Thermodynamic analysis of agonist and antagonist binding to the chicken brain melatonin receptor

Nelson W.S. Chong & David Sugden

Physiology Group, Biomedical Sciences Division, King's College London, Campden Hill Road, London W8 7AH

- 1 The binding of 2-[¹²⁵I]-iodomelatonin to chicken brain membranes, and the inhibition of binding by melatonin, N-acetyltryptamine and luzindole, were examined at temperatures between 4°C and 37°C.
- 2 At all temperatures studied, the binding affinity (K_d or K_i) for 2-[¹²⁵I]-iodomelatonin, melatonin (both agonists) and, to a lesser extent, N-acetyltryptamine (a partial agonist) was reduced by inclusion of guanosine triphosphate (GTP, 1 mM) in the assay. GTP did not affect the K_i for luzindole, a melatonin receptor antagonist.
- 3 The maximal density of binding sites (B_{max}) was not affected by temperature but the K_d showed a peak at 21°C with lower values at both higher and lower temperatures giving curvilinear van't Hoff plots ($\ln K_A$ vs $1/\text{temperature}$).
- 4 Derived changes in entropy (ΔS°) and enthalpy (ΔH°) of binding for all of the melatonin ligands decreased as temperature increased.
- 5 The affinity, and thus the free energy of binding, ΔG° , of these ligands at the melatonin receptor have identical values at several temperatures yet at these temperatures ΔS° and ΔH° were very different, implying that more than one intermolecular force must be involved in the binding of ligand and receptor.
- 6 Conceivably, the large positive ΔS° observed at low temperatures, perhaps as a result of hydrophobic interactions, is compensated by a corresponding, but opposite, change in enthalpy at higher temperatures. However, it is not clear what type of binding force(s) would show such a temperature-dependence.
- 7 These studies suggest that caution must be exercised in the molecular interpretation of derived measures of ΔS° and ΔH° obtained from direct measurements of ΔG° .

Keywords: Thermodynamics; melatonin; 2-[¹²⁵I]-iodomelatonin binding; G-protein coupling

Introduction

In recent years, a number of studies have examined the thermodynamic changes which take place on binding of various neurotransmitters to their specific receptors. In a landmark study on β -adrenoceptors, Weiland *et al.* (1979) observed that agonist binding was enthalpy-driven whereas antagonist binding was entropy-driven. They proposed that enthalpy-driven agonist binding represents a conformational change in the receptor which leads to a biological response, while the entropy increase occurring upon antagonist binding reflects a simple binding event with no transfer of information. Similar thermodynamic changes occur on binding of benzodiazepine receptor agonists and antagonists (Mohler & Richards, 1981). This interpretation has been criticised as it has been pointed out that an agonist-induced conformation change in the receptor is not the only (or likely) cause of the observed negative changes in entropy induced by agonists (Miklavic *et al.*, 1990). Furthermore, it has not been possible to generalise these findings to other receptors. For example, binding of agonists at the dopamine D₂ receptor may be accompanied by positive or negative changes in entropy, and the binding of D₂ antagonists may be entropy or enthalpy-driven (Kilpatrick *et al.*, 1986). Similarly the binding of different muscarinic antagonists can be either entropy or enthalpy-driven (Gies *et al.*, 1986). Nevertheless, thermodynamic analysis may provide information on the inter-molecular forces which are likely to be involved in the binding of ligands to receptors (Testa *et al.*, 1987; Waelbroeck *et al.*, 1993) as the expected signs of changes in entropy and enthalpy accompanying the various individual kinds of interactions that may take place (hydrophobic association, van der Waals, H-bond formation etc.) are known (Ross & Subramanian, 1981).

High affinity, G-protein-coupled binding sites for the pineal hormone, melatonin (5-methoxy N-acetyltryptamine) have been identified in the brain of several species by use of both membrane binding assays and *in vitro* autoradiographic techniques (Morgan & Williams, 1989; Krause & Dubocovich, 1991). Activation of these binding sites has been shown to mediate several biological responses; inhibition of [³H]-dopamine release *in vitro* from neuronal retina (Dubocovich, 1983), aggregation of pigment granules in the melanophores of *Xenopus laevis* (Sugden, 1991), inhibition of adenosine 3':5'-cyclic monophosphate (cyclic AMP) synthesis in pars tuberalis (Morgan *et al.*, 1989; Carlson *et al.*, 1989) and inhibition of gonadotrophin-releasing hormone stimulation of luteinizing hormone release and second messenger responses in neonatal rat pituitary cells (Vanecek & Vollrath, 1989; Vanecek & Klein, 1992). Melatonin, secreted into the circulation from the pineal gland, is known to regulate seasonal changes in various aspects of physiology in photoperiodic species (Bartness & Goldman, 1989) and has been implicated in the mechanisms which regulate circadian rhythms (Armstrong, 1989). Melatonin synthesized locally within the eye regulates various aspects of retinal physiology (Besharse *et al.*, 1988).

