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# BIOASSAY OF OXYTOCIN ON THE SUPERFUSED MAMMARY GLAND OF THE MOUSE, USING AN AUTOMATIC APPARATUS

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1 A sensitive method of assaying oxytocin using the superfused mouse mammary gland is described. The method is shown to be reliable, accurate and precise. Assays are performed automatically and the preparation is stable for at least 18 hours.

2 The preparation is relatively insensitive to bradykinin, histamine, 5-hydroxytryptamine, angiotensin, prostaglandin $F_{2\alpha}$, adrenaline and noradrenaline. The contractions produced by acetylcholine can be abolished by atropine without the sensitivity to oxytocin being affected.

3 Vasopressin has a variable activity on the preparation; its potency can be as high as one-fifth that of oxytocin.

4 It is concluded that this method compares favourably with other published methods.

## Introduction

Bioassays based on the response of mammary tissue to oxytocin have been developed by a number of workers, using tissue from various species both *in vivo* and *in vitro* (see Fitzpatrick & Bentley, 1968, for references), but superfusion of this organ has not been described.

We have developed a method of superfusing a strip of mouse mammary gland, which has certain advantages over existing assays. It has been demonstrated to the Physiological Society (Robinson & Walker, 1973).

## Methods

### *Preparation*

Mice (Tuck No. 1 strain) are used during mid-lactation, the litter having been removed from the mother 3 h before starting the assay to allow the mammary glands to become engorged with milk.

Under light ether anaesthesia, a cut is made along the line of the lower teats on one side, and the mammary gland mass supplying these teats is exposed. A piece of this tissue is cut out, placed on a cork board in a pool of Tyrode solution and trimmed to a thin strip measuring approximately 40 x 3 x 3 mm. The strip is suspended from a strain gauge transducer (Devices ST.01, modified for high sensitivity) so that contractions may be recorded by means of an amplifier with a high frequency filter (Devices S.1) on a potentiometric recorder (Servoscribe RE 511). The lower end of the tissue is fixed by a hook and thread in a hollow support (Figure 1). This arrangement allows the superfusion fluid to flow off the lower end of the tissue in a continuous stream, and the drops ultimately form beneath a second, independent support, where they fall to waste. The superfusion outlet is placed close to the top of the tissue so that drops do not form at this point. In this way, fluctuations in the weight of the fluid on the tissue caused by drops falling on and off the strip are avoided, and the small changes in tension produced by oxytocin can be recorded more clearly.

### *Apparatus*

Since the superfusion must be continuous, a method of delivering small doses of oxytocin to the tissue without interrupting the flow was devised. The strip is superfused at a rate of 0.5 ml/min at room temperature by a roller pump. Fluid is drawn by the pump through a length of vinyl tubing from a reservoir, and modified relays are used to control the flow. A relay moves a brass rod between two stops made of perspex. Tubing passes on either side of the rod and is pinched between it and one of the stops. Whatever the position of the relay, one tube is occluded and the other is open (Figure 2). One of the tubes leads

