# **MOLECULAR PHARMACOLOGY, 25:1-9 The Kinetics of Competitive Radioligand Binding Predicted by the Law of Mass Action<br>Mass Action<br>Mass Action**

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## SUMMARY

Although equilibrium competitive radioligand binding studies are often used to characterize hormone and neurotransmitter receptors, the kinetics of such experiments have SUMMARY<br>SUMMARY<br>Although equilibrium competitive radioligand binding studies are often used to characterize hormone and neurotransmitter receptors, the kinetics of such experiments have<br>not been extensively explored. The i SUMMARY<br>
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not been extensively explored. Th SUMMARY<br>Although equilibrium competitive radioligand binding studies are often used to charac-<br>terize hormone and neurotransmitter receptors, the kinetics of such experiments have<br>not been extensively explored. The interac equation and neurotransmitter receptors, the kinetics of such about doct to sinclude their terize hormone and neurotransmitter receptors, the kinetics of such experiments have not been extensively explored. The interaction Find the extensively explored. The interactions of the radioligand and competitor with the receptors can be described by two differential equations which can be solved to yield a single equation describing the binding of t the receptors can be described by two differential equations which can be solved to yield<br>a single equation describing the binding of the radioligand as a function of time. This<br>equation has several applications: First, it a single equation describing the binding of the radioligand as a function of time. The equation has several applications: First, it can be used to simulate competitive bindir reactions under defined conditions. Second, fi equation has several applications: First, it can be used to simulate competitive binding reactions under defined conditions. Second, fitting experimental data to this equation allows one to determine the association and d before equilibrium is reached. Third, mathematical analysis of the binding equation<br>allows one to determine the association and dissociation rate constants of the competing<br>ligand, parameters that cannot be derived from e Fractional and the association and dissociation rate constants of the competitigand, parameters that cannot be derived from equlibrium experiments. Furthermothis method can be used to determine the  $K_l$  of the competing d ligand, parameters that cannot be derived from equilibrium experiments. Furthermore, this method can be used to determine the  $K_I$  of the competing drug from data acquired before equilibrium is reached. Third, mathematica over time. The answers to these questions depended, to a large extent, which can be defined us to answer two specific questions regarding the kinetics of competitive radioli-<br>gand binding: how long such an incubation take before equilibrium is reached. Third, mathematical analysis of the binding equation<br>allowed us to answer two specific questions regarding the kinetics of competitive radioli-<br>gand binding: how long such an incubation take gand binding: how long such an incubation takes to equilibrate, and how the  $IC_{50}$  varies over time. The answers to these questions depended, to a large extent, on the relative values of the dissociation rate constants o gand binding: how long such an incubation takes to equilibrate, and how the IC<sub>50</sub> varies<br>over time. The answers to these questions depended, to a large extent, on the relative<br>values of the dissociation rate constants of values of the dissociation rate constants of the radioligand and competitor, which can be determined as noted above. When the competitor dissociates from the receptors more rapidly than the radioligand, the IC<sub>50</sub> first d determined as noted above. When the competitor dissociates from the receptors morapidly than the radioligand, the  $IC_{50}$  first decreases and then increases, but never ha value less than the  $K_I$ . At low radioligand conce rapidly than the radioligand, the  $IC_{50}$  first decreases and then increases, but never has a value less than the  $K_I$ . At low radioligand concentrations, equilibrium is reached in the same amount of time required of the value less than the  $K_I$ . At low radioligand concentrations, equilibrium is reached in the same amount of time required of the radioligand to dissociate completely from the receptors as determined in an "off-rate experime than does the radioligand, then the time required to equilibrate depends only on the<br>dissociation rate constant of the competitor, and the  $IC_{50}$  decreases over time.<br>
INTRODUCTION<br>
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11 methods for their analysis (1). In some experimental<br>
situations it is necessary or useful to examine competitive<br>
<sup>2</sup> Recipient of a New Investigator Award from t

<sup>1</sup> Recipient of a New Investigator Award from the National Institutes<br>of Health.<br><sup>2</sup> Recipient of a predoctoral National Institutes of Health Training<br>Grant in hypertension.<br><sup>3</sup> Strictly speaking, equilibrium in never "re

of Health.<br>
<sup>2</sup> Recipient of a predoctoral National Institutes of Health Training<br>
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asymptotically approached. In a practical sens asymptotically approached. In a practical sense, however, equilibrium is reached once the binding deviates from its ultimate equilibrium value by an unmeasurable and trivial amount. After five half-lives, this deviation is

binding experiments before equilibrium is reached, but, although the kinetics of competitive binding have been partially described  $(2-4)$ , several questions remain. Using binding experiments before equilibrium is reached, but,<br>although the kinetics of competitive binding have been<br>partially described (2-4), several questions remain. Using<br>a mathematial expression describing the kinetics of binding experiments before equilibrium is reached, but, although the kinetics of competitive binding have been partially described (2–4), several questions remain. Using a mathematial expression describing the kinetics of binding experiments before equinorium is reached, but,<br>
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partially described (2–4), several questions remain. Using<br>
a mathematial expression describing the kinetics partially described (2–4), several questions remain. Using<br>a mathematial expression describing the kinetics of ra-<br>dioligand binding in the presence of a competing ligand,<br>we addressed the following theoretical questions: dioligand binding in the presence of a competing ligand, we addressed the following theoretical questions: How long does a competitive binding experiment take to reach equilibrium? How does a competitive binding curve chan dioligand binding in the presence of a competing ligand,<br>we addressed the following theoretical questions: How<br>long does a competitive binding experiment take to reach<br>equilibrium? How does a competitive binding curve<br>chan we addressed the following theoretical questions. Trow<br>
long does a competitive binding experiment take to reach<br>
equilibrium? How does a competitive binding curve<br>
change over time? How can the dissociation constant of<br>
a a receptor for a<br>non-equilibrium<br>THE MODEL

 $\mathbf{1}$ 

In this paper we consider only a very simple and widely<br>
used model in which the radioligand and competing drugs<br>
each bind reversibly to the receptors with specified ki-<br>
netic constants and according to the law of mass a THE MODEL<br>In this paper we consider only a very simple and widely<br>used model in which the radioligand and competing drugs<br>each bind reversibly to the receptors with specified ki-<br>netic constants and according to the law of In this paper we consider only a very simple and widely<br>used model in which the radioligand and competing drugs<br>each bind reversibly to the receptors with specified ki-<br>netic constants and according to the law of mass act

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MOTULSKY AND MAHAN<br>using the following symbols<sup>4</sup>:  $R$  = receptor,  $L$  = radio<br>gand,  $I$  = competing drug (or inhibitor);  $RL$  = recept 2 MOTULSKY AND MAHAN<br>using the following symbols<sup>4</sup>:  $R$  = receptor,  $L$  = rad<br>gand,  $I$  = competing drug (or inhibitor);  $RL$  = recept<br>radioligand complex,  $RI$  = receptor-competing drug c 2 MOTULSKY AND MAHAN<br>using the following symbols<sup>4</sup>:  $R$  = receptor,  $L$  = radiol:<br>gand,  $I$  = competing drug (or inhibitor);  $RL$  = receptor<br>radioligand complex,  $RI$  = receptor-competing drug com<br>plex: plex:

$$
R + L \underset{k_2}{\overset{k_1}{\rightleftharpoons}} RL
$$
  

$$
R + I \underset{k_4}{\overset{k_3}{\rightleftharpoons}} RI
$$

 $R + I \underset{k_4}{\rightleftharpoons} RI$ <br>Here  $k_1$  and  $k_3$  are the forward-, association-, or on-rate<br>constants for the respective binding of radioligand and<br>competitor to the receptors (in units of min<sup>-1</sup> M<sup>-1</sup>), and  $R + I \rightleftharpoons RI$ <br>Here  $k_1$  and  $k_3$  are the forward-, association-, or on-rate<br>constants for the respective binding of radioligand and<br>competitor to the receptors (in units of min<sup>-1</sup> M<sup>-1</sup>), and P<br> $k_2$  and  $k_4$  are the r Here  $k_1$  and  $k_3$  are the forward-, association-, or on-rate constants for the respective binding of radioligand and competitor to the receptors (in units of min<sup>-1</sup> M<sup>-1</sup>), and  $k_2$  and  $k_4$  are the respective rever constants for the respective binding of radioligand a<br>competitor to the receptors (in units of min<sup>-1</sup> M<sup>-1</sup>), a<br> $k_2$  and  $k_4$  are the respective reverse-, dissociation-, or o<br>rate constants (in units of min<sup>-1</sup>). The e competitor to the receptors (in units of min<sup>-1</sup>  $M^{-1}$ ), and  $h_2$  and  $h_4$  are the respective reverse-, dissociation-, or off-<br>rate constants (in units of min<sup>-1</sup>). The equilibrium dis-<br>sociation constant of the bindin  $k_2$  and  $k_4$  are the respective reverse-, dissociation-, or o<br>rate constants (in units of min<sup>-1</sup>). The equilibrium d<br>sociation constant of the binding of radioligand to rece<br>tor  $(K_D)$  is defined to be  $k_2/k_1$  (units sociation constant of the binding of radioligand to receptor  $(K_D)$  is defined to be  $k_2/k_1$  (units of molar); the equilibrium dissociation constant of competitor to receptor  $(K_I)$  is likewise defined as  $k_4/k_3$ .

To simplify the equations, we have limited our model<br>to the situation in which only a small fraction  $(\leq 10\%)$ <br>of the radioligand and competitor bind to receptors. Thus equilibrium dissociation constant of competitor to receptor  $(K_I)$  is likewise defined as  $k_4/k_3$ .<br>To simplify the equations, we have limited our model<br>to the situation in which only a small fraction (<10%)<br>of the radioli to the situation in which only a small fraction  $(\leq 10\%)$  petitor, and receptors for any particular set of kinetic of the radioligand and competitor bind to receptors. Thus constants and ligand, competitor, and receptor tor  $(K_I)$  is likewise defined as  $k_4/k_3$ .<br>To simplify the equations, we have limited our model<br>to the situation in which only a small fraction (<10%)<br>of the radioligand and competitor bind to receptors. Thus<br>throughout t To simplify the equations, we have finited our moder<br>to the situation in which only a small fraction  $(<10\%)$ <br>of the radioligand and competitor bind to receptors. Thus<br>throughout the experiment the concentrations of free<br>( of the radioligand and competitor bind to receptors. Thus<br>throughout the experiment the concentrations of free<br>(unbound) radioligand and competitor are constants ap-<br>proximately equal to their respective total concentra-<br> (unbound) radioligand and comproximately equal to their restions. This situation is often referred the following equations:<br>The binding reactions and the following equations:<br> $d(RL)/dt = [LMR]$ 

$d[RL]/dt = [L][R]k_1 - [RL]k_2$	ra
$d[RI]/dt = [I][R]k_3 - [RI]k_4$	is
$[R] = N - [RL] - [RI]$	vi
(here [R] is the concentration of free receptors, N is the total concentration of receptors)	h

the total concentration of receptors)<br>The three equations above completely describe the

 $[R] = N - [RL] - [RI]$ <br>
(here  $[R]$  is the concentration of free receptors, N is per<br>
the total concentration of receptors) has<br>
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netics of a competitive binding incubation. S (here  $[R]$  is the concentration of free receptors,  $N$  is<br>the total concentration of receptors)<br>The three equations above completely describe the<br>kinetics of a competitive binding incubation. Solving<br>these differential eq the total concentration of receptors,  $\lambda$  is the total concentration of receptors) has<br>The three equations above completely describe the be<br>kinetics of a competitive binding incubation. Solving<br>these differential equation The three equations above completely describe the<br>kinetics of a competitive binding incubation. Solving<br>these differential equations (Appendix 1) yields an<br>expression defining the amount of radioligand bound to<br>receptors *Nhetics* of a competitive binding incubation. Solving<br>
these differential equations (Appendix 1) yields are<br>
rexpression defining the amount of radioligand bound to<br>
receptors ([*RL*]) as a function of time:<br>  $[RL] = \frac{Nk_$ 

$$
[RL] = \frac{Nk_1[L]}{K_F - K_S} \left[ \frac{k_4(K_F - K_S)}{K_F K_S} + \frac{(k_4 - K_F)}{K_F} \exp(-K_F t) - \frac{(k_4 - K_S)}{K_S} \exp(-K_S t) \right]^{(1)}
$$

Here the following new variables are used:

$$
K_A = k_1[L] + k_2
$$
  
\n
$$
K_B = k_3[I] + k_4
$$
  
\n
$$
K_F = 0.5[(K_A + K_B + \sqrt{(K_A - K_B)^2 + 4k_1k_3[L][I])}]
$$
  
\n
$$
K_S = 0.5[(K_A + K_B - \sqrt{(K_A - K_B)^2 + 4k_1k_3[L][I])}]
$$
  
\n\*The abbreviations used are: R, receptor; L, radioligand; RL, receptor-radioligand complex; I, competitor; RI, receptor-compactitor com-

The abbreviations used are: R, receptor; L, radioligand; RL, receptor-radioligand complex; I, competitor; RI, receptor-competitor complex;  $k_1$ , association rate of radioligand;  $k_2$ , dissociation rate of radioligand; plex;  $k_1$ , association rate of radioligand;  $k_2$ , dissociation rate of radioligand;  $k_3$ , association rate of competitor;  $k_4$ , dissociation rate of competitor;  $N$ , total concentration of receptors;  $K_A$ ,  $K_B$ ,  $K_F$ [<sup>125</sup>I]iodocyanopindolol.

radioligand binding. As equilibrium is approached, the two exponential terms approach zero and may be ignored.<br>The equation then reduces to: The general properties of Eq. 1 are as expected. At  $me = 0$ , the equation reduces to zero; there is no The general properties of Eq. 1 are as expected. At time  $= 0$ , the equation reduces to zero; there is no radioligand binding. As equilibrium is approached, the The general properties of Eq. 1 are as expected. At time  $= 0$ , the equation reduces to zero; there is no radioligand binding. As equilibrium is approached, the two exponential terms approach zero and may be ignored. The general properties of Eq. 1 are as expected. At<br>time = 0, the equation reduces to zero; there is no<br>radioligand binding. As equilibrium is approached, the<br>two exponential terms approach zero and may be ignored.<br>The eq

The equation then reduces to:  
\n
$$
[RL] = \frac{Nk_1k_4[L]}{K_FK_S} = \frac{N[L]}{K_D \left(1 + \frac{[I]}{K_I} + \frac{[L]}{K_D}\right)}
$$
\nIn order to compare the binding of radioligand in the  
\nresence of competitor with the binding of radioligand

Fresh  $K_D\left(1+\frac{[I]}{K_I}+\frac{[L]}{K_D}\right)$ <br>In order to compare the binding of radioligand in the<br>presence of competitor with the binding of radioligand<br>alone, we also need the equation describing the binding alone, we also need the binding of radioligand in the presence of competitor with the binding of radioligand alone, we also need the equation describing the binding of radioligand alone to the receptors (1): In order to compare the binding of radio<br>presence of competitor with the binding c<br>alone, we also need the equation describin<br>of radioligand alone to the receptors (1):<br> $h_{\text{c}} N[1]$ 

$$
[RL] = \frac{k_1 N[L]}{K_A} [1 - \exp(-K_A t)] \tag{2}
$$

of radioligand alone to the receptors (1):<br>  $[RL] = \frac{k_1 N[L]}{K_A} [1 - \exp(-K_A t)]$  (2)<br>
Using Eqs. 1 and 2, one can easily program a computer<br>
to simulate the competitive interactions of ligand, com- $[RL] = \frac{k_1 N[L]}{K_A} [1 - \exp(-K_A t)]$  (2<br>Using Eqs. 1 and 2, one can easily program a compute<br>to simulate the competitive interactions of ligand, com-<br>petitor, and receptors for any particular set of kineti  $[KL] = \frac{K_A}{K_A} [1 - \exp(-K_A t)]$  (2)<br>Using Eqs. 1 and 2, one can easily program a computer<br>to simulate the competitive interactions of ligand, com-<br>petitor, and receptors for any particular set of kinetic<br>constants and ligand, c Using Eqs. 1 and 2, one can easily program a computo simulate the competitive interactions of ligand, copetitor, and receptors for any particular set of kine constants and ligand, competitor, and receptor concentrations. B Using Eqs. 1 and 2, one can easily program a comput<br>to simulate the competitive interactions of ligand, con<br>petitor, and receptors for any particular set of kinet<br>constants and ligand, competitor, and receptor concer-<br>trat to simulate the competitive interactions of ligand, competitor, and receptors for any particular set of kinetic constants and ligand, competitor, and receptor concentrations. By mathematically manipulating those equations, constants and ligand, competitor, and receptor concentrations. By mathematically manipulating those equations, one can also solve more general problems, as we do under Appendix and discuss below.