In the present study, the agonist 2-[¹²⁵I]-iodomelatonin has been used to investigate the temperature-dependency of binding to chicken brain melatonin sites. The thermodynamic changes induced on binding of 2-[¹²⁵I]-iodomelatonin and melatonin, which are agonists, N-acetyltryptamine, a putative partial agonist (Dubocovich, 1985), and, luzindole (2-benzyl N-acetyltryptamine), an antagonist (Dubocovich, 1988) have been compared.

Methods

Preparation of chicken brain membranes

Chickens (*Gallus domesticus*, White Leghorn) were obtained from Orchard Farms (Buckinghamshire) at 1 day of age and were housed under a diurnal lighting cycle (12:12 L:D, lights on at 06 h 00 min) until killed between 14 h 00 min and 15 h 00 min at 3 months of age. The whole brain was removed, frozen in liquid nitrogen and stored at -70°C . Each brain was homogenized in 20 vols of Tris-HCl (50 mM, pH 7.4) containing phenylmethylsulphonylfluoride (PMSF, 1 mM), leupeptin ($50\text{ }\mu\text{g ml}^{-1}$) and EGTA (1 mM). The homogenate was centrifuged (35,000 g, 30 min, 4°C). The pellet was rehomogenized in the same buffer and centrifuged for a second time. The final membrane pellet was resuspended in Tris-HCl and aliquots frozen at -70°C until used.

2-[^{125}I]-iodomelatonin binding to chicken brain membrane preparations

For saturation analysis of 2-[^{125}I]-iodomelatonin binding to chicken membranes aliquots (70 μg) of membranes were incubated with 8 concentrations (6 to 1100 pM) of 2-[^{125}I]-iodomelatonin. Nonspecific binding was defined in the presence of non-radioactive melatonin (1 μM). Competition assays to determine IC_{50} values of melatonin, N-acetyltryptamine and luzindole were done on duplicate samples using a concentration of 2-[^{125}I]-iodomelatonin between 70 and 105 pM and at least six concentrations of each competing drug. For the time-course experiments, duplicate aliquots of membranes were incubated with 2-[^{125}I]-iodomelatonin (50 to 80 pM) for varying times (0–24 h) at 4, 13, 21, 29 and 37°C . From these experiments, incubation times of 24 h, 4 h, 90 min, 60 min and 30 min were chosen at 4, 13, 21, 29 and 37°C in order to ensure that equilibrium was reached. Reactions were terminated by the addition of 2 ml of ice-cold Tris-HCl buffer to each tube and immediate filtration through glass fibre filters (GF/C; Whatman Ltd., Maidstone, Kent). Each tube was then rinsed with a further 2 ml of buffer and each filter was washed ($2 \times 5\text{ ml}$) and counted in a LKB 1282 compugamma CS counter.

Data analysis

Data from saturation experiments were analysed by non-linear regression analysis using the ENZFITTER programme (Leatherbarrow, 1987) and the equation

$$B = B_{\text{max}} * F / (K_d + F)$$

where B = the concentration of ligand bound to the receptor, F = the concentration of free ligand, K_d = the equilibrium dissociation constant and B_{max} = the maximal concentration of binding sites.

IC_{50} values were determined in competition assays using the ALLFIT programme (De Lean *et al.*, 1978) with the four parameter logistic equation

$$Y = \frac{A - D}{1 + (X/C)^B} + D$$

where X and Y are the concentration of the competing compound and percentage inhibition of 2-[^{125}I]-iodomelatonin binding respectively, and A is the maximal binding (in the absence of competitor), B is the slope factor, C is the IC_{50} and D is the minimal binding (nonspecific binding). Inhibition constants (K_i) were then calculated using the Cheng-Prusoff equation (Cheng & Prusoff, 1973).

Thermodynamic analysis

The Gibb's free energy change of binding (ΔG° , kcal mol^{-1}) was calculated from the equation:

$$\Delta G^{\circ} = -RT \ln K_A \quad (1)$$

where R is the gas constant ($1.99\text{ cal/mol deg}^{-1}$), T is the temperature in degrees Kelvin, and K_A is the reciprocal of the equilibrium dissociation constant (K_d) or inhibition constant (K_i). As the van't Hoff plots for each of the compounds examined were curvilinear we used the procedure of Edelhoch & Osborne (1976) and fitted a second degree polynomial to the graph of ΔG° against temperature ($^{\circ}\text{K}$). From the equation

$$\Delta G^{\circ} = -RT \ln K_A = A + BT + CT^2 \quad (2)$$

three constants were obtained. Values of the standard free enthalpy change ΔH° (Kcal mol^{-1}) and standard entropy change ΔS° ($\text{cal/mol } ^{\circ}\text{K}^{-1}$) were then obtained by use of the following equations:

$$\Delta H^{\circ} = \frac{d(\Delta G^{\circ}/T)}{d(1/T)} = A - CT^2 \quad (3)$$

$$\Delta S^{\circ} = \frac{d\Delta G^{\circ}}{dT} = -B - 2CT \quad (4)$$

Protein determination

Protein was measured by the method of Bradford (1976) with bovine serum albumin as the standard.

