

Kinetics of the Interaction of Dihydroalprenolol with Beta-Adrenergic Receptors in Rat Cerebral Cortex

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Abstract. Time and temperature dependence of the binding of ^3H -dihydroalprenolol (^3H -DHA) to beta-adrenergic receptors in rat cerebral cortex is described. The kinetic data obtained suggest that ^3H -DHA binding proceeds through a two-step reaction scheme consisting of a bimolecular association step followed by an unimolecular internal conversion of the radioligand receptor complex (isomerisation).

Equilibrium thermodynamic analysis provided evidence that the over-all binding process is associated with a small decrease in enthalpy and a substantial increase in entropy.

Within the framework of the two-step binding kinetics, the evaluation of the temperature dependence by the van't Hoff analysis resulted in values for thermodynamic parameters for the single equilibrium steps. The data suggest that the association step can be considered as a bimolecular hydrophobic interaction which is mainly entropy-driven due to the release of structural water, while the isomerisation step is accompanied by a large negative change in both enthalpy and entropy.

The large negative change in the activation entropy for the forward reaction of the isomerisation step, obtained from evaluation of Arrhenius plots, indicates an internal conversion to a highly ordered receptor-ligand complex, while the low activation energy points to a small threshold energy for reaching this structure. Thus, these results support a previous assumption that the hydrophobic center of an adrenergic antagonist interacts with the receptor by entering a pocket (Cherksey et al. 1981).

Key words: Kinetics — Beta-adrenergic receptor — Temperature dependence — van't Hoff plot — Arrhenius parameter

Introduction

Since the conceptual characterisation of the adrenergic receptor by Ahlquist (1948) the use of radiolabelled ligands has led to an increasingly detailed

knowledge of the kinetic and pharmacological properties of the receptor, of its localisation and distribution in a variety of tissues as well as of the existence of subtypes (Williams and Lefkowitz 1978). Radioligand binding studies have been used to investigate the way in which neurotransmitter or drugs exert their physiological or pharmacological action on the receptor. Detailed consideration of the kinetic behaviour of the interaction of ligands with the membrane-bound receptor molecules may further assist the understanding of underlying molecular mechanisms.

Little is known about the energetic aspects of such interactions, and thermodynamic analysis of the binding process may be one way to gain more insight into the elementary steps of this reaction. Investigation of the time dependence of the binding process may elucidate the elementary steps involved in the over-all interaction, while the thermodynamic parameters, such as enthalpy and entropy changes, can be determined from the temperature dependence of the individual reaction steps.

Thermodynamic analysis of ligand binding to mammalian beta-adrenergic receptors has shown that there are fundamental differences between the molecular interactions of agonists and antagonists (Weiland et al. 1979, 1980). Recently, temperature-dependent changes in ligand binding to beta-adrenergic receptors of intact S49 lymphoma cells (Insel and Sanda 1979), the temperature dependence of the benzodiazepine receptor interaction (Quast et al. 1982; Speth et al. 1979) as well as temperature-dependent activation, desensitisation and resensitisation of muscarinic acetylcholine receptor in intact mouse neuroblastoma cells (El-Fakahamy and Richelson 1980) have been reported and the energetic parameters evaluated.

Thermodynamic analysis of the interaction of cholinergic agonists and antagonists with the acetylcholine receptor has provided evidence that the binding process is mainly entropy-driven (Miller et al. 1979; Maelicke et al. 1977).

Distance geometry analysis, as performed recently for the benzodiazepine binding site (Crippen 1982), are dependent on sufficiently exact thermodynamic data, and emphasize the importance of the thermodynamic approach.

On the basis of detailed kinetic studies of the binding of ^{125}I -iodohydroxybenzylpindolol to beta-adrenergic receptors (Ross et al. 1977) and ^3H -quinuclidinyl benzilate to muscarinic acetylcholine receptors (Galper et al. 1977; Jarv et al. 1979) a receptor isomerisation model has been proposed in which the antagonist initially forms with the receptor a rapidly dissociating complex which is then converted into a slowly dissociating ligand-receptor complex. This hypothesis of antagonist-promoted transition of receptor states conflicts with the finding that antagonists do not induce receptor-effector coupling and activation of adenylate cyclase (DeLean et al. 1980). Recently, an alternative explanation has been given for the apparent "two-step" binding kinetics of high-affinity racemic antagonist

radioligands (Bürgisser et al. 1981).

The aim of this paper was to elucidate the molecular mechanisms underlying the interaction of ^3H -dihydroalprenolol (^3H -DHA) with beta-adrenergic receptors in the rat brain cerebral cortex. Kinetic studies at different temperatures were performed and compared with corresponding thermodynamic data obtained from equilibrium binding experiments.

Materials and Methods

Material. 1-[propyl-2,3- ^3H] dihydroalprenolol (^3H -DHA), specific radioactivity 1628 GBq/mmol (37 kBq/ μl), was purchased from the Radiochemical Centre, Amersham, and used without further purification. L-Isoproterenol D-bitartrate was obtained from SIGMA Chemical Co.. All other chemicals used were reagent grade.

