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Temperature-dependence of the kinetics of the binding of [^{3}H]-(+)-N-methyl-4-methyldiphenhydramine to the histamine H_1-receptor: comparison with the kinetics of [^{3}H]-mepyramine

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1 The dissociation of [^{3}H]-(+)-N-methyl-4-methyldiphenhydramine ([^{3}H]-QMDP) from the histamine H_1-receptor was markedly temperature-dependent. The $t_{1/2}$ was 4 min at 37°C and 16 h at 6°C. The association rate constant, k_1, was also temperature-dependent, but not to the same extent as k_{-1}.

2 Plots of the observed rate constant for [^{3}H]-QMDP-receptor complex formation, k_{on}, *versus* [^{3}H-QMDP] were linear at both 30°C and 10°C, consistent with the interaction of [^{3}H]-QMDP with the H_1-receptor being a simple, one-step equilibrium.

3 The ratio of the kinetic constants, k_1/k_{-1}, indicated that the affinity constant of [^{3}H]-QMDP for the H_1-receptor should increase with decreasing temperature. Measurement of (+)-QMDP antagonism of the contraction of longitudinal muscle strips from guinea-pig small intestine induced by histamine at 37°C, 30°C and 25°C provided some evidence that the affinity of (+)-QMDP is greater at 25°C than 37°C. However, the flattening of the concentration-response curves for histamine at low concentrations of (+)-QMDP at 30°C and 25°C is consistent with a slow dissociation of the (+)-QMDP-receptor complex and hence an incomplete equilibration with the agonist.

4 Arrhenius plots for k_1 and k_{-1} for [^{3}H]-QMDP were linear between 37°C and 6°C. The activation energies, E_a, for complex formation and dissociation were 77 ± 4 and $129 \pm 3\,kJ\,mol^{-1}$, respectively.

5 An Arrhenius plot for k_{-1} for the dissociation of [^{3}H]-mepyramine from the H_1-receptor was also linear between 37°C and 6°C. The activation energy was $140 \pm 2\,kJ\,mol^{-1}$.

6 Activation energies for complex formation with the H_1-receptor, E_{af}, and complex dissociation, E_{ad}, were similar for [^{3}H]-QMDP and [^{3}H]-mepyramine. The energy difference, $E_{af} - E_{ad}$, equivalent to the enthalpy change, did not differ significantly for the two ligands (-52 and $-48\,kJ\,mol^{-1}$, respectively). The larger values of k_1 and k_{-1} for [^{3}H]-mepyramine compared to [^{3}H]-QMDP imply the presence of an entropic component in the interaction.

7 The simplest explanation for these observations is that transfer from the aqueous phase into a hydrophobic region is a significant factor in antagonist-H_1-receptor interaction. This would be entropically more favourable for [^{3}H]-mepyramine, a tertiary amine, than for [^{3}H]-QMDP, a quaternary amine.

Introduction

The rate constants for the association of [^{3}H]-mepyramine with the histamine H_1-receptor and for the dissociation of the [^{3}H]-mepyramine-receptor complex are notably temperature-dependent (Wallace & Young, 1983), implying the presence of a significant energy barrier both for association and dissociation. One possible factor in the kinetics, bearing in mind the lipophilic nature of the uncharged forms of most H_1-antihistamines, is that the rate of binding may be governed in part by the rate of transfer of the ligand from the aqueous phase to a hydrophobic region. This might be part of the binding site or it might be the membrane phase. For example, it has been suggested that hydrophobic

antagonists may first partition into the membrane phase and then diffuse laterally to reach their binding site on the receptor protein (Rhodes *et al.*, 1985 and references therein). This can result in a markedly faster rate of receptor complex formation than direct collision coupling with molecules in the aqueous phase. An alternative or additional way in which the membrane might influence antagonist-receptor interaction is via control of receptor conformation. In this case an abrupt change in binding properties, even for antagonists, might occur at the membrane phase-transition temperature. Such discontinuities in van't Hoff plots in the temperature range 15–20°C have been reported for the binding of α_2-antagonists (Lohse *et al.*, 1986) and for the 5-hydroxytryptamine (5-HT) uptake inhibitor paroxetine (Plenge & Mellerup, 1984). There is no direct evidence for any discontinuity in the binding properties of H_1-antagonists, but the observed rate of dissociation of [^{3}H]-mepyramine from the H_1-receptor at 4°C was much slower than would have been predicted from the extrapolation of an Arrhenius plot for k_{-1} constructed between 37°C and 15°C (Wallace & Young, 1983).

If transfer into a hydrophobic region does play a part in the interaction of antagonists with the H_1-receptor, then the kinetics of [^{3}H]-(+)-N-methyl-4-methyldiphenhydramine ([^{3}H]-QMDP), a quaternary ligand for the H_1-receptor (Treherne & Young, 1988), might well differ significantly from those of tertiary amines. To test this we have investigated the temperature-dependence of the kinetics of binding of [^{3}H]-QMDP and made a comparison with the kinetics of [^{3}H]-mepyramine, a tertiary amine of similar size and with a similar equilibrium constant for the H_1-receptor. As part of this study we have re-examined the kinetics of dissociation of [^{3}H]-mepyramine. A preliminary account of some of these results has been given to the British Pharmacological Society (Treherne & Young, 1986).

Methods

Guinea-pig cerebellar homogenate

A washed particulate fraction from guinea-pig cerebellum in 50 mM Na-K phosphate buffer, pH 7.5, (6–10 mg protein ml^{-1}) was prepared as described in the accompanying paper (Treherne & Young, 1988) and stored at −20°C until required for use.

Dissociation of bound [^{3}H]-QMDP and [^{3}H]-mepyramine

Two experimental protocols were used. In the first, 0.7–1.0 nM ^{3}H-ligand was equilibrated with cerebellar homogenate (final protein concentration 0.33–0.35 mg ml^{-1}) in 50–55 ml 50 mM Na-K phosphate buffer, pH 7.5, for 1 h at 30°C. Non-specific binding was determined in a parallel incubation containing either 0.4 μM mepyramine (for [^{3}H]-QMDP) or 2 μM promethazine (for [^{3}H]-mepyramine). The temperature was then either maintained at 30°C or cooled over 15–20 min to temperatures between 25°C and 6°C. Dissociation of the ^{3}H-ligand was induced by addition of 1 μM (+)-QMDP (final concentration, in experiments with [^{3}H]-QMDP) or 1 μM mepyramine (with [^{3}H]-mepyramine). At given times 1 ml aliquots of the incubation mixture were filtered through Whatman GF/B glass-fibre filters (soaked in 0.3% polyethylenimine for 5–16 h previously) on a Shearline R & D (Cambridge, U.K.) 10-place filtration block. Filters were washed 4 times with 4 ml ice-cold buffer and then transferred to scintillation vials and allowed to soak in 10 ml scintillator (Unisolve E (Koch-Light)/water, 95 : 5, v/v, or Quickszint 212 (Zinnser)/water, 95 : 5, v/v) overnight.