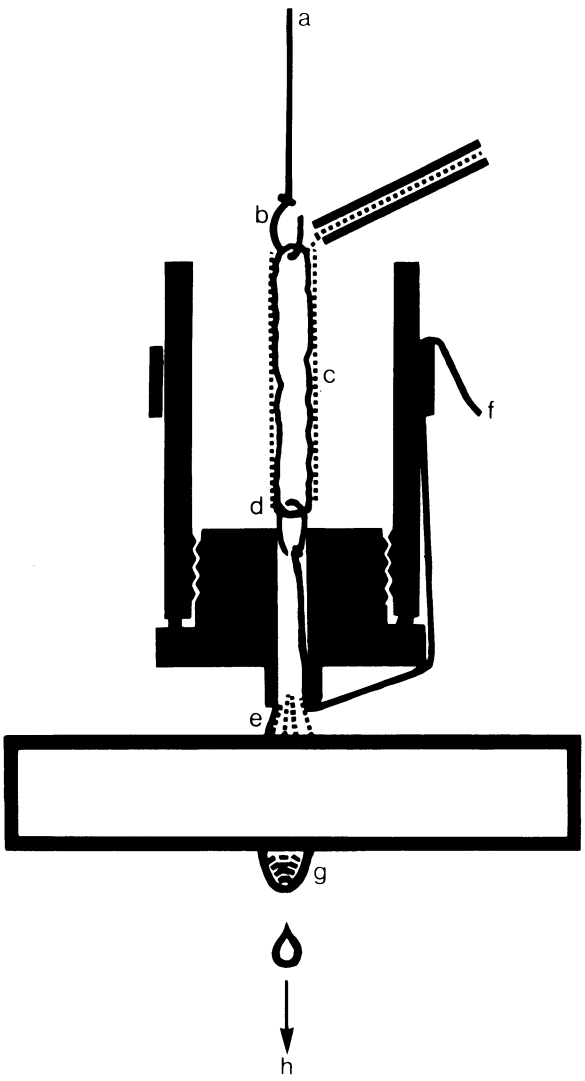


**Fig. 1** Diagram showing strip of mouse mammary gland set up for superfusion. The hollow lower support allows a continuous flow of superfusing fluid. (a) Cotton thread, (b) hook, (c) mammary strip, (d) hollow support, (e) fluid outlet, (f) cotton thread, (g) drop forms, (h) to waste.

from the reservoir, and the other dips into a sample pot. The tubes join together between the relay and the pump; with the pump running continuously, fluid is drawn through whichever tube is open. When the relay is operated, the rod occludes the tube from the reservoir and opens the tube from the sample. At a rate of flow of 0.5 ml/min, operation of the relay for 1 min allows the pump to deliver a dose of 0.5 ml of the sample to the tissue without interruption of flow. In the apparatus shown in Fig. 3, four such relays are used; the tube from the reservoir passes through one side of each in turn, and four tubes leading from different sample pots are occluded by the relays. The tubes from the samples join the reservoir tubing past the relay assembly at a low-volume six-way junction. When any relay is operated, the tube from the reservoir is occluded and 0.5 ml of one of the samples is delivered to the mammary strip.

*Automatic assays*

The relays can be operated by hand, but it is more convenient to operate them automatically. The control unit (Bell, 1973) consists essentially of a clock, two batching counters, a matrix unit, and relay driving circuits. It is possible to set within wide limits both the interval between doses and the amount of each dose, and the relays can be operated independently. The matrix panel enables pulses to be directed to any relay in any order. It is therefore possible to program a full four-point assay of up to 40 randomized doses in one cycle, after which the pattern is repeated if required.

The equipment is reliable, delivers accurate and repeatable doses, and will run unattended for long periods. The preparation takes 10 min to set up, and it is only necessary to place the samples in position, program the design of the assay on the matrix panel and switch on the control unit for the assay to be completed automatically.

*Drugs and solutions*

The superfusion fluid used was modified Tyrode solution described by Roca, Garófalo, Gioia de Coch, Arocena & Coch (1972) as 'Tyrode 90'. It contained (g): NaCl 8.0; KCl 0.20; $MgCl_2 . 6H_2O$ 0.10; $CaCl_2$ anhydrous 0.17; $Na_2HPO_4 . 12H_2O$ 0.0055; $NaHCO_3$ 1.0; glucose 1.0; made up to 1100 ml with distilled water and gassed to pH 7.4 with 5% $CO_2$ and 95% $O_2$. For convenience this is referred to as Tyrode solution.

The following drugs were used: Synthetic oxytocin (Syntocinon, Sandoz) batch No 10272 re-assayed against the Third International Standard for Oxytocic, Vasopressor and Antidiuretic Substances (Bangham & Mussett, 1958) on the rat uterus. Synthetic lysine$^8$-vasopressin (Syntopressin, Sandoz); highly purified arginine$^8$-vasopressin prepared by Dr L.O. Uttenthal in this department and assayed against the International Standard on the blood pressure of the rat; synthetic angiotensin (Val$^5$-angiotensin amide, Hyper-

