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THE ASSAY OF MILK-EJECTING ACTIVITY IN THE LACTATING RAT

BY

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One of the most sensitive methods for assaying oxytocin is to measure the increase in milk-ejection pressure which results from a close arterial injection of the hormone into the lactating mammary gland. This method originates from the work of Cross & Harris (1952) on the role of the neurohypophysis in the milk-ejection reflex. In this work, a teat duct was cannulated in the lactating rabbit and the cannula was partly filled with citrate solution. In response to electrical stimulation of the neurohypophysis or to the intravenous injection of posterior pituitary extract, milk was ejected into the cannula and a volume change was registered by means of a piston recorder and kymograph. Van Dyke, Adamsons & Engels (1955) developed a quantitative assay method in which volume recorders were replaced by highly sensitive pressure transducers and milk ejection pressure was recorded from the cannulated duct with minimal displacement of fluid. Fitzpatrick (1961) achieved an almost 40-fold increase in the sensitivity of the assay in the lactating rabbit by giving close arterial, instead of intravenous, injections of oxytocin. Using a similar technique in the guinea-pig, Tindal & Yokoyama (1962) obtained a further increase in sensitivity and their method of assay has been used to estimate small amounts of oxytocin in blood during suckling and parturition (Folley & Knaggs, 1965a ; Fitzpatrick & Walmsley, 1965).

Many of our investigations have been concerned with the problem of demonstrating independent release of the neurohypophysial hormones (Haldar, 1966 ; Bisset, Hilton & Poisner, 1967 ; Beleslin, Bisset, Haldar & Polak, 1967). In this work an even greater sensitivity than we have been able to obtain with the lactating guinea-pig preparation is often required to exclude the presence of oxytocin in blood extracts. For this purpose the use of the lactating rat has been investigated. Since the work began, Grosvenor (1965) and DeNuccio & Grosvenor (1967) have recorded increases in milk-ejection pressure in the rat in response to oxytocin given by the intravenous route, but the limit of sensitivity

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was not determined. In this paper a quantitative method of assay using the retrograde arterial route is described. In our hands the rat has proved to be considerably more sensitive than the guinea-pig, and has the advantage of being more readily obtainable.

Anatomy of the mammary glands in the lactating rat

The rat possesses six discrete pairs of mammary glands, three thoracic, one abdominal and two inguinal (Myers, 1916). In the lactating animal, the separate glands are indistinguishable although they can be delineated by injecting dye into each teat (Fig. 1). The gland mass appears as a sheet of tissue extending from the neck to the anus interrupted

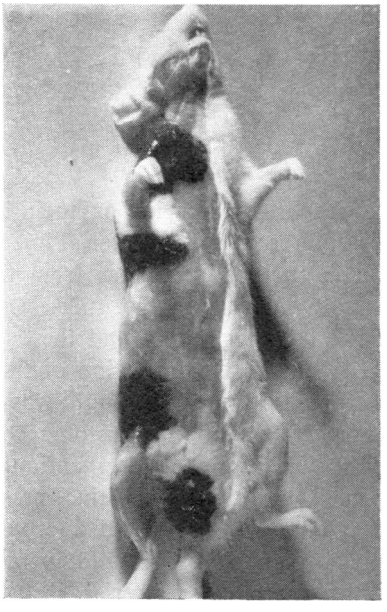

Fig. 1. Delineation of mammary glands in the lactating rat by dyes injected *via* the teats. There are six discrete pairs of glands, three thoracic, one abdominal and two inguinal.

only in the region of the lower ribs. The abdominal portion extends laterally from the mid-line to cover the lateral abdominal wall and is continuous with the thick mass covering the whole of the inguinal region, almost completely encircling the upper part of the thigh. The macroscopic structure of a mammary gland was studied by injecting neoprene latex into the duct system *via* a cannulated teat (Fig. 2). It was found to consist of numerous lobes composed of alveoli drained by lactiferous ducts. These unite to form two secondary ducts which in turn converge to form a single primary duct opening on to the surface of the teat. The appearence of the gland was similar to that of the immature rat described by Myers (1916).

Since there are six pairs of glands from which to record milk-ejection pressure, it was necessary to determine the most effective route for close arterial injection. The vessels to the posterior group of mammary glands were found to be more easily accessible than those to the thoracic group. The blood supply to these glands was therefore studied in greater detail from dissections of animals in which latex had been injected into the abdominal aorta (Fig. 3).