In the second procedure, separate incubations of [^{3}H]-QMDP or [^{3}H]-mepyramine and cerebellar homogenate (0.24–0.35 mg protein), with or without 0.4 μM mepyramine or 2 μM promethazine, in 50 mM Na-K phosphate buffer, pH 7.5, (total volume 1 ml) were set up (5 replicates), allowed to equilibrate for 1 h at 30°C and then cooled or warmed (37°C) to the final incubation temperature as above. Dissociation of the bound ^{3}H-ligand was induced by addition of 1 μM (+)-QMDP or 1 μM mepyramine and incubations terminated after a given time by addition of 4 ml ice-cold buffer and immediate filtration and washing (3 × 4 ml buffer) as above.

The dissociation rate constant, k_{-1}, was obtained by fitting the variation of the 0.4 μM mepyramine-sensitive binding of [^{3}H]-QMDP, or the 2 μM promethazine-sensitive binding of [^{3}H]-mepyramine, with time to the equation:

$$B = B_0 \times \exp(-k_{-1} \times t)$$

where B is the amount of ^{3}H-ligand bound at any time after initiation of dissociation and B_0 is the amount bound at time zero. The best-fit value ± estimated s.e. of k_{-1} was obtained by weighted non-linear regression analysis using a modified Marquardt method as implemented in the Harwell library routine VB01A on the Cambridge IBM 3081. All points were weighted by the inverse of the variance associated with them. An initial estimate of k_{-1} was obtained from the slope of a plot of ln (B/B_0) *versus* time.

Values of k_{-1} for [^{3}H]-QMDP at 30°C obtained using both experimental protocols did not differ significantly.

Dissociation of [^{3}H]-QMDP initiated by dilution

[^{3}H]-QMDP, 10 nM, was incubated with cerebellar homogenate (18.7 mg protein) in the presence or absence of 0.4 μM mepyramine in 50 mM Na-K phosphate buffer, pH 7.5, (total volume 3.56 ml) for 1 h at 30°C. Aliquots (50 μl) were then diluted into 5 ml buffer at the experimental temperature and the whole 5 ml filtered as above at given times after dilution (5 replicates).

The dissociation rate constant, k_{-1}, was obtained by fitting, as above, the variation of the mepyramine-sensitive binding of [^{3}H]-QMDP with time to:

$$B = B_0 \times \exp(-k_{-1} \times t) + B_{min}$$

where B is the amount of [^{3}H]-QMDP bound at any time after dilution, B_0 is the amount bound at time zero and B_{min} is the amount bound at infinite time, B_{min} and k_{-1} were treated as variables.

Association kinetics of [^{3}H]-QMDP

Again two experimental procedures were used. In most experiments binding was initiated by addition of cerebellar homogenate (0.20–0.34 mg protein) to 0.2–3.1 nM [^{3}H]-QMDP in 50 mM Na-K phosphate buffer, pH 7.5, (final incubation volume 1 ml) in the presence or absence of 0.4 μM mepyramine. Incubations (5 replicates) were terminated at various times by addition of 4 ml ice-cold buffer and rapid filtration and washing (3 × 4 ml buffer) as in the dissociation experiments above.

In 2 experiments at 30°C with 1 nM [^{3}H]-QMDP the total incubation volume was 40 ml and 1 ml aliquots (5 replicates) were filtered at various times after homogenate (0.35 mg protein ml^{-1}) addition.

Assuming that the interaction between [^{3}H]-QMDP and the H_1-receptor is a simple one-step equilibrium:

$$A + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} AR$$

and that the binding of [^{3}H]-QMDP to the homogenate does not lead to any appeciable change in the concentration of free [^{3}H]-QMDP (the maximum proportion of total [^{3}H]-QMDP bound was 3%), then the amount of [^{3}H]-QMDP bound to the receptor at any time, B, is given by:

$$B = B_{eq} \times (1 - \exp(-k_{on} \times t))$$

where B_{eq} is the amount of [^{3}H]-QMDP bound at equilibrium and k_{on}, the observed on-rate constant, is given by:

$$k_{on} = k_1 \times [^3\text{H-QMDP}] + k_{-1}$$

Values of k_{on} were determined at each concentration of [^{3}H]-QMDP by fitting the variation of the mepyramine-sensitive binding of [^{3}H]-QMDP to the exponential function using weighted non-linear regression as described above, with k_{on} and B_{eq} as unknowns. An initial estimate of k_{on} was obtained from the slope of a plot of ln $(1 - B/B_{eq})$ *versus* time.

Inhibition of histamine-induced contraction of intestinal smooth muscle

Longitudinal muscle strips from guinea-pig small intestine were suspended in Krebs-Henseleit solution (mM: NaCl 118, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5 and D-glucose 5.5) gassed with 95% O_2 5% CO_2 (v/v) in a conventional organ bath at 30°C or 37°C. In experiments at 25°C the tissue was first equilibrated for 1 h at 37°C before cooling slowly to 25°C. Contractions to histamine were recorded isotonically. (+)-QMDP was present in the Krebs-Henseleit solution, where appropriate, and was allowed to equilibrate with the tissue for at least 40 min at 37°C, 60 min at 30°C or 90 min at 25°C. The affinity constant, K_a, for (+)-QMDP at each temperature was calculated from parallel shifts of the log concentration-response curve to histamine, using the relationship: concentration-ratio = [(+)-QMDP] × Ka + 1, where the concentration-ratio is the ratio of the concentration of histamine required for a given contraction in the presence of (+)-QMDP to the concentration of histamine required for the same response in the absence of antagonist.

Chemicals

[^{3}H]-mepyramine, 26 Ci mmol^{-1}, was obtained from Amersham International and [^{3}H]-QMDP, 83 Ci mmol^{-1}, was prepared as described in the accompanying paper (Treherne & Young, 1988). Mepyramine maleate was purchased from May & Baker Ltd and promethazine hydrochloride from Sigma. (+)-N-methyl-4-methyldiphenhydramine iodide ((+)-QMDP) was a kind gift from Gist-Brocades NV.

