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Anomalous Equilibrium Binding Properties of High-Affinity Racemic Radioligands

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SUMMARY

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In receptor binding studies, high-affinity racemic radioligands are often used as tracers, neglecting the difference in affinity of their stereoisomers. We present an experimental and theoretical study comparing the binding of (±)-[^{3}H]carazolol and (±)-[^{125}I]hydroxybenzylpindolol (HYP) to their pure respective isomers in the frog erythrocyte *beta*-adrenergic system. Saturation binding curves with the racemic radioligands showed deviations from a binding isotherm for a single ligand which were accentuated at higher receptor concentrations. When different affinity constants for both isomers were considered, significant improvement in the fits of the data were obtained by computer-modeling procedures. The $K_D$av (average dissociation constant) obtained by considering the racemic radioligand as a single ligand, as has generally been done in the literature, varied with the receptor concentration from approximately 2 $K_{D(-)}$ at low receptor concentrations to ≅0.5 $K_{D(+)}$ at high receptor concentrations. Thus the generally measured "$K_D$av" of these racemic radioligands is really a hybrid of $K_{D(-)}$ and $K_{D(+)}$. These experimental findings are in very good agreement with Monte Carlo simulations and may help to explain the discrepancies in dissociation constants of high-affinity racemic radioligands reported in the literature. Experimental data and simulations also indicate that information about the $K_{D(-)}$ is greatest at low receptor concentrations, whereas that about $K_{D(+)}$ is greatest at high receptor concentrations. Simultaneous computer fitting of saturation curves from racemic [^{125}I]HYP and the (+)-isomer, isolated by repeated incubations with frog erythrocyte membranes under appropriate conditions, indicates approximately a 20-fold ratio for the individual isomer K_D values. Estimated K_D values of the stereoisomers of [^{125}I]HYP and [^{3}H]carazolol were virtually identical, being $K_{D(-)}$ = 10–50 pM and $K_{D(+)}$ ≅ 400–2000 pM at 25°. Use of the $K_D$av for a racemic radioligand resulted in up to 5-fold systematic underestimation of the affinity of nonracemic competitors. The K_D values of all high-affinity competitors were also found to be misestimated by as much as 10-fold using the commonly employed Cheng and Prusoff [*Biochem. Pharmacol.* 22: 3099–3108 (1973)] approximation when the affinity of the radioligand was significantly lower than that of the competitor. Under such circumstances, slope factors of ≅2 were obtained for competition curves in the absence of cooperativity.

INTRODUCTION

High-affinity radioligands have become widely used tools for the study of drug receptors. In a number of cases the receptors demonstrate stereospecificity, with one optical isomer of a drug being considerably more potent than the other (generally 10 to 100-fold). Several of the most popular and useful radioligands are racemates, e.g., (±)-[^{125}I]HYP[5] (a *beta*-adrenergic antagonist) (1) or (±)-[^{3}H]QNB (a muscarinic cholinergic antagonist) (2). Nonetheless, in almost all published studies with such racemic tracers, the separate individual contribution of each of the two stereoisomers to the over-all

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[5] The abbreviations used are: HYP, hydroxybenzylpindolol; QNB, quinuclidinyl benzilate; DHA, dihydroalprenolol.

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binding obtained has been neglected. Rather, the binding of such racemic radioligands has generally been analyzed as though only a single pure compound were present. This assumption is valid only if binding of the weaker stereoisomer is negligible, and even in this case the concentration of the ligand will be one-half that originally assumed. In fact, as is shown below, both stereoisomers may contribute to the over-all binding.

In this communication we present a detailed experimental and theoretical study of the equilibrium binding of two racemic *beta*-adrenergic ligands, (±)-[^{125}I]HYP and (±)-[^{3}H]carazolol, in comparison with the binding of individual stereoisomers such as (−)-[^{3}H]carazolol and (+)-[^{125}I]HYP. We demonstrate and characterize the deviations of binding of the racemic ligands from the properties expected for binding of a pure[6] isomer and quantitate the errors in estimations of the K_D of the radioligand and competing ligand which result from inappropriate analysis of such data. The results obtained seem to clarify a number of findings in the literature such as the widely varying K_D values of racemic tracers such as IHYP, and the apparent dependence of their K_D values on the receptor concentration R.

METHODS

Materials

The radioligands (−)-[^{3}H]DHA (41 or 58 Ci/mmole), (−)-[^{3}H]carazolol (24 Ci/mmole), (±)-[^{3}H]carazolol (26 Ci/mmole), and (±)-[^{125}I]HYP (2200 Ci/mmole) were obtained from New England Nuclear Corporation, Boston, Mass. (−)-[^{3}H]Carazolol was custom-tritiated by New England Nuclear Corporation. (−)-, (+)-, and (±)-Carazolol (3) were gifts from Boehringer/Mannheim GmbH, Mannheim, Germany. The sources and purity of other materials used in this study have been previously reported (4).

Partially Purified Frog Erythrocyte Membranes

Partially purified frog erythrocyte membranes were prepared as described previously (5). The final membrane pellet was resuspended in the original volume of packed erythrocytes in 25 mM Tris-HCl buffer (pH 7.5) at room temperature containing 2 mM $MgCl_2$, 0.25 M sucrose, 0.01 mM phenylmethylsulfonyl fluoride, and 1 mM Cleland's reagent (DTT). Small aliquots of membrane suspension were frozen in liquid nitrogen and stored at −80°. After up to 2 months' storage there was no observed decrease in antagonist binding.

Equilibrium Radioligand Binding Assays

Partially purified frog erythrocyte membranes with a *beta*-adrenergic receptor binding capacity of approximately 0.2–0.4 pmole/mg of protein (5) were incubated in a final volume of 0.2, 0.5, 1.0, or 5 ml in 75 mM Tris-HCl buffer (pH 7.5) at 25° containing 12.5 mM $MgCl_2$ and 1.5 mM EDTA. After 1 hr the binding incubation was terminated by the addition of 5 ml of ice-cold 75 mM Tris-HCl buffer containing 12.5 mM $MgCl_2$ (pH 7.5) and, by pouring the diluted sample over a Whatman GF/C glass fiber filter and washing the filter under vacuum, with an additional 10–15 ml of buffer. Time course experiments demonstrated that under these conditions equilibrium had been reached. For saturation experiments the binding described in the text and figures refers to specific binding which was defined as the amount of total binding competed for by 10 μM (±)-propranolol. Specific binding for (±)-[^{125}I]HYP was 75% of total binding at the highest receptor concentration (≅1 nM) and greater than 95% at low receptor concentrations (≅20 pM). This corresponded to nonspecific binding of 5%, or less than 1% of total radioligand added. For (±)- or (−)-[^{3}H]carazolol, the specific binding was >95% of total binding (nonspecific <1% of total added radioligand). The nonspecific binding in competition curves was not subtracted but rather was estimated by computer analysis (see below). To minimize the adsorption of the radioligands and the competitors to the polypropylene tubes, dilutions were carried out in 1 mM HCl. In experiments with (±)-[^{125}I]HYP, incubations took place in Beckman Bio-Vials. This allowed the measurement of the actual total radioactivity in each tube. We also counted the empty vials after the assay and were therefore able to correct for the adsorption of (±)-[^{125}I]HYP to the incubation tube. The experiments with tritiated radioligands were carried out in duplicate, and the radioactivity on the filters was measured by adding 10 ml of scintillation fluid and counting in a liquid scintillation spectrometer.

Separation of (±)-[^{125}I]HYP from the Racemic Mixture

By making use of the fact that stereoisomers of antagonists have different affinities for the *beta*-adrenergic receptor, we isolated the enantiomer with the weaker affinity by repeated incubation of [^{125}I]HYP with frog erythrocyte membranes, which should selectively bind the (−)-isomer, leaving the supernatant enriched in (+)-isomer. In order to choose assay conditions which would optimize the yield of a highly enriched (+)-[^{125}I]HYP fraction (which is assumed to be the isomer with the lower affinity), we carried out computer calculations which were based on mass-action law. The three-step separation procedure was the following: 3 nM (±)-[^{125}I]HYP was incubated with frog erythrocyte membranes having a *beta*-adrenergic receptor concentration of 1.5 nM. The incubation buffer was the same as that described for the binding assay. After a 20-min incubation at 25°, the membranes were centrifuged for 10 min at 39,000 × *g*. In the second step, the supernatant was incubated with membranes under the same conditions, except the receptor concentration was 600 pM. After centrifugation, this procedure was repeated at a receptor concentration of 300 pM and again centrifuged at 39,000 × *g* for 10 min. The final supernatant contained 10–20% of the original amount of radioactivity and was used for a saturation binding experiment (see Fig. 2). Calculations according to mass-action law predicted a purity greater than 95% for the final (+)-[^{125}I]HYP fraction. Thin-layer chromatography was performed according to the method of Harden *et al.* (6). The radiochromatogram showed iden-

[6] "Pure" in this context means a single optical isomer rather than chemical or radiochemical purity.

tical spots for both the enriched (+)-[^{125}I]HYP fraction and the racemic radioligand.

