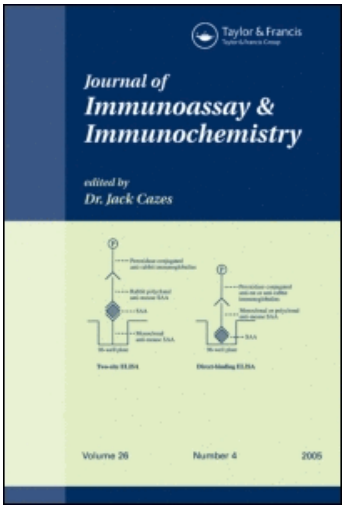

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The Development and Evaluation of a Sensitive and Specific Radioimmunoassay for Oxytocin in Unextracted Plasma

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THE DEVELOPMENT AND EVALUATION OF A SENSITIVE AND SPECIFIC RADIOIMMUNOASSAY FOR OXYTOCIN IN UNEXTRACTED PLASMA

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ABSTRACT

The development and evaluation of a radioimmunoassay for oxytocin is described. High titre antisera were raised to oxytocin coupled to thyroglobulin and tested for specificity with a number of oxytocin analogues and fragments. Two antisera showing high specificity were used to assay plasma directly without extraction. The maximum sensitivity was 0.5pg per tube and the intra- and inter-assay co-efficients of variation were 7.1 and 11.6% respectively. Cross-reactivity studies indicate that the antisera were directed chiefly to the oxytocin side-chain. The antisera could be purified by affinity chromatography using this tripeptide coupled to agarose beads but this did not improve their avidity or specificity. The assay was tested successfully with a number of body fluids and tissue extracts, although human late-pregnancy plasma could not be added directly to the assay. Direct radioimmunoassay was used to estimate the clearance of oxytocin infused into conscious dogs, and good agreement was found when the same samples were also bioassayed. The antisera also efficiently neutralise the biological activity of oxytocin *in vivo* and *in vitro*.

INTRODUCTION

Oxytocin is a difficult peptide hormone to study because the physiological levels in the peripheral circulation are extremely low and almost always below the detection limits of assays currently available. Radicimmunoassays have been developed (3,4,11,12,20)

but their use is not widespread as they are technically very demanding and basal levels of endogenous oxytocin can still only be measured by extraction and concentration of the samples (28). Although immunoassays show better discrimination between arginine vasopressin (AVP) and oxytocin than bioassays and can accept much larger numbers of samples, metabolic degradation products may retain significant immunoreactivity and interfere with assays to an unknown degree. For instance, attempts to apply radioimmunoassay (RIA) to the measurement of oxytocin in human gestation and parturition (3,5,12), have produced conflicting results which are probably due partly to methodological problems and partly to a dissociation of biological and immunological activities which occurs in human pregnancy plasma (10).

This paper describes the evaluation of a sensitive and specific RIA for oxytocin which can be applied to unextracted plasma. The cross-reactivity of the antisera used has been investigated with a variety of related peptides in order to assess the contribution that biologically inactive fragments of oxytocin could make to the total oxytocin-like immunoreactivity measured in samples by this RIA, and the assay has been tested with a variety of biological samples without the use of an extraction procedure.

MATERIALS AND METHODS

Oxytocin (440iu/mg) used for immunisation and iodination was a generous gift from Dr H. Vilhardt (Ferring AB, Malmö). Ferring

also provided [8-lysine]-vasopressin (LVP), (240iu/mg), triglycyl oxytocin and tocinoic acid. [8-arginine]-vasotocin (AVT), (0.1mg/ml), [1-Desamino]-oxytocin and Syntocinon (the oxytocin standard used in the bioassay) were gifts from Sandoz Ltd. Oxytocinoic acid, [9-Desglycineamide]- oxytocin and [8, 9-Desleucylglycineamide]- oxytocin were gifts from the late Professor R. Walter, Department of Physiology and Biophysics, University of Illinois. (9-Gly-O-Methyl)-oxytocin was a gift from Dr T. Barth, the Institute of Organic Chemistry and Biochemistry, Prague. Pro-Leu-Gly-NH_2 (PLG) and N-carbobenzoxy-PLG (z-PLG) were obtained from Sigma Chemical Co. Ltd. International Standards of oxytocin, LVP and AVP were obtained from the National Institute for Biological Standards and Control, Holly Hill, Hampstead, England. Aliquots diluted from the IVth International Standard for oxytocin were used as working standards in the RIA. The contents of the ampoule were diluted to a final concentration of 5ng/ml in RIA buffer (assuming a peptide content of 21μg per ampoule) and stored at -20°C. Samples of human plasma, taken during late pregnancy, were obtained from the Royal Free Hospital, Hampstead, through the courtesy of Professor I. Craft.

