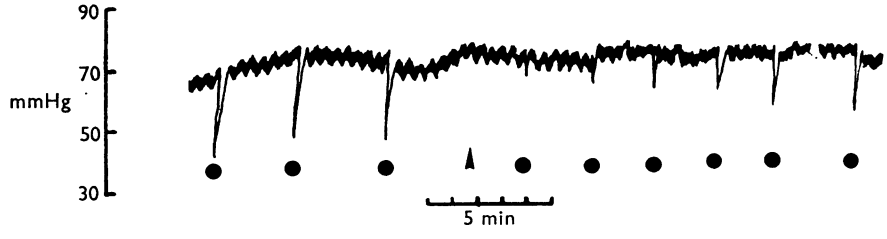

FIG. 10. Blood pressure of chicken. At dots, 30 mu. pituitary (posterior lobe) extract and, at arrow, 60 μg carbamoyl-methyloxytocin injected intravenously.

of 80 and 160 μg analogue was not attributed to the fall of blood pressure since a dose of 40 μg induced a similar fall without inhibition.

Discussion

The work reported here has confirmed that carbamoyl-methyloxytocin inhibits the action of oxytocin and the vasopressins on the uterus, the mammary gland and the vasculature over a wide range of conditions. For completeness it may be noted that it also inhibits the action of oxytocin on rat epididymal adipose tissue (Braun, Hechter & Rudinger, 1969). In the water-loaded rat under ethanol anaesthesia, carbamoyl-methyloxytocin has an oxytocin-like antidiuretic effect which, however, is attended by tachyphylaxis and inhibition of the response to oxytocin (V. Pliška, unpublished results).

The inhibition by carbamoyl-methyloxytocin is reversible and surmountable. The parallel displacement by the analogue of the log dose-response curves for oxytocin on the preparations *in vitro* and the selectivity of the inhibitory effect, together with the structural relation to oxytocin and methyloxytocin, provide strong evidence that carbamoyl-methyloxytocin acts as a competitive antagonist at the tissue receptors for the neurohypophysial hormones. In addition, the equal inhibition of oxytocin and vasopressin by the analogue on the isolated uterus and mammary gland strip confirms the generally held assumption that in these tissues the two peptides act on the same receptors.

The place of carbamoyl-methyloxytocin in the general scheme of structure-activity relations for oxytocin analogues is best discussed with reference to the properties of methyloxytocin and other derivatives modified in sequence position 2.

On the rat uterus methyloxytocin reproducibly acts as an inhibitor only if the organ is taken from a spayed rat and the medium contains a low concentration of

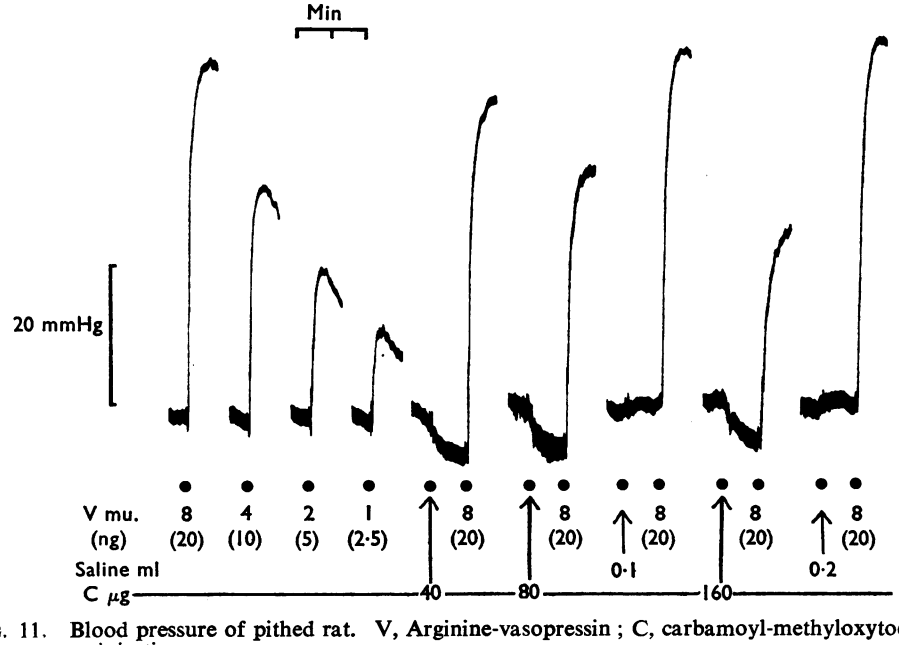

FIG. 11. Blood pressure of pithed rat. V, Arginine-vasopressin ; C, carbamoyl-methyloxytocin. Intravenous injections.

calcium and no magnesium (Rudinger, Krejčí, Poláček & Kupková, 1968 ; Poláček, Krejčí & Rudinger, unpublished). Under other conditions it may either act as an inhibitor, or show fully oxytocin-like properties, or exhibit intermediate behaviour Krejčí, Poláček & Rudinger, 1967b). The higher homologue [2-O-ethyltyrosine]-oxytocin (ethyloxytocin) causes inhibition, sometimes preceded by rudimentary contractions which are not dose-dependent (Poláček, Krejčí & Rudinger, unpublished). Like oxytocin, methyloxytocin intensifies the spontaneous contractions of the rat uterus elicited *in vitro* by high concentrations of calcium and magnesium (see Rudinger, 1969 ; Krejčí, Poláček & Rudinger, unpublished) and induces or intensifies contractions of the rat uterus *in situ*. By contrast, carbamoyl-methyl-oxytocin has no contractile effect and inhibits the action of oxytocin on the uterus over the whole range of experimental conditions *in vitro* and *in vivo*.

