ELSEVIER

European Journal of Pharmacology 310 (1996) 13–17

ejp

# Thermodynamics of the interaction of *d*-tubocurarine with nicotinic receptors of mammalian skeletal muscle in vitro

Bisweswar Banerjee, Dilip K. Ganguly *

*Division of Pharmacology and Experimental Therapeutics, Indian Institute of Chemical Biology, 4 Raja S.C. Mullick Road, Calcutta 700032, India*

Received 6 May 1996; accepted 7 May 1996

**Abstract**

Thermodynamic study is important for defining drug receptor interactions, and denervated rat hemidiaphragm is a unique preparation for such a study on nicotinic receptors. As a continuation of our earlier study with acetylthiocholine on the same preparation, we now report on the characteristics of temperature-dependent binding of *d*-tubocurarine, a reversible antagonist. The O. Arunlakshana and H.O. Schild (1959, Br. J. Pharmacol. 14, 48) equation, as improved by D.R. Woud and R.B. Parker (1971, J. Pharmacol. Exp. Ther. 177, 13), was used to calculate the dissociation constant of *d*-tubocurarine at various temperatures (10–37°C) from the parallel shift of the acetylcholine dose-response curve to the right by effective doses of *d*-tubocurarine. It was observed that the values of the dissociation constant increased with a decrease in temperature. Both the enthalpy ($\Delta H°$) and entropy ($\Delta S°$) changes as evaluated from the van't Hoff plot ($\ln K_d$ vs. $1/T$) were found to be positive and their relative value ($\Delta H° - T\Delta S°$) produced a negative free energy change which characterises the binding of *d*-tubocurarine as an entropy-controlled process. This finding is in agreement with the neurotoxin binding reported earlier. The present finding and earlier observations with acetylthiocholine reveal that agonist and antagonist binding to the nicotinic receptor may differ depending on the experimental conditions.

*Keywords:* Nicotinic receptor; Thermodynamics; *d*-Tubocurarine; Skeletal muscle; Entropy; Enthalpy

## 1. Introduction

Determination of the equilibrium constant is essential for the study of drug-receptor interactions and provides a useful tool for classifying drugs and receptors (Tallarida et al., 1979). Thermodynamic studies are being increasingly used to provide more information about the biophysical phenomena that occur during drug-receptor interaction and which are beyond the resolving power of the equilibrium constant. Ligand binding in the case of β-adrenoceptors is thermodynamically distinct as is revealed from the pioneering work of Weiland et al. (1979, 1980), who reported that antagonist binding is favoured by a positive change of entropy with little change of enthalpy and that agonist binding is accompanied by a large decrease in enthalpy. Maelicke et al. (1977), however, could not find any thermodynamic differences between the binding of agonist and antagonist to the nicotinic receptor isolated from the extracts of electric organ of *Electrophorus electricus*, indicating that thermodynamic characteristics differ with different receptors. Since radioligand binding studies are not always sensitive to functional differences between the interactions of the agonist and antagonist with a receptor (Duarte et al., 1988), we used in vitro denervated rat hemidiaphragm and the Furchgott (1966) method. This had allowed us to calculate the dissociation constant of acetylthiocholine, a specific nicotinic receptor agonist, at various temperatures. It was found then that the interaction occurred through negative enthalpy and entropy changes (Banerjee and Ganguly, 1995). In order to see whether the binding of the reversible nicotinic receptor antagonist, *d*-tubocurarine, differs from binding of the agonist, we now carried out a thermodynamic study with *d*-tubocurarine on the in vitro denervated rat hemidiaphragm preparation at different temperatures. The dissociation constant at different temperatures was calculated using the Arunlakshana and Schild (1959) equation as improved statistically by Woud and Parker (1971). The advantage of the method is that from the $ED_{30}$ values of the dose-response curve, one can calculate the dissociation constant by iteration thus avoiding the interference by desensitisa-

* Corresponding author. Tel.: +91 33 4735112; fax: +91 33 4735112.


*PII* S0014-2999(96)00360-3

tion which is caused by higher doses of the drug. We have selected the isolated denervated rat diaphragm preparation for the study, since it is perhaps the only in vitro mammalian preparation which responds to nicotinic receptor agents over a wide range of temperatures.

## 2. Materials and methods

### 2.1. Materials

*d*-Tubocurarine, acetylcholine and physostigmine were obtained from Sigma.