Results

Kinetics of dissociation of [^{3}H]-QMDP from the histamine H_1-receptor

The rate of dissociation of [^{3}H]-QMDP from the histamine H_1-receptor, initiated by the addition of 1 μM (+)-QMDP, was markedly temperature-dependent (Figure 1). At 30°C the binding of [^{3}H]-QMDP insensitive to inhibition by 0.4 μM mepyramine ('non-specific' binding) did not change over the period measured (1–100 min), but the mepyramine-insensitive binding had declined to zero by 80 min

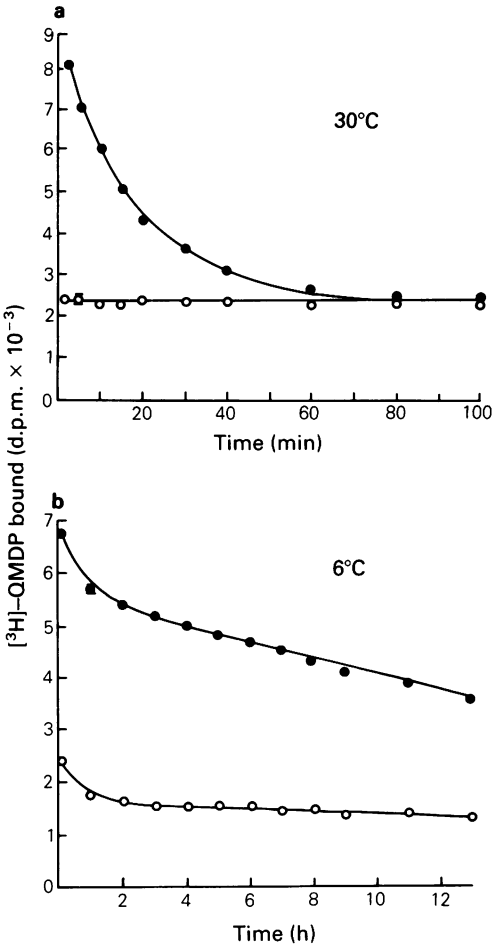

Figure 1 Kinetics of dissociation of [^{3}H]-N-methyl-4-methyldiphenhydramine ([^{3}H]-QMDP) at (a) 30°C and (b) 6°C. [^{3}H]-QMDP, 0.50 nM in (a) and 0.55 nM in (b), was incubated with guinea-pig cerebellar homogenate (0.35 mg protein ml^{-1}) in 50 mM Na-K phosphate buffer, pH 7.5, for 60 min in the presence or absence of 0.4 μM mepyramine. In (b) the incubations were then cooled to 6°C over a 15 min period. Dissociation of bound [^{3}H]-QMDP was initiated by addition of unlabelled (+)-QMDP at time zero to give a final concentration of 1 μM and [^{3}H]-QMDP remaining bound at various times measured as described under Methods. Each point is the mean of 5 replicates, vertical bars show s.e. mean. Where no error bars are shown the error was within the size of the symbol. The curves have been drawn by inspection. Best-fit values for the dissociation constant, k_{-1}, obtained by fitting the difference between each pair of points to an exponential function (see Methods), were: 30°C, $5.5 \pm 0.2 \times 10^{-2}$ min^{-1}; 6°C, $8.0 \pm 0.3 \times 10^{-4}$ min^{-1}. (●), [^{3}H]-QMDP bound in the absence of mepyramine; (○), [^{3}H]-QMDP bound in incubations containing 0.4 μM mepyramine (non-specific binding).

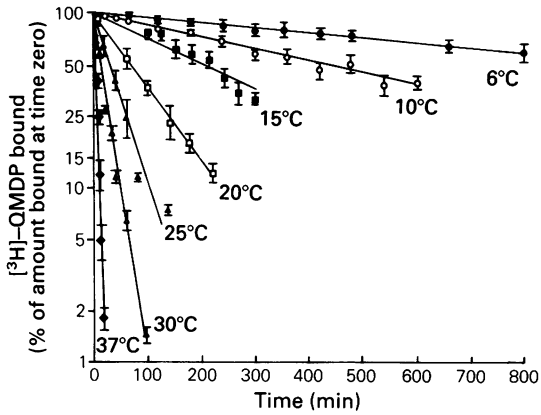

Figure 2 Temperature-dependence of the dissociation of [^{3}H]-N-methyl-4-methyldiphenhydramine ([^{3}H]-QMDP) from the H_1-receptor. The rate of decline of the 0.4 μM mepyramine-sensitive binding of [^{3}H]-QMDP, determined from experiments such as those shown in Figure 1, is shown as a plot of log([^{3}H]-QMDP bound × 100/[^{3}H]-QMDP bound at time zero) *versus* time. Each point is the mean from 5 replicate determinations within a single experiment; s.e. mean shown by vertical bars. Some points at <50% dissociation have been omitted for the sake of clarity. Best-fit lines were determined by weighted linear regression. The best-fit values of the dissociation rate constant, k_{-1}, obtained from the data as described under Methods, are set out in Table 1. Temperatures: (●), 6°C; (○), 10°C; (■), 15°C; (□), 20°C; (▲), 25°C; (△), 30°C; (◆), 37°C.

(Figure 1a). In contrast, at 6°C less than half of the receptor-specific binding of [^{3}H]-QMDP had been lost over the period of the experiment, 13 h (Figure 1b). At the lower temperature there was a small initial decline in the level of the mepyramine-insensitive binding of [^{3}H]-QMDP (Figure 1b). This component was also apparent in experiments at 10°C and 15°C, but not at higher temperatures.

The value of k_{-1} for [^{3}H]-QMDP at 6°C was not significantly altered when the non-specific binding was defined by 1 μM temelastine and dissociation was induced by addition of 1 μM mepyramine (2 experiments).

At all temperatures at which measurements were made, the decline in the receptor-specific binding of [^{3}H]-QMDP with time approximated well to a single exponential, as demonstrated by the linearity of plots of $\log(B/B_0)$ *versus* time (Figure 2), where B_0 is the amount bound at time zero. The values of the rate constant of dissociation, k_{-1}, obtained from these experiments by fitting the data directly to an exponential function (see Methods) are set out in Table 1, with the corresponding values of $t_{1/2}$, the half-time for dissociation.

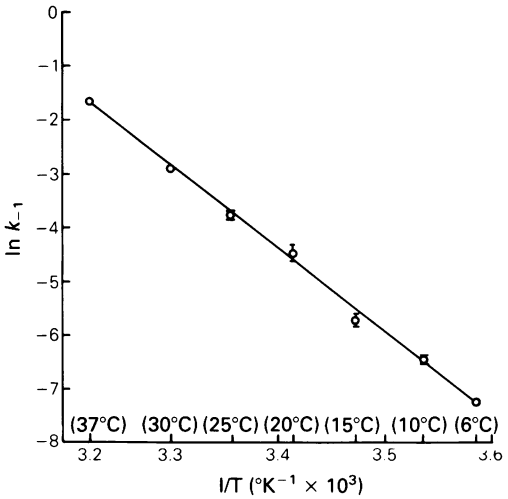

Figure 3 Arrhenius plot of the variation of the rate constant for dissociation of the [^{3}H]-N-methyl-4-methyldiphenhydramine ([^{3}H]-QMDP)-receptor complex, k_{-1}, with temperature. Values of k_{-1} (±s.e. mean) are taken from Table 1. The best-fit line drawn was obtained by linear regression analysis. Activation energy, E_a, 129 ± 3 kJ mol^{-1}.

An Arrhenius plot of the dissociation data, ln k_{-1} *versus* 1/T, was linear over the temperature range studied (37°C to 6°C) (Figure 3) and yielded an activation energy, E_a, of 129 ± 3 kJ mol^{-1}.

