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Temperature Dependence of the Binding of [^{3}H]Mepyramine and Related Compounds to the Histamine H_1 Receptor

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SUMMARY

The extent of the promethazine-sensitive binding of [^{3}H]mepyramine to a washed fraction from guinea pig cerebellum was little altered between 4° and 30°. The dissociation of [^{3}H]mepyramine from the H_1 receptor was markedly temperature-sensitive. An Arrhenius plot of the variation of the dissociation rate constant, k_{-1}, with temperature was linear over the range 37°–15° (E_a = 160 kJ mole^{-1}). The association rate constant, k_1, was also temperature-dependent, and an Arrhenius plot approximated well to a straight line between 30° and 15° (E_a = 112 kJ mole^{-1}). The linear relationship may hold down to 4°. A consequence of the slow dissociation at 4° is that the IC_{50} for mepyramine inhibition of promethazine-sensitive [^{3}H]mepyramine binding at 4° is independent of the concentration of [^{3}H]mepyramine if the cerebellar homogenate is first incubated with the nonradioactive antagonist before addition of the ^{3}H-ligand. Promethazine binding showed a temperature dependence similar to that of mepyramine, and for both antagonists and for chlorpromazine the affinity constant was not greatly increased (equal to or less than a factor of 2) at 4° compared with 30°. Tripelennamine showed a more rapid dissociation at 4° than the other antagonists. Mequitazine dissociated slowly at 4°, but the affinity constant was lower at 4° than at 30°.

INTRODUCTION

There are indications in the literature that the interaction of antagonists with the histamine H_1 receptor is sensitive to changes in temperature. Changes in the affinity of H_1 antagonists have been inferred from changes in the potency of antagonists as inhibitors of the histamine-induced contraction of guinea pig intestinal smooth muscle. Originally it was proposed that there was a temperature-dependent interconversion of H_1 and H_2 receptors (1), but later data were not all consistent with this simple model (2). At temperatures below 20° the antagonism produced by promethazine appeared to become noncompetitive and irreversible, whereas tripelennamine remained competitive. In seeming contrast to these observations is the report that the rate of recovery of the histamine-induced contraction of guinea pig ileum from blockade by diphenhydramine could be increased by briefly reducing the temperature of the bathing solution to 4° (3). However, the effects of temperature on tissue response may be complex, and it is not easy to establish that changes in antagonist action are the result of a change in the drug-receptor interaction.

A much more direct approach to the temperature dependence of the antagonist-receptor interaction is offered by measurement of ^{3}H-ligand binding. No attempt has been made to make any extensive study of this kind for the H_1 receptor, but there are observations in the literature which suggest that changes in temperature could have a marked effect on ^{3}H-ligand-receptor interaction. The affinity of [^{3}H]mepyramine binding to H_1 receptors in tissue sections from guinea pig brain changes little between 4° and 30°, but the kinetics of binding seem to be slowed considerably at the lower temperature, since under these conditions [^{3}H]mepyramine can be used successfully for the autoradiographic localization of H_1 receptors (4). [^{3}H]Doxepin binding to guinea pig brain membranes seems to show similar properties, since the dissociation from the H_1 receptor is much slower at 25° than at 37° (5).

Marked changes in the rate at which ligands equilibrate with the receptor can have important implications for the interpretation of binding data, and in view of this we have made a more detailed investigation of the effect of temperature on the binding of [^{3}H]mepyramine to histamine H_1 receptors in guinea pig cerebellum. A preliminary report of some of these results has been made to the British Pharmacological Society (6).