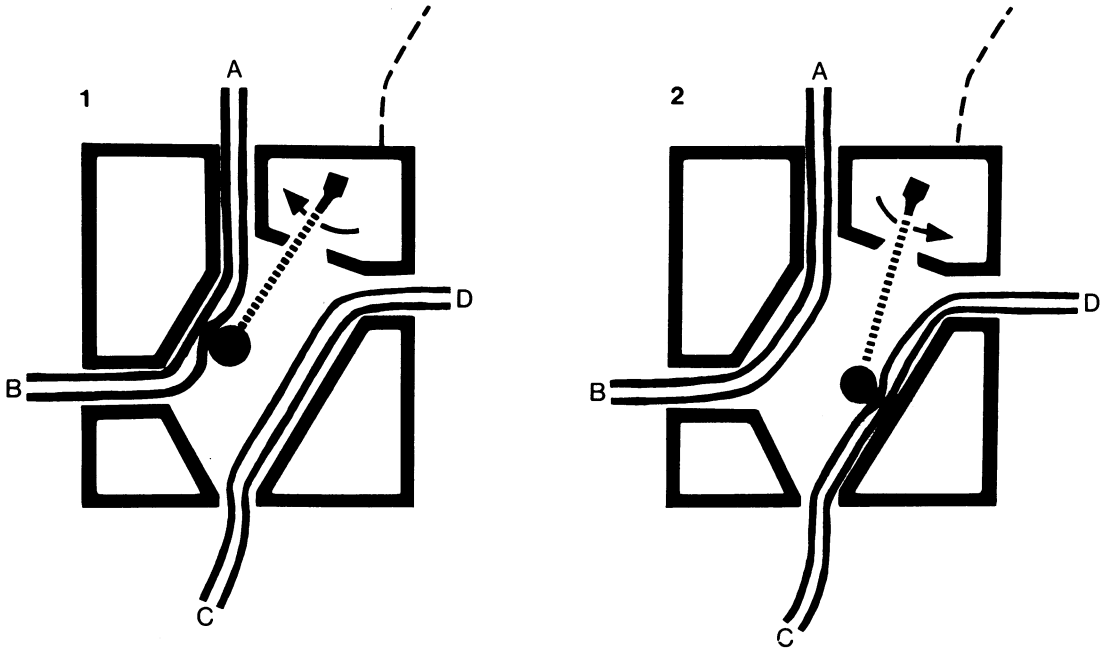


**Fig. 2** Diagram showing the mechanism of a relay. (1) At rest, tube AB is occluded and superfusion fluid is pumped through tube DC. (2) When the relay is operated, tube DC is occluded and the sample is pumped through tube AB. At the end of the dose, the relay is switched back to position 1 and superfusion fluid is pumped once more.

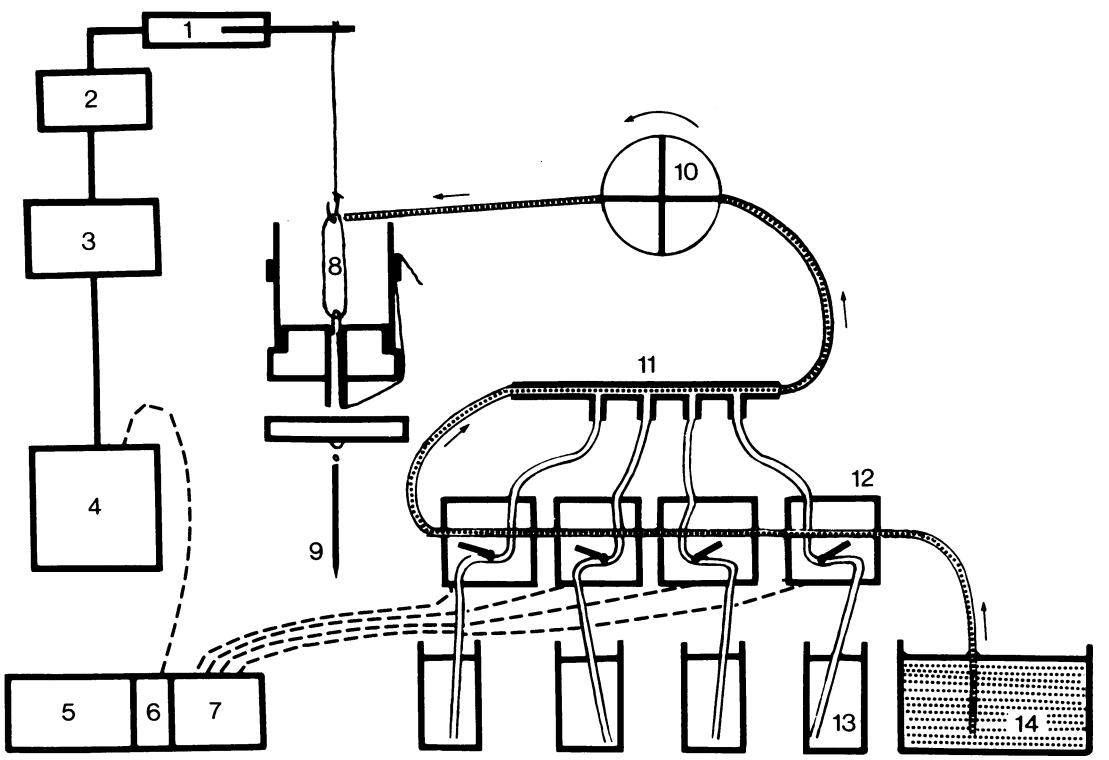


**Fig. 3** Diagram of the automatic assay apparatus. (1) Strain-gauge transducer; (2) high-frequency filter; (3) amplifier; (4) potentiometric recorder; (5) control unit; (6) marker; (7) relay drivers; (8) mammary strip; (9) to waste; (10) roller pump; (11) low-volume junction; (12) electromagnetic relays; (13) assay solutions; (14) superfusion fluid.