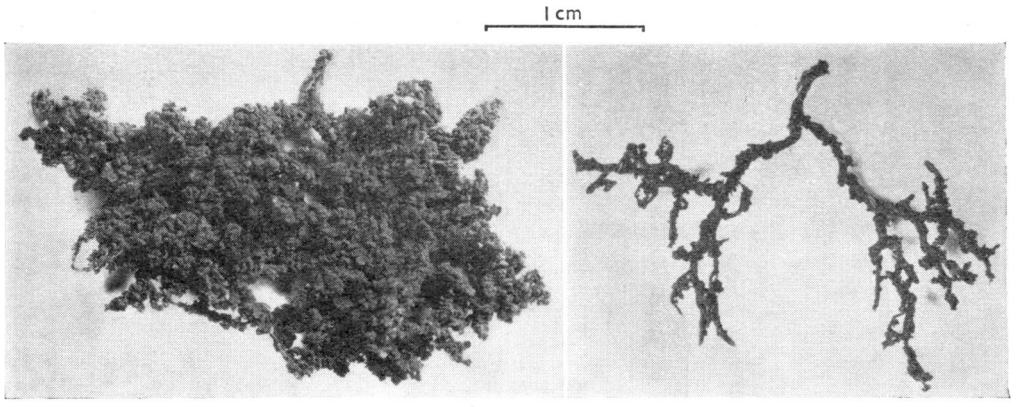

Fig. 2. Latex cast of an abdominal mammary gland in the lactating rat. There is one primary duct opening on to the surface of the teat.

The abdominal and inguinal glands receive their blood supply from the superficial epigastric artery, the superficial and deep external pudendal arteries and occasionally from the iliac branch of the ilio-lumbar artery. It was demonstrated that solutions of dye in 0.9% NaCl solution injected into either the superficial epigastric or the external pudendal vessels spread uniformly throughout the abdominal and the inguinal glands. It appeared therefore that there was free anastomosis between branches of the vessels within the gland mass. In addition, the milk-ejection pressures recorded from two inguinal glands simultaneously, following retrograde arterial injections *via* the saphenous artery, were not significantly different. No one gland therefore is preferable to another.

METHODS

Recording milk-ejection pressure in the lactating rat

Lactating rats (250–350 g) of the Sprague-Dawley strain, usually with their first but occasionally with their second or third litters, were used from 3 to 21 days after parturition. The number of young in the litters varied from 8–10.

The rats were anaesthetized, within 30–60 min after separation from their litters, by intraperitoneal injection of sodium pentobarbitone, 4.5 mg (in 0.25 ml.)/100 g. This was supplemented as necessary by slow intravenous injections of 1.8–3.6 mg (in 0.1–0.2 ml.) or, in some experiments, by continuous intravenous infusion at the rate of 0.04 mg (in 0.022 ml.)/min. 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, designed by the Electronics Division at the National Institute for Medical Research, Mill Hill, is similar in principle to that described by Krnjević & Mitchell (1961).

The trachea was cannulated to ensure a free airway but artificial respiration was not applied. For intravenous injections the external jugular vein was cannulated with polyethylene tubing (Portex; PP 25). For retrograde arterial injections, a polyethylene cannula (Portex; PP 10) was inserted into the saphenous artery and then, if possible, pushed up into the femoral artery until its tip lay between the origins of the superficial epigastric and popliteal arteries (see Fig. 3). In some experiments in which the cannula did not extend into the femoral artery, the popliteal artery was

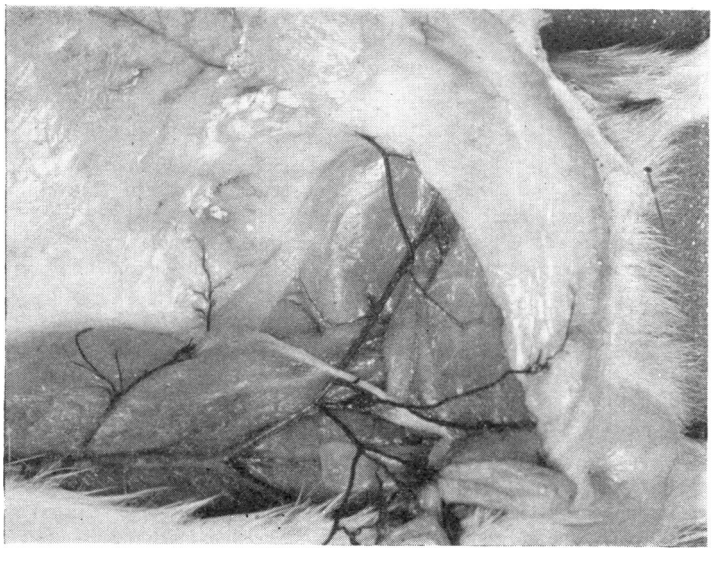

Fig. 3. Arterial blood supply to the abdominal and inguinal mammary glands in the lactating rat. From a dissection following the injection of latex solution into the abdominal aorta.

ligated. To facilitate cannulation, the saphenous artery was dilated by applying a pad of cotton wool soaked in a warm solution of papaverine hydrochloride (1 mg/ml.). Correct alignment of the cannula was ensured by securing it with a ligature to the underlying muscles. The skin incision was closed with sutures.