METHODS

Preparation of washed cerebellar homogenate. Cerebella from two guinea pigs (Hartley strain, males) were homogenized in 5 volumes of 50 mM sodium-potassium phosphate buffer (37.8 mM Na_2HPO_4, 12.2 mM KH_2PO_4),

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pH 7.5, in a Teflon-glass homogenizer and then centrifuged at 6000 × *g* for 20 min. The pellet was resuspended in phosphate buffer and centrifuged at 8700 × *g* for 1 min in a Beckman Microfuge B. The resulting pellet was resuspended in 50 mM phosphate buffer to give a protein concentration, measured by the method of Lowry *et al.* (7), of 10–15 mg/ml and stored at −20° until required. Where binding was to be measured in Krebs-phosphate solution, pH 7.2 [containing (millimolar) NaCl, 133; KCl, 4.7; $MgSO_4$, 1.2; $CaCl_2$, 2.5; Na_2HPO_4, 5; and glucose, 5.5], the final pellet was resuspended in 5 mM phosphate buffer, pH 7.5. There was no obvious decrease in the receptor-specific binding of [^{3}H]mepyramine after storage for a period of up to 2 months.

A washed homogenate of the longitudinal muscle from guinea pig small intestine was prepared as described previously (8).

Measurement of [^{3}H]mepyramine binding. The composition of the incubation mixtures containing [^{3}H]mepyramine and washed tissue homogenate are given in the figure legends or in the following section on the measurement of rate constants. In early experiments, bound [^{3}H]mepyramine was measured using a microcentrifugation assay essentially as described earlier (8). In the majority of experiments the incubation mixture was filtered rapidly through Whatman GF/B glass-fiber filters under vacuum and the filters were washed twice with 4 ml of ice-cold medium (normally 50 mM phosphate buffer, pH 7.5) containing 1 μM mepyramine. Scintillator (10 ml), toluene/ethoxyethanol/2-[1,1′-biphenyl]-4-yl-5-[4-(1,1-dimethylethyl)phenyl]-1,3,4-oxadiazole (70:30:0.6, v/v/w), or toluene/emulsifier mixture No. 1 (Fisons)/water/2-[1,1′-biphenyl]-4-yl-5-[4-(1,1-dimethylethyl) phenyl]-1,3,4-oxadiazole (70:30:10:0.6,v/v/v/w) was added and the filters were allowed to stand overnight in the latter scintillator or disintegrated by vigorous shaking in the former. Tritium was measured by liquid scintillation counting, and counts per minute were converted into distintegrations per minute by the channels-ratio method using an external radioactive source.

Evidence has been presented in earlier studies (8, 9) that the binding of [^{3}H]mepyramine sensitive to inhibition by 2 μM promethazine can be equated with binding to histamine H_1-receptors.

Measurement of rate constants. If mepyramine interacts with the receptor according to the equilibrium

$$D + R \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} DR$$

then, at any time t, for complex formation

$$DR_t = DR_\infty[1 - \exp(-k_{on} \cdot t)]$$

where

$$k_{on} = k_1[D] + k_{-1}$$

For dissociation of the drug-receptor complex following removal of the free drug

$$DR_t = DR_0 \exp(-k_{-1} \cdot t)$$

Dissociation of receptor-bound [^{3}H]mepyramine was induced by 100-fold dilution (20-fold in early experiments) into buffer with or without added nonradioactive ligands. Details are given in the legends to Figs. 1 and 2. The rate constant, k_{-1}, was determined from the slope of a plot of log ([^{3}H]mepyramine bound) versus time.

For determinations of k_1, measurements were first made of the variation of [^{3}H]mepyramine bound with time following addition of the ^{3}H-ligand. Incubations in 50 mM phosphate buffer, pH 7.5 (total volume 15 or 20 ml), contained 0.1–2.5 nM [^{3}H]mepyramine, cerebellar homogenate (0.24–0.41 mg of protein per milliliter) with or without 2 μM promethazine. Samples (1 ml) were removed at intervals and filtered through Whatman GF/B filters as described above. The time course of the receptor-specific binding was obtained by subtraction of the curves in the presence and absence of promethazine and k_{on} ± estimated standard error determined by fitting these data to the equation $B_t = B_\infty[1 - \exp(-k_{on} \cdot t)]$, where B_t and B_∞ are the amounts of [^{3}H]mepyramine bound at times t and ∞. The best-fit values of B_∞ and k_{on} were obtained by nonlinear regression analysis using a modified Marquardt procedure as implemented in the Harwell Library routine VB01A on the Cambridge IBM 370/165. Each point was weighted by the reciprocal of the variance associated with it. The rate constant for complex formation, k_1, was determined by weighted linear regression analysis of a plot of k_{on} versus [[^{3}H]mepyramine].