Experimental Data Analysis

Curve fitting. All of the ligand binding data were expressed as the molar concentration of radioligand bound versus total concentration of varying ligand. Estimates of the parameters for the different models used were obtained by nonlinear least-squares curve fitting according to the method of Marquardt and Levenberg, as described by Fletcher and Schrager (7). In order to correct for the lack of homogeneity of the variance, the square of the deviations of the points from their predicted value $\hat{Y}$ were weighted according to the reciprocal of their expected variance according to ref. 8: var $(\hat{Y}) = a_0 + a_1 Y^{a_2}$, where a_1 is a proportionality constant roughly equal to the square of the relative error of the measured response Y, the exponent a_2 is usually between 1 and 2, and a_0 is a small constant in the range of the square of the standard error at the lowest response value Y. The appropriateness of the variance function is verified by checking that the weighted residual variance of the curve fitting is in the neighborhood of 1.

The goodness of the fit according to different models is compared by using the "extra sum of squares" principle (9) as applied by Rodbard (10):

$$F = [(SS_1 - SS_2)\ (df_1 - df_2)]/(SS_2/df_2) \qquad (1)$$

where SS_1 and SS_2 are the sum of squares of residuals for the fit with the simpler model and the more complex model, respectively, and df_1 and df_2 are the corresponding degrees of freedom of the fits. The F ratio has $(df_1 - df_2)$ and df_2 degrees of freedom for the numerator and denominator, respectively.

The model. Both saturation binding curves and competition curves are analyzed according to a general model for the interactions of several ligands with several classes of sites on the basis of the mass-action law (11). In the case of the binding of n ligands to a single class of sites, the model reduces to

$$B_i = K_i F_i R/(1 + \sum_{a=1}^{n} K_a F_a) + N_i F_i \qquad (2)$$

$$L_i = F_i + B_{i_{(i=1,\ 2\ \ldots\ n)}} \qquad (3)$$

where B_i is the concentration of ligand i bound, F_i or F_a is the corresponding free concentration of ligand i or a, L_i is the total concentration of ligand i, K_i or K_a is the affinity constant for ligand i or a, N_i is a proportionality factor for nonspecific binding of ligand i, and R is the total receptor concentration. Since the total concentration L_i of each ligand is known and is generally different from free ligand concentration F_i, Eq. 2 is supplemented with Eq. 3 for conservation of mass of each ligand. For convenience, the dissociation constants $K_{D_i} = 1/K_i$ are reported in the following text. We assumed that nonspecific binding was not stereoselective. Therefore the same proportionality factor N was estimated for both stereoisomers.

In the case of a single isomer, attempts to fit the saturation curve according to a model for one or two ligands is used to check that the one-ligand model (n = 1, Model I) is sufficient, i.e., that the two-ligand model (n = 2, Model II) does not significantly improve the goodness of the fit as tested according to Eq. 1. For a racemic radioligand, attempts to fit the saturation binding curve according to Model I provide estimates of an apparent dissociation constant ($K_D$av), whereas the use of a model for two ligands (n = 2) provides estimates for the microscopic dissociation constants $K_{D(-)}$ and $K_{D(+)}$ for each isomer. A significant improvement ($p < 0.05$) of the fit with Model II is indicative of the heterogeneity of the binding of the stereoisomers to the receptors.

Competition curves are first analyzed according to a four-parameter logistic equation (12):

$$Y = d + [(a - d)/(1 + (X/c)^b] \qquad (4)$$

where X and Y the total concentrations of competitor and radioligand bound, respectively, c is the 50% effective concentration of competitor EC_{50}, b is the slope factor ("pseudo-Hill coefficient"), and a and d are the extrapolated upper and lower limits for the observed value of Y when X is 0 and infinite, respectively. The EC_{50} values of the competition curves are used for calculation of apparent dissociation constants K_{D_2} of the competitor according to the approximation of Cheng and Prusoff (13):

$$K_{D_2} \simeq EC_{50}/(1 + L_1/K_{D_1}) \qquad (5)$$

where L_1 is the total radioligand concentration and K_{D_1} is the dissociation constant of the radioligand. In the case where the radioligand used is racemic, the dissociation constant K_{D_1} is the average dissociation constant obtained by fitting the racemic radioligand saturation curve according to Model I.

The competition curves were also analyzed according to exact mass-action law using Eq. 2 and 3. In this case the competition curve was described in terms of the concentration of radioligand bound B_1 versus the total concentration of the competitor L_2. When a racemic ligand was used either as the radioligand or as its competitor, only the average dissociation constant $K_D$av was considered.

Monte Carlo simulations. Repeated saturation curves for racemic radioligand binding at different concentrations of receptor sites were simulated and then analyzed according to the same modeling technique as that used for experimental data. The dissociation constants assumed were analogous to those of (±)-carazolol, with a $K_{D(-)}$ of 20 pM and a $K_{D(+)}$ of 400 pM. For each curve, 12 concentrations of radioligand, evenly spaced on a logarithmic scale, covered a range corresponding from 0.05 to 0.95 of receptor occupancy according to Eq. 2 and 3. To each calculated value was added a normally distributed random error with variance coefficients in Eq. 1 of $a_0 = 10^{-4} \times R^2$, $a_1 = 0.01$, and $a_2 = 1.5$. These coefficients correspond to the sum of a constant error equal to 1% of the receptor concentration and a relative error up to ≅10%. Use of such a variance function generated simulated "data" with a distribution of error similar to that commonly observed in the frog erythrocyte *beta*-adrenergic receptor. For each receptor concentration, 30

curves were generated and individually analyzed according to Model I or Model II, as explained above for experimental data. The resulting parameter estimates (binding capacity R, average dissociation constant $K_D$av and the individual affinity constants $K_{D(-)}$ and $K_{D(+)}$ are then averaged and their means are compared with the true parameter values used to generate the simulated curves. All successful attempts to fit the curves with Model II were compared with the corresponding fits with a single ligand model to test for a significant ($p < 0.05$) improvement of the goodness of the fit. All calculations and simulations were performed using computer programs in PL/1 for a PDP 11/45.

Application of information theory. By utilizing the theoretical approach of information theory, first applied for binding studies by Weber (14), we evaluated which receptor concentrations would provide the best estimates for the binding parameters $K_{D(-)}$ and $K_{D(+)}$ in the case of a racemic radioligand. The general equations for the content of information, I, as a function of the probability, p, of an outcome, j, is:

$$-I(p) = \sum_{j=1}^{m} p_j \log_2 p_j \qquad (6)$$

(For further details see ref. 15). Since the number of outcomes, m, in a binding experiment is 2 (the ligand can be either bound or free), Eq. 6 reduces to

$$-I(p) = p \log_2 p + (1-p) \log_2 (1-p) \qquad (7)$$

In accordance with Weber (14), we define the binding probability for each ligand L_i as

$$p_i = \frac{\text{actual conc. of } L_iR}{\text{maximal possible conc. of } L_iR} = \frac{B_i}{B_{i,\max}} \qquad (8)$$

Whereas the actual concentration B_i of each L_iR complex can be immediately calculated according to Eq. 2 and 3, the maximal possible complex concentration $B_{i,\max}$ has to be determined

$$B_{i,\max} = \begin{cases} B_i + E & \text{if } E \le F_i \\ L_i & \text{if } E > F_i \end{cases} \quad \text{for } i = 1,2,\ldots n \qquad (9)$$

where E = empty receptor concentration ($E = R - \sum_{i=1}^{n} B_i$), F_i = free concentration of ligand i, and L_i = total concentration of ligand i. The information for an individual ligand and particular data point was computed from Eq. 7, 8, and 9. In analogy with the Monte Carlo simulations, the over-all information from a saturation binding experiment at various, fixed, receptor concentrations has been approximated by averaging 12 data points, which were evenly spaced on a logarithmic scale, covering a range of 0.05 to 0.95 of receptor occupancy.