Immunisation

For each immunisation oxytocin was coupled to porcine thyroglobulin (TGG) (Sigma, Type 2) essentially as described for AVP by Moore et al (14). 2mg oxytocin and 5mg TGG were dissolved in 3mlH_2O, and 7mg N-ethyl-N'-(3-dimethylaminopropyl)-carbodiimide hydrochloride (BDH) was added in 1ml H_2O with vigorous mixing.

After 24h at 22°C, 7mg hydroxylamine-HCl was added. After a further 5h, the mixture was emulsified in an equal volume of complete Freunds adjuvant (Difco).

Four rabbits were immunised by multiple intradermal injections each receiving between 0.25 - 0.5mg oxytocin as conjugate, spread over 20-30 sites as an initial injection. Three such injections were given monthly, after which the first bleeds were obtained. The rabbits were then given three booster injections of 0.1 - 0.2 mg conjugated oxytocin followed by bleeding at monthly intervals, after which they were rested for a three to four month period before further injections. The sera obtained were stored at -20°C in small aliquots.

Iodination procedure

Initially, oxytocin was iodinated using the Chloramine-T method (8). In more recent experiments the iodogen method as described by Salacinsky et al (19) was used as follows: Oxytocin (5μg) and $Na^{125}I$(1mCi) were added in 0.2M phosphate buffer pH 7.4 to a film of Iodogen reagent (2.5μg) deposited in a small glass tube, and incubated for 20 min. The final reaction mixture was applied to a 1x20cm column of DEAE-Sephadex A-25 (Pharmacia, G.B.) equilibrated with RIA buffer. Unreacted ^{125}Iodide was retained on the top of this column and the iodinated peptide emerged as a single broad peak. This material was rechromatographed on a smaller column (1 x 10cm) of DEAE Sephadex A25 under the same conditions, and the fractions tested for binding to excess antibody. The

fraction showing the best immunoreactivity was divided into 1μCi aliquots and stored at -20°C.

Radioimmunoassay procedure

The buffer used throughout (RIA buffer) was 0.1M tris-(hydroxymethyl)-methylamine adjusted to pH 7.4 with 5M HCl, and containing 3mg/ml bovine serum albumin, Sigma Fraction V. (BSA).

Antibody titration curves were constructed by incubating 400 μl of doubling dilutions of antibody with 50μl ^{125}I-oxytocin diluted to contain 2-3000 cpm. Standard curves were obtained by adding 200μl of doubling dilutions of standard oxytocin (1-250pg), 50μl ^{125}I-oxytocin (2-3000cpm) and 200μl of antisera diluted to produce between 35-50 per cent binding when no unlabelled oxytocin was present. Samples (200μl) were assayed in duplicate in place of standard. Control tubes containing either tracer alone, buffer in place of antibody, or buffer in place of sample, were included in every assay. When different types of biological sample were assayed, control tubes with excess antibody were included to assess incubation damage. Incubation was carried out for 2-4 days at 4°C. Separation of bound from free ^{125}I-oxytocin was achieved by the rapid addition of 900μl absolute ethanol, mixing and centrifugation at 3000g for 10 min at 4°C. The supernatants were removed by aspiration, and radioactivity in the pellets was estimated using a Packard gamma scintillation spectrophotometer. Counts bound in sample or standards were expressed as a percentage of the counts bound in the zero standard tubes, after correction for counts non-specifically trapped in the pellet.

Tests of specificity

A variety of peptide analogues were tested for immunoreactivity. Each substance was tested over a wide range of concentrations in order to estimate parallelism and the degree of cross-reaction. Oxytocin was also treated in the following ways to attempt to alter its immunoreactivity (7,9,10):-

(a) Incubation of 5µg oxytocin with 1ml 10% thioglycollic acid for 1hr at 22°C.