Milk-ejection pressure was recorded usually from the most distal (lower inguinal) teat, but occasionally from the upper inguinal or abdominal teat. The surrounding skin was shaved and the tip of the teat excised. A polyethylene cannula (Portex ; PP 10) was inserted 3–5 mm into the primary duct (see Fig. 2) and tied firmly in position with a ligature round the teat. The cannula was connected with a strain gauge transducer (Statham P 23 D o ; 0–75 cm Hg), the whole system being filled with a 3.8% solution of sodium citrate to prevent clotting of milk. After cannulation a small volume (0.05–0.2 ml.) of citrate solution was injected into the duct through the transducer to clear milk from the tip of the cannula. Occasionally, 0.2 ml. citrate solution was injected in this way during the course of an experiment in order to raise the intramammary pressure and so increase the sensitivity of the preparation. The strain gauge was mounted in such a way as to preserve the natural alignment of the teat and to apply slight tension. The gauge was connected with a potentiometric recorder (Goerz type RE 511 or Leeds Northrup: Speedomax ; Type H) adjusted to give a full scale deflection for an increase in pressure of 10 or 20 mm Hg.

Intravenous injections were given in a volume of 0.05–0.4 ml. washed in with 0.1 ml. 0.9% NaCl solution. For retrograde arterial injections the volume was kept constant at 0.2 ml. and the injection was given rapidly in 1–2 sec. After the response had subsided the arterial cannula was washed through by injecting 0.1 ml. heparin solution. Injections were given by both routes at intervals of 5 min.

Materials

Drugs used were synthetic oxytocin (Syntocinon, Sandoz) ; synthetic arginine vasopressin (Sandoz) ; pituitary (posterior lobe) extract (Third International Standard for Oxytocic, Vasopressor and Anti-diuretic Substances (Bangham & Mussett, 1958)) ; synthetic angiotensin (val^5-hypertensin II-Asp-β-amide (CIBA) ; synthetic bradykinin (Parke-Davis) ; adrenaline hydrogen tartrate (British Drug Houses) ; 5-hydroxytryptamine creatinine sulphate (May and Baker) ; acetylcholine chloride (Roche Products) ; histamine acid phosphate (British Drug Houses) and heparin (Pularin, Evans Medical).

Doses of adrenaline, 5-hydroxytryptamine, acetylcholine and histamine are expressed in terms of base. All dilutions of adrenaline were made in 0.9% NaCl solution containing 0.01% ascorbic acid (British Drug Houses). Heparin 50 u./ml. was injected in 0.9% NaCl solution.

Doses of oxytocin, vasopressin and pituitary (posterior lobe) extract are given in units. The weight of oxytocin corresponding with a given number of units was calculated on the basis that pure synthetic oxytocin has an oxytocic activity of 450 u./mg (Boissonnas, Guttman, Berde & Konzett, 1961).

The anaesthetic used was pentobarbitone sodium, BP (Nembutal (Veterinary), Abbott).

RESULTS

Characteristics of milk-ejection responses in the lactating rat

Milk-ejection responses to the retrograde arterial injection of oxytocin or pituitary (posterior lobe) extract were observed in 115 rats. The threshold dose was not determined in every rat ; nevertheless, 61% of the rats responded to 10 μ-u. or less and 30% to 5 μ-u. or less. In six rats the threshold was 2–2.5 μ-u. and in three rats 1–1.25 μ-u. When a retrograde arterial injection was given, part of the injection could be seen to enter the popliteal artery. The effect on the sensitivity of the preparation of occluding this artery during the injection was investigated. Six series of three injections were made. In each series, an injection of 40 μ-u. oxytocin with the popliteal artery occluded was made between two injections of 40 and 80 μ-u. without occlusion. By means of a statistical analysis similar to that used by Vos (1943), it was shown that the effect of occlusion was to increase the response to 40 μ-u. oxytocin by 33%.

Intravenous injections of oxytocin were tested in 20 rats. Sixty per cent responded to 100 μ-u. or less and of these two responded to 10 μ-u.