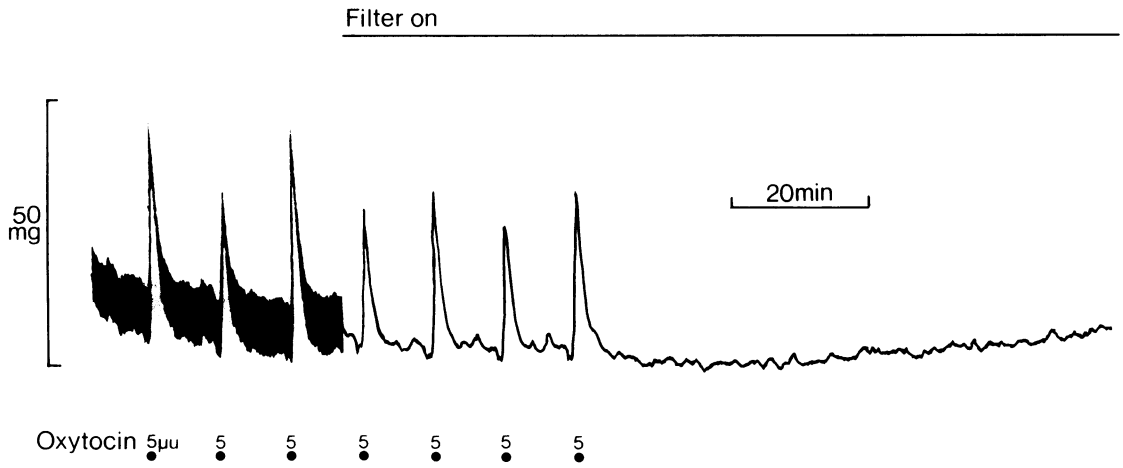


**Fig. 4** Superfused mouse mammary gland. Contractions produced by repeated doses of 5 μu oxytocin. The figure shows the effect of the high-frequency filter on the record obtained.

tensin, CIBA); atropine sulphate, bradykinin triacetate, acetylcholine chloride, 5-hydroxytryptamine creatinine sulphate, histamine, noradrenaline bitartrate (Sigma); adrenaline (Koch-Light). Prostaglandin $F_{2\alpha}$ was supplied by Professor J.R. Vane.

All drugs were diluted in Tyrode solution immediately before use. The concentrations of the salts are expressed in terms of the free base.

## Results

### *Responses to oxytocin*

Responses could be obtained over a wide range of initial tensions, and the usual tension used was 400 to 500 mg. Alteration of the tension during the assay usually reduced the responses and so this was avoided. At the highest recording sensitivity used,