RESULTS

Saturation binding experiments with racemic radioligands. A close analysis of saturation binding data obtained with a racemic radioligand reproducibly showed a deviation from the theoretical binding isotherm expected for a single ligand binding to a single class of sites.

In Fig. 1A–C, saturation binding curves with the high-affinity *beta*-adrenergic radioligands (±)-[^{3}H]carazolol (panel A) and (±)-[^{125}I]HYP (panel C) are compared with that obtained with the pure enantiomer (−)-[^{3}H]carazolol (panel B). Computer curve fitting was performed according to mass-action law as described in detail under Methods. For the racemic radioligands (Fig.

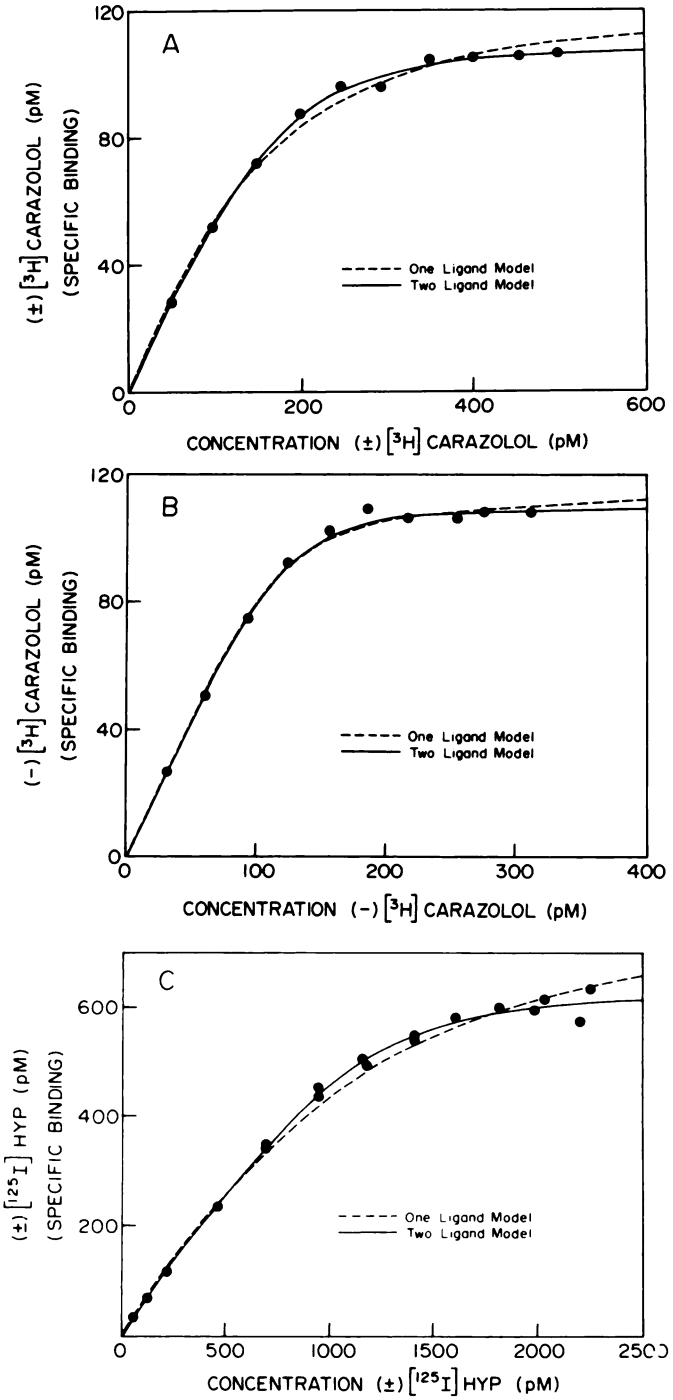

FIG. 1. *Computer curve fitting according to Model I and Model II for saturation binding experiments with racemic and nonracemic radioligands*

Frog erythrocyte membranes were incubated with increasing concentrations of (±)-[^{3}H]carazolol (panel A), (−)-[^{3}H]carazolol (panel B), and (±)-[^{125}I]HYP (panel C). A significant improvement of the computer fit ($p < 0.05$) for Model II over Model I was achieved in the case of the racemic radioligand (panels A and C). No significant improvement for Model II over Model I could be observed for (−)-[^{3}H]carazolol (panel B). The parameter values are given in Table 1 (panel A corresponds to experiment 9, panel B to experiment 13, and panel C to experiment 5).

1A and C), we observed a small, but significant, deviation in the shape of the fitted curve from the experimental data points when Model I was applied (- - -). A slight underestimation in the most curved part of the plot and an overestimation of binding in the saturation region are the characteristics of the improper Model I binding isotherm. For the pure stereoisomer (−)-[^{3}H]carazolol (Fig. 1B), Model I was appropriate and, as expected, fitted very well the experimental data. This is also true for saturation binding experiments with (−)-[^{3}H]DHA (data not shown). However, a significant improvement in the "goodness of fit" was obtained when Model II, as opposed to Model I, was applied to the data for the racemic radioligands (Fig. 1A and C). However, there was no improvement with Model II when the pure isomer (−)-[^{3}H]carazolol was used (Fig. 1B).

Since the individual stereoisomers of [^{125}I]HYP are not available, we made an attempt to isolate the component with the lower affinity, which is assumed to be the (+)-enantiomer, from its racemic mixture by repeated incubation with frog erythrocyte membranes under appropriate conditions (for details see Methods). Saturation binding experiments with such highly enriched fractions of (+)-[^{125}I]HYP yielded binding isotherms which were much different from those obtained with the original racemic mixture (Fig. 2). Simultaneous analysis of these two particular saturation experiments by computer curve-fitting methods, in which the values for $K_{D(+)}$ and receptor concentration (R) were shared by the two curves, yielded $K_{D(-)}$ = 18 ± 2 pM and $K_{D(+)}$ = 380 ± 23 pM for the two stereoisomers, respectively. Since the (+)-[^{125}I]HYP fraction may still have been slightly contaminated with the (−)stereoisomer, the value for $K_{D(+)}$ may be somewhat underestimated. In Table 1, which summarizes results from a number of saturation binding experiments with several radioligands, the average K_D value for (+)-[^{125}I]HYP was in the region of 2 nM, whereas for the (−)-isomer an approximately 100-fold higher affinity was observed ($K_{D(-)} \cong$ 20 pM).

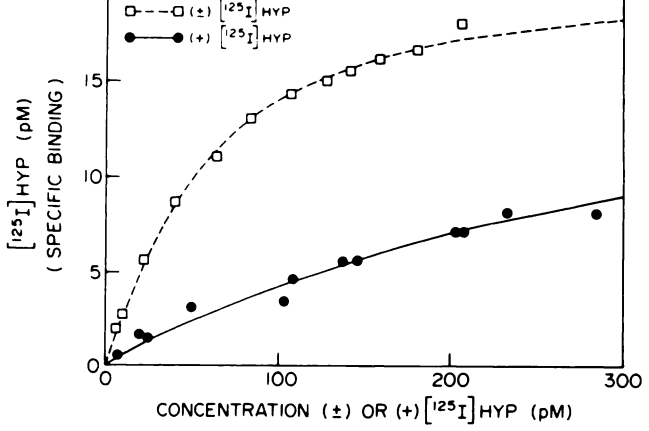

FIG. 2. *Comparison of binding of (±)-[^{125}I]HYP with* (+)-[^{125}I]-*HYP*

(+)-[^{125}I]HYP (—), which was isolated from a racemic mixture by repeated incubation with frog erythrocyte membranes as described under Methods, exhibits much lower affinity as compared with (±)-[^{125}I]HYP (- - -). Both curves were analyzed simultaneously. Assuming Model I for (±)-[^{125}I]HYP and Model II for the racemic radioligand, while sharing $K_{D(+)}$ and R for both curves, yielded $K_{D(-)}$ = 18 ± 2 pM and $K_{D(+)}$ = 380 ± 23 pM, respectively.

