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Measurement of specific radioactivities in labelled hormones by self-displacement analysis

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A graphical method is described that allows the determination of specific radioactivites of radioactively labelled hormones. This method combines the self-displacement technique, plotting bound/free ratios versus mass of unlabelled hormone or total radioactivity of labelled preparation added to the receptor preparation, and the maximal binding capacity of the labelled hormone. The procedure presented herein provides a more realistic specific radioactivity for use in all binding experiments. Application of the method is demonstrated for ^{125}I-labelled ovine prolactin, and data are presented for ^{125}I-labelled human choriogonadotropin and [^{3}H]testosterone.

Hormone binding studies are performed with radioactively labelled hormones and it is necessary to know their specific radioactivities in order to obtain the binding parameters such as affinity constant and binding sites in terms of concentration. Until now, most of the investigators obtained the specific radioactivity data from purification steps after hormone labelling, data that represent a source of error because, as has been demonstrated for insulin (Cresto *et al.*, 1981), the iodination procedure may yield monoiodinated hormone, as well as di- or non-iodinated molecules, and, unless one could purify the different fractions, the specific radioactivity thus obtained represents an average from different species, not all with the same biological activity.

Other authors have used a self-displacement technique to obtain a more realistic specific radioactivity (Catt *et al.*, 1974; Moyle *et al.*, 1980), which assumes that the mass of the tracer in the labelled stock solution was that which gave an equal B/F ratio as unlabelled hormone in a plot of B/F versus log [mass (ng) unlabelled hormone] or volume (μl) of ^{125}I-labelled hormone. This assumption is true only when the mass of the tracer is negligible compared with that of the unlabelled hormone added, as will be proved in the present paper.

Abbreviation used: B/F ratio, ratio of bound hormone to free hormone.

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In the present work we provide a useful and simple technique to obtain specific radioactivities of labelled hormones which uses the self-displacement method, and present a detailed description of the procedures employed and the mathematical principles involved.

Materials and methods

Iodination of ovine prolactin

Ovine prolactin (NIH-P-S 12) was iodinated with Na^{125}I (New England Nuclear NEZ-033A; carrier-free; 17 Ci/mg), at room temperature by using the lactoperoxidase/perhydrol method of Thorell & Johansson (1971), except that the reaction was allowed to proceed at pH 7.4 for 2 min. A first fractionation of the iodinated hormone was carried out on a column (0.5 cm × 20 cm) of Sephadex G-75 equilibrated with 50 mM-sodium phosphate buffer, pH 7.5, containing 0.2% bovine serum albumin (Sigma, fraction V), 100 mM-NaCl and 0.05% NaN_3.

Binding experiments

Ventral prostates from young male Wistar rats were homogenized at 0°C in 4 vol. of phosphate-buffered saline (0.01 M-sodium phosphate buffer, containing, per litre, 8 g of NaCl, 0.2 g of KCl and 0.1 g of $CaCl_2$, and 0.5 mM-$MgCl_2$), pH 7.4, filtered through a nylon cloth and centrifuged at 27000 *g* in an International B-20 refrigerated centrifuge at 2–4°C for 30 min. The pellet was suspended in 4 vol.

of phosphate-buffered saline and used for the binding studies.

Two sets of binding experiments were performed. In one set, 100 μl of membrane preparation was equilibrated with different concentrations of labelled hormone (between 15000 and 400000 c.p.m.), in a final volume of 200 μl, at room temperature for 20 h. Non-specific binding was assayed in parallel incubations containing 5 μg of unlabelled ovine prolactin. In the second set, 100 μl of membrane preparation was equilibrated with 50000 c.p.m. of ^{125}I-labelled ovine prolactin and different concentrations of unlabelled hormone (0–20 ng/tube), in a final volume of 200 μl, at room temperature for 20 h. Non-specific binding was evaluated with 5 μg of unlabelled hormone.

At the end of the reaction the incubation mixture was diluted with 2 ml of cold phosphate-buffered saline and the tubes were centrifuged at 5000 *g* at 2–4°C for 15 min. The pellets were washed twice with the same buffer, re-centrifuged as above, and the radioactivity in the remaining pellets was determined with a Beckman Auto-Gamma 4000 spectrometer with 60% efficiency.

There were no losses during washing, as evaluated by protein determination by the Lowry method. There was complete recovery of bound radioactivity in the pellet as evaluated by washing a known amount of bound radioactivity pipetted into a clean tube.

