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# SYNTHETIC ANALOGUES OF OXYTOCIN ACTING AS HORMONOGENS

BY

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In the study of synthetic hormone analogues attention has usually been concentrated on their effects on the target organs or tissues. However, the metabolic aspects of hormone regulation should offer a promising alternative approach to the rational design of hormone analogues since the hormone levels in blood and tissues are governed under physiological conditions not only by the release of the hormones and their excretion but also by their metabolic inactivation. Two groups of analogues were therefore prepared and examined which were expected to show modified metabolic behaviour.

In the first group, structural modifications were introduced which would be expected to stabilize the compounds against the enzyme systems believed to be responsible for the inactivation of oxytocin. It has been shown that the oxytocin- and vasopressin-inactivating enzyme of pregnancy serum (" serum oxytocinase ") acts primarily as a (hemi)cystine aminopeptidase, splitting the bond between the amino-terminal hemicystine residue and the tyrosine (Tuppy & Nesvadba, 1957 ; Beránková, Rychlík & Šorm, 1959, 1960, 1961). In (N-activation) by tissue extracts an aminopeptidase is again implicated, but this seems to require preliminary reduction of the disulphide bond ; both stages have been defined for liver cell sap (Rychlík, 1964 ; Bartošek, Pliška, Rychlík & Šorm, 1964). If the results of these *in vitro* experiments are relevant to conditions *in vivo* and the physiological inactivation of oxytocin is due primarily to aminopeptidase action, then analogues of oxytocin resistant to aminopeptidase would be expected to show protracted biological effects *in vivo*. As typical compounds of this kind, analogues with the terminal amino group methylated (1-*N*-methylhemicystine-oxytocin ; MeCys[1]-oxytocin), and with the terminal hemicystine residue in the D configuration (1-D-hemicystine-oxytocin ; D-Cys[1]-oxytocin) were synthesized (Jošt, Rudinger & Šorm, 1961, 1963). The last of these analogues has since been prepared independently by Hope, Murti & du Vigneaud (1963).

The second group of analogues was designed to act as synthetic " hormonogens " ; a hormonogen is defined as a derivative whose biological effects are wholly or largely due to its conversion to the active hormone under biological conditions. In these particular analogues amino-acid residues or short peptide chains were attached to the terminal amino group of oxytocin on the assumption that they might be gradually

removed *in vivo* by enzyme action—specifically by the action of aminopeptidases—with the continuous release of the parent hormone. This group includes glycyl-, leucyl-, phenylalanyl-, prolyl-, glycyl-glycyl-, leucyl-leucyl, and leucyl-glycyl-glycyl-oxytocin. For comparison two additional analogues, sarcosyl and D-leucyl-oxytocin, were prepared in which the attached substituents would not be expected to be susceptible to aminopeptidase action *in vivo* (Jošt, Rudinger & Šorm, 1961, 1963; Kasafírek, Jošt, Rudinger & Šorm, 1965). Glycyl-oxytocin has also been prepared and studied by du Vigneaud, Fitt, Bodanszky & O'Connell (1960).

The analogues were examined for oxytocic and antidiuretic activity and for their effects on the mammary gland and chicken blood pressure. A preliminary pharmacological characterization of most of these compounds has already been given (Bisset, 1964; Rychlík, 1964; Beránková-Ksandrová, Rychlík & Šorm, 1964).

## METHODS

### *Assay of oxytocic activity*

The isolated rat uterus and the rat uterus *in situ* were used. Oxytocic activity was assayed on the isolated rat uterus by the procedure of Holton (1948) using organs taken from ovariectomized virgin rats of the Wistar strain (200–250 g body weight) 48 hr after treatment with 20 $\mu$g oestradiol. The solution in the organ bath contained 0.6 mM $Ca^{2+}$ and the temperature was kept at 30°. Contractions were measured isometrically and recorded on a smoked drum. A solution of synthetic oxytocin (SPOFA), assayed against the Third International Standard for Oxytocic, Pressor and Antidiuretic Substances (Bangham & Musset, 1958), was used for comparison.

Oxytocic activity on the uterus *in situ* was determined using virgin Wistar rats in natural pro-oestrus under urethane anaesthesia (1.75 g/kg). After laparotomy the uterus was exposed, an incision was made and a glass cannula was inserted for a short distance into the distal end of one of the uterine horns so as to give a tight fit. The cannula was then secured with a ligature. The uterine horn and cannula were filled with saline at an excess pressure of 5–10 mm of water and the volume changes were registered on a smoked drum using a float type manometer as described by Režábek & Souček (1962). Oxytocin and its analogues were injected into the femoral vein through a polyethylene cannula. The area under the trace on the smoked drum as determined with a planimeter was taken as a measure of the response. Synthetic oxytocin (SPOFA) was used as standard.

### *Assay of antidiuretic activity*

Female Wistar rats (170–230 g body weight) with 10% excess water load under ethanol anaesthesia were used (Sawyer, 1958). After laparotomy, the neck of the bladder was incised, and a polyethylene cannula was threaded from the bladder through the urethra. The cannula was secured by ligating the bladder just below the openings of the ureters. The hydration was maintained at 10% of the body weight throughout the experiment by administering aqueous ethanol by stomach tube from a micropump actuated through a relay system by a contact whenever the rat (placed on a scale pan) lost weight through urine excretion. The aqueous alcohol was either 1% or 2% according to the requirement for maintaining anaesthesia. Oxytocin and its analogues were injected in 0.5 ml. of solution through a polyethylene cannula into the femoral vein. The conductivity of the urine was measured continuously and the rate of urine flow was automatically recorded at 1 min intervals. Details of the preparation and apparatus are given elsewhere (Pliška & Rychlík, unpublished). In calculating numerical values, the volume readings alone were used. The response was expressed in terms of the total antidiuretic effect as $E\zeta=(V_0-V)/V_0$ where V is the volume excreted during the experimental period and $V_0$ the volume excreted in an equal control period. According to the duration of the response to a particular analogue time periods $\zeta$ of 60 or 90 min were chosen and the response to doses of the standard (synthetic oxytocin SPOFA) were evaluated

over the corresponding time periods. From the responses to four or more different doses of the analogue and to a similar number of doses of the standard obtained in the same experimental animal, log-dose-response curves were plotted. Since the curves obtained for the hormonogen group of analogues, using the total antidiuretic effect as a measure of the response, were not parallel to that for oxytocin the activities given in Table 2 were determined by extrapolating the curves to zero response (threshold dose) and comparing the intercepts for analogue and standard on the log-dose axis. Alternatively, the response was expressed in terms of the maximal intensity of the antidiuretic effect as $E_D=(I_o-I)/I_o$ where $I_o$ is the rate of urine flow in the control period (volume per unit time) and I the minimum rate of flow reached during the experimental period. Log-dose-response-curves plotted in terms of the intensity were parallel for the analogue and standard in all cases and the numerical values for the antidiuretic activities were identical, within the limits of experimental error, with those derived as above from extrapolation to threshold dose of the curves obtained by plotting log-dose against total antidiuretic effect. In order to obtain a measure of the prolongation of response the index of persistence, introduced by Pliška (1966), was used. This index, $I_p$, was derived as $I_p=k_s/k_p$, the ratio of the exponential elimination constants for standard and analogue. The exponential elimination constants are dose-independent parameters characterizing the duration of the effect and can be calculated in several ways from the relation between the intensity and the total effect; details of their derivation, and a discussion of the methods for expressing antidiuretic activity are given elsewhere (Pliška, 1966).

