J. Steroid Biochem. Molec. Biol. Vol. 39, No. 3, pp. 299–302, 1991
Printed in Great Britain.

0960-0760/91 $3.00 + 0.00

QUANTITATIVE CHARACTERIZATION OF HORMONE RECEPTORS BY A NONLINEAR REGRESSION APPROACH

BODO PROBST*† and ANNEMARIE HERMKES†
Department of Animal Physiology, University of Bayreuth, 8580 Bayreuth, Fed. Rep. Germany

(Received 16 February 1991)

Summary—This paper describes a mathematical model for the quantification of receptors based only upon the total bound values as a function of the total ligand concentration. In contrast to methods relying on linearization transformations, this nonlinear model requires more sophisticated computation, however, avoids loss of material for determination of nonspecific binding in competed tubes. Monte-Carlo simulation indicated high stability of this model against random experimental error. The androgen receptor of the male gerbil (*Meriones unguiculatus*) ventral prostate is characterized using the described nonlinear computation.

INTRODUCTION

Reversible associations between hormones and binding proteins or receptors are investigated in many basic endocrinological and clinical disciplines [1]. Binding characteristics are usually determined by equilibrium saturation analysis, which provides the maximum binding capacity of a binding protein and the strength of the binding reaction expressed by the equilibrium association constant. Assuming a binding protein has a constant number of binding sites, each capable of an independent, reversible interaction with a single ligand characterized by an association constant, the type of binding is described as noninteracting or independent binding [2, 3]. The binding parameters of this type of binding may be calculated from equilibrium binding analysis data. However, the optimal data evaluation method for this type of data is still under discussion [4, 5]. We now present a computational method, which according to the law of mass action calculates binding parameters from saturation data without the requirement of linearization transformations. Thus the problems occurring with the Scatchard evaluation method of parameter estimation are avoided, and lower sample sizes are required for performing saturation analysis. The results obtained using this model with published data are compared to calculations using the Scatchard analysis and Monte-Carlo simulations.

*To whom correspondence should be addressed.
†Present address: Department of Animal Physiology, University of Tübingen, 7400 Tübingen, Germany.

MATHEMATICAL MODEL

Despite the widespread knowledge of the mathematical model underlying the independent and reversible binding of a ligand to a binding site [2, 5], for the purpose of reference and clarity we start the deduction of our model from the law of mass action. The relationship between a ligand L and a single kind of binding site B by a reversible association may be symbolized according to the law of mass action as:

$$B + L \underset{k_d}{\overset{k_a}{\rightleftharpoons}} BL \qquad (1)$$

k_a and k_d represent the kinetic association and dissociation constants, respectively, of the ligand–receptor complex. The equilibrium association constant K is given by the ratio of these constants.

$$K = \frac{k_a}{k_d} = \frac{[BL]}{[B]\cdot[L]} \qquad (2)$$

where $[B]\cdot[L]$ and [BL] are the concentrations of unoccupied binding sites [B], sites occupied by the ligand [BL] and the free ligand [L] at equilibrium.

Under experimental conditions, it is usually not possible to measure the concentration of

unoccupied binding sites as well as the concentration of free ligand. Substituting

$$[B] = [B_0] - [BL] \quad (3)$$

$$[L] = [L_0] - [BL], \quad (4)$$

where B_0 is the total number of binding sites available and L_0 is the total concentration of the ligand, into equation (2) results in:

$$K \cdot [L] = \frac{[BL]}{[B_0] - [BL]} \quad (5)$$

$$K \cdot [L] \cdot [B_0] - K \cdot [L] \cdot [BL] = [BL] \quad (6)$$

$$K \cdot [L] \cdot [B_0] = [BL] + K \cdot [L] \cdot [BL]$$

$$= [BL] \cdot (1 + K \cdot [L]) \quad (7)$$

$$[BL] = \frac{K \cdot [L] \cdot [B_0]}{1 + K \cdot [L]} \quad (8)$$

$$[BL] = \frac{K \cdot [B_0] \cdot ([L_0] - [BL])}{1 + K \cdot ([L_0] - [BL])}. \quad (9)$$

Equation (9) describes the binding of a ligand to a binding site in terms of the total amount of ligand $[L_0]$, the total number of binding sites $[B_0]$ and the association constant K. From equation (9) various transformations lead to Scatchard, Michaelis–Menten or Lineweaver–Burk relations.