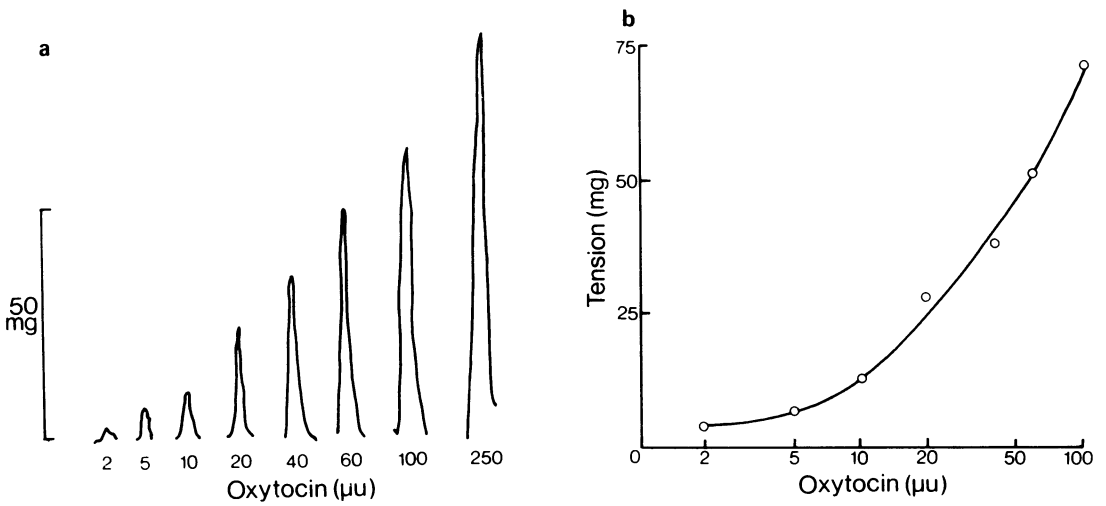


**Fig. 5** Superfused mouse mammary strip. (a) Representative contractions to a wide range of doses of oxytocin given during an experiment. Trace not continuous; the responses are arranged in order of increasing dose. (b) A dose-response curve constructed from similar results from another experiment. Each point is the mean of three responses.

the baseline would sometimes show a slow drift, but the responses obtained were unaffected by drifts of up to 10% of full scale deflection per hour.

Minor mechanical disturbances caused by the flow of superfusion could be seen on the trace, particularly if the superfusion outlet had been incorrectly placed, causing small drops to form. Figure 4 shows the effects of passing the signal through a high frequency filter. The fast fluctuations on the baseline have been eliminated, and the peaks are seen more clearly. All experiments were recorded using this filter.

The sensitivity of the preparation to oxytocin varied from assay to assay. The lowest dose that gave consistent responses was 0.44 μu, but the lowest dose satisfactory for assay purposes was about 2 μu, which requires a concentration in the sample of 4 μu/ml. This is the same sensitivity that was obtained in preliminary experiments in which the mammary strip was suspended in a bath of 4 ml capacity, but since 16 μu are required to achieve this concentration by this method, superfusion effectively increases the sensitivity eight times. It is possible to increase the effective sensitivity still further by reducing the rate of superfusion. The results presented here were obtained at a rate of 0.5 ml/minute.

The mammary strip contracts to oxytocin over a wide range of concentrations. The responses to increasing doses are seen in Fig. 5a, and a dose-response curve obtained in another experiment is plotted in Figure 5b. The responses were always single peaks; no spontaneous activity was ever observed.

The contractions to repeated doses of oxytocin were relatively constant over a long period, though there was often a gradual increase in sensitivity until about 8 h after the start of the assay.

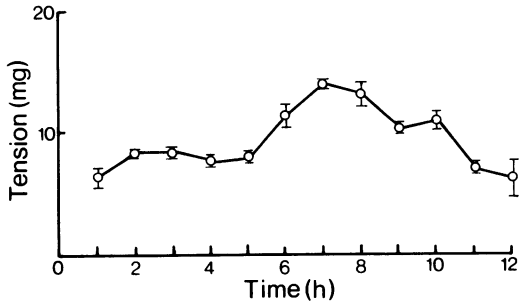


**Fig. 6** Contractions of the superfused mammary strip to oxytocin. Doses of 2 μu were given at 10 min intervals over a period of 12 hours. Each point is the mean ± s.e. of six responses.

Figure 6 shows an experiment in which a low dose of oxytocin (2 μu) was given at 10 min intervals over a period of 12 hours.

It was usually necessary to wait 10 min between doses, since if they were given more frequently tachyphylaxis often occurred. The disadvantage of the long interval required between doses in an assay is partially offset by this ability of the preparation to respond over a long period, and most assays were run for at least 18 hours.

The results of eight four-point assays, in which solutions containing known amounts of oxytocin were assayed against each other, are shown in Table 1. The mean error, i.e. the difference between the estimated and known concentration expressed as a percentage, was 2.6 ± 0.37% (s.e.), the mean index of precision (λ) was 0.0447 ± 0.0057 (s.e.), the mean fiducial limits at $P = 0.05$ were ±11.3%, and there was no significant deviation from parallelism in any of the assays. These