Materials

2-[^{125}I]-iodomelatonin (2200 Ci mmol^{-1}) was purchased from DuPont, Stevenage, Herts. Unlabelled 2-iodomelatonin was from Research Biochemicals Inc., Natick, MA, U.S.A. Melatonin and GTP were from Sigma Chemical Co., Poole, Dorset. N-acetyltryptamine was synthesized as described previously (Sugden, 1991) and luzindole (2-benzyl N-acetyltryptamine) was obtained from Dr S.M. Reppert, Boston, U.S.A. All drugs were dissolved in methanol at a stock concentration of 10 mM and stored at -30°C .

Results

Control experiments for temperature dependence of specific 2-[^{125}I]-iodomelatonin binding to chicken brain membrane preparations

Thermodynamic analysis of binding data requires that the system studied is truly at equilibrium. The time-course of association of 2-[^{125}I]-iodomelatonin (50 to 80 pM) to chicken

Figure 1 Kinetics of specific 2-[^{125}I]-iodomelatonin binding to chicken brain membranes at different temperatures. The concentration of 2-[^{125}I]-iodomelatonin used was 50 to 80 pM and melatonin (1 μM) was used to define specific binding. The temperatures used were 4°C ($\blacktriangle$, inset), 13°C ($\times$), 21°C ($\triangle$), 29°C ($\circ$) and 37°C ($\bullet$). Each point represents the mean of duplicate determinations which differed by $<10\%$.

Table 1 Changes in K_d and B_{max} of 2-[^{125} I]-iodomelatonin binding to chicken brain membranes with temperature

	Temperature ($^{\circ}$ C)				
	4	13	21	29	37
K_d (pM)	64.6 ± 2.9	41.5 ± 1.3	20.2 ± 1.1	50.5 ± 4.6	103.2 ± 5.9
B_{max} (fmol mg^{-1} protein)	27.4 ± 0.7	25.8 ± 0.4	27.6 ± 0.4	25.1 ± 0.4	26.2 ± 0.7

K_d and B_{max} were calculated using non-linear regression analysis from data obtained in saturation experiments. Data given are the mean $\pm$ s.e.mean of 4 to 6 determinations. One way analysis of variance indicated that K_d values, but not B_{max} , varied significantly with temperature ($P < 0.05$).

Figure 2 Scatchard analysis of specific 2-[^{125} I]-iodomelatonin binding to chicken brain membranes at different temperatures. Each line shows a representative Scatchard plot at 4° C (▲), 13° C (X), 21° C (Δ), 29° C (○) and 37° C (●). Specific binding was defined using melatonin ($1 \mu M$).

Figure 3 (a) van't Hoff plot of the dependence of the K_A for 2-[^{125} I]-iodomelatonin binding on temperature. Each triangle represents a K_A value ($K_A = K_d^{-1}$) from an individual saturation experiment. Data were fitted using a second degree polynomial equation. (b) Dependence of the standard Gibbs free energy change (ΔG°) on temperature for binding of 2-[^{125} I]-iodomelatonin to chicken brain membranes. Each circle represents ΔG° calculated from an individual saturation curve. Data were fitted with a second degree polynomial equation and the constants A, B and C were obtained and used in the equations given in Methods to calculate changes in enthalpy (ΔH°) and entropy (ΔS°).

brain membranes was measured at 4, 13, 21, 29 and 37° C to ensure equilibrium (Figure 1). The rate of association increased as the temperature of incubation increased. The following incubation times which allow apparent equilibrium at each temperature were chosen for saturation and competition studies: 30 min at 37° C, 60 min at 29° C, 90 min at 21° C, 4 h at 13° C, and 24 h at 4° C.

Changes in affinity with temperature were reversible and not an artifact of the longer times required to reach equilibrium at the lower temperature. This was clear since the affinity of the receptor for 2-[^{125} I]-iodomelatonin measured at 37° C, after preincubation of membranes at 4° C for 24 h, was the same as its affinity after the 37° C incubation alone; similarly the affinity for 2-[^{125} I]-iodomelatonin at 4° C was not changed by prior incubation at 37° C for 30 min (data not shown).

Changes in affinity are not a reflection of changes in the pH of the Tris-HCl incubation buffer as separate buffers were used which were adjusted to pH 7.4 at each incubation temperature. Furthermore, K_d and B_{max} values were not significantly different when assays were done at pH 6.5 or 8.5 (data not shown).

