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Validation and Statistical Analysis of a Computer Modeling Method for Quantitative Analysis of Radioligand Binding Data for Mixtures of Pharmacological Receptor Subtypes

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SUMMARY

Accurate and reliable assessment of mixtures of pharmacological receptor subtypes depends on the use of optimally quantitative data analysis of radioligand binding in competition with selective drugs. We have statistically tested the validity and determined the limits of a computer modeling method for the analysis of heterogeneous receptor populations which we have previously applied to the study of *alpha*- and *beta*-adrenergic receptor subtypes. The method was checked by testing its ability to provide accurate estimates in the analysis of binding data from experimentally derived and simulated *beta*-adrenergic receptor subtype mixtures. Such mixtures of receptor subtypes were created by combining in various proportions previously characterized pure $beta_1$- and $beta_2$-adrenergic receptor preparations from turkey and frog erythrocyte membranes and by using Monte Carlo simulation methods. Both methods illustrate that, with currently available *beta*-adrenergic ligands, the resolution limit of the method is a mixture of about 90%/10% or 10%/90% high- and low-affinity receptor subtypes. With such mixtures, computer analysis of competition curves for tritiated dihydroalprenolol ([^{3}H]DHA) binding by Zinterol (MJ9184) (200-fold $beta_2$-adrenergic-selective), or norepinephrine (70-fold $beta_1$-adrenergic selective) results in the observation of significant discrimination of two sites in two-thirds of the experiments. Simultaneous analysis of competition curves from the same experiment using ligands of different selectivities provides more accurate estimates of the contribution of each component to the receptor mixture as well as the affinity constant of the ligands for each receptor subtype. With an "ideal" mixture of equal proportions of each receptor subtype, competition curves for drugs with only a 6-fold selectivity would be sufficient to document both receptor subtypes. These results confirm the validity of the computer modeling method that we have used for analyzing mixtures of adrenergic receptor subtypes. In addition, the same validation approach can be applied to other receptor subtype systems in order to estimate their resolution limit and aid in the development of new selective drugs.

INTRODUCTION

Recent progress in the study of hormone and neurotransmitter receptor heterogeneity in various tissues has been achieved by the application of radioligand binding techniques. Since the original proposal (1-3) that competitive binding of nonselective radioligands versus selective competitors may be used to quantitate receptor subtypes, many applications of the technique have provided quantitative estimates of the relative proportions of such receptor subtypes in various preparations (4-11). The same methodology has also been used to characterize agonist-induced multiple forms of receptor populations in systems regulated by guanine nucleotides (9, 10, 12, 13). Alternatively, ligand binding methods based on the use of highly selective radiolabeled drugs have been described in cases where such compounds were available (14, 15). However, radiolabeled drugs are more generally nonselective for receptor subtypes (e.g., the *beta*-adrenergic antagonists [^{3}H]DHA[4] and [^{125}I]hydroxybenzylpindolol) and most unlabeled compounds are less than

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[4] The abbreviations used are: DHA, dihydroalprenolol; p(NH)ppG, guanylimidodiphosphate.

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100-fold selective, making the experimental approach involving nonselective radioligands more feasible.

Recently, several different analytical methods for quantitating the proportions of receptor subtypes and the affinity of selective drugs for each subtype have been proposed. Some of these methods are graphical (2, 3) whereas others are computer-based (4–12). Several approaches have been based on the use of Eadie-Hofstee plots (2–6). We have been using a method based on computer modeling of untransformed binding data, using a general model following mass-action law principles and incorporating the binding characteristics of both the radioligand and the competitor (4, 8, 9, 13). Ultimately the appropriateness and the advantages of this or any other analytical method should be judged by its ability to provide reliably accurate estimates of the proportion of receptor subtypes and of the binding characteristics of selective competitors. Nonetheless, to date, few if any of the analytical methods proposed for quantitation of receptor subtypes have been rigorously validated or evaluated either experimentally or statistically. Accordingly, the present studies were undertaken to define the accuracy and the limits of resolution of the computer modeling approach which we have proposed for analysis of receptor subtypes. This has been accomplished by both experimental and computer simulation methods. Mixtures of *beta*-adrenergic receptor subtypes of known proportions and binding characteristics have been created by combining turkey ($beta_1$-adrenergic) and frog ($beta_2$-adrenergic) erythrocyte membranes and then subjecting binding data obtained with such mixtures to our analytical method. In addition, binding data from such mixtures of receptor subtypes have been simulated by Monte Carlo technique and subjected to the same analysis. Both approaches indicate that this analytical technique is accurate even in extreme cases with small contributions from one receptor subtype with low selectivity for the competing drug.

METHODS

Turkey erythrocyte membrane preparation. Purified membrane preparations from turkey erythrocytes were prepared as previously described (16), with minor modifications. Blood, obtained from Dr. C. Parkhurst (Featherdown Farms, Raleigh, N. C.) was washed three times by centrifugation (400 × *g* for 5 min) and resuspension in cold Buffer A (150 mM NaCl and 10 mM Tris-HCl, pH 7.4 at 25°), aspirating the supernatant and buffy coat each time. Packed erythrocytes were then lysed by Vortex mixing for 1 min in cold hypotonic Buffer B [10 mM Tris-HCl, 5 mM $MgCl_2$, and 2 mM dithiothreitol (Sigma Chemical Company, St. Louis, Mo.), pH 7.4 at 25°], in a ratio of 5 ml of Buffer B per milliliter of packed whole cells. A pellet obtained by centrifugation at 39,000 × *g* for 10 min at 4° was resuspended in Buffer C (75 mM Tris-HCl and 25 mM $MgCl_2$, pH 7.65 at 25°), 5 ml of buffer per milliliter of packed cells. This mixture was then sonicated-homogenized on ice using a Brinkman Polytron at maximal speed for 10–15 sec, followed by vigorous Dounce homogenization in the cold. The homogenate was diluted 1:1 in Buffer C with centrifugation at 1200 × *g* for 10 min over a layer of 7–10 ml of 35% (w/v) sucrose. The supernatant was harvested and recentrifuged at 39,000 × *g* for 10 min. This final pellet was resuspended in a small volume (approximately 1 ml/ml of original packed cells) of cold Buffer D (75 mM Tris-HCl, 12.5 mM $MgCl_2$, and 1.5 mM EDTA, pH 7.65 at 25°). Small aliquots of the membrane preparation were stored in liquid nitrogen until used. No differences in [^{3}H]DHA binding were observed between duplicate lots of membranes utilized immediately or those which had been frozen prior to use.

Frog erythrocyte membrane preparation. Purified membrane preparations from frog erythrocytes were prepared as previously described (13), with slight modifications. Blood was obtained from southern grass frogs (*Rana pipiens*, obtained from Nasco-Steinhilber, Fort Atkinson, Wisc.) as previously reported (13). Cells were washed three times by centrifugation (400 × *g* for 10 min) and resuspension in Buffer E (100 mM NaCl and 10 mM Tris-HCl, pH 7.4 at 25°), aspirating the supernatant and buffy coat each time. Packed erythrocytes were lysed by brief Vortex mixing in 5 ml of ice-cold water per milliliter of packed cells followed by a 10-min lysing period on ice. The preparation was then homogenized using a Potter-Elvehjem homogenizer, maintaining the membranes on ice at all times. The homogenate was diluted 1:1 with Buffer F [25 mM Tris-HCl, 0.25 M sucrose, 10 mM $MgCl_2$, 2.5 mM EDTA, and 0.01 mM phenylmethylsulfonyl fluoride (Sigma Chemical Company), pH 7.6 at 25°]. The pellet obtained by centrifugation at 39,000 × *g* for 15 min at 4° was resuspended in 10 volumes of Buffer F per milliliter of original packed cells, and 20 ml of the suspension were layered over a 10-ml cushion of 50% sucrose in 50 mM Tris-HCl and 10 mM $MgCl_2$ (pH 8.1 at 25°). Membranes were obtained by harvesting the supernatant and interface following a 12-min centrifugation at 1,200 × *g* in the cold. The membranes were centrifuged at 39,000 × *g* for 15 min, washed, and recentrifuged at 39,000 × *g* for 15 min. The final pellet was resuspended in 1 ml of Buffer F per milliliter of original packed cells and kept frozen in small aliquots in liquid nitrogen until used. No differences in [^{3}H]DHA binding were observed between duplicate lots of membranes utilized immediately and those which were frozen prior to use.