*Assay on the mammary gland*

Two methods were used. In the one, milk-ejecting activity was assayed by measuring milk-ejection pressure in the mammary gland of the lactating guinea-pig. In the other, activity was assayed by measuring tension in isolated strips of mammary gland from rats or guinea-pigs.

Milk-ejecting activity was assayed according to the method originally described for the rabbit by van Dyke, Adamsons & Engel (1955) and later applied with modifications to the guinea-pig (Bisset, 1962). A guinea-pig (850 to 1,000 g), taken from its litter about 7 days after parturition, was anaesthetized by intraperitoneal injection of urethane (1.25–1.5 g/kg). The trachea was cannulated but artificial respiration was not applied. The body temperature was kept constant at 38° C by means of a rectal thermometer consisting of a germanium transistor with open circuit base. The leakage current from this transistor was fed into a voltage amplifier followed by a current amplifier to provide a heating current to a resistance mat on which the animal was laid. The circuit was designed by the Electronics Division at the National Institute for Medical Research, Mill Hill. After the tip of one nipple had been cut off, polyethylene tubing of 0.5 mm internal diameter was inserted into the milk-duct and connected with a Statham strain gauge transducer to measure milk-ejection pressure which was registered on a 1 mV potentiometric recorder (Speedomax: Type H, Leeds & Northrup). Oxytocin and its analogues were given by injection into the external jugular vein or by retrograde injection into the internal saphenous artery after ligation of all branches other than that to the mammary gland (Tindal & Yokoyama, 1962). In some experiments oxytocin was given by slow infusion into the external jugular vein by means of a syringe and infusion pump.

The method of measuring tension in isolated strips of mammary gland was that described for the rat by Rydén & Sjöholm (1962). From the mammary glands of lactating rats and guinea-pigs strips 4 cm in length were cut radially from the nipple and suspended in an 11 ml. bath of Tyrode's solution at 38° C. A mixture of 5% carbon dioxide and 95% oxygen was bubbled continuously through the solution in the organ bath and reservoir. Tension in the strips was recorded isometrically by an RCA 5734 transducer with the same potentiometric recorder as that used for the mammary gland *in situ*. An initial tension of about 160 mg was applied. The strips of guinea-pig gland were taken from animals which had been used on the previous day for assaying milk-ejecting activity *in vivo*. These strips were stored overnight in Tyrode's solution at 4° C.

The standard used for all assays on the mammary gland was synthetic oxytocin ("Syntocinon," Sandoz).

*Assay on chicken blood pressure*

Avian depressor activity was determined by the method of Coon (1939) on Leghorn cocks, 2–3 kg in weight, anaesthetized with phenobarbitone (120 mg/kg) intravenously. The arterial pressure was recorded on a smoked drum with a mercury manometer. Oxytocin and its analogues were injected into the vein of one wing. In several experiments in which the effect of an infusion of oxytocin on the response to the analogues was investigated, the oxytocin was infused from a syringe and pump into the vein of the other wing. The Czechoslovak National Posterior Pituitary Standard (assayed against the Third International Standard) was used for comparison; synthetic oxytocin (SPOFA) was used in the infusion experiments.

*Analogues of oxytocin*

All the peptides were products purified by countercurrent distribution, isolated by lyophilization and characterized by elemental analysis and amino-acid analysis (Jošt, Rudinger & Šorm, 1963; Kasafírek, Jošt, Rudinger & Šorm, 1965). The peptide concentrations were calculated from the nitrogen content. The materials were kept at −15°, or in dilute sterile solution (*p*H 3.5) at 0°. With samples of the same analogue which were synthesized in two separate runs identical results were obtained in pharmacological studies.

## RESULTS

### *Oxytocic activity*

MeCys[1]-oxytocin showed weak activity on the rat uterus *in situ*. The effect was not protracted, but after several doses had been given the main response was followed by a number of contractions resembling spontaneous activity; when oxytocin was then given its main response was also followed by such contractions. With oxytocin alone this occurred only after a considerably greater number of injections. The actions of D-Cys[1]-oxytocin and sarcosyl-oxytocin were similar to that of MeCys[1]-oxytocin. The last two analogues were also tested on the isolated uterus and their activities were of the same order as on the uterus *in situ* (Table 1).

Of the analogues of the second structural type which were designed to act as hormonogens, glycyl, glycyl-glycyl, and leucyl-glycyl-glycyl-oxytocin showed a protracted action on the uterus *in situ* even at low dose levels, whereas phenylalanyl-oxytocin had a protracted effect only at higher dose levels. With leucyl-, leucyl-leucyl, and prolyl-oxytocin there was no clearly protracted effect. The activities of the hormonogen group

TABLE 1

EFFECT OF OXYTOCIN ANALOGUES ON THE RAT UTERUS ISOLATED AND *IN SITU*
Activities are given in u./μmole. The ratios in the last column are calculated from the mean value of the activity on the uterus *in situ*

| Peptide | Oxytocic activity | | Ratio |
|---|---|---|---|
| | (*a*) Isolated uterus* | (*b*) Uterus *in situ* | (*b*)/(*a*) |
| Oxytocin | 450 | 450 | |
| MeCys[1]– | 0·25 | 0·5–1 | 3 |
| Phenylalanyl– | 3 | 45–60 | 17 |
| Leucyl– | 25 | 70–100 | 3·5 |
| Glycyl– | 0·33 | 3–5 | 12 |
| Prolyl– | 0·65 | 5–10 | 11 |
| Leucyl–leucyl– | 16 | 80–100 | 5 |
| Glycyl–glycyl | 0·8 | 5–10 | 6 |
| Leucyl–glycyl–glycyl– | 0·25 | 15–25 | 80 |
| Sarcosyl– | 2·3 | 1·5–2 | 0·8 |

* Weighted mean of 3–5 assays

of analogues on the rat uterus *in situ* and on the isolated uterus are given in Table 1. Although the activities of these analogues on the uterus *in situ* could be determined only approximately it is evident that their activity is 3.5 to 80 times higher on the uterus *in situ* than on the isolated organ.

### *Antidiuretic activity*

In the assay on the water-loaded rat, MeCys[1]-oxytocin, D-Cys[1]-oxytocin and sarcosyl-oxytocin exhibited effects which were qualitatively indistinguishable from those of oxytocin itself. The log-dose response plots (with the effect expressed conventionally in terms of total antidiuresis) for the analogues were parallel with those for oxytocin. The antidiuretic activities were low (Table 2).