Tissue preparation. Rats (strain BD III) of either sex were reared under normal animal house conditions and were killed by decapitation at ages of 15–16 weeks. The brains were rapidly removed, placed on ice and the cerebral cortex isolated. Tissue samples were homogenised in 9 ml ice-cold 50 mmol/l Tris-HCl, pH 8.0, containing about 300 mg tissue wet weight, centrifuged at 45,000 g for 20 min. The resulting pellet was washed once, resuspended in Tris buffer and stored at -20°C until use less than one week later.

Equilibrium binding experiments. Equilibrium binding studies were carried out by the filtration method described by Bylund (1978). Briefly, binding assays were run in triplicate in glass tubes which contained 900 μl of the membrane suspension corresponding to about 200 μg protein. The reactions were initiated by the addition of 100 μl ^3H -DHA to the tubes to give final concentrations ranging between 0.1 and 4 nmol/l. Samples were incubated for 15 min at 35°C , 20 min at 30°C ; 30 min at 25°C ; and 45 min at 20°C . Under these conditions, the binding of ^3H -DHA was shown to reach equilibrium. Reactions were stopped by the addition of 3 ml ice-cold buffer to each assay tube, and the samples were rapidly filtered through glass fibre filters NK VIII (VEB Feinpapierfabrik Neu-Kaliss, G. D. R.) and rinsed by two aliquots of 4 ml ice-cold buffer. The filters were placed in scintillation vials and the bound radioactivity determined.

The specific binding of ^3H -DHA was defined as the amount of ^3H -DHA bound in the absence of competing ligand minus the amount bound in the presence of 100 $\mu\text{mol/l}$ L-isoproterenol. Specific binding was about 50–60 % of total ^3H -DHA binding. Under these assay conditions less than 10 % of ^3H -DHA was bound in each experiment performed.

Kinetic binding experiments. Kinetic studies were performed in a thermostated 100 ml reaction vessel. The temperature of the reaction mixture was kept within 0.1°C during the reaction. The cerebral cortex membrane suspension (40 ml, containing 0.5 mg protein/ml) and the ^3H -DHA solution (5 ml, to give final concentrations ranging from 0.2 to 3.8 $\mu\text{mol/l}$) were separately warmed to the required temperature shortly prior to the initiation of incubation. The reaction was started by rapidly mixing the ^3H -DHA solution into the membrane suspension. At appropriate time intervals, 200 μl aliquots of the reaction mixture were removed, filtered through glass fibre filters NK VIII under vacuum and immediately rinsed with 8 ml ice-cold buffer. Bound radioactivity was measured by liquid scintillation counting. The decrease in volume of the reaction mixture (at the end of the reaction) was kept to less than 10 %. The kinetic measurements were carried out at three different temperatures ranging between 25 and 35°C . To determine nonspecific binding a 1 ml aliquot of the reaction mixture was incubated in the presence of 100 $\mu\text{mol/l}$ L-isoproterenol under the same conditions and treated as described.

Calculation procedures. To evaluate equilibrium constants from the equilibrium binding studies, the Scatchard plot was used:

$$\frac{b_{sp}}{f} = -\frac{1}{K_D} b_{sp} + \frac{K_D}{b_{max}} \quad (1)$$

where b_{sp} denotes the specific binding; f the free ligand concentration; K_D , the equilibrium dissociation constant; b_{max} the maximum receptor number. Both K_D and b_{max} can be calculated from the slope and intercept, respectively, by applying least squares regression analysis.

For the determination of equilibrium thermodynamic parameters, the classical thermodynamic equations were used. The standard Gibbs free energy change, ΔG° , was calculated from the equation

$$\Delta G^\circ = -RT \ln K_D \quad (2)$$

where R is the gas constant (8.29 J/mol degree), and T the temperature in degrees Kelvin.

The standard enthalpy change, ΔH° , was calculated according to the van't Hoff equation of the dependence of K_D on temperature:

$$\log K_D = -\frac{\Delta H^\circ}{2.303 R} \frac{1}{T} + \frac{\Delta S^\circ}{2.303 R} \quad (3)$$

The slope of the van't Hoff plot $\log K_D$ versus T^{-1} is $-\Delta H^\circ/2.303 R$. The standard entropy change, ΔS° , was estimated from the Gibbs-Helmholtz equation

$$\Delta G^\circ = \Delta H^\circ - T \Delta S^\circ \quad (4)$$

after determining first ΔG° and ΔH° as described above.

The kinetics of the binding of ^3H -DHA to beta-adrenergic receptors were studied under pseudo first-order conditions involving a 100 to 2000-fold excess of ligand (^3H -DHA) over receptor concentration which allowed the data of the time dependence of the binding of ^3H -DHA to be fitted by the first-order rate equation

$$\log \frac{b_{t,eq} - b_t}{b_{t,eq} - b_i} = \frac{k_{obs}}{2.303} t + \log F \quad (5)$$

with

$$F = 1 - b_{nonsp}/b_{t,eq}$$

and

$$k_{obs} = k_1[L] + k_{-1}$$

where b_t is the total bound amount at time t ; $b_{t,eq}$ designates the total amount of ^3H -DHA bound at equilibrium; b_{nonsp} denotes the nonspecific binding and k_{obs} is the pseudo first-order rate constant. b_i was the sum of the specific and nonspecific binding at time t . Because in all cases the nonspecific binding was constant by 1 min of incubation, the total change in radioligand binding reflected only the specific binding. The quantity $b_{t,eq}$ was estimated from the plot b_t versus t ; k_{obs} and b_{nonsp} were evaluated from the slope and intercept of the plot $\log b_{t,eq}/(b_{t,eq} - b_t)$ versus t , respectively, using least squares regression analysis.