[^{3}H]DHA binding assays. All assays were performed in duplicate in a final volume of 900 μl containing the following components: (a) 75 μl of various concentrations of adrenergic agonists or antagonists in ascorbic acid (final concentration, 5×10^{-4} M), or ascorbic acid alone; (b) 75 μl of p(NH)ppG (Boehringer Mannheim Biochemicals, Indianapolis, Ind., final concentration 1×10^{-4} M); (c) 150 μl of [^{3}H]DHA (0–25 nM in saturation experiments, 2–4 nM in competition studies; (d) 600 μl of membrane preparation as described above. The guanine nucleotide p(NH)ppG was included in all binding assays in order to prevent the formation of agonist-specific high-affinity ligand receptor complex, as described for both $beta_1$ (16) and $beta_2$ (13) adrenergic receptors. Agonist competition curves in the presence of p(NH)ppG thus document only the low-affinity conformation of each *beta*-adrenergic receptor subtype. Incubations were carried out for 15 min at 37° and were terminated by rapid vacuum filtration over Whatman GF/C filters which

were washed with 25 ml of cold Buffer C. Radioactivity bound to membranes adhering to the filters was counted at 47% efficiency in a toluene-Triton X-100-based fluor.

Specific binding was defined as the difference between radioactivity bound in the absence and presence of 10^{-6} M (±)-propranolol. Specific binding was greater than 90% in the turkey and about 95% of total binding in the frog erythrocyte membranes at a concentration of radioligand of 2–4 nM. All data and figures are presented in terms of specific binding of [^{3}H]DHA. Sources of adrenergic ligands used in this study have been previously documented (17).

Preparation of mixtures of adrenergic receptor subtypes. Aliquots of pure turkey and pure frog erythrocyte membrane preparations were used in order to document the affinity constant of [^{3}H]DHA and the receptor density of each pure preparation. Protein was determined by the method of Lowry *et al.* (18), using bovine serum albumin as a standard. Assay of turkey erythrocyte membranes (1–2 mg/ml) in Buffer D indicated an affinity constant for [^{3}H]DHA of 0.18 ± 0.02 nM^{-1} and a binding capacity of 566 ± 49 fmoles/mg of protein (n = 8). Assay of frog erythrocyte membranes (0.3–0.8 mg/ml) showed an affinity constant for [^{3}H]DHA of 0.64 ± 0.05 nM^{-1} and a binding capacity of 871 ± 79 fmoles/mg of protein (n = 9). The 3 to 4-fold higher affinity of [^{3}H]DHA for frog than for turkey membrane preparations was taken into account in all further data analysis, as described below. Aliquots of previously assayed turkey ($beta_1$-adrenergic) and frog ($beta_2$-adrenergic) erythrocyte membranes were appropriately combined in order to prepare receptor mixtures of known composition: (a) 10% $beta_1$/90% $beta_2$; (b) 50% $beta_1$/50% $beta_2$; and (c) 90% $beta_1$/10% $beta_2$ adrenergic receptors.

Monte Carlo simulations of receptor subtype mixtures. Theoretical competition curves were generated using standard methods (19). The mathematical model described below for the competitive binding of a fixed concentration of radioligand and varying concentrations of a selective drug to a mixture of receptor subtypes was used. Each curve included 20 points equidistant on a logarithmic scale over 6 orders of magnitude of concentration of the competitor. The parameters used for the model were chosen in order to closely resemble typical binding data of [^{3}H]DHA to mixtures of $beta_1$- and $beta_2$-adrenergic receptors: total radioligand = 2 nM, affinity constants K_{beta_1} and K_{beta_2} for radioligand = 1 nM^{-1}, sum of $beta_1$- and $beta_2$- adrenergic receptor concentrations = 0.3 nM, nonspecific binding = 0.6% of total radioligand added. Random normal noise was then added to each point calculated on the theoretical curve so that the expected variance of each determination (B_i) of the concentration (nanomolar) of radioligand bound was increasing with B_i according to the formula $\text{var}(B_i) = 10^{-6} + 3 \times 10^{-3} (B_i)^{1.5}$. This variance function was chosen in order to reproduce experimental data scatter typically encountered in *beta*-adrenergic receptor systems such as frog and turkey erythrocyte membranes. For each choice of receptor subtype mixture and competing drug selectivity, a total of 30 curves were simulated. A total of 90 sets of 30 replicate experiments were obtained for various conditions of receptor subtype mixtures ranging from 10% $beta_1$/90% $beta_2$ to 90% $beta_1$/10% $beta_2$-adrenergic receptor subtypes and for competing drugs with 2- to 100-fold selectivity for one of the receptor subtypes.

Data analysis. Experimental and simulated competition curves were analyzed by nonlinear least-squares curve fitting according to the method of Marquardt and Levenberg (20). Groups of competition curves obtained from the same experiment were routinely analyzed simultaneously, whereas simulated binding curves were studied individually.

The competition curves were initially analyzed according to a four-parameter logistic equation as previously described (21):

$$Y = d + (a - d)/(1 + (X/c)^b)$$

where X and Y are the concentration of the competitor and radioligand bound, respectively; c is the 50% effective concentration of competitor (ED_{50}); b is the steepness factor; 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 steepness factor b is used as a preliminary index of receptor site heterogeneity and of drug selectivity. A steepness factor $b = 1$ is expected for a competition curve of a nonselective drug, whereas $b < 1$ is typical of competition curves for selective drugs.

Subsequently, the general model of Feldman (22), involving the interaction of several ligands (L_i) with several independent classes of sites (R_j) according to mass-action law, was used as described previously (4, 8, 13, 23). When applied to competition curves, the model can be schematized as in Fig. 1, where L_1 and L_2 are the known total concentration of radioligand and competing drug, respectively; R_1 and R_2 are the unknown concentrations of $beta_1$- and $beta_2$-adrenergic receptor subtypes, respectively; K_{11} and K_{12} are the predetermined affinity constants of radioligand L_1 for each receptor subtype R_1 and R_2; and K_{21} and K_{22} are the unknown affinity constants of competing drug L_2 for both receptor subtypes. The additional terms N_1 and N_2 included in the model accommodate nonspecific binding of each ligand and correspond to the ratio of the concentration of ligand nonspe-

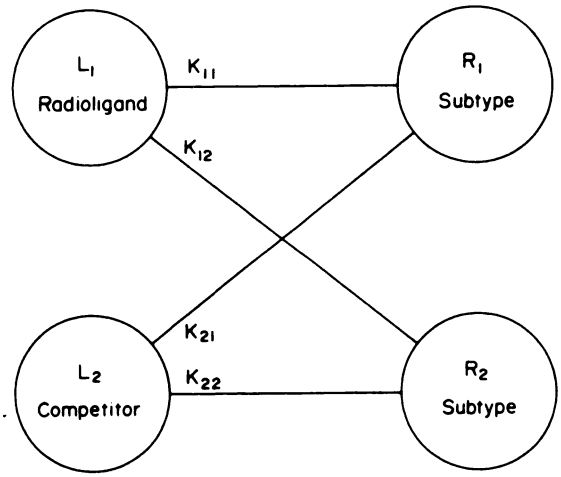

FIG. 1. *Schematic diagram of the model used for the competitive binding of a radioligand L_1 and a selective competitor L_2 to two receptor subtypes R_1 and R_2*

cifically bound divided by the free ligand concentration. Feldman's model distinguishes free ligand concentration F_i from total ligand concentration L_i and thus inherently provides correction for the effect of the mass of radioligand on the apparent potency of the competitor, and also for radioligand depletion when a large percentage of radioligand is bound to the receptor. Following Feldman's nomenclature, the concentration of ligand i bound B_i can be explicitly expressed as a function of not only the corresponding free ligand F_i but of all free ligands F_a according to:

$$B_i = \sum_{b=1}^{2} K_{ib} \cdot F_i \cdot R_b / S_b + N_i F_i$$

where $S_b = 1 + \sum_{a=1}^{2} K_{ab} \cdot F_a$. The concentration F_u of free ligand u can in turn be calculated by solving the implicit function $G_u = 0$:

$$G_u = G(F_u) = L_u - F_u \cdot T_u$$

where $T_u = 1 + \sum_{b=1}^{2} K_{ub} \cdot R_b / S_b + N_b$, and L_u is the total concentration of ligand u. The method involves iterative approximations of F_i using a Newton-Raphson procedure:

$$F_{i_{new}} = F_{i_{old}} - \sum_{u=1}^{2} (A_{ui})^{-1} \cdot G_u$$

where the matrix $A_{ui} = dG_u/dF_i = F_u \cdot \sum_{b=1}^{2} K_{ib} \cdot K_{ub} \cdot R_b / S_b^2 - \delta_{ui} \cdot T_u$, and δ_{ui} is a Kronecker delta ($\delta_{ui} = 1$ if $u = i$; otherwise $\delta_{ui} = 0$). Calculation of partial derivatives of B_i with respect to any parameter Z (i.e., K, R, N) of the model is required by the nonlinear least-squares procedure and is obtained from:

$$dB_i/dZ = -dF_i/dZ = \sum_{u=1}^{2} (A_{ui})^{-1} \cdot (dG_u/dZ)$$

where dG_u/dZ for specific parameters of the model are obtained from the following equations:

$$dG_u/dK_{vj} = (F_u \cdot R_j / S_j) \cdot (-\delta_{uv} + K_{uj} \cdot F_v / S_j)$$

$$dG_u/dR_j = -F_u \cdot K_{uj} / S_j$$

$$dG_u/dN_v = -\delta_{uv} \cdot F_u$$

The deviations of the points from their predicted value on the fitted curve (residuals) were weighted according to the reciprocal of their expected variance according to the formula $\text{var}(B) = a_0 + a_1 \cdot B^{a_2}$, where $a_0 = 10^{-6}$, $a_1 = 3 \times 10^{-3}$, and $a_2 = 1.5$ for the purpose of the analysis of binding data expressed in nanomolar concentration units (4, 8, 13). The parameters of the variance function were estimated so as to reproduce the average experimental fluctuations in the radioligand binding assay. The appropriateness of this variance function was checked by calculating the weighted residual variance of the least-squares fit (average weighted residual variance). A residual variance in the neighborhood of 1 indicates that the residuals are properly weighted according to the reciprocal of their expected variance.

Computer modeling of each competition curve provides estimates and standard error for the affinity constant of the competitor for each receptor subtype (K_{21}, K_{22}), the concentration of each receptor subtype (R_1, R_2), and the coefficient for nonspecific binding for each ligand (N_1, N_2). Estimates for the affinity constants of the radioligand (K_{11}, K_{12}) are separately determined for each membrane preparation and set constant to the predetermined values during the analysis of competition curves. For the purpose of this report, R_1 and R_2 are expressed as percentages of the total concentration of the binding sites $R_1 + R_2$.

Testing for statistically significant resolution of two receptor subtype components is obtained by comparing the residual variance of the fit obtained when the data are analyzed according to a model for a single class (Model 1) or two classes (Model 2) of receptor subtypes, using a partial F-test (23, 24):

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

where SS_1 and SS_2 are the sum of squares of residuals with Models 1 and 2, and df_1 and df_2 are the corresponding degrees of freedom (number of data points minus number of parameters estimated).

Arithmetic means are reported for the average of the percentage of each receptor subtype from replicate experiments and simulations. However, geometric means are shown for average estimates of affinity constants. For that purpose, the arithmetic mean of the logarithmic transform of individual estimates of the affinity constants was calculated and its antilogarithmic transform used as the geometric mean. The standard error of the geometric mean of the affinity constants was calculated by multiplying the value of the geometric mean by the standard error of the averaged logarithmic transform. Statistical testing for differences between average estimates of affinity constants in turkey and frog erythrocyte membrane preparations was performed using Student's t-test. Similarly, average estimates of affinity constants and receptor subtype concentrations obtained from replicate simulated competition curves were compared with the "true" values for these parameters used for generating the Monte Carlo simulations.

All calculations were performend by using interactive computer programs written in PL/1 for a PDP 11/45 minicomputer. An earlier version of the computer modeling program written in BASIC was originally developed in collaboration with Munson and Rodbard (23). They have reported the application of the method to various examples of radioligand binding data (23).

RESULTS

Characterization of pure turkey and frog erythrocyte membrane beta-adrenergic receptors. Contrary to many animal tissues which contain mixtures of receptor subtypes, turkey and frog erythrocyte membranes each contain a pure population of *beta*$_1$- and *beta*$_2$-adrenergic receptors, respectively. As shown below, these nonmammalian *beta*-adrenergic receptor systems demonstrate a potency order for agonists which satisfies the criteria of Lands *et al.* (25) for receptor subpopulations. However, they display affinity constants for some subtype-selective ligands which are at variance with those reported for

mammalian species (26). They are simply used in this study as two distinct receptor populations which reproduce typical mixtures of naturally occurring pharmacological receptor subpopulations.

Figure 2 illustrates the characteristics of [^{3}H]DHA binding to pure frog (2A and C) and turkey (2B and D) erythrocyte membranes in competition experiments using various *beta*-adrenergic ligands. In all cases, for agonists, antagonists, or partial agonists, the steepness factors of the competition curves were indistinguishable from 1.0 in the presence of p(NH)ppG, consistent with a single subpopulation of *beta*-adrenergic receptors in each pure preparation. In all examples shown in Fig. 2, computer modeling of either agonist or antagonist competition curves with a model for a single homogeneous receptor subpopulation was appropriate, as indicated by the goodness of fit of the computer-drawn fitted curve through the points. There was thus no need to consider the presence of high- and low-affinity forms of each *beta*-adrenergic receptor subpopulation usually observed when agonist competition curves are obtained in the absence of guanine nucleotide in both frog (13) and turkey (16) erythrocyte membrane preparations. The ability of agonists to compete for [^{3}H]DHA binding follows the expected *beta*$_2$-adrenergic potency series in frog erythrocyte membranes (Fig. 2A), with isoproterenol 7-fold more potent than epinephrine, which is in turn 10-fold more potent than norepinephrine. In Fig. 2B, a *beta*$_1$-adrenergic potency ratio for the agonists is illustrated for turkey erythrocyte membranes. Isoproterenol is 10-fold more potent than epinephrine, and norepinephrine is slightly more potent than epinephrine. The average estimates of the potency of these agonists in replicate experiments using pure turkey and pure frog erythrocyte membrane preparations are shown in Table 1, Column 1.

Figure 2 also illustrates competition for [^{3}H]DHA binding by two antagonists (propranolol and butoxamine) and a partial agonist (MJ9184 or Zinterol). As summarized in Table 1, propranolol and butoxamine were approximately equipotent for competing against [^{3}H]DHA in the frog and turkey erythrocyte membranes. Epinephrine and isoproterenol were slightly selective for turkey erythrocyte receptor subpopulation (5- and 8-fold, respectively). In contrast, norepinephrine is approximately 69-fold more potent at *beta*$_1$-adrenergic receptors from turkey erythrocytes than at *beta*$_2$-adrenergic receptors from frog erythrocytes, whereas MJ9184 exhibits a 194-fold selectivity for the frog *beta*$_2$-adrenergic receptor subpopulation. Of more than 15 adrenergic drugs tested, only epinephrine, isoproterenol, norepinephrine, and MJ9184 exhibited a statistically significant difference between the affinity constants obtained in pure turkey and frog erythrocytes. As noted under Methods, the radioligand [^{3}H]DHA itself was slightly (3- to 4-fold) but significantly selective for the frog as opposed to the turkey erythrocyte *beta*-adrenergic receptor subpopula-