There was no spontaneous activity in the preparation and no tachyphylaxis on repeating injections at intervals of 5 min. Typical milk-ejection responses to retrograde arterial and intravenous injections of oxytocin in the same rat are illustrated in Fig. 4. The figure shows graded responses to four doses of oxytocin increasing in the ratio 1 : 1.5 for each

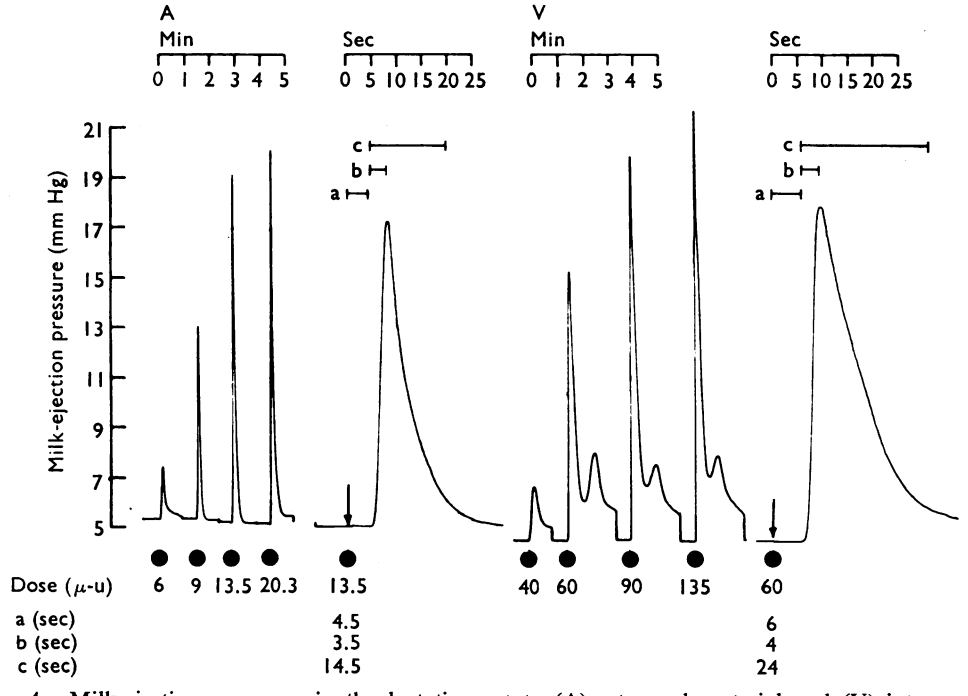

Fig. 4. Milk-ejection responses in the lactating rat to (A) retrograde arterial and (V) intravenous injections of oxytocin. The recorder was switched off 1–2 min after each injection. One injection by each route was repeated with a faster speed of recording: a = latent period; b = time to peak response; c = duration of response (for definition of these parameters see text).

route of injection. In response to arterial injections, the pressure rose and fell sharply in a single peak. After intravenous injections of 60–135 μ-u. the rapid initial rise in pressure was followed by a small secondary rise and the pressure took 2 to 3 min to return to the baseline. Three parameters of the milk-ejection response, which are illustrated in Fig. 4, were considered: (a) the latent period from the injection to the beginning of the pressure rise; (b) the time elapsing from this point until the peak pressure was reached; and (c) the duration of the response, measured from the beginning of the pressure rise to the point at which the pressure had returned to one-tenth of its peak value. In an experiment in which 21 retrograde arterial injections of 7.5 to 12 μ-u. oxytocin were given, the ranges, means and S.E.'s of these parameters were as follows: (a) 3 to 8 (4.7 ± 0.34) sec, (b) 2 to 10 (6.2 ± 0.49) sec and (c) 17 to 29 (21.7 ± 0.91) sec.

In the experiment shown in Fig. 4, the ratio of the threshold dose by intravenous injection to that by retrograde arterial injection was 7. In six other experiments, this

ratio was 5, 5, 20, 10, 10 and 10. However the comparison of threshold doses gives only an approximate measure of the relative effectiveness of the two routes of injection. In two experiments, one of which is illustrated in Fig. 5, a more precise comparison was made by means of a six-point assay. Three doses of oxytocin were given by the arterial, and three by the intravenous route, the dose ratio being the same for the two routes. Both the routes and the doses were assigned to the animal in random order. The results were submitted to an analysis of variance. In each experiment the regression was highly significant ($P<0.001$) for both routes and the regression lines for the two routes showed no significant deviation from parallelism ($P>0.05$). The sensitivity to retrograde arterial injection was 24.7 (95% fiducial limits: 20.1–30.8) times greater than that to intravenous injection in the experiment of Fig. 5 but only 9.1 (95% fiducial limits: 7.4–11.1) times greater in the other experiment. These results show that there is a wide variation in the relative effectiveness of the two routes of injection.

