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Conditions for Biphasic Competition Curves in Radioligand Binding for Ligands Subjected to Metabolic Transformation

Jarl E. S. Wikberg, Maija Dambrova, Staffan Uhlén and Peteris Prusis*

Department of Pharmaceutical Biosciences, Division of Pharmacology, Uppsala University, Uppsala, Sweden

ABSTRACT. The conditions affording biphasic competition curves in radioligand binding for ligands subjected to metabolic transformation was analyzed theoretically. It was shown that when a competing ligand was subjected to transformation to a ligand that showed higher affinity than the parent compound, biphasic competition curves, which might wrongly be interpreted as indicating the presence of two receptor sites, could be observed in binding assays containing a homogenous receptor population. Biphasic competition curves were seen if the conversion of the competitor occurred according to zero and second order kinetics, as well as by enzymatic catalytic processes. However, when the conversion occurred according to a first order kinetics, the competition curves were uniphasic and resolved only into one-site fits, with the apparent affinity of the competitor reflecting the degree of conversion of the competitor to its metabolite. When the metabolic conversion resulted in a metabolite that showed lower affinity for the receptor than the parent compound, the competition curves became supersteep for conversions according to zero and second order kinetics, as well as for conversion by enzymatic catalytic processes. BIOCHEM PHARMACOL **56**;9:1129–1137, 1998. © 1998 Elsevier Science Inc.

KEY WORDS. radioligand binding; metabolic transformation; biphasic competition; mathematical simulation

Radioligand binding has become a standard technique for the analysis of the interactions of ligands with their receptors. The technique is fast and simple and allows the investigation of the pharmacological properties of many drugs within a short time. Used in conjunction with modern data analysis techniques [1, 2], the results obtained are generally very accurate and highly reproducible. However, in the past we grossly misinterpreted radioligand binding data when we suggested the presence of novel subtypes of α_2-adrenoceptors (α_{2A1}, α_{2A2}, α_{2B1}, and α_{2B2}), based on the observation that some hydroxyguanidine compounds yielded strongly biphasic competition curves in certain α_2-adrenoceptor binding assays [3–5]. We then provided data indicating that the biphasic competition curves were instead due to a transformation of the hydroxyguanidines into metabolites that showed higher affinity for the α_2-adrenoceptor than the hydroxyguanidines themselves [6]. Recently, we showed that guanoxabenz,† one of the hydroxyguanidines used in these studies, was converted to guanabenz in the binding assays, the guanabenz showing *ca.* 100-fold higher affinity for the α_2-adrenoceptors compared to guanoxabenz itself [7].

The competition curves obtained with guanoxabenz in the α_2-adrenoceptor binding assays were sometimes biphasic, whereas in other cases they were uniphasic. These differences seemed to be due to a varying capacity of the membranes isolated from different tissues to metabolize the guanoxabenz. For example, when cerebral cortex membranes were used, the guanoxabenz competition curves were uniphasic, with the data analysis indicating that guanoxabenz bound only to one low affinity α_2-adrenoceptor site. However, when spleen membranes were used, guanoxabenz usually gave biphasic competition curves, the data analysis indicating the presence of both high and low affinity binding sites. Moreover, the assay conditions strongly affected the shape of the competition curves. For example, in the spleen, allopurinol decreased the apparent affinity of guanoxabenz, whereas xanthine increased it. These effects were attributed to the inhibition and augmentation of the guanoxabenz-metabolizing activity, respectively, which was presumed to reside in xanthine oxidase [8].

In the present study, we theoretically analyzed the effect of metabolic conversion of ligands on the shape of radioligand binding curves. The data showed that biphasic competition curves could be seen when a ligand was converted into another ligand showing higher affinity than the parent compound when the conversion proceeded according to

* Corresponding author: Dr. Jarl E. S. Wikberg, Pharmaceutical Pharmacology, BMC, Box 591, SE-751 24 Uppsala, Sweden. Tel. +46-18-4714238; FAX +46-18-559718; E-mail: Jarl.Wikberg@farmbio.uu.se

† *Abbreviations:* guanoxabenz, 1-(2,6-dichlorobenzylidene-amino)-3-hydroxyguanidine; guanabenz, 1-(2,6-dichlorobenzylidene-amino)-3-guanidine.

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zero and second order kinetics. In addition, catalytic conversion of a competing ligand under Michaelis–Menten type of kinetics gave rise to biphasic competition curves, whereas first order kinetics yielded only uniphasic competition curves. On the other hand, if the metabolic activity led to inactivation of a compound (i.e. the metabolite showing lower affinity for the receptor than the parent compound), a completely different pattern was seen, the curves becoming supersteep for zero and second order, as well as catalytic type of kinetics. Our results should be useful for the interpretation of radioligand binding data, when a ligand is suspected of being altered by metabolic processes during its assaying.

MATERIALS AND METHODS

The kinetic properties of a number of models were evaluated, their common feature being that a substrate, S, was irreversibly converted to a product, P, by some reaction. The reaction was allowed to proceed for the time t, thereafter the reaction was stopped. The concentration of P would then be dependent upon the concentration of S according to the equation: $P = S_0 - S$ (Eqn 1) where P is the concentration of P and S_0 and S are the concentrations at $t = 0$ and at time t of substrate S.