### 2.2. Denervated hemidiaphragm preparations

Experiments were performed on denervated hemidiaphragm preparations of albino rats of Sprague-Dawley strain (150–200 g) of either sex. Denervation of the left hemidiaphragm was carried out by sectioning the left phrenic nerve according to the method of Mitchell and Silver (1963) and the preparations were set up as described earlier (Vedasiromoni and Ganguly, 1984). The diaphragm was suspended in a 25-ml double-walled organ bath containing Kreb's solution (mmol: NaCl 118, KCl 4.7, $CaCl_2 \cdot 6H_2O$ 2.5, $NaHCO_3$ 25, $KH_2PO_4$ 1.2, $MgSO_4 \cdot 7H_2O$ 1.2 and dextrose 11.1) continuously aerated with 95% $O_2$ and 5% $CO_2$ and the pH was kept at 7.4.

### 2.3. Experimental protocols

The tissue preparations were kept in the bath solution for 2–2.5 h for adaptation to the desired temperature before the addition of any drug. The muscle contractions were recorded isometrically by means of a force-displacement transducer and pen recorder. Responses to each concentration of the agonist (acetylcholine) added to the organ bath were used to construct the control dose-response curve. A second dose-response curve was obtained by addition of each concentration of the agonist in presence of an effective fixed concentration of the antagonist (*d*-tubocurarine). An interval of 5 min was always allowed between the addition of the antagonist and agonist. Likewise, a third and fourth dose-response curves were obtained in presence of higher concentrations of the antagonist. The concentration range of the effective doses of the antagonist at various temperatures in the second, third and fourth dose-response curves were 0.05–0.6, 0.1–2.3 and 0.4–4.5 $\mu$M, respectively.

### 2.4. Calculation and statistics

The dissociation constant of the antagonist was calculated from the Arunlakshana and Schild (1959) equation

$$\log(\mathrm{DR}-1) = -\log(K_d) + \log([B]) \quad (1)$$

where DR = dose ratio which indicates the degree of shift, $K_d$ = antagonist dissociation constant and $[B]$ = antagonist dose. In the present study, using acetylcholine as agonist, the dissociation constant of *d*-tubocurarine was determined using Eqn. 1 where dose ratios were statistically evaluated with the method developed by Woud and Parker (1971). The logistic function used as the relationship between the effect and dose is of the following type

$$E = (M[A]^P)/([A]^P + K^P) \quad (2)$$

where $E$ = effect, [A] = dose, $M$ = maximal response, $P$ = slope and $K = \mathrm{ED}_{30}$. $M$ and $P$ should be the same for all curves whereas $K$ will be different for each curve. Initial estimates of $P$ and $K_i$ are required. $\mathrm{ED}_{30}$ values are the reasonable starting estimate of $K_i$. The exact values of $P$ and $K_i$ were calculated with an iterative process. The dose ratios required for the calculation of the dissociation constant of the antagonist were determined from the ratios of the estimated $K$ values

$$\mathrm{DR}_i = K_{i+1}/K_1 \quad (3)$$

From the regression plot of $\log(\mathrm{DR}-1)$ vs. $\log([B])$, which corresponds to the linear equation

$$\log(\mathrm{DR}-1) = a + b\log([B]) \quad (4)$$

where $a$ = constant and $b$ = slope.

The dissociation constant can be calculated by means of the following three processes

1. By comparison of Eqns. 4 and 1, $K_d = 10^{-a}$.
2. By putting $\log(\mathrm{DR}-1) = 0$, $K_d = 10^{-a/b}$.
3. On theoretical grounds '$b$' should be unity. In that case

$$K_d = 10\exp\{\Sigma\log([B]) - \Sigma\log(\mathrm{DR}-1)\}/(N-1)$$

In the present study, $K_d$ was calculated as the mean of the above three processes. This constant ($K_d$) was used to get the standard free energy change

$$\Delta G^\circ = -RT\ln(1/K_d) = RT\ln(K_d) \quad (5)$$

where $R$ = universal gas constant (8.314 J/mol/°) and $T$ = absolute temperature. The enthalpy of binding ($\Delta H^\circ$) can be evaluated from the van't Hoff equation

$$\ln K_d = (\Delta H^\circ/R)(1/T) - \Delta S^\circ/R \quad (6)$$

From the intercept of Eqn. 6, the standard entropy change ($\Delta S^\circ$) can be calculated. The slope of the plot of $\ln K_d$ vs. $1/T$ is $\Delta H^\circ/R$. Determination of $\Delta H^\circ$ and $\Delta G^\circ$ also allows the calculation of entropy ($\Delta S^\circ$) at each temperature, from the equation

$$\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ \quad (7)$$

## 3. Results

Rat hemidiaphragm is a unique preparation which responds to agonists and antagonists over a wide range of temperatures (5–40°C). The responses of denervated di-