Independent estimates of b_{nonsp} obtained in parallel equilibrium binding experiments were in good agreement with those obtained in kinetic studies. The temperature dependence of the rate constants was fitted by the Arrhenius equation

$$\log k = -\frac{E_a}{2.303 R} \frac{1}{T} + \log A \quad (6)$$

where E_a designates the activation energy, the energy required to initiate the reaction; and A is the

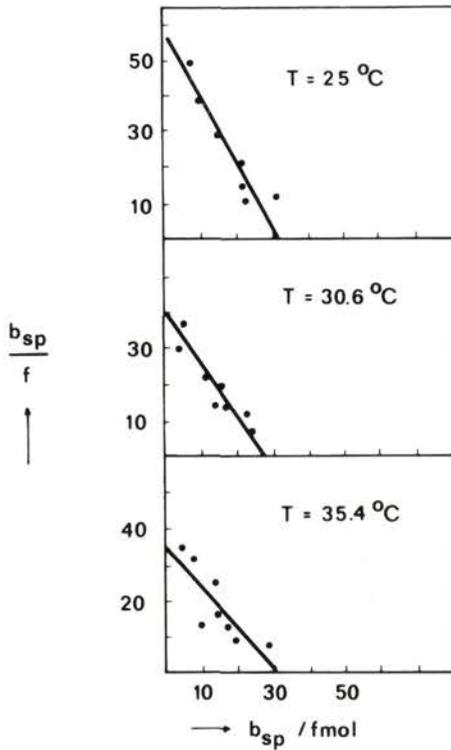


Fig. 1. Representative binding of ^3H -DHA to beta-adrenergic receptors in rat cerebral cortex membranes at various temperatures. Data are presented according to the method of Scatchard (1949). b_{sp} -specifically bound ^3H -DHA, f -free ligand concentration. For evaluation of parameters, see Table 1 and 2.

preexponential factor. Within the framework of the absolute rate theory (Glasstone et al. 1941) the preexponential factor A is related to the activation entropy change, ΔS^\ddagger , a measure for the molecular rearrangement during the reaction process in the following way:

$$A = e k_B T / h e^{\Delta S^\ddagger / R} \quad (7)$$

with k_B being the Boltzmann constant; and h the Planck constant.

Results

Equilibrium binding studies

Equilibrium binding of ^3H -DHA to beta-adrenergic receptors was investigated at different temperatures from 20 to 35 °C. Figure 1 shows representative experiments plotted according to the method of Scatchard (1949). The plots yield straight

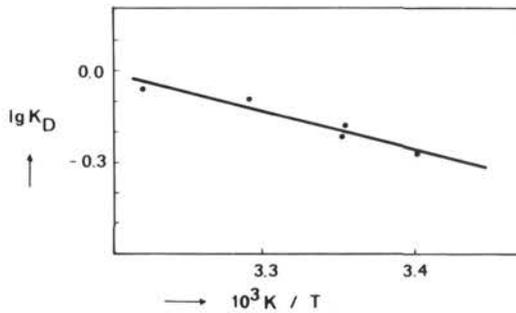


Fig. 2. Plot of the equilibrium dissociation constant versus reciprocal temperature T in degrees Kelvin on a semilogarithmic scale (van't Hoff). The values of K_D were obtained from Scatchard analysis of equilibrium binding experiments (see Table 1).

lines indicating that the ligand binds to a single class of binding sites. The Hill coefficients were near unity in all cases as would be expected for a competitive interaction with one class of binding site.

With increasing temperature, the binding affinity ($1/K_D$) decreased (Table 1). As expected no significant alteration of receptor number with changes in incubation temperature could be detected.

In order to determine the changes in thermodynamic parameters the equilibrium dissociation constants, K_D , were plotted on a semilogarithmic scale versus T^{-1} (van't Hoff plot). As shown in Figure 2, the van't Hoff plot was linear over the temperature range examined. From the slope of this line, a value for the standard enthalpy change, ΔH° , of 26.9 kJ/mol was calculated using eqn. (3). The standard entropy change, ΔS° , was estimated to be -85.5 J/mol degr. using eqn. (4) (also see Table 2).

Kinetic experiments

The binding of $^3\text{H-DTA}$ to rat cerebral cortex membrane preparations is rapid, reaching equilibrium within 5–6 min at 30.8 °C. Figure 3 shows results of a representative experiment carried out with different ligand concentrations. The kinetic data have been used to calculate the rate constants k_1 and k_{-1} assuming the following reaction scheme



for the binding of the ligand, L, ($^3\text{H-DHA}$) to the beta-adrenergic receptor, R, by forming a receptor-ligand complex RL. The reaction is considered to be a pseudo first-order reversible reaction (since $[\text{R}]/[\text{L}] \ll 1$) and allows thus the kinetic data to

Table 1. Summary of data obtained by the evaluation of the plot k_{obs} versus ligand concentration applying nonlinear least squares regression analysis