(b) Incubation of 5µg oxytocin with 1ml 50% mercaptoethanol overnight at 22°C, followed by addition of a molar excess of iodoacetamide.

(c) 100ng oxytocin was incubated with 1ml human late pregnancy plasma at 37°C, and 100µl samples withdrawn at various time intervals, diluted to 1ml with 0.25% acetic acid and boiled for 3 minutes to destroy enzyme activity.

(d) Incubation of 125µg oxytocin with 1.5mg chymotryspin (Worthington) in 1ml phosphate buffer pH 7.4 at 37°C. Samples were withdrawn at various time intervals and snap-frozen in solid CO_2. Just prior to assay they were diluted to 1ml with 0.25% acetic acid, boiled for 3 minutes, diluted with RIA buffer and assayed.

Neutralisation of biological activity

Samples of oxytocin and vasopressin were incubated for 30 and 60 minutes with antiserum RI_4 diluted 1:2500 in Tyrode's solution, at pH 7.5. 0.5ml aliquots were assayed on an *in vitro* mouse mammary strip bioassay for oxytocin (16).

Purification of anti-oxytocin antisera by affinity chromatography

The tripeptide Pro-Leu-$GlyNH_2$ (15mg) was covalently attached to 4g cyanogen bromide activated agarose beads (Pharmacia, G.B.), following the instructions supplied by the manufacturers. A (3ml) column of this material was equilibrated in RIA buffer and used for affinity chromatography of anti-oxytocin antiserum RI_4. 800µl of a 1:50 dilution of this antiserum was applied to the top of the column and washed through with RIA buffer. The column was then eluted with 6ml of RIA buffer adjusted to pH 3.5 and finally with 1 M HCl. Fractions of 3ml were collected and titrated for anti-oxytocin antibodies.

Oxytocin infusions

Oxytocin was infused via chronically implanted venous catheters into conscious unrestrained dogs for 1h using a portable continuous infusion pump (15) as described by Stevenson et al (25). Blood samples (1ml) were withdrawn into heparinised syringes, centrifuged at 10000g for 2 min, and stored at -20°C until assay. Control plasma from the same dogs was used for the oxytocin standard curves.

RESULTS

Iodination

The iodinated peptide emerged as a single broad peak from the second DEAE-Sephadex column, fractions in the descending part usually showing the best immunoreactivity. The specific activity averaged around 1000 Ci/mmole. 90-95% of the tracer could be pre-

cipitated in antibody excess, whereas 3-7% of the counts were non-specifically precipitated by ethanol in the absence of antibody. Most preparations could be used for up to eight weeks. Both the Chloramine T and Iodogen methods produced equally good tracers as judged by maximum binding to antibody, and standard curves. The Iodogen method gave higher yields and was more convenient and is now the method of choice.

Antisera

All the rabbits produced low titre antibodies to oxytocin after three injections. With continued boosting, two produced considerable increases in titre. Following a rest period of three to four months, a further booster injection produced high titres in all four rabbits and one month later the rabbits were bled out. Table 1 shows the main characteristics of some of these later bleeds. Rabbits R1 and R3 produced the most sensitive antisera and these discriminated well between oxytocin and AVP. R2 and R4 produced less sensitive antisera which were also less able to discriminate between oxytocin and AVP. Three representative antisera ($R1_4$, $R2_6$, $R3_5$,) were chosen for further study.

Sensitivity

The smallest amount of oxytocin which could be detected under optimal conditions with $R1_4$, was 2pg added in 200µl. This produced an inhibition of binding distinguishable both from zero and from 4pg ($P<0.01$). $R3_5$ was more sensitive (1pg/200µl) whereas $R2_6$ was relatively insensitive.

TABLE 1

Characteristics of Some Antisera to Oxytocin

Anti-serum	Titre (1)	pg Oxytocin for 50% B_o (2)	Cross-reaction with AVP (3)
Rl_3	78000	40	<0.001%
Rl_4	110000	46	<0.001%
Rl_5	110000	36	0.005%
$R2_5$	150000	80	0.13%
$R2_6$	300000	60	2.3%
$R3_5$	230000	20	0.004%
$R4_5$	32000	95	0.04%

1. Titre was determined as the dilution which binds 50% of a trace amount of ^{125}I-oxytocin.
2. The amount of oxytocin required to inhibit binding of ^{125}I-oxytocin by 50%.
3. The amount of oxytocin as a percentage of the amount of AVP necessary to cause the same fall in binding.