## WHEN IS EQUILIBRIUM ESTABLISHED?

In the absence of inhibitor, the rate at which the radioligand binds to receptor is determined by the exponential term  $\exp(-K_A t)$ . The half-life for this binding is  $0.69/K_A$ . After five half-lives,  $3.5/K_A$ , equilibrium is WHEN IS EQUILIBRIUM ESTABLISHED!<br>In the absence of inhibitor, the rate at which the<br>radioligand binds to receptor is determined by the ex-<br>ponential term  $\exp(-K_A t)$ . The half-life for this binding<br>is  $0.69/K_A$ . After five h In the absence of inhibitor, the rate at which the radioligand binds to receptor is determined by the exponential term  $\exp(-K_A t)$ . The half-life for this binding is  $0.69/K_A$ . After five half-lives,  $3.5/K_A$ , equilibrium in radioligand binds to receptor is determined by the exponential term  $exp(-K_A t)$ . The half-life for this bindin<br>is  $0.69/K_A$ . After five half-lives,  $3.5/K_A$ , equilibrium<br>virtually reached as binding deviates from its true equ<br> ponential term  $\exp(-K_A t)$ . The half-life for this binding<br>is  $0.69/K_A$ . After five half-lives,  $3.5/K_A$ , equilibrium is<br>virtually reached as binding deviates from its true equi-<br>librium value by less than  $3\%$ . In the prese is  $0.69/K_A$ . After five half-lives,  $3.5/K_A$ , equilibrium is virtually reached as binding deviates from its true equilibrium value by less than  $3\%$ . In the presence of competitor the situation is more complicated. Severa virtually reached as binding deviates from its true equi-<br>librium value by less than 3%. In the presence of com-<br>petitor the situation is more complicated. Several authors<br>have pointed out that it takes longer for equilibr librium value by less than  $3\%$ . In the presence of competitor the situation is more complicated. Several author have pointed out that it takes longer for equilibrium to be established when an inhibitor is present  $(2-4)$ lished. we pointed out that it takes longer for equilibrium to established when an inhibitor is present  $(2-4)$ , but no neral rule defining how long it takes has been pubhed.<br>Competitive binding experiments are commonly per-<br>rmed

be established when an inhibitor is present (2–4), but no<br>general rule defining how long it takes has been pub-<br>lished.<br>Competitive binding experiments are commonly per-<br>formed with a single concentration of radioligand an general rule defining how long it takes has been pub<br>lished.<br>Competitive binding experiments are commonly per<br>formed with a single concentration of radioligand and a<br>variety of concentrations of competitor in order to genlished.<br>Competitive binding experiments are commonly per-<br>formed with a single concentration of radioligand and a<br>variety of concentrations of competitor in order to gen-<br>erate a competitive binding curve. The time require Competitive binding experiments are commonly per-<br>formed with a single concentration of radioligand and a<br>variety of concentrations of competitor in order to gen-<br>erate a competitive binding curve. The time required for<br>th the concentration of competitor in order to generate a competitive binding curve. The time required for<br>the incubations to reach equilibrium depends, in part, on<br>the concentration of competitor present. We first con-<br>side erate a competitive binding curve. The time required for<br>the incubations to reach equilibrium depends, in part, on<br>the concentration of competitor present. We first con-<br>sider the approach to equilibrium when the competit the incubations to reach equilibrium depends, in part, on<br>the concentration of competitor present. We first con-<br>sider the approach to equilibrium when the competitor<br>concentration is equal to its equilibrium IC<sub>50</sub>. The extreme the approach to equilibrium when the competitor<br>concentration is equal to its equilibrium  $IC_{50}$ . The time<br>required for this to occur depends on the relative values<br>of  $k_2$  and  $k_4$  (Appendix 2), and we conside ncentration is equal to its equilibrium IC<sub>50</sub>. The time<br>quired for this to occur depends on the relative values<br> $k_2$  and  $k_4$  (Appendix 2), and we consider the two<br>tremes: first when  $k_4 \ll k_2$  and then when  $k_4 \gg k_2$ 

required for this to occur depends on the relative values<br>of  $k_2$  and  $k_4$  (Appendix 2), and we consider the two<br>extremes: first when  $k_4 \ll k_2$  and then when  $k_4 \gg k_2$ .<br>When the dissociation rate of the unlabeled comp of  $k_2$  and  $k_4$  (Appendix 2), and we consider the two extremes: first when  $k_4 \ll k_2$  and then when  $k_4 \gg k_2$ .<br>When the dissociation rate of the unlabeled competitor is much slower than that of the radioligand  $(k_4 \ll k$ extremes: first when  $k_4 \ll k_2$  and then when  $k_4 \gg k_2$ .<br>When the dissociation rate of the unlabeled competitor<br>is much slower than that of the radioligand  $(k_4 \ll k_2)$ ,<br>equilibrium at the IC<sub>50</sub> is reached at 1.75/ $k_4$ . equilibrium at the  $IC_{50}$  is reached at 1.75/ $k<sub>4</sub>$ . Note that is much slower than that of the radioligand  $(k_4 \ll k_2)$ , equilibrium at the  $IC_{50}$  is reached at  $1.75/k_4$ . Note that in this case the concentration and kinetic constants of the radioligand do not matter. This relationsh the radioligand do not matter. This relationship is only useful experimentally when  $k_4$  is known or can be estimated (see below).<br>In many experimental protocols the radioligand dissociates from receptors more slowly tha this case the concentration and kinetic constants of<br>this case the concentration and kinetic constants of<br>e radioligand do not matter. This relationship is only<br>eful experimentally when  $k_4$  is known or can be esti-<br>ated

the radioligand do not matter. This relationship is onluseful experimentally when  $k_4$  is known or can be est mated (see below).<br>In many experimental protocols the radioligand dissociates from receptors more slowly than petitor  $(k_2 \ll k_4)$ . As shown in Appendix 2, the length of time required to reach equilibrium at the  $IC_{50}$  depends mated (see below).<br>In many experimental protocols the radioligand dis-<br>sociates from receptors more slowly than does the com-<br>petitor  $(k_2 \ll k_4)$ . As shown in Appendix 2, the length of<br>time required to reach equilibrium a In many experimental protocols the radioligand dis-<br>sociates from receptors more slowly than does the com-<br>petitor  $(k_2 \ll k_4)$ . As shown in Appendix 2, the length of<br>time required to reach equilibrium at the IC<sub>50</sub> depend petitor  $(k_2 \ll k_4)$ . As shown in Appendix 2, the length of time required to reach equilibrium at the IC<sub>50</sub> depends on the radioligand concentration. When the radioligand concentration is low  $([L] \ll K_D)$ , the time required

same in the absence of competitor as in the presence of competitor,  $3.5/k_2$ . As the radioligand concentration is k<br>same in the absence of competitor as in the presence of<br>competitor,  $3.5/k_2$ . As the radioligand concentration is<br>increased infinitely, the time required to reach equilibsame in the absence of competitor as in the presence<br>competitor,  $3.5/k_2$ . As the radioligand concentration<br>increased infinitely, the time required to reach equil<br>rium is only halved to  $1.75/k_2$  (Fig. 1; Appendix 2). same in the absence of competitor as in the presence of competitor,  $3.5/k_2$ . As the radioligand concentration is increased infinitely, the time required to reach equilibrium is only halved to  $1.75/k_2$  (Fig. 1; Appendix same in the absence of competitor as in the presence<br>competitor,  $3.5/k_2$ . As the radioligand concentration<br>increased infinitely, the time required to reach equil<br>rium is only halved to  $1.75/k_2$  (Fig. 1; Appendix 2).<br>the competitor,  $3.5/k_2$ . As the radioligand concentration is<br>increased infinitely, the time required to reach equilib-<br>rium is only halved to  $1.75/k_2$  (Fig. 1; Appendix 2). In<br>the absence of competition, however, the rate o tration. Thus the time required to reach equilibration.<br>Thus is only halved to  $1.75/k_2$  (Fig. 1; Appendix 2).<br>the absence of competition, however, the rate of radiologand binding increases linearly with radioligand conce rium is only halved to  $1.75/k_2$  (Fig. 1; Appendix 2). In the absence of competition, however, the rate of radioligand binding increases linearly with radioligand concentration. Thus the higher the radioligand concentrati the absence of competition, however, the rate of radio gand binding increases linearly with radioligand concentration. Thus the higher the radioligand concentration to be reached in the presence of the compitor and the tim The above analyses assumed that the competitor. Thus the higher the radioligand concentration, e greater the disparity between the time required for ullibrium to be reached in the presence of the competitor and the time r the greater the disparity between the time required for<br>equilibrium to be reached in the presence of the compet-<br>itor and the time required in its absence.<br>The above analyses assumed that the competitor was<br>present at its

equilibrium to be reached in the presence of the competitor and the time required in its absence.<br>The above analyses assumed that the competitor was<br>present at its equilibrium  $IC_{50}$ . As seen in Fig. 2, the<br>slope of the itor and the time required in its absence.<br>The above analyses assumed that the competitor was<br>present at its equilibrium  $IC_{50}$ . As seen in Fig. 2, the<br>slope of the competitive binding curve decreases slightly<br>over time; present at its equilibrium IC<sub>50</sub>. As seen in Fig. 2, the<br>slope of the competitive binding curve decreases slightly<br>over time; thus the periphery of the curve may not be as<br>close to equilibrium as is the middle of the cur over time; thus the periphery of the curve may not be as close to equilibrium as is the middle of the curve. The time required for the entire curve to reach equilibrium completely depends on the slower of the two dissocia close to equilibrium as is the middle of the curve. The<br>time required for the entire curve to reach equilibrium<br>completely depends on the slower of the two dissociation<br>rate constants  $k_2$  and  $k_4$ . When the radioligand time required for the entire curve to reach equilibrium<br>completely depends on the slower of the two dissociation<br>rate constants  $k_2$  and  $k_4$ . When the radioligand disso-<br>ciates more slowly  $(k_2 < k_4)$ , full equilibrium completely depends on the slower of the two dissociat<br>rate constants  $k_2$  and  $k_4$ . When the radioligand dis<br>ciates more slowly  $(k_2 < k_4)$ , full equilibrium is reach<br>at 3.5/ $k_2$ ; equilibrium is reached most slowly at h rate constants  $k_2$  and  $k_4$ . When the radioligand dissociates more slowly  $(k_2 < k_4)$ , full equilibrium is reached radioliant 3.5/ $k_2$ ; equilibrium is reached most slowly at high concentrations of competitor, where ver at 3.5/ $k_2$ ; equilibrium is reached most slowly at high concentrations of competitor, where very little radioli-<br>gand ever binds. Conversely, in situations where the competitor dissociates more slowly  $(k_4 < k_2)$ , equilib concentrations of competitor, where very little ragand ever binds. Conversely, in situations wher competitor dissociates more slowly  $(k_4 < k_2)$ , equililis reached at  $3.5/k_4$ , and equilibrium is reached slowly at very low gand ever binds. Conversely, in situations where the competitor dissociates more slowly  $(k_4 < k_2)$ , equilibrium is reached most slowly at very low concentrations of competitor.<sup>5</sup> These mathematical relationships can read

is reached at 3.5/ $k_4$ , and equilibrium is reached m<br>slowly at very low concentrations of competitor.<sup>5</sup><br>These mathematical relationships can readily be<br>plied in an experimental context. The value of  $k_2$ <br>routinely dete gand is bound to tissue and the rate at which is bounded in the rate mathematical relationships can readily be applied in an experimental context. The value of  $k_2$  routinely determined in "off-rate" experiments; radioly These mathematical relationships can readily be applied in an experimental context. The value of  $k_2$  is routinely determined in "off-rate" experiments; radioli-<br>gand is bound to tissue and the rate at which it disso-<br>ci routinely determined in "off-rate" experiments; radioli-<br>gand is bound to tissue and the rate at which it disso-<br>ciates is determined after diluting the incubation mixture<br>eor after adding an excess of an unlabeled recept roductly determined in on rate experiments, radiom<br>gand is bound to tissue and the rate at which it disso-<br>ciates is determined after diluting the incubation mixture<br>or after adding an excess of an unlabeled receptor-speciates is determined after diluting the incubation mixture<br>or after adding an excess of an unlabeled receptor-spe-<br>cific drug. The time for essentially all (97%) of the<br>radioligand to dissociate is  $3.5/k_2$ . After incubat or after adding an excess of an unlabeled receptor-spe-<br>cific drug. The time for essentially all  $(97%)$  of the<br>radioligand to dissociate is  $3.5/k_2$ . After incubating that<br>long, all competitive binding experiments in whic cific drug. The time for essentially all  $(97\%)$  of the radioligand to dissociate is  $3.5/k_2$ . After incubating that long, all competitive binding experiments in which  $k_2 \leq k_4$  (regardless of radioligand concentration) long, all competitive binding experiments in which  $k_2 \le k_4$  (regardless of radioligand concentration) will have reached equilibrium. When high concentrations of radioligand ( $\ge 10$   $K_D$ ) are used and  $k_2 \ll k_4$ , equili (regardless of radioligand concentration) will have<br>ached equilibrium. When high concentrations of radiation and  $k_2 \ll k_4$ , equilibrium<br>e IC<sub>50</sub> will have been reached by half that much tim<br>For some radioligands there wi

reached equilibrium. When high concentrations of radioligand  $(\geq 10 K_D)$  are used and  $k_2 \ll k_4$ , equilibrium a<br>the IC<sub>50</sub> will have been reached by half that much time.<br>For some radioligands there will be no problem foll bilgand  $(\geq 10 K_D)$  are used and  $k_2 \ll k_4$ , equilibrium at rece<br>the IC<sub>50</sub> will have been reached by half that much time.<br>For some radioligands there will be no problem follow-<br>ing the guideline derived above. For other the IC<sub>50</sub> will have been reached by half that much time. ut<br>For some radioligands there will be no problem follow-<br>ing the guideline derived above. For other radioligands,<br>however, it may not be feasible to allow an incu For some radioligands there will be no problem following the guideline derived above. For other radioligand however, it may not be feasible to allow an incubation proceed that long. For example, the dissociation raconstan ing the guideline derived above. For other radioligands,<br>however, it may not be feasible to allow an incubation to<br>proceed that long. For example, the dissociation rate<br>constant  $(k_2)$  of  $[^{125}I]ICYP$  from *beta*-adrenerg proceed that long. For example, the dissociation rate<br>constant  $(k_2)$  of  $[^{125}]$ ICYP from *beta*-adrenergic recep-<br>tors on intact S49 lymphoma cells is 0.0045 min<sup>-1</sup> (5). A<br>competitive binding experiment, using a high r constant  $(k_2)$  of  $[^{125}][ICYP]$  from *beta*-adrenergic receptors on intact S49 lymphoma cells is 0.0045 min<sup>-1</sup> (5). A competitive binding experiment, using a high radioligano concentration, would require nearly 400 min t tors on intact S49 lymphoma cells is  $0.0045$  min<sup>-1</sup> (5). A competitive binding experiment, using a high radioligand ti concentration, would require nearly 400 min to reach equilibrium. In many contexts this would be imp concentration, would require nearly 400 min to reach equilibrium. In many contexts this would be impractical. con<br>As can be seen in Fig. 4, the  $IC_{50}$  can change very slowly is required the final phases of the experiment equilibrium. In many contexts this would be impractic As can be seen in Fig. 4, the IC<sub>50</sub> can change very slow during the final phases of the experiment, and an a ceptable approximation of the equilibrium IC<sub>50</sub> may atta during the final phases of the experiment, and an acceptable approximation of the equilibrium  $IC_{50}$  may be attained in less than half the time required for equilibrium to be established. This is best determined experime