Dissociation of [^{3}H]-QMDP induced by dilution

In the experiments described above dissociation of bound [^{3}H]-QMDP was initiated by addition of an excess (1 μM) of unlabelled (+)-QMDP. There were some differences in the pattern of the decline of [^{3}H]-QMDP binding observed when dissociation was induced by 100 fold dilution into phosphate buffer. An experiment at 30°C is shown in Figure 4. There was a fall in the mepyramine-insensitive binding of [^{3}H]-QMDP over a period of *circa* 30 min (Figure 4a, cf. Figure 1a) and in incubations not containing 0.4 μM mepyramine the level of [^{3}H]-QMDP bound failed to fall to the same level as in the presence of mepyramine. This difference in levels at 100 min can be accounted for by the residual binding of 0.1 nM [^{3}H]-QMDP (the final concentration after dilution). When the 0.4 μM mepyramine-sensitive binding of [^{3}H]-QMDP (Figure 4b) was fitted, allowing the amount of [^{3}H]-QMDP bound at infinite time to be a variable (see Methods), the value of k_{-1} obtained, 5.5 ± 0.4 × 10^{-2} min^{-1}, was not significantly different from that obtained when dissociation was induced by addition of 1 μM (+)-QMDP, 5.4 ± 0.1 × 10^{-2} min^{-1} (Table 1). Similarly,

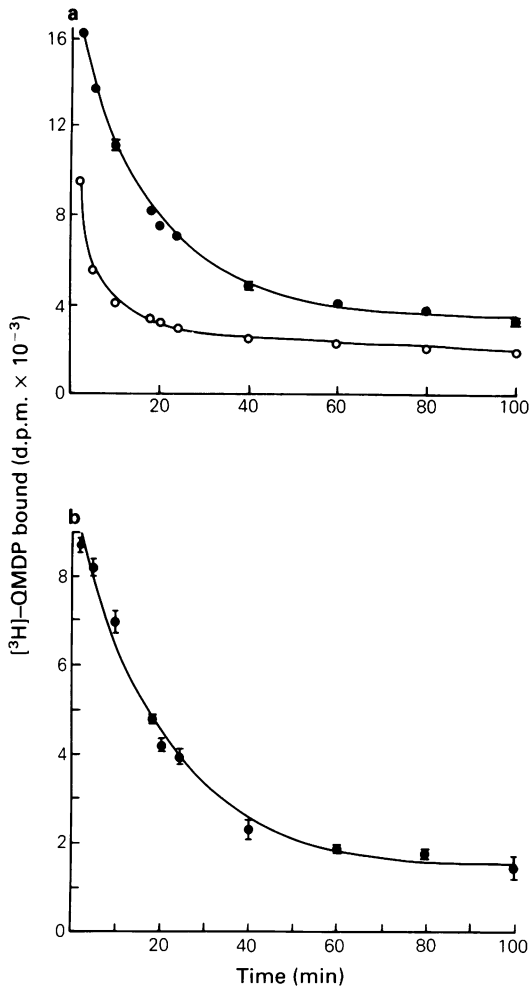

Figure 4 Kinetics of dissociation of [^{3}H]-N-methyl-4-methyldiphenhydramine ([^{3}H]-QMDP) initiated by dilution. (a) [^{3}H]-QMDP (10 nM) and cerebellar homogenate (18.7 mg protein) were incubated in 3.56 ml 50 mM Na-K phosphate buffer, pH 7.5, for 60 min at 30°C in the presence and absence of 0.4 μM mepyramine. Dissociation was initiated at time zero by diluting 50 μl aliquots to 5 ml with buffer (final concentrations: protein 0.052 mg ml^{-1}, [^{3}H]-QMDP 0.1 nM) at 30°C. Bound [^{3}H]-QMDP was measured as described under Methods. Each point is the mean of 5 replicates; s.e. mean shown by vertical bars. Where no error bars are shown the error was within the size of the symbol. The curves have been drawn by inspection. (●), No mepyramine present in the initial incubation; (○), 0.4 μM mepyramine present during the initial incubation (non-specific binding). (b) Time-course of the dissociation of H_1-receptor bound [^{3}H]-QMDP. The best-fit curve drawn was obtained by fitting an exponential function (see Methods) to the difference between each pair of points in (a) (k_{-1} 5.5 ± 0.4 × 10^{-2} min^{-1}).

Table 1 Rate constants for [^{3}H]-N-methyl-4-methyldiphenhydramine ([^{3}H]-QMDP) binding to the histamine H_1-receptor

Temperature (°C)	k_1 ($M^{-1}\,min^{-1}$)	k_{-1} (min^{-1})	$t_{1/2}$ (min)
37	$1.6 \pm 0.1 \times 10^8$	$1.9 \pm 0.1 \times 10^{-1}$	4
30	$6.1 \pm 0.4 \times 10^7$	$5.4 \pm 0.1 \times 10^{-2}$	13
25	$5.6 \pm 0.5 \times 10^7$	$2.3 \pm 0.2 \times 10^{-2}$	30
20	$3.2 \pm 0.6 \times 10^7$	$1.1 \pm 0.2 \times 10^{-2}$	61
15	$1.5 \pm 0.1 \times 10^7$	$3.4 \pm 0.3 \times 10^{-3}$	206
10	$8.7 \pm 0.1 \times 10^6$	$1.6 \pm 0.1 \times 10^{-3}$	441
6	$5.5 \pm 0.8 \times 10^6$	$7.2 \pm 0.2 \times 10^{-4}$	960

Values of k_{-1} and, hence, $t_{1/2}$ were determined from the dissociation of the mepyramine-sensitive binding of [^{3}H]-QMDP as described under Methods. Values of k_{-1} at 30°C and 6°C are the weighted means ±s.e. mean from 3 experiments. See text for other determinations of k_{-1} at 30°C, 25°C and 10°C. Values of k_1 at 30°C and 10°C were obtained from plots of [[^{3}H]-QMDP] *versus* k_{on}, the observed rate constant for complex formation (Figure 6). At other temperatures k_1 was calculated from k_{on} using the values of k_{-1} given above (see Methods for details).

in an experiment at 25°C the values of k_{-1} obtained from dilution and (+)-QMDP addition, $1.9 \pm 0.2 \times 10^{-2}\,min^{-1}$ and $2.3 \pm 0.2 \times 10^{-2}\,min^{-1}$, respectively, did not differ significantly.