Delaying the addition of tracer produced a small increase in sensitivity but it was not routinely used. In fact, for small assays, it was found possible to mix antibody and tracer together immediately before addition to the assay tubes without materially affecting the sensitivity of the assay and this manoeuvre considerably reduced the time to set up an assay. The assay volumes could be reduced by half and as little as 0.5pg/100µl could be detected using $R3_5$ (Figure 1).

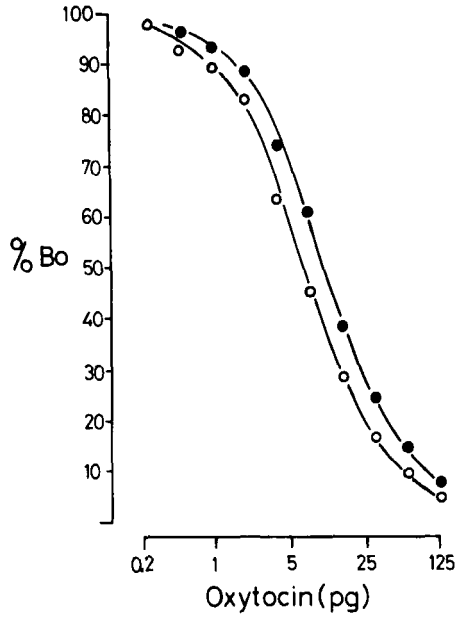

FIGURE 1. Delayed addition of tracer. Two standard curves are shown using antiserum $R3_5$. The total assay volume of 250µl contained 100µl doubling dilutions of oxytocin, 100µl antiserum and 50µl ^{125}I-oxytocin added simultaneously ●——●, or after 2 days of the 4 day incubation period o——o.

A series of closely related peptides were tested for cross-reactivity and the results are assembled in Table 2. Modifications to the ring or amino terminus of oxytocin have little effect on binding, whereas analogues with a basic residue at position 8 have much reduced ($R2_6$) or virtually abolished ($R1_4$, $R3_5$) crossreactivity. Two different sets of analogues were tested to define more closely the antigenic determinant within the oxytocin molecule. Removal of the terminal amide profoundly reduced binding to all sera whereas replacing the amide with a methyl ester was much less detrimental.

TABLE 2

Specificity of Antisera $R1_4$, $R2_6$ and $R3_5$

Substance tested		% Cross-Reactivity		
		$R1_4$	$R2_6$	$R3_5$
Oxytocin	Cys-Tyr-Ileu-Gln-Asn-Cys-Pro-Leu-GlyNH$_2$	100	100	100
Arginine Vasotocin	Cys-Tyr-Ileu-Gln-Asn-Cys-Pro-Arg-GlyNH$_2$	<0.001	1.2*	<0.001
Arginine Vasopressin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Arg-GlyNH$_2$	<0.001	2.3*	0.005
Lysine Vasopressin	Cys-Tyr-Phe-Gln-Asn-Cys-Pro-Lys-GlyNH$_2$	<0.002	0.12*	0.006
Tri-glycyl Oxytocin	Gly-Gly-Gly-Cys-Tyr-Ileu-Gln-Asn-Cys-Pro-Leu-GlyNH$_2$	75	52	76
Desamino-oxytocin	H-Cys-Tyr-Ileu-Gln-Asn-Cys-Pro-Leu-GlyNH$_2$	100	23	100
[Gly-oMe]-oxytocin	Cys-Tyr-Ileu-Gln-Asn-Cys-Pro-Leu-Gly-oMe	3.3	4.0	2.25
Oxytocinoic acid	Cys-Tyr-Ileu-Gln-Asn-Cys-Pro-Leu-Gly	0.031	0.051*	0.016
DesGlyNH$_2$-oxytocin	Cys-Tyr-Ileu-Gln-Asn-Cys-Pro-Leu	0.001	0.003*	0.001
Desleu,DesGly-NH$_2$-oxytocin	Cys-Tyr-Ileu-Gln-Asn-Cys-Pro	<0.001	<0.001	<0.001
Tocinoic acid	Cys-Tyr-Ileu-Gln-Asn-Cys	<0.001	0.004*	<0.001
Prolyl-leucyl-glycineamide(PLG)	Pro-Leu-GlyNH$_2$	<0.005	0.013	0.18
N-carbobenzoxy-PLG	Z-Pro-Leu-GlyNH$_2$	0.03	2.0	0.38
N-Carbobenzoxy-S-Benzyl-Cys-PLG	Z-Cys(Bz)-Pro-Leu-GlyNH$_2$	0.13	8.0	18.3