In order to obtain the maximal binding capacity of the labelled hormone preparation, different samples of the membrane preparation were equilibrated with approx. 30000 c.p.m. of labelled hormone and the non-specific binding was obtained from the radioactivity bound in the presence of 5 μg of unlabelled ovine prolactin. From the ordinate intercept of a plot of 1/(radioactivity specifically bound) versus 1/(receptor volume added) it was possible to determine the fraction of ovine prolactin that would bind at infinite acceptor concentration (maximal binding capacity).

Other binding studies

Human choriogonadotropin (kindly provided by Dr. E. Passeron, Laboratorios Elea, Buenos Aires, Argentina) (11000 i.u./mg) was labelled as described for ovine prolactin and similar binding experiments were conducted with a collagenase-dispersed Leydig-cell preparation (Mendelson *et al.*, 1975) as receptor source.

For [1,2-^{3}H(n)]testosterone (NET-387; 40–60 Ci/mmol; New England Nuclear), the binding studies were performed with a specific antibody (obtained in our laboratories by active immunization of rabbit with bovine-albumin-coupled testosterone) as receptor system, and again two sets of experiments were performed, one with different concentrations of labelled hormone and the other with various masses of unlabelled testosterone.

In both cases, human choriogonadotropin and testosterone, the maximal binding capacity was 100%.

Description and application of the method

When only a single order of reaction sites is present, one may write the following Scatchard (1949) equation:

$$\frac{B}{F} = Kq - KB$$

where K is the affinity constant and q is the total number of binding sites.

The saturation analysis can be performed by varying the concentration of labelled hormone or by a competition study in which a constant amount of radioactive ligand is mixed with different concentrations of the same, but unlabelled, reactant. For both cases, one can obtain two plots: B/F versus amount (ng) of unlabelled hormone added (Ekins *et al.*, 1968) and B/F versus total radioactivity (c.p.m.) added to the incubation mixture.

If the receptor system is the same in both cases, that is, the same K and q parameters apply (which is always true when the same receptor or antibody preparation are used and when both experiments are performed at the same time), and the radioactive as well as the unlabelled hormone show identical binding behaviour, then, for a given B/F value the mass of hormone bound to the receptor must be the same in each case. The equation that reflects this statement is:

$$\frac{B_1}{T_1} \cdot p_1^* = \frac{B_2}{T_2}(p_2^* + p) \qquad (1)$$

where, for the first experiment, B_1 is radioactivity specifically bound, T_1 is (total radioactivity − non-specific radioactivity) × maximal binding capacity, and p_1^* is the mass of radioactive hormone corresponding to T_1. B_2, T_2 and p_2^* are comparable parameters for the second experiment, and p is the mass of unlabelled hormone added.

In eqn. (1), p_1^*/T_1 and p_2^*/T_2 are equal to the reciprocal of the specific radioactivity (S.R.) of the labelled hormone, in terms of c.p.m./ng, so:

$$\frac{B_1}{\text{S.R.}} = \frac{B_2}{\text{S.R.}} + \frac{B_2 \cdot p}{T_2} \qquad (2)$$

From eqn. (2):

$$\text{S.R.} = \frac{(B_1 - B_2)T_2}{B_2 \cdot p} = \frac{B_1 T_2 - B_2 T_2}{B_2 \cdot p} \qquad (3)$$

The original assumption was that:

$$\frac{B_1}{F_1}=\frac{B_2}{F_2}$$

where F_1 is the amount of free hormone (T_1-B_1) and F_2 is T_2-B_2.

Then:

$$\frac{B_1}{T_1-B_1}=\frac{B_2}{T_2-B_2} \quad (4)$$

Rearranging eqn. (4):

$$B_1(T_2-B_2)=B(T_1-B_1) \quad (5)$$

$$B_1T_2-B_1B_2=B_2T_1-B_2B_1 \quad (6)$$

$$B_1T_2=B_2T_1 \quad (7)$$

Replacing eqn. (7) in eqn. (3):

$$\text{S.R.}=\frac{B_2T_1-B_2T_2}{B_2\cdot p}=\frac{B_2(T_1-T_2)}{B_2\cdot p} \quad (8)$$

Then:

$$\text{S.R.}=\frac{T_1-T_2}{p}$$

Eqn. (8) reflects the fact that the specific radioactivity could be obtained from the graphs at equal B/F values and that it is necessary to subtract the total radioactivity used as tracer when the unlabelled hormone is the variable.