$T/^\circ\text{C}$	$K_A/\text{nmol l}^{-1}$	K_{iso}	$K_D/\text{nmol l}^{-1}$		k_2/min^{-1}	k_{-2}/min^{-1}
			kinetics	equil. exp.		
20.2				0.53 ± 0.05		
25.0	0.484 ± 0.080	0.0437 ± 0.0629	0.0211 ± 0.0338	0.63 ± 0.06	0.616 ± 0.362	0.0269 ± 0.0229
30.8	0.458 ± 0.041	0.0920 ± 0.120	0.0422 ± 0.0588	0.80 ± 0.08	0.744 ± 0.374	0.0685 ± 0.0548
35.4	0.334 ± 0.057	0.0962 ± 0.128	0.0321 ± 0.0482	0.86 ± 0.16	0.995 ± 0.408	0.0975 ± 0.0897

K_A , K_{iso} and K_D denote equilibrium constants within the model (II) as described in the text; k_2 and k_{-2} are rate constants for the isomerisation step in model (II).

The errors cited for K_A , k_2 and k_{-2} as well as $K_D(\text{equ.})$ are standard errors as obtained from the nonlinear and linear regression analysis, respectively. The standard errors of the derived constants, K_{iso} and $K_D(\text{kin.})$, were calculated according to the error propagation law.

Table 2. Summary of thermodynamic and Arrhenius parameters obtained from the temperature dependence experiments

Quantity	K_A	K_{iso}	K_D^*		k_2	k_{-2}
			kin.	equ.		
ΔG°	53.2 ± 8.8	7.5 ± 10.8	60.8 ± 97.4	52.4 ± 5.0		
ΔH°	-29.9 ± 1.1	66.0 ± 2.4	36.1 ± 3.5	26.9 ± 0.5		
ΔS°	-279 ± 56	196 ± 289	-83.0 ± 140	-85.5 ± 9.7		
E_a					34.1 ± 2.7	105.2 ± 1.1
ΔS^\ddagger					-176 ± 180	70 ± 14
$\log(A/\text{s}^{-1})$					4.0 ± 4.1	16.9 ± 3.4

ΔG° — standard free enthalpy change; ΔH° — standard enthalpy change; E_a — activation energy in kJ/mol; ΔS° — standard entropy change; and ΔS^\ddagger activation entropy change (in $\text{J mol}^{-1} \text{degr.}^{-1}$). K_A , K_{iso} , and K_D are equilibrium constants; k_2 and k_{-2} are rate constants of the two-step reaction scheme (II), see text. A — preexponential factor.

* K_D obtained by both equilibrium binding experiments (equ.) and kinetic studies (kin.): $K_D = K_A K_{iso}$.

The values for the enthalpy changes, activation energies and preexponential factors were obtained from weighed least squares regressions of the corresponding van't Hoff and Arrhenius plots (see Table 1, weight = 1/variance). All errors cited are standard errors as obtained from least squares analyses or according to the error propagation law.

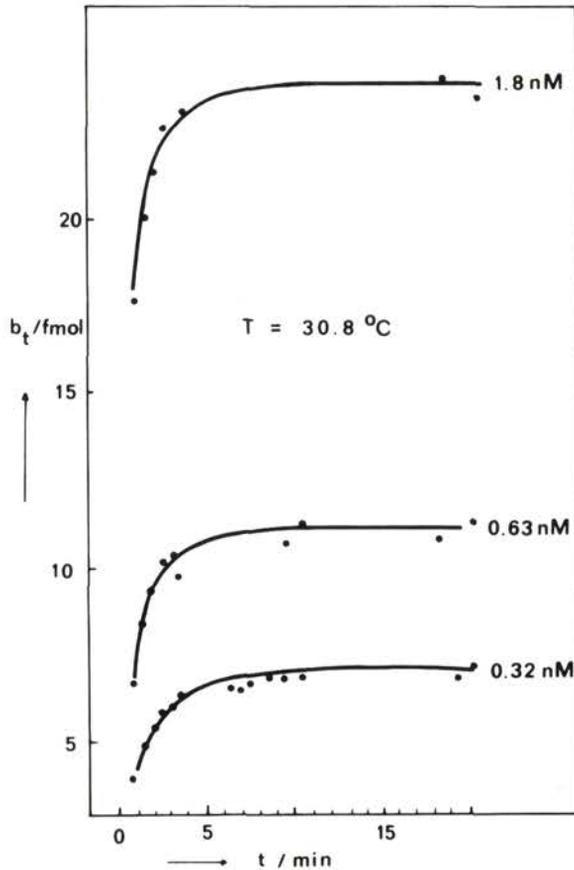


Fig. 3. Plot of a representative experiment showing time dependence of the binding of ^3H -DHA to beta-adrenergic receptors in rat cerebral cortex membranes at various ligand concentrations. b_t -totally bound amount of ^3H -DHA at time t .

be fitted according to the rate equation (5) as plotted in Figure 4. Hence, the slopes (k_{obs}) of the lines in Figure 4, which were obtained by assaying ^3H -DHA binding at different ligand concentrations, should provide an estimate for the rate constants, k_1 and k_{-1} using the relation

$$k_{\text{obs}} = k_1[\text{L}] + k_{-1} \quad (8)$$

where k_{obs} denotes the apparent pseudo first-order rate constant. In Figure 5, the plot of k_{obs} versus the ligand concentration $[\text{L}]$ is shown. As can be seen, no linear correlation between k_{obs} and $[\text{L}]$ could be detected within the ligand concentration range of 0.1 and 3.8 nmol/l. The hyperbolic shape of the plot k_{obs} versus $[\text{L}]$ shows the assumed reaction mechanism (I) to be inadequate to describe the binding of

