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METHODS OF STEROID RECEPTOR CALCULATION: AN INTERLABORATORY STUDY*

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SUMMARY

Data from a determination of progesterone receptors in a human endometrial cytosol preparation were sent to 28 specialist laboratories with a request for details of calculations of receptor parameters normally carried out. The 18 replies received included data from two further laboratories in contact with one of the recipients of the questionnaire, and 12 recipients did not reply. Our own results have been included in the survey. Six different methods of calculation were used and up to four points of a seven point assay were rejected. The estimates of receptor binding site concentration varied between 3.3 and 29 nM (or, rejecting the highest value, between 3.3 and 5.2 nM) and those of the dissociation constant varied between 0.43 and 59 nM (or, rejecting the highest value, between 0.43 and 7.8 nM). The shapes of the binding curves and the methods of calculation are discussed.

INTRODUCTION

Studies on steroid receptors in a variety of cell preparations have moved from a qualitative into a quantitative era. Estimates of receptor binding site concentrations may be important in the prediction of the response of breast tumours to endocrine therapy [1–3] and the elucidation of steroid hormone and antihormone mechanisms requires accurate quantitative data. The assessment of methods of subcellular fractionation and of estimating bound as well as unbound receptor sites also requires reliable quantitation.

Apart from differences in analytical techniques, it seemed possible that methods of calculation used by different laboratories may affect results. In most publications, it is stated that non-specifically bound is subtracted from total bound steroid prior to calculation of receptor parameters. Such a statement does not clarify the exact method of calculation. It does not specify how the unbound steroid moiety is arrived at and whether or not the non-specifically bound steroid concentration has been adjusted to that of free steroid with which the specifically bound moiety was in equilibrium [4]. The combination of estimated values used in the calculation of receptor parameters [5] may lead to outlying points or curvature or both, but the rejection of data and its possible effects on estimates of receptor concentration or affinity is rarely discussed.

In order to establish to what extent methods of calculation differ between laboratories and what effects, if any, such differences might have, a questionnaire together with data from an analysis were sent to 28 specialist laboratories. This paper reports the results of the survey.

THE SURVEY

Progesterone receptor binding sites in human endometrial cytosol (100 mg tissue/ml of buffer) were determined using [1α, 2α-^{3}H]-progesterone (Radiochemical Centre, Amersham, Bucks.), equilibration at 4°C for 16 h and precipitation of bound steroid with polyethylene glycol [6]. This method does not dissociate progesterone from its receptor during separation of bound from unbound steroid. The heat lability of these receptors was used to estimate and correct for "non-specific" binding components. The data in Table 1 show that at 40°C, some binding components are denatured rapidly, leaving heat stable component(s) which bind an amount of [^{3}H]-progesterone comparable with that bound by the unheated preparation in the presence of excess radioinert steroid. Dissociation constants of the heat-labile progesterone receptor complexes ranged from 2–10 nM as compared with a value 2.9 nM obtained by Bayard *et al.*[7] and values in the range 0.05–1.2 nM reported by Pollow *et al.*[8] for "saturable" progesterone binding sites. Thus the binding sites estimated as heat-labile material have properties (affinity, saturability) commonly ascribed to steroid receptors. Corticosteroid-binding globulin, derived from blood and possibly saturable, is stable at 40°C and, if present, was estimated as "non-specific" binding in the experiment

* This survey is the result of cooperation of: Dr. Diana M. Barnes, Manchester, England; Dr. F. Bayard, Toulouse, France; Dr. G. Daxenbichler, Innsbruck, Austria; Dr. D. Egert, Hamburg, Germany; Dr. H. Friesen, Winnipeg, Canada; Dr. R. A. Hawkins, Edinburgh, Scotland; Dr. A. J. Koenders, Nijmegen, The Netherlands; Dr. R. Leake, Glasgow, Scotland; Dr. M. E. Lippman, Bethesda, U.S.A.; Professor W. L. McGuire, San Antonio, U.S.A.; Dr. H. Magdelenat, Paris, France; Dr. Betty G. Mobbs, Toronto, Canada; Dr. J. Poortman, Utrecht, The Netherlands; Dr. Geraldine Rosset, Geneva, Switzerland; Dr. E. J. Van Doorn, Edmonton, Canada.