TABLE 2

ANTIDIURETIC ACTIVITIES OF OXYTOCIN ANALOGUES IN THE WATER-LOADED RAT

Antidiuretic activities at threshold dose are given in m-u./μmole; the confidence limits of the mean (68·3% probability) are shown in parentheses

| Peptide | No. of experiments | Antidiuretic activity | Index of persistence |
|---|---|---|---|
| Oxytocin | | 3,000 | |
| MeCys[1]– | 2 | 3·9 ( 1·5 – 6·1 ) | 1·0 |
| D–Cys[1]– | 2 | 3·9 ( 2·1 – 4·5 ) | 1·0 |
| Phenylalanyl | 3 | 205·0 (130 –310 ) | 2·6 |
| Leucyl– | 2 | 270·0 (120 –400 ) | 2·9 |
| Glycyl– | 4 | 132·0 ( 94 –180 ) | 3·2 |
| Prolyl– | 5 | 5·0 ( 2·6 – 8·4 ) | 4·3 |
| Leucyl–leucyl– | 4 | 270·0 (150 –480 ) | 3·0 |
| Glycyl–glycyl– | 4 | 25·0 ( 14 – 31 ) | 4·0 |
| Leucyl–glycyl–glycyl– | 6 | 3·9 ( 3·2 – 4·7 ) | 5·0 |
| Sarcosyl– | 4 | 25·0 ( 17 – 37 ) | 1·0 |
| D-Leucyl | 5 | 0·54 ( 0·44– 0·67) | 2·6 |

On the other hand, all the analogues of the second structural type, designed to act as hormonogens, showed distinctly protracted effects. This is shown for prolyl- and leucyl-oxytocin in Fig. 1, which gives the conductivity of the urine and the rate of flow. When the response was expressed in terms of the total antidiuretic effect, the log-dose-response plots for the analogues showed steeper slopes than the plot for oxytocin (Fig. 2,*a*); however, when the maximal intensity of antidiuresis produced by a given dose was used as the measure of response, the log-dose response curves were parallel in all cases (Fig. 2,*b*).

For all the analogues tested the antidiuretic activities (calculated by extrapolation to threshold dose) and indexes of persistence are given in the last two columns of Table 2.

These results show that both the intensity of the antidiuretic effect and its duration, as measured by the index of persistence, are dependent on the structure of the additional amino acid or peptide chain. Within the hormonogen group there is an inverse linear relation between the logarithm of antidiuretic activity and the index of persistence. (Rychlík, 1964). In contrast, the two analogues of the first group, MeCys[1]-oxytocin and D-Cys[1]-oxytocin and the two additional analogues, sarcosyl-oxytocin and D-leucyl-oxytocin, which were synthesized for comparison with the hormonogen group, do not fall into line with this group. The effect of D-leucyl-oxytocin did appear to be somewhat protracted by all the criteria used, but its antidiuretic activity was only about 0.1% of that shown by the L-leucyl derivative.

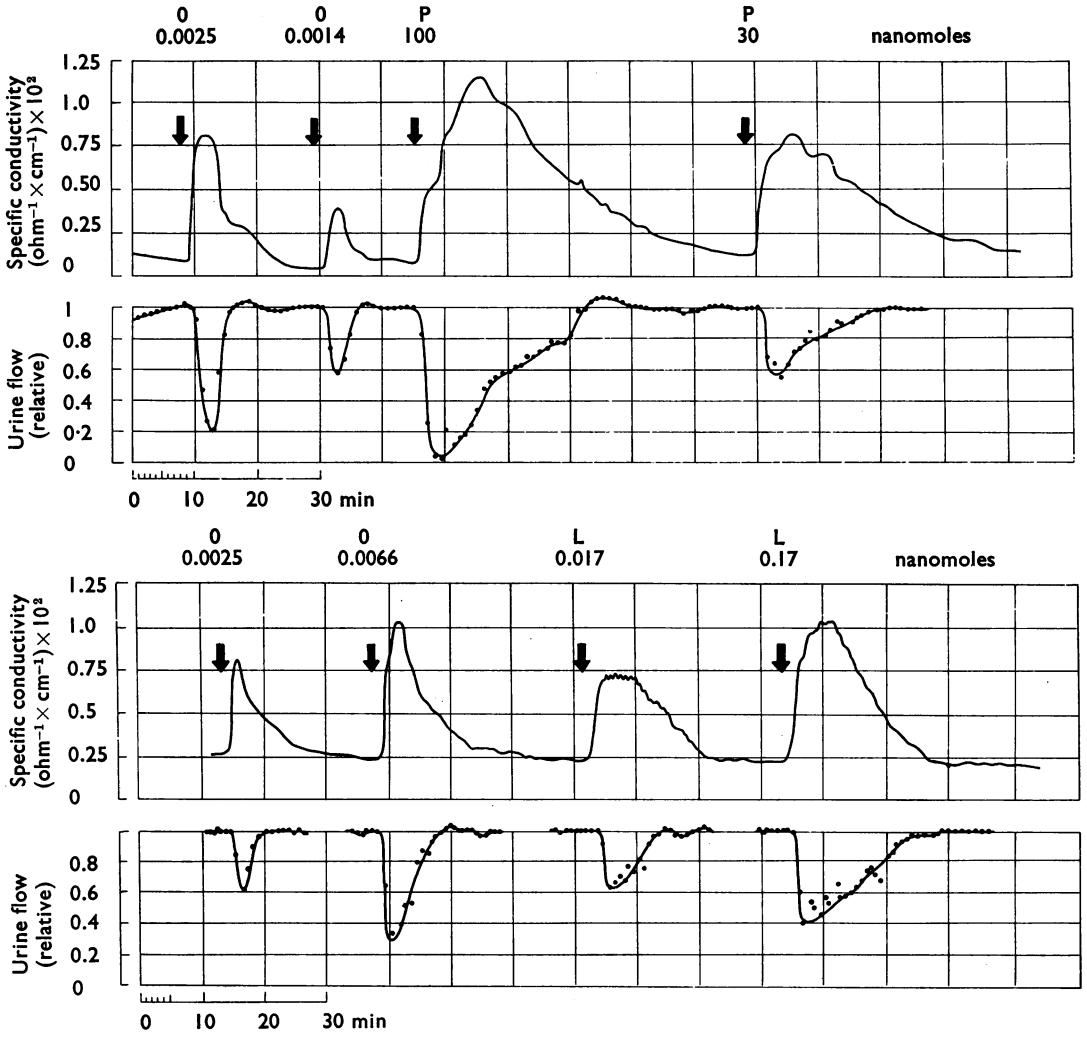


Fig. 1. Antidiuretic effects of oxytocin (O), prolyl-oxytocin (P), and leucyl-oxytocin (L). The upper curve of each pair is a tracing of urine conductivity, the lower curve a plot of urine flow. Doses are given in nanomoles assuming an oxytocic activity of 450 u/mg for oxytocin.

## *Effects on the mammary gland*

### *Milk-ejecting activity*

In experiments on the mammary gland of the guinea-pig *in situ*, no spontaneous changes in milk-ejection pressure were observed. The threshold dose of oxytocin for eliciting an increase in pressure was 0.5–2.0 m-u* by intravenous, and 0.05–0.1 m-u by retrograde arterial injection. With both routes of injection, the pressure increased in a single peak; it reached a maximum within 45 sec, then fell and at the end of 1 min

* 1 m-u is equivalent to 2.2 ng or 0.0022 nanomoles of pure oxytocin, assuming an oxytocic-activity of 450 u/mg.