FIG. 1. *Effect of radioligand concentration on the time required for a competitive binding experiment to reach equilibrium*<br>The time required for radioligand binding to reach equilibrium in<br>the presence of an IC<sub>80</sub> conc FIG. 1. *Effect of radioligand concentration on the time required for a competitive binding experiment to reach equilibrium* The time required for radioligand binding to reach equilibrium in the presence of an  $IC_{80}$  con the presence of an IC<sub>80</sub> concentration of competitor is plotted against radioligand concentration ([L]). When  $k_4 \leq k_2$  this time does not depend on [L]; if  $k_4 \ll k_2$  the equilibration time is  $1.75/k_4$ ; if  $k_4 = k_2$  radioligand concentration ([L]). When  $k_4 \le k_2$  this time does not depend<br>on [L]; if  $k_4 \ll k_2$  the equilibration time is  $1.75/k_4$ ; if  $k_4 = k_2$  the<br>equilibration time is  $3.5/k_4$ . When  $k_2 \ll k_4$  the situation is more<br> on [*L*]; if  $k_4 \ll k_2$  the equilibration time is 1.75/k<sub>i</sub>; if  $k_4 = k_2$  the equilibration time is 3.5/k<sub>i</sub>. When  $k_2 \ll k_4$  the situation is more complicated. At low radioligand concentrations the time required to reach equilibration time is 3.5/ $k_1$ . When  $k_2 \ll k_1$  the situation is more complicated. At low radioligand concentrations the time required to reach equilibrium is 3.5/ $k_2$ ; this is the same as the time required for 97% of t complicated. At low radioligand concentrations the time required to reach equilibrium is  $3.5/k_2$ ; this is the same as the time required for 97% of the radioligand to dissociate in an "off rate" experiment. When very larg reach equilibrium is  $3.5/k_2$ ; this is the same as the time required for 97% of the radioligand to dissociate in an "off rate" experiment. When very large amounts of radioligand are used, the  $IC_{50}$  is much higher and th 197% of the radioligand to dissociate in an "off rate" experiment. Where we was the radioligand to dissociate in an "off rate" experiment. Where we was the equilibration time is halved. Also shown is the time required fra very large amounts of radioligand are used, the  $IC_{60}$  is much higher and<br>the equilibration time is halved. Also shown is the time required for<br>radioligand binding to reach equilibrium in the absence of competition.<br>time

the equilibration time is halved. Also shown is the time required for<br>radioligand binding to reach equilibrium in the absence of competition.<br>time required for competitive binding curves to reach<br>equilibrium (as long as th radiologiana binding to reach equilibrium in the absence of competition.<br>
time required for competitive binding curves to reach<br>
equilibrium (as long as the system in in a zone A). Nor<br>
does altering the receptor concentra equilibrium (as long as the system in in a zone A). Nor does altering the receptor concentration affect the time required for equilibrium to be reached when radioligand alone binds to receptors. This is because altering th equilibrium (as long as the system in in a zone A). Note altering the receptor concentration affect the time-<br>required for equilibrium to be reached when radioligan<br>alone binds to receptors. This is because altering the<br>re does altering the receptor concentration affect the time<br>required for equilibrium to be reached when radioligand<br>alone binds to receptors. This is because altering the<br>receptor concentration does not change any of the time required for equilibrium to be reached when radioligand<br>alone binds to receptors. This is because altering the<br>receptor concentration does not change any of the time-<br>dependent terms in Eqs. 1 and 2. If, for example, one<br>d receptor concentration does not change any of the time-<br>dependent terms in Eqs. 1 and 2. If, for example, one<br>doubles the number of receptors present, the number of<br>receptors bound by radioligand or competitor each min-<br>ut dependent terms in Eqs. 1 and 2. If, for example, one doubles the number of receptors present, the number of receptors bound by radioligand or competitor each minute will be doubled. But, since there are twice as many rece doubles the number of receptors present, the number of receptors bound by radioligand or competitor each minute will be doubled. But, since there are twice as many receptors present, the time required to reach equilibrium ute will be doubled. But, since there are twice as many<br>receptors present, the time required to reach equilibrium<br>is unchanged.<br>COMPETITIVE BINDING CURVES BEFORE EQUILIBRIUM IS<br>REACHED

## REACHED

during the final phases of the experiment, and an ac-<br>ceptor concentrations of pre-equilibrium<br>ceptable approximation of the equilibrium  $IC_{50}$  may be binding reactions. They concluded that the  $IC_{50}$  increases<br>attained unchanged.<br>MPETITIVE BINDING CURVES BEFORE EQUILIBRIUM IS<br>ACHED<br>Now that we have derived expressions defining the<br>ne required for competitive binding incubation to reach COMPETITIVE BINDING CURVES BEFORE EQUILIBRIUM IS<br>REACHED<br>Now that we have derived expressions defining the<br>time required for competitive binding incubation to reach<br>equilibrium, we turn to the next question: What do EXAMPLETTIVE BINDING CORVES BEFORE EQUILIBRIOM IS<br>REACHED<br>Now that we have derived expressions defining the<br>time required for competitive binding incubation to reach<br>equilibrium, we turn to the next question: What do<br>compe Now that we have derived expressions defining the<br>time required for competitive binding incubation to reach<br>equilibrium, we turn to the next question: What do<br>competitive binding curves look like before equilibrium<br>is reac time required for competitive binding incubation to reach<br>equilibrium, we turn to the next question: What do<br>competitive binding curves look like before equilibrium<br>is reached? Ehlert *et al.* (3) approached this question equilibrium, we turn to the next question: What do competitive binding curves look like before equilibrium is reached? Ehlert *et al.* (3) approached this question by performing numerical simulations of pre-equilibrium bi competitive binding curves look like before equilibrium<br>is reached? Ehlert *et al.* (3) approached this question by<br>performing numerical simulations of pre-equilibrium<br>binding reactions. They concluded that the IC<sub>50</sub> inc is reached? Ehlert *et al.* (3) approached this question by performing numerical simulations of pre-equilibrium binding reactions. They concluded that the  $IC_{50}$  increases over time if  $k_2 < k_4$  and decreases if  $k_4 < k_2$ performing numerical simulations of pre-equilibri<br>binding reactions. They concluded that the  $IC_{50}$  increa<br>over time if  $k_2 < k_4$  and decreases if  $k_4 < k_2$ . Th<br>generalizations were based on simulations of seve<br>binding c binding reactions. They concluded that the IC<sub>50</sub> increases<br>over time if  $k_2 < k_4$  and decreases if  $k_4 < k_2$ . These<br>generalizations were based on simulations of several<br>binding curves at a few time points. We used an ana points. Incruinductions were stated on simulations of see<br>Inding curves at a few time points. We used an an<br>al approach to prove these generalizations. As show, the situation is more complicated at early<br>ints.<br>To understand the ch ical approach to prove these generalizations. As shown<br>below, the situation is more complicated at early time<br>points.<br>To understand the changing positions of non-equilib-<br>rium competition curves, it is instructive to compa

below, the situation is more complicated at early time<br>points.<br>To understand the changing positions of non-equilib-<br>rium competition curves, it is instructive to compare first<br>the kinetics of radioligand binding in the abs points.<br>To understand the changing positions of non-equi-<br>rium competition curves, it is instructive to compare if<br>the kinetics of radioligand binding in the absence and<br>the presence of competitor (Fig. 3A). Because comp<br>t rium competition curves, it is instructive to compare first<br>the kinetics of radioligand binding in the absence and in<br>the presence of competitor (Fig. 3A). Because competi-<br>tion binding curves are displayed as the ratio of the kinetics of radioligand binding in the absence and in the presence of competitor (Fig. 3A). Because competi-

Altering the receptor concentration does not affect the<br><sup>5</sup> Our definition of equilibrium,  $3.5/k_2$  and  $3.5/k_4$ , may be practically<br>irrelevant at extreme concentrations of competitor. At very high com-<br>petitor concentrat Findering the receptor concentration does not arect the<br><sup>6</sup> Our definition of equilibrium,  $3.5/k_2$  and  $3.5/k_4$ , may be practical<br>irrelevant at extreme concentrations of competitor. At very high com-<br>petitor concentratio <sup>6</sup> Our definition of equilibrium,  $3.5/k_2$  and  $3.5/k_4$ , may be practically irrelevant at extreme concentrations of competitor. At very high competitor concentrations, virtually no radioligand will *ever* bind, and  $\frac{1$ <sup>6</sup> Our definition of equilibrium,  $3.5/k_2$  and  $3.5/k_4$ , may be practivelevant at extreme concentrations of competitor. At very high opetitor concentrations, virtually no radioligand will *ever* bind, "equilibrium" will <sup>6</sup> Our definition of equilibrium,  $3.5/k_2$  and  $3.5/k_4$ , may be practically irrelevant at extreme concentrations of competitor. At very high competitor concentrations, virtually no radioligand will *ever* bind, and "equi equilibration to concentrations, virtually no radioligand will ever bind, and "equilibrium" will be reached instantaneously. At very low concentrations of competitor, the competitor can be essentially ignored and the equi **Produce the equilibrium**" will be reached instantaneously. At very low concentrations of competitor, the competitor can be essentially ignored and the equilibration time is that of the radioligand alone,  $3.5/K_A$ . However Expansion will be competitor can be essentially ignoted.<br>
Equilibration time is that of the radioligand alone,  $3.5/K$ ,<br>
by deriving the equilibration time for these extreme cases<br>
that all parts of the competition curve wi



*time*

I og COMPETITORJ (M)<br>FIG. 2. Change in apparent "slope factor" or "psuedo-Hill slope" of<br>Simulated competitive binding experiments are shown on a normal-<br>discale (top) and on an absolute scale (bottom). To calculate the Simulated competitive binding experiments are shown on a normalized scale *(top)* and on an absolute scale *(bottom)*. To calculate these curves, the following values were used: [radioligand] =  $35 \text{ pm}$ ,  $k_1 = 5.86$ curves, the following values were used: [radioligand] = 35 pM,  $k_1 = 5.86$ <br>x 10<sup>8</sup> min<sup>-1</sup> M<sup>-1</sup>,  $k_2 = 0.0045$  min<sup>-1</sup> [thus  $K_D = 7.6$  pM; these constants<br>are those of  $\binom{125}{1}$ [CYP binding to *beta*-adrenergic recept Simulated competitive binding experiments are shown on a normalized scale (top) and on an absolute scale (bottom). To calculate these<br>curves, the following values were used: [radioligand] = 35 pM,  $k_1 = 5.86 \times 10^8$  min<sup>-</sup> ized scale (top) and on an absolute scale (bottom). To calculate these<br>curves, the following values were used: [radioligand] = 35 pM,  $k_1 = 5.86 \times 10^8$  min<sup>-1</sup> M<sup>-1</sup>,  $k_2 = 0.0045$  min<sup>-1</sup> [thus  $K_D = 7.6$  pM; these const  $f(x)$   $f(x)$ are those of  $[{}^{125}]$ ICYP binding to be<br>cells (5)],  $k_3 = 1000k_1$ ,  $k_4 = 100k_2$ . Th<br>following time points (minutes:  $a = 1$ ,<br>1296. The respective "slope factors"<br>curve) are 1.1, 1.0, 1.2, 1.2, and 1.0. Sens (3)],  $\kappa_3 = 1000\kappa_1$ ,  $\kappa_4 = 100\kappa_2$ . The curves were calculated at the following time points (minutes:  $a = 1$ ,  $b = 6$ ,  $c = 36$ ,  $d = 216$ , and  $e = 1296$ . The respective "slope factors" (calculated at the IC<sub>50</sub>

1296. The respective "slope factors" (calculated at the  $IC_{50}$  of eac<br>curve) are 1.1, 1.0, 1.2, 1.2, and 1.0.<br>3B. At the very earliest time points the binding of radio<br>oligand is unaffected by the presence of (unbound) c petitor; thus binding in the presence of competitor; thus binding in the presence of (unbound) competitor; thus binding in the presence of competitor is  $100\%$  of the binding in its absence. This ratio immedi-3B. At the very earliest time points the binding of repligand is unaffected by the presence of (unbound) copetitor; thus binding in the presence of competito:<br>100% of the binding in its absence. This ratio immediely decrea 3B. At the very earliest time points the binding of radi-<br>oligand is unaffected by the presence of (unbound) com-<br>ompetitor; thus binding in the presence of competitor is<br> $100\%$  of the binding in its absence. This ratio value. titor; thus binding in the presence of competitor is 0% of the binding in its absence. This ratio immedi-<br>by decreases and eventually reaches its equilibrium<br>lue.<br>When the radioligand dissociates from the receptors<br>pre ra