Kinetics of association of [^{3}H]-*QMDP with the* H_1-*receptor*

The rate of association of [^{3}H]-QMDP with the H_1-receptor was also markedly temperature-dependent (Figure 5). At 30°C, equilibration with 0.56 nM [^{3}H]-QMDP was practically complete in 30–40 min (Figure 5a), but at 6°C at least 6 h were required for 0.93 nM [^{3}H]-QMDP (Figure 5b). The equilibrium level of the 0.4 μM mepyramine-insensitive binding (non-specific binding) was also reached more rapidly at 30°C than 6°C, but even at 30°C some 5–10 min were necessary. Values of the observed rate constant for complex formation, k_{on}, were obtained by fitting curves of the mepyramine-sensitive binding of [^{3}H]-QMDP *versus* time to an exponential function as described under Methods. If the interaction between [^{3}H]-QMDP and the H_1-receptor is a simple one-step equilibrium then $k_{on} = k_1 \times$ [^{3}H-QMDP] + k_{-1} and k_{on} should be a linear function of the concentration of [^{3}H]-QMDP. This was tested at two temperatures, 30°C and 10°C (Figure 6). The large proportion of non-specific binding at higher concentrations of [^{3}H]-QMDP (80% with 5.3 nM [^{3}H]-QMDP at 10°C) limited the range over which the relationship could be tested,

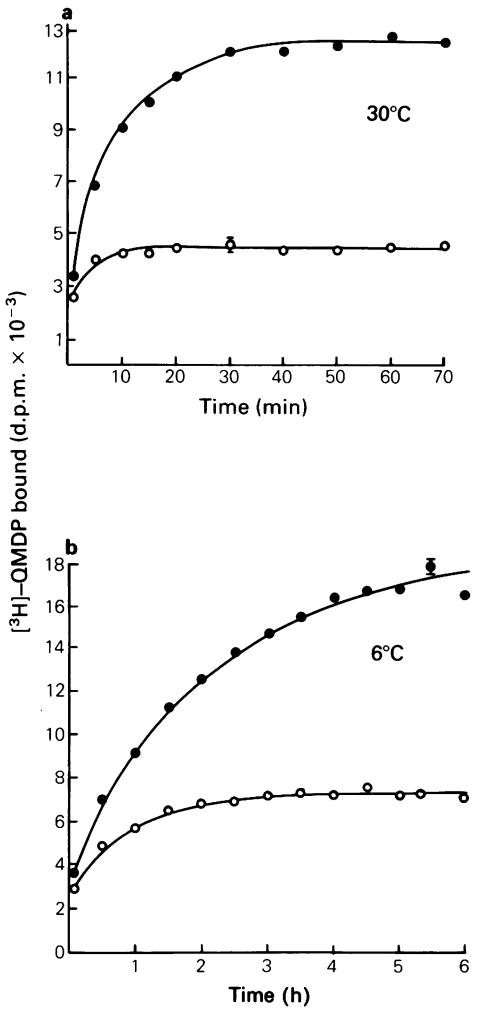

Figure 5 Kinetics of association of [^{3}H]-N-methyl-4-methyldiphenhydramine ([^{3}H]-QMDP) at (a) 30°C and (b) 6°C. The amount of [^{3}H]-QMDP bound at various times after addition of cerebellar homogenate (0.35 mg protein) and 0.56 nM [^{3}H]-QMDP (at 30°C) or 0.93 nM [^{3}H]-QMDP (at 6°C) in 50 mM Na-K phosphate buffer in the presence or absence of 0.4 μM mepyramine (total incubation volume 1 ml) was determined as described under Methods. Each point is the mean of 5 replicate determinations within the same experiment; s.e. mean shown by vertical bars. Where no error bars are shown the error was within the size of the symbol. The curves have been drawn by inspection. Best-fit values of the association rate constant, k_1, obtained by fitting the difference between each pair of points to an exponential function to give k_{on} (see Methods) and taking the value of k_{-1}, given in Table 1, were: 30°C, $6.8 \pm 0.4 \times 10^7\,M^{-1}\,min^{-1}$; 6°C, $5.5 \pm 0.8 \times 10^6\,M^{-1}\,min^{-1}$. (●), No mepyramine present; (○), 0.4 μM mepyramine present in the incubation (non-specific binding).

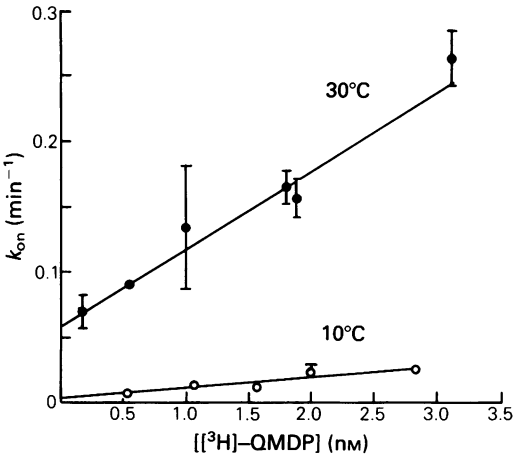

Figure 6 Variation of the observed rate constant for complex formation, k_{on}, with the concentration of [^{3}H] - N - methyl - 4 - methyldiphenhydramine ([^{3}H]-QMDP). Each value of k_{on} was obtained from an experiment such as that shown in Figure 5a or 5b, in which the time-course of the binding of [^{3}H]-QMDP was measured in the presence and absence of 0.4 μM mepyramine and k_{on} determined by fitting an exponential function to the curve of mepyramine-sensitive binding *versus* time (see Methods). The lines drawn were obtained from weighted linear regression analysis. Best-fit parameters: 30°C, k_1 $6.1 \pm 0.4 \times 10^7$ M^{-1} min^{-1}, k_{-1} $5.8 \pm 0.3 \times 10^{-2}$ min^{-1}; 10°C, k_1 $8.7 \pm 0.1 \times 10^6$ M^{-1} min^{-1}, k_{-1} $2.6 \pm 1.2 \times 10^{-3}$ min^{-1}. (●), 30°C; (○), 10°C.

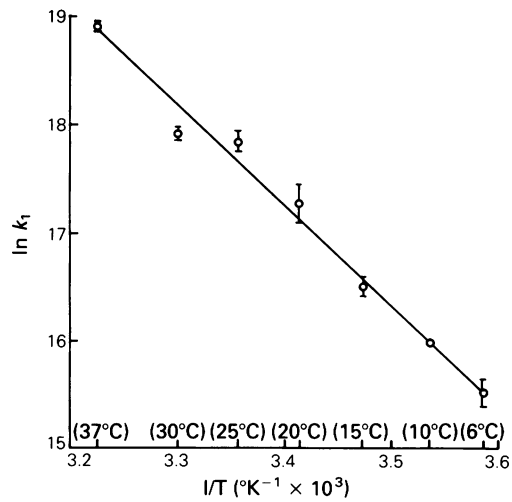

Figure 7 Arrhenius plot of the variation of the rate constant for [^{3}H]-N-methyl-4-methyldiphenhydramine ([^{3}H]-QMDP)-receptor formation, k_1, with temperature. The values of k_1 are taken from Table 1. The best-fit line drawn was obtained by linear regression analysis. Activation energy, E_a, 77 ± 4 kJ mol^{-1}.

but there was no evidence of non-linearity at either temperature (Figure 6).