**Table 1** Statistical analysis of a series of four-point assays of oxytocin

| Assay | Dose (μu) low | Dose (μu) high | Potency ratio true | Potency ratio found | % Error | Index of precision (λ) | ± % Fiducial limits (P = 0.05) | Number of groups |
|---|---|---|---|---|---|---|---|---|
| 1 | 5.5 | 11 | 1 | 0.985 | 1.5 | 0.0620 | 13.2 | 6 |
| 2 | 5 | 10 | 1 | 0.969 | 3.1 | 0.0134 | 3.41 | 4 |
| 3 | 10 | 20 | 1 | 1.030 | 3.0 | 0.0440 | 13.74 | 3 |
| 4 | 100 | 200 | 1 | 1.013 | 1.3 | 0.0415 | 7.35 | 7 |
| 5 | 3.3 | 5 | 1 | 1.016 | 1.6 | 0.0412 | 11.0 | 4 |
| 6 | 3.7 | 5.5 | 0.9 | 0.932 | 3.6 | 0.0361 | 9.23 | 5 |
| 7 | 10 | 15 | 1 | 0.959 | 4.1 | 0.0621 | 16.7 | 4 |
| 8 | 17.5 | 25 | 1 | 0.973 | 2.7 | 0.0571 | 15.42 | 4 |
| | | | | Mean | 2.6 | 0.0447 | 11.3 | |
| | | | | ±s.e. | 0.37 | 0.0057 | 1.57 | |

figures indicate that automatic assays carried out under these conditions are reliable, accurate and precise.

*Responses to other substances*

The specificity of the assay was tested by giving doses of oxytocin and various other substances alternately. Each substance was tested on at least three different occasions, and the results are shown in Table 2.

It will be seen that the preparation is relatively specific for oxytocin; the only substances likely to be present in body fluids in sufficient quantities to interfere with assays are acetylcholine and vasopressin. The responses to as much as 500 ng acetylcholine are completely abolished by the addition of atropine 20 μg/ml to the superfusing fluid, and this does not affect the responses to small amounts of oxytocin. Vasopressin produced contractions similar to those of oxytocin. Its potency, however, could not be expressed as a single value in terms of activity of oxytocin on this tissue, since the dose-response curves for both lysine$^8$-vasopressin and arginine$^8$-vasopressin were not parallel to those of oxytocin, and thus the apparent potency depends upon the dose at which

**Table 2** Threshold doses of substances contracting the superfused mammary strip

| *Substance* | *Threshold (ng)* |
|---|---|
| Oxytocin | $10^{-3}$ |
| Arg$^8$-Vasopressin | $11 \times 10^{-3}$ |
| Lys$^8$-Vasopressin | $8.5 \times 10^{-3}$ |
| Acetylcholine | 0.2 |
| Bradykinin | $3.5 \times 10^3$ |
| Val$^5$-Angiotensinamide | 125 |
| Adrenaline | $>5 \times 10^2$ * |
| Noradrenaline | $>5 \times 10^2$ * |
| Histamine | $2.5 \times 10^4$ |
| 5-Hydroxytryptamine | $>5 \times 10^4$ * |
| Prostaglandin $F_{2\alpha}$ | $10^3$ |

The doses were given in volumes of 0.5 ml.
* No contraction was observed at this dose.

**Table 3** Some assays for oxytocin using mammary tissue

| | | *Oxytocin* | | | | | |
|---|---|---|---|---|---|---|---|
| *Method* | * *Ref.* | *Threshold dose (μu)* | *Threshold conc. (μu/ml)* | *Lowest dose (μu) for routine assay* | *Min. conc. (μu/ml) required in assay sample* | λ | *% Error* |
| (1) *In vivo* | | | | | | | |
| Guinea-pig | (1)(2) | 1 | – | 16 | 32 | 0.080 | 3.7 |
| Rat | (3) | 1 | – | 5 | 25 | 0.074 | 7.1 |
| Rat | (4) | 0.5 | – | 5 | 25 | 0.079 | – |
| (2) *In vitro* | | | | | | | |
| Rabbit strip | (5) | 100 | 100 | 500 | $10^4$ | 0.993 | – |
| Rat strip | (6) | $2 \times 10^3$ | 200 | $4 \times 10^3$ | – | – | 3.9 |
| Rat strip | (7) | 2 | 0.5 | 50 | 5† | 0.115 | – |
| Rat strip | (4)(8) | 1 | 1 | 25 | 25 | 0.087 | – |
| Rat milk expulsion | (9) | $10^{-10}$ | $10^{-8}$ | $10^{-6}$ | $10^{-4}$ | 1.026 | – |
| Rat milk expulsion | (10) | $4.4 \times 10^{-5}$ | $4.4 \times 10^{-3}$ | $2.2 \times 10^{-2}$ | 2.2 | – | – |
| Rat transparency | (11) | 0.25 | 0.25 | 0.5 | 5 | 0.108 | 2.5 |
| Mouse strip | (12) | 10 | 5 | 20 | 10 | 0.046 | – |
| Mouse strip | (13) | 0.2 | 0.1 | 2 | 1 | – | – |
| Superfused mouse strip | (14) | 0.44 | 0.88 | 2 | 4 | 0.045 | 2.6 |