Under experimental conditions additional nonspecific binding of the ligand to other binding sites occurs, which is also subject to the law of mass action. Consequently, the nonspecific binding could be described by adding a second term similar to equation (9), which differs in parameters for K and B_0. However, nonspecific binding is usually much weaker than specific binding. Therefore, in the range investigated in saturation analysis nonspecific binding may well be approximated by a straight line through the origin.

$$[NL] = m \cdot [L_0]. \quad (10)$$

Equation (10) describes the concentration of nonspecific bound ligand [NL] as a function of the slope of the straight line (m) and the total amount of ligand available $[L_0]$. The total amount of ligand bound by specific as well as nonspecific binding sites, which is usually measured during experiments, is given as the sum of specifically and nonspecifically bound ligand.

$$[TL] = [BL] + [NL]. \quad (11)$$

Substituting the specifically bound ligand concentration [BL] in (4) and, consequently also in (9), by total bound ligand concentration [TL] and adding the nonspecifically bound ligand term (10) results in equation (12).

$$[TL] = \frac{K \cdot [B_0] \cdot ([L_0] - [TL])}{1 + K \cdot ([L_0] - [TL])} + m \cdot [L_0]. \quad (12)$$

Equation (12) corresponds to a model of the binding reaction that according to the law of mass action allows us to calculate the binding parameters K and $[B_0]$ together with the nonspecific binding in terms of the slope m from total ligand bound [TL] and total ligand added $[L_0]$, which are easily available in standard experimental designs. After separation of the variables [TL] and $[L_0]$, this quadratic equation results in (13).

$$[TL] = \frac{-\sqrt{1 + 2a + a^2 + (2K - 2mK - 2aK + 2amK) \cdot L_0 + (K^2 - 2mK^2 + m^2K^2) \cdot L_0^2} + 1 + a + KL_0 + mKL_0}{2K} \quad (13)$$

where $a = K \cdot [B_0]$. Equation (13) can not be solved with standard mathematical methods to estimate binding constants and nonspecific binding. However, nonlinear regression calculation of this function with the Marquard–Levenberg algorithm [6] provides binding constants together with estimates of precision of the parameters which are useful for means of quality control.

RESULTS

Monte-Carlo Simulation

The technique of Monte-Carlo simulation enables one to verify the parameter estimates. To compare the nonlinear analysis with the Scatchard analysis we used data [7], which were generated utilizing known values of the parameters in the specific model. Assuming that there is random normal error associated with the measurement of the bound ligand, these artificial data were repeatedly modified by addition of a normally distributed error term with a mean of zero and a standard deviation of 10% of the expected bound ligand concentration during the execution of the program. In the case of the Scatchard analysis, the nonspecifically

even worse in this respect and therefore are used much less in receptor biochemistry.

A number of more refined approaches have therefore been developed especially by Rodbard *et al.* [5, 13, 14] and others [4, 12], which probably due to the required computational power have not been very successful in the laboratory until now. However, the use of modern desktop computers permits one to avoid these oversimplifications by using correct mathematical methods for the evaluation of binding data for the identification of binding parameters in receptor studies.

The approach presented here allows us to omit the determination of nonspecific binding, since the receptor parameters are calculated from total binding as the dependent variable and the ligand concentration as the independent variable. These concentrations can usually be measured or are known precisely. Therefore more data points of total binding can be obtained from a given amount of tissue and the binding parameters can consequently be calculated more reliably. Furthermore, the use of untransformed raw data avoids the simplifications imposed by the Scatchard-plot method.