100% of the binding in its absence. This ratio immediately decreases and eventually reaches its equilibrium calculum.<br>
When the radioligand dissociates from the receptors  $\frac{m_0}{m_0}$  or rapidly than does the competitor value. When the radioligand dissociates from the receptors<br>more rapidly than does the competitor  $(k_2 > k_4)$ , the<br>binding of radioligand in the presence of the competitor<br>overshoots its equilbrium value: at some intermedia When the radioligand dissociates from the receptors<br>more rapidly than does the competitor  $(k_2 > k_4)$ , the<br>binding of radioligand in the presence of the competitor<br>overshoots its equilbrium value: at some intermediate<br>time When the radionigand dissociates from the receptors<br>
more rapidly than does the competitor  $(k_2 > k_4)$ , the<br>
binding of radioligand in the presence of the competitor<br>
overshoots its equilibrium value: at some intermediate<br> absence of competitor, however, the radioligand binding presence of rapidly dissociating (upper curve) or slowly dissociating<br>in the presence of the competitor constantly decreases (lower curve) competition is displayed as overshoots its equilbrium value: at some intermediate time points there is more radioligand bound to receptors than there will be at equilibrium (ref. 2; Appendix 3). Fixtynessed as a percentage of radioligand binding in than there will be at eq<br>Expressed as a percentag<br>absence of competitor, h<br>in the presence of the co<br>as is shown in Fig. 3B.<br>When the radioligand pressed as a percentage of radioligand binding in the<br>sence of competitor, however, the radioligand binding<br>the presence of the competitor constantly decreases,<br>is shown in Fig. 3B.<br>When the radioligand dissociates more s

absence of competitor, however, the radioligand binding<br>in the presence of the competitor constantly decreases,<br>as is shown in Fig. 3B.<br>When the radioligand dissociates more slowly than the<br>competitor  $(k_2 < k_4)$ , the spec as is shown in Fig. 3B.<br>
When the radioligand dissociates more slowly than the used<br>
competitor  $(k_2 < k_4)$ , the specific binding does not over-<br>
shoot its equilibrium value but rather monotonically<br>
approaches that equili When the radioligand dissociates more slowly than the micrompetitor  $(k_2 < k_4)$ , the specific binding does not overshoot its equilibrium value but rather monotonically approaches that equilibrium (Fig. 3A). Expressed as a competitor  $(k_2 < k_4)$ , the specific binding does not over-<br>shoot its equilibrium value but rather monotonically<br>approaches that equilibrium (Fig. 3A). Expressed as a For-<br>percentage of the radioligand binding in the absen

percentage drops, then it increases, as shown in Fig. 3B.<br>At equilibrium the properties of a competitive binding<br>curve are determined by the  $K_I$  of the competitor, and percentage drops, then it increases, as shown in Fig. 3B.<br>At equilibrium the properties of a competitive binding<br>curve are determined by the  $K_I$  of the competitor, and<br>every competitor with a given  $K_I$  will yield the sa percentage drops, then it increases, as shown in Fig. 3B.<br>At equilibrium the properties of a competitive binding<br>curve are determined by the  $K_I$  of the competitor, and<br>every competitor with a given  $K_I$  will yield the sa percentage drops, then it increases, as shown in Fig. 3B.<br>At equilibrium the properties of a competitive binding<br>curve are determined by the  $K_I$  of the competitor, and<br>every competitor with a given  $K_I$  will yield the sa curve are determined by the  $K_I$  of the competitor, and<br>every competitor with a given  $K_I$  will yield the same<br>equilibrium competitive binding curve regardless of the<br>individual values of  $k_3$  and  $k_4$  ( $K_I = k_4/k_3$ ). Be every competitor with a given  $K_I$  will yield the same<br>equilibrium competitive binding curve regardless of the<br>individual values of  $k_3$  and  $k_4$  ( $K_I = k_4/k_3$ ). Before equi-<br>librium is reached, however, the kinetics of



**FIG.** 3. *Binding of a radioligand in the absence and presence of a competitor*

FIG. 3. Binding of a radioligand in the absence and presence of competitor<br>A. The binding of a radioligand to receptor is shown in the absence for competitor (top curve), in the presence of a competitor that disso-<br>ciates competitor<br>A. The binding of a radioligand to receptor is shown in the absence<br>of competitor (*top curve*), in the presence of a competitor that disso-<br>ciates from the receptors more slowly than does the radioligand ( $k_4$ A. The binding of a radioligand to receptor is shown in the absence<br>of competitor (*top curve*), in the presence of a competitor that disso-<br>ciates from the receptors more slowly than does the radioligand  $(k_4 < k_2)$ ; midd ciates from the receptors more slowly than does the radioligand  $(k_4 < k_2;$  middle curve), and in the presence of a competitor that dissociates more rapidly than does the radioligand  $(k_2 < k_4;$  bottom curve). The vertical shows the curve), and in the presence of a competitor that dissociates<br>more rapidly than does the radioligand  $(k_2 < k_4)$ ; bottom curve). The<br>vertical axis is radioligand binding relative to the equilibrium binding<br>of radi the presence of competitor. In the presence of competitor, the vertical axis is radioligand binding relative to the equilibrium binding of radioligand alone. Note that equilibrium is reached more slowly in the presence of wertical axis is radioligand binding relative to the equilibrium binding<br>of radioligand alone. Note that equilibrium is reached more slowly in<br>the presence of competitor. In the case of the slowly dissociating<br>competitor, version and a shown; and alone. Note that equilibrium is reached more slowly in the presence of competitor. In the case of the slowly dissociating competitor, equilibrium has not yet been established at the right of the cu rapidly dissociating competitor. In the case of the slowly dissociating competitor, equilibrium has not yet been established at the right of the curve shown; at equilibrium this curve will merge with the curve of the rapid

competitor, equilibrium has not yet been established at the right of the curve shown; at equilibrium this curve will merge with the curve of the rapidly dissociating competitor.<br>B. At each time point the amount of radiolig rapidly dissociating competitor.<br> **EXECUTE:** B. At each time point the amount of radioligand binding in the<br>
presence of rapidly dissociating (*upper curve*) or slowly dissociating<br>
(*lower curve*) competitior is displayed B. At each time point the amount of radioligand binding in the presence of rapidly dissociating (*upper curve*) or slowly dissociating (*lower curve*) competitior is displayed as a percentage of the binding of the radioli the radioligand alone at that time point. The following values we<br>used to calculate these curves:  $k_1 = 1.0 \times 10^8$  min<sup>-1</sup>  $M^{-1}$ ,  $k_2 = 0.05$ <br>min<sup>-1</sup>, [radioligand] = 3 nM, [competitor] = 100 nM. [These values a<br>those o lets (8) the rapidle of these curves:  $k_1 = 1.0 \times 10^8$  min<sup>-1</sup>  $M^{-1}$ ,  $k_2 = 0.037$  min<sup>-1</sup>, [radioligand] = 3 nM, [competitor] = 100 nM. [These values are those of [<sup>3</sup>H]yohimbine binding to *alpha*<sub>2</sub>-adrenergic recept min<sup>-1</sup>, [radioligand] = 3 nM, [competitor] = 100 nM. [These values are those of [<sup>3</sup>H]yohimbine binding to *alpha*<sub>2</sub>-adrenergic receptors on platelets (8)]. For the rapidly dissociating competitor,  $k_3 = k_1$  and  $k_4 = 1$ 





**curves over time**<br>**curves over time**<br>**curves over time**<br>**curves over time**<br>**curves over time**<br>**Maintaining a constant**  $K_I$ **, the association and dissociation rates of** FIG. 4. Change in the position of competitive radioligand binding<br>curves over time<br>The  $IC_{80}$  of a competitive binding curve is plotted against time.<br>Maintaining a constant  $K_I$ , the association and dissociation rates of the competitive binding curve is plotted against time.<br> **Competitive varied to create the family of curves shown.** In the top curve, the competitor associates and dissociates slowly; these rates the family of curves shown *turves over time*<br>
The  $IC_{50}$  of a competitive binding curve is plotted against time.<br>
Maintaining a constant  $K_I$ , the association and dissociation rates of<br>
the competitor were varied to create the family of curves sh The IC<sub>50</sub> of a competitive binding curve is plotted against time.<br>Maintaining a constant  $K_I$ , the association and dissociation rates of<br>the competitor were varied to create the family of curves shown. In the<br>*top curve* Maintaining a constant  $K_I$ , the association and dissociation rates of<br>the competitor were varied to create the family of curves shown. In the<br>top curve, the competitor associates and dissociates slowly; these rates<br>are p top curve, the competitor associates and dissociates slowly; these rates<br>are proportionately more rapid in the *lower curves*. To calculate the<br>curves shown, the following values were used:  $k_1 = 5.86 \times 10^8$  min<sup>-1</sup><br> $M^{-1$ are proportionately more rapid in the *lower curves*. To calculate the curves shown, the following values were used:  $k_1 = 5.86 \times 10^8$  min<sup>-1</sup>,  $k_2 = 0.0045$  min<sup>-1</sup>, and [radioligand] = 35 pM (same as Fig. 2). In the *t* the top curve,  $k_3 = 10^3$  min<sup>-1</sup>,  $M_4 = 10^{-3}$  min<sup>-1</sup>, and the  $K_l$  is therefore  $10^{-6}$  M. Each succeeding curve was generated by increasing both  $k_3$  and  $k_4$  by half an order of magnitude. The bottom curve, theref  $10^{-6}$  M. Each succeeding curve was generated by increasing both  $k_3$  and =  $10^7$  min<sup>-1</sup> M<sup>-1</sup> and  $k_4 = 10$  min<sup>-1</sup>. A computer calculated the entire competitive binding curve for each set of rate constants at each time point using Eq. 1, and found the IC<sub>80</sub>. At equilibrium all of the curve point using Eq. 1, and found the  $IC_{50}$ . At equilibrium all of the curves

converge with an  $IC_{50}$  of 5.6  $\mu$ M. The last time point shown is 200 min.<br>The lowest curves shown ("rapid  $k_3$ ,  $k_4$ ") represent a common situation: the radioligand dissociates much more slowly than the competitor, b converge with an IC<sub>80</sub> of 5.6  $\mu$ M. The last time point shown is 200 min.<br>
The lowest curves shown ("rapid  $k_3$ ,  $k_4$ ") represent a common<br>
situation: the radioligand dissociates much more slowly than the com-<br>
petito  $K_i$  at early time points and gradually increases to its equilibrium value defined by the Cheng and Prussoff equation (9),  $IC_{50} = K_I(1 + [L]/K_D)$ . Thus, when  $[L] \ll K_D$ , the  $IC_{50}$  will be nearly constant over time. value defined by the Cheng and Prussoff equation (9), IC<sub>50</sub> =  $K_I(1 + [L]/K_D)$ . Thus, when  $[L] \ll K_D$ , the IC<sub>50</sub> will be nearly constant over time.<br>combinations of  $k_4$  and  $k_3$  yielding the same  $K_I$  (Fig. 4).<br>In all of t

In all of the curves the inhibition of radioligand binding<br>at equilibrium is identical; only the kinetics of inhibition<br>differ. As  $k_3$  and  $k_4$  increase, the initial decrease in the combinations of  $k_4$  and  $k_3$  yielding the same  $K_I$  (Fig. 4).<br>In all of the curves the inhibition of radioligand binding<br>at equilibrium is identical; only the kinetics of inhibition<br>differ. As  $k_3$  and  $k_4$  increase, In all of the curves the inhibition of radioligand binding<br>at equilibrium is identical; only the kinetics of inhibition<br>differ. As  $k_3$  and  $k_4$  increase, the initial decrease in the<br>IC<sub>50</sub> becomes more pronounced. In t differ. As  $k_3$  and  $k_4$  increase, the initial decrease in the  $IC_{50}$  becomes more pronounced. In the most extreme case, when  $k_3$  and  $k_4$  are extremely fast, the minimum  $IC_{50}$  occurs instantaneously and has a val  $\prod_{60}^{100}$  becomes models and  $\prod_{60}^{100}$  occurs instand pendix 4). In all ot and occurs later.<br>These findings case, when  $k_3$  and  $k_4$  are extremely fast, the minimum  $IC_{50}$  occurs instantaneously and has a value of  $K_I$  (Appendix 4). In all other cases, that minimum  $IC_{50}$  is larger and occurs later.<br>These findings are exten  $\prod_{s=0}^{\infty}$  occurs instantaneously and has a value of  $K_I$  (*A* pendix 4). In all other cases, that minimum IC<sub>50</sub> is larend occurs later.<br>and occurs later.<br>These findings are extended to entire competitive binding cur

pendix 4). In all other cases, that minimum  $IC_{50}$  is larger<br>and occurs later.<br>These findings are extended to entire competitive<br>binding curves in Fig. 5. As Ehlert *et al.* (3) demonstrated, for<br>when  $k_4 < k_2$ , the  $IC_{5$ and occurs later. 20<br>
These findings are extended to entire competitive<br>
binding curves in Fig. 5. As Ehlert *et al.* (3) demonstrated, for<br>
when  $k_4 < k_2$ , the IC<sub>50</sub> of the competitive binding curve<br>
yeradually decrease binding curves in Fig. 5. As Ehlert *et al.* (3) demonstrated, for when  $k_4 < k_2$ , the IC<sub>50</sub> of the competitive binding curve value gradually decreases over time; the curve moves to the The left. If, however,  $k_4 > k_2$ , when  $k_4 < k_2$ , the IC<sub>50</sub> of the competitive binding curve<br>gradually decreases over time; the curve moves to the<br>left. If, however,  $k_4 > k_2$ , then the IC<sub>50</sub> will first decrease<br>and later increase. That initial decrease explanally decrease. That initial decrease in the  $IC_{50}$  will first decrease<br>and later increase. That initial decrease in the  $IC_{50}$  may<br>occur quickly and one may therefore observe only the<br>later increase, as Ehlert *et* and later increase. That initial decrease in the  $IC_{50}$  may<br>occur quickly and one may therefore observe only the<br>later increase, as Ehlert *et al.* (3) did. In this case the<br>minimum (leftmost) value of the  $IC_{50}$  will b *K1.*

ETICS OF COMPETITIVE RADIOLIGAND BINDING 5<br>"Slope factors" or "pseudo-Hill slopes" are used to<br>scribe the shape of a competitive binding curve. We INETICS OF COMPETITIVE RADIOLIGAND BINDING 5<br>
"Slope factors" or "pseudo-Hill slopes" are used to<br>
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have simulated many pre-equilibrium competition curves INETICS OF COMPETITIVE RADIOLIGAND BINDING 5<br>
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on a compute



The constants used in Fig. 4 were used to calculate competitive<br>ding curves over time<br>The constants used in Fig. 4 were used to calculate competitive<br>ding curves at the following times (minutes: 2, 5, 10, 20, 30, 50, 100, FIG. 5. Change in competitive binding curves over time<br>The constants used in Fig. 4 were used to calculate competitive<br>binding curves at the following times (minutes: 2, 5, 10, 20, 30, 50, 100,<br>200, 400, 800, and 10,000. FIG. 5. *Change in comp*<br>The constants used in<br>binding curves at the follo<br>200, 400, 800, and 10,000.<br>A. The change in the co FIG. 5. Change in competitive binding curves over time<br>The constants used in Fig. 4 were used to calculate competitive<br>iding curves at the following times (minutes: 2, 5, 10, 20, 30, 50, 100,<br>0, 400, 800, and 10,000.<br>A. T The constants used in Fig. 4 were used to calculate competitive<br>binding curves at the following times (minutes: 2, 5, 10, 20, 30, 50, 100,<br>200, 400, 800, and 10,000.<br>A. The change in the competitive binding curve over tim

binding curves at the following times (minutes: 2, 5, 10, 20, 30, 50, 100, 200, 400, 800, and 10,000.<br>
A. The change in the competitive binding curve over time is shown<br>
for the case where  $k_4 < k_2$ . These curves were gen 200, 400, 800, and 10,000.<br>A. The change in the competitive binding curve over time is shown<br>for the case where  $k_4 < k_2$ . These curves were generated using the<br>values noted above for the *topmost curve* of Fig. 4  $(k_4 = 1$ A. The cl<br>for the case<br>values noted<br>The *heavy li*<br>over time.<br>B. Compo the case where  $k_4 < k_2$ . These curves were generated using the ues noted above for the *topmost curve* of Fig. 4  $(k_4 = 10^{-3} \text{ min}^{-1})$ .<br>e *heavy line* connects the IC<sub>80</sub> values; note that these values decrease er time.<br>B values noted above for the *topmost curve* of Fig. 4  $(k_4 = 10^{-3} \text{ min}^{-1})$ .<br>The *heavy line* connects the IC<sub>50</sub> values; note that these values decrease over time.<br>B. Competitive binding curves were generated to match the