We wondered about the contribution of the individual stereoisomers having different affinities for the receptors in a saturation binding experiment. For this reason, we generated a theoretical binding isotherm following mass-action law with $K_{D(-)}$ = 20 pM, $K_{D(+)}$ = 400 pM, and R = 1000 pM. This result is shown in Fig. 3. The curve representing the contribution of the weaker isomer reaches a maximum when the total radioligand concentration is slightly greater than R and then falls off. At infinitely higher radioligand concentrations, the ratio of the binding from both isomers $B_{(+)}/B_{(-)}$ tends to the limit $K_{D(-)}/K_{D(+)}$. The amplitude of the maximum for the binding of the (+)-stereoisomer is a function of the receptor concentration and is more pronounced at higher receptor concentration (for further details see Appendix).

The influence of receptor concentration on the binding parameter estimates. For a racemic radioligand the receptor binding site concentration R chosen for a saturation binding experiment affects the estimate for the dissociation constant, when Model I is applied. Since this "constant" is varying and does not represent a microscopic dissociation constant, we call it an average dissociation constant $K_D$av. The parameter estimates from saturation binding experiments with (±)-[^{125}I]HYP, which are summarized in Table 1 (experiments 1–6) clearly demonstrate a systematic increase in $K_D$av as the receptor concentration increases. A $K_D$av of approximately 50 pM at very low R, varying up to 1 nM for very high R, was obtained when the (±)-[^{125}I]HYP saturation experiments were analyzed as though only a single ligand was present (Model I). Similar results have been obtained using (±)-[^{3}H]carazolol (see Table 1, experiments 7–12).

In contrast, no systematic trend in the estimates for $K_{D(-)}$ or $K_{D(+)}$ as a function of the binding capacity was observed when the saturation curves were analyzed according to the appropriate Model II. In addition, from a comparison of the estimates of the receptor concentrations obtained from Model I with those obtained from Model II, it can be concluded that the analysis according to Model I results in a slight overestimation of R. The extent of this deviation is not only dependent on the number and the accuracy of the data points but also on the concentration range covered by the radioligand and the distribution of the data points. In our experiments (see Table 1) this overestimation of R was usually in the range of 10%.

Monte Carlo simulations. In order to elucidate further the dependence of $K_D$av, $K_{D(-)}$, and $K_{D(+)}$ on R in a more systematic manner, we simulated saturation binding experiments assuming a racemic radioligand and analyzed them with the same methods as used for real data, first according to Model I and then applying the correct two ligand assumptions (Model II) (see Methods for details). Each point in Figs. 4 and 5A represents the averaged parameter estimates obtained from 30 simulated saturation binding experiments. The $K_{D(-)}$ and $K_{D(+)}$ for the stereoisomers of the radioligand had been set to 20 pM and 400 pM, respectively. The receptor concentration was, for a particular experiment, constant, and covered a range of 10 to 1000 pM in the entire study. The variation of the average dissociation constant ($K_D$av) determined by application of Model I to these simulated binding experiments, as a function of R, is shown in Fig. 4. If the ratio $K_{D(+)}/K_{D(-)}$ is large, then $K_D$av approaches $\cong 2\,K_{D(-)}$

TABLE 1

Summary of parameter estimates from saturation binding experiments with different radioligands

The saturation binding experiments with (±)-[^{125}I]HYP, (±)-[^{3}H]carazolol, and j(−)-[^{3}H]carazolol in frog erythrocyte membranes were carried out as described under Methods. All parameter estimates derived according to either Model I or Model II were obtained by computer analysis following the mass-action law as described under Methods and are given with their standard errors. The dependence of $K_D$av on the receptor concentration is obvious, whereas no systematic trend for $K_{D(-)}$ and $K_{D(+)}$ was observed. A slight overestimation of R was the result when the experimental data were analyzed according to Model I.

Experiment	Radioligand	Model I		Model II			Remarks
		R	$K_D$av	R	$K_{D(-)}$	$K_{D(+)}$	
		pM	pM	pM	pM	pM	
1	(±)-[^{125}I]HYP	21.7 ± 0.4	48 ± 3.0	20.6 ± 1.2	16 ± 2.0	[a]	[b]
2		94.3 ± 2.3	79 ± 5.0	73.4 ± 1.0	8.6 ± 0.09	1230 ± 490	[c]
3		284 ± 6.0	375 ± 19	244 ± 5.0	75 ± 6.0	[a]	[b]
4		503 ± 10	322 ± 18	448 ± 9.0	60 ± 13	1130 ± 226	[c]
5		902 ± 36	840 ± 12	661 ± 13	22 ± 5.6	3160 ± 547	[c]
6		877 ± 33	1070 ± 90	806 ± 72	370 ± 233	2610 ± 1830	[b]
7	(±)-[^{3}H]Carazolol	14.5 ± 0.3	28 ± 2.0	14.2 ± 0.3	11 ± 3.0	141 ± 180	[b]
8		109 ± 3.0	29 ± 9.0	106 ± 2.0	6.3 ± 1.9	[a]	[b]
9		127 ± 4.0	61 ± 6.0	111 ± 2.0	6.8 ± 1.8	279 ± 50	[c]
10		135 ± 4.0	62 ± 8.0	129 ± 10.0	23 ± 24	139 ± 97	[b]
11		170 ± 3.0	154 ± 10	166 ± 5.0	72 ± 26	379 ± 197	[b]
12		249 ± 10	177 ± 16	211 ± 3.0	23 ± 2.0	1100 ± 140	[c]
13	(−)-[^{3}H]Carazolol	115 ± 2.0	9.1 ± 1.5[d]				
14		127 ± 3.0	9.6 ± 3.1[d]				

[a] Value for $K_{D(+)}$ reached infinite value (no affinity for (+)-isomer).
[b] No significant improvement for Model II over Model I ($p > 0.05$).
[c] Significant improvement for Model II over Model I ($p < 0.05$).
[d] Represents $K_{D(-)}$.

at low and ≅ $K_{D(+)}/2$ at high R (for further details see Appendix). These relationships may be used to approximate $K_{D(-)}$ and $K_{D(+)}$ without the necessity of computer analysis. The relative error of the mean for $K_D$av was not affected by R and remained, under these conditions, constantly ≅ 10% (Fig. 4).

A quite different picture was obtained when the same data from simulated saturation binding experiments were analyzed according to Model II (Fig. 5A). The average estimated values for $K_{D(-)}$ and $K_{D(+)}$ with their respective standard errors of the mean are shown in Fig. 5A. In the intermediate region, when R ≅ 100 pM, the estimates obtained for $K_{D(-)}$ and $K_{D(+)}$ were very close to their true

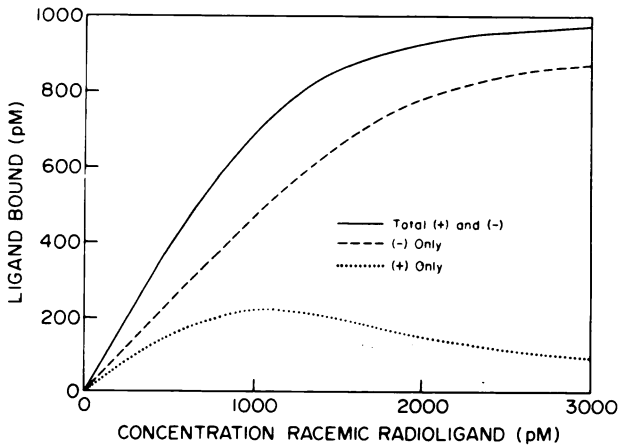

FIG. 3. *Theoretical saturation binding isotherm for a racemic radioligand*

Computer-generated drawing of a saturation binding experiment with a racemic radioligand. The parameters used were $K_{D(-)}$ = 20 pM, $K_{D(+)}$ = 400 pM, and R = 1000 pM. The over-all binding is divided into the contribution of each isomer. It should be noted that the total concentration of the individual isomers are one-half those indicated on the horizontal axis.

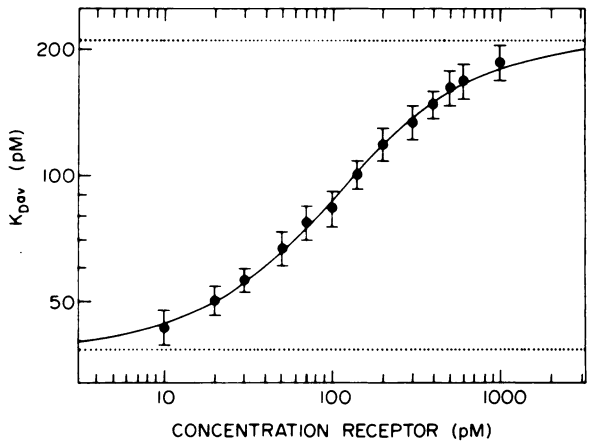

FIG. 4. *Average dissociation constant K_D(av) of a racemic ligand as a function of receptor concentration*

Each point represents the mean of $K_D$av from 30 Monte Carlo simulations of saturation binding experiments with the corresponding standard error of the mean. The true dissociation constants for the isomers of the racemic radioligand were set at $K_{D(-)}$ = 20 pM and $K_{D(+)}$ = 400 pM. The $K_D$av at different R was obtained from the application of Model I instead of the proper Model II. ····, Theoretical limits for $K_D$av (see text for further details).