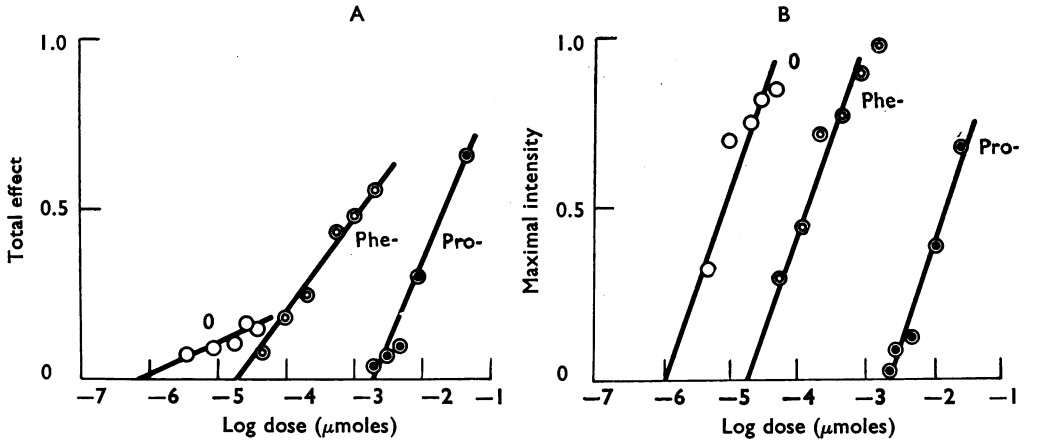


Fig. 2. Log-dose—response plots for oxytocin (O), phenylalanyl-oxytocin (Phe), and prolyl-oxytocin (Pro), with the total antidiuresis (A) and the maximal intensity of antidiuresis (B) as a measure of the response.

had subsided almost completely. Injections of oxytocin could be given repeatedly at 5-min intervals without inducing tachyphylaxis.

MeCys[1]-oxytocin, D-Cys[1]-oxytocin and sarcosyl-oxytocin produced the same type of response as oxytocin and when assayed for milk-ejecting activity possessed only 0.3–1.0 u/μ-mole.

D-Leucyl-oxytocin was inactive and the remaining analogues, which comprise the hormonogen group, produced a response different from that to oxytocin. Increases in milk-ejection pressure caused by these analogues were usually protracted and repeated injections tended to produce tachyphylaxis followed by inhibition of the response to oxytocin. They sometimes inhibited the response to oxytocin without first eliciting an increase in pressure. It was not possible to assign a definite potency to these analogues, but when they produced an increase in pressure at the beginning of an experiment the threshold dose was at least 100 times greater than that of oxytocin.

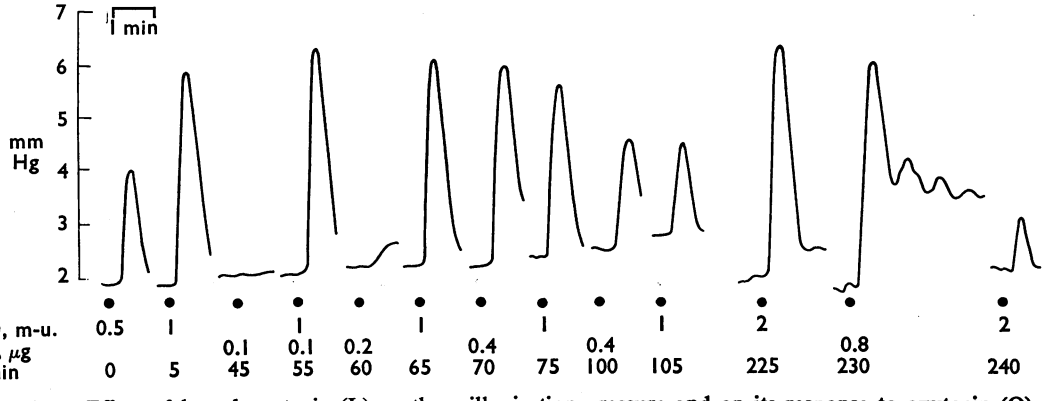


Fig. 3. Effect of leucyl-oxytocin (L) on the milk-ejection pressure and on its response to oxytocin (O).

D-*Leucyl-oxytocin:* Injected intravenously in doses from 1 to 64 μg, increasing by factors of 4, this analogue failed to elicit an increase in milk-ejection pressure and did not inhibit the response to 1 m-u of oxytocin given five min after the last injection of the analogue.

*Leucyl-oxytocin:* In all five experiments in which it was tested the intravenous injection of this analogue caused an increase in milk-ejection pressure. A typical result is illustrated in Fig. 3. The threshold dose in this experiment was 0.2 μg; with 0.4 μg there was a delay in the return of the pressure to normal; after 0.8 μg an initial large increase in pressure was followed by a series of small increases and the pressure did not return to the baseline until 10 min after the injection. Repeated doses of 0.4–0.8 μg induced tachyphylaxis and the response to subsequent doses of oxytocin were then inhibited. Inhibition occurred only after doses of the analogue which were sufficiently large to cause an increase in milk-ejection pressure. A subthreshold dose of 0.1 μg did not inhibit the response to 1 m-u oxytocin injected simultaneously. Inhibition caused by the analogue could be surmounted by injecting twice to four times the original dose of oxytocin.

*Leucyl-glycyl-glycyl-oxytocin:* In three out of eight experiments this analogue did not itself elicit an increase in milk-ejection pressure when injected intravenously but it strongly inhibited the response to oxytocin. Such an experiment is illustrated in Fig. 4. With increasing doses of the analogue up to 10 μg, there was a progressive decline in the response of the gland to oxytocin and 35 min after the injection of 10 μg, oxytocin in a dose of 16 m-u was required to match the initial response to 2 m-u. In one of these three experiments the time course of recovery from inhibition was studied. Ten min after the injection of 2 μg of the analogue, the response to 4 m-u of oxytocin was

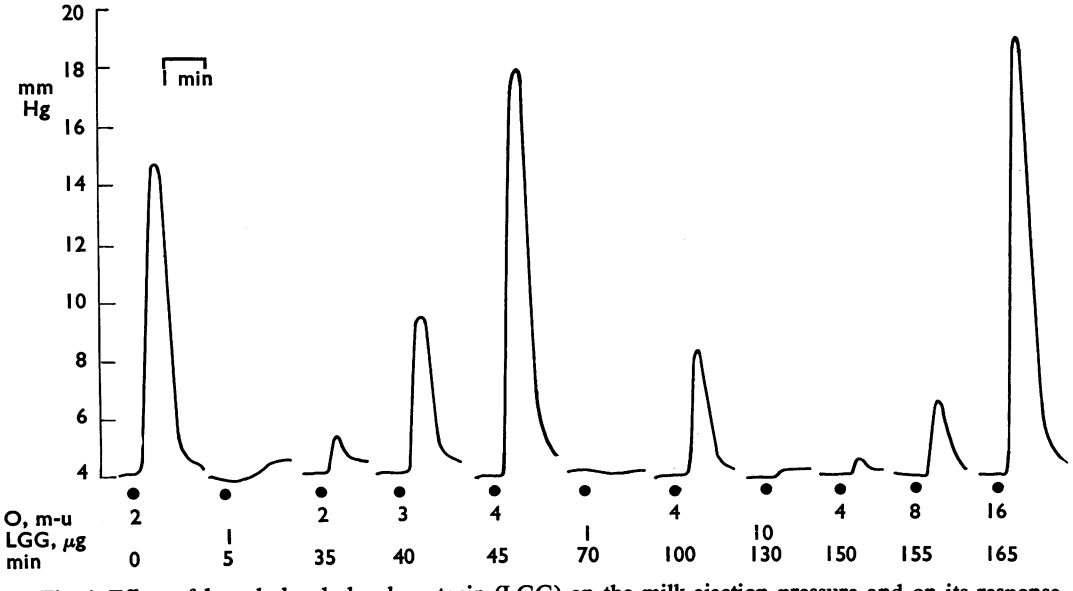


Fig. 4. Effect of leucyl-glycyl-glycyl-oxytocin (LGG) on the milk-ejection pressure and on its response to oxytocin (O).

smaller than that originally obtained with 2 m-u. Doses of 4 m-u were then injected at intervals of 10 min: recovery of the response occurred slowly and was almost complete after 90 min. In the other five experiments in which an increase in milk-ejection pressure did occur after intravenous injection of the analogue, the threshold dose was 2 μg. With doses of 4 μg and above the pattern of response was similar to that observed with leucyl-oxytocin—i.e., a prolonged increase in pressure followed by tachyphylaxis and inhibition of the response to oxytocin. In one of these experiments the effects of intravenous and retrograde arterial injections were compared. It was found that, in the relatively low dose (0.4 μg) required to elicit a milk-ejection response by the arterial route, the analogue did not cause tachyphylaxis, nor did it inhibit the response to oxytocin. Its milk-ejecting activity was equivalent to 0.6 u/μ-mole.