The effect of temperature on specific 2-[^{125} I]-iodomelatonin binding to chicken brain membrane preparations

Non-linear regression analysis of saturation studies of specific 2-[^{125} I]-iodomelatonin binding to chicken brain membranes showed no significant change in apparent B_{max} values with temperature (Table 1; Figure 2). K_d values did vary significantly with temperature (Table 1). The apparent affinity of 2-[^{125} I]-iodomelatonin (K_d) was greatest at 21° C; at 4° C, 13° C, 29° C and 37° C the apparent affinity was reduced. At each of the temperatures tested Hill coefficients (n_H) were

Table 2 Thermodynamic parameters for 2-[¹²⁵I]-iodomelatonin binding in chicken brain membranes

	Temperature (°C)				
	4	13	21	29	37
ΔG° (Kcal mol ⁻¹)	-12.94 ± 0.03	-13.60 ± 0.02	-14.41 ± 0.03	-14.26 ± 0.05	-14.19 ± 0.03
ΔH° (Kcal mol ⁻¹)	+23.5	+9.2	-4.0	-17.6	-31.5
ΔS° (cal/mol deg ⁻¹)	+132.7	+81.6	+36.2	-9.3	-54.7

ΔG° , standard Gibbs free energy of binding; ΔH° standard free enthalpy change; ΔS° standard free entropy change. ΔG° was calculated using the equation $\Delta G^\circ = -RT \ln K_A$ where R is the gas constant (1.99 cal/mol deg⁻¹) and T is the temperature in °K, and $K_A = 1/\text{mean} \pm \text{s.e. mean } K_d$ obtained in saturation studies. Data given are the mean $\pm$ s.e. mean of 4 to 6 determinations. One way ANOVA indicated that ΔG° varied significantly with temperature.

Table 3 Effect of temperature and GTP (1 mM) on inhibition constant (K_i) values of melatonin analogues binding to chicken brain membranes

Compound	Conditions	K_i (nM)		
		4°C	21°C	37°C
Melatonin	Control	0.33 ± 0.03	0.07 ± 0.004	0.76 ± 0.04
	+ GTP	1.85 ± 0.23	0.71 ± 0.10	2.80 ± 0.39
N-acetyltryptamine	Control	2200 ± 101	280 ± 14	2160 ± 110
	+ GTP	3070 ± 237	1030 ± 78	2460 ± 180
Luzindole	Control	2220 ± 270	573 ± 72	3130 ± 369
	+ GTP	1550 ± 122	581 ± 44	1850 ± 140

K_i values were determined from IC_{50} values obtained in a single competition experiment using the Cheng-Prusoff equation (1973). Errors given are the standard errors of the computer derived estimates.

Figure 4 Changes in the thermodynamic parameters of 2-[¹²⁵I]-iodomelatonin binding with temperature in chicken brain membranes. The standard free enthalpy change (ΔH° , a) and the standard free entropy change (ΔS° , b) were calculated from the curve of ΔG° against temperature (Figure 3) as described in Methods.

close to unity (0.99–1.06) and Scatchard plots were linear, suggesting a single class of binding sites.

Thermodynamic parameters of 2-[¹²⁵I]-iodomelatonin binding

The van't Hoff plot for 2-[¹²⁵I]-iodomelatonin binding was clearly not linear (Figure 3a). The curve of ΔG° against

temperature (Figure 3b) fitted well ($r = 0.989$) to a second order polynomial equation allowing the thermodynamic parameters ΔH° and ΔS° to be calculated by the method of Edelhoch & Osborne (1976). Both ΔH° and ΔS° , calculated from the curve of ΔG° against temperature as described in Methods, varied over the temperature range studied (Figure 4; Table 2). Large positive changes in entropy and enthalpy were found at the lower temperatures (<21°C). An alternative analysis of ΔG° against temperature as two straight lines, the first over the temperature range 4 to 21°C and the second from 21 to 37°C, is unsatisfactory because only 5 temperatures were studied, and the inflection occurs in the centre of the temperature range.

Temperature-dependence and effect of GTP on affinity of melatonin, N-acetyltryptamine and luzindole

The K_d values obtained for 2-[¹²⁵I]-iodomelatonin in saturation experiments at 4°C, 21°C and 37°C were used to calculate K_i values for melatonin, N-acetyltryptamine and luzindole using the Cheng-Prusoff equation and IC_{50} values obtained in competition experiments at these temperatures (Table 3). All three compounds inhibited 2-[¹²⁵I]-iodomelatonin binding completely with pseudo Hill coefficients close to unity (0.81–1.09). K_A values varied with temperature (Figure 5). ΔH° and ΔS° were calculated from the curves of ΔG° against temperature. As for 2-[¹²⁵I]-iodomelatonin, positive changes in entropy and enthalpy were found at 4°C, but these thermodynamic parameters became negative at 37°C (Table 4).