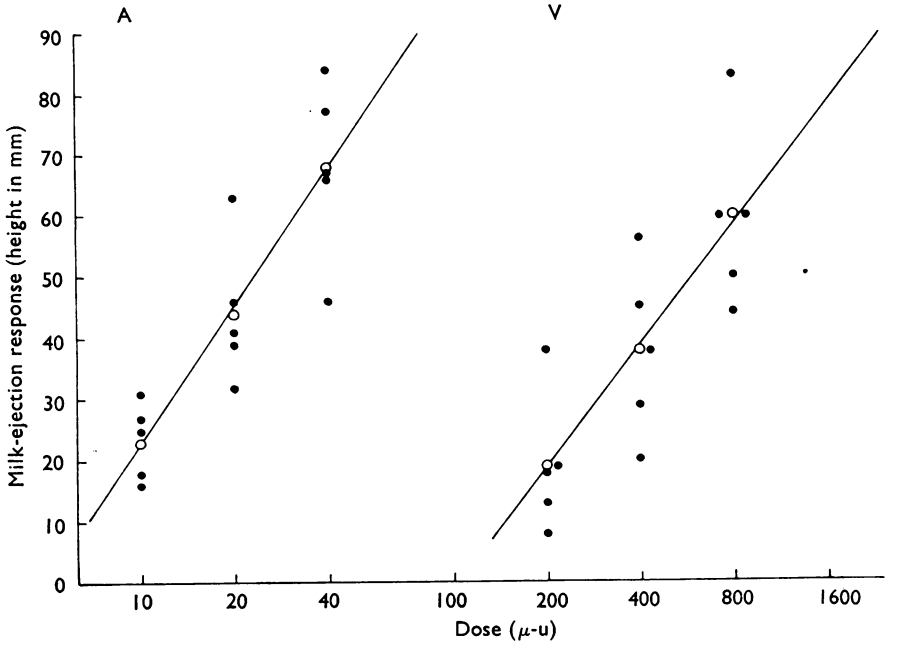

Fig. 5. Six-point assay to compare the effectiveness of retrograde arterial (A) and intravenous (V) injections of oxytocin. The dots represent individual responses and the circles, mean responses. The regression lines were fitted by the method of least squares. The common slope (b)=70.42, the experimental error (s)=8.26 and the index of precision ($\lambda=s/b$)=0.117. The sensitivity was 24.7 (95% fiducial limits: 20.1–30.8) times greater by the arterial than the intravenous route.

The accuracy of four-point assays using both retrograde arterial and intravenous injections was investigated. The results, which were submitted to analyses of variance, are shown in Table 1. The mean error—that is, the difference between the actual and the estimated potencies of the test solutions—was in per cent, 7.1 ± 2.40 (S.E.) for the arterial, and 4.5 ± 0.80 (S.E.) for the intravenous route. The corresponding values for the index of precision were 0.074 ± 0.0014 (S.E.) and 0.032 ± 0.0037 (S.E.). The highest percentage error was 17.5; this occurred in assay No. 3 in which only two groups of

TABLE 1

STATISTICAL ANALYSIS OF 4-POINT ASSAYS BY RETROGRADE ARTERIAL (A) OR INTRAVENOUS (V) INJECTION IN THE LACTATING RAT

The Test (T) solutions consisted of known dilutions of the standard (S) solutions

Route of injection	Assay no.	No. of blocks of 4 doses	Doses of S (μ-u.) Low	Doses of S (μ-u.) High	Ratio of potencies (T/S) Actual ratio =A	Ratio of potencies (T/S) Estimated ratio =R	95% fiducial limits of R R1	95% fiducial limits of R R2	% Error= 100 (A–R)/A	Exp. error (s)	Common Slope of Regression lines (b)	Index of precision (λ)= s/b
A	1	4	30	60	0·67	0·72	0·46	0·98	7·5	8·05	69·3	0·116
A	2	4	10	15	0·80	0·71	0·55	0·83	11·2	9·66	172·5	0·056
A	3	2	5	10	0·80	0·94	0·46	1·73	17·5	16·5	178·6	0·092
A	4	4	9	18	0·89	0·80	0·68	0·93	10·1	9·61	171·9	0·056
A	5	5	30	40	0·90	0·92	0·85	0·98	2·2	15·54	538·7	0·029
A	6	7	50	100	0·80	0·79	0·59	0·98	1·3	16·56	135·2	0·122
A	7	5	20	30	0·80	0·80	0·69	0·90	0	2·64	53·9	0·049
							Mean S.E.		7·1 ±2·40	Mean S.E.		0·074 ±0·0014
V	8	6	200	300	0·90	0·88	0·80	0·96	2·2	4·03	95·6	0·042
V	9	4	180	240	0·83	0·87	0·80	0·92	4·8	9·95	429·2	0·023
V	10	4	150	200	0·90	0·95	0·85	1·05	5·6	6·96	187·1	0·037
V	11	4	100	150	0·90	0·96	0·87	1·06	6·7	5·47	154·0	0·035
V	12	4	150	180	0·90	0·87	0·78	0·93	3·3	2·64	112·1	0·024
							Mean S.E.		4·5 ±0·80	Mean S.E.		0·032 ±0·0037

four injections were given; in all other assays four to seven groups were obtained and the highest percentage error was 11.2. There was no significant deviation from parallelism in any of the assays ($P>0.05$) except No. 2 ($0.05>P>0.01$).