*Leucyl-leucyl-oxytocin:* The type of response obtained after intravenous injections of this analogue appeared to depend on the size of the initial dose. An increase in milk-ejection pressor occurred only when the initial dose was large (5 μg); the response was then followed by inhibition. When the initial dose was small (0.5 μg) no increase in pressure was produced and none then occurred on progressively raising the dose even to 10 μg, but inhibition of the response to oxytocin developed. For instance, in one experiment in which the initial dose was 5 μg the analogue caused a prolonged increase in pressure; 40 min after the injection, when the pressure had returned to normal, the smallest dose of oxytocin which was effective in eliciting a response was 16 m-u although the gland had originally responded to 1 m-u. On the other hand, in another experiment, after successive injections of 0.5, 1, 2, 4 and 10 μg, which produced no rise of milk-ejection pressure, the response to 64 m-u of oxytocin was no greater than that originally obtained with 2 m-u.

After retrograde arterial injections of 0.4–0.8 μg a protracted response occurred followed by 50% inhibition of the response to 0.1 m-u of oxytocin.

*Glycyl- and phenylalanyl-oxytocin:* Injected intravenously in doses of 1 μg or less these analogues produced a pattern of response which conformed with that described for leucyl-oxytocin. The phenlyalanyl analogue in a dose of 1 μg elicited a particularly protracted response, a series of small increases in pressure occurring over a period of 25 minutes.

*Glycyl-glycyl-oxytocin:* In the single experiment in which it was tested, this analogue had an action strikingly different from that of the leucyl-leucyl derivative. The threshold dose on intravenous injection was 1.6 μg. It produced an increase in milk-ejection pressure which was not prolonged, even with doses of 12 μg. Moreover, repeated doses of 3–6 μg induced only a moderate degree of tachyphylaxis and the response to oxytocin was finally inhibited by not more than 50%. When leucyl-leucyl-oxytocin was tested later on the same preparation, it produced a typically protracted milk-ejection response and strongly inhibited the response both to oxytocin and to the glycyl-glycyl analogue.

*Prolyl-oxytocin:* This analogue was also exceptional in eliciting a milk-ejection response which was not protracted, and the threshold dose on intravenous injection was relatively high (4 μg). Larger doses inhibited subsequent responses to oxytocin. No inhibition occurred when a subthreshold dose was injected one min before or simultaneously with oxytocin.

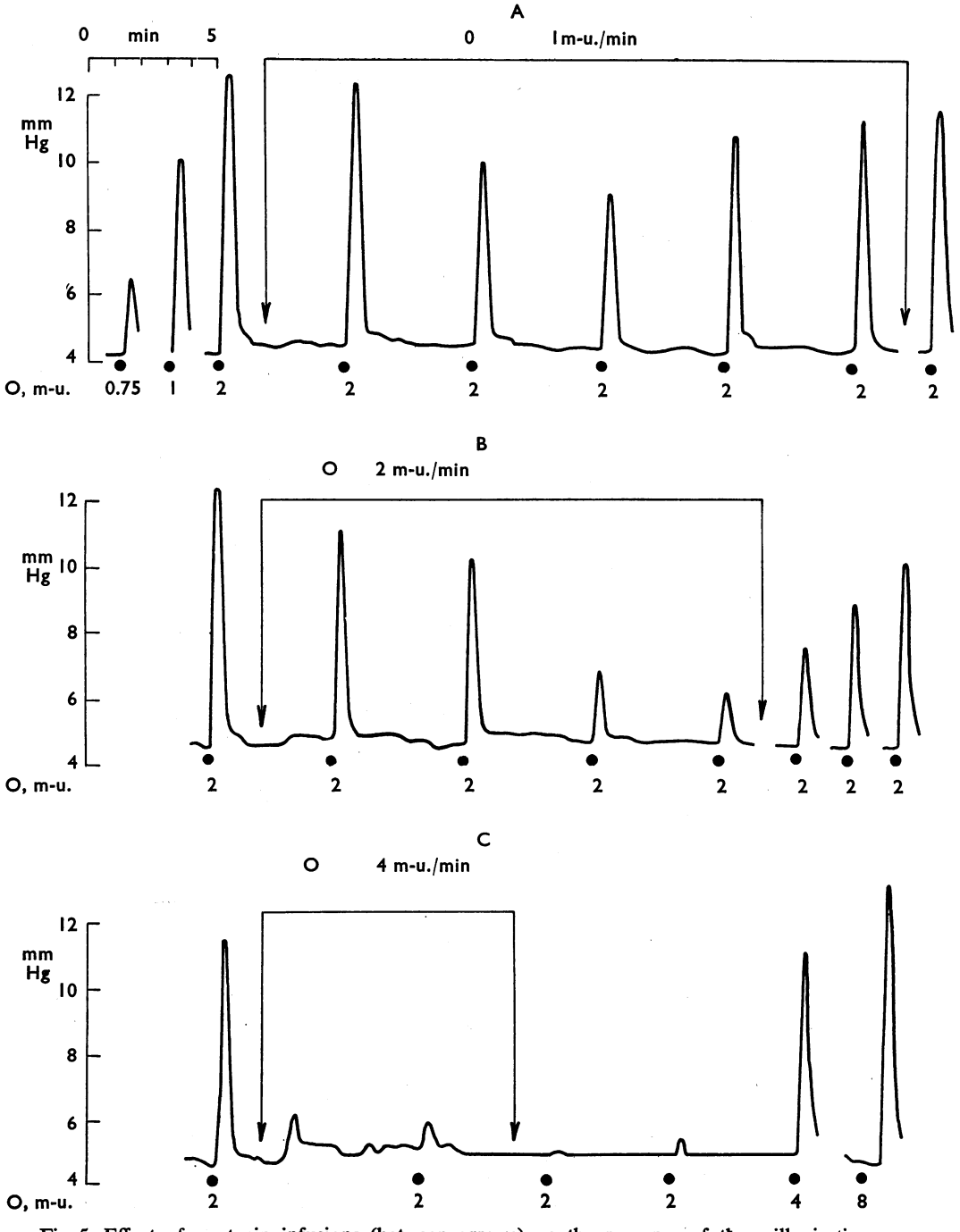


Fig. 5. Effect of oxytocin infusions (between arrows) on the response of the milk-ejection pressure to single doses of oxytocin (O). Dots mark challenge doses of oxytocin. In the discontinuous parts of the tracing, injections were given at intervals of 5 min.