As expected, guanosine triphosphate (GTP, 1 mM) decreased the affinity of melatonin for the 2-[¹²⁵I]-iodomelatonin binding site. Preliminary experiments indicated that GTP was equipotent with GTP γ S, a non-hydrolysable GTP analogue, and that a higher concentration of GTP (10 mM) did not shift melatonin affinity more than 1 mM GTP at either 4, 21 or 37°C (data not shown). This finding agrees with previous results on 2-[¹²⁵I]-iodomelatonin binding in chicken retina which show that the inhibitory effect of 10 mM GTP was not significantly greater than 1 mM GTP (Chong & Sugden, 1991). The inhibitory effect of GTP was clearly seen at each of the temperatures studied (4, 21 and 37°C) (Figure 5; Table 3). The affinity of luzindole, reported to be a melatonin receptor antagonist (Dubocovich, 1988), was not reduced by

Table 4 Effect of GTP on the thermodynamic parameters of the binding interactions of melatonin analogues with the 2-[¹²⁵I]-iodomelatonin binding site of chicken brain membranes.

Compound	Conditions	Thermodynamic parameters at:-								
		4°C			21°C			37°C		
		ΔG°	ΔH°	ΔS°	ΔG°	ΔH°	ΔS°	ΔG°	ΔH°	ΔS°
Melatonin	Control	-12.08 ± 0.07	+36.3	+174.5	-13.73 ± 0.08	-6.9	+23.4	-12.91 ± 0.14	-49.8	-118.9
	+ GTP	-11.08 ± 0.07	+21.0	+116.2	-12.32 ± 0.09	-3.5	+30.1	-12.15 ± 0.09	-28.0	-50.9
N-acetyltryptamine	Control	-7.18 ± 0.03	+40.4	+172.0	-8.83 ± 0.03	-2.4	+22.0	-8.05 ± 0.03	-45.0	-119.3
	+ GTP	-7.00 ± 0.04	+20.3	+98.5	-8.06 ± 0.05	-0.04	+27.3	-7.97 ± 0.05	-20.3	-39.7
Luzindole	Control	-7.18 ± 0.07	+28.4	+128.7	-8.41 ± 0.08	-3.7	+16.3	-7.82 ± 0.08	-35.6	-89.5
	+ GTP	-7.38 ± 0.04	+20.3	+99.7	-8.40 ± 0.05	-2.2	+20.9	-8.14 ± 0.05	-24.6	-53.2

Units are: ΔG° , Kcal mol⁻¹; ΔH° , Kcal mol⁻¹; ΔS° , cal/mol deg⁻¹. Errors on ΔG° are calculated from the standard errors of the estimates of IC₅₀ values.

Figure 5 van't Hoff plots of the dependence of K_A on temperature for melatonin (a), N-acetyltryptamine (b) and luzindole (c) in chicken brain membranes in the absence (■) or presence (▲) of GTP (1 mM). K_A values were calculated from K_i values obtained in competition experiments using 2-[¹²⁵I]-iodomelatonin (70 to 105 pM). Data were fitted with a second order polynomial equation and ΔH° and ΔS° calculated as described in Methods.

GTP at any of the temperatures studied (Figure 5; Table 3). N-acetyltryptamine, which has been reported to be a partial agonist at the melatonin receptor (Dubocovich, 1985; Sugden, 1991), did show some shift in affinity with GTP but not as dramatic a shift as melatonin, a full agonist at the receptor.

Discussion

A number of studies have now reported specific binding of 2-[¹²⁵I]-iodomelatonin in brain, pituitary and retinal tissue using both radioreceptor assays and *in vitro* autoradiography (Morgan & Williams, 1989; Krause & Dubocovich, 1991). The 2-[¹²⁵I]-iodomelatonin binding site has a high affinity for its endogenous ligand, melatonin, suggesting that it is physiologically relevant to the range of melatonin concentrations normally found in the circulation, and has a unique pharmacology quite distinct from that of 5-hydroxytryptamine and other neurotransmitters (Sugden & Chong, 1991). In addition, the pharmacological and biochemical characteristics of the site appear to be very similar in marsupial and eutherian mammals, birds and reptiles suggesting that the melatonin receptor has been well-conserved through evolution (Rivkees *et al.*, 1989; Sugden & Chong, 1991; Paterson *et al.*, 1992).

In the present study, the affinity of 2-[¹²⁵I]-iodomelatonin clearly changed with temperature. The highest affinity was seen at 21°C with a reduced affinity both at lower (4°C and 13°C) and higher (29°C and 37°C) temperatures. This is in contrast to a recent study (Dubocovich, 1991) which reported that 2-[¹²⁵I]-iodomelatonin binding to chicken brain and retinal membranes had a substantially lower affinity at 0°C ($K_d \sim 340$ –440 pM) than at 25°C and 37°C ($K_d \sim 60$ –100 pM). In the study by Dubocovich (1991), binding at 0°C was allowed only 5 h to reach equilibrium. In our study a much longer period was required to reach equilibrium at this temperature (Figure 1) so that it seems likely that the results obtained at 0°C in Dubocovich's study have underestimated the true affinity of 2-[¹²⁵I]-iodomelatonin.