In the lactating guinea-pig (Bisset, 1964) methyloxytocin, like oxytocin, increases milk-ejection pressure. It also contracts the mammary gland strip *in vitro,* but the rate of contraction is slower than with oxytocin and the onset of tachyphylaxis is more rapid ; with ethyloxytocin these anomalies become more pronounced (Poláček *et al.*, 1967). On the mammary gland, carbamoyl-methyloxytocin, though it invariably inhibits the response to oxytocin, does retain some remnants of oxytocin-like activity. In isolated instances, a milk-ejection response has been seen *in vivo* and the mammary-gland strip will generally contract in response to the analogue in the presence (but not in the absence) of magnesium. When such contractions occur they are rudimentary, or at any rate submaximal, show no regular dependence on dose and are attended by inhibition of the response to oxytocin. In these features they resemble the responses sometimes observed with methyloxytocin or ethyloxytocin on the isolated rat uterus, as described above. In both cases the occurrence of such responses is difficult to predict and varies even under apparently identical experimental conditions, and in both cases the presence of magnesium favours the contractile over the inhibitory effect (Krejčí *et al.*, 1967b ; Krejčí, Poláček & Rudinger, unpublished).

Like carbamoyl-methyloxytocin, methyloxytocin has no rat pressor activity and inhibits the pressor response to oxytocin and the vasopressins (Law & du Vigneaud, 1960 ; Krejčí, Kupková & Vávra, 1967a). However, methyloxytocin does retain some avian depressor activity, althought this activity is absent in ethyloxytocin.

It has earlier been shown (Zhuze, Jošt, Kasafírek & Rudinger, 1964 ; Cort, Lichardus, Rudinger & Hagemann, 1966 ; Rudinger & Krejčí, 1968 ; Poláček, Rudinger & Krejčí, in preparation) that a bulky substituent in the *para* position of the side-chain in position 2 extends the range of biological preparations in which the analogues show incipient or full antagonism. Evidently introduction of the carbamoyl substituent into the terminal amino group of methyloxytocin has the same effect to an even greater extent.

The structural cause of this effect is not clear. Analogues of oxytocin substituted in the terminal amino group with methyl (Jošt, Rudinger & Šorm, 1963 ; Beránková-Ksandrová, Bisset, Jošt, Pliška, Rudinger, Rychlík & Šorm, 1966 ; Yamashiro, Aanning, Branda, Cash, Murti & du Vigneaud, 1968), isopropyl (Hruby & du Vigneaud, 1969), acetyl (Boissonnas, Pechère & Guttmann, unpublished ; compare Boissonnas *et al.*, 1961 ; Smyth, 1967b), sarcosyl (Jošt *et al.*, 1963 ; Beránková-Ksandrová *et al.*, 1966) or carbamoyl (Smyth, 1967a) groups show negligible or low but, as far as has been recorded, qualitatively normal oxytocin-

like activity on the isolated rat uterus and on the mammary gland although N-acetyloxytocin does inhibit the avian depressor response to oxytocin. In view of their low activity, these analogues do not appear to have been studied in detail. Low to moderate protracted activity is found in derivatives substituted with enzymically removable amino-acyl or peptidyl groups (Beránková-Ksandrová *et al.*, 1966).

On the other hand, N,O-disubstitution of oxytocin (Smyth, 1967a, b, 1970) affords derivatives which, like carbamoyl-methyloxytocin, inhibit the action of oxytocin on the isolated rat uterus under the standard assay conditions (the presence or absence of magnesium is not specified) without showing any uterotonic action of their own. It seems, therefore, that simultaneous substitution in the amino group and modification of the side-chain in sequence position 2 generally leads to effective inhibitors of the neurohypophysial hormones.

Carbamoyl-methyloxytocin has already proved invaluable for identifying oxytocin in extracts of blood samples collected during suckling (Bisset, Clark & Haldar, 1970) and electrical stimulation of the paraventricular nucleus (Bisset & Clark, 1968; Bisset, Clark & Errington, 1970) when these extracts are assayed for milk-ejecting activity in the lactating rat. Another interesting application of the analogue has been for distinguishing between the receptors for oxytocin and insulin on isolated fat cells from rat epididymal adipose tissue where the "insulin-like" action of oxytocin is inhibited but not the effect of insulin itself (Braun *et al.*, 1969). However, although carbamoyl-methyloxytocin behaves almost as a pure antagonist, high concentrations are required for effective inhibition and a more potent and longer lasting antagonist would be desirable for blocking the response of target organs in the whole animal to oxytocin released endogenously, for example, during suckling or parturition.

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