*Effect on the isolated strip*

A strip of guinea-pig mammary gland exposed to 0.5–2.0 m-u of oxytocin underwent an almost immediate and rapid increase in tension, which reached a maximum within 1 min and was then followed by gradual relaxation. In contrast, leucyl-oxytocin and leucyl-leucyl-oxytocin in threshold doses of 0.01 to 0.05 $\mu$g produced an increase in tension only after a latent period of about 1 min and the tension then increased slowly and continuously until the analogue was washed out of the organ bath a few min later.

On the other hand, leucyl-glycyl-glycyl-oxytocin elicited responses similar in character to the response to oxytocin. No tachyphylaxis occurred, and the log-dose response curves for the analogue and for oxytocin were parallel. A similar effect was also obtained on the isolated strip of the mammary gland of the rat. The estimates of potency for leucyl-glycyl-glycyl-oxytocin were 0.6 u/$\mu$-mole on a guinea-pig preparation and 1.7 to 2.6 u/$\mu$-mole on two rat preparations.

*The milk-ejection response to intravenous injections of oxytocin during its continuous infusion*

In support of the idea that analogues of the hormonogen group act by continuously releasing oxytocin *in vivo* was the finding that the inhibitory action of these analogues in the lactating guinea-pig could be mimicked by infusing oxytocin intravenously. The result of such an experiment is shown in Fig. 5. Oxytocin was infused at three different rates. The infusion of 1 m-u/min (at A) or 2 m-u/min (at B) did not increase the milk-ejection pressure and the base-line remained unchanged, but the responses to 2 m-u of oxytocin injected intravenously during these infusions were partially inhibited. The inhibition was greater at the higher than at the lower rate of infusion. In addition, when the infusion at the higher rate was stopped, recovery of the response to intravenous injections of oxytocin was slow and, as shown by the last response in B, it was not complete 15 min later. The infusion of 4 m-u/min (at C) produced a small transient increase in milk-ejection pressure ; when this had passed off the response to intravenous injections of 2 m-u of oxytocin was completely inhibited and remained so for several min after the infusion was stopped. The inhibition could be surmounted by increasing the dose of injected oxytocin to 4 and 8 m-u, as shown by the last two responses at C. Control infusions of 0.9% NaCl solution did not produce inhibition of the response to intravenous injections of oxytocin.

*Effects on chicken blood pressure*

Oxytocin, in doses of 10–40 m-u, caused a brief fall in arterial blood pressure, amounting to 20–60 mm Hg and lasting generally no more than 30 sec. MeCys$^1$-oxytocin, D-Cys$^1$-oxytocin and sarcosyl-oxytocin gave qualitatively the same response as oxytocin ; neither tachyphylaxis nor inhibition was produced.

All the analogues of the second structural type—i.e., the hormonogen group, evoked responses distinctly different in character from those to oxytocin. The blood pressure generally fell more gradually and recovery was always more prolonged. Each of the analogues produced tachyphylaxis and caused a varying degree of inhibition of the response to subsequent doses of oxytocin. It was therefore not possible to determine

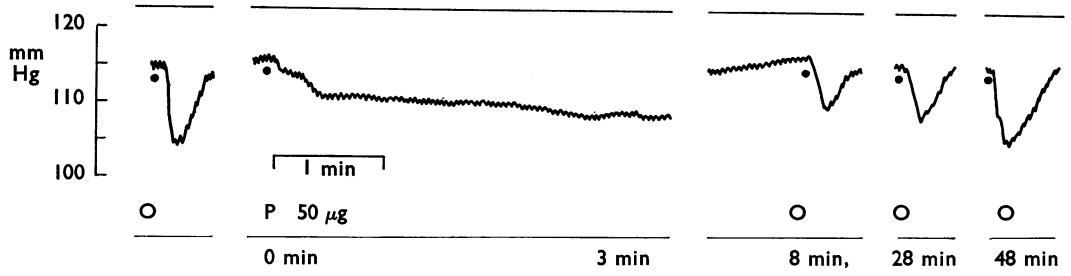


Fig. 6. Effect of phenylalanyl-oxytocin (P) on the blood pressure of the fowl and on its response to oxytocin (O). Challenge doses of oxytocin 30 m-u.

the activities by the usual procedure and they were estimated by comparing the decrease in blood pressure caused by a suitable dose of the analogue with the effect of standard doses of oxytocin injected before the analogue.

*Phenylalanyl-oxytocin.* The estimated activity was 0.2–0.4 u/μ-mole. In doses from 20 to 100 μg this analogue caused a fall of blood pressure, which reached its lowest value in about 4 min, followed by a return to normal in 5–8 min. With repeated injections of the same dose of the analogue its depressor action became progressively smaller as tachyphylaxis developed and the response to oxytocin was inhibited by 30–60%. The sensitivity to oxytocin returned gradually within 1 to 2 hr. Fig. 6 shows the prolonged depressor action of an intravenous injection of 50 μg of phenylalanyl-oxytocin followed by partial inhibition of the response to 30 m-u of oxytocin. As shown by the repeated injections of oxytocin at 20-min intervals recovery of sensitivity required about 50 min.

*Leucyl-oxytocin and leucyl-leucyl-oxytocin.* The estimated activity was 2 u/μ-mole for the leucyl and 0.5–1 u/μ-mole for the leucyl-leucyl analogue. In order to produce a fall of 20–60 mm Hg, as obtained with 10–40 m-u of oxytocin, 5–20 μg of the leucyl and 10–80 μg of the leucyl-leucyl analogue had to be injected. The fall produced by the analogues was similar to that seen after the injection of phenylalanyl-oxytocin in that the blood pressure fell gradually and recovered slowly. It took 4–6 min before the blood pressure returned to normal. Tachyphylaxis and inhibition of the response to oxytocin developed to about the same degree as after phenylalanyl-oxytocin.

*Glycyl-glycyl-oxytocin.* The estimated activity was 0.3–0.5 u/μ-mole. In doses of 20 to 80 μg the analogue produced a rapid fall in blood pressure like that seen with

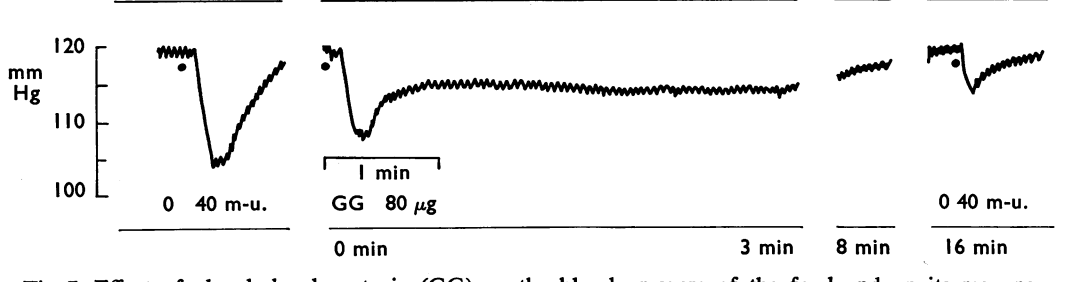


Fig. 7. Effect of glycyl-glycyl-oxytocin (GG) on the blood pressure of the fowl and on its response to oxytocin (O).

oxytocin but the recovery, which was initially as rapid as with oxytocin, slowed down to the extent that a period of 6–8 min was required before the blood pressure returned to normal. Tachyphylaxis as well as inhibition of the response to oxytocin occurred. The experiment of Fig. 7 illustrates the typical depressor response to the analogue, injected in a dose of 80 $\mu$g, and the subsequent partial inhibition of the response to oxytocin.