Each analogue was tested over a wide range of concentrations, and the ratio of the weight of peptide to the weight of oxytocin required to cause an equal fall in binding is expressed as a percentage cross-reactivity relative to oxytocin. * indicates non-parallel inhibition curves.

Stepwise shortening of the side chain by removal of the glycine, leucine and proline residues lowered the crossreactivity to negligible proportions even though the entire ring portion of the oxytocin molecule remains intact. On the other hand, the tripeptide side chain, PLG, had weak and variable cross reactivity with all three sera; addition of the N-carbobenzoxy group increased the cross-reactivity and the further addition of a fully protected cysteine residue increased very significantly the cross reactivity with all three sera (Table 2).

Whereas reducing the disulphide bridge destroys the biological activity of oxytocin, the immunoreactivity was virtually unaltered. Thioglycollate-treated oxytocin had a slightly enhanced immunoreactivity although this did not dilute in parallel with a standard curve of oxytocin. Mercaptoethanol-treated oxytocin had 96% immunoreactivity which did dilute parallel to the standard curve.

The effects of different enzymes capable of destroying oxytocin were also investigated. Human late-pregnancy plasma oxytocinase reduced the immunoreactivity of oxytocin by only 25% after 1 hour, whereas biological activity was fully abolished, indicating that the fragments produced retained significant immunoreactivity. In contrast, chymotrypsin destroyed immunoreactivity very rapidly, less than 0.1% remaining after 10 minutes incubation.

Incubation Conditions

Binding of Rl_4 was markedly temperature sensitive, dropping from 50% at $4^{\circ}C$ to 10% at $22^{\circ}C$ for the same antiserum dilution.

There was no significant improvement in binding to any of the sera when the incubation time was extended beyond four days. The separation procedure was quick and reliable and did not appear to disrupt the binding equilibrium. Essentially the same binding was observed using dextran-coated charcoal to precipitate the free fraction.

The precision and reproducibility of the assay was tested by repeated assays of two different samples distributed in small aliquots and frozen. Within-assay and between-assay coefficients of variation were 7.1% and 11.6% respectively (n=8).

Biological Samples

Human, dog, rat and guineapig plasma were tested with Rl_4 and $R3_5$. Figure 2 shows two standard curves of oxytocin with antiserum Rl_4, one in buffer and one with the incorporation of 50µl dog plasma per tube. Plasma causes a small increase in the non-specific trapping of counts in the larger pellet and thus in the apparent binding of the zero standard tubes. When the plasma standard curve is corrected for this, the curves obtained are virtually superimposable (Figure 2b). Similar results were obtained with $R3_5$ which would tolerate up to 40% plasma without loss of sensitivity. Human late pregnancy plasma could not be assayed directly as the high concentration of oxytocinase damaged the tracer, as reflected in a reduction of counts precipitated with antibody excess. All control plasma samples showed no detectable endogenous oxytocin (i.e. <1pg/200µl). Endogenous oxytocin released during suckling in guineapigs was readily detectable by