Table 1. Heat denaturation of endometrial progesterone receptors

Time (min)	[^{3}H]-P Bound (nM) [^{3}H]-P	[^{3}H]-P + excess P
0	0.47	0.23
5	0.30	0.24
10	0.26	0.24
20	0.24	0.24
30	0.23	0.25
45	0.24	0.24
60	0.25	0.24
90	0.25	0.25

Cytosol was heated at 40°C for the times indicated and then equilibrated either with 2 nM [^{3}H]-progesterone or with 2 nM [^{3}H]-progesterone in the presence of 100-fold excess radioinert steroid. A 50-fold excess of cortisol was present in each case.

used for the survey. In our normal assays we now include cortisol since glucocorticoid receptor could be present and may bind progesterone. In the heating experiment (Table 1) cortisol had been used. Duplicate analyses were carried out at seven concentrations of [^{3}H]-progesterone (0.14–12 nM). The results (Table 2) together with a questionnaire (Table 3) were sent to 28 specialist laboratories. Participants were not told which steroid receptor was being estimated and by what methods the data were obtained, but were informed that the results represented duplicate assays, that the total counts were determined by counting duplicate aliquots of standards and that samples were not quenched with respect to the results obtained for total and for non-specific binding. Further information supplied was that corrections for blanks and for removal of aliquots during assay had already been made, that the specific activity of the steroid was 58 Ci/mmol, that counter efficiency was 40% and that the assay volume was 100 μl.

RESULTS AND DISCUSSION

Figure 1 shows plots of total and non-specifically bound steroid against total steroid concentrations in terms of count rates. Scatchard[5] plots for total, non-specific and specific binding, the latter calculated by the method of Rosenthal[4], are shown in Fig. 2. It is evident that in this case the non-receptor binding component was relatively unimportant and saturable, even at the low total concentrations of progesterone used (up to 12 nM). The data had been chosen for this survey because, in spite of good duplicate results, "anomalous" behaviour (i.e. not compatible with generally assumed models) was obvious.

Eighteen laboratories returned the questionnaire. Six different methods of calculating results were used and these were as follows: (T = total counts present, B_T = total bound steroid and B_{NS} = non-specifically bound steroid). For Method A, $(B_T - B_{NS})/(T - B_T)$ was plotted against $(B_T - B_{NS})$. For Method B, $(B_T - B_{NS})/[T - (B_T - B_{NS})]$ was plotted against

Table 2. Data sent to participants

Standard	Total counts (T, c.p.m.)	Total binding (B_T, c.p.m.)	Non-specific binding (B_{NS}, c.p.m.)
1	60223	17174	3775
	61788	17822	3687
2	28368	11639	3143
	28613	11429	3191
3	14178	7413	1943
	14010	7278	1965
4	7031	3861	1026
	7059	3899	996
5	3192	1770	477
	3147	1801	466
6	1586	826	272
	1563	812	260
7	736	417	154
	709	417	145

Table 3. Questionnaire sent to participants

1. Calculate the dissociation constant using the given data.
2. Calculate the receptor site concentration using the given data.
3. Describe, giving an example for one point, exactly how the calculations were performed.
4. Do you usually use a computer or a programmable calculator?
5. Sketch or enclose your Scatchard plot and give the data used for plotting it.
6. How often do you see imperfect Scatchard plots?
7. Have you any comments regarding these data and calculations?

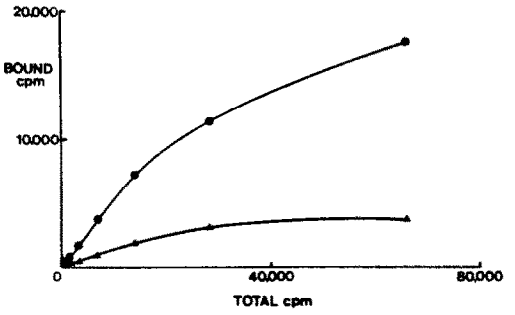

Fig. 1. Total (●) and heat-stable (▲) bound steroid in relation to total steroid present.