The *theory* and connects the rogy values, note that these values decrease<br>over time.<br>B. Competitive binding curves were generated to match the middle<br>curve of Fig. 4 ( $k_4 = 10^{-1}$  min<sup>-1</sup>). Note that the IC<sub>60</sub> first dec

*b* curve of Fig. 4  $(k_4 = 10^{-1} \text{ min}^{-1})$ . Note that the IC<sub>50</sub> first decreases,<br>then increases.<br>C. Here the competitive binding curves are plotted to match the<br>bottom curve in Fig. 4  $(k_4 = 10 \text{ min}^{-1})$ . Here the initial de once in Fig. 1.1.4 The initial the IC<sub>50</sub> instructed is,<br>then increases.<br>C. Here the competitive binding curves are plotted to match the<br>bottom curve in Fig. 4 ( $k_4 = 10 \text{ min}^{-1}$ ). Here the initial decreases in IC<sub>50</sub><br>occ



*ing experiments*

To demonstrate the feasibility of determining the  $K_l$  of a competing ligand from non-equilibrium data, we analyzed the kinetics of **[1251]** FIG. 6. Determining the  $K_l$  from non-equilibrium competitive binding experiments<br>To demonstrate the feasibility of determining the  $K_l$  of a competing<br>ligand from non-equilibrium data, we analyzed the kinetics of  $\binom{12$ Elignal from non-equilibrium data, we analyzed the kinetics of  $[126]$ <br>ICYP binding to *beta*-adrenergic receptors on intact S49 lymphoma<br>cells in the presence of 1 nM propranolol. Using methods published<br>elsewhere (5), I CCYP binding to *beta*-adrenergic receptors on intact S49 lymphoma<br>cells in the presence of 1 nM propranolol. Using methods published<br>elsewhere (5), ICYP and propranolol were added simultaneously to the<br>cells and the spec between the presence of 1 nM propranolol. Using methods published elsewhere (5), ICYP and propranolol were added simultaneously to the cells and the specific ICYP binding was determined at various times between 0.5 and 4 m belsewhere (5), ICYP and propranolol were added simultaneously to the cells and the specific ICYP binding was determined at various times between 0.5 and 4 min thereafter. The data were fit to Eq. 1 in the text using a Ma From Tektronix (3), or a time precific ICYP binding was determined at various times<br>between 0.5 and 4 min thereafter. The data were fit to Eq. 1 in the text<br>using a Marquardt nonlinear least-squares regression program ava between 0.5 and 4 min thereafter. The data were fit to Eq. 1 in the text using a Marquardt nonlinear least-squares regression program available from Tektronix (10). The program was given the following constants that were from Tektronix (10). The program was given the following constants<br>that were determined previously or set experimentally:  $k_1 = 2.05 \times 10^9$ <br> $M^{-1}$  min<sup>-1</sup> (determined in a parallel experiment),  $k_2 = 0.0045$  min<sup>-1</sup>,<br> $[R]$ 

how much the radioligand binding wateled assuming would differ if *k<sub>3</sub>* and *k<sub>4</sub>* were different. The *dotted lines* show the binding predicted assuming that the *K<sub>1</sub>* was the same, but that the values of both *k<sub>3</sub>* a To demonstrate the sensitivity of the technique, we also have shown<br>how much the radioligand binding would differ if  $k_3$  and  $k_4$  were<br>different. The *dotted lines* show the binding predicted assuming that<br>the  $K_I$  was From much the radioligand binding would differ if  $k_3$  and  $k_4$  were indifferent. The *dotted lines* show the binding predicted assuming that the  $K_I$  was the same, but that the values of both  $k_3$  and  $k_4$  were varie the  $K_f$  was the same, but that the values of both  $k_3$  and  $k_4$  were varied either a half-order magnitude higher (above) or lower (below) than the values determined by the program. These curves are clearly resolved fro predicted if *k<sub>4</sub>* alone were increased *(above)* or lower *(below)* than the values determined by the program. These curves are clearly resolved from the experimental points. The *dashed lines* show the binding predicte from the experimental points. The *dashed thes* show the binding<br>predicted if  $k_4$  alone were increased (below) or decreased (above) half<br>an order of magnitude, thus altering the  $K_I$ .<br>for each. These simulations used a

an order of magnitude, thus altering the  $K_t$ .<br>
for each. These simulations used a variety of kinetic a constants, and the slopes (calculated at the  $IC_{50}$ ) were palways between 1.0 and 1.3. It is noteworthy that the sig ally see the simulations used a variety of kinetic constants, and the slopes (calculated at the  $IC_{50}$ ) were palways between 1.0 and 1.3. It is noteworthy that the signe-equilibrium slope factor was never less than 1 for for each. These simulations used a variety of kinetic constants, and the slopes (calculated at the  $IC_{50}$ ) were always between 1.0 and 1.3. It is noteworthy that the gre-equilibrium slope factor was never less than 1 for for each. These simulations used a variety of kinetic an constants, and the slopes (calculated at the  $IC_{50}$ ) were  $PC_$ always between 1.0 and 1.3. It is noteworthy that the sit pre-equilibrium slope factor was never less constants, and the slop<br>always between 1.0 and<br>pre-equilibrium slope factories that at equilibriu<br>also be seen in Fig. 2.<br>presenting and *x* presenting and *x* presenting and *x* present pre-equilibrium slope factor was never less than<br>curves that at equilibrium have a slope of 1.0. Thi<br>also be seen in Fig. 2.<br>DETERMINING THE  $K_l$  FROM KINETIC DATA<br>Fquilibrium competitive binding curves are often

rves that at equilibrium have a slope of 1.0. This can<br>so be seen in Fig. 2.<br>TERMINING THE  $K_l$  FROM KINETIC DATA in<br>Equilibrium competitive binding curves are often used co<br>determine the dissociation constant  $(K_l)$  of a also be seen in Fig. 2.<br>DETERMINING THE  $K_l$  FROM KINETIC DATA<br>Equilibrium competitive binding curves are often used<br>to determine the dissociation constant  $(K_l)$  of a receptor<br>for an unlabeled ligand. As shown above, seve DETERMINING THE  $K_l$  FROM KINETIC DATA<br>Equilibrium competitive binding curves are often used<br>to determine the dissociation constant  $(K_l)$  of a receptor<br>for an unlabeled ligand. As shown above, several hours<br>may elapse bef DETERMINING THE  $R_I$  FROM KINETIC DATA<br>Equilibrium competitive binding curves are often us<br>to determine the dissociation constant  $(K_I)$  of a recept<br>for an unlabeled ligand. As shown above, several hou<br>may elapse before eq Equilibrium competitive binding curves are often used<br>to determine the dissociation constant  $(K_I)$  of a receptor<br>for an unlabeled ligand. As shown above, several hours<br>may elapse before equilibrium is achieved in some rec for an unlabeled ligand. As shown above, several hours order to attain equilibrium. The analyses of this paper<br>may elapse before equilibrium is achieved in some recep-<br>tor systems. During these hours other unavoidable even for an unlabeled ligand. As shown above, several hours compared and some property may elapse before equilibrium is achieved in some receptors when they cover that may affect the results. For example, the thigand or recepto may elapse before equilibrium is achieved in some receptor systems. During these hours other unavoidable events<br>may occur that may affect the results. For example, the<br>ligand or receptors may degrade, target cells may die, tor systems. During these hours other unavoidable events W<br>may occur that may affect the results. For example, the the<br>ligand or receptors may degrade, target cells may die, or<br>extend to determine the compositions it woul may occur that may affect the results. For example, the tigand or receptors may degrade, target cells may die, or the composition of the incubation mixture may change. In these situations it would be desirable to determin ligand or receptors may degrade, target cells may die, or<br>the composition of the incubation mixture may change.<br>In these situations it would be desirable to determine the<br> $K_l$  in a shorter period of time. Equation 1 makes In these situations it would be desirable to determine the to d  $K_l$  in a shorter period of time. Equation 1 makes this is the possible. The kinetics of radioligand binding in the pres-<br>ence of a competitor can be measure

are readily determined in standard experiments and the are readily determined in standard experiments and the<br>concentrations of radioligand, competitor, and receptor<br>are set by the experimenter. Any general-purpose curveare readily determined in standard experiments and t<br>concentrations of radioligand, competitor, and recept<br>are set by the experimenter. Any general-purpose curv<br>fitting algorithm can therefore be used to fit Eq. 1 to t are readily determined in standard experiments and the concentrations of radioligand, competitor, and receptor are set by the experimenter. Any general-purpose curve-<br>fitting algorithm can therefore be used to fit Eq. 1 t concentrations of radio<br>are set by the experiment<br>fitting algorithm can the<br>experimental data and<br>gether yield  $K_I$  ( $k_4/k_3$ ).<br>In Fig. 6 we illustrate Exercise the experimenter. Any general-purpose curve-<br>ting algorithm can therefore be used to fit Eq. 1 to the<br>perimental data and determine  $k_3$  and  $k_4$ , which to-<br>ther yield  $K_I$  ( $k_4/k_3$ ).<br>In Fig. 6 we illustrate t

fitting algorithm can therefore be used to fit Eq. 1 to the experimental data and determine  $k_3$  and  $k_4$ , which together yield  $K_I$  ( $k_4/k_3$ ).<br>In Fig. 6 we illustrate the feasibility of this approach for determining gether yield  $K_I$  ( $k_4/k_3$ ).<br>In Fig. 6 we illustrate the feasibility of this approach<br>for determining  $K_I$ . Here we have determined the  $K_I$  of<br>*beta*-adrenergic receptors on S49 lymphoma cells for (-)-<br>propranolol in a In Fig. 6 we illustrate the feasibility of this approach<br>for determining  $K_l$ . Here we have determined the  $K_l$  of<br>*beta*-adrenergic receptors on S49 lymphoma cells for  $(-)$ -<br>propranolol in a 4-min experiment. The result for determining  $K_l$ . Here we have determined the  $K_l$  of *beta*-adrenergic receptors on S49 lymphoma cells for  $(-)$ -<br>propranolol in a 4-min experiment. The result  $(0.3 \text{ nM})$  is similar to that determined in conventiona propranolol in a 4-min experiment. The result  $(0.3 \text{ nM})$ <br>is similar to that determined in conventional equilibrium<br>competitive binding experiments lasting 2 hr  $(0.2 \text{ nM})$ <br>ref. 5). Moreover, the kinetic analysis yielde is similar to that determined in conventional equilibric<br>competitive binding experiments lasting 2 hr (0.2 r<br>ref. 5). Moreover, the kinetic analysis yielded values<br> $k_3$  (3.1  $\pm$  0.5 × 10<sup>9</sup> min<sup>-1</sup> M<sup>-1</sup>) and  $k_4$  (1.0 ments.  $k_3$  (3.1  $\pm$  0.5  $\times$  10<sup>9</sup> min<sup>-1</sup> M<sup>-1</sup>) and  $k_4$  (1.0  $\pm$  0.26 min<sup>-1</sup>)<br>that can not be determined from equilibrium experi-<br>ments.<br>DISCUSSION<br>The equations and simulations were based on a simple<br>molecular model in

from Tektronix (10). The program was given the following constants simultaneously to a single receptor binding site; (d) only<br>that were determined previously or set experimentally:  $k_1 = 2.05 \times 10^9$  a small fraction of t ments.<br>DISCUSSION<br>The equations and simulations were based on a sim<br>molecular model incorporating the following assum<br>tions: (a) a single class of noninteracting receptor DISCUSSION<br>The equations and simulations were based on a simple<br>molecular model incorporating the following assump-<br>tions: (a) a single class of noninteracting receptors is<br>present that binds the radioligand and competitor presents and simulations were based on a simple<br>molecular model incorporating the following assump-<br>tions: (a) a single class of noninteracting receptors is<br>present that binds the radioligand and competitor re-<br>versibly; ( The equations and simulations were based on a simple<br>molecular model incorporating the following assump-<br>tions: (a) a single class of noninteracting receptors is<br>present that binds the radioligand and competitor re-<br>versib molecular model incorporating the following assumptions: (a) a single class of noninteracting receptors is<br>present that binds the radioligand and competitor re-<br>versibly; (b) these binding reactions follows the law of<br>mass present that binds the radioligand and competitor reversibly; (b) these binding reactions follows the law of mass action; (c) radioligand and competitor cannot bind to receptors (zone A); (e) radioligand and competitor are versibly; (b) these binding reactions follows the law of<br>mass action; (c) radioligand and competitor cannot bind<br>simultaneously to a single receptor binding site; (d) only<br>a small fraction of the radioligand and competitor mass action; (c) radioligand and competitor cannot bind<br>simultaneously to a single receptor binding site; (d) only<br>a small fraction of the radioligand and competitor binds<br>to receptors (zone A); (e) radioligand and competi simultaneously to a single receptor binding site; (d) only<br>a small fraction of the radioligand and competitor binds<br>to receptors (zone A); (e) radioligand and competitor are<br>simultaneously exposed to the receptors; and (f) a small fraction of the radioligand and competitor binds<br>to receptors (zone A); (e) radioligand and competitor are<br>simultaneously exposed to the receptors; and (f) the<br>properties of all free receptors are identical whether to receptors (zone A); (e) radioligand and competitor are<br>simultaneously exposed to the receptors; and (f) the<br>properties of all free receptors are identical whether or<br>not they once bound ligand or competitor. This is a<br>s properties of all free receptors are identical whether or<br>not they once bound ligand or competitor. This is a<br>simple model, and a more complex model may be required<br>in some experimental situations. Nevertheless, the model<br> not they once bound ligand or competitor. This is a not they once bound ligand or competitor. This is<br>simple model, and a more complex model may be require<br>in some experimental situations. Nevertheless, the mode<br>of simple competitive interactions incorporating thes<br>assumpti simple model, and a more complex model may be requi<br>in some experimental situations. Nevertheless, the mo<br>of simple competitive interactions incorporating th<br>assumptions is commonly accepted as the basis of st<br>dard methods ments. simple competitive interactions incorporating thes<br>sumptions is commonly accepted as the basis of stan<br>rd methods for analyzing competitive binding experi-<br>ents.<br>Our analyses and discussion were based around radi-<br>igand bi

assumptions is commonly accepted as the basis of standard methods for analyzing competitive binding experiments.<br>Our analyses and discussion were based around radioligand binding experiments. The mathematics, however, are dard methods for analyzing competitive binding experiments.<br>
Our analyses and discussion were based around radi-<br>
oligand binding experiments. The mathematics, however,<br>
are identical for any situation in which two ligands ments.<br>
Our analyses and discussion were based around radio<br>
oligand binding experiments. The mathematics, however<br>
are identical for any situation in which two ligand<br>
compete for binding to a single population of recepto Our analyses and discussion were based around radioligand binding experiments. The mathematics, however are identical for any situation in which two ligand compete for binding to a single population of receptors and the bi oligand binding experiments. The mathematics, however,<br>are identical for any situation in which two ligands<br>compete for binding to a single population of receptors,<br>and the binding of one of those ligands is measured.<br>Port are identical for any situation in which two ligano<br>compete for binding to a single population of receptor<br>and the binding of one of those ligands is measure<br>Portions of our discussion may therefore apply to othe<br>situation compete for binding to a single population of rece<br>and the binding of one of those ligands is mea<br>Portions of our discussion may therefore apply to<br>situations such as radioimmunoassays, fluorescent<br>ing assays, and competit and the bind<br>Portions of o<br>situations suc<br>ing assays, an<br>ical responses<br>The theore Portions of our discussion may therefore apply to other situations such as radioimmunoassays, fluorescent binding assays, and competitive antagonism of pharmacological responses.<br>The theoretical analyses described in this