The binding affinity of melatonin, N-acetyltryptamine and luzindole to the 2-[¹²⁵I]-iodomelatonin binding site was also temperature-dependent (Figure 5). Like 2-[¹²⁵I]-iodomelatonin, each of these compounds showed the highest affinity at 21°C and lower affinities at 4°C and 37°C. For melatonin, an agonist, GTP reduced affinity at each temperature by 3.5 to 11 fold. For N-acetyltryptamine, a putative partial agonist, GTP also shifted affinity but to a lesser extent (1.1 to 3.7 fold). The melatonin receptor antagonist, luzindole (Dubocovich, 1988), did not show any reduction in affinity with GTP at any temperature (ratio K_d (+ GTP)/ K_d (-GTP) = 0.6 to 1.0). GTP was as potent as GTPγS, a stable analogue of GTP which is resistant to metabolism, in inhibiting 2-[¹²⁵I]-

iodomelatonin binding in chicken brain membranes (Chong & Sugden, unpublished data) suggesting that the differences in the GTP-shift observed at different temperatures do not reflect the metabolism of GTP.

Calculation of the thermodynamic parameters ΔH° and ΔS° for all four compounds used in these studies reveals that as temperature increases there are reductions in both enthalpy and entropy of binding. Because binding was studied only at 5 temperatures, analysis of the data as two straight lines is unsatisfactory. However, it is possible that the inflection in the van't Hoff plot at around 21°C may, in fact, represent a 'break' which could be explained by a phase transition in the lipid bilayer containing the receptor, as has been suggested for [³H]-flunitrazepam binding to rat brain benzodiazepine receptors (Speth *et al.*, 1979). Whether a phase transition of the lipid bilayer can account for the curvilinear van't Hoff plot can only be resolved by further determinations of 2-[¹²⁵I]-iodomelatonin binding affinity at temperatures between 13 and 29°C. It would also be interesting to determine if an Arrhenius plot obtained by measuring the dissociation rate constant (K_{off}) at different temperatures showed a sudden break indicative of a phase transition. There are several other examples of curvilinear van't Hoff plots for ligand-receptor-binding in the literature (Gies *et al.*, 1986; Mei *et al.*, 1987; Borea *et al.*, 1988). In one case, the hypothesis that a phase transition in the lipid bilayer might be responsible for the break in the van't Hoff plot was ruled out as an Arrhenius plot was linear (Waelbroeck *et al.*, 1985).

In previous thermodynamic studies of protein-ligand association, positive changes in entropy have generally been interpreted to reflect a decrease in the local ordering of water molecules adsorbed on both the ligand and receptor (Kilpatrick *et al.*, 1986; Gies *et al.*, 1986; Ross & Subramanian, 1981) when ligand and receptor associate. However, as pointed out by Miklavic *et al.* (1990) the fundamental properties of water would suggest that this type of hydrophobic binding would be strongly temperature-dependent. Direct experimental evidence confirms that this is the case for model hydrophobic interactions (Abraham & Matteoli, 1988). In such model systems, curvilinear van't Hoff plots are obtained and ΔH° and ΔS° do change with temperature. In the present experiments, ΔH° and ΔS° also changed with temperature, consistent with the interpretation that a hydrophobic interaction may drive the binding of melatonin ligands to the receptor.

However, for 2-[¹²⁵I]-iodomelatonin binding to the melatonin receptor this interpretation is not entirely satisfactory and cannot completely explain the observed changes in affinity with temperature. The affinity, and free energy of binding, ΔG° , have equal values at a number of temperatures within the range studied (4° to 37°C). For example, for 2-[¹²⁵I]-iodomelatonin the data in Figure 3 indicate that at 4°C and 33°C the affinity of the ligand for the receptor is equal. Yet at these two temperatures ΔS° is markedly different; at 4°C,

ΔS° is +133 cal/mol deg⁻¹, while at 33°C ΔS° (determined from Figure 4) is -31 cal/mol deg⁻¹. At 4°C, it could be argued that the large positive change in ΔS° , perhaps as a result of hydrophobic interaction, may drive binding of ligand and receptor. At 33°C this cannot be the case as ΔS° is negative. Nevertheless, the affinity of the ligand for the receptor is identical at both temperatures. Thus at 33°C some other intermolecular interaction must compensate for the reduction in hydrophobic binding. Clearly, more than one factor must be involved in the binding of 2-[¹²⁵I]-iodomelatonin to its receptor so that, in this particular case, the negative change of entropy with increasing temperature is compensated by a corresponding, but opposite, change in enthalpy with temperature. What type of binding force(s) would show this difference in temperature dependence is not clear.

An additional complication is that in the absence of GTP, 2-[¹²⁵I]-iodomelatonin binds not to the isolated receptor protein but probably to a melatonin receptor-G protein-GDP complex, the so-called ternary complex. The contribution of these additional components to the measured free energy of binding, and whether this contribution changes with temperature, is not known. However, the changes in affinity and ΔG° with temperature are qualitatively similar for both the agonists investigated (melatonin, 2-[¹²⁵I]-iodomelatonin) and for the antagonist, luzindole. In this latter case, binding to the receptor would not be complicated by interactions with the G protein-GDP complex. Thus it may be that the contribution of the binding forces arising from these elements of the ternary complex is minor.