For all models evaluated, it was further assumed that both the S and P would bind reversibly to some receptor, R. In order to measure the binding of S and P, the radioligand binding technique was used, employing a radioactive ligand (radioligand), L, which also binds reversibly to R. Thus, a reaction mixture containing S, P, and L was added to a suspension of R for sufficient time to allow binding to proceed to equilibrium. The amount of radioligand-receptor complex, LR, was then measured. The procedure was repeated for a number of different initial concentrations of the substrate.

The models explored differed by the type of kinetics describing the conversion of S to P. The evaluated models were zero, first and second order kinetics, and catalytic (Michaelis–Menten) type of kinetics.

For zero and first order kinetics, the reaction can be described as shown in scheme (I):

$$\mathrm{S} \xrightarrow{k} \mathrm{P} \qquad \text{(I)}$$

For zero order kinetics, the rate of reaction is constant: hence $dS/dt = -k$ (Eqn 2). For first order kinetics the rate depends linearly on the concentration of the substrate: hence $dS/dt = -k \cdot S$ (Eqn 3).

Second order kinetics involves two reactants, S and another reactant X as described by scheme (II):

$$\mathrm{X} + \mathrm{S} \xrightarrow{k} \mathrm{P} \qquad \text{(II)}$$

If we assume that X and S react equimolarly, then the concentration of X is related to the concentration of S as follows: $X = X_0 - P = X_0 - S_0 + S$ (Eqn 4). The rate of the reaction could then be described by: $dS/dt = -k \cdot S \cdot X = -k \cdot S \cdot (X_0 - S_0 + S)$ (Eqn 5).

For catalytic (Michaelis–Menten) type of kinetics, the conversion of the S to P is catalyzed by a catalyst, E. The catalyst-substrate complex, ES, is formed by a reversible reaction which then breaks down to P and free catalyst E as shown in scheme (III):

$$\mathrm{S} + \mathrm{E} \underset{k_{E-}}{\overset{k_{E+}}{\rightleftharpoons}} \mathrm{ES} \xrightarrow{k} \mathrm{E} + \mathrm{P} \qquad \text{(III)}$$

The rate of the reaction for scheme (III) is described by: $dS/dt = -k_{E+} \cdot S \cdot E + k_{E-} \cdot ES$ (Eqn 6), where k_{E+} and k_{E-} are rate constants and S, E, and ES denote the concentrations of S, E, and catalyst-substrate complex ES. However, the rate of change in the concentration of ES is time-dependent and described by: $dES/dt = k_{E+} \cdot S \cdot E - k_E \cdot ES - k \cdot ES$ (Eqn 7). The concentration of free catalyst is calculated as follows: $E = E_0 - ES$ (Eqn 8). Moreover, to allow using Eqn 1 for calculation of the concentration of P, we assumed that when the reaction was stopped, the catalyst-substrate complex ES was destroyed.

The amount of radioligand bound to the receptor in the presence of the competing substrate and product is obtained by considering scheme (IV):

$$\begin{aligned} &\mathrm{L} + \mathrm{R} \overset{K_L}{\rightleftharpoons} \mathrm{LR} \\ &\mathrm{S} + \mathrm{R} \overset{K_S}{\rightleftharpoons} \mathrm{SR} \\ &\mathrm{P} + \mathrm{R} \overset{K_P}{\rightleftharpoons} \mathrm{PR} \end{aligned} \qquad \text{(IV)}$$

The concentration of radioligand-receptor complex LR would then be given by equation system (9):

$$\begin{cases} LR = \dfrac{K_L \cdot L_f \cdot R_0}{1 + K_L \cdot L_f + K_S \cdot S_f + K_P \cdot P_f} \\ SR = \dfrac{K_S \cdot S_f \cdot R_0}{1 + K_L \cdot L_f + K_S \cdot S_f + K_P \cdot P_f} \\ PR = \dfrac{K_P \cdot P_f \cdot R_0}{1 + K_L \cdot L_f + K_S \cdot S_f + K_P \cdot P_f} \end{cases} \qquad (9)$$

where LR, SR, and PR are the concentrations of the ligand-receptor complexes of respectively L, S, and P, respectively, with R, K_L, K_S, and K_P affinity constants, R_0 the total (initial) concentration of receptor R, L_f the concentration of free (unbound) radioligand, S_f the concentration of free substrate S, and P_f the concentration of free product P.

The free concentrations of L, P and S (L_f, P_f, and S_f, respectively) may be calculated from the total concentrations of, L, P, and S (L, S, and P, respectively) as follows:

$$\begin{cases} L_f = L - LR \\ S_f = S - SR \\ P_f = P - PR \end{cases} \quad (10)$$

As equation systems 9 and 10 proved to be quadratic, the solution was achieved numerically by using the Newton–Raphsons algorithm, essentially as described [9]. Differential equations were solved analytically, except the catalytic (Michaelis–Menten) kinetics model which was solved numerically by using Euler's method [10].

A set of binding data was generated for each model that generally contained 18 initial concentrations (within the relevant concentration range) of substrate S, assuming a fixed concentration of the radioligand L. The concentrations of radioligand-receptor complex were plotted (y-axis) versus initial concentrations of substrate (x-axis), thus creating so called competition curves. The value of the rate constant k of the conversion reaction of S to P was varied systematically to generate sets of competition curves. In the case of second order kinetics, we also calculated sets of competition curves by systematically varying the initial concentrations of reactant X. Moreover, in the case of catalytic (Michaelis–Menten) kinetics, we calculated sets of competition curves by systematically varying the initial concentration of E.