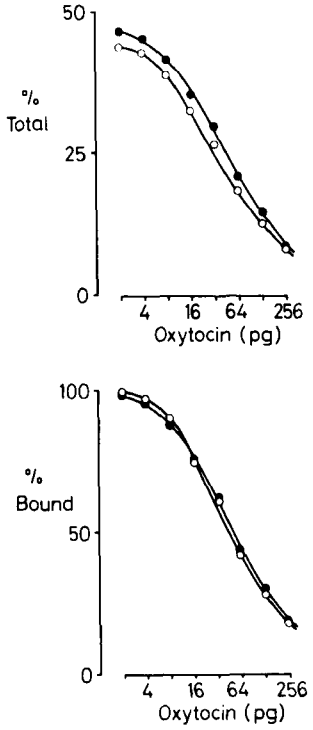

FIGURE 2. Standard curves were obtained using antiserum $R1_4$, one in buffer o——o and one containing 50µl dog plasma per tube ●——● (final concentration 11%). In A, the counts precipitated are expressed as a percentage of the total counts added. In B, the same data are expressed as a percentage of the counts bound in the zero standard tubes after correction for the non-specific counts precipitated in the buffer blank or plasma blank tubes as appropriate.

direct RIA (18). Oxytocin was stable in dog and guineapig plasma when stored at -20°C for at least six months. Cerebrospinal fluid and dilute human urine could be included up to 200µl/tube with no interference in the assay. It was possible to detect endogenous immunoreactivity in both types of sample on occasion,

and oxytocin added to these fluids gave dilution curves which superimposed standard curves in RIA buffer.

Homogenates of guineapig or rat neurohypophyses were assayed for oxytocin content by RIA and gave dilution curves completely parallel to the standard curve throughout its range (Figure 3).

Neutralisation of Biological activity

Antiserum Rl_4 was tested for its ability to neutralise oxytocin in an *in vitro* bioassay. Figure 4 shows that a 30 minute incubation with Rl_4 completely abolished the biological effects of oxytocin whilst leaving the response to vasopressin unaltered. A final dilution of 1 in 2500 of Rl_4 was sufficient to neutralise more than 40pg/ml oxytocin which is five times more

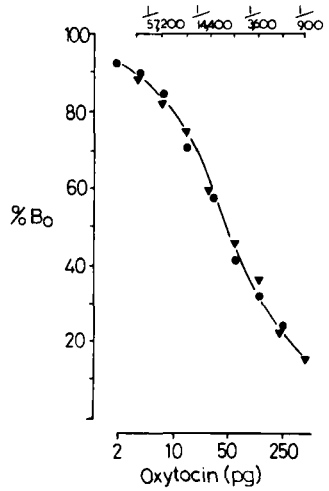

FIGURE 3. Doubling dilutions of an 0.1M HCl extract of one guineapig neural lobe (▼——▼) are compared to doubling dilutions of oxytocin in a standard curve with Rl_4 ●——●.

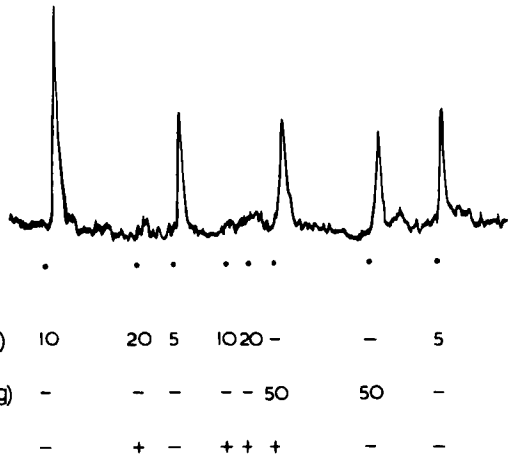

FIGURE 4. Neutralisation of the biological activities of oxytocin and vasopressin on the mouse mammary strip in vitro. At the dots, doses of oxytocin (OT) and vasopressin (AVP) with or without prior incubation with antiserum Rl_4 at 1:2500, were given to the bioassay preparation. Contractions of the myoepithelial cells produce increase in tension in response to the neurohypophysial hormones; on this preparation, AVP was 10-fold less potent than oxytocin but its activity was unaffected by incubation with an amount of Rl_4 which completely neutralised the effect of oxytocin.

than the concentration necessary to produce a threshold response in the bioassay.