The specificity of the lactating rat for oxytocin

The threshold dose of oxytocin eliciting a milk-ejection response by the retrograde arterial route of injection was compared with that of some other naturally occurring substances. The results are shown in Table 2, which includes for comparison threshold

TABLE 2

THRESHOLD DOSES OF SOME NATURALLY OCCURRING SUBSTANCES FOR STIMULATION OF THE MAMMARY GLAND AND UTERUS

Substance	Mammary gland: *In situ* (retrograde arterial injection) Guinea-pig[1] (μg)	Mammary gland: *In situ* (retrograde arterial injection) Rat[2] (μg)	Mammary gland: Isolated strip Rabbit[3] (μg/ml.)	Mammary gland: Isolated strip Guinea-pig[4] (μg/ml.)	Mammary gland: Isolated strip Rat[5] (μg/ml.)	Rat uterus: *In situ*[6] (intravenous injection) (μg)	Rat uterus: Isolated[6] (μg/ml.)
Oxytocin	$1·1–2·2\times10^{-5}$	$1·1–2·2\times10^{-5}$	$2·2\times10^{-4}$	2.2×10^{-5}	1×10^{-6}	$1·1\times10^{-3}$	$2·2\times10^{-4}$
Acetylcholine (base)	0·016–0·024	0·004–0·016	25	3×10^{-3}	1×10^{-6}–1×10^{-5}	—	—
5-Hydroxytryptamine (base)	11	0·1–0·2	50*	3×10^{-3}	0·01–1	0·11	9×10^{-4}
Histamine (base)	18	20*	125*	3×10^{-2}	1×10^{-5}–1×10^{-2}	—	—
Bradykinin	1	0·1–0·2	—	3×10^{-3}	—	0·4	2×10^{-4}
Angiotensin	1–2	15–20†	—	3×10^{-3}	—	0·01	8×10^{-4}

Data from: [1] Folley & Knaggs, 1965b; [2] this paper; [3] Moore & Zarrow, 1965; [4] Martinet & Lis (personal communication); [5] Rydén & Sjöholm, 1962; ([3 4 5] assuming doses expressed in terms of base); [6] Bisset, Haldar & Lewin, 1966.

* No response to dose shown; † equivocal responses to doses shown.

doses for a number of other assay preparations. The milk-ejection responses to acetylcholine and 5-hydroxytryptamine were always identical in character with that produced by oxytocin ; bradykinin sometimes elicited a prolonged and fluctuating response lasting up to 5 min. For all three substances a dose-response relationship could be established. The most potent was acetylcholine but the lowest threshold dose recorded for this substance was 5 ng, which was almost 500 times greater than that of oxytocin (0.011 ng or 5 μ-u.) in the same experiment. When oxytocin and bradykinin were injected in a mixture their individual effects were additive. The milk-ejecting activity of bradykinin, unlike that of oxytocin, was not inhibited following incubation with 0.01 M sodium thioglycollate. The milk-ejection response to 5-hydroxytryptamine was abolished by the intravenous injection of BOL (50 μg), and that to acetylcholine by the intravenous injection of atropine (1 mg), without affecting the response to oxytocin.

Histamine (20 μg), which was tested on three rats, did not produce a response. Angiotensin (15–20 μg) caused a small, gradual increase in milk-ejection pressure in one rat but produced no response in two other rats.

Adrenaline was also tested. It did not produce an increase in milk-ejection pressure but doses of 5–20 ng injected simultaneously with oxytocin (20–40 μ-u.) inhibited the response to oxytocin by 50% to 75%. This effect of adrenaline was inhibited by β-receptor blocking agents, and is the subject of the following paper.

In assaying oxytocin in blood, it is important to take into account the intrinsic milk-ejecting activity of any vasopressin which is present. In nine experiments a sample of synthetic arginine vasopressin was assayed for milk-ejecting activity by retrograde arterial injection, against pituitary (posterior lobe) extract. The antidiuretic activity of the sample had been previously assayed against the same sandard by intravenous injection in the water-loaded rat under ethanol anaesthesia (Bisset, 1962a). The milk-ejecting activity of the sample, expressed as a percentage of its antidiuretic activity, varied from 11.4% to 15.7% (mean = 13.8% $\pm$ 0.57 S.E.).