1. When establishing an experimental protocol for It is assays, and competitive antagonism of pharmacolog-<br>
In the sponses.<br>
The theoretical analyses described in this paper apply<br>
four experimental situations:<br>
1. When establishing an experimental protocol for<br>
mpetitive ical responses.<br>The theoretical analyses described in this paper apply<br>in four experimental situations:<br>1. When establishing an experimental protocol for<br>competitive radioligand binding experiments one must<br>decide how long The theoretical analyses described in this paper apply<br>in four experimental situations:<br>1. When establishing an experimental protocol for<br>competitive radioligand binding experiments one must<br>decide how long to allow the in in four experimental situations:<br>1. When establishing an experimental protocol for<br>competitive radioligand binding experiments one must<br>decide how long to allow the incubation to proceed in<br>order to attain equilibrium. The 1. When establishing an experimental protocol for competitive radioligand binding experiments one mudecide how long to allow the incubation to proceed is order to attain equilibrium. The analyses of this pape make it clear decide how long to allow the incubation to proceed in decide how long to allow the incubation to proceed in<br>order to attain equilibrium. The analyses of this paper<br>make it clear how to set the duration of the incubation.<br>When the competitor dissociates from the receptor faste order to attain equilibrium. The analyses of this paper<br>make it clear how to set the duration of the incubation.<br>When the competitor dissociates from the receptor faster<br>than does the radioligand, the time required to atta When the competitor dissociates from the receptor faste<br>than does the radioligand, the time required to attai<br>equilibrium is determined by the dissociation rate of th<br>radioligand. Thus the time required for the radioligan<br> than does the radioligand, the time required to atts equilibrium is determined by the dissociation rate of t radioligand. Thus the time required for the radioligato dissociate from receptors in an "off-rate" experime is th radioligand. Thus the time required for the radioligand<br>to dissociate from receptors in an "off-rate" experiment<br>is the same as the time required for a competitive exper-<br>iment to reach equilibrium. Often investigators use radioligand. Thus the time required for the radioligand<br>to dissociate from receptors in an "off-rate" experiment<br>is the same as the time required for a competitive exper-<br>iment to reach equilibrium. Often investigators use to dissociate from receptors in an "off-rate" experiment<br>is the same as the time required for a competitive exper-<br>iment to reach equilibrium. Often investigators use an<br>"on-rate" experiment to determine the time required. is the same as the time required for a competitive experiment to reach equilibrium. Often investigators use an "on-rate" experiment to determine the time required.<br>This will yield the correct result only if a very low conc

rate" is  $K_A$  [=  $k_1[L] + k_2$ ], which approximates  $k_2$  only APPI<br>when  $[L] \ll K_D$ ). As noted above and in the legend to<br>Fig. 4, an acceptable approximation of the IC<sub>50</sub> can be Fig. 4, an acceptable approximates  $k_2$  only API<br>when  $[L] \ll K_D$ ). As noted above and in the legend to<br>Fig. 4, an acceptable approximation of the IC<sub>50</sub> can be<br>attained in less than half the time required to reach rate" is  $K_A$   $[= k_1[L] + k_2]$ , which approximates  $k_2$  only API<br>when  $[L] \ll K_D$ ). As noted above and in the legend to<br>Fig. 4, an acceptable approximation of the IC<sub>50</sub> can be<br>attained in less than half the time required to equilibrium. when  $[L] \ll K_D$ ). As noted above and in the legend to<br>
Fig. 4, an acceptable approximation of the IC<sub>50</sub> can be<br>
attained in less than half the time required to reach<br>
acquilibrium.<br>
2. Several authors have demonstrated tha

Fig. 4, an acceptable approximation of the  $IC_{50}$  can be<br>
attained in less than half the time required to reach I<br>
equilibrium.<br>
2. Several authors have demonstrated that it can take<br>
longer for radioligand binding to re equilibrium.<br>
2. Several authors have demonstrated that it can take<br>
longer for radioligand binding to reach equilibrium in the<br>
presence of a competing drug than in its absence, and we<br>
have now quantitated the relationsh 2. Several authors have demonstrated that it can take training longer for radioligand binding to reach equilibrium in the presence of a competing drug than in its absence, and we have now quantitated the relationship. Thus presence of a competing drug than in its absence, and we have now quantitated the relationship. Thus, if an experimental protocol is based on the minimal time required for the binding of the radioligand alone to reach equi have now quantitated the relationship. Thus, if an experimental protocol is based on the minimal time required for the binding of the radioligand alone to reach equilibrium, competitive binding experiments will be terminat quired for the binding of the radioligand alone to reach equilibrium, competitive binding experiments will be terminated before equilibrium is established. However, this fact is not well known, and some published competit equilibrium, competitive binding experiments will be ter-<br>minated before equilibrium is established. However, this<br>fact is not well known, and some published competitive<br>binding curves may have been obtained under non-equi fact is not well known, and some published competitive<br>binding curves may have been obtained under non-equi-<br>librium conditions. The relationship derived in this paper<br>allow one to determine whether the apparent  $K_I$  valu binding curves may have been obtained under<br>librium conditions. The relationship derived in<br>allow one to determine whether the apparent<br>determined by these non-equilibrium curves<br>be over- or underestimates of the true  $K_I$ From conditions. The relationship derived in this paper<br>ow one to determine whether the apparent  $K_I$  values<br>termined by these non-equilibrium curves are likely to<br> $K_I$ <br>over- or underestimates of the true  $K_I$ .<br>3. Recent

allow one to determine whether the apparent  $K_I$  values ven<br>determined by these non-equilibrium curves are likely to  $K_S$ <br>be over- or underestimates of the true  $K_I$ . (2)<br>3. Recent experiments by ourselves and others have determined by these non-equilibrium curves are likely to<br>be over- or underestimates of the true  $K_l$ .<br>3. Recent experiments by ourselves and others have<br>demonstrated that *beta*-adrenergic agonists appear to<br>bind transien be over- or underestimates of the true  $K_I$ .<br>3. Recent experiments by ourselves and others high affinity to *beta*-adrenergic agonists appear<br>bind transiently to *beta*-adrenergic receptors on int<br>cells with a high affini 3. Recent experiments by ourselves and others have<br>demonstrated that *beta*-adrenergic agonists appear to 2<br>bind transiently to *beta*-adrenergic receptors on intact<br>cells with a high affinity, and that this binding "dese demonstrated that *beta*-adrenergic agonists appear to bind transiently to *beta*-adrenergic receptors on intacells with a high affinity, and that this binding "deser sitizes" the receptors so as to decrease their later af cells with a high affinity, and that this binding "desensitizes" the receptors so as to decrease their later affinity for the agonists  $(5-7)$ . This transient high-affinity binding is observed during the first few minutes cells with a high affinity, and that this binding "desensitizes" the receptors so as to decrease their later affinity and for the agonists  $(5-7)$ . This transient high-affinity bind-<br>ing is observed during the first few m for the agonists (5–7). This transient high-affinity bind-<br>ing is observed during the first few minutes of the com-<br>petition between agonist and ligand, long before equilib-<br>for<br>inum is reached. At equilibrium the agonist action. between agonist and ligand, long before equilib-<br>petition between agonist and ligand, long before equilib-<br>rium is reached. At equilibrium the agonist appears to  $[L]$ <br>compete for radioligand binding with a low aff rium is reached. At equilibrium the agonist appears to compete for radioligand binding with a low affinity and in a manner essentially consistent with the law of mass action. The anomolous behavior of agonist binding is ob compete for radioligand binding with a low affinity and<br>in a manner essentially consistent with the law of mass<br>action. The anomolous behavior of agonist binding is<br>observed only in kinetic experiments. A full theoretical<br> in a manner essentially consistent with the law of mass<br>action. The anomolous behavior of agonist binding is<br>observed only in kinetic experiments. A full theoretical<br>analysis of this transient high-affinity binding has not action. The anomolous behavior of agonist binding is<br>observed only in kinetic experiments. A full theoretical<br>analysis of this transient high-affinity binding has not<br>yet been published. As a first step in analyzing such d observed only in kinetic experiments. A full theoretical panalysis of this transient high-affinity binding has not cyet been published. As a first step in analyzing such data, wit is necessary to demonstrate that the data analysis of this transient high-affinity binding has not yet been published. As a first step in analyzing such data, it is necessary to demonstrate that the data are not compatible with a simple model of competitive bindin it is necessary to demonstrate that the data are not I.<br>compatible with a simple model of competitive binding itive<br>based on the law of mass action. The best way to dem-<br>part<br>onstrate that the early competition data canno based on the law of mass action. The best way to dem-<br>onstrate that the early competition data cannot be ex-<br>plained by the law of mass action is to compare directly<br>low a<br>the observed data with the theoretical prediction plained by the law of mass action is to compare directly lothe observed data with the theoretical predictions (5). In addition, the generalizations derived in this paper allow  $K_i$  one to be certain immediately that an ea the observed data with the theoretical predictions (5). In addition, the generalizations derived in this paper allow  $K$  one to be certain immediately that an early competition curve is inconsistent with the law of mass a addition, the generalizations derived in this paper allow<br>one to be certain immediately that an early competition<br>curve is inconsistent with the law of mass action if the<br>early  $IC_{50}$  is less than equilibrium  $K_I$ , or if curve is inconsistent with the law of mass action if the early  $IC_{50}$  is less than equilibrium  $K_I$ , or if the early slope factor is less than 1.0 (and the equilibrium slope factor is equal to 1).<br>4. It may not be feasib

factor is less than 1.0 (and the equilibrium slope factor is equal to 1).<br>4. It may not be feasible to allow an incubation to proceed long enough for equilibrium to be established if, for example, the ligand or receptor d for example, the ligand or receptor degrades, or the target is equal to 1).<br>4. It may not be feasible to allow an incubation t<br>proceed long enough for equilibrium to be established if<br>or example, the ligand or receptor degrades, or the targe<br>cells die. Under such circumstances, the 4. It may not be feasible to allow an incubation to proceed long enough for equilibrium to be established if, for example, the ligand or receptor degrades, or the target cells die. Under such circumstances, the experimente proceed long enough for equilibrium to be established if, only for example, the ligand or receptor degrades, or the target cells die. Under such circumstances, the experimenter may be forced to terminate the binding incub for example, the ligand or receptor degrades, or the target cells die. Under such circumstances, the experimenter may be forced to terminate the binding incubations before equilibrium is established. We have shown how to cells die. Under such circumstances, the experimenter<br>may be forced to terminate the binding incubations be-<br>fore equilibrium is established. We have shown how to<br>determine the  $K_l$  using an experimental protocol that<br>can may be forced to terminate the binding incubations b<br>fore equilibrium is established. We have shown how<br>determine the  $K_I$  using an experimental protocol th<br>can be completed long before equilibrium is reache<br>Moreover, thi fore equilibrium is established. We have shown how to determine the  $K_I$  using an experimental protocol that can be completed long before equilibrium is reached. Moreover, this technique uniquely allows one to determine t can be completed long before equilibrium is reached.<br>Moreover, this technique uniquely allows one to determine the individual values of the association and dissociation rate constants of an unlabeled compound that determi

## ACKNOWLEDGMENTS

thank Vincent Dionne, Leslie Morrow, and Paul Insel for helpful<br>We thank Vincent Dionne, Leslie Morrow, and Paul Insel for helpful<br>nments, Sandra Dutky for preparing the manuscript, and Arlene determine the A<sub>J</sub>.<br>ACKNOWLEDGMENTS<br>We thank Vincent Dionne, Leslie Morrow, and Paul Insel for helpful<br>comments, Sandra Dutky for preparing the manuscript, and Arlene<br>Koachman for performing the experiment shown in Fig. 6. ACKNOWLEDGMENTS<br>We thank Vincent Dionne, Leslie Morrow, and Paul Insel f<br>comments, Sandra Dutky for preparing the manuscript, an<br>Koachman for performing the experiment shown in Fig. 6.

FITICS OF COMPETITIVE RADIOLIGAND BINDING<br>
PENDIX: MATHEMATICAL DETAILS<br>
Solution to the Differential Equations<br>
Defining y as [RL] and x as [RI], and setting both<br>
ual to zero initially, the differential equations were APPENDIX: MATHEMATICAL DETAILS<br>1. Solution to the Differential Equations<br>Defining  $y$  as  $[RL]$  and  $x$  as  $[RI]$ , and setting both<br>equal to zero initially, the differential equations were<br>transformed by the method of Lapla 1. Solution to the Differential Equations<br>
Defining y as [RL] and x as [RI], and setting<br>
equal to zero initially, the differential equations<br>
transformed by the method of Laplace:<br>  $s\hat{y} = Nk_1[L]/s - k_1[L]\hat{x} - k_1[L]\hat{y} - k_2\$ 

$$
s\hat{y} = Nk_1[L]/s - k_1[L]\hat{x} - k_1[L]\hat{y} - k_2\hat{y}
$$
  

$$
s\hat{x} = Nk_3[I]/s - k_3[I]\hat{x} - k_3[I]\hat{y} - k_4\hat{y}
$$

Solving the second equation for  $\hat{x}$  and inserting into  $s\hat{x} = Nk_3[I]/s - k_3[I]\hat{x} - k_3[I]$ <br>Solving the second equation for  $\hat{x}$  as<br>the first yields (after some rearranging)<br> $NK_1[L]$ 

$$
\hat{y} = \frac{NK_1[L]}{(s + K_F)(s + K_S)} + \frac{NK_1K_4[L]}{s(s + K_F)(s + K_S)}
$$

Back-transforming yields Eq. 1 in the text. The inter-<br>vening algebraic steps make use of the facts that  $K_F$  +  $\hat{y} = \frac{NK_1[L]}{(s + K_F)(s + K_S)} + \frac{NK_1K_4[L]}{s(s + K_F)(s + K_S)}$ <br>Back-transforming yields Eq. 1 in the text. The inter-<br>vening algebraic steps make use of the facts that  $K_F + K_S = K_A + K_B$  and  $K_F K_S = K_A K_B - k_1 k_3[L][I]$ . Arányi<br>(2) has publish Back-transforming yields Eq. 1 in t<br>vening algebraic steps make use of  $K_S = K_A + K_B$  and  $K_F K_S = K_A K_B$ -<br>(2) has published a similar derivation. Back-transforming yields Eq. 1 in the text. The inter-<br>vening algebraic steps make use of the facts that  $K_F + K_S = K_A + K_B$  and  $K_F K_S = K_A K_B - k_1 k_3[L][I]$ . Arányi<br>(2) has published a similar derivation.<br>2. How Long Does It Take fo *Ing algebraic steps make use of the facts that*  $K_F$ <br>  $= K_A + K_B$  and  $K_F K_S = K_A K_B - k_1 k_3[L][I]$ . Arai<br>
has published a similar derivation.<br> *Incubation to Reach Equilibrium at the IC<sub>50</sub>?*<br>
At the IC<sub>50</sub>, [*I*] =  $(k_4/k_3)(1 + [L]/$ 