The plots of k_{on} against [^{3}H-QMDP] yield values both of k_1 (from the slope) and k_{-1} (from the intercept on the ordinate). The value of k_{-1} at 30°C obtained in this way, $5.8 \pm 0.3 \times 10^{-2}$ min^{-1}, was in close agreement with the value obtained from direct measurement of dissociation, $5.4 \pm 0.1 \times 10^{-2}$ min^{-1} (Table 1). At 10°C the error on the intercept of the plot (Figure 6) is much larger, but the value obtained for k_{-1}, $2.6 \pm 1.2 \times 10^{-3}$ min^{-1}, was not significantly different from that obtained by direct measurement, $1.6 \pm 0.1 \times 10^{-3}$ min^{-1} (Table 1). This agreement of the values of k_{-1} from the two methods of measurement is again consistent with the equilibrium between [^{3}H]-QMDP and the H_1-receptor being a simple one-step equilibrium.

Values of k_1 at temperatures other than 30°C and 10°C were calculated using the values of k_{-1} determined directly. The values of k_1 obtained are set out in Table 1. An Arrhenius plot of ln k_1 *versus* 1/T (Figure 7) gave no evidence of any discontinuity in the temperature range 37°C to 6°C. The activation energy, E_a, determined from the slope of this plot was 77 ± 4 kJ mol^{-1}.

Temperature-dependence of K_a *for* [^{3}*H*]*-QMDP*

The ratio of k_1/k_{-1} (values in Table 1) gives an affinity constant of $1.12 \pm 0.08 \times 10^9$ M^{-1} for the binding of [^{3}H]-QMDP to the histamine H_1-receptor at 30°C. This is in close agreement with the value of K_a of $1.14 \pm 0.03 \times 10^9$ M^{-1} from the equilibrium binding of [^{3}H]-QMDP at 30°C (Treherne & Young, 1988) and is similar to the values from (+)-QMDP inhibition of the binding of [^{3}H]-mepyramine, 2.0×10^9 M^{-1} (Hill & Young, 1981) and 0.7×10^9 M^{-1} (Treherne & Young, 1988). However, the difference in the slopes of the Arrhenius plots for k_1 and k_{-1} indicates that the affinity of [^{3}H]-QMDP should increase as the temperature falls. Calculated from the values of k_1 and k_{-1} in Table 1, the ratio k_1/k_{-1} should rise from $0.8 \pm 0.1 \times 10^9$ M^{-1} (mean ± approximate s.e. mean) at 37°C to $6.8 \pm 1.0 \times 10^9$ M^{-1} at 6°C.

There are potential problems in checking these values against affinity constants determined from inhibition of histamine-induced contraction of the guinea-pig ileum. Even at 37°C, $t_{1/2}$ for [^{3}H]-QMDP dissociation is 4 min (Table 1) and complete equilibrium would not be achieved during the normal period of application of the agonist, say 1 min. At lower temperatures it seems even less

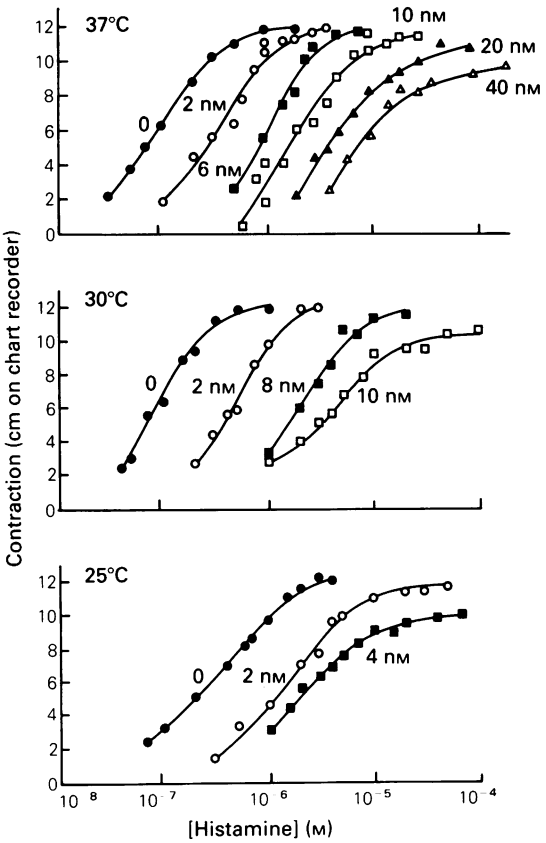

Figure 8 The effect of temperature on (+)-N-methyl-4-methyldiphenhydramine ((+)-QMDP) inhibition of histamine-induced contraction of intestinal smooth muscle. Measurements on longitudinal muscle strips from guinea-pig small intestine were made as described under Methods. Concentration-response curves have been drawn by inspection. (●), Control responses to histamine. Concentrations of (+)-QMDP: 37°C, (○) 2 nM; (■) 6 nM, (□) 10 nM, (▲) 20 nM and (△) 40 nM; 30°C (○) 2 nM, (■) 8 nM and (□) 10 nM; 25°C (○) 2 nM and (■) 4 nM.

likely, on the basis of the values of $t_{1/2}$ in Table 1, that equilibrium could be achieved.

This prediction appears to be borne out by the effect of (+)-QMDP on concentration-response curves for histamine-induced contraction of longitudinal muscle strips from guinea-pig small intestine at 37°C, 30°C and 25°C (Figure 8). The most obvious feature of these traces is that at 37°C concentration-response curves to histamine are shifted to the right in a parallel fashion by (+)-QMDP until at a concentration of 40 nM some depression of the maximum occurs, while at 25°C there is a clear depression of the maximum at low concentrations, 2–4 nM, of (+)-QMDP. However, the pattern of response observed varied between experiments. In 4 of 10 experiments at 30°C, 7–20 nM (+)-QMDP failed to depress the maximum response and this was also the case in 2 experiments (of 5) at 25°C in which 8 and 10 nM (+)-QMDP were used. Nor did flattening of the concentration-response curves, where it was observed, always occur at the same histamine concentration, as the experiments in Figure 8 might suggest. Interpretation of the effect of temperature is further complicated by the observation that lowering the temperature from 37°C to 25°C caused a rightward shift and a decrease in the slope of the concentration-response curve to histamine, but without reducing the maximum contraction obtained. However in 2 experiments in which the concentation-response curve to histamine was shifted to the right in a parallel fashion by 7.5 nM (+)-QMDP at 37°C, subsequent cooling to 25°C produced a decrease in the maximum response, which could be restored by rewarming to 37°C. The flattening was apparently not due to a non-specific action on the smooth muscle, since in experiments in which comparison was made the concentration-response curve to carbachol at 30°C was not affected by 10–40 nM (+)-QMDP.