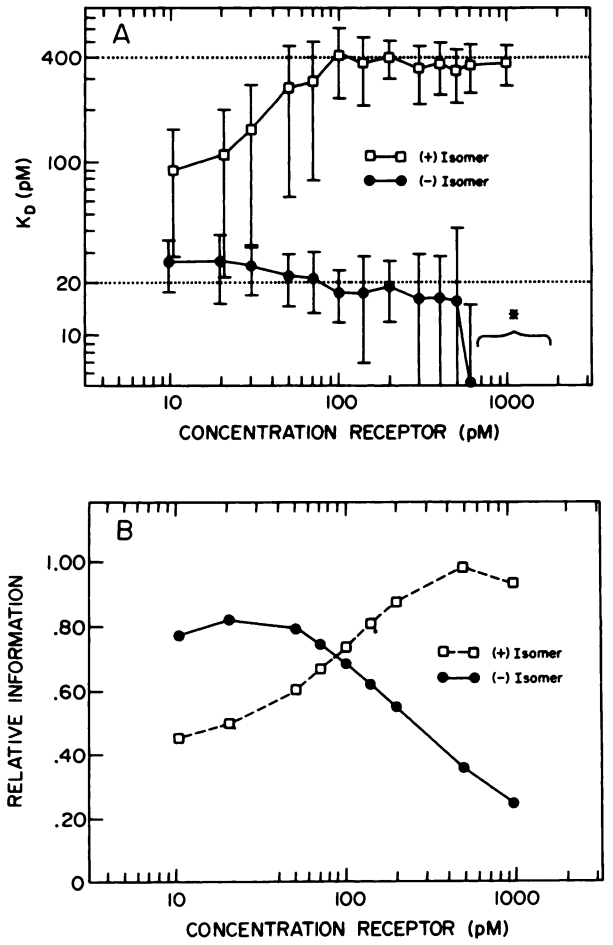

FIG. 5. *Estimates of $K_{D(-)}$ and $K_{D(+)}$ of a racemic radioligand as a function of receptor concentration derived from Monte Carlo simulations of saturation binding experiments and application of the information theory to the estimates*

A. The same binding data from Monte Carlo simulations as shown in Fig. 4 were analyzed using Model II to yield estimates for $K_{D(-)}$ and $K_{D(+)}$. The deviation from the true values of these parameters and the relative standard errors of the mean were strongly affected by the receptor concentration. Because of the logarithmic scale of the ordinate, the error bars appear asymmetric. ···· Corresponds to the true $K_{D(-)}$ and $K_{D(+)}$. The *asterisk* indicates that the value for $K_{D(-)}$ at $R > 600$ pM was tremendously underestimated, accompanied by an extremely high error exceeding the coordinate limits of this figure.

B. The theoretical information for $K_{D(-)}$ and $K_{D(+)}$ obtained from saturation binding experiments with a racemic tracer at various, fixed, receptor concentrations was calculated as described under Methods. The qualitative agreement with the results obtained from Monte Carlo simulations (panel A) is obvious.

values and demonstrated comparable and low relative standards errors. At low receptor concentrations ($R \cong 10$ pM), the estimated ratio $K_{D(+)}/K_{D(-)}$ was found to be ≅ 3–4, which was primarily caused by a systematic underestimated value of $K_{D(+)}$. In this region the standard error of the mean for $K_{D(+)}$ was considerable, but small for $K_{D(-)}$. On the other hand, when the receptor concentration was high ($R > 600$ pM), terribly inaccurate and unreliable estimates for $K_{D(-)}$ were obtained. Thus, at low receptor concentrations the $K_{D(-)}$ was well determined, and at high R the $K_{D(+)}$ was better determined.

Similar results have been obtained by the application of information theory, as is shown in Fig. 5B. Each point is an average of the theoretical information of the same saturation binding experiments as in Fig. 5A (see Methods for details). The values for the information for the stereoisomer K_D values reaches a maximum at a receptor concentration $R = K_{D(-)}$ or $R = K_{D(+)}$, respectively. At low R, the information with respect to $K_{D(+)}$ was low, and even lower at high R for $K_{D(-)}$. The intercept of both curves was located at $R \cong 100$ pM. These findings are in very good agreement with the results obtained by Monte Carlo simulations as described above (Fig. 5A). Thus, both analytic approaches indicate that for a high-affinity racemic radioligand the optimal information about the K_D values for the two stereoisomers is obtained when a binding experiment is carried out at receptor concentrations which are intermediate between $K_{D(-)}$ and $K_{D(+)}$.

Competition binding experiments with high-affinity racemic radioligands. In order to study the effect of high-affinity racemic radioligands on competition binding curves, we constructed curves, using as radioligands, (a) (±)-[^{3}H]carazolol, a high-affinity racemic radioligand; (b) (−)-[^{3}H]carazolol, a high-affinity pure stereoisomeric radioligand; and (c) (−)-[^{3}H]DHA, also a pure stereoisomer but with lower affinity. The competitors used were (−)-, (+)-, and (±)-carazolol. When (±)-[^{3}H]carazolol was the radioligand (Fig. 6A) a family of parallel competition curves appeared with slope factors in the neighborhood of one (experiments 4–6 in Table 2). By contrast, when (−)-[^{3}H]DHA was the tracer (Fig. 6B), the competition curve of (−)-carazolol was very steep with a slope factor of 2, whereas the slope factor for the much weaker competitor (+)-carazolol was approximately 1 (experiment 1 in Table 2). In Table 2 the competitors' K_D values as calculated by the commonly used Cheng and Prusoff (13) approximation are compared with those calculated by computer modeling according to mass-action law (see Methods for details). The Cheng and Prusoff approximation yielded poor estimates under certain conditions. This was especially true for the K_D of (−)-carazolol, when either the lower affinity (−)-[^{3}H]DHA (experiment 2 in Table 2 and Fig. 6B) or racemic (±)-[^{3}H]carazolol (experiment 5 in Table 2 and Fig. 6A) were used as the radioligands. This problem was not noted when the competitor was of lower affinity, e.g., (+)-carazolol (experiments 1, 4, and 7 in Table 2). The best agreement between Cheng and Prusoff approximation and computer modeling was noted when (−)-[^{3}H]carazolol was used as the radioligand (experiments 7–9 in Table 2).

Thus, these studies identify the following potential problems in the analysis of competition curves with very high-affinity compounds. First, "hypersteep" curves (slope factor ~ 2) are obtained if the competitor is considerably more potent than the radioligand. Under these circumstances the Cheng and Prusoff approximation for determining K_D of the competitor is inaccurate. Second, this approximation is also inaccurate when the radioligand is racemic. These points are discussed further below.