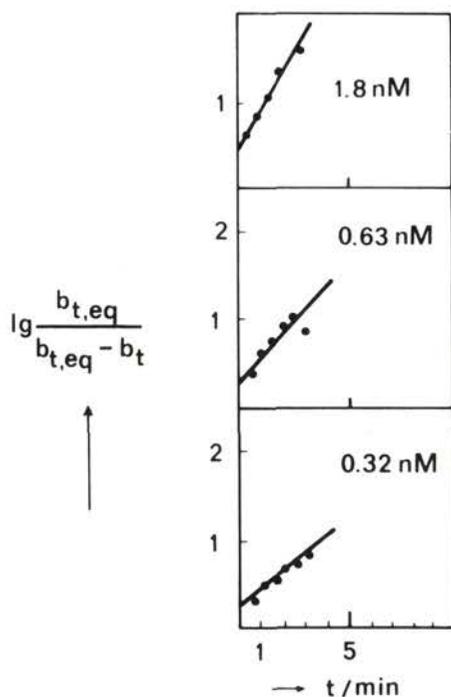


Fig. 4. Kinetics of the binding of ^3H -DHA to beta-adrenergic receptors in rat cerebral cortex membranes under pseudo first-order conditions at various ligand concentrations. The data of the time dependence are fitted by the first-order rate equation (5) as described in Materials and Methods. $b_{t,\text{eq}}$ -totally bound amount of ^3H -DHA at time t . The slopes of the lines correspond to the pseudo first-order rate constant k_{obs} and the nonspecific binding can be calculated from the intercepts (see Materials and Methods).

^3H -DHA to beta-adrenergic receptors in rat cerebral cortex.

The simplest mechanism that could describe these results would be that involving the assumption of a "two-step" binding kinetics:



Under equilibrium conditions, where $k_{-1} > k_2$ (Strickland et al. 1975), the following relation holds:

$$k_{\text{obs}} = \frac{k_2 [\text{L}]}{K_A + [\text{L}]} + k_{-2} \quad (9)$$

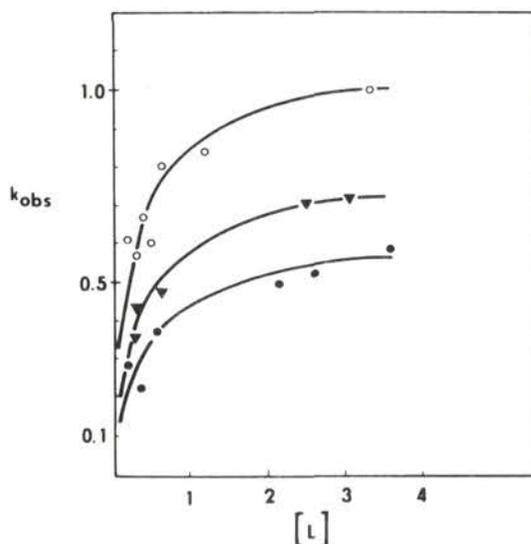


Fig. 5. Plot of the pseudo first-order rate constant k_{obs} versus ligand concentration $[L]$ (nmol l^{-1}) at different temperatures. k_{obs} was obtained by fitting the kinetic data to the pseudo first-order rate equation (5) as shown in Figure 4. The lines were calculated by nonlinear least squares fit of the data to equation (9).

In this equation, $K_A = k_{-1}/k_1$ denotes the dissociation equilibrium constant for the formation of the first receptor-ligand complex (RL), and k_2 and k_{-2} designate the respective rate constants for the isomerisation step in model (II).

The values of K_A , k_2 and k_{-2} were calculated by nonlinear least squares fit of the data on Figure 5, and are listed in Table 1. According to the reaction scheme (II) the constant K_D obtained from the equilibrium binding studies has to hold the following relation:

$$K_D = K_A K_{\text{iso}} \quad (10)$$

where $K_{\text{iso}} = k_{-2}/k_2$ represents the equilibrium constant of the isomerisation step.

As shown in Table 1, the values of K_D obtained from equilibrium binding studies differ from those obtained from kinetic experiments by a factor of about 20, while the observed temperature dependence of K_D is similar in both cases (see also Table 2).

With increasing temperature, the value of K_A decreased while the values of K_D and K_{iso} increased. Analysis of van't Hoff plots (Figure 6) give values for the standard enthalpy and entropy changes of -29.9 kJ/mol and -279 J/mol degr., respectively, for K_A and 66.0 kJ/mol and 196 J/mol degr., respectively for K_{iso} (Table 2).