A more reliable value for the specific radioactivity is attained as follows. From eqn. (8):

$$\text{S.R.}=T_2/p_2^*=\frac{T_1-T_2}{p}$$

Rearranging eqn. (8):

$$T_1=T_2+\frac{T_2}{p_2^*}\cdot p \quad (9)$$

This is the equation of a straight line whose variables are T_1 and p. On the other hand, the ordinate is T_2, that is, the total labelled tracer used when the unlabelled hormone was varied, and the slope is the specific radioactivity of the radioactive preparation. Plotting T_1 versus p for different B/F ratios, one can obtain a straight line using unweighed linear regression, which is a simple method whose errors can be easily calculated by using a desk-top calculator.

Results and discussion

Fig. 1 shows the purification profile observed after the labelling of ovine prolactin with $Na^{125}I$. The specific radioactivity of this hormone preparation [calculated from the labelling efficiency (96%) obtained from the elution profile] was 32.6 μCi/μg.

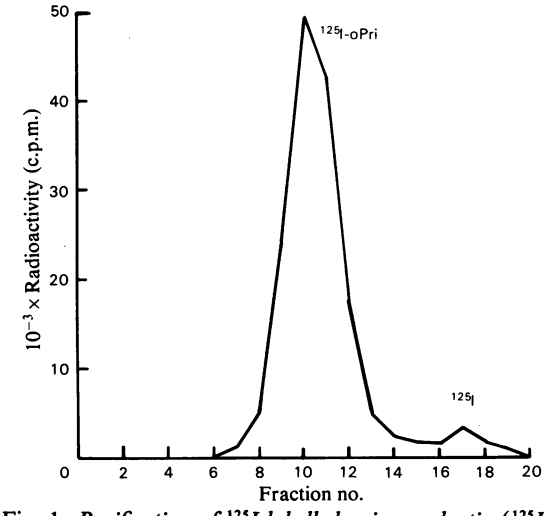

Fig. 1. *Purification of ^{125}I-labelled ovine prolactin (^{125}I-oPrl) through a Sephadex G-75 column*
Iodination mixture was applied to a column (0.5 cm x 20 cm) of Sephadex G-75 equilibrated with 50 mM-phosphate buffer pH 7.5, containing 0.2% bovine serum albumin, 100 mM-NaCl and 0.05% NaN_3. In all, 20 fractions were collected and two peaks (labelled hormone and free ^{125}I) were distinguished. Specific radioactivity calculated from the elution profile was 32.6 μCi/μg.

Fig. 2 shows the self-displacement assay in which unlabelled ovine prolactin or the radioactive preparation were varied and the B/F ratios calculated in both cases. For reasons that will be discussed further, a maximal binding capacity of 100% was assumed.

From these representations and using a B/F range from 0.08 to 0.045 we obtained T_1 and p and plotted them as shown in Fig. 3. The specific radioactivity, calculated from the slope of this line, was 33.3 μCi/μg, which is in good agreement with the above-mentioned value of 32.6 μCi/μg.

But, as we discussed in the introduction, this is not the real value, but an average of different specific radioactivities from labelled molecules. In order to obtain the more accurate specific radioactivity we considered the maximal binding capacity of the hormone preparation.

Fig. 4 shows the plot of 1/[radioactivity (c.p.m.) bound] versus 1/[volume (ml) of receptor added], where the reciprocal of the ordinate gives the maximal binding capacity (64% in this case).

Assuming this value, we replotted the corrected B/F ratios against the unlabelled ovine prolactin or

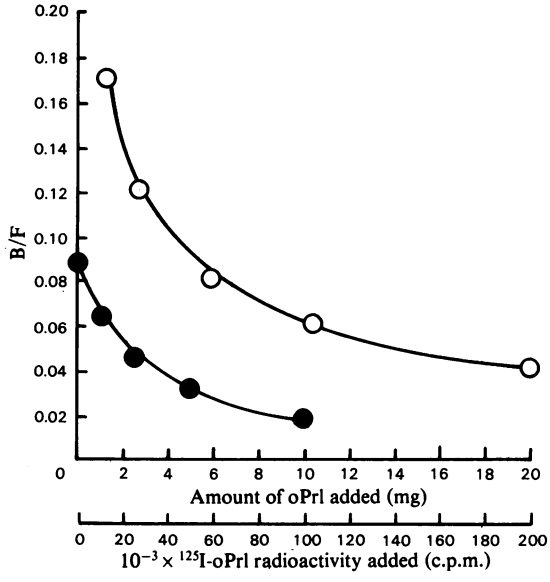

Fig. 2. *Displacement curves obtained from binding experiments*

Data, obtained from binding experiments performed as described in the Materials and methods section, were transformed into B/F ratios (a maximal binding capacity for the labelled hormone of 100% being assumed) and plotted against the amount (ng) of ovine prolactin added (●) or total radioactivity of ^{125}I-labelled ovine prolactin (^{125}I-oPrl) added to the receptor preparation (○).

