CROSS-REACTIVITY OF ANTI-OXYTOCIN SERA

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Two anti-oxytocin sera, raised using thyroglobulin–oxytocin conjugates as immunogens in a rabbit and a pig, have been compared. Differences in assay conditions gave rise to large differences in the apparent titre of the antisera, and altered the specificity of the resulting assays.

Radioimmunoassays for oxytocin have recently been established in two different laboratories using porcine[1] and rabbit[2] antisera, with sensitivities of 5 pg and 1 pg per tube respectively. The specificity of both antisera have been evaluated with limited number of oxytocin analogues in their laboratories of origin. In an attempt to examine further the cross-reactivity of our antisera, we engaged in an exchange of oxytocin analogues and an inter-laboratory comparison of antisera.

In this note we describe how small differences in assay conditions required large differences in antiserum dilution and resulted in significant alteration in the apparent specificity of the antisera. Our experiences may be of interest to others using multiple antisera from different sources.

MATERIALS AND METHODS

Porcine anti-oxytocin serum (code A1) and rabbit anti-serum (code $RIII_5$) were obtained as described[1,2]. Oxytocin was obtained from Ferring, Malmö (440 IU/mg) or Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, Prague (450 IU/mg). For assays in London, the IVth International Standard for oxytocin (National Institute for Biological Standards and Control, Holly Hill, Hampstead, U.K.) was used assuming an ampoule content of 21 μg peptide.

Deamino-1-carba-oxytocin, carba-1-oxytocin, deamino-6-carba-oxytocin, deamino-dicarba--oxytocin, deamino-1-carba-oxytocin sulfoxide and [9-glycine-O-methyl]oxytocin were synthesised at the Department of Organic Synthesis of Prague's Institute, their properties were the same as previously published. Tocinoic acid was obtained from Ferring, Malmö. Oxytocinoic acid, [9-desglycinamide]oxytocin and [8-desleu-9-desglycinamide]oxytocin were gifts from the late

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Professor R. Walter, Department of Physiology and Biophysics, University of Illinois. Prolyl--leucyl-glycinamide was obtained from Sigma Chemical, London.

([S-*carboxamidomethylcysteine*^{1,6}]*oxytocin*) was prepared by incubating oxytocin (5 μg) with 1 ml 50% mercaptoethanol overnight at 22°C. After addition of a molar excess of iodoacetamide, the reaction mixture was diluted with RIA buffer and assayed. Reduced oxytocin was devoid of uterotonic activity *in vitro*. Na ^{125}I was obtained from the Radiochemical Centre, Amersham, U.K., or from Isotop, Hungary. The following buffer systems were used: *a*) 0·1M-Tris-HCl, 0·3% BSA, pH 7·4 (London), *b*) 0·1M-Tris-HCl, 0·2% BSA, pH 7·7 (Prague), *c*) 0·2M Na-barbital 0·14M-NaCl, 0·01M-EDTA, 0·07 mM L-cystine and 0·25% BSA, pH 7.7.

^{125}I-*labelled oxytocin* was prepared as previously described using Chloramine-T (Prague) or Iodogen (London). Antiserum titration curves were constructed by incubating doubling dilutions of antiserum with ^{125}I-oxytocin, standard curves were obtained by incubating double dilutions of standard oxytocin (1—1 000 pg), ^{125}I-oxytocin (2 000—5 000 cpm) and aliquots of antiserum diluted to produce 35—50% binding when no unlabelled oxytocin was present. Total volume was 450 μl and all tubes (in duplicate) were incubated at 4°C for 2—4 days. Separation of bound from free oxytocin was achieved either by (*i*) rapid addition of 900 μl ethanol, mixing and centrifugation at 3 000*g* for 10 min at 4°C, or (*ii*) addition of 1 ml dextran-coated charcoal, mixing, and centrifugation after 30 min delay at 4°C. The radioactivity in the pellet and/or the supernatant was counted in a Packard gamma scintillation spectrometer or an ISOCAP 300, Nuclear, Chicago. Results are expressed as a percentage of the counts bound, after correction for counts non-specifically trapped in the pellet. To test antiserum specificity, analogues were added in place of standards. Each substance was tested over a wide range of concentrations in order to estimate parallelism and the degree of cross-reactivity.

RESULTS AND DISCUSSION

Preliminary experiments with antisera exchanged between our laboratories gave remarkable differences in the apparent titres of the antisera (10—100 fold). These

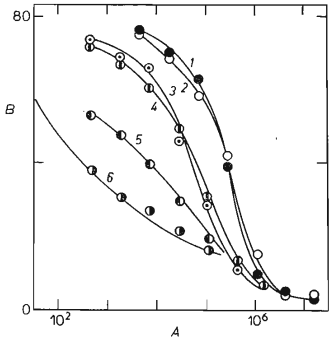

FIG. 1

Antiserum titration curves obtained using different assay conditions.