Acknowledgements—This work was supported by a grant from the Deutsche Forschungsgemeinschaft (Pr 285/1-1). The authors thank Ms Ines Kuchenbäcker and Mr Harald Müller for their reliable performance of binding assays. A versatile computer program is currently available for calculation of binding parameters from raw data. Different approaches like Scatchard, Lineweaver–Burk and Hyperline have been implemented together with Hill-evaluation of cooperativity: all results are presented graphically for printout. At present, the program can be obtained for the ATARI-1040 computer series from the authors.

REFERENCES

1. Barsano C. P. and Baumann G.: Simple algebraic and graphic methods for the apportionment of hormone (and receptor) into bound and free fractions in binding equilibria: or how to calculate bound and free hormone? *Endocrinology* **124** (1989) 1101–1106.
2. Feldman H. A.: Mathematical theory of complex ligand-binding systems at equilibrium: some methods for parameter fitting. *Analyt. Biochem.* **48** (1972) 317–338.
3. Hrdina P. D.: General principles of receptor binding. In *Neuromethods 4, Receptor Binding* (Edited by A. A. Boulton and G. B. Baker). Humana Press, Clifton, NJ (1986) pp. 1–22.
4. Jawny J., Jochum P. and Eiermann W.: Sensitivity and optimal performance in steroid receptor analysis. *J. Steroid Biochem.* **20** (1984) 595–603.
5. Rodbard D.: Mathematics of hormone–receptor interaction: 1. Basic principles. In *Receptors for Reproductive Hormones* (Edited by B. W. O'Malley and A. R. Means). Plenum Press, New York (1973) pp. 289–326.
6. McIntosh J. E. A. and McIntosh R. P.: *Mathematical Modelling and Computers in Endocrinology*. Springer, Heidelberg, New York (1980).
7. Schwarz S.: A simple computer program for Scatchard plot analysis of steroid receptors including non-specific binding correction on a low cost desk top calculator. *J. Steroid Biochem.* **11** (1979) 1641–1646.
8. McGinnis M. Y., Davies P. G., Meaney M. J., Singer M. and McEwen B. S.: *In vitro* measurement of cytosol and cell nuclear androgen receptors in male rat brain and pituitary. *Brain Res.* **275** (1983) 75–82.
9. Shain S. A., Boesel R. W., Lamm D. L. and Radwin H. M.: Characterization of unoccupied (R) and occupied (RA) androgen binding proteins of the hyperplastic human prostate. *Steroids* **32** (1978) 541–556.
10. Gallon Ch., Veyssiere G., Berger M., Jean-Faucher Ch., De Turckheim M. and Jean Cl.: Age-related changes in the concentration of cytosolic androgen receptors in the epididymis vas deferens and seminal vesicle of maturing male mice. *J. Androl.* **10** (1989) 188–194.
11. Hicks L. L. and Walsh P. C.: A microassay for the measurement of androgen receptors in human prostatic tissue. *Steroids* **33** (1979) 389–406.
12. Kuss E.: Kritische Anmerkungen zur Auswertung von Rezeptoranalysen. *J. Clin. Chem. Clin. Biochem.* **20** (1982) 227–234.
13. Munson P. J. and Rodbard D.: Ligand: a versatile computerized approach for characterization of ligand-binding systems. *Analyt. Biochem.* **107** (1982) 220–239.
14. Thakur A. K., Jaffe M. L. and Rodbard D.: Graphical analysis of ligand-binding systems: evaluation by Monte-Carlo simulation. *Analyt. Biochem.* **107** (1980) 279–295.

Table 1. Comparison of results from Monte-Carlo simulation

	Scatchard	Hyperline	
True	1.81	1.81	k_d (10^9 mol/l)
	1.11	1.11	R_0 (10^{-10} l/mol)
	0.007	0.007	m (Slope of NB)
Estimated	1.47 ± 0.35	1.73 ± 0.43	k_d ($\pm$SD, $n = 50$)
	1.24 ± 0.17	1.13 ± 0.16	R_0 ($\pm$SD, $n = 50$)
	0.007	0.007 ± 0.001	m ($\pm$SD, $n = 50$)
% Bias	−18.3	−5.0	k_d
	+10.5	+2.0	R_0
		−5.0	m

Results from Monte-Carlo simulation of 50 experiments using generated data and 10% normally distributed random error. Indicated are the initial "true" parameter values, the mean and standard deviation of estimated parameters after 50 simulated experiments and percent deviation of parameter estimates from the initial values.

bound ligand was calculated using the same procedure also with a 10% error level.