($B_T - B_{NS}$). Method C is that described by Rosenthal[4]. For Method D, $B_T/(T - B_T)$ was plotted against B_T. For Method E, B_{NS} was plotted against T and a straight line fitted, giving an intercept, a, and a slope, b, for the equation $B_{NS} = a + bT$. Then, using calculated values of B_{NS} for each total ligand concentration, specific binding was calculated by subtraction from B_T. Method F was similar to Method E but the straight line was assumed to pass through the origin, i.e. $B_{NS} = bT$. Users of Methods E and F appear to have employed $T - B_T$ to estimate unbound ligand (i.e. analogous to Methods A, C and D in this respect). The estimates of the receptor binding site concentration, [R], and the dissociation constant, K_d, obtained by 19 laboratories including our own are shown in Table 4. The results for K_d ranged from 0.43 to 59 nM, those for [R] from 3.3 to 29 nM. Most laboratories used Method B and it is evident that the arbitrary rejection of experimental results is a major cause of variation whichever method of calculation is used (Table 5). Inclusion of the "anomalous" point at 0.31 nM progesterone markedly altered estimates of K_d and [R]. Rejection of data at low concentrations

Table 4. Estimates of Receptor Parameters obtained in 19 laboratories

Method	Laboratory	K_d*	[R]*	N*
A	1†	3.4	3.6	5
	2†	3.4	3.6	5
	3†	3.4	3.7	5
	4†	3.3	3.6	5
B	5	5.4	4.2	5
	6†	7.6	5.1	7
	7	4.6	3.8	4
	8†	5.1	4.1	5
	9	3.9	3.3	3
	10†	5.5	4.3	5
	11†	7.8	5.2	7
	12	5.3	4.2	5
	13	5.4	4.2	5
C	14†	3.0	3.6	6
	15	3.3	3.8	6
D	16	3.3	4.6	6
	17†	5.3	3.9	5
E	11	58.5	29.0	7
	11	0.43	3.9	5
F	18†	2.4	3.0	5
	19	2.4	3.4	6

* K_d = dissociation constant (nM); [R] = receptor binding site concentration (nM); N = number of points used.

† Laboratory normally employs a programmable calculator or computer.

decreased the estimates while rejection at high concentration had inconsistent effects. Method A is not correct from a theoretical point of view since B_T and B_{NS}, used to calculate specific binding, were not in equilibrium with the same concentration of unbound ligand. Use of Method C (4) removes this objection. In this case, however, Methods A and C gave very similar results.

The erroneous but common calculation of unbound ligand as $T - (B_T - B_{NS})$ (Method B) leads to flatten-

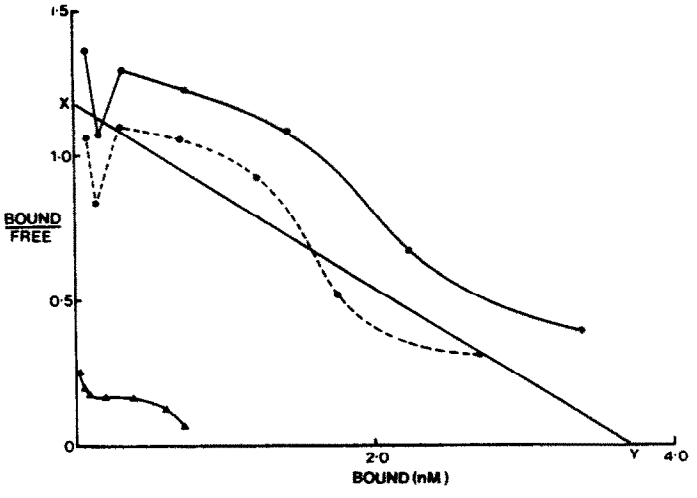

Fig. 2. Scatchard plots obtained for total (●——●), non-specific (▲) and specific (●- - -●) binding; the latter was determined by the method of Rosenthal[4]. The regression line XY was calculated using all points except that corresponding to a total steroid concentration of 0.31 nM.