Clearly care must be taken not to overinterpret the changes in ΔH° and ΔS° ; the original experimental measurements of binding constants lead directly to the determination of ΔG° and it is the value of the Gibbs free energy which determines the extent of binding. The van't Hoff plot allows an easy numerical partitioning of ΔG° between ΔH° and ΔS° . However, care must be taken in associating these derived changes in enthalpy and entropy with the particular molecular events involved in ligand-receptor binding. Melatonin, by contrast with many neurotransmitter amines, does not have highly favourable sites for hydrogen or ionic bond formation. In comparison to its metabolic precursor, 5-hydroxytryptamine, the amine is transformed to an amide and the hydroxyl group methylated. This may account for the inflection of the van't Hoff plots occurring in the observed temperature range. The data presented here show that caution must be exercised in the molecular interpretation of thermodynamic parameters derived from ΔG° (Raffa & Porreca, 1989).

We would like to thank Drs Peter Garratt and Michael Ewing, Chemistry Department, University College London for their helpful advice on the interpretation of the thermodynamic data and Dr Steve Reppert for providing a sample of luzindole.

References

- ABRAHAM, M.H. & MATTEOLI, E. (1988). The temperature variation of the hydrophobic effect. *J. Chem. Soc. Faraday Trans.1*, **84**, 1985-2000.
- ARMSTRONG, S.M. (1989). Melatonin: the internal zeitgeber of animals? In *Pineal Research Reviews*, Vol. 7, ed. Reiter, R.J. pp. 157-202. New York: Alan R. Liss.
- BARTNESS, T.J. & GOLDMAN, B.D. (1989). Mammalian pineal melatonin: a clock for all seasons. *Experientia*, **45**, 939-945.
- BESHARSE, J.C., IUVONE, P.M. & PIERCE, M.E. (1988). Regulation of rhythmic photoreceptor metabolism: a role for post-receptoral neurons. In *Progress in Retinal Research*, ed. Osborne, N. & Chader, G.J. pp. 21-61. Oxford: Pergamon Press.
- BOREA, P.A., BERTELLI, G.M. & GILLI, G. (1988). Temperature dependence in the binding of μ , δ and κ agonists to the opiate receptors in guinea-pig brain. *Eur. J. Pharmacol.*, **146**, 247-252.
- BRADFORD, M.M. (1976). A rapid and sensitive method for the quantification of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal. Biochem.*, **72**, 248-254.
- CARLSON, L.L., WEAVER, D.R. & REPERT, S.M. (1989). Melatonin signal transduction in hamster brain: inhibition of adenylyl cyclase through a pertussis toxin-sensitive G protein. *Endocrinology*, **125**, 2670-2676.
- CHENG, Y.C. & PRUSOFF, W.H. (1973). Relationship between the inhibition constant (K_i) and the concentration of inhibitor which causes 50% inhibition (IC_{50}) of an enzymatic reaction. *Biochem. Pharmacol.*, **22**, 3099-3108.
- CHONG, N.W.S. & SUGDEN, D. (1991). Guanine nucleotides regulate 2-[¹²⁵I]-iodomelatonin binding sites in chick retinal pigment epithelium but not in neuronal retina. *J. Neurochem.*, **57**, 685-689.