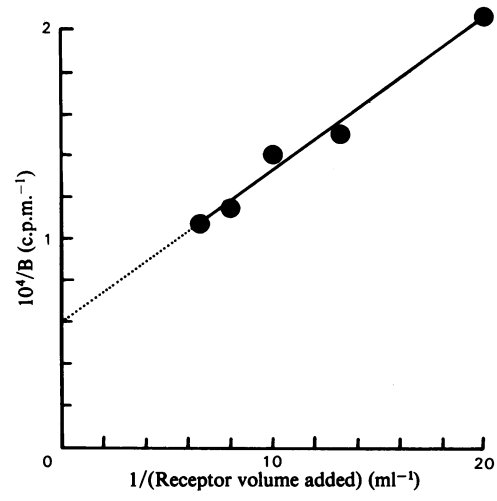

Fig. 4. *Determination of the maximal binding capacity of the radioactive hormone preparation*

Five portions of receptor preparation were incubated with a fixed amount of ^{125}I-labelled ovine prolactin (approx. 30000 c.p.m.) and 1/(radioactivity specifically bound, B) was plotted versus 1/(receptor volume added). From the ordinate intercept of the graph the fraction of the ovine prolactin that would bind at infinite acceptor concentration was determined and found to be 64% of the total radioactivity added.

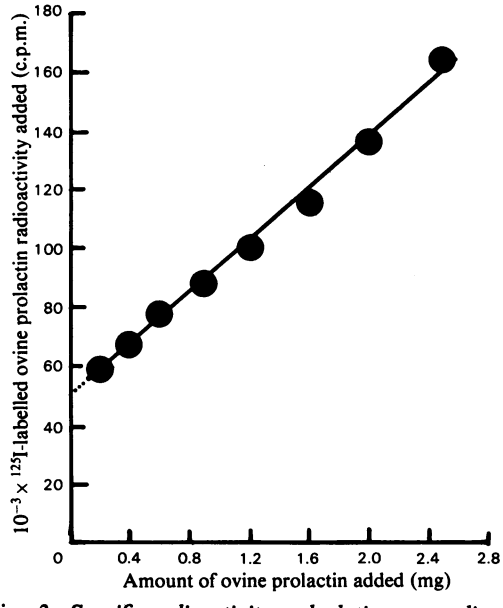

Fig. 3. *Specific-radioactivity calculation according to eqn. (9)*

The total radioactivity added to the binding system was plotted against the corresponding mass of the new total radioactivity added (Fig. 5) and by using these plots and the straight line obtained from them, we calculated a specific radioactivity of 12.4 μCi/μg.

In order to observe the effect of considering one specific radioactivity or the other, we performed the corresponding Scatchard plots (Fig. 6) using the B/F ratios and the specific radioactivity bound when radioactive hormone was the variable. It can be observed that the affinity constant is maintained ($K_a = 5.4 \times 10^9$ M^{-1} for sp. radioactivity = 33.3 μCi/μg, and $K_a = 4.7 \times 10^9$ M^{-1} for sp. radioactivity = 12.4 μCi/μg), but there is a unlabelled hormone, both obtained from the graphs presented in Fig. 2, utilizing several points where the B/F ratio was the same for both curves. The slope of the straight line observed is the specific radioactivity of the labelled hormone and resulted in 33.3 μCi/μg (in good agreement with the 32.6 μCi/μg obtained from Fig. 1).