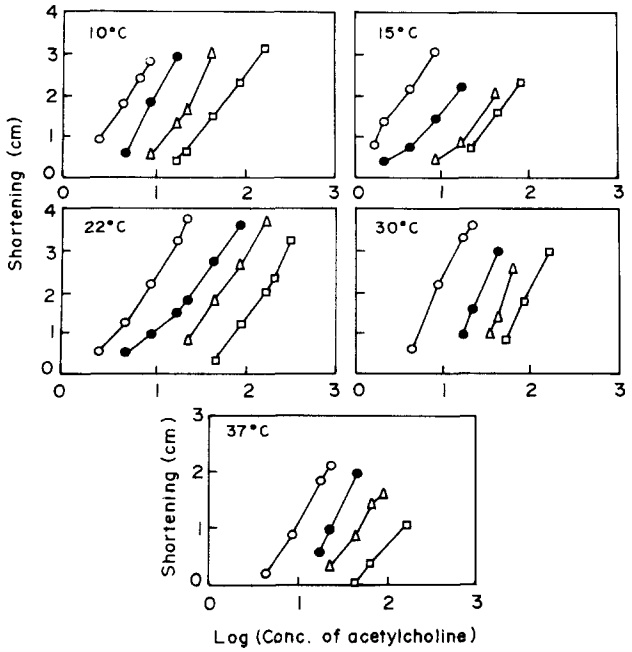


Fig. 1. Isometric contraction of the rat denervated hemidiaphragm preparations in response to different concentrations of acetylcholine ($\mu$M) measured at various temperatures in the absence (o) and in the presence (●, △ and □) of a reversible antagonist, *d*-tubocurarine. Physostigmine (8 $\mu$M) was included in all bathing fluids. Single representative experiment out of six experiments is shown for each temperature used.

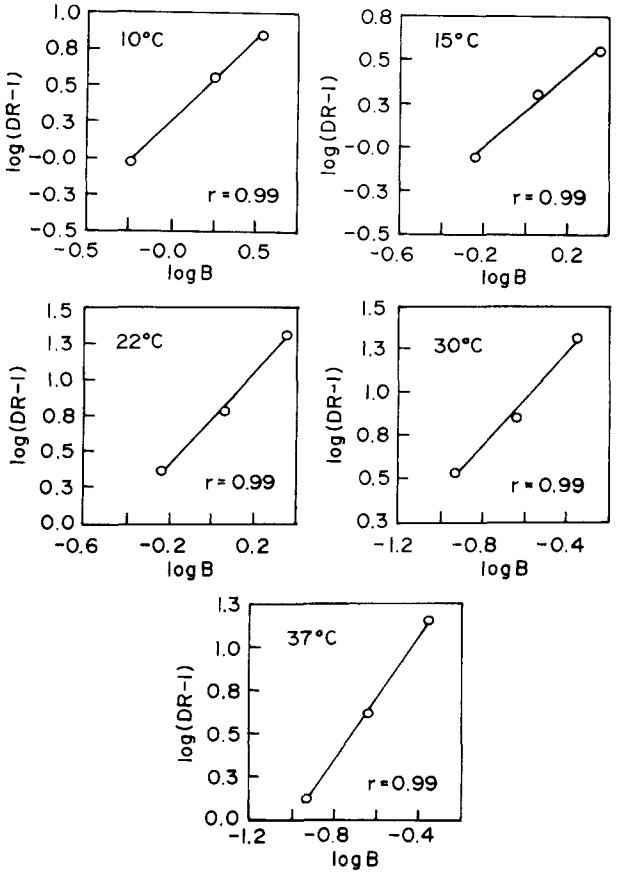


Fig. 2. Arunlakshana-Schild plot of log (DR − 1) vs. log $B$ shown with linear regression ($r = 0.99$) at different temperatures. The dissociation constant can be calculated from the intercept of the line.