## (2) has published a similar derivation.<br>
2. *How Long Does It Take for a Competitive Binding*<br> *Incubation to Reach Equilibrium at the*  $IC_{50}$ ?<br>
At the  $IC_{50}$ ,  $[I] = (k_4/k_3)(1 + [L]/K_D)$ . In Eq. 1, [*I*]<br>
and  $k_3$  only appe

rate constant,  $k_3[I]$ . Thus, for a known  $K_D$ , a fixed [L] *rate constant, iii*  $\frac{1}{2}$ . *How Long Does It Take for a Competitive Binaing Incubation to Reach Equilibrium at the*  $IC_{50}$ *?*<br>At the  $IC_{50}$ ,  $[I] = (k_4/k_3)(1 + [L]/K_D)$ . In Eq. 1,  $[I]$  and  $k_3$  only appear as a product, t *Incubation to Reach Equitorium at the*  $IC_{50}$ :<br>At the  $IC_{50}$ ,  $[I] = (k_4/k_3)(1 + [L]/K_D)$ . In Eq. 1,  $[I]$ <br>and  $k_3$  only appear as a product, the pseudo-first-order<br>rate constant,  $k_3[I]$ . Thus, for a known  $K_D$ , a fixed  $[L]$ and  $k_3$  only appear as a product, the pseudo-first-order<br>rate constant,  $k_3[I]$ . Thus, for a known  $K_D$ , a fixed [*L*]<br>and [*I*] = IC<sub>50</sub>,  $k_3[I]$  is a simple function of  $k_4$ . Similarly,<br>for a radioligand of known  $K_D$ for a radioligand of known  $K_D$  and fixed concentration [*L*], the pseudo-first-order association rate constant,  $k_1[L]$ , is a simple function of  $k_2$ . Therefore the kinetics of binding may be described in terms of  $k_2$ r a radioligand of known  $K_D$  and fixed concentration <br>], the pseudo-first-order association rate constant,<br>[*L*], is a simple function of  $k_2$ . Therefore the kinetics<br>binding may be described in terms of  $k_2$  and  $k_4$ 

[*L*], the pseudo-first-order association rate constant,  $k_1[L]$ , is a simple function of  $k_2$ . Therefore the kinetics of binding may be described in terms of  $k_2$  and  $k_4$ . The time required for equilibrium to be achi which  $k_2 \gg k_4$ . The time required for equilibrium to be achieved<br>rds heavily on the relative values of  $k_2$  and  $k_4$ .<br>msider first the case in which  $k_2 \ll k_4$ , then the case<br>ich  $k_2 \gg k_4$ .<br>*I.*  $k_2 \ll k_4$ . The amount of time required

pends heavily on the relative values of  $k_2$  and  $k_4$ . We<br>consider first the case in which  $k_2 \ll k_4$ , then the case in<br>which  $k_2 \gg k_4$ .<br>*I.*  $k_2 \ll k_4$ . The amount of time required for a compet-<br>itive binding incubati consider first the case in which  $k_2 \ll k_4$ , then the case in<br>which  $k_2 \gg k_4$ .<br>I.  $k_2 \ll k_4$ . The amount of time required for a compet-<br>itive binding incubation to reach equilibrium depends, in<br>part, on the radioligand c which  $k_2 \gg k_4$ .<br>
I.  $k_2 \ll k_4$ . The amount of time required for a competitive binding incubation to reach equilibrium depends, in part, on the radioligand concentration. We consider the two extremes, when the radioligan itive binding incubation to reach equilibrium depends, in part, on the radioligand concentration. We consider the two extremes, when the radioligand concentration is very low and when it is very high.<br>(a) Very low radioli  $part, on the radioligand concentration. We consider the$ 

(a) Very low radioligand concentration: here  $[L] \ll \frac{1}{2}$ 

$$
[I] = IC_{50} = K_I([L]/K_D + 1) \simeq K_I
$$

early  $IC_{50}$  is less than equilibrium  $K_I$ , or if the early slope<br>factor is less than 1.0 (and the equilibrium slope factor<br>is equal to 1).<br>4. It may not be feasible to allow an incubation to<br>proceed long enough for equi (a) Very low radioligand concentration: here  $[L] \ll$ <br>  $[I] = IC_{50} = K_I([L]/K_D + 1) \approx K_I$ <br>
Because  $k_3[I] (= k_4)$  is much larger than  $k_1[L]$ , the mpetitor will bind rapidly and the radioligand binding  $K_D$ :<br>  $[I] = IC_{50} = K_I([L]/K_D + 1) \approx K_I$ <br>
Because  $k_3[I] (= k_4)$  is much larger than  $k_1[L]$ , the<br>
competitor will bind rapidly and the radioligand binding<br>
will take longer. Thus the competitor will always be  $[I] = IC_{50} = K_I([L]/K_D + 1) \approx K_I$ <br>Because  $k_3[I] (= k_4)$  is much larger than  $k_1[L]$ , the<br>competitor will bind rapidly and the radioligand binding<br>will take longer. Thus the competitor will always be<br>nearly at equilibrium with fre Because  $k_3[I] (= k_4)$  is much larger than  $k_1[L]$ , the<br>competitor will bind rapidly and the radioligand binding<br>will take longer. Thus the competitor will always be<br>nearly at equilibrium with free receptors, and we need<br>on Because  $k_3[I] (= k_4)$  is much larger the competitor will bind rapidly and the radioli<br>will take longer. Thus the competitor will nearly at equilibrium with free receptors,<br>only consider the binding of the radioligand only consider the binding of the radioligand:

$$
d[RL]/dt = k_1[L][R] - k_2[RL]
$$
  
[R] = (N - [RL])/2

*d*[*RL*]/*dt* =  $k_1[L][R] - k_2[RL]$ <br>  $[R] = (N - [RL])/2$ <br>
(half of the receptors not occupied by radioligand will be bound to competitor because the competitor is present  $d[KL]/dt = k_1[L][R] - k_2[KL]$ <br>  $[R] = (N - [RL])/2$ <br>
(half of the receptors not occupied by radioligand will be<br>
bound to competitor because the competitor is present<br>
at its  $K_l$  and equilibrates rapidly).  $[R] = (N - [RL])/2$ <br>(half of the receptors not occupied bound to competitor because the<br>at its  $K_l$  and equilibrates rapidly).<br>Solving for  $[RL]$ :  $[R] = \sqrt{R}$ <br>(half of the receptors<br>bound to competitor<br>at its  $K_I$  and equilibright solving for  $[RL]$ :

or 
$$
[RL]
$$
:  
\n $[RL] = N/2(1 - \exp(-k_1[L] - k_2t))$   
\n $\approx N/2(1 - \exp(-k_2t))$ 

 $[RL] = N/2(1 - \exp(-k_1[L] - k_2t))$ <br>  $\approx N/2(1 - \exp(-k_2t))$ <br>
The half-life is 0.69/k<sub>2</sub>; equilibrium is achieved at 3.5/<br>  $k_2$ . This is the same amount of time required for the  $[RL] = N/2(1 - \exp(-k_1[L] - k_2t))$ <br>  $\approx N/2(1 - \exp(-k_2t))$ <br>
The half-life is 0.69/k<sub>2</sub>; equilibrium is achieved at 3.5/<br>  $k_2$ . This is the same amount of time required for the

B MOTULSKY AND MAHAN<br>binding of radioligand alone, when it is present at very su<br>low concentration.  $=$ <br>(b) Very high radioligand concentration: here  $[L] \gg ex$ 

MOTULSKY AND MAHAN<br>
binding of radioligand alone, when it is present at very subst<br>
low concentration.  $= k$ <br>
(b) Very high radioligand concentration: here  $[L] \gg \exp(-k)$ <br>  $K_D$ : *[I]* <sup>=</sup> IC50 <sup>=</sup> *K,([L]/K +* 1)

$$
[I] = IC_{50} = K_I([L]/K_D + 1)
$$

(b) Very high radioligand concentration: here  $[L] \gg K_D$ :<br>  $[I] = IC_{50} = K_I([L]/K_D + 1)$ <br>
The pseudo-first-order on-rate of the radioligand,  $k_1[L]$ ,<br>
can be expressed as  $k_2[L]/K_D$ ). Similarly, the pseudo- $K_D:$ <br>  $[I] = IC_{50} = K_I([L]/K_D + 1)$ <br>
The pseudo-first-order on-rate of the radioligand,  $k_1$ <br>
can be expressed as  $k_2[L]/K_D$ ). Similarly, the pseu<br>
first-order on-rate of the competitor,  $k_3[I]$ , can be  $[I] = IC_{50} = K_I([L]/K_D + 1)$ <br>The pseudo-first-order on-rate of the radioligand,  $k_1[L]$ ,<br>can be expressed as  $k_2[L]/K_D$ ). Similarly, the pseudo-<br>first-order on-rate of the competitor,  $k_3[I]$ , can be ex-<br>pressed as  $k_4([L]/K_D + 1)$ The pseudo-first-order on-rate of the radioligand,  $k_1[L]$ ,<br>can be expressed as  $k_2[L]/K_D$ ). Similarly, the pseudo-<br>first-order on-rate of the competitor,  $k_3[I]$ , can be ex-<br>pressed as  $k_4([L]/K_D + 1)$ . Given that  $k_4 \gg k_2$ The pseudo-first-order on-rate of the radioligand,  $k_1[L]$ ,<br>can be expressed as  $k_2[L]/K_D$ . Similarly, the pseudo-<br>first-order on-rate of the competitor,  $k_3[I]$ , can be ex-<br>pressed as  $k_4([L]/K_D + 1)$ . Given that  $k_4 \gg k_2$ can be expressed as  $k_2[L]/K_D$ . Similarly, the pseudo-<br>first-order on-rate of the competitor,  $k_3[I]$ , can be ex-<br>pressed as  $k_4([L]/K_D + 1)$ . Given that  $k_4 \gg k_2$ , the<br>competitor will therefore bind to the receptor much fa first-order on-rate of the competitor,  $k_3[I]$ , can be expressed as  $k_4([L]/K_D + 1)$ . Given that  $k_4 \gg k_2$ , the receptor will therefore bind to the receptor much faster p than will the radioligand. Thus again the competito pressed as  $k_4([L]/K_D + 1)$ . Given that  $k_4 \gg k_2$ , the receptors. Another approach is to analyze the time decompetitor will therefore bind to the receptor much faster pendence of Eq. 1. The binding described by that equati than will the radioligand. Thus again the competitor will rapidly than the radioligand will. Equilibrium will be  $e$ stablished as the radioligand reaches equilibrium with

$$
d[RL]/dt = k_1[L][R] - k_2[RL]
$$

$$
[R] = N - [RL] - [RI]
$$

 $B_1 = N - [RL] - [RL]$ <br>Because the competitor will always be virtually at<br>when is  $K_S > k_4$ ? Expanding<br>willibrium with the free receptors,<br> $[RI] = [R][I]/K$ .<br>Therefore,  $-K_A K_B + k_1[L]k_3[I]$  $d[RL]/dt = k_1[L][R]$ <br>  $[R] = N - [RL] - [R]$ <br>
Because the competitor will a<br>
equilibrium with the free receptors<br>  $[RL] = [R][L]/R$ Because the competitor wi<br>equilibrium with the free recep<br> $[RI] = [R]$ <br>From the definition of IC<sub>50</sub>,<br> $[II/K] = [I][K_R]$ 

$$
[RI] = [R][I]/K_i
$$

$$
[RI] = [R][I]/K_I
$$
  
inition of IC<sub>50</sub>,  

$$
[I]/K_I = [L]/K_D + 1 \simeq [L]/K_D
$$
  

$$
\Rightarrow K_{\sim}
$$
 Substituting

(because  $[L] \gg K_D$ ). Substituting

$$
[R] = [RI]K_I/I = [RI]K_D/[L],
$$
  
\n
$$
[R] = (N - [RL])K_D/[L]
$$
  
\n
$$
d[RL]/dt = k_2N - 2k_2[RL]
$$

After integrating,

$$
[RL] = N/2(1 - \exp(-2k_2t))
$$

 $a[RL]/dt = k_2N - 2k_2[RL]$ <br>
ter integrating,<br>  $[RL] = N/2(1 - \exp(-2k_2t))$ <br>
Therefore, the half-life is 0.35/k<sub>2</sub> and equilibrium is the<br>
ached at 1.75/k<sub>2</sub> min. Thus, by increasing the radioli-After integrating,<br>  $[RL] = N/2(1 - \exp(-2k_2 t))$ <br>
Therefore, the half-life is  $0.35/k_2$  and equilibrium<br>
reached at  $1.75/k_2$  min. Thus, by increasing the radioly<br>
gand concentration, the time required to reach equil  $[RL] = N/2(1 - \exp(-2k_2 t))$ <br>Therefore, the half-life is 0.35/ $k_2$  and equilibrium<br>reached at 1.75/ $k_2$  min. Thus, by increasing the radi<br>gand concentration, the time required to reach equi<br>rium is halved. Why cannot the react Therefore, the half-life is  $0.35/k_2$  and equilibrium is<br>reached at  $1.75/k_2$  min. Thus, by increasing the radioli-<br>gand concentration, the time required to reach equilib-<br>rium is halved. Why cannot the reaction be "pushe Therefore, the half-life is  $0.35/k_2$  and equilibrium is<br>reached at  $1.75/k_2$  min. Thus, by increasing the radioli-<br>gand concentration, the time required to reach equilib-<br>rium is halved. Why cannot the reaction be "pushe reached at 1.75/ $k_2$  min. Thus, by increasing the radioli-<br>gand concentration, the time required to reach equilib-<br>rium is halved. Why cannot the reaction be "pushed"<br>faster? The rate at which the radioligand binds is pr gand concentration, the time required to reach equilibrium is halved. Why cannot the reaction be "pushed" faster? The rate at which the radioligand binds is proportional to both its concentration and the number of free rec faster? The rate at which the radioligand binds is pro-<br>portional to both its concentration and the number of<br>free receptors. When the radioligand concentration is<br>increased, the concentration of competitor must also be<br>i faster? The rate at which the radioligand binds is pro-<br>portional to both its concentration and the number of<br>free receptors. When the radioligand concentration is<br>increased, the concentration of competitor must also be<br>i portional to both its concentration and the number of<br>free receptors. When the radioligand concentration is<br>increased, the concentration of competitor must also be<br>increased (so that it remains as its  $IC_{50}$ ), and the nu free receptors. When the radioligand concentration is<br>increased, the concentration of competitor must also be<br>increased (so that it remains as its  $IC_{50}$ ), and the number<br>of free receptors decreases. The product of radio increased, the concentration of competitor must also be<br>increased (so that it remains as its  $IC_{50}$ ), and the number<br>of free receptors decreases. The product of radioligand<br>concentration times free receptor concentration radioligand. Free receptors decreases. The product of radioligand<br>ncentration times free receptor concentration can at<br>st be doubled by increasing the concentration of the<br>dioligand.<br>II.  $k_2 \gg k_4$ . In this case the competitor will bi concentration times free receptor concentration can at<br>best be doubled by increasing the concentration of the<br>radioligand.<br> $II. k_2 \gg k_4$ . In this case the competitor will bind much<br>more slowly than radioligand. We can ther

more slowly than radioligand. We can therefore consider radioligand.<br>II.  $k_2 \gg k_4$ . In this case the competitor will bind much<br>more slowly than radioligand. We can therefore consider<br>the free receptors and radioligand always to be at equi-<br>librium. The time required for the e II.  $k_2 \gg k_4$ . In this case the competitor will bind much<br>more slowly than radioligand. We can therefore consider<br>the free receptors and radioligand always to be at equi-<br>librium. The time required for the entire competi more slowly than radioligand. We can therefore consider<br>the free receptors and radioligand always to be at equi-<br>librium. The time required for the entire competitive<br>binding incubation to reach equilibrium is therefore t the free receptors and radioligand always to be at equilibrium. The time required for the entire competitive binding incubation to reach equilibrium is therefore the time required for the competitor to reach equilibrium w binding incubation to reach equilbrium is therefore the time required for the competitor to reach equilibrium with the free receptors. The math is similar to that above:<br> $d[RI]/dt = k_3[I][R] - k_4[RI]$ 

$$
d[RI]/dt = k_3[I][R] - k_4[R]
$$
  
[R] = N - [RI] - [RL]  
[RL] = [R][L]/K<sub>D</sub>

substituting,  $[R] = (N - [RI]/(1 + [L]/K_D)$  and  $d[RI]/dt$ <br>=  $k_4N - 2k_4[RI]$ . Integrating,  $[RI] = N/2(1 - exp(-2k_4t))$ . bstituting,  $[R] = (N - [RI]/(1 + [L]/K_D)$  and  $d[RI]/dt$ <br>  $k_4N - 2k_4[RI]$ . Integrating,  $[RI] = N/2(1 - p(-2k_4t))$ .<br>
Equilibrium is therefore reached in  $3.5/2k_4 = 1.75/k_4$ <br>
in. Note that in this case the concentration of radiolisubstituting,  $[R] = (N - [RI]/(1 + [L]/K_D)$  and  $d[RI]$ <br>=  $k_4N - 2k_4[RI]$ . Integrating,  $[RI] = N/2(1$ <br>exp(-2 $k_4 t$ )).<br>Equilibrium is therefore reached in 3.5/2 $k_4 = 1.75$ <br>min. Note that in this case the concentration of radio<br>gand is