Monte Carlo simulations of competition curves using a racemic radioligand. How does the estimate of a K_{D}av

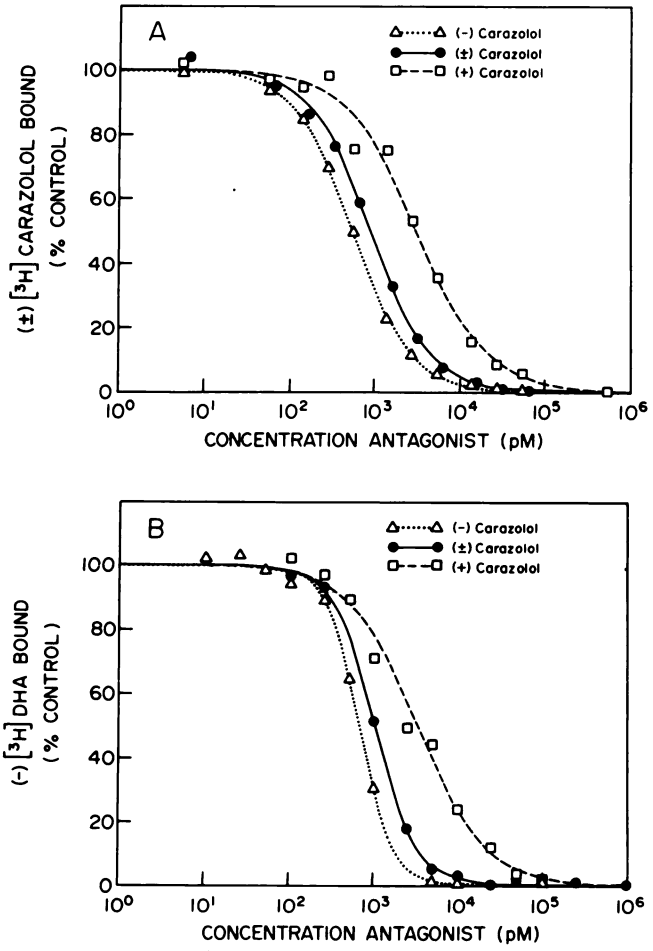

FIG. 6. *Competition curves of* (−)-, (+)-, *and* (±)-*carazolol with* (+)-*[^{3}H]carazolol and* (−)-*[^{3}H]DHA as the radioligand*

Competition binding assays were carried out as desribed under Methods. Competition curves of the stereoisomers as well as the racemate of carazolol were obtained by using either (±)-[^{3}H]carazolol (panel A) or (−)-[^{3}H]DHA (panel B) as tracers. The values for the dissociation constants of the competitors, EC_{50} values, and slope factors are given in Table 2. The *lines* through the *points* are computer-drawn according to a four-parameter logistic equation as described under Methods (Eq. 4).

for a racemic radioligand affect the estimate of a K_D for a competing nonracemic ligand? To elucidate this point, we carried out Monte Carlo simulations for competition binding experiments (see Methods for details). The same K_D values for the labeled stereoisomers ($K_{D(-)}$ = 20 pM and $K_{D(+)}$ = 400 pM) were chosen as used previously to generate the simulated data points. For different assay conditions with varying, fixed receptor concentration and tracer concentration, the K_D values for various competitors (K_D values spanning a range of values from 20 to 100,000 pM) have been analyzed in two ways: once using the true values for $K_{D(-)}$ and $K_{D(+)}$ of the racemic radioligand and, on the other hand, using a $K_D$av of 50 pM or 200 pM, respectively. These values are approximately the limits of the $K_D$av calculated from a saturation binding experiment (see above). The results of these simulated competition experiments are summarized in Table 3. No significant deviations from the true K_D values for the competitors were observed when the exact values for $K_{D(-)}$ and $K_{D(+)}$ for the racemic tracer were used. However, depending on the chosen conditions, the use of $K_D$av for the racemic radioligand resulted in up to a 4- to 5-fold deviation of the estimates from the true competitor's K_D.

DISCUSSION

The present studies indicate that there are potential problems in the interpretation of both saturation and competition experiments with racemic radioligands. These problems have not been previously explicitly addressed in the literature. For example, a remarkably wide range for the dissociation constant of the high-affinity racemic tracer (±)-[^{125}I]HYP for *beta*-adrenergic receptors has been reported in the literature (10–2000 pM) (16). By contrast, the values reported for the K_D of (−)-[^{3}H]DHA for *beta*-adrenergic receptors in several tissues are more tightly clustered (15). In one study the apparent K_D of (±)-[^{125}I]HYP for turkey erythrocyte membrane *beta*-adrenergic receptors was noted to vary depending on the receptor concentration, the ligand appearing to be of higher affinity at low receptor concentration (17). Similar observations have been made for the high-affinity racemic muscarinic cholinergic ligand (±)-[^{3}H]QNB (18).

In the present studies we have documented the dependence of the $K_D$av of (±)-[^{125}I]HYP for frog erythrocyte *beta*-adrenergic receptors on receptor concentration when data are analyzed as if the tracer represented a single species of molecule (Model I). The $K_D$av values spanned a range from 50 pM to 1000 pM (Table 1), in close agreement with the range of values reported in the literature (16). Our experimental results were also in good agreement with the results of Monte Carlo simulations (Fig. 4). The application of this method confirmed and expanded the experimental results. The simulations based on the assumption of different affinities for the (−)- and the (+)-stereoisomers of the racemic radioligand ($K_{D(-)}$ = 20 pM, $K_{D(+)}$ = 400 pM) demonstrated the relationship between $K_D$av and R, when analyzed according to Model I. This phenomenon is the result of the differential contribution of the two stereoisomers to the overall binding at the different receptor concentrations. The (−)-isomer binds preferentially at low R and the (+)-isomer contributes increasingly as R increases. Thus, the $K_D$av reflects the varying contributions of $K_{D(-)}$ and $K_{D(+)}$ at the different receptor concentrations (see Fig. 3). By contrast, $K_{D(-)}$ and $K_{D(+)}$ estimated by application of Model II were more or less constant and independent of R except at extreme values of R.

Our analysis indicates that the limits for $K_D$av are approximately $K_{D(+)}/2$ at very high and 2 $K_{D(-)}$ at very low receptor concentrations (exact equations given in Appendix). Thus, if binding saturation experiments with a racemic tracer were performed at a very low and a very high receptor concentration, then analyzed according to Model I (e.g., with Scatchard analysis) it should be possible to approximate the $K_{D(-)}$ and $K_{D(+)}$. It should be noted that the real dependence on the receptor concentration of $K_D$av of a racemic radioligand as determined by Monte Carlo simulations according to Model I (see Fig. 4) has nothing to do with the variation of the concentration of a radioligand required for half-maxi-

TABLE 2

Competition of (−), (+), and (±)-carazolol with different radioligands for frog erythrocyte membrane beta-adrenergic receptors

The competition binding experiments were carried out as described under Methods. The experimental data and the fitted competition curves for the experiments using (±)-[^{3}H]carazolol or (−)-[^{3}H]DHA as tracers are shown in Fig. 6. The parameter estimates, given with their standard errors, were obtained by the application of two different computer analysis methods: (a) EC_{50} and slope factors were determined by the four-parameter logistic Eq. 1. By using this EC_{50} value, the K_D was calculated according to the Cheng and Prusoff (13) approximation. (b) Computer curve fitting was performed according to the mass-action law as described under Methods. A discrepancy between Cheng and Prusoff approximation and mass-action law in the estimates for the K_D values of the competitors was noted when either a racemic radioligand (experiments 4–6) or a tracer with affinity lower than that of the competitor (experiment 2) was used.

Experiment	Radioligand[a]	Competitor	R	EC_{50}	Slope factor	K_D of competitor from	
						Cheng and Prusoff approximation[b]	Mass-action law
			pM	pM		pM	pM
1	(−)-[^{3}H]DHA, T = 5600 pM, B_0 = 463 pM	(+)-Carazolol	920	2960 ± 410	1.01 ± 0.07	1390	1130 ± 110
2		(−)-Carazolol	920	670 ± 40	2.07 ± 0.21	315	56 ± 8.0
3		(±)-Carazolol[c]	920	1030 ± 100	1.74 ± 0.16	484	130 ± 22
4	(±)-[^{3}H]Carazolol, T = 142 pM, B_0 = 88 pM	(+)-Carazolol	340	3020 ± 240	1.04 ± 0.04	1640	440 ± 40
5		(−)-Carazolol	340	524 ± 42	1.23 ± 0.05	285	40 ± 4.0
6		(±)-Carazolol[c]	340	870 ± 66	1.25 ± 0.06	473	169 ± 17
7	(−)-[^{3}H]Carazolol, T = 205 pM, B_0 = 109 pM	(+)-Carazolol	122	2460 ± 250	0.73 ± 0.04	170	336 ± 97
8		(−)-Carazolol	122	402 ± 43	1.13 ± 0.06	28	15 ± 40
9		(±)-Carazolol[c]	122	367 ± 47	0.96 ± 0.05	25	26 ± 2.0

[a] T, Total concentration; B_0, initial binding.

[b] K_D of radioligand used for Cheng and Prusoff approximation is equal to the value obtained by mass-action law.

[c] Racemic carazolol is considered as one-ligand with an average dissociation constant $K_D$av.

mum receptor occupancy. Rather this latter relationship, described by Chang et al. (19), is related to the case where free and total radioligand concentrations are erroneously equated.