The shapes of the resulting competition curves are dependent on the affinity of P for R compared to the affinity of S for R. In the following, "activation" will refer to the case when $K_P > K_S$ and "deactivation" to the case when $K_P < K_S$. The affinities of K_S and K_P were, for the activation cases, set close to the values observed experimentally for guanoxabenz and its product guanabenz. (In the following, the units "concentration" and "time" will be used, which may (arbitrarily) be taken to correspond to nM and hr, respectively, in which case the selected numbers scale to values in the range of those often found in real radioligand binding situations). Thus, the following selections were made: $K_S = 2.5 \times 10^{-4}$ concentration^{-1} (i.e. $K_d = 4000$ concentration) and $K_p = 2.5 \times 10^{-2}$ concentration^{-1} (i.e. $K_d = 40$ concentration). For the deactivation cases, the affinities were set so that $K_S = 2.5 \times 10^{-2}$ concentration^{-1} (i.e. $K_d = 40$ concentration) and $K_p = 2.5 \times 10^{-4}$ concentration^{-1} (i.e. $K_d = 4000$ concentration). The affinity of the radioligand was for all cases set to $K_L = 1$ concentration^{-1}, which scales it in the range of that observed for many radioligands when concentration units correspond to nM. The reaction time was, unless otherwise stated in the text, set to 1 time unit.

In order to characterize the simulated radioligand binding data objectively, they were fitted into the equation

$$B_i = \sum_{b=1}^{n} \frac{K_{ib} F_i R_b}{1 + \sum_{a=1}^{m} K_{ab} F_a} \quad (11)$$

where B_i is the amount of ligand i binding to the receptors, K_{ib} and K_{ab} the affinity of, respectively, ligand i and a for receptor b, F_i and F_a the free concentration of ligand i and a, respectively, and R_b the concentration of receptor b, by using non-linear regression analysis, according to the method generally referred to as computer modeling, essentially as described [1]. Equation 11 assumes that reactants bind reversibly to receptor sites according to the law of mass action. Moreover, the free concentrations of ligands in Eqn 11 were calculated from their total concentration present in the assay, as described [1]. The apparent binding constants of S and P for the receptor obtained from the computer modeling calculations are given as dissociation constants K_d (i.e. $K_d = 1/K$).

The data were also fitted to the four parameter logistic function

$$Y = B + \frac{A - B}{1 + \left(\frac{X}{IC_{50}}\right)^{n_H}} \quad (12)$$

In order to estimate IC_{50} values and slope factors (pseudo Hill coefficients; n_H). Nonlinear regressions were performed by using Marquardt's algorithm [11]. All calculations were based on programs written in Metrowerks Pascal on an SPC TS30DO computer.

RESULTS

Analysis of the Effect on the Competition Curves of Ligands Subjected to Activation under Zero, First, and Second Order Kinetics

The effect of metabolic activation (i.e. the affinity of the product being higher than the substrate for the receptor) was studied by assuming that the product was formed with different types of kinetics. In the following examples, it was assumed that the affinity of the substrate and product for the receptor were 2.5×10^{-4} concentration^{-1} and 2.5×10^{-2} concentration^{-1}, respectively.

For zero order kinetics, the concentration of substrate S at time t is obtained by solving Eqn 2, the analytical solution of which is given by

$$S = \begin{cases} S_0 - k \cdot t, & if \quad S > 0 \\ 0, & if \quad S = 0 \end{cases} \quad (13)$$

where S is the concentration of substrate S, S_0 the initial concentration of substrate S, k the rate constant, and t the time.

The appearance of the competition curves resulting from systematic variation of the rate constant, k, in Eqn 13 is illustrated in Fig. 1A (e.g. applying Eqn 13 with Eqn 9 and Eqn 10). As can be seen the competition curves are strongly biphasic. When the data were subjected to standard radio-