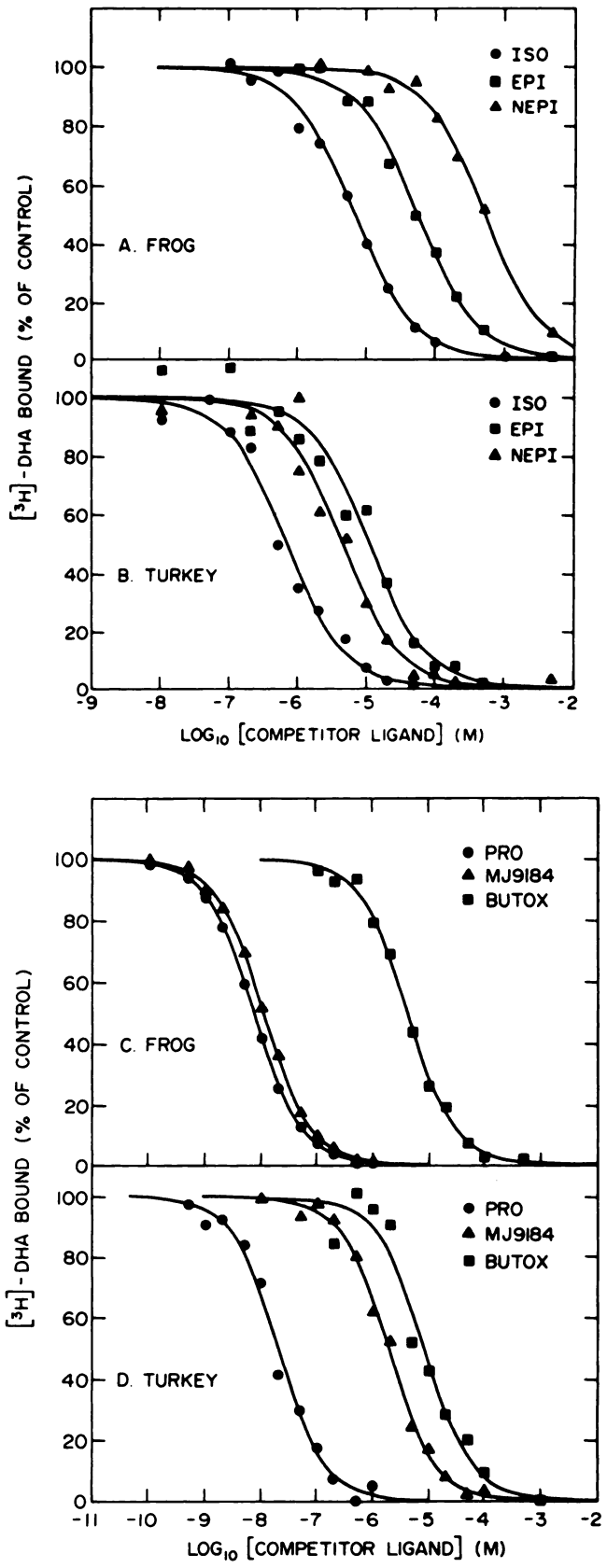

FIG. 2. *Competition curves for [^{3}H]DHA beta-adrenergic agonists in frog (A and C) and turkey (B and D) erythrocyte membranes*

Data points are indicated by *symbols*, whereas the *lines* represent computer-fitted lines according to a model for a single receptor population. The ordinate is the percentage specific binding and the abscissa is the log-molar concentration of competitors: isoproterenol (*ISO*), epinephrine (*EPI*), norepinephrine (*NEPI*), propranolol (*PRO*), MJ9184, and butoxamine (*BUTOX*). These data are representative of three or four similar experiments. The affinity constants derived from computer modeling are shown in Table 1, Column 1. In no case did a model for two receptor subtypes significantly improve the fit.

TABLE 1

Computer estimates of affinity constants (M^{-1})

Computer estimates of affinity constants of adrenergic ligands for turkey ($beta_1$) or frog ($beta_2$) receptors alone or in various mixtures are presented. Affinity constants for ligands are given by K_{B_1} for $beta_1$-receptors (turkey) or K_{B_2} for $beta_2$-receptors (frog). Affinity constants were derived by computer analysis of competition curves as shown in Figs. 1–4. Geometric means (± standard error when appropriate) are given. Numbers in parentheses indicate the number of experiments performed.

Ligand and affinity constant	Receptor			
	Pure turkey ($beta_1$) or pure frog ($beta_2$)	10% Turkey and 90% frog	50% Turkey and 50% frog	90% Turkey and 10% frog
MJ9184 (Zinterol)				
K_{B_1}	$1.1 \pm 0.03 \times 10^6$ (3)	$1.5 \pm 0.5 \times 10^7$ (2)	$1.6 \pm 0.03 \times 10^6$ (3)	$1.9 \pm 0.2 \times 10^6$ (3)
K_{B_2}	$2.2 \pm 0.1 \times 10^8$ (3)[a]	$2.1 \pm 0.1 \times 10^8$ (2)	$1.1 \pm 0.2 \times 10^8$ (3)	$1.3 \pm 0.6 \times 10^8$ (3)
(−)-Norepinephrine				
K_{B_1}	$3.1 \pm 0.1 \times 10^5$ (3)	$6.9 \pm 2.0 \times 10^5$ (2)	$3.1 \pm 0.5 \times 10^5$ (3)	$3.5 \pm 0.2 \times 10^5$ (3)
K_{B_2}	$4.6 \pm 0.3 \times 10^3$ (3)[a]	$4.9 \pm 0.2 \times 10^3$ (2)	$4.8 \pm 0.4 \times 10^3$ (3)	$1.1 \pm 0.2 \times 10^4$ (3)
(−)-Epinephrine				
K_{B_1}	$2.6 \pm 0.3 \times 10^5$ (3)	ND[b]	$4.3 \pm 0.9 \times 10^5$ (2)	ND
K_{B_2}	$4.6 \pm 0.5 \times 10^4$ (3)[a]	ND	$8.3 \pm 0.09 \times 10^4$ (2)	ND
(−)-Isoproterenol				
K_{B_1}	$2.3 \pm 0.2 \times 10^6$ (4)	ND	$6.2 \pm 1.3 \times 10^6$ (3)	ND
K_{B_2}	$2.8 \pm 0.2 \times 10^5$ (3)[a]	ND	$7.1 \pm 0.3 \times 10^5$ (3)	ND
Butoxamine				
K_{B_1}	$1.7 \pm 0.1 \times 10^5$ (3)	ND	2.6×10^5 (1)	ND
K_{B_2}	$3.4 \pm 0.1 \times 10^5$ (3)	ND	2.6×10^5 (1)[c]	ND
(±)-Propranolol				
K_{B_1}	$1.8 \pm 0.2 \times 10^8$ (3)	ND	$2.6 \pm 0.04 \times 10^8$ (2)	ND
K_{B_2}	$2.0 \pm 0.3 \times 10^8$ (3)	ND	$2.6 \pm 0.04 \times 10^8$ (2)[c]	ND

[a] K_{B_2} is significantly different from K_{B_1}.
[b] ND, Not determined.
[c] Two components could not be resolved.

tion. This slight selectivity of the radioligand itself has been taken into account in the computer modeling of the competition curves shown in Figs. 2–4 and in Table 1.