Table 2 Comparison of affinity constants for (+)-N-methyl-4-methyldiphenhydramine ((+)-QMDP) determined from kinetic constants and from inhibition of histamine-induced contraction of intestinal smooth muscle

Temperature (°C)	*Affinity constant* ($M^{-1} \times 10^{-9}$) *from* k_1/k_{-1}	*Histamine contraction*
37	0.8 ± 0.1	1.2 ± 0.1
30	1.1 ± 0.1	1.8 ± 0.2
25	2.5 ± 0.3	2.8 ± 0.5

Values of k_1/k_{-1} ± approximate s.e. mean were calculated from the values of k_1 and k_{-1} for [^{3}H]-QMDP given in Table 1. Affinity constants ± s.e. mean from the inhibition by (+)-QMDP of histamine-induced contraction of strips of the longitudinal smooth muscle of guinea-pig small intestine were determined from parallel shifts of the log concentration-response curve to histamine in 4 (25°C) or 8 (30°C and 37°C) independent experiments as described under Methods.

In view of the apparent complexity of the effect of temperature, it is notable that when affinity constants were calculated for (+)-QMDP from those curves which showed a parallel shift, the values obtained at 37°C, 30°C and 25°C (Table 2) were similar to the values calculated from the ratio k_1/k_{-1}.

Table 3 Variation of the dissociation rate constant for [^{3}H]-mepyramine with temperature

Temperature (°C)	k_{-1} (min^{-1})	$t_{1/2}$ (min)
37	$7.2 \pm 0.2 \times 10^{-1}$	1
30	$1.8 \pm 0.1 \times 10^{-1}$	4
22	$4.5 \pm 0.3 \times 10^{-2}$	16
15	$1.0 \pm 0.1 \times 10^{-2}$	69
6	$1.7 \pm 0.1 \times 10^{-3}$	415

Values of k_{-1} were obtained from fitting an exponential function (see Methods) to the rate of decline of the promethazine-sensitive binding of [^{3}H]-mepyramine with time, determined from experiments analogous to those shown in Figure 1, but with the non-specific binding of [^{3}H]-mepyramine determined by 2 μM promethazine. Dissociation of bound [^{3}H]-mepyramine was initiated by addition of 1 μM mepyramine. The value at 6°C is the weighted mean ±s.e. mean from 3 independent experiments.

Kinetics of dissociation of [^{3}H]-*mepyramine*

The Arrhenius plot for k_{-1} for [^{3}H]-QMDP (Figure 3) shows no evidence of any discontinuity between 37°C and 6°C. In earlier experiments with [^{3}H]-mepyramine, in which dissociation was induced by dilution, the rate of dissociation at 4°C appeared to be much slower than would have been expected from extrapolation of an Arrhenius plot measured between 37°C and 15°C, raising the possibility of a discontinuity in the plot (Wallace & Young, 1983). In view of the more accurate data obtained in the present series of experiments, in which dissociation was initiated by addition of excess unlabelled ligand rather than by dilution (Figures 1 and 4), we have re-examined the kinetics of dissociation of [^{3}H]-mepyramine induced by 1 μM mepyramine. The values of k_{-1} obtained at temperatures from 37°C to 6°C are set out in Table 3 and presented as an Arrhenius plot in Figure 9. The data obtained by Wallace & Young (1983) using the dilution method are shown in Figure 9 for comparison. The agreement between the two sets of values is good (Figure 9) and confirms that at all temperatures [^{3}H]-mepyramine dissociates from the receptor faster than [^{3}H]-QMDP (compare $t_{1/2}$ values in Tables 1 and 3). However, the Arrhenius plot is linear down to 6°C and provides no evidence for any discontinuity in the properties of [^{3}H]-mepyramine binding to the histamine H_1-receptor.

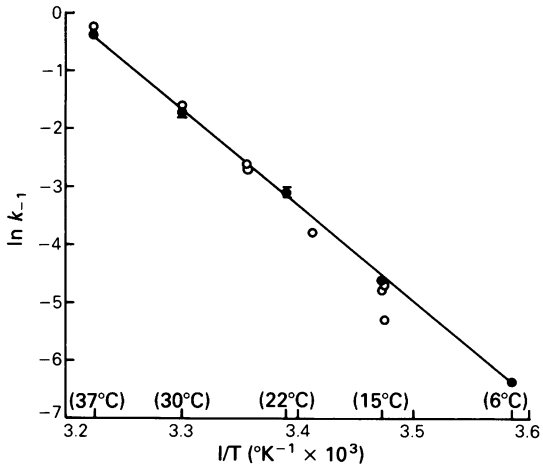

Figure 9 Arrhenius plot of the variation of the rate constant for dissociation of the [^{3}H]-mepyramine-receptor complex with temperature. (●) Values of k_{-1} taken from Table 3. The best-fit line to these points was drawn by linear regression. Activation energy, E_a, 144 ± 3 kJ mol^{-1}. (○) Values of k_{-1} taken from Wallace & Young (1983).

Discussion

It is evident that [^{3}H]-QMDP shows the same marked temperature-dependence of the kinetics of binding to the histamine H_1-receptor as does [^{3}H]-mepyramine. Thus, for [^{3}]-QMDP the $t_{1/2}$ for dissociation increases from 4 min at 37°C to 16 h at 6°C, a 240 fold change. However, a point of particular interest is that at any given temperature the magnitude of k_1 or k_{-1} for [^{3}H]-QMDP is consistently less than the corresponding value for [^{3}H]-mepyramine. Moreover, this difference is not reflected by any marked difference in the activation energies (Table 4). Indeed, if anything the values of E_a for association and dissociation of [^{3}H]-mepyramine are greater than those for [^{3}H]-QMDP. However, the difference is small and it is notable that E_a (dissociation) − E_a(association), equivalent to the enthalpy change for the interaction, is the same, within error, for both ligands (−52 kJ mol^{-1} for [^{3}H]-QMDP and −48 kJ mol^{-1} for [^{3}H]-mepyramine). Given the similar size and, presumably, shape

Table 4 Activation energies, E_a, for ^{3}H-ligand-receptor complex formation and dissociation

^{3}*H-ligand*	E_a (kJ mol^{-1}) k_1	k_{-1}	ΔE_a
[^{3}H]-QMDP	77 ± 4	129 ± 3	−52 ± 5
[^{3}H]-mepyramine	92 ± 9*	140 ± 2	−48 ± 9

* Taken from Wallace & Young (1983).

of [^{3}H]-QMDP and [^{3}H]-mepyramine, this implies that the difference in magnitude of the two rate constants for the two ligands at a given temperature must be due to an entropic term. Thus the association of [^{3}H]-mepyramine with the H_1-receptor is entropy driven to a greater extent than for [^{3}H]-QMDP. This would be consistent, for example, with the two-step model for ligand-protein interaction proposed by Ross & Subramanian (1981), whereby the ligand first becomes partially immobilised in a primarily hydrophobic association step. Whether the membrane phase is involved, in the sense that ligands might first insert in the membrane and then diffuse laterally to the receptor binding site (Rhodes *et al.*, 1985) or whether it is purely a function of the character of the binding site on the receptor remains unknown. Studies of the kinetics of binding to the solubilised receptor might help to clarify this. It is interesting to note that the aspartate residue in the β_2-receptor which is suggested to be involved in binding the amino function of β_2-ligands is located within one of the hydrophobic domains of the protein (Strader *et al.*, 1987). This is not unexpected if the energy gain from the ionic interaction is to be maximised, but it serves to underline the possibility that the whole of the H_1-antagonist binding site could be within some hydrophobic pocket.