* (1) Tindal & Yokoyama, 1962 (2) Folley & Knaggs, 1965 (3) Bisset *et al.*, 1967 (4) Fabian, Forsling, Jones & Lee, 1969 (5) Moore & Zarrow, 1965 (6) Smith, 1961 (7) Rydén & Sjöholm, 1962 (8) Brovetto, Olhaberry, Gioia de Coch, Coda, Fielitz, Cabot, Fraga & Coch, 1967 (9) Van Dongen & Hays, 1966 (10) Van Dongen & Marshall, 1967 (11) Bilek *et al.*, 1971 (12) Fielitz, Roca, Mattei, Melander, Garófalo, Gioia de Coch & Coch, 1970 (13) Roca *et al.*, 1972 (14) present paper.

the comparison is made. In some assays its apparent potency was as much as one-fifth that of oxytocin.

## Discussion

Most methods of assaying oxytocin biologically depend on its action in lowering the blood-pressure of the fowl, contracting the uterus, or contracting the myoepithelial elements in the mammary gland. The blood-pressure method (Coon, 1939) is insensitive and useful only for the assay of the large amounts of oxytocin that occur in extracts of the neurohypophysis or for determining the oxytocic potency of synthetic peptides. Methods using the uterus (Holton, 1948) are somewhat more sensitive, particularly if the animal is pretreated with stilboestrol (Follett & Bentley, 1964) and the uterus is superfused (Fitzpatrick, 1961), but the organ responds in addition to many other substances which are likely to be present in concentrated extracts of body fluids (Bisset & Lewis, 1962). Only methods using the mammary gland are sensitive and specific enough for the detection and assay of the small quantities of the hormone present in plasma and urine (Fitzpatrick & Bentley, 1968).

Many methods using the mammary gland involve the measurement of intramammary pressure *in vivo*. The teat of the anaesthetized lactating animal is cannulated, oxytocin is injected into the blood stream and changes in the pressure are recorded. Suitable animals are the rabbit (Fitzpatrick, 1961), guinea-pig (Tindal & Yokoyama, 1962) and rat (Bisset, Clark, Haldar, Harris, Lewis & Rocha e Silva, 1967). It is possible to obtain greater sensitivity by giving injections directly into the arterial supply, so that the oxytocin reaches its site of action in higher concentrations (Fitzpatrick, 1961; Bisset *et al.*, 1967). Other workers have used the mammary gland *in vitro*; a strip of tissue is suspended in an organ bath, and the increases in tension caused by oxytocin are recorded (Méndez-Bauer, Cabot & Caldeyro-Barcia, 1960; Rydén & Sjöholm, 1962; Roca *et al.*, 1972). Alternatively, fragments of gland may be observed under a microscope; the latent period

| *Approx. 95% fiducial limits ±* | *Approx. potency of AVP relative to oxytocin (%)* | *Other substances* | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | *Ach* | *Br* | *Ang* | *A* | *NA* | *Hist* | *5-HT* |
| | | (1) *Minimum effective dose (ng)* | | | | | | |
| 18% | – | 16 | $10^3$ | $10^3$ | $5 \times 10^3$ | – | $1.8 \times 10^4$ | $1.1 \times 10^4$ |
| 26% | 14 | 4 | 100 | $1.5 \times 10^4$ | – | – | $>2 \times 10^4$ | 100 |
| – | 25** | – | – | – | – | – | – | – |
| | | (2) *Minimum effective concentration (ng/ml)* | | | | | | |
| – | 15 | $2.5 \times 10^4$ | – | – | 100 | – | $1.25 \times 10^5$ | $>5 \times 10^4$ |
| 15% | 3 | – | – | – | – | – | – | – |
| 11% | – | $10^{-3}$ | – | – | $10^{-2}$ | $10^{-2}$ | $10^{-2}$ | 10 |
| – | 10** | – | $>10^3$ | $>10^3$ | – | – | – | – |
| – | – | – | – | – | – | – | – | – |
| – | 100 | $1.3 \times 10^{-3}$ | 450 | – | >80 | >74 | >50 | >77 |
| 34% | 38** | 0.6 | 2 | 250 | – | – | $10^3$ | 0.12 |
| 15% | 20 | 20 | $10^3$ | – | $>10^5$ | $>10^5$ | $>10^4$ | $>10^6$ |
| – | – | – | – | – | – | – | – | – |
| 11% | 20** | 0.4 | $7 \times 10^3$ | 250 | $>10^3$ | $>10^3$ | $5 \times 10^4$ | $>10^5$ |

AVP, arginine[8]-vasopressin; Ach, acetylcholine; Br, bradykinin; Ang, angiotensin; A, adrenaline; NA, noradrenaline; Hist, histamine; 5-HT, 5-hydroxytryptamine.