Resolution of beta-adrenergic receptor subtypes in mixtures of turkey and frog erythrocyte membranes. Preliminary determination of the binding properties of the radioligand [^{3}H]DHA and of various selective and nonselective competing drugs in pure turkey and frog erythrocyte membranes provided us with the unique opportunity to test the ability of the computer modeling method to estimate accurately those binding properties using receptor subtype mixtures of known composition. Figures 3 and 4 illustrate results obtained when various mixtures of turkey and frog erythrocyte membrane preparations were used for [^{3}H]DHA binding in the presence of competing ligands. Figure 3 represents experiments performed on a mixture of approximately equal proportions (50%/50%) of turkey and frog *beta*-adrenergic receptors. In Fig. 3A, the curves illustrate the results obtained with a $beta_1$ (norepinephrine) and a $beta_2$ (MJ9184) adrenergic selective drug compared with a nonselective drug (propranolol). The curves for the two selective drugs are biphasic and shallow (steepness factor = 0.46 and 0.47), and are indicative of more than one receptor subpopulation. In contrast, the competition curve for propranolol is uniphasic and steep (steepness factor = 1.02), and does not suggest heterogeneity of receptors. In Fig. 3B, three additional drugs are compared with propranolol. The competition curves for butoxamine and epinephrine are steep (steepness factors = 1.0 and 0.97, respectively) and do not reveal receptor heterogeneity. In contrast, the curve for isoproterenol is less steep (steepness factor = 0.83), indicative of the mixture of receptor subtypes. The results of computer analysis of these data are listed in Table 1, Column 3. The affinity constants of each competing ligand for each receptor subtype and their proportion in the mixture were estimated by computer modeling of the competition curves. As shown, competition curves for propranolol and butoxamine found to be nonselective between turkey and frog beta-adrenergic subpopulations (Table 1, Column 1) were best fit to a model for equal affinities of the competing drugs for the constituents of the receptor mixture. The other four selective ligands were best fit to a model for distinct affinities of the competitor for the two receptor subpopulations. A comparison of the affinity constants estimated from the 50%/50% mixture (Table 1, Column 3) with those in Table 1, Column 1 indicates that the computer modeling method can provide accurate estimates of affinity constants of a ligand for the receptor subpopulations, even with weakly selective ligands such as epinephrine (5-fold selective) and isoproterenol (8-fold selective). More accurate estimates of the affinity constants for each receptor subtype were obtained with the more highly selective ligands norepinephrine (67-fold) and MJ9184 (200-fold) binding. With the less discriminatory ligands (epinephrine and isoproterenol), the binding data were significantly resolved by computer modeling into two components in only some of the experiments. In three similar experiments, a significantly better fit of the data to a model for two receptor subtypes was obtained in only one experiment with epinephrine and two with isoproterenol. Simultaneous computer modeling of competition curves for weakly selective drugs improved

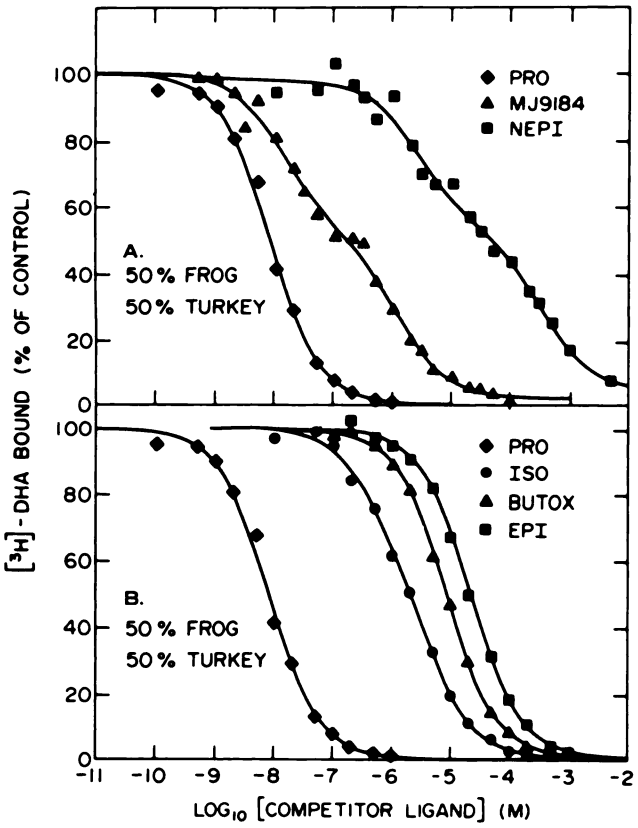

FIG. 3. *Competition curves for [^{3}H]DHA and beta-adrenergic ligands for a mixture of equal (50%/50%) proportions of beta$_1$-adrenergic (turkey) and beta$_2$-adrenergic receptors (frog)*

Coordinates as in Fig. 1.

A. These data are representative of two [propranolol (*PRO*)] or three [MJ9184, norepinephrine (*NEPI*)] similar experiments.

B. These data are representative of one [Butoxamine (*BUTOX*)], two [Propranolol (*PRO*)], epinephrine (*EPI*)], or three (isoproterenol (*ISO*)] similar experiments. The corresponding affinity constants obtained by computer modeling according to a model for one or two receptor subpopulations are given in Table 1, Column 3.

the resolution of the binding components by pooling information from the curves about the proportion of receptor subtypes. When several curves were analyzed simultaneously, the epinephrine and isoproterenol competition curves were significantly better fit to a model for two receptor subpopulations in all experiments.

The computer modeling method also provides estimates for the proportions of receptor subtypes in the mixture. In a series of three experiments similar to those shown in Fig. 3, the computer estimates of 54.1 ± 1.5% of *beta*$_1$- and 45.9 ± 1.5% of *beta*$_2$-adrenergic receptor subtypes closely fit the true values of 55.5% *beta*$_1$ (turkey) and 44.5% *beta*$_2$ (frog) adrenergic receptors derived from the actual determination of receptor densities in the pure preparations, as measured prior to their mixing. Resolution of receptor subtype components in mixtures of equal proportions of *beta*$_1$- and *beta*$_2$-adrenergic receptors offers optimal conditions for estimating all of the binding parameters since there are identical contributions from each component to the mixture. Testing the computer modeling technique with such mixtures permits a determination of the threshold of resolving power for selective drugs. As shown in Fig. 3 and Table 1, a minimum of 5- to 8-fold selectivity is required in order to resolve successfully the components of the mixture.

Additional experiments were also performed with receptor subtype mixtures containing only 10% of one of the components in order to determine the limit of resolution of the minor component of a mixture and the accuracy of its corresponding binding parameters. Since epinephrine and isoproterenol were only marginally able to demonstrate heterogeneous binding in 50%/50% mixtures, they were not used in subsequent experiments with 10%/90% mixtures. Instead, only the two ligands found to be most discriminatory, norepinephrine and MJ9184, were utilized in subsequent studies. Results from a typical set of experiments are illustrated in Fig. 4 and summarized in Table 1, Columns 2 and 4. Fig. 4A shows competition curves obtained with a mixture of 10% *beta*$_1$- and 90% *beta*$_2$-adrenergic receptors. The heterogeneity of the sites is revealed by the "foot" on the competition curve for the *beta*$_2$-selective drug MJ9184, which has a low affinity for the minor *beta*$_1$-adrenergic component, and by a "shoulder" on the curve for the *beta*$_1$-selective drug norepinephrine owing to the higher affinity of the drug for the minor *beta*$_1$-adrenergic component.

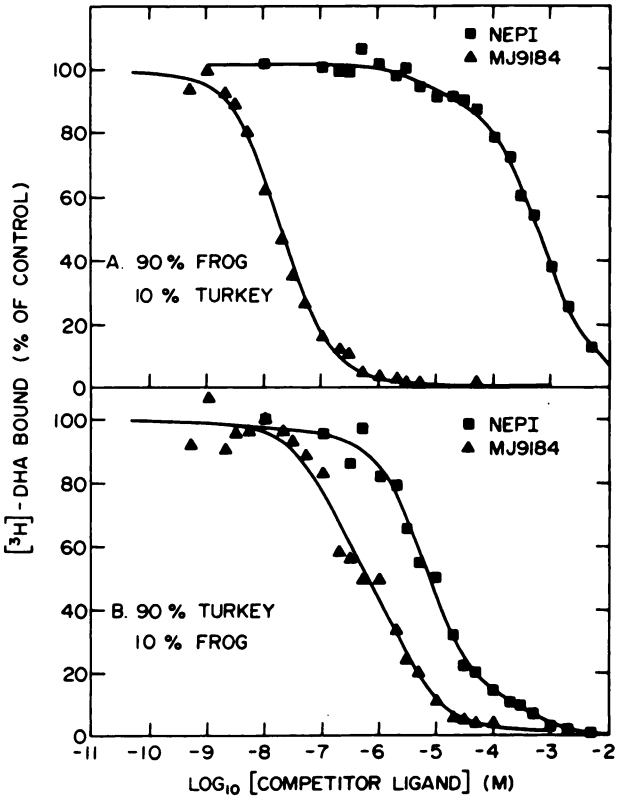

FIG. 4. *Competition curves for [^{3}H]DHA, norepinephrine (NEPI), and MJ9184 in mixtures of unequal proportions of beta$_1$-adrenergic (turkey) and beta$_2$-adrenergic receptors (frog)*

Coordinates as in Fig. 1.