DISCUSSION

The anatomy of the mammary glands in the rat is well adapted to the measurement of milk-ejection pressure. Cannulation of the duct system is relatively simple because, as in the guinea-pig there is only one duct opening on to the surface of the teat. Either of the two inguinal glands or the abdominal gland may be used for recording milk-ejection pressure, since a retrograde injection into the saphenous artery reaches all three glands through the superficial epigastric and external pudendal arteries. Ligation of the popliteal artery increases the response by one-third but there is no other major vessel which requires to be ligated. Several features commend the preparation for the bioassay of oxytocin. The increase in milk-ejection pressure in response to both intravenous and retrograde arterial injections of oxytocin has a duration of less than 1 min ; injections can be given at intervals of 5 min for several hours without inducing tachyphylaxis and the dose-response curve is steep. Estimates of potency by four-point assays are reasonably accurate, as indicated by the index of precision, λ. According to Holton (1948), for a satisfactory assay, λ should not be much more than 0.05. In our series of assays, the value of λ was 0.032 for the intravenous, and 0.074 for the retrograde arterial route.

For the estimation of small amounts of oxytocin in blood, an assay must be both sensitive and specific. The most sensitive method which has been developed so far for

assaying milk-ejecting activity is that of Tindal & Yokoyama (1962) using retrograde arterial injections in the lactating guinea-pig. Discounting initial responses which were sometimes obtained with 1 μ-u. oxytocin but could not be repeated, Tindal & Yokoyama give a figure of 10 μ-u. for the threshold by this method; the usual range of doses in four-point assays is 15–60 μ-u. The index of precision, λ, is 0.080. For the same method, Folley & Knaggs (1965a, b) quote a threshold of 5–10 μ-u. Other workers have been unable to obtain this degree of sensitivity in the guinea-pig (Fitzpatrick & Walmsley, 1965; Beránková-Ksandrová, Bisset, Jošt, Krejčí, Pliška, Rudinger, Rychlík & Šorm, 1966). In our experience the rat has proved to be 5 to 10 times more sensitive than the guinea-pig under the same experimental conditions. In our series of 115 rats, 30% responded to 5 μ-u. or less and 61% to 10 μ-u. or less. It can be seen from Table 2 that both the guinea-pig and the rat are relatively specific for oxytocin, but there are differences in their responses to other pharmacologically active substances. The rat appears to be about 10 times more sensitive than the guinea-pig to bradykinin and 100 times more sensitive to 5-hydroxytryptamine but 10 times less sensitive to angiotensin.

Assay of the milk-ejecting activity of oxytocin in the lactating rat appears to be more specific than methods based on isolated organ preparations and in most cases, it is equally or more sensitive. The isolated rat uterus in an organ bath used according to the method of Holton (1948) usually responds to a concentration of about 100 μ-u./ml. oxytocin. By giving repeated injections of stilboestrol to the rats before use, Follett & Bentley (1964) were able to obtain threshold responses to 25–50μ-u. The superfused rat uterus responds to 1–8 μ-u. (Fitzpatrick, 1961). However, the isolated rat uterus discriminates poorly between different pharmacologically active substances (Bisset & Lewis, 1962; Bisset, Haldar & Lewin, 1966). Table 2 shows that on a weight basis, the threshold doses of 5-hydroxytryptamine, bradykinin and angiotensin do not differ from that of oxytocin by more than a factor of 4. The sensitivity of the isolated uterus to bradykinin is a serious disadvantage, since it is difficult to avoid the release or formation of plasma kinins in the preparation of blood extracts and this factor has interfered with past attempts to assay oxytocin in blood (Bisset, 1961). Blood extracts may also contain an unidentified oxytocic substance (UOS) which causes contraction of the isolated uterus but has no milk-ejecting activity (Hawker, 1961).

In contrast with the isolated organ, the rat uterus *in situ* discriminates reasonably well between oxytocin and a number of other substances (Table 2) but, even with sensitive methods of recording, the minimum threshold dose of oxytocin is 500 μ-u. (Bisset, Haldar & Lewin, 1966) and this is too high for estimation of the hormone in blood.

In 1960, Méndez-Bauer, Cabot & Caldeyro-Barcia showed that isolated strips of mammary gland from lactating rabbits developed a measurable increase in tension when exposed to oxytocin. This preparation shows a high degree of specificity for oxytocin (Méndez-Bauer *et al.*, 1960; Moore & Zarrow, 1965) but the lowest concentration which can be detected is 100 μ-u./ml. The isolated strip of rat mammary gland is much more sensitive. The lowest concentration of oxytocin which has been detected by this method is 0.5 μ-u./ml. but the usual range of concentrations in four-point assays is 5–100 μ-u./ml. (Rydén & Sjöholm, 1962). This preparation is relatively non-specific for oxytocin (Table 2). For example, the threshold doses of oxytocin and acetylcholine, on a weight basis, are about the same, whereas the threshold dose of acetylcholine for causing milk-ejection in

the rat by retrograde arterial injection is about 500 times higher than that of oxytocin. The isolated mammary gland of the guinea-pig, which has been investigated recently by Martinet & Lis (personal communication), appears to bear a closer similarity to the rat gland than to that of the rabbit.