aphragm, in terms of shortening (cm), to various concentrations of acetylcholine at different temperatures (10, 15, 22, 30 and 37°C) are shown in Fig. 1. The qualitative nature of the tissue responsiveness remained the same in the temperature range (10–37°C) used. Though the tissue responds at 5°C as well as at 40°C, the dissociation constant was calculated only between 10 and 37°C (Table 1). The nature of the action of a competitive antagonist is that it will produce a shift of the agonist dose-response curve to the right. At all temperatures studied by us, three effective doses of the reversible antagonist *d*-tubocurarine produced a parallel shift of the dose-response curve to the right (Fig. 1). The curves had a slope ($P$) somewhere between 1 and 4. The plot of log(DR − 1) vs. log([$B$]) yielded a straight line with linear regression ($r = 0.99$) according to Eqn. 1 (Fig. 2). The dissociation constant was calculated from the intercept of this straight line as well as with the statistical procedure developed by Woud and Parker (1971). Dissociation constant ($K_d$) values for *d*-tubocurarine obtained at each temperature between 10 and 37°C were used to construct a van't Hoff plot of $\ln K_d$ vs. $1/T$ (Fig. 3). The best straight line through the data points was determined by linear regression analysis ($r = 0.90$). Enthalpy and entropy changes were calculated from the slope and the intercept of this straight line, respectively. Free energy at each temperature was calculated from the

Table 1
Dissociation constant ($K_d$) measured at different temperatures ($n = 6$)

| Temperature (°C) | 10 | 15 | 22 | 30 | 37 |
|---|---|---|---|---|---|
| $K_d$ ($\mu$M) | 0.518 | 0.523 | 0.327 | 0.032 | 0.047 |
| ±S.E.M. ($\mu$M) | 0.034 | 0.05 | 0.07 | 0.003 | 0.01 |

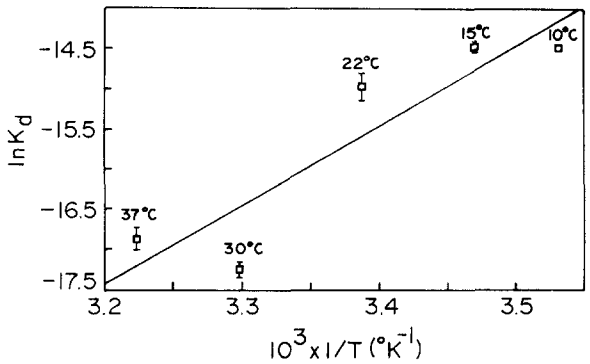


Fig. 3. Van't Hoff plot of $\ln K_d$ vs. $1/T$ shown with linear regression ($r = 0.90$). The dissociation constant was measured at five temperatures, viz. 10, 15, 22, 30 and 37°C. Slope and intercept of the line yielded the enthalpy and entropy values, respectively. Each point show the mean ± S.E.M. of six experiments. The S.E.M. of point with no error bar indicated was less than the size of the symbol.

Table 2
Values of thermodynamic parameters

| Temperature (°C) | 10 | 15 | 22 | 30°C | 37 |
|---|---|---|---|---|---|
| $\Delta G^\circ$ (kJ/mol) | $-34.07 \pm 0.16$ | $-34.66 \pm 0.23$ | $-36.77 \pm 0.55$ | $-43.52 \pm 0.26$ | $-43.56 \pm 0.56$ |
| $\Delta H^\circ$ (kJ/mol) | | | 82.41 ± 21.81 [a] | | |
| $\Delta S^\circ$ (kJ/deg/mol) | | | 0.408 ± 0.073 [a] | | |

[a] Calculated from the van't Hoff plot: $\Delta H^\circ = R$ (slope) and $\Delta S^\circ = R$ (intercept).

dissociation constant values using the equation $\Delta G^\circ = RT \ln K_d$ (Table 2).

## 4. Discussion

The present study showed that the affinity of *d*-tubocurarine for the nicotinic receptor is temperature-dependent, as seen from the values of the dissociation constant measured at different temperatures ranging from 10 to 37°C. It is interesting that the affinity of the reversible antagonist, *d*-tubocurarine, increases with an increase in temperature. This is in agreement with reports on other drug-receptor interactions (Weiland et al., 1980; Maelicke et al., 1977; Zahniser and Molinoff, 1983). However, our results differ from those of Jenkinson (1960) and Van Maanen (1950) who reported that tubocurarine binding to frog rectus was temperature-independent. The difference between the results obtained from denervated rat hemidiaphragm and those from frog rectus may be due to species differences (mammalian vs. amphibian) and some other variables (methods, denervation vs. non-denervation, etc.). Besides, in the present study the dissociation constant was calculated with the Arunlakshana and Schild (1959) equation statistically improved by Woud and Parker (1971) while in the earlier studies the method developed by Gaddum (1943) was used. The nature of the temperature-dependent binding of *d*-tubocurarine is different from that of acetylthiocholine binding reported earlier by us (Banerjee and Ganguly, 1995). It was found that the affinity of acetylthiocholine increased with a decrease in temperature. This indicates that agonist and antagonist respond in opposite ways to changes in temperature and leads to the concept that thermodynamic analysis of pharmacological data provides meaningful information regarding the molecular events that occur during drug-receptor interactions.