*Leucyl-glycyl-glycyl-oxytocin.* The estimated activity was 0.3–0.5 u/$\mu$-mole. The depressor response to 20–80 $\mu$g of the analogue resembled either that to phenylalanyl-oxytocin or, less frequently, that to glycyl-glycyl-oxytocin. In both instances tachyphylaxis developed with inhibition of the response to oxytocin.

*Prolyl-oxytocin.* The estimated activity was less than 0.05 u/$\mu$-mole. This analogue had scarcely any depressor effect. In doses up to 160 $\mu$g, it produced a fall of less than 5 mm Hg, but doses as low as 20 $\mu$g inhibited the action of subsequent injections of oxytocin.

*Glycyl-oxytocin.* This analogue has been studied by du Vigneaud *et al.* (1960). Like them we found that it produced a prolonged fall in arterial blood pressure followed by tachyphylaxis with inhibition of the responses to oxytocin.

*The avian depressor response to intravenous injections of oxytocin during its continuous infusion*

An intravenous infusion of oxytocin caused some inhibition of the responses to single intravenous injections of oxytocin given during, or even after, the infusion. The extent of the inhibition depended on the rate of infusion. Infusion at a rate of 8–16 m-u/min caused either no change in arterial blood pressure or a slight fall: during such an infusion, however, the response to a challenge dose of oxytocin (10–40 m-u) decreased by 20–30%. In some experiments the sensitivity to oxytocin returned within ten min of the cessation of the infusion but in others recovery was still incomplete after 1 to 2 hr, when the experiment was terminated. Infusion at a rate of 20 m-u/ml. always caused a fall of arterial blood pressure, which varied from animal to animal, but the blood pressure usually returned to normal before the end of the infusion period (15–20 min). Inhibition of the response to a challenge dose of oxytocin was more pronounced, and recovery of sensitivity after cessation of the infusion was slower than with the lower

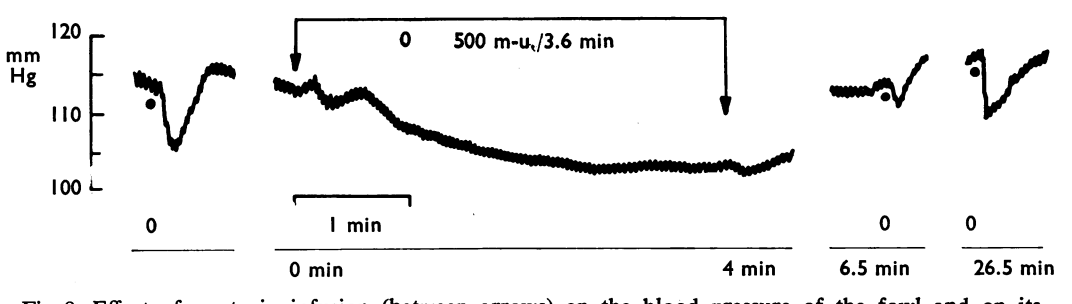


Fig. 8. Effect of oxytocin infusion (between arrows) on the blood pressure of the fowl and on its response to oxytocin. Challenge doses of oxytocin 30 m-u. This experiment was arranged for direct comparison with the effect of phenylalanyl-oxytocin shown in Fig. 6.

rate of infusion. Finally infusion at a rate of 70–150 m-u/min caused a pronounced fall in blood pressure. In the experiment illustrated in Fig. 8 infusion was at the rate of 140 m-u/min for 3.6 min. Challenge doses of oxytocin were tested in this instance only after cessation of the infusion and recovery of the blood pressure, which required about 4 min. The response to the challenge doses was then depressed. In some experiments the sensitivity had not fully recovered after 1 hr.

## DISCUSSION

The oxytocin analogues of the first group examined, with the terminal amino group methylated or the amino-terminal hemicystine residue replaced by D-hemicystine, are thought to be resistant to degradation by aminopeptidases, since it was found earlier that *S*-benzyl-D-cysteinyl-L-tyrosine and *N*-substituted derivatives of *S*-benzyl-L-cysteinyl-L-tyrosine such as the benzyl-oxycarbonyl derivative were stable to both pregnancy serum and the oxytocin-inactivating system of liver cell sap (Beránková, Rychlík & Šorm, 1961). However, neither MeCys$^1$-oxytocin nor D-Cys$^1$-oxytocin exhibited a protracted action in any of the *in vivo* systems examined. Furthermore, deamino-oxytocin, an analogue which lacks the terminal amino group altogether (Hope, Murti & du Vigneaud, 1962) and is therefore not susceptible to the action of leucine aminopeptidase or pregnancy serum (Golubow & du Vigneaud, 1963 ; Golubow, Chan & du Vigneaud, 1963), showed a normal time course of the response in the avian depressor and milk ejection assays (Chan & du Vigneaud, 1962). Making an analogue stable to aminopeptidase action is therefore not in itself sufficient to confer upon that analogue a protracted type of action.

A protracted action was found in the second group of analogues, in which the substituent group was an amino-acid or a peptide. Since the protracted action of these analogues appears to be due to the liberation or " generation " of oxytocin from them by enzymes, we propose the term " synthetic hormonogen " to describe such analogues.

As the nature of the substituent group in a hormonogen must influence the rate of release of oxytocin, the responses to different hormonogen analogues should differ in their time course. The simplest system to analyse in this respect is the antidiuretic effect, since it is uncomplicated by tachyphylaxis and can be measured quantitatively. In the present experiments, a protracted antidiuretic effect was found to be most pronounced in those analogues in which glycine or a glycine peptide was the substituent, and least marked in those in which the substituent was leucine or phenylalanine. This is in accordance with the high levels of leucine aminopeptidase activity which are generally found in blood and tissues (see, e.g., Blech, Hermann & Kleine, 1962) since leucyl derivatives should be split more rapidly than glycyl derivatives. The antidiuretic activity, when it is expressed in terms of the maximal intensity of the effect or in terms of the threshold dose, must depend on the speed with which an effective concentration of oxytocin is built up (against an essentially constant rate of elimination and inactivation), and should therefore be higher for leucyl than for glycyl derivatives. On the other hand, the response should be less protracted with the leucyl analogues because they are more rapidly exhausted. The striking inverse relation found, over the whole range of hormonogens, between the index of persistence—which is a measure of prolongation—and antidiuretic activity constitutes strong evidence for a causal relationship between the two parameters.

If the protracted action of these aminoacyl analogues is due to the release of oxytocin it should not be observed with two additional analogues of the same structural type, sarcosyl-oxytocin and D-leucyl-oxytocin, since neither the *N*-methylamino acid, sarcosine, nor D-leucine would be expected to be split off at an appreciable rate by the common aminopeptidases. In fact sarcosyl-oxytocin did not show a protracted action on any of the systems in which it was tested. D-Leucyl-oxytocin did produce a slightly protracted antidiuretic response but there is reason to believe that this may have been due to contamination with the L-isomer.

Release of oxytocin by the hormonogen group of analogues *in vivo* would explain why their activities relative to oxytocin are higher *in vivo* than on isolated organs. Measured on the rat uterus, the ratio of activity *in situ* to that on the isolated organ was 5 or higher for all but one of the hormonogens, whereas for sarcosyl-oxytocin this ratio was about 1 and for MeCys[1]-oxytocin about 3. Other oxytocin analogues are known to exhibit a higher activity *in vivo* than on the isolated organ, but the difference is not as great as for most of the hormonogens. For instance, for 3-valine-oxytocin the corresponding ratio of activities in both the rat and the cat is about 3 (Berde, Doepfner & Konzett, 1957; Smith & Ginsburg, 1961).