Analysis of curves of inhibition of [^{3}H]mepyramine binding. Curves of percentage of uninhibited binding of [^{3}H]mepyramine versus concentration of inhibitor (15 points/curve) were fitted to a Hill equation:

$$\%\ \text{Uninhibited binding of [}^3\text{H]mepyramine} = \frac{100 - NS}{[([A]/IC_{50})^n + 1]} + NS$$

where n is the Hill coefficient, $[A]$ is the concentration of inhibitor, IC_{50} is the concentration of inhibitor required for 50% inhibition of the inhibitor-sensitive binding, and NS is the percentage of the inhibitor-insensitive binding. The best-fit values ± estimated standard error of n, IC_{50}, and NS were obtained using the routine VB01A (see above). Each point was weighted by the reciprocal of the variance associated with it.

If equilibration occurs between [^{3}H]mepyramine and inhibitor, the affinity constant of the inhibitor, K_a, is related to the IC_{50} by the relationship $K_a = ([[^3H]\text{mepyramine}] \cdot K_{mep} + 1)/IC_{50}$, where K_{mep} is the affinity constant for [^{3}H]mepyramine.

Drugs. [^{3}H]Mepyramine (24.1 Ci/mmole) was obtained from the Radiochemical Centre, Amersham. Mepyramine maleate and promethazine hydrochloride were purchased from May & Baker, and chlorpromazine hydrochloride was obtained from Sigma Chemical Company. Gifts of mequitazine (Berk Pharmaceuticals) and tripelennamine (CIBA-Geigy) are gratefully acknowledged.

RESULTS

Amount of H_1 receptor binding. The amount of promethazine-sensitive binding of [^{3}H]mepyramine to cerebellar homogenates varied somewhat between preparations from different batches of guinea pigs, but there was no obvious difference in the extent of binding at temper-

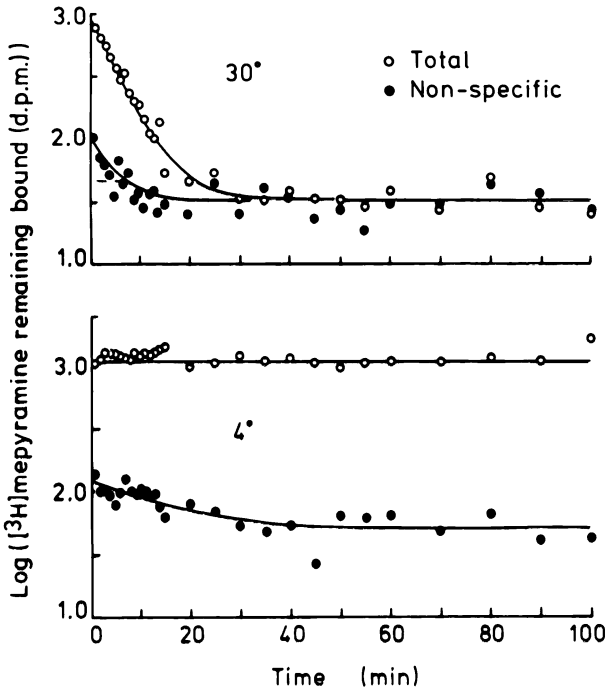

FIG. 1. *Kinetics of dissociation of [^{3}H]mepyramine from cerebellar homogenates at 4° and 30°*

[^{3}H]Mepyramine (nominal free concentration 5 nM) and cerebellar homogenate (6.5 mg of protein) in 50 mM Na/K-phosphate buffer, pH 7.5 (total volume 2.0 ml), were incubated for 60 min at 4° or 30°. At the end of this period the mixture was diluted in buffer (38 ml), at the same temperature, containing 2 μM promethazine. Samples (1 ml) were taken at intervals, and bound [^{3}H]mepyramine was determined by a filtration assay as described under Methods. The time course of nonspecific binding of [^{3}H]mepyramine (●) was determined by having 2 μM promethazine present in the initial incubation. Note that the scale of the ordinate is logarithmic. The value of k_{-1} at 30° was 2.0×10^{-1} min^{-1}.

atures between 4° and 37°. In three independent experiments in which the binding of 5 nM [^{3}H]mepyramine was measured in the presence and absence of 2 μM promethazine at 4° and 30° on the same homogenate, the promethazine-sensitive binding at 30° was 84%, 88%, and 90% of that at 4°. The expected value, if the maximal possible binding were the same, is 98% (using the affinity constants in Table 4).