After performing 50 simulated experiments, the deviation from the known true parameter values was compared (Table 1). Although the same number of data points were used in both simulation runs, the percent bias in the Hyperline was less as compared to the Scatchard method. Furthermore, a subsequent Hill-analysis [3] was performed using the results of Scatchard and Hyperline evaluation, respectively. With these data [7], the Hill coefficient calculated from Scatchard analysis deviated from unity ($h = 0.72$), whereas the Hill coefficient calculated from Hyperline analysis was 0.995. Since both calculations, Scatchard and Hyperline, are based upon the assumption of the existence of a single and noninteracting binding site, the significant deviation from unity occurring with Scatchard analysis may be an indication of an incorrect measurement of nonspecific binding, which is not required for Hyperline calculation.

Androgen binding in the gerbil ventral prostate

After homogenization and ultracentrifugation (130,000 *g*, 4°C) of the tissue in Tris–HCl buffer [8] a saturation analysis was performed using 9 concentration steps between 0.3 and 12.2 nM ^{3}H-labelled Mibolerone (Amersham). After 24 h incubation at 1°C the steady state is reached and the bound steroid was separated on LH-20 minicolumns. The radioactivity was counted in a liquid scintillation counter (Beckman, 47% counting efficacy). This results in a total binding curve of labelled steroid, which is sufficient for evaluation using the Hyperline method.

The mean androgen receptor concentration was 21.1 ± 5.4 (pmol/mg wet weight, mean $\pm$ SEM, $n = 8$), the mean association constant was

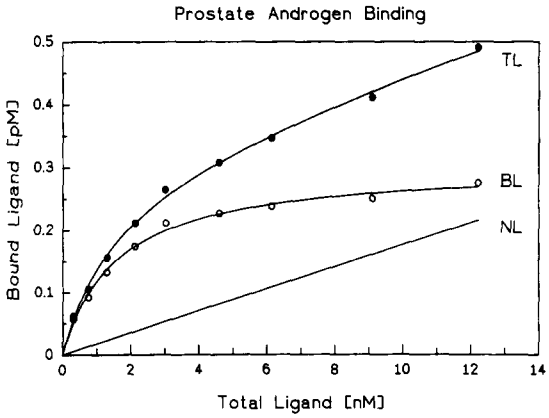

Fig. 1. Typical example of a saturation experiment. The (●) indicate the measured total bound ligand. The (○) show the calculated specific binding to the prostate androgen receptor as calculated from the total bound values. $R_0 = 15.8$ pmol/mg wet weight; $k_a = 5.9 \times 10^8$ l/mol.

$0.72 \pm 0.2 \times 10^9$ l/mol (mean $\pm$ SEM, $n = 8$), which is in the range reported by others for human [9] or mouse [10] prostate cytosolic androgen receptors. Figure 1 shows a typical saturation curve. The standard deviation of the parameter estimates as calculated by the nonlinear regression method, which can be used for control of assay performance, was in the range of 18–76% for the association constant and 20–77% for the receptor concentration.

DISCUSSION

Receptor studies in clinical and biological research are commonly used for a wide range of applications. The determination of receptor, association constants and concentrations using equilibrium techniques such as saturation assays with increasing amounts of radioligand added to a constant volume of cytosol are routine in receptor studies. Serious drawbacks are met from two aspects in this approach. First, in most cases only a very limited amount of tissue is available for receptor analysis. This resulted in the application of so-called "one point" assays [11], where only a little reliable information can be obtained. The second problem concerns the kind of evaluation method to be used for calculation of receptor characteristics. Most commonly, the Scatchard-plot is used despite its serious disadvantages. These include: its high sensitivity with respect to data scattering, being a relation between two dependent variables, which is unsuited for linear regression, and its unequal weighting of data points [4, 12, 13]. Other linearization transformations like the Lineweaver–Burk plot or graphical methods are