At each temperature, the dose-response curve for acetylcholine was correspondingly shifted to the right in presence of effective doses of the reversible antagonist, *d*-tubocurarine. The equi-effective doses calculated from these dose-response curves yielded a preliminary estimate of dose ratios, the exact values of which were determined by the iterative method developed by Woud and Parker (1971). Such a procedure eliminates the possibility of desensitisation, enzyme activity, muscle mechanics, etc. The advantages of using denervated rat hemidiaphragm preparations for thermodynamic study are (a) an increase in the population of free receptors, resulting in higher sensitivity which favours equilibrium conditions of binding, (b) it is perhaps the only mammalian preparation which responds over a wide range of temperature sufficient for the calculation of thermodynamic parameters and (c) the time course of desensitisation is slower in the denervated than in the innervated end plate (Axelsson and Thesleff, 1959).

The magnitude of the dissociation constant obtained by us is in agreement with those obtained by Colquhoun and Rang (1976) though they carried out their experiments using a homogenised preparation of denervated rat hemidiaphragm, [$^{125}$I]$\alpha$-bungarotoxin, and a different method of analysis. Gibbs free energy calculated from the dissociation constant in the present study at each temperature was found to be negative, which indicates that the drug binding to the receptor is a spontaneous process.

Though the van't Hoff plot (Fig. 3) appears non-linear, the regression coefficient of the plot was found to be around 0.90. In a biological preparation, like ours (in vitro isolated tissue preparation), it is quite rare to get perfect linearity. A similar type of van't Hoff plot ($r = -0.90$) was reported by Raffa et al. (1985). However, the enthalpy change ($\Delta H^\circ$) which was measured using a van't Hoff plot was found to be positive in the present study, which indicates that *d*-tubocurarine binding to the receptor is endothermic in nature. A thermodynamically favourable increase in entropy change ($\Delta S^\circ$) compensates for the positive enthalpy change leading to a negative free energy change ($\Delta G^\circ = \Delta H^\circ - T\Delta S^\circ$, since $T\Delta S^\circ > \Delta H^\circ$; $\Delta G^\circ < 0$), indicating that the *d*-tubocurarine binding to the receptor is entropically controlled. This result is similar in sign and magnitude to the results of neurotoxin binding studies with extracts of nicotinic receptors from *E. electricus* (Maelicke et al., 1977).

The energetics of the binding of *d*-tubocurarine are the opposite of our earlier finding with acetylthiocholine where the binding occurred by negative $\Delta H^\circ$ and $\Delta S^\circ$ and the unfavourable decrease in entropy was compensated for by an enthalpy change (Banerjee and Ganguly, 1995). This indicates that acetylthiocholine binding to the nicotinic receptor is enthalpy-controlled whereas that of *d*-tubocurarine is entropy-controlled. These results are in conformity with the report of Weiland et al. (1979, 1980) that agonist and antagonist binding to the receptor may be differentiated by thermodynamic parameters in that antagonist binding was favoured by a large entropy component

and agonist binding was favoured by a large decrease in enthalpy component.

The thermodynamics of antagonist binding are similar to those of many other protein binding reactions (Miklavc et al., 1990) where information transfer is not involved. The increase in entropy and enthalpy in antagonist binding can be explained by hydrophobic and ionic interactions (Ross and Subramanian, 1981). The iceberg around the ligand and the receptor may melt as the 'hydrophobically associated' species is formed. The process is associated with the positive change of entropy and enthalpy. The favourable entropy-controlled association may be applicable to an amphiphyle molecule, such as *d*-tubocurarine. Theoretical studies to examine the structural features of *d*-tubocurarine and acetylthiocholine are currently in progress.

In spite thermodynamic parameters not being directly measurable, the results obtained by us appear to be quite interesting. Application of the statistical method of obtaining dose ratios, as done in the present study, eliminates biological interference, such as desensitisation, etc. Comparison of the observed binding characteristics of *d*-tubocurarine to the nicotinic receptors of denervated rat hemidiaphragm at various temperatures with these characteristics for acetylthiocholine reported by us earlier (Banerjee and Ganguly, 1995) suggests that the energetics of the binding of the agonist and the antagonist may differ.