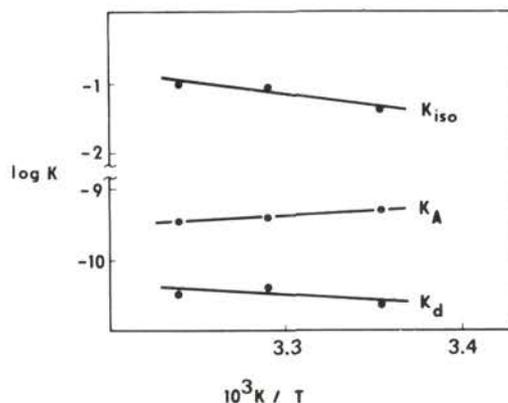


Fig. 6. Van't Hoff plots of the dependence of the equilibrium constants for the association step (K_A), isomerisation step (K_{iso}), and for the kinetically determined dissociation constant $K_d = K_A K_{iso}$ on temperature T (for the reaction scheme (II), see text). The values of K_A and K_{iso} were obtained by evaluating the plot k_{on} versus $[L]$ according to eqn. (9) (also see Table 1 and Figure 5). The slope of the line is $-\Delta H^\circ/R$ and the intercept corresponds to $\Delta S^\circ/R$ (see text).

As expected, the values of k_2 and k_{-2} increase with the rising temperature. The plot of the rate constants on a semilogarithmic scale versus T^{-1} (Arrhenius plot) was linear over the temperature range studied (Figure 7). Using eqn. (6) the activation energy E_a and the preexponential factor A can be calculated from the slope and intercept of these Arrhenius plots. As indicated in Table 2, the activation energies amount to 34.1 kJ/mol for k_2 and 105.2 kJ/mol for k_{-2} . The preexponential factors were calculated to be $1.0 \times 10^4 \text{ s}^{-1}$ for k_2 and $7.9 \times 10^{16} \text{ s}^{-1}$ for k_{-2} . Within the framework of the absolute rate theory (Glasstone et al. 1941) the preexponential factor is related to the change in the activation entropy, ΔS^\ddagger , according to eqn. (7). Hence the values for the activation entropy change, a measure for the molecular rearrangement which occurred during the reaction process, were calculated to be -176 J/mol degr. for reaction step (2) and 70 J/mol degr. for step (-2) within the reaction scheme (II).

Discussion

In this paper we describe the temperature and time dependence of the ^3H -DHA binding to the beta-adrenergic receptor in rat cerebral cortex. The obtained kinetic data suggest that ^3H -DHA binding proceeds through a two-step mechanism as already shown for the binding of hydroxybenzylpindolol to beta-adrenergic receptors (Ross et al. 1977), and for quinuclidinyl benzilate to muscarinic acetylcholine

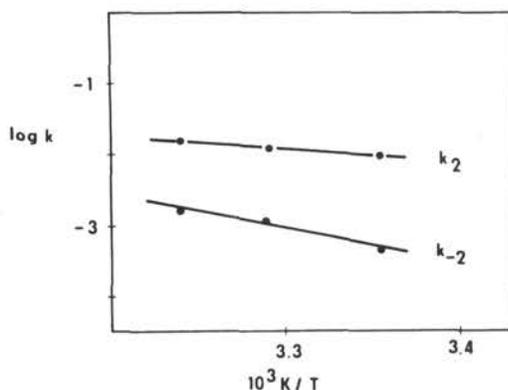


Fig. 7. Arrhenius plot of the dependence of the rate constants for the forward (k_2) and reverse reaction (k_{-2}) of the isomerisation step (according to the reaction scheme (II) as mentioned in the text) on temperature T . The values of k were obtained by evaluating the plots k_{obs} versus $[L]$ as shown in Figure 5, and they are given in Table I. From the slope and intercept of the line the activation energy and the preexponential factor can be calculated, respectively (see Materials and Methods).

receptors (Galper et al. 1977; Jarv et al. 1979). At 35 °C and under steady-state conditions, approximately 90 % of the bound ³H-DHA is attached to the converted receptor molecule R^+ , formed through the isomerisation step (2). Furthermore, the data of the equilibrium-binding studies do not reflect any inhomogeneity of the receptor population. Indeed, the assumption concerning a two-step reaction mechanism has been shown to be compatible with homogenous binding data as derived from theoretical considerations (Janin 1973). The observed difference in the values of K_D obtained from equilibrium binding experiments and kinetic studies seem to indicate that the assumption of a two-step reaction mechanism could possibly be incorrect. The good agreement of the thermodynamic parameters for K_D determined by the two independent binding experiments seems to indicate, however, a systematic underestimation of k_{-2} (or overestimation of k_2) rather than the invalidity of the assumed reaction scheme. This will also be emphasized taking into consideration the error estimation of the constants determined. The data obtained from the equilibrium experiments obviously allow a rather reliable estimation of the thermodynamic parameters. Otherwise, the way in which the kinetic data had to be elaborated necessarily implies an error increment with each following step of the data treatment. Because of the very small physiological temperature range in which kinetic measurements seem to be reasonable, the estimation of preexponential factors of the kinetic constants by linear extrapolation of the $k(T)$ -values to $1/T \rightarrow 0$ (Arrhenius plot) involves larger uncertainties of the obtained values. Therefore, the activation entropy calculated by taking into

account the preexponential factor should be used more as a qualitative rather than a quantitative measure of the occurring thermodynamic processes. Otherwise, data estimated from the slopes of the van't Hoff and Arrhenius lines, respectively, such as enthalpy and activation energy, obviously provide more reliable values. Despite of these limitations mentioned, the presented data should allow a qualitative interpretation of the processes occurring during the receptor-ligand interaction.