- DELEAN, A.P., MUNSON, P.J. & ROBBARD, D. (1978). Simultaneous analysis of families of sigmoidal curves: application to bioassay, radioligand assay, and physiological dose-response curves. *Am. J. Physiol.*, **235**, E97–E102.
- DUBOCOVICH, M.L. (1983). Melatonin is a potent modulator of dopamine release in the retina. *Nature*, **306**, 782–784.
- DUBOCOVICH, M.L. (1985). Characterization of a retinal melatonin receptor. *J. Pharmacol. Exp. Ther.*, **234**, 395–401.
- DUBOCOVICH, M.L. (1988). Luzindole (N-0774): a novel melatonin receptor antagonist. *J. Pharmacol. Exp. Ther.*, **246**, 902–910.
- DUBOCOVICH, M.L. (1991). Pharmacological characterization of melatonin binding sites. *Adv. Pineal Res.*, **5**, 167–173.
- EDELHOCH, H. & OSBORNE, J.C. Jr. (1976). The theoretical basis of the stability of proteins, nucleic acids and membranes. *Adv. Protein Chem.*, **30**, 183–250.
- GIES, J.-P., ILIEN, B. & LANDRY, Y. (1986). Muscarinic acetylcholine receptor: thermodynamic analysis of the interaction of agonists and antagonists. *Biochim. Biophys. Acta*, **889**, 103–115.
- KILPATRICK, G.J., EL TAYAR, N., VAN DE WATERBEEMD, H., JENNER, P., TESTA, B. & MARSDEN, C.D. (1986). The thermodynamics of agonist and antagonist binding to dopamine D-2 receptors. *Mol. Pharmacol.*, **30**, 226–234.
- KRAUSE, D.N. & DUBOCOVICH, M.L. (1991). Melatonin receptors. *Annu. Rev. Pharmacol. Toxicol.*, **31**, 549–568.
- LEATHERBARROW, R.J. (1987). *ENZFITTER – A Non-Linear Regression Data Analysis Program for the IBM PC*. Amsterdam: Elsevier-Biosoft.
- MEI, L., WANG, J.-X., ROESKE, W.R. & YAMAMURA, H.I. (1987). Thermodynamic analyses of pirenzepine binding to membrane-bound and solubilized muscarinic receptors from rat forebrain and heart. *J. Pharmacol. Exp. Ther.*, **242**, 991–1000.
- MIKLAVIC, A., KOCJAN, D., MAVRI, J., KOLLER, J. & HADZI, D. (1990). 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**, 663–669.
- MOHLER, H. & RICHARDS, J.G. (1981). Agonist and antagonist benzodiazepine receptor interaction *in vitro*. *Nature*, **294**, 763–765.
- MORGAN, P.J., LAWSON, W., DAVIDSON, G. & HOWELL, H.E. (1989). Melatonin inhibits cyclic AMP production in cultured ovine pars tuberalis cells. *J. Mol. Endocrinol.*, **3**, R5–R8.
- MORGAN, P.J. & WILLIAMS, L.M. (1989). Central melatonin receptors: implications for a mode of action. *Experientia*, **45**, 955–965.
- PATERSON, A., CHONG, N.W.S., BRINKLOW, B.R., LOUDON, A.S.I. & SUGDEN, D. (1992). Characterization of 2-[¹²⁵I]-iodomelatonin binding sites in the brain of a marsupial, Bennett's wallaby (*Macropus rufogriseus rufogriseus*). *Comp. Biochem. Physiol.*, **102A**, 55–58.
- RAFFA, R.B. & PORRECA, F. (1989). Thermodynamic analysis of the drug-receptor interaction. *Life Sci.*, **44**, 245–258.
- RIVKEES, S.A., CARLSON, L.L. & REPPERT, S.M. (1989). Guanine nucleotide-binding protein regulation of melatonin receptors in lizard brain. *Proc. Natl. Acad. Sci. U.S.A.*, **86**, 3882–3886.
- ROSS, P.D. & SUBRAMANIAN, S. (1981). Thermodynamics of protein association reactions: Forces contributing to stability. *Biochemistry*, **20**, 3096–3102.
- SPETH, R.C., WASTEK, G.J. & YAMAMURA, H.I. (1979). Benzodiazepine receptors: Temperature dependence of [³H]flunitrazepam binding. *Life Sci.*, **24**, 351–358.
- SUGDEN, D. (1991). Aggregation of pigment granules in single cultured *Xenopus laevis* melanophores by melatonin and analogues. *Br. J. Pharmacol.*, **104**, 922–927.
- SUGDEN, D. & CHONG, N.W.S. (1991). Pharmacological identity of 2-[¹²⁵I]-iodomelatonin binding sites in chicken brain and sheep pars tuberalis. *Brain Res.*, **539**, 151–154.
- TESTA, B., JENNER, P., KILPATRICK, G.J., EL TAYAR, N., VAN DE WATERBEEMD, H. & MARSDEN, C.D. (1987). Do thermodynamic studies provide information on both the binding to and the activation of dopaminergic and other receptors? *Biochem. Pharmacol.*, **36**, 4041–4046.
- VANECEK, J. & KLEIN, D.C. (1992). Melatonin inhibits gonadotropin-releasing hormone-induced elevation of intracellular Ca^{2+} in neonatal rat pituitary cells. *Endocrinology*, **130**, 701–707.
- VANECEK, J. & VOLLRATH, L. (1989). Melatonin inhibits cyclic AMP and cyclic GMP accumulation in the rat pituitary. *Brain Res.*, **505**, 157–159.
- WAELEBROECK, M., CAMUS, J., TASTENOY, M., LAMBRECHT, G., MUTSCHLER, E., KROPFGANS, M., SPERLICH, J., WIESENBERGER, F., TACKE, R. & CHRISTOPHE, J. (1993). Thermodynamics of antagonist binding to rat muscarinic M_2 receptors: antimuscarinics of the pridinol, sila-pridinol, diphenidol and sila-diphenidol type. *Br. J. Pharmacol.*, **109**, 360–370.
- WAELEBROECK, M., ROBBERECHT, P., CHATELAIN, P., DE NEFF, P. & CHRISTOPHE, J. (1985). Effects of temperature and ethanol on agonist and antagonist binding to rat heart muscarinic receptors in the absence and presence of GTP. *Biochem. J.*, **231**, 469–476.
- WEILAND, G.A., MINNEMAN, K.P. & MOLINOFF, P.B. (1979). Fundamental difference between the molecular interactions of agonists and antagonists with the β -adrenergic receptor. *Nature*, **281**, 144–147.

(Received August 19, 1993

Revised September 20, 1993

Accepted September 21, 1993)