A. Competition curves for norepinephrine and MJ9184 in a mixture of approximately 10% *beta*$_1$- and 90% *beta*$_2$-adrenergic receptors. These data are representative of two similar experiments.

B. Competition curves for norepinephrine and MJ9184 in a mixture of approximately 90% *beta*$_1$- and 10% *beta*$_2$-adrenergic receptors. These data are representative of three similar experiments.

When turkey and frog *beta*-adrenergic receptors were mixed in a ratio of 90%/10% or 10%/90%, respectively, computer analysis of the results indicated that the data could be significantly better fit to a model for two receptor subtypes; the estimates of the affinity constants of the selective drugs for each receptor subpopulation are listed in Table 1. Comparison of the values obtained from computer analysis of these curves with the data in Table 1, Column 1 demonstrates that the computer method can detect a small (10%) component of a receptor subpopulation mixture and delineate binding characteristics of the selective drug used. However, it should be noted that the estimates of the affinity constants for the minor component of the system are more subject to variation. Nonetheless, the average estimates of the affinity constants are unbiased and acceptable approximations of the true values.

The computer estimates of the proportions of frog and turkey receptors were also obtained from the data in Fig. 4. In the experiment represented in Fig. 4A (10% turkey, 90% frog), the computer estimates of the mixture composition were 14.8 ± 4.5% $beta_1$- and 85.2 ± 2.2% $beta_2$-adrenergic receptors. In the experiment shown in Fig. 4B (90% turkey/10% frog), the computer estimates of the mixture composition were 90.8 ± 3.7% $beta_1$- and 9.2 ± 2.2% $beta_2$-adrenergic receptors. In one experiment of three with a 90%/10% mixture of $beta_1$- and $beta_2$-adrenergic receptors, there was no statistically significant improvement of the fit of the data to a model for two receptor subtypes. Similar findings were observed in one experiment of two with a 90%/10% mixture of $beta_2$- and $beta_1$-adrenergic receptors. Hence the ability of the computer modeling method to resolve the components of a receptor subtype mixture when there is only a minor contribution (10%) of one of the components approaches a limit with 70- to 200-fold selective ligands (norepinephrine and MJ9184, respectively).

Resolution of receptor subtypes in simulated binding experiments (Monte Carlo simulations). The application of the computer modeling method to experimentally derived competition binding data using mixtures of *beta*-adrenergic receptor subtypes of known composition has revealed that the method can successfully resolve the components of the mixture and provide accurate estimates of the affinity constants of the selective competitor for each receptor subtype. As noted above, the experimental data indicated that, as a limit, a 5- to 8-fold selective competitor could be used to resolve the two receptor subpopulations from a mixture of equal proportions, whereas a 70- to 200-fold selective competitor was required to resolve the components in extreme cases of mixtures with minor contributions (~10%) from one of the receptor subtypes. Further studies of the resolution limits of the computer modeling technique were not experimentally feasible because of the exceedingly large number of experiments required and the lack of ligands required to cover evenly a broad range of receptor subtype selectivity. For this purpose, the Monte Carlo simulation method could be advantageously used, as exemplified by the following results based on more than 2700 simulated experiments.

Figure 5 shows a typical example of a simulated competitive binding experiment. The *solid line* corresponds to the ideal binding curve of a selective drug with a mixture of receptor subtypes. The *closed circles* demonstrate an example of simulated binding data incorporating random normal deviation around the ideal binding curve. The *shaded area* represents the range of values calculated for each binding data point in 30 replicate competition binding curves.

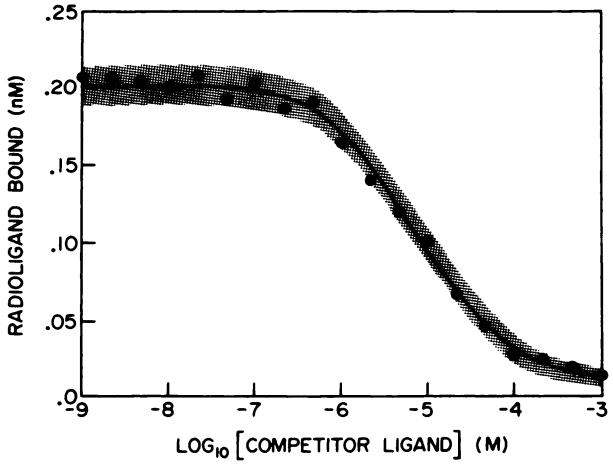

FIG. 5. *Example of a computer simulation of competition binding data*

The data points (●) randomly deviate from the true model (——). The *shaded background* shows the range of values of 30 similar simulations.

For each set of conditions specifying the proportion of the receptor components in the mixture and the affinity constants of the selective drug for each receptor subtype, 30 replicate experiments were simulated and individually analyzed by computer modeling according to models for one and two receptor subpopulations. Since the receptor subpopulations considered for the simulations were arbitrary, their corresponding concentrations as well as their affinity constants for the selective ligand were simply identified according to the component with higher affinity (R_H, K_H) and lower affinity (R_L, K_L) for the selective ligand.

The effect of varying the ratio of affinity constants K_H and K_L of the selective competitor (i.e., the selectivity) on the percentage of the 30 simulated experiments which are statistically significantly better fit to the model for two receptor subtypes is shown in Fig. 6. In the case of receptor subtype mixtures of nearly equal proportions (40% R_H and 60% R_H), the resolving power of the computer modeling method is acutely sensitive to changes in the selectivity ratio K_H/K_L, with significant resolution of the two components occurring in less than 10% of the experiments for a 3-fold selective drug, but 50% significant success for a 6-fold selective compound and 90% significant resolution for a 9-fold selective ligand (Fig. 6). In extreme cases of mixtures with only 10% of one of the components (10% R_H and 90% R_H), significant resolution of the two receptor subtypes is achieved only with more selective ligands (Fig. 6). A 20-fold selectivity ratio is required for 50% successful resolution of the components in the case of a ligand with high affinity for the predominent component (90% R_H), whereas a 40-fold selectivity is required for the same degree of resolution in the case

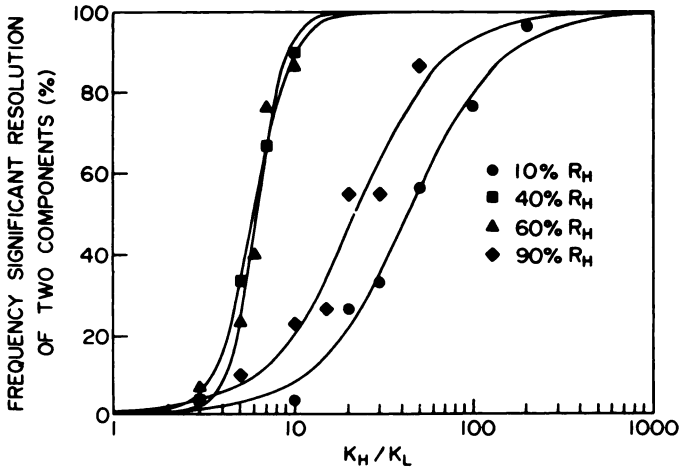

FIG. 6. *Relationship between the frequency of statistically significant resolution of the receptor subpopulations and the ratio (K_H/K_L) of their affinity constants for the selective ligand*

Representative examples of intermediate and extreme cases of receptor mixtures are shown. Each *point* represents the average frequency of success obtained in 30 simulated experiments.

of a ligand with higher affinity for the minor component of the mixture (10% R_H).