The main result of the equilibrium thermodynamic analysis is that the ^3H -DHA binding to beta-adrenergic receptors is associated with a small decrease in enthalpy and a substantial increase in entropy. These data are in agreement with the finding that antagonist binding to beta-adrenergic receptor in erythrocyte membranes and mammalian tissues is largely entropy-driven with only a small enthalpy component (Weiland et al. 1979; 1980). As reported for the reaction of ovalbumin with rabbit antibodies (Singer and Campbell 1955), and for the binding of organic ions by proteins (Klotz and Urquhardt 1949), a large positive entropy change coupled with a small enthalpy change could be detected. Ross and Subramanian (1981) presented a hypothetical thermodynamic model for the reaction of protein with other molecules (proteins, ligand molecules etc.) in which protein association is thought to occur in two steps, consisting of a hydrophobic association, followed by the intermolecular interaction (process of internal conversion). Water tends to form a more ordered structure in the vicinity of nonpolar hydrocarbon groups which results in the mutual penetration of the hydration layers of the two species forming a hydrophobically associated complex. The release of structural water due to the hydrophobic interaction, leads to a substantial increase in entropy, and only to a slight increase in enthalpy, which is due to the expenditure of energy in excluding the water molecules. The second step of the intermolecular conversion involves ionic and van der Waals interactions as well as hydrogen bonds, and it is mainly associated with a negative enthalpy change (also see Kauzmann 1959).

The kinetic data obtained in this work will be tentatively discussed in terms of this thermodynamic two-step model: The association step (1) can be considered as a hydrophobic interaction resulting in a large positive change in entropy and a slight change in enthalpy, which is in agreement with the data found by Ross and Subramanian (1981). As shown by Janin and Chothia (1981), hydrophobicity provides the major source of stabilisation free energy in the protein association complex. The loss of translational and rotational degrees of freedom of the ligand during the binding to the protein must be compensated for the removal of its accessible surface from contact with water (release of structural water; for quantitative estimates, see Janin and Chothia, 1981).

The isomerisation step is accompanied by a large negative change in both enthalpy and entropy. One can only speculate on the nature of the processes which will cause this reversal of changes in enthalpy and entropy. The decrease in

enthalpy could be brought about by the formation of more favourable contacts (ionic, or H-bonds) between ligand and receptor or within the receptor molecule itself. The loss of internal degrees of freedom of the protein by conformational changes, the exposure of previously buried parts of the protein to the solvent, or generally, the formation of a more ordered structure (e. g. the hydrophobic centre of the antagonist enters a pocket in the receptor (Cherksey et al. 1981)) can lead to a decrease in entropy. This is emphasized by the large negative change in the activation entropy for the reaction step (2), indicating the formation of a highly ordered complex. The low value of the activation energy of about 35 kJ/mol means that only a small amount of energy is required to form this complex. In contrast, the reverse reaction (-2) is associated with a high threshold energy and a large positive activation entropy change.

Weiland et al. (1979) suggested that only agonists should be able to induce a conformational change of the receptor R to R⁺, which would lead to the activation of adenylate cyclase. Furthermore, Bürgisser et al. (1981) demonstrated that the complex binding kinetics found for the radioligands (\pm) hydroxybenzylpindolol and (\pm) quinuclidinyl benzilate could be explained by simultaneous binding of both isomers of the ligand rather than by receptor state transitions.

In contrast to that, the data found in our kinetic studies also suggest complex binding behavior for the nonracemic radioligand ³H-dihydroalprenolol.

The different binding behaviour of agonists and antagonists of the adrenergic system could at least in part be due to their structural differences. The antagonists are characterised by replacement of the catechol entity with a strongly hydrophobic group. Fluorescence studies on the beta-adrenergic receptor topology of frog erythrocyte membranes have been interpreted as indicating that the hydrophobic centre of the antagonists enters a pocket in the receptor which cannot be occupied by the hydrophilic catechol (Cherksey et al. 1981). In this way, the binding of adrenergic antagonists may also proceed through a two-step scheme: the bimolecular hydrophobic association step is followed by the hydrophobic entity entering a receptor pocket (isomerisation, or unimolecular internal conversion). The binding data presented in this paper could support, in part, this interpretation.

However, in such a complex system as studied here, careful consideration of the experimental data as outlined above is necessary. A number of other processes affecting the binding behaviour, e. g. the role of hydrogen ion concentration, the concentration of guanine nucleotides (Lefkowitz et al. 1976; U'Prichard and Snyder 1978; William and Lefkowitz 1977), ionic forces (Bird and Maguire 1978), salt concentrations etc., must be considered in order to get a more detailed picture of the interaction of receptor protein with ligand molecules.