Temperature dependence of [^{3}H]mepyramine dissociation from histamine H_1 receptors. The rate of dissociation of [^{3}H]mepyramine from cerebellar H_1 receptors was markedly temperature-dependent. At 30° dissociation was reasonably fast, but at 4° there was no measurable dissociation over a 2-hr period (Fig. 1). In this experiment the initial [^{3}H]mepyramine/homogenate incubation mixture was diluted 20-fold into buffer containing 2 μM promethazine, but essentially the same pattern was observed when dissociation was initiated solely by 100-fold dilution. In one experiment in which samples were taken up to 6 hr after dilution at 4° there was no obvious dissociation of the bound [^{3}H]mepyramine. A similar result was obtained when a washed homogenate of the longitudinal muscle from guinea pig small intestine was used in place of the cerebellar preparation.

The temperature dependence of the rate constant for dissociation of the [^{3}H]mepyramine-receptor complex, k_{-1}, is shown as an Arrhenius plot in Fig. 2. The very slow dissociation at lower temperatures limits the range over which accurate measurement can be made, but between 37° and 15° the plot is clearly linear. In the majority of experiments dissociation was initiated by dilution into a 100-fold excess of buffer, but the rate constants were not appreciably altered if the diluting buffer contained 2 μM mepyramine, 2 μM promethazine, or 0.1 mM histamine (Fig. 2). Similarly, k_{-1} at 30° and 15° was not significantly altered if the initial incubation and 100-fold dilution were made in Krebs-phosphate medium rather than 50 mM phosphate buffer. The activation energy of the dissociation of the [^{3}H]mepyramine-receptor complex, taken from the slope of the Arrhenius plot, was 160 kJ mole^{-1} (38 kcal mole^{-1}).

The rate of dissociation of receptor-bound [^{3}H]mepyramine depended solely on the temperature of the diluting buffer. Thus if the [^{3}H]mepyramine-receptor complex was allowed to form at 30° and then diluted 100-fold into buffer at 4°, the dissociation over a 120-min period was negligible. Similarly, initial incubation at 4° followed by dilution into buffer at 30° yielded the kinetics of dissociation (▲, Fig. 2) previously observed at 30°.

Temperature dependence of the rate constant for [^{3}H]mepyramine-receptor complex formation. If there is a simple equilibrium between [^{3}H]mepyramine and the receptor, then the over-all rate constant for complex

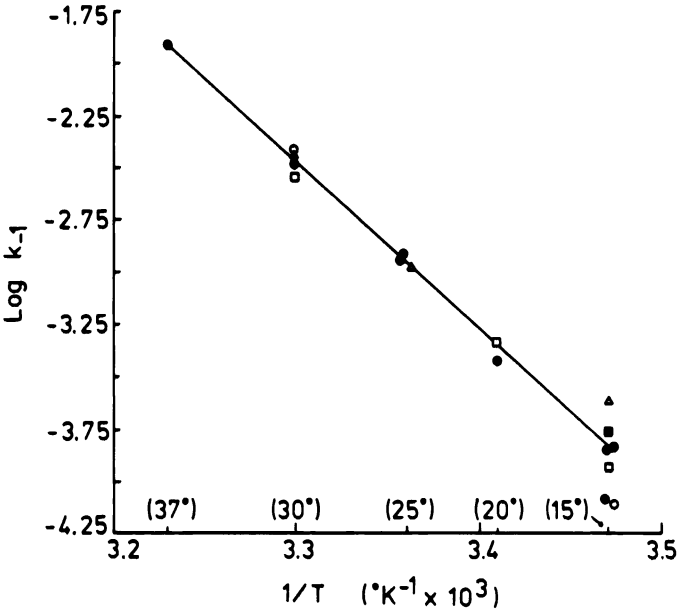

FIG. 2. *Arrhenius plot of the variation of the rate constant for dissociation of the [^{3}H]mepyramine-receptor complex, k_{-1}, with temperature*