Purification of anti-oxytocin antiserum

Antiserum Rl_4 was subjected to affinity chromatography on a column of agarose-Pro-Leu-Gly-NH_2. More than 98% of the titratable antibody adsorbed to the column which was then eluted with RIA buffer adjusted to pH 3.5, at which pH, no binding is observed at the dilutions used for RIA. 28% of the antibody originally loaded was recovered in the eluate at pH 3.5, and a further 3% was obtained by a subsequent wash with 1M HCl. When the material

eluting at pH 3.5 was used to set up a standard curve, the results were very similar to those obtained using the crude antiserum, 50% displacement requiring 36pg oxytocin.

Oxytocin Infusions

The half-life of oxytocin infused into conscious dogs was estimated by direct RIA using unextracted plasma (Figure 5). Infusion rates of 7.67 and 23ng $kg^{-1}min^{-1}$ achieved steady state

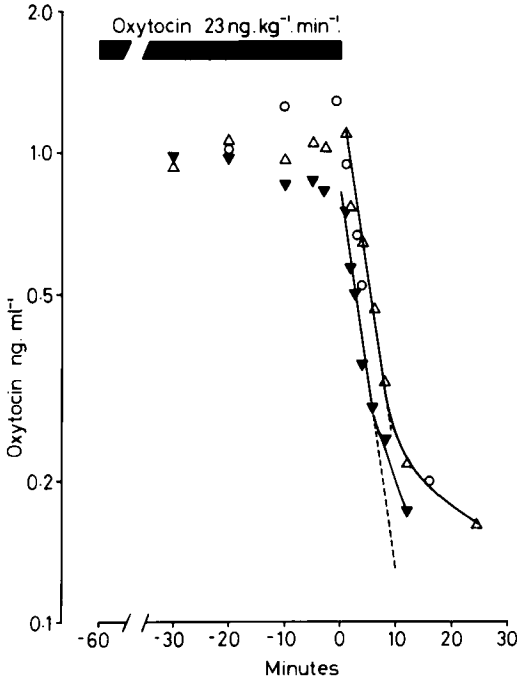

FIGURE 5. Oxytocin infusions in conscious dogs. The clearance of oxytocin from the circulation was measured by direct RIA of serial blood samples obtained during and after intravenous infusions of oxytocin in dogs. The data shown here were obtained in three different experiments (O,Δ,▼), performed and assayed on separate occasions.

levels of 0.37 and 1.02ng/ml immunoreactive oxytocin. The mean (± SD) half-life estimated from the initial rate of disappearance of oxytocin from the plasma was 4.34 ± 1.5 minutes (n=5). The metabolic clearance rate and apparent distribution space (24) were 20.5±3.6 ml/kg/min and 124.6±30 ml/kg respectively. 23 of these samples, shown to contain 0.1 - 1.5ng/ml oxytocin by RIA, were also tested by bioassay. The ratio of bio-to immunoreactivity was 1.06± 0.1 (S.E.M.).

DISCUSSION

The assay of the very low circulating concentrations of oxytocin (circa 10^{-12}M) requires iodinated oxytocin of very high specific activity and extremely avid antibodies able to discriminate between closely related peptides. AVT and AVP differ from oxytocin by only one and two amino acid residues, respectively, and all of these peptides can be broken down rapidly by a variety of enzymes present in tissues, and the degradation products may retain significant immunoreactivity. Furthermore, it is possible that a carboxy-terminal fragment of the oxytocin molecule may have an independent physiological role (2), and such a fragment should be distinguishable from oxytocin in plasma or tissue extracts.

In the present work, the methods used for the iodination and purification of labelled oxytocin produced a satisfactory tracer of high specific activity, which could not be improved by further purification using gel filtration or affinity chromatography (17).

It would seem that conjugation to thyroglobulin (22) followed by multiple-site intradermal injection with a suitable adjuvant is a reliable method of raising antibodies to neurohypophysial peptides (14,23,28). A simple immunisation regime using an oxytocin-thyroglobulin conjugate, produced high titres in all four rabbits, two of which produced antisera specific and avid enough for RIA. The pattern of response was interesting in that the rabbits achieved high titres at quite different rates, rises occurring in some cases during the 3-month rest period after the initial course of immunisation. Oxytocin will have coupled to thyroglobulin primarily via the α-amino group of cysteine in position 1, leaving the oxytocin side-chain free. This selective attachment may have directed the antibody reaction to the free side-chain, as such specificity was observed with all of the present antisera.