The present investigations were triggered by our observations that binding saturation data obtained with the racemic *beta*-adrenergic tracers (±)-[^{125}I]HYP and (±)-[^{3}H]carazolol showed small but significant deviations from predicted mass-action law binding isotherms according to Model I. Such behavior was not observed with the pure stereoisomers (−)-[^{3}H]DHA and (−)-[^{3}H]carazolol. Nonetheless, as can be observed from the data in Table 1, analyzing racemic radioligand and saturation binding data according to the more appropriate Model II led to a statistically significant improvement in the goodness of fit in about one-half of the experiments. This observation is in good agreement with results of Monte Carlo simulations (not shown), which also indicated significant improvements in the goodness of fit in approximately 50% of simulated experiments at optimal receptor

TABLE 3

Monte Carlo simulations of competition binding experiments with a racemic radioligand

The simulations were performed as described under Methods. The competitors are assumed to be nonracemic compounds having true K_D values ranging from 20 to 100,000 pM. The K_D values of these competitors were determined by analysis of their simulated competition curves in two different ways. First, the curves were analyzed assuming a $K_D$av for the racemic radioligand, arbitrarily set to $K_D$av = 50 pM for low receptor concentration and at 200 pM for high receptor concentrations. Second, the simulated competition curves were modeled assuming a racemic radioligand and knowing the true $K_{D(-)}$ = 20 pM and $K_{D(+)}$ = 400 pM. The parameter estimates from the computer analysis according to mass-action law are given with their standard errors. The considerably better agreement of the values obtained by Model II, especially at high receptor concentration, is apparent.

Simulation	Parameters used for Monte Carlo simulation				Estimate of competitor's K_D from computer modeling	
	Receptor concentration	Racemic Radioligand		K_D of competitor	Using $K_D$av for radioligand	Using exact $K_{D(-)}$ and $K_{D(+)}$ for radioligand
		Total concentration	$K_D$av			
	pM	pM	pM	pM	pM	pM
1	50	100	50	20	26.5 ± 0.6	19.7 ± 0.5
2	50	100	50	100	135 ± 3.0	101 ± 2.0
3	50	100	50	400	535 ± 11	400 ± 7.0
4	50	100	50	100,000	133,000 ± 3,100	101,000 ± 2,200
5	200	400	200	20	69.9 ± 2.7	19.9 ± 0.3
6	200	400	200	100	351 ± 9.0	101 ± 1.0
7	200	400	200	400	1,500 ± 40	402 ± 7.0
8	200	400	200	100,000	337,000 ± 9,000	102,000 ± 2,000
9	500	100	200	100,000	418,000 ± 60,000	97,000 ± 15,000

concentration. However, since it is known that we are dealing with a racemic radioligand, we know that Model II is the more appropriate one. Thus, even in those cases where a statistically significant improvement in the fit does not occur with Model II, usable and reasonable values for the binding parameters $K_{D(-)}$ and $K_{D(+)}$ may still be obtained (Table 1).

The optimal receptor concentration for obtaining most information about both isomers in one binding curve seems to be in the neighborhood of the geometric mean of $K_{D(-)}$ and $K_{D(+)}$ (see Fig. 5A). Below the optimal receptor concentration, the error for the estimate of $K_{D(+)}$ increased drastically, owing to the small contribution of the (+)-stereoisomer to the binding at low R. At high R, the error for $K_{D(-)}$ is very large. This is a reflection of the fact that at high R virtually all of the (−)-isomer is bound in the low concentration part of a saturation binding curve. This result is in accordance with the information theory (Fig. 5B) which predicts that at very high R only little information can be obtained about the (−)-isomer.

In certain cases the estimates for $K_{D(+)}$ from computer modeling reached an infinite value [no affinity for the (+)-isomer]. This occurred more frequently at very low receptor concentrations, where the contribution of the (+)-isomer to the binding is very low. This special case is analogous to the approach taken by other investigators (20), who have assumed that only the (−)-isomer of a racemic mixture would bind, considering the (+)-isomer as a radiochemical impurity with no affinity (21, 22). Although this method might be sufficient at low receptor concentrations, it should not be applied at high R, where the contribution of the (+)-isomer is not negligible.

Attempts to analyze the binding data from a racemic tracer with the usual graphical methods (e.g., Scatchard plots) according to Model II would fail. The slightly downward concave curvature in the Scatchard plot might not even be detected. In addition, the slope of such a plot is not simply related to the individual K_D values. Therefore, optimal data analysis of experiments with racemic radioligands requires (a) binding data with high accuracy, (b) an appropriate receptor concentration as demonstrated above, and (c) a nonlinear least-squares computer curve fitting method. Our computer modeling techniques based on the generalized model for complex ligand binding systems described by Feldman (11), accompanied with appropriate statistics, have also been previously successfully applied in our laboratory to the analysis of multiple-site systems (23, 24).

Errors are also introduced in the interpretation of competition binding experiments with racemic radioligands if the tracer is considered as having a single $K_D$av. We found by Monte Carlo simulations that, depending on the ligand and receptor concentration, the consequences of using a $K_D$av for calculation of competitor K_D values according to the Cheng and Prusoff (13) approximation was up to a 5-fold underestimation of competitors' affinity (see Table 3).

Another interesting problem in interpretation of competition binding data was uncovered in the present studies and relates to the case of very high-affinity competitors, be they racemic or not. It was found that, when the competitor has appreciably higher affinity than the radioligand, the slope factor of the competition curve is greater than 1. This result can be predicted by calculations based simply on the law of mass action.[7] Under such circumstances, the EC_{50} of the competitor does not directly reflect its potency and is no longer a reliable parameter to use for calculations of K_D values according to the Cheng and Prusoff relationship. A theoretical study of high-affinity competitors was derived by Rodbard and Lewald (25) a number of years ago (see appendix III of ref. 25). This point is exemplified by experiment 2 of Table 2 in which a serious error resulted when the Cheng and Prusoff approximation was used to calculate the K_D of (−)-carazolol from its EC_{50} for competition with the less potent radioligand (−)-[^{3}H]DHA. Similar findings and conclusions have been reported by Hulme *et al.* (26) for brain muscarinic receptors. The Cheng and Prusoff approximation, as noted above, fails when the radioligand is racemic even if its potency is equal to or greater than that of the competitor (see Table 2, experiment 5).

The racemic radioligand model may be considered as a special case of ligand heterogeneity. These studies demonstrate the potential problems which may occur when a heterogenous ligand mixture is used. Another important group of labeled ligands are iodinated polypeptides which usually are a mixture of mono-, di-, and poly-labeled ligand species with different K_D values and specific radioactivities, and of unknown proportions. Even with sophisticated computer modeling, such cases would be very difficult to analyze properly according to the mass-action law.

Finally, recent studies in our laboratory indicate that, in addition to the anomalous equilibrium binding data delineated here, the use of racemic radioligands may also lead to a variety of potentially misleading kinetic properties. Most notable is the seeming presence of rapidly and slowly dissociable forms of a receptor, the proportions of which appear to change with time.[8]

In summary, our studies indicate a number of potential pitfalls in the interpretation of equilibrium binding data obtained with high-affinity racemic radioligands which largely relate to the contribution of both isomers to the over-all binding isotherm. The binding properties of high-affinity radioligands are thus only apparently anomalous and unexpected when the ligands are used as though they were a single enantiomer. These properties have been largely neglected in previous literature. In addition to leading to erroneous estimation of the absolute value of radioligand affinities and the affinity of competing ligands, ignorance of these properties of high-affinity racemic radioligands can magnify daily and between laboratory variability in the assessment of ligand-binding properties. Although these pitfalls can be circumvented by appropriate analytical techniques (computer curve fitting) and experimental methods (very low receptor concentrations), the best approach is probably the avoidance of ligand heterogeneity when possible. For this reason, very high-affinity pure stereoisomer radioligands such as (−)-[^{3}H]carazolol should be particularly useful.

[7] Bürgisser E., manuscript in preparation.

[8] Bürgisser E., A. De Lean, and R. J. Lefkowitz, submitted for publication.

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APPENDIX

Theoretical Properties of Racemic Radioligand Binding

The binding of a racemic radioligand to a single class of sites can be considered as a specific example of ligand heterogeneity, if the individual stereoisomers bind to the receptor with different affinities. The specific radioactivity for both isomers of a racemate (1:1 mixture) is assumed to be equal.