A antiserum dilution, B % bound ^{125}I--oxytocin. ● $RIII_5$, London buffer, ethanol separation; ○ $RIII_5$, Prague buffer, ethanol separation; ◑ $RIII_5$, Prague buffer, charcoal separation; ⊙ Al, London buffer, ethanol separation; ⓘ Al Prague buffer, ethanol separation; ◐ Al, Prague buffer, charcoal separation

differences were not due to storage or shipment conditions. In the case of $RIII_5$ there was very little difference ($\pm 15\%$) in the titre whether the antiserum was stored for a year at $-20°C$, lyophilised, reconstituted and left at room temperature for 6 days or repeatedly frozen and thawed over a three month period.

Titre determinations of oxytocin antisera A1 and $RIII_5$ were therefore compared in both laboratories by combining separately the components of each assay system (buffers, iodinated oxytocin, separation method, *etc.*).

It was found that the value of titre obtained in either laboratory with a given buffer system was little influenced by the source of iodinated peptide indicating that the different labelling methods produced tracers giving comparable results with each antiserum. Changing the buffer systems from tris to phosphate gave rise to small differences in titres (2–5 fold), but the major cause of the discrepancies was the different separation procedures routinely employed in the two laboratories. The ethanol separation method gave titres 20 fold higher than the charcoal method with A1 and up to 100 fold higher with $RIII_5$. Fig. 1 illustrates this in a single experiment (Prague) in which both separation methods were used with both antisera.

Table I summarises the results obtained in our two laboratories. The rabbit antiserum was more susceptible to the disruption of equilibrium binding by charcoal than the porcine antiserum but it is not possible to generalise since on the one hand an antiserum to arginine vasopressin raised in a similar way in Prague[3] showed similar binding whether dextrancoated charcoal, ethanol, polyethylene glycol or a double antibody method was used, and on the other hand, human plasmacoated charcoal prepared as described by Möhring[4] gave similar results to ethanol precipitation using $RIII_5$ in London. Obviously, each step in the assay must be considered when different antisera are to be compared. We also examined the cross-reactivities of both $RIII_5$ and A1 against oxytocin analogues modified in the "ring" or "tail"

TABLE I

Values of the titre obtained using different assay conditions

Sera	Ethanol precipitation			Charcoal separation	
	Tris (London)	Tris (Prague)	Veronal	Tris (Prague)	Veronal
$RIII_5$	1 : 230 000	1 : 140 000	1 : 95 000	1 : 100	1 : 500
A1	1 : 25 000	1 : 32 000	1 : 36 000	1 : 1 000	1 : 2 000

moieties, and the results are shown in Table II. At high dilutions, using ethanol separation, both antisera were extremely sensitive to changes in the oxytocin side-chain and somewhat less affected by alterations in the ring structure. However, comparison of the same sera at much higher concentrations necessary to give the same apparent binding ($B/F = 1$) with charcoal separation demonstrated that the specificity altered considerably. In particular, $RIII_5$ showed much greater ability to detect alterations in the oxytocin ring. The most likely explanation of this finding is that there is a heterogenous population of antibodies in $RIII_5$ showing different specificities, and these are revealed at widely differing dilutions of the same antiserum. This has particular significance if the antisera are subsequently used in immuno-histochemical procedure at much higher concentrations than are used in radioimmunoassays.

We have shown that small differences in the assay procedures may have a marked effect on the binding of antisera exchanged between laboratories, and if the sera are used at different dilutions, the resulting assay may show a considerable difference in specificity. The exchange of analogues is also likely to reveal much more information about the characteristics of antisera to small peptides.

TABLE II

Cross-reactivity of oxytocin analogues relative to oxytocin using different antisera dilutions

Compound	A 1		R III 5	
	1 : 1 000—2 000	1 : 20 000—45 000	1 : 100	1 : 230 00
Oxytocin	100	100	100	100
Deaminooxytocin	50	20	—	100
Carba-1-oxytocin	9·1	8·2	2·0	28·7
Deamino-1-carba-oxytocin	6·25	8·8	9·5	65
Deamino-6-carba-oxytocin	1·56	1·66	5·4	—
Deamino-di-carba-oxytocin	0·75	—	1·7	110
[9-Glycine-O-methyl]oxytocin	—	10	—	2·25[a]
Oxytocinoic acid	—	0·015	—	0·016[a]
[9-Desglycinamide]oxytocin	—	0·001	—	0·001[a]
[8,9-Desleucylglycinamide]oxytocin	—	0·001	—	0·001[a]
Tocinoic acid	—	0·001	—	0·001[a]
Prolyl-leucyl-glycinamide	—	0·001	—	0·18[a]
Reduced oxytocin	100	96·0	—	98·0[a]

[a] Values taken from ref.[2].

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