Measurements were made as described in the legend to Fig. 1, except that dilution was into 198 ml of 50 mM phosphate buffer containing: ●, no additions; ■, 2 μM mepyramine; □, 2 μM promethazine; or △, 0.1 mM histamine. Correspondingly larger samples (5 ml) were applied to the GF/B filters. The dissociation rate constant, k_{-1}, units min^{-1}, was determined from the slope of a plot of log (promethazine-sensitive [^{3}H]mepyramine binding) against time. Other experimental conditions: ▲, initial incubation at 4° followed by dilution into buffer at 30°; ○, initial incubation and dilution in Krebs-phosphate medium.

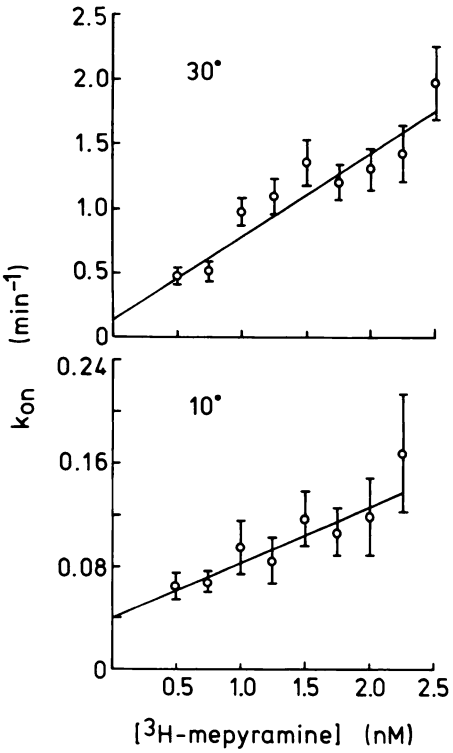

FIG. 3. *Concentration dependence of the overall-rate constant for [^{3}H]mepyramine-receptor complex formation, k_{on}, at 30° and 10°*

Each point represents the best-fit value of k_{on} ± estimated standard error obtained from weighted nonlinear regression analysis of curves of the variation with time of the promethazine-sensitive binding of [^{3}H]mepyramine to a cerebellar homogenate (see Methods). The lines drawn were obtained by weighted linear regression. Note the different scales on the ordinates of the two graphs.

formation, k_{on}, is given by

$$k_{on} = k_1 \cdot [D] + k_{-1}$$

(see Methods). If this model holds, then at any given temperature a plot of k_{on} against [[^{3}H]mepyramine] should be linear, with slope k_1 and intercept k_{-1}. At all temperatures the promethazine-insensitive binding of [^{3}H]mepyramine reached its maximal value within 1 min and thereafter remained constant. In contrast, the rate of equilibration of the total binding, and hence of the promethazine-sensitive binding, depended both on the temperature and on the concentration of [^{3}H]mepyramine. Representative plots of k_{on} against [[^{3}H]mepyramine] at 30° and 10° are shown in Fig. 3. The experimental error is greater than that in the simpler dissociation experiments, but the data are fitted reasonably well by a linear relationship. The same was true at the other temperatures.