=  $k_4N - 2k_4[RI]$ . Integrating,  $[RI] = N/2(1 - \exp(-2k_4 t))$ .<br>
Equilibrium is therefore reached in  $3.5/2k_4 = 1.75/k_4$ <br>
min. Note that in this case the concentration of radioli-<br>
gand is irrelevant.<br>
Determining the duration of

Equilibrium is therefore reached in  $3.5/2k_4 = 1.75/k_4$ <br>min. Note that in this case the concentration of radioli-<br>gand is irrelevant.<br>Determining the duration of time required to reach<br>equilibrium depended largely on cons min. Note that in this case the concentration of radioli-<br>gand is irrelevant.<br>Determining the duration of time required to reach<br>equilibrium depended largely on considering the relative<br>rates at which radioligand and compe gand is irrelevant.<br>Determining the duration of time required to reach<br>equilibrium depended largely on considering the relative<br>rates at which radioligand and competitor bind to the<br>receptors. Another approach is to analyz Determining the duration of time required to readequilibrium depended largely on considering the relation<br>rates at which radioligand and competitor bind to the<br>receptors. Another approach is to analyze the time d<br>pendence equilibrium depended largely on considering the relative rates at which radioligand and competitor bind to the receptors. Another approach is to analyze the time dependence of Eq. 1. The binding described by that equation rates at which radioligand and competitor bind to the receptors. Another approach is to analyze the time dependence of Eq. 1. The binding described by that equation will reach equilibrium as the slower exponential term in receptors. Another approach is to analyze the time dependence of Eq. 1. The binding described by that equation will reach equilibrium as the slower exponential term involving  $K_s$  reaches equilibrium. Evaluating  $K_s$  nume pendence of Eq. 1. The binding described by that equation will reach equilibrium as the slower exponential term involving  $K_S$  reaches equilibrium. Evaluating  $K_S$  numerically with various values for the kinetic constants above. and [L] yielded conclusions identical with those derived when is  $K_S > k_4$ ,  $k_F$ ,  $K_S$ <br>When is  $K_S > k_4$ ? Expanding  $K_S$  and rearranging yields<br> $\sqrt{(K_{1} + K_{2})^2 - 4K_{1}K_{2} + 4h_{1}L_{1}h_{2}L_{2} + 2h_{2}L_{1}K_{2} + 4h_{2}L_{2}h_{2} + 4h_{2}L_{2}h_{1}L_{2}h_{2} + 4h_{2}L_{2}h_{2}h_{1}L_{1}h_{2}h_{2}h_{1}h_{2$ 

When is 
$$
K_S > k_4
$$
? Expanding  $K_S$  and rearranging yields  
\n $\sqrt{(K_A + K_B)^2 - 4K_AK_B + 4k_1[L]k_3[I]} < 2k_4 - (K_A + K_B)$   
\nTherefore,  $-K_AK_B + k_1[L]k_3[I] > (k_4)^2 - k_4K_A - k_4K_B$ .  
\nNote that squaring the negative expressions caused the  
\nsign of the inequality to change. Simplifying this expres-

$$
\sqrt{(K_A + K_B)^2 - 4K_AK_B + 4k_1[L]k_3[I] < 2k_4 - (K_A + K_B)}
$$
\nTherefore,  $-K_A K_B + k_1[L]k_3[I] > (k_4)^2 - k_4K_A - k_4K_B$ .\nNote that squaring the negative expressions caused the sign of the inequality to change. Simplifying this expression yields  $k_4 < k_2$ . Similarly  $K_S > k_2$  when  $k_4 > k_2$ . When is  $K_F > k_4$  or  $K_F > k_2$ ? Similar algebra leads to a tautology; therefore,  $K_F$  is always greater than  $k_2$  and

a tautology; therefore,  $K_F$  is always greater than  $k_2$  and *k4.* In yields  $k_4 < k_2$ . Similarly  $K_S > k_2$  when  $k_4 > k_2$ .<br>When is  $K_F > k_4$  or  $K_F > k_2$ ? Similar algebra leads to tautology; therefore,  $K_F$  is always greater than  $k_2$  and  $K_F$  is always greater than  $k_2$  and  $k_4$ ;  $K_S$ 

between  $k_2$  and  $k_4$ . 4. tautology; therefore,  $R_F$  is always greater than  $k_2$  and  $k_4$ .<br>
Thus  $K_F$  is always greater than  $k_2$  and  $k_4$ ;  $K_S$  is always<br>
between  $k_2$  and  $k_4$ .<br>
4. Proof That the Binding of Radioligand "Overshoots"<br>
Its *If* Its Equilibrium Indian in the Sinding of Radiolignum Value if  $k_4$  *Zequilibrium Value if*  $k_4 < k_2$ <br> *Its Equilibrium Value if*  $k_4 < k_2$ <br> *In Eq. 1 the [RL]* is defined by the s

tween  $k_2$  and  $k_4$ .<br>*Proof That the Binding of Radioligand "Overshot*<br>*Its Equilibrium Value if*  $k_4 < k_2$ <br>In Eq. 1 the [*RL*] is defined by the sum of its equi<br>im value plus two exponential terms. When th 4. Proof That the Binding of Radioligand "Overshoots"<br>Its Equilibrium Value if  $k_4 < k_2$ <br>In Eq. 1 the [RL] is defined by the sum of its equilib-<br>rium value plus two exponential terms. When these<br>terms are positive, [RL] w 4. Proof I nat the Binaing of Radioligana Overshoots<br>
Its Equilibrium Value if  $k_4 < k_2$ <br>
In Eq. 1 the [RL] is defined by the sum of its equilib-<br>
rium value plus two exponential terms. When these<br>
terms are positive, [RL Its Equilibrium value  $y_{k_4}$   $\leq$ <br>In Eq. 1 the [RL] is defined b<br>rium value plus two exponent<br>terms are positive, [RL] will value. This will occur when<br> $h = K_2$ 

$$
\frac{k_4-K_F}{K_F}\exp(-K_Ft)-\frac{k_4-K_S}{K_S}\exp(-K_St)>0
$$

value. I has will occur when<br>  $\frac{k_4 - K_F}{K_F} \exp(-K_F t) - \frac{k_4 - K_S}{K_S} \exp(-K_S t) > 0$ <br>
Because  $K_F$  is always greater than  $k_4$ , the first term will<br>
always be negative. The second term will make a positive  $\frac{k_4 - K_F}{K_F}$  exp( $-K_F t$ )  $-\frac{k_4 - K_S}{K_S}$  exp( $-K_S t$ ) > 0<br>Because  $K_F$  is always greater than  $k_4$ , the first term will<br>always be negative. The second term will make a positive<br>contribution when  $k_4 < K_S$ ; this occurs when Exp( $-K_F t$ ) -  $K_S$  exp( $-K_S t$ ) > 0<br>Because  $K_F$  is always greater than  $k_4$ , the first term will<br>always be negative. The second term will make a positive<br>contribution when  $k_4 < K_S$ ; this occurs when  $k_4 < k_2$ .<br>This is a su Because  $K_F$  is always greater than  $k_4$ , the first term will always be negative. The second term will make a positive contribution when  $k_4 < K_S$ ; this occurs when  $k_4 < k_2$ . This is a sufficient condition for the entire Because  $K_F$  is always greater than  $k_4$ , the first term will always be negative. The second term will make a positive contribution when  $k_4 < K_S$ ; this occurs when  $k_4 < k_2$ . This is a sufficient condition for the entire always be negative. The seconorchibution when  $k_4 < K_S$ ;<br>This is a sufficient condition<br>positive at long time points<br>approach zero at these times. contribution when  $R_4 < R_5$ ; this occurs when  $R_4 < R_2$ .<br>This is a sufficient condition for the entire sum to be<br>positive at long time points, because  $\exp(-K_F t)$  will<br>approach zero at these times.<br>5. What Is the Minimum Ra

## *is* is a surficient condition for the entire sum to be<br>sitive at long time points, because  $exp(-K_F t)$  will<br>proach zero at these times.<br>What Is the Minimum Ratio of Binding of Radioligand<br>in the Presence of Competitor Compa Filive at long time<br>*in Its Abe Minim*<br>*in the Presence of Un Its Absence*?<br>As shown in Fig. 31 What Is the Minimum Ratio of Binding of Radioligand<br>in the Presence of Competitor Compared with Binding<br>in Its Absence?<br>As shown in Fig. 3B, the binding ratio dips below its<br>uilibrium value only if  $k_2 < k_4$ . This dip is

Equilibrium value of Competitor Compared with Binding<br>in the Presence of Competitor Compared with Binding<br>in Its Absence?<br>As shown in Fig. 3B, the binding ratio dips below its<br>equilibrium value only if  $k_2 < k_4$ . This dip in the Presence of Competitor Compared with Binding<br>in Its Absence?<br>As shown in Fig. 3B, the binding ratio dips below its<br>equilibrium value only if  $k_2 < k_4$ . This dip is most<br>pronounced when  $k_3$  and  $k_4$  are large, as In the most extreme case,  $K_B \gg K_A$ . This dip is most<br>pronounced when  $k_3$  and  $k_4$  are large, as seen in Fig. 4.<br>In the most extreme case,  $K_B \gg K_A$  and  $K_S = K_A$  and  $K_F = K_B$ . We cannot evaluate the binding ratio at time z As shown in Fig. 3B, the binding ratio dips below its<br>equilibrium value only if  $k_2 < k_4$ . This dip is most<br>pronounced when  $k_3$  and  $k_4$  are large, as seen in Fig. 4.<br>In the most extreme case,  $K_B \gg K_A$  and  $K_S = K_A$  and equilibrium value only if  $k_2 < k_4$ . This dip is most<br>pronounced when  $k_3$  and  $k_4$  are large, as seen in Fig. 4.<br>In the most extreme case,  $K_B \gg K_A$  and  $K_S = K_A$  and  $K_F = K_B$ . We cannot evaluate the binding ratio at time In the most extreme case,  $K_B \gg K_A$  and  $K_S = K_A$  and  $K_F = K_B$ . We cannot evaluate the binding ratio at time zero because there is no binding (division by zero), but we can evaluate the ratio at the earliest time dt. Because  $= K_B$ . We cannot evaluate the binding ratio at time zero because there is no binding (division by zero), but we can evaluate the ratio at the earliest time *dt*. Because  $[RL] = 0$  at time zero,  $[RL]$  will equal the derivat

Without competitor,  $[RL]$  at early time points will be<br> $[RL] = d[RL]/dt = Nh_{\perp}[L] = e^{n\pi r}(-K_{\perp}) \approx Nh_{\perp}[L]$ 

Without competitor, [RL] at early time points will be 
$$
[RL] = d[RL]/dt = Nk_1[L](1 - \exp(-K_A)) \simeq Nk_1[L]
$$
\nIn the presence of competitor, [RL] will be  $[BL] = d[BL]/dt = (NK[1]/(K - K_1))[(h -$ 

in the presence of competitor, 
$$
[KL]
$$
 will be  
\n
$$
[RL] = d[RL]/dt = (NK[L]/(K_F - K_S))[(k_4 - K_S)\exp(-K_S t) - (k_4 - K_F)\exp(-K_F t)]
$$
\nIn the most extreme case,  $K_F \gg K_S$  and at the earliest time point  $\exp(-K_F) \approx 0$  and  $\exp(-K_S t) \approx 1$ . Therefore,

 $[RL] = d[RL]/dt = (NK[L]/(K_F - K_S))[(k_4 - K_S)(K_S - K_S)]$ <br>  $K_S) \exp(-K_S t) - (k_4 - K_F) \exp(-K_F t)$ <br>
In the most extreme case,  $K_F \gg K_S$  and at the earliest<br>
time point  $\exp(-K_F) \approx 0$  and  $\exp(-K_S t) \approx 1$ . Therefore,<br>  $[RL] = NK_1[L](k_4 - K_S)/(K_F - K_S) \approx Nk_1[L]k_4/K_F$ . Th  $K_S$ ) $\exp(-K_S t) - (k_4 - K_F)\exp(-K_F t)$ <br>
In the most extreme case,  $K_F \gg K_S$  and at the earliest<br>
time point  $\exp(-K_K) \approx 0$  and  $\exp(-K_S t) \approx 1$ . Therefore,<br>  $[RL] = NK_1[L](k_4 - K_S)/(K_F - K_S) \approx Nk_1[L]k_4/K_F$ . The  $\frac{R_{2.6} \cdot 6.124-36}{3.0333}$ <br>
Ther In the most extreme case,  $K_F \gg K_S$  and at the earliest<br>time point  $\exp(-K_F) \approx 0$  and  $\exp(-K_S t) \approx 1$ . Therefore,<br> $[RL] = NK_1[L] (k_4 - K_S)/(K_F - K_S) \approx Nk_1[L]k_4/K_F$ . The<br>ratio therefore is  $\approx k_4/K_F \approx k_4/K_B \approx K_1/([I] + K_1)$ . This<br>ratio will b  $[RL] = NK_1[L](k_4 - K_S)/(K_F - K_S) \approx Nk_1[L]k_4/K_F$ . The<br>ratio therefore is  $\approx k_4/K_F \approx k_4/K_B \approx K_I/([I] + K_I)$ . This<br>ratio will be 1:2 when  $[I] = K_I$ . By definition, when this<br>ratio is 1:2,  $[I] = IC_{50}$ . In other words, at the earliest<br>time poin ratio will be 1:2 when  $[I] = K_I$ . By definition, when this as metric is 1:2,  $[I] = IC_{50}$ . In other words, at the earliest  $B_{50}$  and  $IC_{50} = K_I$ . This relationship was derived for and the extreme case in which  $K_B \gg K_A$ . In l ratio is 1:2,  $[I] = IC_{l}$ <br>time points  $IC_{50} = K_{l}$ ,<br>the extreme case in v<br>cases,  $IC_{50} > K_{l}$  initial<br>be less than the  $K_{l}$ .

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