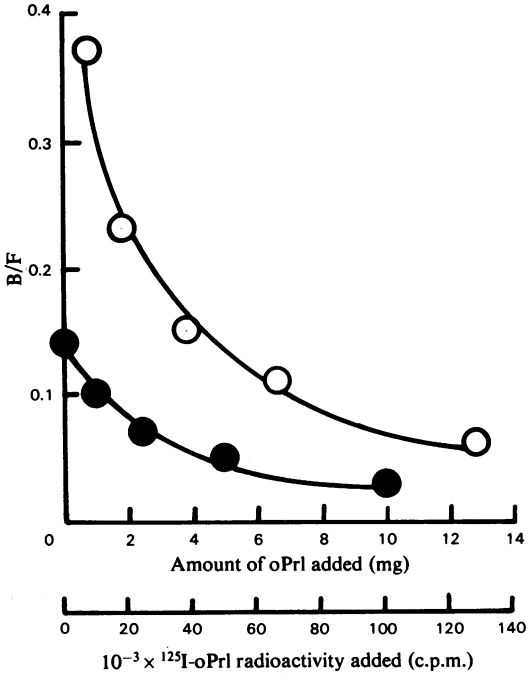

Fig. 5. *Displacement curves obtained from the same binding experiments as Fig. 2, but with data corrected by using the maximal binding capacity*

Total radioactivity was corrected with the maximal binding capacity, and thus a new B/F ratio was obtained for each point. These new values were plotted against the amount (ng) of ovine prolactin (oPrl) added (●) or total ^{125}I-labelled ovine prolactin (^{125}I-oPrl) radioactivity added to the receptor preparation (○). From these two curves a new straight line similar to that in Fig. 3 was drawn and a corrected specific-radioactivity value was obtained (12.4 μCi/μg).

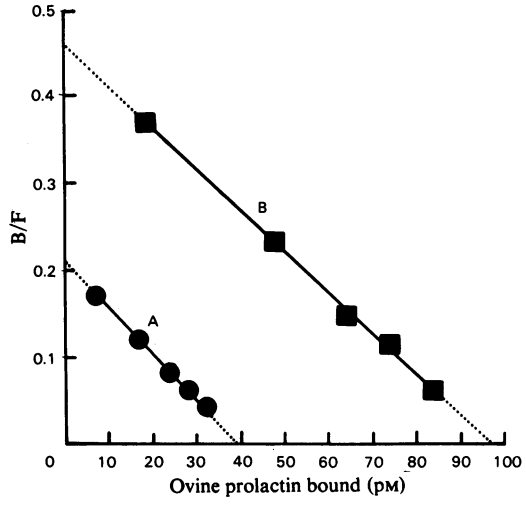

Fig. 6. *Scatchard plots of data obtained from binding studies using the specific radioactivities calculated from Figs. 1 and 5*

The B/F data obtained from the binding experiment where the receptor was incubated with increasing concentrations of labelled hormone, as shown in Fig. 2 (assuming a maximal binding capacity of 100%) and in Fig. 5 (assuming the real maximal binding capacity of 64%), were plotted versus the concentration of hormone bound in each case, using the corresponding specific radioactivity of 33.3 μCi/μg in plot A and 12.4 μCi/μg in the plot B. In both cases the affinity constant (that is, the slope of the Scatchard plot) is the same ($K_a = 5.4 \times 10^9$ M^{-1} for A, and 4.7×10^9 M^{-1} for B), but there is a significant increase in the concentration of binding sites (39 pM for A and 97 pM for B). We consider a specific radioactivity of 12.4 μCi/μg to be more realistic for binding studies because, as explained in the text, its calculation combines the self-displacement assay with the maximal binding capacity of the labelled hormone.

significant increase in the concentration of binding sites (39 pM–97 pM for the lower specific radioactivity).

We propose to use as specific radioactivity that obtained by self-displacement analysis (which provides the true mass of active tracer, calculated from the competition studies), using the maximal binding capacity (the real radioactivity corresponding to the tracer mass) for all the binding studies.

The values obtained for ^{125}I-labelled human choriogonadotropin, using rat Leydig cells as receptor system, and for [^{3}H]testosterone, utilizing a specific antibody, are as follows: for human choriogonadotropin, the specific radioactivity determined by the elution profile of the column chromatography after its labelling was 12.5 μCi/μg, whereas the result obtained by the method described herein was 11.5 μCi/μg. On the other hand, the results for [^{3}H]testosterone were: 36.0 Ci/nmol, when the specific radioactivity was calculated from data obtained from New England Nuclear (40–60 Ci/mmol), recalculated for the ^{3}H decay, and 37.0 Ci/mmol when the self-displacement technique was utilized. In both cases a maximal binding capacity of 100% was considered.

These results confirmed the method proposed and also provided evidence that it may be used for peptide and steroid hormones and in radioligand–receptor assays as well as in radioimmunoassay experiments.

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