In two respects the interaction between [^{3}H]-QMDP and the H_1-receptor, and most probably between [^{3}H]-mepyramine and the receptor, is not as complex as it might be. First, there is no indication that antagonists induce a conformational change in the receptor on binding, such as appears to occur in the interaction of quinuclidinyl benzilate and N-methyl-4-piperidinyl benzilate with the muscarinic receptor (Järv *et al.*, 1979; Schreiber *et al.*, 1985). This conformational change is evidenced most simply by hyperbolic plots of k_{on}, the observed rate constant of complex formation, against ^{3}H-ligand. For [^{3}H]-QMDP such a plot is linear, within experimental error, both at 30°C and at 10°C (Figure 6), although the concentration-range of [^{3}H]-QMDP over which the relationship could be tested was limited. Similar plots for [^{3}H]-mepyramine are also consistent with a linear relationship (Wallace & Young, 1983). This means that the observed activation energy apparently does not include a term for an 'induced-fit'.

The second simplifying feature of the kinetics of both [^{3}H]-QMDP and [^{3}H]-mepyramine is that Arrhenius plots for k_1 and k_{-1} are linear over the temperature range studied. There is no indication of any discontinuity in the region of the membrane-transition temperature, such is observed with α_2 antagonists (Lohse *et al.*, 1986) or of a more complex situation, such as that indiated by curvilinear Arrhenius plots for glucocorticoid binding to the cytosolic receptor (Eliard & Rousseau, 1984). The linearity of the Arrhenius plots for k_{-1} for both [^{3}H]-QMDP and [^{3}H]-mepyramine is particularly clear (Figures 3 and 9). This resolves the uncertainty in the earlier study with [^{3}H]-mepyramine (Wallace & Young, 1983) of whether a discontinuity might have been observed in the plot for k_{-1} if values had been obtained at temperature below 15°C. At the same time it is not clear why the dissociation of [^{3}H]-mepyramine initiated by dilution (Wallace & Young, 1983) was so extremely slow at 4°C, compared to the slow, but measurable, rate at 6°C in the present study, in which dissociation was induced by addition of excess unlabelled antagonist. At other temperatures the rate constants determined by dilution or by addition of excess antagonist compared closely (Figure 9). Similarly, in a limited number of experiments with [^{3}H]-QMDP rate constants measured by the two methods at 30°C and 25°C were in reasonable accord. The same is true of the values of k_{-1} obtained from association data (Figure 6). In the earlier study with [^{3}H]-mepyramine the value of k_{-1} determined directly from measurement of dilution-induced dissociation of the [^{3}H]-ligand-receptor complex was shown to be independent of the temperature at which [^{3}H]-mepyramine was equilibrated with the receptor and depended solely on the temperature of the diluting buffer. Thus incubation at 4°C followed by dilution at 30°C gave the same dissociation kinetics as observed after preliminary incubation at 30°C (Wallace & Young, 1983). Similarly, equilibration at 30°C and dilution into buffer at 4°C gave the same very slow dissociation as observed in experiments conducted at 4°C throughout. It therefore seems that the temperature of the initial incubation to form the ^{3}H-ligand-receptor complex is not the source of the anomaly with [^{3}H]-mepyramine. The very slow rate of formation of the [^{3}H]-QMDP-receptor complex at low temperature (Figure 5) and the extremely long experimental time that would be necessary if complex formation and then dissociation were to be carried out at 6°C have discouraged us from attempting a similar experiment with [^{3}H]-QMDP. Consequently, for dissociation measurements the preliminary incubation was carried out at a standard temperature of 30°C and the complex then cooled or warmed to the temperature at which dissociation was to be measured. There is no indication that this procedure influences the dissociation kinetics of [^{3}H]-QMDP any more than those of [^{3}H]-mepyramine. In particular, the value of k_{-1} at 10°C determined in this way was in reasonable agreement with the value from the variation of the observed rate constant, k_{on}, with [^{3}H-QMDP] (Figure 6), where measurements were made at 10°C throughout. The consistency of the kinetic behaviour of [^{3}H]-QMDP, including the mono-

phasic dissociation plots (Figure 2), gives no indication of a slow conformational change at any temperature.

Slow equilibration can pose a number of problems. This is well illustrated by the action of [^{3}H]-QMDP on intestinal smooth muscle (Figure 8). Even at 30°C, k_{-1} for [^{3}H]-QMDP is 4 min (Table 1) and implies that complete equilibration between (+)-QMDP and histamine will not have been achieved in the time, normally <1 min, that histamine was present. At 30°C it is clear, given a $t_{1/2}$ of 13 min for [^{3}H]-QMDP, that the parallel shifts of the concentration-response curve represent the action of an effectively irreversible antagonist on a tissue with an H_1-receptor reserve. In these circumstances a value of the affinity can be determined from parallel shifts (Rang, 1966), but strictly this is only accurate if there is an appreciable receptor reserve remaining (Rang, 1966). At 25°C there is a further complication, in that the concentration-response curve for histamine is shallower than at 37°C, and even low concentrations of (+)-QMDP cause a decrease in the maximum response obtainable. This might mean that the apparent receptor reserve is less at lower temperatures. All of this discussion assumes, of course, that the kinetics of antagonist-H_1-receptor interaction are the same in cerebellum and intestine. What the observations do make clear is that there are pitfalls in the use of antagonists, or the determination of their equilibrium affinities, without a knowledge of the kinetics of their action. With very slow antagonists, such as iodobolpyramine (Korner *et al.*, 1986), the problems of obtaining meaningful data from organ bath experiments may be obvious (Blakemore *et al.*, 1987), but for other compounds the difficulties may be less overt. The apparent change in the mode of action of promethazine as an H_1-antagonist on the guinea-pig ileum as the temperature is lowered can be explained most simply on the basis of its kinetic properties (Wallace & Young, 1983; Cook *et al.*, 1985).

No detailed kinetic studies have been carried out on H_1-antagonists other than [^{3}H]-QMDP and [^{3}H]-mepyramine and consequently it is uncertain to what extent their properties are shared by other compounds. It is clear that both [^{3}H]-doxepin (Tran *et al.*, 1981) and [^{125}I]-iodobolpyramine (Korner *et al.*, 1986) show temperature-dependent kinetics, but preliminary data suggests that others, such as tripelenamine may dissociate from the receptor within 60 min at 4°C (Wallace & Young, 1983). The very slow kinetics of very hydrophobic antagonists such as astemizole (Laduron *et al.*, 1982) and iodobolpyramine, an 'extended' mepyramine (Korner *et al.*, 1986), even at 25–30°C, is of particular interest in view of the indication from the present study that an initial hydrophobic interaction may play a significant role in the kinetics of binding of [^{3}H]-mepyramine. This merits further investigation.

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