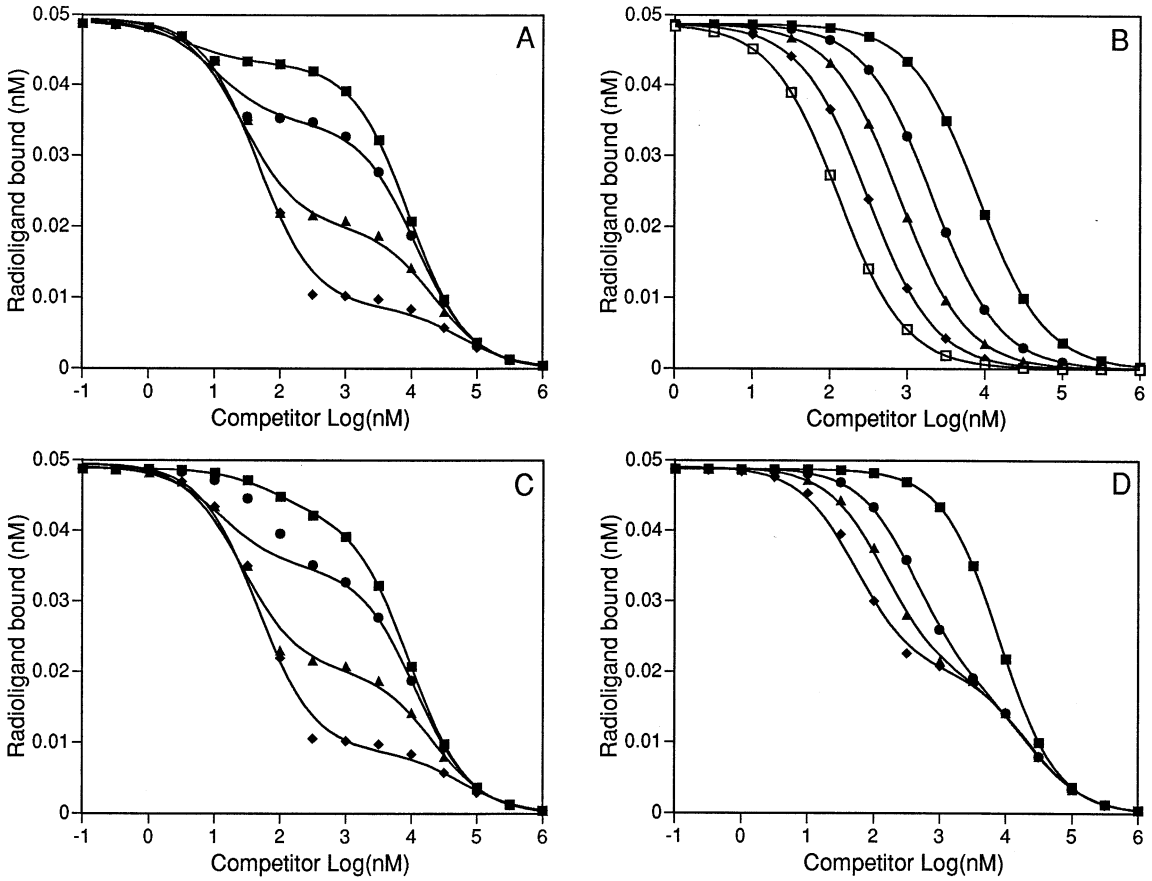

FIG. 1. Simulated radioligand binding competition curves for a substrate subjected to transformation into a product showing higher affinity for a receptor than the original substrate. For the simulations shown in all panels, the affinity of the radioligand for the receptor (K_L) was assumed to be 1 concentration^{-1}, the affinity of the substrate for the receptor (K_S) 2.5 × 10^{-4} concentration^{-1}, and the affinity if the product for the receptor (K_P) 2.5 × 10^{-2} concentration^{-1}. The reaction time for conversion of substrate to product was assumed to be one time unit. The concentration of radioligand added to the assay was set to 1 concentration unit. Shown in panel A are simulations assuming conversion according to zero order kinetics (Eqn 13) with the rate constant (k) being 10 (■), 20 (●), 100 (▲), and 300 (♦) concentration × time^{-1}. Shown in panel B are simulations assuming conversion according to first order kinetics (Eqn 14) with the rate constants (k) being 0 (■), 0.03 (●), 0.1 (▲), 0.3 (♦), and 1 (□) time^{-1}. Shown in panels C and D are simulations assuming conversion according to second order kinetics (Eqn 15). In C the rate constant, k, was held constant at 0.01 concentration x time$^{-1,}$ while the concentration of X was varied systematically as follows: X = 10 (■), 20 (●), 100 (▲), and 300 (♦) concentration units. In D, the concentration of X was held constant at 100 concentration units, while the rate constant k was varied systematically as follows: k = 0 (■), 0.001 (●), 0.003 (▲), and 0.01 (♦) concentration × time^{-1}.

ligand binding data computer modeling (see Materials and Methods), the curves were resolved into two-site fits (Table 1A). As can be seen from Fig. 1A and Table 1A, the apparent proportion of sites varies with the rate of conversion of the competitor. The slope factors (n_H), obtained from fitting the data to the logistic function, were also less than unity, reflecting the biphasicity of the competition curves (Table 1A). It may be noted from Eqn 13 that the variation of incubation time has the same effect as the variation of the rate constant k.

When the metabolic conversion occurs according to first order kinetics, the concentration of substrate S at time t is obtained by solving equation [3], the solution being given by

$$S = S_0 \cdot e^{-k \cdot t} \qquad (14)$$

where S is the concentration of substrate S at time t, S_0 the initial concentration of substrate S and k the rate constant.

The appearance of competition curves resulting from systematic variation of the rate constant, k, in Eqn 14 is illustrated in Fig. 1B. As can be seen from the figure, increasing the rate of metabolic conversion (i.e. increasing k) causes a leftward shift of the competition curves. The competition curves are also steep and uniphasic and resolved into one-site fits by computer modeling, the slope factors of all the competition curves being close to unity (Table 1B). It may be noted from Eqn 14 that varying the incubation time t has the same effect as varying the rate constant k.