Limits for average dissociation constants ($K_D$av). The limiting value of $K_D$av at infinitesimal receptor concentration ($R \to 0$) and at infinitely large receptor concentration ($R \to \infty$) can be derived by expressing the total concentration of radioligand bound ($B = B_{(-)} + B_{(+)}$) according to the formulation of Scatchard (26):

$$B/F = \frac{R - B}{K_D\text{av}} \tag{1}$$

where F is the combined free concentration of both stereoisomers. Owing to the requirement of a constant proportion of ($L_{(-)} = L_{(+)}$) of the total concentration of the isomers, the $K_D$av can be simply expressed as

$$K_D\text{av} = \frac{K_{D(+)}(K_{D(-)} + E) + K_{D(-)}(K_{D(+)} + E)}{(K_{D(-)} + E) + (K_{D(+)} + E)} \tag{2}$$

where $K_{D(-)}$ and $K_D(+)$ are the dissociation constants for the corresponding isomers and E is the concentration of empty receptor sites. The limits of $K_D$av are then easily obtained:

$$\lim_{R \to 0} K_D\text{av} = \frac{2K_{D(-)}K_{D(+)}}{K_{D(-)} + K_{D(+)}} \tag{3}$$

and

$$\lim_{R \to \infty} K_D\text{av} = \frac{1}{2} \cdot (K_{D(-)} + K_{D(+)}) \tag{4}$$

Paton and Rang (27) derived an equation similar to Eq. 3 for the case of low receptor concentration.

Limits for the contribution of each isomer to the overall binding. The contribution of each isomer to the overall binding of a racemic radioligand is given by

$$B_{(-)} = \frac{L_{(-)}E}{K_{D(-)} + E} \tag{5}$$

and

$$B_{(+)} = \frac{L_{(+)}E}{K_{D(+)} + E} \tag{6}$$

where $L_{(-)} = L_{(+)}$ is the total concentration of each isomer. Figure 3 compares the contribution of both isomers to the over-all binding in a saturation binding curve for a given set of parameters. At high concentration of the racemic radioligand, the ratio $B_{(+)}/B_{(-)}$ is expressed as

$$\lim_{L_{(-)} = L_{(+)} \to \infty} B_{(+)}/B_{(-)} = K_{D(-)}/K_{D(+)} \tag{7}$$

At infinitesimal radioligand concentration the limit of the ratio $B_{(+)}/B_{(-)}$ is dependent on the receptor concentration R:

$$\lim_{L_{(-)} = L_{(+)} \to 0} B_{(-)}/B_{(+)} = \frac{K_{D(-)} + R}{K_{D(+)} + R} \tag{8}$$

It is obvious from Eq. 8 that at low radioligand concentrations the contribution of the weaker isomer [(+)-isomer] to the over-all binding is dependent on R and relatively high if $R \gg K_{D(-)}$ (see Fig. 3).

REFERENCES

1. Aurbach, G. D., S. A. Fedak, C. J. Woodward, J. S. Palmer, D. Hauser, and T. Troxler. Beta-adrenergic receptor: stereospecific interaction of iodinated beta-blocking agent with high affinity site. *Science (Wash. D. C.)* **186**:1223–1224 (1974).
2. Yamamura, H. I., and S. H. Snyder. Muscarinic cholinergic binding in rat brain. *Proc. Natl. Acad. Sci. U. S. A.* **71**:1725–1729 (1974).
3. Bartsch, W., K. Dietmann, H. Leinert, and G. Sponer. Cardiac action of carazolol and methypranol in comparison with other beta receptor blockers. *Arzneim Forsch.* **27**:1022–1026 (1977).
4. Mukherjee, C., M. G. Caron, M. Coverstone, and R. J. Lefkowitz. Identification of adenylate cyclase-coupled β-adrenergic receptors in frog erythrocytes with (−)-[^{3}H]alprenolol. *J. Biol. Chem.* **250**:4869–4876 (1975).
5. Stadel, J. M., and R. J. Lefkowitz. Multiple reactive sulfhydryl groups modulate the functions of adenylate cyclase-coupled *beta* adrenergic receptors. *Mol. Pharmacol.* **16**:709–718 (1979).
6. Harden, T. K., B. B. Wolfe, and P. B. Molinoff. Binding of iodinated *beta* adrenergic antagonists to proteins derived from rat heart. *Mol. Pharmacol.* **12**:1–15 (1976).
7. Fletcher, J. E., and R. I. Schranger. A user's guide to least squares model fitting. *U.S. Department of Health, Education and Welfare, Tech. Rep.* 1 (1973).
8. Rodbard, D., and H. A. Feldman. Theory of protein-ligand interaction. *Methods Enzymol.* **36**:3–16 (1975).
9. Draper, N. R., and H. Smith. *Applied Regression Analysis.* New York, Wiley (1966).
10. Rodbard, D. Statistical quality control and routine data processing for radioimmunoassays and immunoradiometric assays. *Clin. Chem.* **20**:1255–1270 (1974).
11. Feldman, H. A. Mathematical theory of complex ligand-binding systems at equilibrium. *Anal. Biochem.* **48**:317–338 (1972).
12. De Lean, A., P. J. Munson, and D. Rodbard. Simultaneous analysis of families of sigmoidal curves: application of bioassay, radioligand assay and physiological dose-response curves. *Am. J. Physiol.* **4**:E97–E102 (1978).
13. Cheng, Y.-C., and W. H. Prusoff. Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (I_{50}) of an enzymatic reaction. *Biochem. Pharmacol.* **22**:3099–3108 (1973).
14. Weber, G. The binding of small molecular to proteins, in *Molecular Biophysics* (B. Pullman and M. Weissbluth, eds.). Academic Press, New York, 369–396 (1965).
15. Brillouin, L. *Science and Information Theory*, Ed. 2. Academic Press, New York (1962).
16. Maguire, M. E., E. M. Ross, and A. G. Gilman. Beta-adrenergic receptor: ligand binding properties and the interaction with adenylyl cyclase. *Adv. Cyclic Nucleotide Res.* **8**:1–83 (1977).
17. Tolkovsky, A. M., and A. Levitzki. Mode of coupling between the beta-adrenergic receptor and adenylate cyclase in turkey erythrocytes. *Biochemistry* **17**:3795–3810 (1978).
18. Fields, T. Z., W. R. Roeske, E. Morkin, and H. I. Yamamura. Cardiac muscarinic cholinergic receptors. Biochemical identification and characterization. *J. Biol. Chem.* **253**:3251–3258 (1978).
19. Chang, K.-T., S. Jacobs, and P. Cuatrecasas. Quantitative aspects of hormone-receptor interaction of high affinity. Effect of receptor concentration and measurement of dissociation constants of labelled and unlabelled hormones. *Biochim. Biophys. Acta* **406**:294–303 (1975).
20. Brown, E. M., S. A. Fedak, C. T. Woodward, G. D. Aurbach, and D. Rodbard.

β-Adrenergic receptor interactions: direct comparison of receptor interaction and biological activity. *J. Biol. Chem.* **251**:1239–1246 (1976).
21. Builder, S. E., and I. H. Segel. Equilibrium ligand binding assays using labelled substrates: nature of the errors introduced by radiochemical impurities. *Anal. Biochem.* **85**:413–424 (1978).
22. Taylor, S. I. Binding of hormones to receptors: an alternative explanation of non-linear Scatchard plots. *Biochemistry* **14**:2357–2361 (1975).
23. Hancock, A. A., A. L. De Lean, and R. J. Lefkowitz. Quantitative resolution of *beta* adrenergic receptor subtypes by selective ligand binding: application of a computerized model fitting technique. *Mol. Pharmacol.* **16**:1–9 (1979).
24. Hoffman, B. B., A. DeLean, C. L. Wood, D. D. Schocken, and R. J. Lefkowitz. Alpha-adrenergic receptor subtypes: quantitative assessment by ligand binding. *Life Sci.* **24**:1739–1746 (1979).
25. Rodbard, D., and J. E. Lewald. Steroid assay by protein binding. *Acta Endocrinol. Suppl. 147* **64**:79–103 (1970).
26. Hulme, E. C., N. J. M. Birdsall, A. S. V. Burgen, and P. Mehta. The binding of antagonists to brain muscarinic receptors. *Mol. Pharmacol.* **14**:737–750 (1978).
27. Paton, W. D. M., and H. P. Rang. The uptake of atropine and related drugs by intestinal smooth muscle of the guinea-pig in relation to acetylcholine receptors. *Proc. R. Soc. B Lond. Biol.* **163**:1–44 (1965).

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