Table 5. Effect of rejection of experimental data

Range of concentrations (nM)	N	Method A K_d (nM)	Method A [R] (nM)	Method C K_d (nM)	Method C [R] (nM)
2.76–11.8	3	3.6	3.7	2.7	3.5
1.38–11.8	4	3.4	3.6	2.7	3.5
0.62–11.8	5	3.6	3.7	2.9	3.6
0.31–11.8	6	4.4	4.1	3.7	4.1
0.14–11.8	7	4.6	4.2	6.7	5.6
0.14–11.8*	6	4.0	3.9	3.3	3.8
0.62–5.58	4	3.0	3.2	2.7	3.4
0.62–2.76	3	5.2	5.2	5.3	6.1

* Omitting the point at a total steroid concentration of 0.31 nM.

ing of the curve and over-estimation of [R]. Thus for 5 points, Method A gave $K_d = 3.4$ and [R] = 3.6 whereas Method B gave $K_d = 5.3$ and [R] = 4.2. The error incurred by Method B would be greater at low receptor concentrations and for relatively higher non-specific binding.

We believe Method C to be the correct calculation, though very few laboratories use it. It makes no assumptions about the shape of the curve for B_{NS}/F_{NS} and is widely applicable.

Most people would object to Method D, but it is apparently used to detect receptors in tumour tissue. We would not advocate its use. The magnitude of error (overestimate) will obviously depend on the proportion of non-specific binding (low in the present case).

We do not think Methods E and F were applicable in this case, since the plot of B_{NS} against T was not linear. Hence the strange results returned by laboratory 11 (Table 4). Even where a linear plot is obtained, the same theoretical objection as that for Method A is applicable.

Participants' comments indicated that non-linear Scatchard plots are quite common; proportions depend on the receptors under study and vary between 5 and 33%. One contributor asked "Have you ever seen a perfect Scatchard plot?"

Several collaborators commented that curvature of the Scatchard plot was seen because the range of concentrations of ligand was too wide, or the concentration of receptors too high. One contributor said that in their laboratory, tissue preparations producing curved plots were re-assayed at higher dilution. Our total range of concentrations was 0.14–12 nM in the estimates of both the total and the non-specific (heat stable) binding components. Most workers use 0.3 or 0.5–5 or 6 nM steroid with and without 50–100-fold excess of radioinert steroid. Thus the range of total concentration commonly extends up to 250 or even 500 nM with two specific activities of the radioactive species in parallel assays. Dilution of a receptor preparation and re-assay may merely conceal the curved portion of the plot to produce more assay points on the linear part.

Curvature of Scatchard plots for non-specific components is common, but may escape detection in many laboratories. Calculations of $B_{NS}/(T - B_{NS})$ from data for non-specific binding [9] or plots of $B_{NS}/(T - B_{NS})$ against B_{NS} [4], which would reveal non-linearity, are apparently not used by most workers. In assays of progesterone receptors in human endometrium, we have obtained curves as frequently as straight lines, when non-specific binding is estimated using either excess radio-inert steroid or heat denaturation. When such curvature is present, the method of calculation proposed by Chamness and McGuire [9] is not directly applicable and this was not used by any of the contributors to this survey. The method of Rosenthal[4], on the other hand, can be applied in this situation.

This survey has revealed considerable uncertainty among investigators with regard to receptor binding site calculations, as well as arbitrary rejection of valid experimental points. As a result, receptor parameters showed variation which, in interlaboratory comparisons, would be superimposed upon differences between analytical methods used. In the present case, calculation by Methods A and C gave similar results because receptor binding was high in relation to non-specific (heat-stable) binding. In less favourable situations, larger errors may be incurred by erroneous use of Method A. More serious attention might be paid to the existence and possible reasons for curvature of Scatchard plots, both for the non-specific and the receptor components of tissue preparations, and publications should contain full details of the frequency of "anomalous" plots and criteria for estimating receptor parameters in such situations. It is not known at present whether such findings are due to methodological artefacts, cooperativity (positive or negative) or the presence of several classes of binding sites and some elucidation of this problem would be desirable. It is hoped, therefore, that this report will result in a more careful choice of appropriate methods of calculation and fuller data on their validity.

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