For second order kinetics, the concentration of substrate S at time t is obtained by solving Eqn 5, the analytical solution of which is given by

TABLE 1. Results of computer analysis of the simulated competition curves shown in Fig. 1

A. Zero order

k	k_{hi}	k_{lo}	% *Hi*	IC_{50}	n_H
10	1.80	4,580	12.5	7,500	0.89
30	5.14	5,840	30.2	4,550	0.61
100	13.0	11,100	60.3	313	0.31
300	22.6	29,600	83.1	56.6	0.42

B. First Order

k	K_d	IC_{50}	n_H
0	3,990	8,080	0.99
0.03	1,020	2,060	0.99
0.1	384	780	1.00
0.3	150	304	0.99
1.0	63	128	1.00

C. Second order; variation of X_0; $k = 0.01$

X_0	K_{hi}	k_{lo}	% *Hi*	IC_{50}	n_H
10	38.0	4,620	12.8	8,650	0.87
30	32.9	5,880	30.5	4,240	0.64
100	27.1	10,600	59.9	537	0.37
300	25.7	27,200	82.4	43.4	0.43

D. Second order; variation of k; $X_0 = 100$

k	k_{hi}	K_{lo}	% *hi*	IC_{50}	n_H
0	—	3,990	0	8,082	0.99
0.001	218	11,800	64.5	1,710	0.57
0.003	73.7	11,000	61.2	972	0.46
0.01	27.1	10,600	59.7	542	0.37

The data for sections A, C, and D were analyzed by fitting to a model that assumed that ligands bound to two independent receptor sites according to the law of mass action, the K_{hi} and K_{lo} representing the dissociation constants for the apparent high and low affinity sites of the competing, respectively. The % *Hi* represents the apparent proportion of the high affinity site. The data for section B were analyzed by fitting to a model that assumed that ligands bound to one receptor site according to the law of mass action, the K_d representing the apparent dissociation constant of the competitor for the receptor. The data for all sections were also fitted to the logistic function to obtain IC_{50} and n_H values.

$$S = \frac{S_0 \cdot (X_0 - S_0)}{X_0 \cdot e^{k \cdot t \cdot (X_0 - S_0)} - S_0} \qquad (15)$$

where S is the concentration of substrate S at time t, S_0 the initial concentration of substrate S, X_0 the initial concentration of reactant X, and k the rate constant.

The competition curves resulting from systematic variations of X_0 and k in Eqn 15 are illustrated in Fig. 1, panels C and D, respectively. The results of the data analysis are shown in Table 1, sections C and D, respectively. As can be seen from the figures and the table, systematic variation of X_0 results in changes in the apparent proportion of sites, whereas systematic variation of k results primarily in changes in the slope factors, the latter having an effect on the apparent affinity of the competitor for the apparent high affinity site. It can be noted from Eqn 15 that, similarly to the case for first order kinetics the variation of incubation time has the same effect as would the variation of the rate constant k.

Analysis of the Effect on the Competition Curves of Ligands Subjected to Deactivation with Zero, First, and Second Order Kinetics

The effects of metabolic deactivation (i.e. the affinity of the product for the receptor being less than that of the substrate) was studied in an analogous fashion as for metabolic activation in the preceding section. Thus, Eqns 13, 14, and 15 were applied in conjunction with equation Eqn 9 and Eqn 10, assuming that the affinity of the substrate and product were 2.5×10^{-2} concentration^{-1} and 2.5×10^{-4} concentration^{-1}, respectively. A similar set of data as for Fig. 1 was obtained by systematically varying parameters, the results of which are shown in Fig. 2A-D and Table 2. As can be seen from the figures and the table, the metabolic deactivation according to zero and second order kinetics results in supersteep competition curves (i.e. $n_H > 1$). In the case of first order kinetics, the competition curves are shifted in a parallel fashion to the right, the slope factors being close to unity.

Analysis of the Effect on the Competition Curves of Ligands Subjected to Activation According to Catalytic Kinetics

In the case of catalytic (Michaelis–Menten) kinetics, the analytical solution proved difficult to find. The concentration of substrate S after some time t was therefore calculated numerically by solving Eqns 6, 7, and 8 using Euler's method. The competition curves found for different values of rate constant k as well as for different initial concentrations of catalyst E, for the case of metabolic activation, are shown in Fig. 3. The results of the data analysis of the competition curves are shown in Table 3. As can be seen from the figure, competition curves are strongly biphasic for all cases. As can be seen from Table 3, the apparent proportion of high affinity sites are increased with increasing rate constant or increasing concentration of enzyme. Moreover, a similar effect could be shown by increasing the time of incubation (data not shown).

If similar simulations were undertaken under the assumption that the metabolic conversion resulted in deactivation, supersteep competition curves were instead obtained, analogous to the case for zero and second order kinetics (data not shown).