Few authors have tested the cross-reactivity of their anti-oxytocin antisera in any systematic manner (4). In the present work, the structural requirements for immunoreactivity were studied in some detail for three different antisera, with particular attention to likely metabolic degradation products. Changes in the amino-terminal portion had little or no effect on immunoreactivity and the ring structure itself seems unnecessary for binding, since reduction of the disulphide bridge had only small effects on immunoreactivity, and the tocinoic acid ring itself was virtually devoid of any immunoreactivity. In contrast, modifications to the side-chain profoundly altered immunoreactivity.

Substitution of a basic residue in position 8 caused a large drop in immunoreactivity, as did the progressive removal of side-chain residues. This renders the assay virtually free from interference from AVP or AVT.

Even removal of the terminal amide caused a large fall in immunoreactivity, which could be partially restored by replacing the amide with a methyl ester. Although the side-chain tri-peptide, Pro-Leu-GlyNH_2, showed very low cross-reactivity, a fully protected tetra-peptide sequence corresponding to residues 6 to 9 was significantly immunoreactive. Similar results were obtained from the studies of enzyme degradation. Chymotrypsin virtually destroyed immunoreactivity by removing the terminal glycinamide residue, whereas the degradation products remaining after the complete destruction of the biological activity of oxytocin by oxytocinase, retained strong immunoreactivity. This enzyme opens the ring structure by peptide bond cleavage and the final end products (21) are somewhat analogous to the synthetic protected tetra-peptide sequence, shown here to have considerable immunoreactivity.

This information is necessary when considering the possible interference of fragments or degradation products of oxytocin which may be present in biological samples. In the present assay any breakdown of the side-chain sequence (27) or the presence of the intact side-chain would not interfere significantly. On the other hand, fragments with quite drastic alterations to the oxytocin ring still retain high immunoreactivity and for this

reason the present assay should not be applied directly to the assay of human pregnancy plasma. Indeed, it is arguable that the present state of uncertainty concerning the oxytocin concentrations in human gestation and parturition is due in large part to the effects of oxytocinase (9) and the fragments it produces on the immunoreactivity measured in the various assay systems (11).

Anti-oxytocin antisera were subjected to affinity chromatography on agarose-PLG. Although the corresponding soluble analogue, N-Carbobenzoxy-PLG, had a very low cross-reactivity compared with oxytocin, the much lower affinity actually favours the practical use of affinity chromatography; recoveries of avid antisera are much lower if oxytocin is used as the immobilised ligand (I.C.A.F. Robinson & J. Möhring, unpublished). Although the titratable antibodies were quantitatively retained on agarose-PLG columns, the resulting purified antibodies showed no improved specificity or sensitivity compared with the original serum, indicating that there were little or no ring-directed antibodies in the sera. The fact that such weakly cross-reactive ligands can retain antisera so effectively when immobilised on a solid support should also be borne in mind when assessing the specificity of antisera for use in immunohistochemical procedures.

The sensitivity of the present assay is comparable with other recently reported assays (5,6,20,28), although each group have used their own ill-defined peptide standards. The new IVth International Standard for oxytocin has been used in the present assay which should allow comparison in future between different

RIAs. Plasma caused no interference or reduction in sensitivity of the antisera. Oxytocin levels have been determined during suckling (18) or following infusions of exogenous hormone, by direct assay of the plasma without extraction. Lauson (13) has comprehensively reviewed the available data on the clearance of oxytocin in a number of mammalian species, but there is little previous work reported on conscious dogs. In the present work, the values for metabolic clearance and half-life obtained by RIA lie well within the range reported for other species. It is possible that during constant infusion over 1 hour, significant amounts of immunoreactive metabolites may have accumulated to give falsely high values. However, essentially similar values were obtained by bioassay of some of the samples, indicating that any biologically inactive degradation products will have interfered only to a small extent.

Finally, the antisera efficiently neutralise the biological activity of oxytocin, both in vivo (18) and in vitro (26). Antiserum Rl_4 has already been used in this way to confirm the presence of oxytocin in the urine of Brattleboro rats (1) and in extra-hypothamamic areas (Robinson & Möhring, unpublished). The specificity of the antiserum complements that of the bioassay and aids the identification of oxytocin in biological samples.

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