## Acknowledgements

The authors gratefully acknowledge the help rendered by Dr. J.R. Vedasiromoni in the preparation of the manuscript. The technical assistance rendered by S.C. Sharma is acknowledged. B. Banerjee is the Recipient of a University Grant Commission (India) Fellowship.

## References

Arunlakshana, O. and H.O. Schild, 1959, Some quantitative uses of drug antagonists, Br. J. Pharmacol. 14, 48.

Axelsson, J. and S. Thesleff, 1959, A study of supersensitivity in denervated mammalian skeletal muscle, J. Physiol. 147, 178.

Banerjee, B. and D.K. Ganguly, 1995, Thermodynamic studies with acetylthiocholine on nicotinic receptors of mammalian skeletal muscle in vitro, Biochem. Pharmacol. 49, 1713.

Colquhoun, D. and H.P. Rang, 1976, Effects of inhibitors on the binding of iodinated α-bungarotoxin to acetylcholine receptors in rat muscle, Mol. Pharmacol. 12, 519.

Duarte, E.P., C.R. Oliveira and A.P. Carvalho, 1988, Thermodynamic analysis of antagonist and agonist interactions with dopamine receptors, Eur. J. Pharmacol. 147, 227.

Furchgott, R.F., 1966, The use of β-haloalkylamines in the differentiation of receptors and in the determination of dissociation constants of receptor-agonist complexes, in: Advances in Drug Research, Vol. 3, eds. N.J. Harper and A.B. Simmonds (Academic Press, New York) p. 21.

Gaddum, J.H., 1943, Symposium on Chemical Constitution and Pharmacological Action, Trans. Faraday Soc. 39, 323.

Jenkinson, D.H., 1960, The antagonism between tubocurarine and substances which depolarize the motor end plate, J. Physiol. 152, 309.

Maelicke, A., B.W. Fulpius, R.P. Klett and E. Reich, 1977, Acetylcholine receptor: responses to drug binding, J. Biol. Chem. 252, 4811.

Miklavc, A., D. Kocjan, J. Mavri, J. Koller and D. Hadzi, 1990, Commentry: on the fundamental difference in the thermodynamics of agonist and antagonist interactions with β-adrenergic receptors and the mechanism of entropy driven binding, Biochem. Pharmacol. 40, 663.

Mitchell, J.F. and A. Silver, 1963, The spontaneous release of acetylcholine from the denervated hemidiaphragm of the rat, J. Physiol. (London) 165, 117.

Raffa, R.B., J.F. Aceto and R.J. Tallarida, 1985, Measurement of thermodynamic parameters of isolated rabbit thoracic aorta, J. Pharmacol. Exp. Ther. 235, 596.

Ross, P.D. and S. Subramanian, 1981, Thermodynamics of protein association reactions: forces contributing to stability, Biochemistry 20, 3096.

Tallarida, R.J., A. Cowan and M.W. Alder, 1979, Minireview: pA2 and receptor differentiation: a statistical analysis of competitive antagonism, Life Sci. 25, 637.

Van Maanen, E.F., 1950, The antagonism between acetylcholine and the curare alkaloids D-tubocurarine, c-curarine-I, c-toxiferine-II and β-erythroidine in the rectus abdominis of the frog, J. Pharmacol. 99, 255.

Vedasiromoni, J.R. and D.K. Ganguly, 1984, N-Carbamoyl-2-(2,6-dichlorophenyl) acetamidine hydrochloride (LON-954), a tremorogen, on rat diaphragm, Jpn. J. Pharmacol. 34, 353.

Weiland, G.A., K.P. Minneman and P.B. Molinoff, 1979, Fundamental difference between the molecular interactions of agonists and antagonists with the β-adrenergic receptor, Nature (London) 281, 114.

Weiland, G.A., K.P. Minneman and P.B. Molinoff, 1980, Thermodynamics of agonist and antagonist interactions with mammalian β-adrenergic receptors, Mol. Pharmacol. 18, 341.

Woud, D.R. and R.B. Parker, 1971, Pharmacological estimation of drug receptor dissociation constants. Statistical evaluation. II. Competitive antagonists, J. Pharmacol. Exp. Ther. 177, 13.

Zahniser, N.R. and P.B. Molinoff, 1983, Thermodynamic differences between agonist and antagonist interactions with binding sites for [$^3$H]spiroperidol in rat striatum, Mol. Pharmacol. 23, 303.