DISCUSSION

As summarized in Table 4, we have shown here that different reaction mechanisms result in biphasic competition curves in radioligand binding assays when a competing ligand is converted into a product that shows higher affinity for the receptor than the original competitor. However, in these cases, supersteep competition curves instead resulted when the product showed lower affinity than the parent compound. An exception to these changes in the slopes of

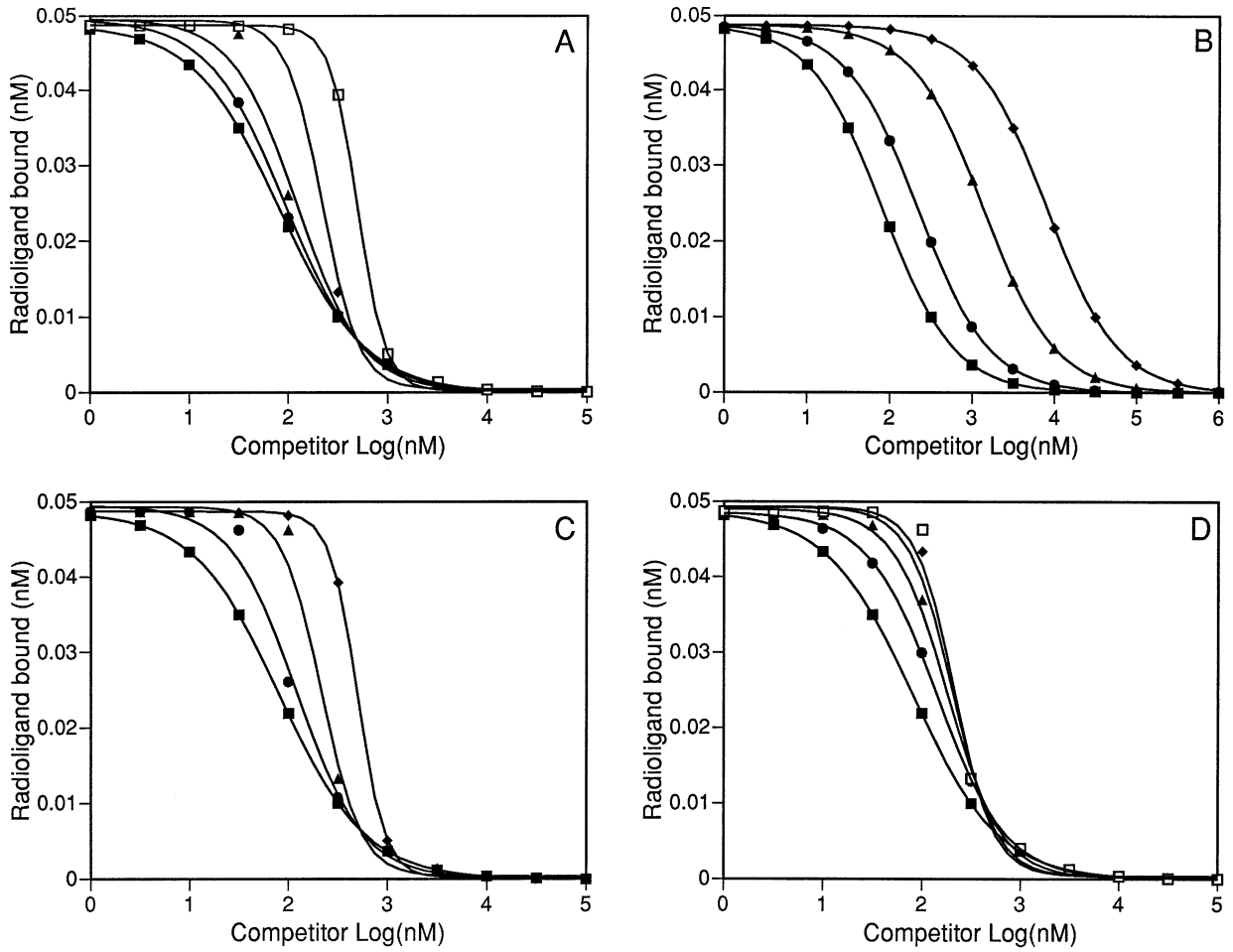

FIG. 2. Simulated radioligand binding competition curves for a substrate subjected to transformation into a product showing lower affinity for a receptor than the original substrate. For the simulations shown in all panels, the affinity of the radioligand for the receptor (K_L) was assumed to be 1 concentration^{-1}, the affinity of the substrate for the receptor (K_S) 2.5 × 10^{-2} concentration^{-1}, and the affinity of the product for the receptor (K_P) 2.5 × 10^{-4} concentration^{-1}. The reaction time for conversion of substrate to product was assumed to be one time unit. The concentration of radioligand added to the assay was set to 1 concentration unit. Panel A shows simulations with zero order, B first order, and C and D second order kinetics. The parameters and symbols for panels A–D were identical to those described in the corresponding section of the legend to Fig. 1.

the competition curves was observed when the metabolism occurred according to first order kinetics. In this case, uniphasic competition curves were seen for both activation and deactivation, resulting only in parallel shifts in the competition curves. The reasons for the difference for first order kinetics versus zero, second order, and catalytic kinetics is that the proportion of the product formed relative to the amount of original compound remaining at the time of assay is always constant for first order kinetics, but not for the other types of kinetics. In fact, first order kinetics results in the same effect as in a dilution series of a mixture of two compounds that are added to a competition assay. In addition, for a dilution series, a uniphasic competition curve with slope factor close to unity results, the apparent affinity of a mixture being dependent on the affinities of the compounds, as well as their proportions in the mixture. (A good example of the latter is racemic mixtures of stereoisomeric compounds. Despite the fact that the isomers most often show different affinities for a receptor, the racemates give uniphasic competition curves that resolve into one-site fits in radioligand binding assays, when one receptor site is present).