** Where a range of figures was given by the authors, the highest values is shown here.

† Assuming a 10 ml organ bath.

between the application of oxytocin and the expulsion of droplets of milk is related to the concentration of hormone applied (Van Dongen & Hays, 1966). It is also possible to measure the changes in transparency induced by oxytocin in a flat segment of mammary gland (Bílek, Mikuláš, Slaba & Skála, 1971).

Methods *in vivo* are less satisfactory than those *in vitro* in a number of ways. They are more difficult to set up and require care over the preparation and maintenance of the animal. Further, the depth of anaesthesia and rate of injection of oxytocin are critical factors. With most of these methods, the responses are rapid and reliable, but the 95% fiducial limits are rather wide. Of the *in vitro* methods, the droplet expulsion method of Van Dongen & Hays (1966), though very sensitive, can be criticized on a number of grounds (Cowie & Tindal, 1971), not least because the index of precision ($\lambda$) is more than 1, which makes it unsuitable for routine assays. The transparency method described by Bílek *et al.* (1971) is more promising; it is sensitive and has a satisfactory index of precision. On the other hand the preparation reacts to small quantities of other substances as well as to oxytocin. Similarly, the isolated mammary strip of the rat is sensitive but relatively non-specific (Rydén & Sjöholm, 1962). That of the mouse is far more specific, and maintains its reactivity and stability at room temperature for long periods (Roca *et al.*, 1972). We have therefore selected the mouse mammary strip for superfusion.

Most workers have avoided superfusion of this organ, apparently because of the difficulty of preventing the fluctuations in weight of the tissue from distorting the record at the high sensitivity of recording necessary (Fitzpatrick & Bentley, 1968). We have shown that this difficulty can be overcome by a simple method of fixing the tissue and the introduction of a high-frequency damping device. Superfusion has the advantage that doses are dissolved in a small volume instead of being diluted in the fluid in an organ-bath; thus the effective sensitivity is increased.

The method described here is sensitive; it can be used to assay fluids containing as little as 4 $\mu$u per ml. Roca *et al.* (1972) have reported that the mouse mammary strip suspended in a bath is even more sensitive than this, but we have failed to obtain responses below a threshold dose of about 0.4 $\mu$u, possibly because we used a different strain of mouse. The sensitivity remains high for at least 18 hours. When controlled by the automatic apparatus, assays of a large number of samples can be conveniently performed. A disadvantage is that doses cannot be given at short intervals without the occurrence of tachyphylaxis. The preparation is relatively insensitive to other substances; vasopressin is the main exception. The dose-response curves for oxytocin and vasopressin are not parallel, and it is therefore difficult to express the activity of vasopressin in terms of oxytocin. It follows that the assay of oxytocin in a sample containing vasopressin is complicated, and it would be desirable first to remove the vasopressin e.g. by incubation with trypsin (Bisset, Errington & Richards, 1973) or by selective extraction.

Table 3 gives details of other methods using the mammary gland. It will be seen that the method described here compares favourably with most of the others in accuracy, sensitivity and specificity. The chief merit of this method of superfusion is that it is possible to obtain an assay response routinely with only 0.5 ml of a sample containing as little as 4 $\mu$u/ml. If the tissue were suspended in an organ bath of 10 ml, a dose of 50 $\mu$u would be required to achieve the same concentration; alternatively, the bath would have to be entirely filled with the sample to be assayed, which would necessitate the use of very large samples. By concentrating samples before assay, it should be possible to measure with reasonable accuracy the oxytocin content of plasma in the physiological range. Experiments on these lines are in progress.

Radio-immunoassays of oxytocin have recently been developed (for review see Chard, 1973). Although such methods are not affected by large amounts of vasopressin, they are at present not as sensitive as the best biological methods. Furthermore, it has been shown that antibodies to oxytocin may react with fragments or derivatives of oxytocin, which have no biological activity. Thus, immunological methods may give falsely high levels where breakdown of oxytocin is rapid, e.g. in human pregnancy plasma (Chard, Forsling, James, Kitau & Landon, 1970). Sensitive bioassays remain therefore an essential alternative.

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