The value of the ratio K_H/K_L for a selective ligand corresponding to a 50% chance of significantly resolving a given mixture into its components defines the lower resolution limit of the method. Under such conditions, there is a 94% chance of observing a statistically significant resolution of the two components of the mixture in at least one of four replicate experiments using a selective ligand at the resolution limit. Figure 7 summarizes the operational range of selectivity ratio K_H/K_L, of the competing ligand and mixture composition $\%R_H$. The boundary between the resolution area (*clear*) and the unresolved area (*cross-hatched*) corresponds to the limiting case of 50% probability of significant resolution of the components in each experiment. A 10-fold selective drug can be used to characterize receptor mixtures with 20% or more of the lesser component. However, a ligand up to a 100-fold selective was required to resolve successfully the receptor subtype mixtures with a smaller (less than 20%) minor component. The lack of symmetry of the diagram in Fig. 7 is related to the fact that the random normal deviation of the points in the *upper part* of the competition curves (Fig. 5) is larger than that at the *bottom* of the curves. This renders the accurate assessment of a minor component at the *top* of the competition curve (low $\%R_H$) more subject to error than is the case for a minor component with lower affinity for the selective ligand (high $\%R_H$).

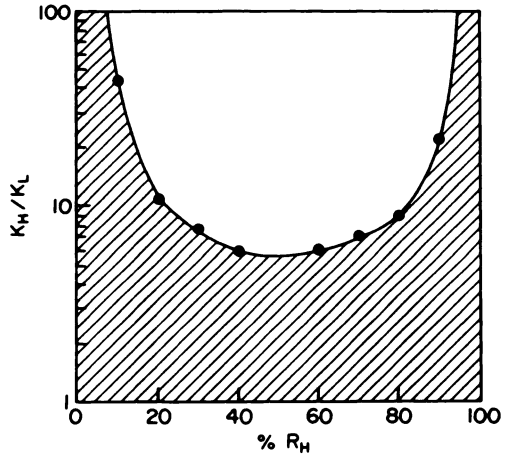

FIG. 7. *Phase diagram of the resolution limit of the computer modeling technique as determined by Monte Carlo simulations described in Figs. 5 and 6*

The *points* represent the selectivity ratio K_H/K_L of a selective ligand able to resolve significantly R_H and R_L in 50% of the simulated experiments for a given proportion of the mixture ($\%R_H$).

The accuracy of the estimation of the affinity constants K_H and K_L of the selective ligand and the relative concentration of receptor subtypes R_H and R_L varies with the selectivity ratio of the ligand and the mixture composition. Under conditions corresponding to the resolution boundary shown in Fig. 7 (*solid line*), the size of the relative error of the parameter estimates for K_H, K_L, R_H, and R_L is documented in Fig. 8A and B. In general, the relative error of the concentration of the major receptor

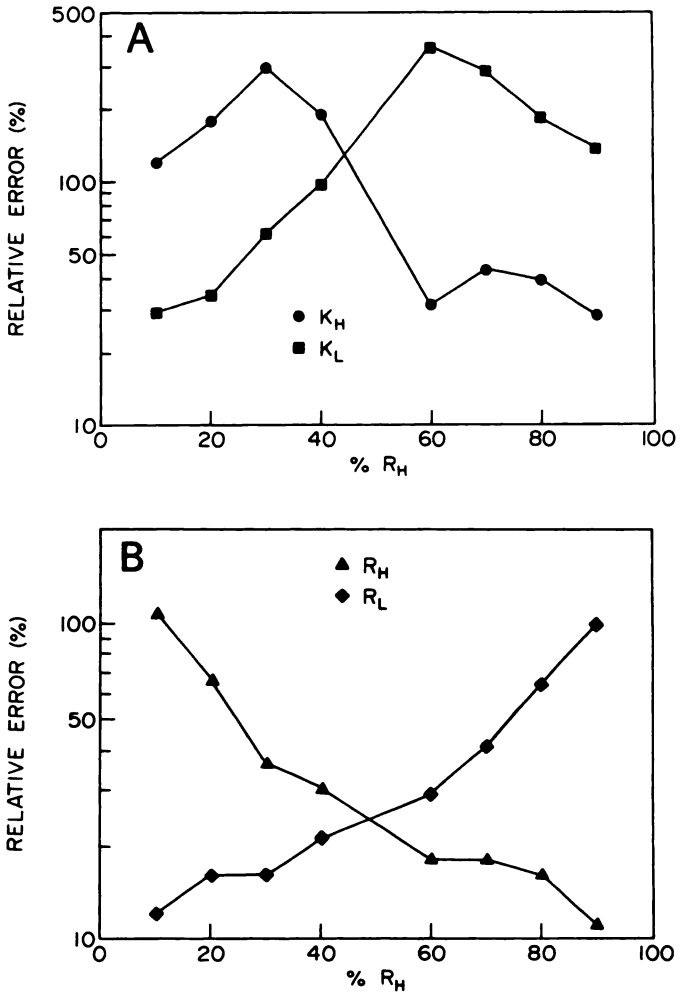

FIG. 8. *Relationship between the relative error of the estimates of the affinity constants K_H and K_L (A) and the receptor subtype concentrations R_H and R_L (B) with the composition of the receptor subtype mixture ($\%R_H$)*

The relative errors are calculated by dividing the standard error of 30 estimates from simulated experiments by their arithmetic average under conditions corresponding to the resolution boundary shown in Fig. 7 (——).

subpopulation and of its affinity constant for the selective ligand is smaller than the relative error of the corresponding parameter estimates for the lesser component of the mixture. For example, more accurate estimates of K_H (Fig. 8A) and R_H (Fig. 8B) are obtained from the analysis of a mixture with a high percentage of R_H than at a low percentage of R_H. The relative errors of the corresponding parameters K_H and K_L (Fig. 8A) and R_H and R_L (Fig. 8B) are approximately equal when both components of the mixture are about equal (50% R_H). In general, the estimates of the affinity constants K_H and K_L are subject to a larger error than R_H and R_L, with relative errors typically above 100% under conditions corresponding to the 50% resolution boundary (Fig. 8A). However, under conditions resulting in greater than 90% significant resolution of the two components (*clear area* in Fig. 7), the relative errors for all four parameter estimates K_H, K_L, R_H, and R_L drop to 25% or less (data not shown).

Another important finding from the Monte Carlo simulation studies is that, in contrast to the estimates of receptor subtype concentrations R_H and R_L, estimates of the affinity constants K_H and K_L of the selective ligand were not normally distributed, but were more accurately considered as log-normally distributed. Figure 9 compares the average estimates of affinity constants obtained by computer modeling of simulated competition curves with their true values used in the simulation process. Each *point* corresponds to the geometric average (Fig. 9A) or to the arithmetic average (Fig. 9B) of estimates from 30 replicate experiments. As can be seen, the geometric mean provides a strikingly better estimate of the true parameter value of the affinity constant.

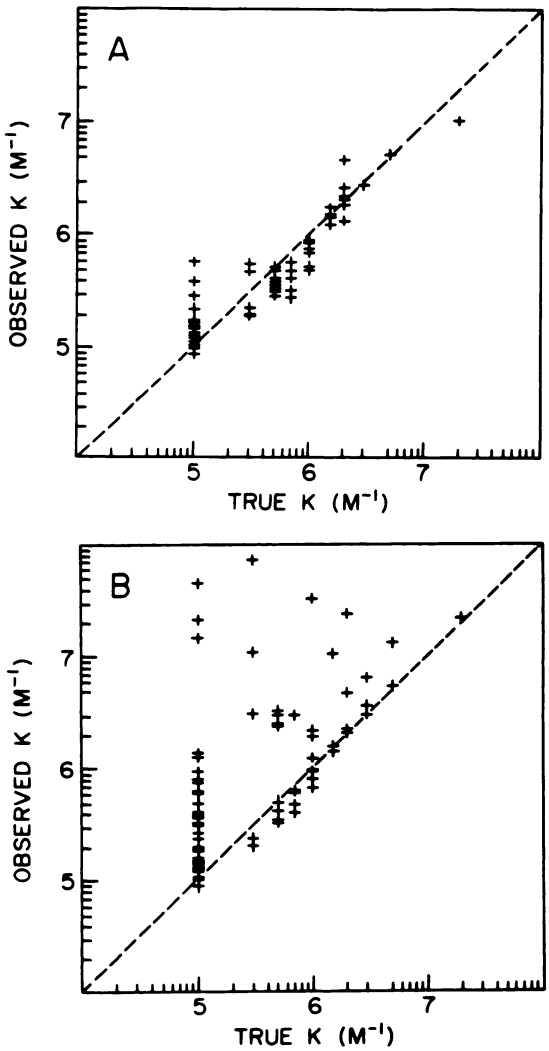

FIG. 9. *Comparison of the geometric mean (A) or the arithmetic mean (B) of the estimates of the affinity constants with their true values*

Each *point* represents the average of 30 determinations obtained by Monte Carlo simulations.