From our earlier studies (see Introduction), it is clear that the low affinity α_2-adrenoceptor binder guanoxabenz is converted to the high affinity α_2-adrenoceptor binder guanabenz by an enzymatic process. The above analysis shows that when the conversion occurs under the influence of an enzymatic process, based on Michaelis–Menten type of kinetics, biphasic competition curves are the expected result. This behavior is due to the fact that the rate of conversion of the substrate to its product becomes limited by the saturation of the catalyst above certain concentrations of the substrate. As the conversion of a substance according to first and second order kinetics pertains primarily to non-enzymatic chemical reactions, the generation of biphasic competition curves seems to be the expected behavior of a radioligand binding competition assay when the competitor is subjected to metabolic activation, as this

TABLE 2. Results of computer analysis of the simulated competition curves shown in Fig. 2

A. Zero order

k	IC_{50}	n_H
0	81.2	1.00
10	93.8	1.11
30	122	1.33
100	221	2.38
300	498	3.11

B. First order

k	IC_{50}	n_H
0	81.2	1.00
1	217	1.00
3	1,370	0.99
10	8,000	0.99

C. Second order; variation of X_0; $k = 0.01$

X_0	IC_{50}	n_H
0	81.2	1.00
30	121	1.31
100	215	2.18
300	496	3.10

D. Second order; variation of k; $X_0 = 100$

k	IC_{50}	n_H
0	81.2	1.00
0.01	142	1.24
0.03	182	1.58
0.1	206	1.95
0.3	215	2.18

The data were fitted to the logistic function to obtain IC_{50} and n_H values.

activation would be expected to be catalyzed by an enzymatic activity.

It is interesting to note that for the second order case, the proportion of apparent high and low affinity sites remains almost constant with variation of the rate constant k, when X is held constant, the primary effect being on the apparent affinity of the competitor for the apparent high affinity site (Table 1D). By contrast, variation of X while holding k constant primarily affects the apparent proportions of high and low affinity sites (Table 1C). This behavior could be useful for the design of experiments for the classification of the reaction mechanisms involved in the activation of a ligand in a radioligand binding assay. Moreover, as pointed out in the Results section, variation in reaction time t has the same effect as the variation in k and will be a simpler parameter to vary experimentally.

At this point, we would also like to mention that the binding of ligands to the receptor according to Eqns 9 and 10 assumes that the binding is at its equilibrium. In a real experiment, where the substrate conversion may not have gone to completion at the time of separation of the bound radioligand from the free, this may not be entirely the case. In this case, complex concomitant differential equations would have to be solved in order to exactly describe the binding curves. However, the presently used equations would be approximately valid if the ligand binding occurred rapidly in comparison to the conversion process. Moreover, experiments could quite easily be designed where the conversion is achieved in a separate reaction which is quenched after a certain time. The binding reaction could thereafter be allowed to proceed to equilibrium.

It should also be pointed out that the data of our earlier studies indicate that the conversion of guanoxabenz to guanabenz is a reductive process that requires an additional electron-donating compound (e.g. xanthine or NADH) serving as a co-factor. If the enzymatic reaction requires an