Direct evidence for a hormonogen mechanism of the protracted action is derived from enzymic experiments *in vitro* carried out by Beránková, Rychlík & Šorm (1964; and by Pliška, Cort, Douša, Rychlík & Šorm (unpublished experiments). During the incubation of analogues of this group with leucine aminopeptidase or with tissue homogenates under conditions where the inactivation of oxytocin itself is minimal, the biological activity of the incubate as measured by different biological assays was found to increase and a concomitant release of the " extra " amino-acid was demonstrated chromatographically.

The view that hormonogens act by release of oxytocin does not exclude the possibility that they possess in addition some inherent activity of their own. The fact that sarcosyl-oxytocin and MeCys[1]-oxytocin, as well as other oxytocin derivatives substituted in the amino group with metabolically relatively inert substituents such as acetyl (Boissonnas, Pechère & Guttmann, unpublished; cf. Boissonnas, Guttmann, Berde & Konzett, 1961) and carbamyl (Bisset, Poisner & Smyth, 1963; Smyth, personal communication), all show definite, if low, oxytocin-like activity in one or more assay systems, indicates that substitution in the amino group by itself need not lead to complete loss of activity. On the other hand, the action of hormonogens on isolated organs is not necessarily a sign of inherent activity, since oxytocin may be released from the analogue by the action of aminopeptidases in the isolated tissues. Certain features of the responses found on isolated organs are strongly suggestive of such a mechanism, as for example, the long latent period and continuous increase in tension of the isolated strip of the mammary gland exposed to leucyl- or leucyl-leucyl-oxytocin. The analogues which showed this kind of behaviour *in vitro* are those which appear from other evidence also to be the most susceptible to enzymic activation.

The action of analogues of the hormonogen type on the blood pressure of the fowl and also on milk-ejection pressure in the mammary gland of the lactating guinea-pig is complicated by the appearance of tachyphylaxis and inhibition of the action of oxytocin. As these features were observed only with the analogues of the hormonogen group and

not with sarcosyl- or D-leucyl-oxytocin nor with the analogues of the first group, they also may be the result of a continuous release of oxytocin. It had already been shown that a single large dose of oxytocin injected into the lactating guinea-pig caused a large increase in milk-ejection pressure followed by a prolonged period during which the mammary gland was refractory to small doses of oxytocin (Bisset, 1964). In the present experiments the effect of a gradual release of oxytocin from a hormonogen was more closely imitated by slow intravenous infusion of oxytocin into the lactating guinea-pig and the chicken. By varying the rates of infusion, the effects of the various types produced by the hormonogen analogues were mimicked. Thus infusion of oxytocin at subthreshold rates, which did not itself cause an increase in milk-ejection pressure or a fall of blood pressure in the chicken, produced tachyphylaxis to intravenous injections of previously effective doses of oxytocin. At higher rates of infusion there was an initial response, which gradually faded, resembling the " protracted " effect of the analogues, and the subsequent tachyphylaxis was more pronounced.

Responses to a hormonogen analogue may thus involve its inherent activity as well as its hormonogen action, and the latter may appear as a protracted effect, or as inhibition resulting from a tachyphylactic mechanism, or as a combination of both. The actual pattern of response in any particular case will be determined by the structure of the hormonogen and the biological properties of the assay system. For example, the behaviour of gylcyl-glycyl-oxytocin, which on the mammary gland showed no protracted action and in the chicken in some experiments an initial oxytocin-like time course of the response followed by a hormonogen-like phase, suggests that this compound has a relatively high inherent activity in these two systems coupled with a low rate of release of oxytocin. The same explanation could apply to prolyl-oxytocin, the action of which on the mammary gland was found not to be protracted. On the other hand, both glycyl-glycyl-oxytocin and prolyl-oxytocin conformed with the hormonogen pattern in the antidiuretic assay, and for prolyl-oxytocin this applied also to the chicken blood pressure.

Again, by considering the probable time course of oxytocin release, the finding can be explained that, in small doses, leucyl-glycyl-glycyl-oxytocin sometimes inhibited the response to oxytocin in the lactating guinea-pig and prolyl-oxytocin that in the fowl without themselves first evoking a response, while analogues such as phenylanyl- or leucyl-oxytocin invariably evoked a positive response before causing noticeable inhibition. Activation of the more susceptible hormonogens results in a rapid build-up of the oxytocin concentration to the threshold for response ; on the other hand, the sluggish release of oxytocin from the more slowly activated hormonogens maintains a subthreshold concentration of the hormone for some time, so that tachyphylaxis is established before the threshold for response is reached. In agreement with this interpretation, very large doses of prolyl-oxytocin were found to cause a prolonged drop in the blood pressure of the fowl, and large initial doses of leucyl-glycyl-glycyl-oxytocin showed the same action on the milk-ejection pressure as, e.g., leucyl-oxytocin ; here the slow rate of release of oxytocin appears to be compensated by the increased concentration of the hormongen.

Evidently the hormonogen concept affords a satisfactory general explanation of the varied biological properties of this group of hormone analogues. A detailed interpretation, however, may well require consideration of additional factors such as the distribution

behaviour of such analogues, differences in the enzyme spectra of individual organs and possibly species, and the effects of the rate at which the hormone concentration changes on the character of the response in individual biological preparations.

SUMMARY

1. Two series of synthetic analogues of oxytocin, in which the amino terminal hemicystine residue was modified with the aim of producing an alteration in metabolic behaviour, were tested for oxytocic and antidiuretic activity and for their action on the mammary gland and chicken blood pressure.

2. One series was designed to be resistant to the aminopeptidases which are believed to inactivate oxytocin *in vivo*. In one analogue of this series the terminal amino group was methylated and in the other the terminal hemicystine residue was in the D configuration. The responses to these analogues *in vivo* were not prolonged, indicating that resistance to aminopeptidases in itself is not sufficient to confer a protracted action.

3. In the other series of analogues an amino-acid or a short peptide chain was attached to the terminal amino group of oxytocin in the expectation that the substituent group would be split off by aminopeptidases *in vivo*, causing a continuous liberation of oxytocin. The term " hormonogen " has been introduced for analogues thought to act in this way. The series includes glycyl-, leucyl-, phenylalanyl-, prolyl-, glycyl-glycyl, leucyl-leucyl and leucyl-glycyl-glycyl-oxytocin. In contrast with the analogues of the first series the hormonogens usually produced a protracted action *in vivo*. The protracted responses of the mammary gland and of chicken blood pressure were followed by tachyphylaxis and inhibition of subsequent responses to oxytocin. In some instances these analogues inhibited oxytocin without first eliciting a response. On the uterus *in situ* the activity was higher than on the isolated organ. Variations in the pattern of response seemed to depend on a number of factors, including the nature of the substituent group, the species and target organ in the assay system, the route of injection and the size of the initial dose.

4. Two additional analogues, sarcosyl- and D-leucyl-oxytocin, in which the terminal amino group was substituted by an amino-acid which was not expected to be split off by aminopeptidases *in vivo*, did not exhibit protracted or inhibitory effects.

5. The typical actions of the hormonogens were mimicked by continuously infusing oxytocin into assay preparations at rates comparable with those at which it might theoretically be liberated from the analogues.

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