Recently a method has been developed for assaying oxytocin on the mammary gland *in vitro* which is based on measurement of the time required for the expulsion of milk to occur when minute pieces of gland are placed in solutions of oxytocin and examined microscopically (van Dongen & Hays, 1966). This is the most sensitive method so far devised; the minimum threshold dose of oxytocin for quantitative purposes is 1×10^{-6} μ-u./ml. However, 13 observations are required to establish the significance of a 10-fold increase in dose, and the index of precision, λ, is high (1.02). In addition, although the preparation is insensitive to bradykinin, histamine, 5-hydroxytryptamine and catecholamines, there is no difference in its sensitivity to oxytocin and vasopressin (van Dongen & Marshall, 1967).

Although the lactating mammary gland of the rat *in situ* is sensitive to oxytocin and responds only to very high doses of most other naturally occurring substances which might be present in blood extracts, it is important to remember that vasopressin possesses a considerable degree of milk-ejecting activity. This activity varies according to the species and the method of assay (for review, see Bisset, 1967). The intrinsic milk-ejecting activity of arginine vasopressin, tested by retrograde arterial injection in the lactating rat, was found to be equivalent to 13.8% of its antidiuretic activity. The importance of taking this factor into account is exemplified by some recent work in which both milk-ejecting and antidiuretic activities were estimated in extracts of blood collected during carotid occlusion in the cat (Clark & Rocha e Silva, 1966). The method described in this paper was used for the assay of milk-ejecting activity. Many of the extracts were found to contain milk-ejecting activity which might have been attributed to oxytocin but it was shown that the whole of this activity could be accounted for by their content of vasopressin and, in fact, the experiments provided evidence for the release of vasopressin independently of oxytocin. In this work the blood was extracted by the method of Bisset, Hilton & Poisner (1967). The combination of this method of extraction with the assay of milk-ejecting activity in the lactating rat permits the detection of oxytocin in blood at levels of less than 10 μ-u./ml.

Assay of milk-ejecting activity in the lactating rat should also be useful for testing synthetic analogues of oxytocin. It is usual to assay the oxytocic activity of these analogues on the isolated rat uterus and their milk-ejecting activity in the rabbit or guinea-pig. To compare these activities, and to draw conclusions regarding structure-activity relationships or the nature of the tissue receptors for oxytocin in various organs, may be misleading, since the assays involve two or three different species and one is carried out *in vivo* whereas the other is based on an isolated organ. As an example of the type of discrepancy which may arise, (O-Me)Tyr^2-oxytocin has been found to act as a specific competitive inhibitor of oxytocin on the isolated rat uterus under certain experimental conditions, whereas *in situ* it acts as a pure agonist both on the guinea-pig mammary gland and the rat uterus (Beránková, Rychlík, Jošt, Rudinger & Šorm, 1961; Rudinger & Krejčí, 1962; Krejčí, Poláček, Kupová & Rudinger, 1964; Bisset, 1962b, 1964, 1967). The present method for assaying oxytocin in the lactating rat, used in

conjunction with the method which has already been developed for the rat uterus *in situ* (Bisset, Haldar & Lewin, 1966), now makes it possible to estimate both milk-ejecting and oxytocic activities *in situ* in the same species.

SUMMARY

1. A method is described for assaying milk-ejecting activity in the lactating rat.

2. Milk-ejection pressure is recorded from the cannulated teat duct of an abdominal or inguinal mammary gland. Drugs are given either intravenously or by retrograde injection into the saphenous artery.

3. The preparation does not exhibit spontaneous activity or tachyphylaxis and provides a sensitive method for the assay of oxytocin. Thirty per cent of the rats tested responded to retrograde arterial injections of 5 μ-u. or less and 61% to 10 μ-u. or less; in a series of four-point assays, the mean index of precision, λ, was 0.074 ± 0.0014.

4. Arginine vasopressin, given by arterial injection, has an intrinsic milk-ejecting activity equivalent to $13.8 \pm 0.57\%$ of its antidiuretic activity. Bradykinin, angiotensin, 5-hydroxytryptamine, histamine and acetylcholine, injected arterially, produced an increase in milk-ejection pressure only at relatively high doses.

5. The usefulness of the preparation for estimating small amounts of oxytocin in blood is discussed.

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