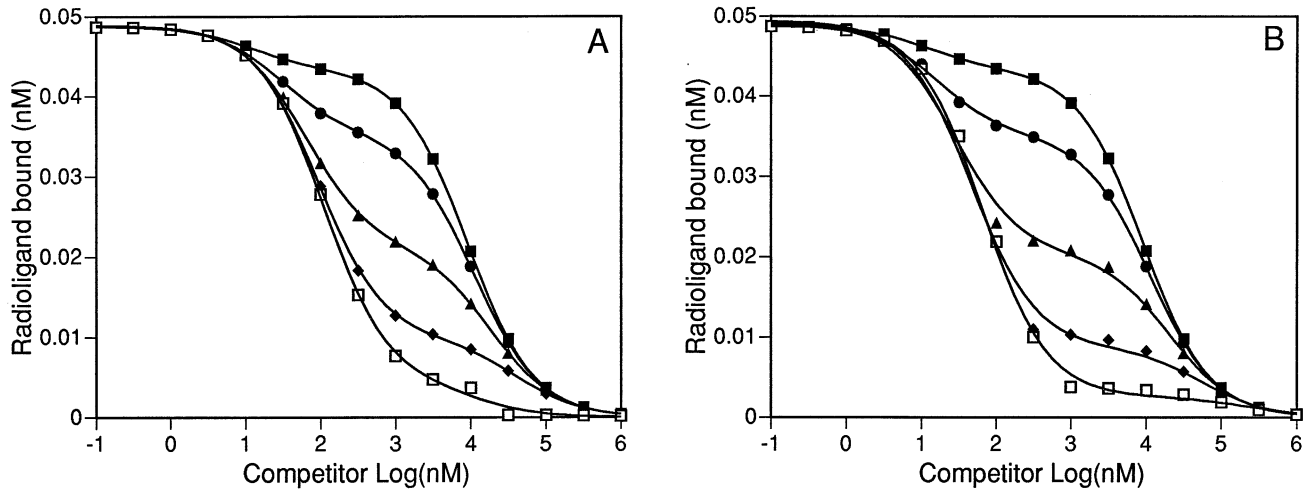

FIG. 3. Radioligand binding competition curves for a substrate subjected to transformation into a product showing higher affinity for a receptor than the original substrate according to catalytic (Michaelis–Menten) type of kinetics obtained from simulations using equations 6–10. For the simulations shown in all panels, affinity of the radioligand for the receptor (K_L) was assumed to be 1 concentration^{-1}, the affinity of the substrate for the receptor (K_S) 2.5 × 10^{-4} concentration^{-1}, and the affinity if the product for the receptor (K_P) 2.5 × 10^{-2} concentration^{-1}. The reaction time for conversion of substrate to product was assumed to be one time unit and the concentration of the radioligand 1 concentration unit. Shown in panel A are simulations where E was set to 0.1, K_{E+} to 10, K_{E-} to 10, and k varied systematically as follows: 100 (■), 300 (●), 1000 (▲), 3000 (♦), and 10000 (□). Shown in panel B are simulations where k was set to 100, K_{E+} to 10, K_{E-} to 10, and E varied systematically as follows: 0.1 (■), 0.3 (●), 1 (▲), 3 (♦), and 10 (□).

TABLE 3. Results of computer analysis of the simulated competition curves shown in Fig. 3

A. Systematic variation of *k*					
k	K_{hi}	K_{lo}	% *Hi*	IC_{50}	n_H
100	6.24	4,520	11.4	7,180	0.88
300	15.1	5,590	28.1	4,480	0.65
1000	31.3	9,430	57.0	722	0.39
3000	45.9	21,500	80.3	156	0.46
10000	53.8	6,030	90.2	129	0.79
B. Systematic variation of *E*					
E	K_{hi}	K_{lo}	% *Hi*	IC_{50}	n_H
0.1	6.24	4,520	11.4	7,180	0.88
0.3	8.02	5,670	28.8	4,490	0.63
1	13.9	10,400	59.3	329	0.32
3	22.9	28,600	82.9	58.3	0.45
10	32.2	128,000	95.0	73.5	0.99

The data were analyzed by fitting to a model that assumed that ligands bound to two independent receptor sites according to the law of mass action, the K_{hi} and K_{lo} representing the dissociation contants for the apparent high and low affinity sites, respectively. The % *Hi* represents the apparent proportion of the high affinity site. The data were also fitted to the logistic function to obtain IC_{50} and n_H values.

additional compound, the above model (III) (see Materials and Methods section) may not be entirely correct, as the enzyme is also expected to bind the co-factor and convert it into a new product. The kinetics is thus expected also to be influenced by the amount of co-factor available. Nevertheless, it is still clear that for such cases as well the competition curves for a compound that becomes metabolically activated should become biphasic. This is because the rate of conversion of the competing ligand is in this case not only restricted by the concentration of the enzyme, the concentration of the substrate and the rate constants for conversion of the substrate, but also on the concentration of the co-factor. Although models taking into account several substrates would not be difficult to construct and might be helpful in explaining the reaction mechanisms of our previous studies on guanoxabenz, we considered them to be beyond the scope of the present study, where we wanted to discover the simplest reaction mechanisms that could generate biphasic competition curves.

TABLE 4. Summary of the properties of radioligand competition assays for a ligand subjected to metabolic transformation

	Activation	Deactivation
Zero order	Shallow	Supersteep
	$n_H < 1$	$n_H > 1$
	Two apparent sites	—
First order	Uniphasic	Uniphasic
	$n_H = 1$	$n_H = 1$
	One apparent site	One apparent site
Second order	Shallow	Supersteep
	$n_H < 1$	$n_H > 1$
	Two apparent sites	—
Catalytical	Shallow	Supersteep
	$n_H < 1$	$n_H > 1$
	Two apparent sites	—

Indicated are the slopes and the slope factors (n_H) of the competition curves and their ability to be resolved into apparent one- or two-site fits by radioligand binding law of mass action computer modeling. It is intended by activation that the affinity of the ligand for the receptor is increased by the metabolic transformation. It is intended by deactivation that the affinity is decreased.

We have extensively searched the literature for examples of metabolic conversion of ligands in radioligand binding assays that resulted in biphasic competition curves. However, we have failed to find any single example, except those from our own studies on guanoxabenz [5] and a hydroxyguanidine analogue of guanoxabenz called LT11 [4], both of which gave biphasic competition curves in assays that utilized single populations of α_2-adrenoceptors. We find the lack of other examples in the literature quite surprising. Metabolic activation of a substance yielding a more active principle is a well-known phenomenon in pharmacology. For example, proguanil (chloroguanide) is converted to the active compound cycloguanil [12, 13] and α-methyldopa is converted to α-methyldopamine and α-methylnoradrenaline, and there exist numerous other similar examples. It is possible that biphasic or shallow competition curves due to metabolic activation in radioligand binding assays have in the past been misinterpreted or overlooked, and we hope that the present study could help in the interpretation of ligand binding data when such metabolic activation is suspected. However, a more common situation is probably that metabolic deactivation takes place in radioligand binding. Many ligands are well known to be subjected to metabolic degradation (e.g. peptide hormones and other natural products used in ligand binding have often been demonstrated to be subjected to degradation by specific enzymatic processes). As shown above, the shape of the competition curves will in these cases become steeper, and the apparent affinity of the ligand accordingly reduced. Of course, the estimation of the affinities of the competitor will in these cases become biased. This source of error in radioligand binding seems in the past to have attracted surprisingly little attention.

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