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References

- Ahlquist R. P. (1948): Study of adrenergic receptors. *Am. J. Physiol.* **153**, 586—600
- Bird S. J., Maguire M. E. (1978): The agonist-specific effect of magnesium ion on binding by beta-adrenergic receptors in S49 lymphoma cells. *J. Biol. Chem.* **253**, 8826—8834
- Bürgisser E., Lefkowitz R. J., DeLean A. (1981): Alternative explanation for the apparent "two-step" binding kinetics of high affinity racemic antagonist radioligands. *Molec. Pharmacol.* **19**, 509—512
- Bylund D. B. (1978): Beta-adrenergic receptor binding in guinea pig cerebral cortex. *Brain Res.* **152**, 391—395
- Cherksey B. D., Murphy R. B., Zadunaisky J. A. (1981): Fluorescence studies of the beta-adrenergic receptor topology. *Biochemistry* **20**, 4278—4283
- Crippen G. M. (1982): Distance geometry analysis of the benzodiazepine binding site. *Molec. Pharmacol.* **22**, 11—19
- DeLean A., Stadel J. M., Lefkowitz R. J. (1980): A ternary complex model explains the agonist-specific binding properties of the adenylate cyclase-coupled beta-adrenergic receptor. *J. Biol. Chem.* **255**, 7108—7117
- El-Fakahamy E., Richelson E. (1980): Temperature dependence of muscarinic acetylcholine receptor, activation, desensitization and resensitization. *J. Neurochem.* **34**, 1288—1295
- Glasstone S., Laidler K. J., Eyring H. (1941): *The Theory of Rate Processes*. Wiley Interscience, New York
- Galper J. B., Klein W., Catterall W. A. (1977): Muscarinic acetylcholine receptors in developing chick heart. *J. Biol. Chem.* **252**, 8692—8699
- Insel P. A., Sanda M. (1979): Temperature-dependent changes in binding to beta-adrenergic receptors of intact S49 lymphoma cells. *J. Biol. Chem.* **254**, 6554—6559
- Janin J. (1973): The study of allosteric proteins. *Progr. Biophys. Mol. Biol.* **27**, 77—120
- Janin J., Chothia C. (1981): Role of hydrophobicity in the binding of coenzymes. *Biochemistry* **17**, 2943—2948
- Jarv J., Hedlund B., Bartfai T. (1979): Isomerization of the muscarinic receptor-antagonist complex. *J. Biol. Chem.* **254**, 5595—5598
- Kauzmann W. (1959): Some factors in the interpretation of protein denaturation. *Adv. Prot. Chem.* **14**, 1—63
- Klotz I. M., Urquhart J. M. (1949): The binding of organic ions by proteins. Effect of temperature. *J. Am. Chem. Soc.* **71**, 847—851
- Lefkowitz R. J., Mullikin D., Caron M. G. (1976): Regulation of beta-adrenergic receptors by guanyl-5'-yl imidodiphosphate and other purine nucleotides. *J. Biol. Chem.* **251**, 4686—4692
- Maelicke A., Fulpus B. W., Klett R. P., Reich E. (1977): Acetylcholine receptor. Responses to drug binding. *J. Biol. Chem.* **252**, 4811—4830
- Miller J., Witzemann V., Quast U., Raftery M. A. (1979): Proton magnetic resonance studies of cholinergic ligand binding to the acetylcholine receptor in its membrane environment. *Proc. Nat. Acad. Sci. U. S. A.* **76**, 3580—3584
- Quast U., Mählmann H., Volmer K. O. (1982): Temperature dependence of the benzodiazepine-receptor interaction. *Molec. Pharmacol.* **22**, 20—25
- Ross D., Subramanian S. (1981): Thermodynamics of protein association: forces contributing to stability. *Biochemistry* **20**, 3096—3102

- Ross E. M., Maguire M. E., Sturgill T. W., Biltonen R. L., Gilman A. G. (1977): Relationship between the beta-adrenergic receptor and adenylate cyclase. *J. Biol. Chem.* **252**, 5761—5775
- Scatchard G. (1949): The attraction of proteins for small molecules and ions. *Ann. N. Y. Acad. Sci.* **51**, 660—672
- Singer S. J., Campbell D. H. (1955): Physical chemical studies of soluble antigen-antibody complexes. V. Thermodynamics of the reaction between ovalbumin and its rabbit antibodies. *J. Am. Chem. Soc.* **77**, 4851—4855
- Speth R. C., Wastek, G. J., Yamamura, H. I. (1979): Benzodiazepine receptor: temperature dependence of ³H-flunitrazepam binding. *Life Sci.* **24**, 351—358
- Strickland S., Palmer G., Massey V. (1975): Determination of dissociation constants and specific rate constants of enzyme-substrate (or protein-ligand) interactions from rapid reaction kinetic data. *J. Biol. Chem.* **250**, 4048—4052
- U'Prichard D. C., Snyder S. H. (1978): Guanyl nucleotide influences on ³H-ligand binding to alpha-noradrenergic receptors in calf brain membranes. *J. Biol. Chem.* **253**, 3444—3452
- Weiland G. A., Minneman K. P., Molinoff P. B. (1979): Fundamental difference between the molecular interactions of agonists and antagonists with the beta-adrenergic receptor. *Nature* **281**, 114—117
- Weiland G. A., Minneman K. P., Molinoff P. B. (1980): Thermodynamics of agonist and antagonist interactions with mammalian beta-adrenergic receptors. *Molec. Pharmacol.* **18**, 341—347
- Williams L. T., Lefkowitz R. J. (1977): Slowly reversible binding of catecholamine to nucleotid-sensitive state of the beta-adrenergic receptor. *J. Biol. Chem.* **252**, 7207—7213
- Williams L. T., Lefkowitz R. J. (1978): *Receptor Binding Studies in Adrenergic Pharmacology*. Raven Press, New York

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