DISCUSSION

Using a dual approach with experimentally derived and simulated binding data, we have demonstrated that computer modeling of competition binding data for selective ligands based on Feldman's general ligand binding model (22) can accurately and reliably provide estimates of the proportions of receptor subtypes and of the affinity constants of selective ligands. The unique advantage of prior knowledge of the binding characteristics of each pure receptor subtype constituent (frog and turkey erythrocyte membranes) and the original experimental approach in creating receptor subpopulation mixtures of predetermined composition, together with the extensive Monte Carlo simulations (2,700 experiments) provide an unprecedented opportunity to test the statistical properties of the parameter estimates of the computer modeling method. Such prospective studies cannot be performed with animal tissue containing given mixtures of receptor subtypes unless each constituent receptor subpopulation can be isolated and assayed separately. Similar studies have not been previously reported for any of the methods currently used to analyze complex binding curves. Our use of controlled mixtures of pure receptor subtypes proved to be essential in the experimental validation of the computer modeling method. A similar experimental approach could be extremely useful for testing the selectivity of drugs for receptor subtypes by using aliquots of a large-scale frozen receptor subtype mixture of known composition.

In the case of experimental data typical of *beta*-adrenergic receptors in pure erythrocyte membranes, characterized by low, nonspecific binding and small standard error of replicate determinations, a 6- to 10-fold selective ligand could be successfully used to resolve various receptor subtype mixtures when the minor component accounts for at least 20% of the mixture. However, a 30- to 100-fold selective ligand was required to resolve mixtures with a smaller contribution of the minor constitutent (<20%). The remarkable agreement between the resolution limits of the computer modeling method delineated by the experimental and simulated binding data strengthens our conclusions and suggests that, in other systems in which extensive testing with multiple replicate experiments would be prohibitive, Monte Carlo simulation methods could be reliably used to define the resolution limits of the system. In cases where there is a larger proportion of nonspecific binding or larger standard error of replicate determinations, the resolution

TABLE 2

Methods for quantitative analysis of receptor subtypes by ligand binding

	Method		
	Analysis of "pseudo-Scatchard" transform of % inhibition of radioligand binding (refs. 2, 3, 5, 6)	Computerized analysis of % inhibition of radioligand binding (refs. 7, 10–12)	Computer modeling of untransformed data on basis of mass-action law (refs. 4, 8, 9, 13, 23)
Data transform	% Inhibition of radioligand binding and "pseudo-Scatchard" plot	% Inhibition of radioligand binding	None
Presence of radioligand	Ignored during data analysis	Ignored during data analysis	Integral part of model
Correction of estimates for affinity constants K	Required after data analysis; Cheng and Prusoff approximate correction (ref. 27)	Required after data analysis; Cheng and Prusoff approximate correction (ref. 27)	Not required
Appropriateness of coordinate system for curve fitting	No: both coordinate variables subject to error and are correlated	Yes	Yes
Statistical estimate of "goodness of fit"	No	Yes	Yes
Comparison of one- and two-component models	No	No	Yes
Simultaneous analysis of several curves	No	No	Yes
Applicability in case of selective radioligand	No	No	Yes
Correction for nonhomogeneity of variance	No	Yes	Yes
Correction for "radioligand depletion"	No	Requires special variant of the method (ref. 28)	Accounted for by model
Minimal computer requirement	Hand calculator or desk-top calculator	Desk-top calculator or minicomputer	Minicomputer or large-scale computer

boundary shown in Fig. 7 would be raised toward higher selectivity ratios of the ligand. In such systems, more selective ligands would be required in order to achieve the same degree of resolution of the components of receptor subtype mixtures.

The computer modeling method based on mass-action law principles used here offers a number of advantages over other methods proposed for the analysis of complex binding isotherms in the context of receptor subtypes. These are summarized in Table 2. Briefly stated, the method we applied is statistically more appropriate because untransformed binding data are used and weighted according to the reciprocal of the expected variance of replicate determinations. The model also includes the radioligand among its components (Fig. 1), contrasting with the other methods which do not and which therefore require correction of the apparent potency estimates of the competing ligand, subsequent to their determination (27). The general model used here (22) also encompasses cases in which the free radioligand concentration varies, as when a significant proportion of the radioligand is bound ("radioligand depletion"). Such conditions are frequently encountered in the use of very high-affinity radioiodinated ligands (28, 29). The model also uniquely accommodates conditions in which the radioligand itself demonstrates different affinity constants for each receptor subtype, as is the case for [^{3}H]DHA in the mixture of turkey and frog erythrocyte membranes used in this study. The failure of other methods to correct for differences in radioligand affinity for the different receptor subtypes can lead to major misestimations of the binding characteristics of the receptor constituent with lower affinity for the radioligand.

Another advantage of the computer modeling approach is that increased statistical reliability can be gained by analyzing several curves simultaneously, irrespective of the competitor ligand used. This is of paramount importance when extreme cases of receptor subtype mixtures are analyzed. As stated under Results, in several cases of mixtures of 90%/10% or 10%/90% adrenergic receptor subtypes, an individual data curve analyzed separately could not document a significant resolution with two components. However, in general more than one ligand was assayed in each experiment, and all competition curves were analyzed simultaneously, providing a common estimate of the mixture composition. Under these circumstances, a trend toward greater accuracy of the estimates of the affinity constant for each competitor could be observed. The accuracy of the parameter estimates could be optimally enhanced when two competitors with reciprocal selectivity for the two receptor subtypes were studied simultaneously, as, for example, in Fig. 4. An additional advantage of simultaneous analysis of several selective competitors is that their potency relationship for each receptor subpopulation can be more accurately defined or verified, as, for example, in Fig. 3 and Table 1.

We have also documented by Monte Carlo simulations the log-normal distribution of estimates of affinity constants about their true mean, as shown in Fig. 9. For this reason, we have reported in Table 1 and utilized for statistical tests the geometric means of experimentally

derived affinity constants. However, the distribution of the estimates of receptor subtype proportions appears to be normally distributed (data not shown) and, therefore, arithmetic means of the receptor proportions were utilized for statistical purposes. It should be recalled that log-normal distribution of efficacy in biological systems has been previously reported (30), but the geometric mean has been seldom utilized in statistical analysis of binding data (7, 13). This possible source of analytical error should be minimized by utilizing geometric means for tests when drug efficacy or potency is involved. The apparently large (>100%) relative error of the estimates of the affinity constants observed under limit conditions for resolution of the two receptor subpopulations (Fig. 8A) was also currently observed in the analysis of individual experimental competition curves. When reinterpreted on a more appropriate logarithmic scale, the size of the errors of the affinity constants more closely match the size of the errors for the concentrations of each receptor subpopulation (Fig. 8B).

In summary, our computer modeling method for the analysis of competition binding data for drugs selective for receptor subtype mixtures has been statistically validated and its resolution limits have been defined. The method has been tested for its ability to provide accurate estimates for the binding parameters corresponding to experimentally derived and simulated mixtures of receptor subpopulations with predetermined characteristics. The method can reliably resolve the components of the mixture using competition curves for even weakly selective ligands, provided that the experimental data are not subject to excessive replicate variation. A similar approach can be used to study the resolution limits of the method in other systems, thereby providing predictions of requirements for the resolution of pharmacological receptor subtypes and the development of new selective drugs. Similar conclusions obtain when the method is applied to the study of high- and low-affinity forms of a single receptor population discriminated by agonists.

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