The variation of k_1, obtained from the slope of plots such as those in Fig. 3, with temperature in the range 30°–4°, is shown in the form of an Arrhenius plot in Fig. 4. At 37° the rate of complex formation was too fast for accurate measurement. Again the data are fitted reasonably well by a single straight line, equivalent to an activation energy of 92 kJ mole^{-1} (22 kcal mole^{-1}), but the possibility that there might be a change of slope at lower temperatures cannot be excluded. If only the first six points are considered (30°–15°) the slope of the

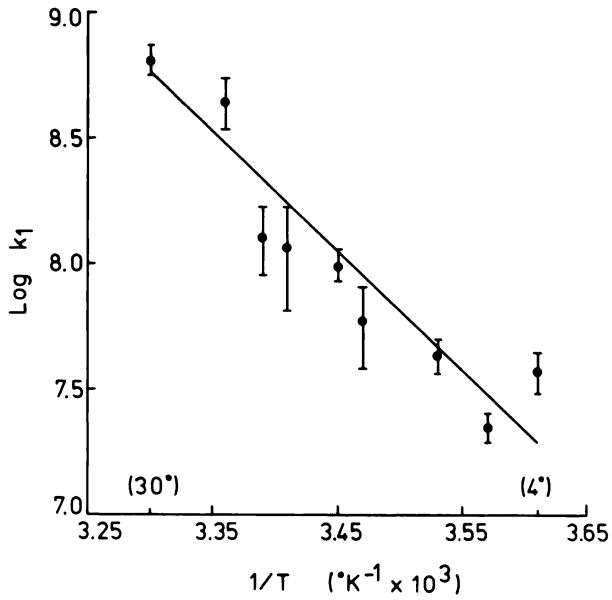

FIG. 4. *Arrhenius plot of the variation of the rate constant for [^{3}H]mepyramine-receptor complex formation, k_1, with temperature*

Each point (± standard error) represents the best-fit value, determined by weighted linear regression, of the slope of a plot of the variation of k_{on} with the concentration of [^{3}H]mepyramine at a given temperature (see Fig. 3). The line drawn was calculated from weighted linear regression analysis. The units of k_{on} are min^{-1}.

weighted best-fit line increases, giving E_a = 112 kJ mole^{-1} (27 kcal mole^{-1}).

The plots of k_{on} against [[^{3}H]mepyramine] (e.g., Fig. 3) contain further information in that they yield values of both k_1 and k_{-1}, and consequently the affinity constant at the various temperatures can be obtained from the ratio k_1/k_{-1} (Table 1). This is not the best experimental method for determining the affinity, since both k_1 and k_{-1} have an appreciable uncertainty associated with them and consequently the error of the ratio is large. However, it is clear that the affinity constant determined in this way alters very little between 30° and 4°.

Determination of the affinity constant for mepyramine from inhibition curves. A consequence of the very slow dissociation of mepyramine from the H_1 receptor at low temperatures is that the relationship between the affinity constant and the IC_{50} obtained from curves of mepyramine inhibition of [^{3}H]mepyramine binding will depend

TABLE 1

Affinity constants for [^{3}H]mepyramine derived from the kinetics of complex formation

The rate constants, k_1 and k_{-1}, were determined from plots of the observed rate constant for complex formation, k_{on}, against the concentration of [^{3}H]mepyramine, as described under Methods.

Temperature	k_1/k_{-1}
	M^{-1}
4°	$4.2 \pm 2.2 \times 10^9$
7	$6.5 \pm 1.3 \times 10^8$
10	$1.1 \pm 0.3 \times 10^9$
15	$3.3 \pm 1.1 \times 10^8$
17	$2.9 \pm 1.4 \times 10^9$
20	$1.2 \pm 1.0 \times 10^9$
22	$1.1 \pm 0.5 \times 10^9$
25	$1.4 \pm 0.8 \times 10^9$
30	$4.7 \pm 3.0 \times 10^9$

TABLE 2

Effect of temperature on the IC_{50} for the inhibition of [^{3}H] mepyramine binding by mepyramine

Cerebellar homogenate (0.31 or 0.37 mg of protein) and nonradioactive mepyramine were incubated for 1 hr on ice in 50 mM phosphate buffer (1 ml), pH 7.5, before the addition of 0.5 or 3 nM [^{3}H]mepyramine (5 or 30 μl); the incubation then was continued for 1 hr at 4° or 30°. The incubation was terminated by the addition of 4 ml of ice-cold buffer containing 1 μM mepyramine, and the bound [^{3}H]mepyramine was separated by filtration as described under Methods. The IC_{50} values were obtained from nonlinear regression analysis (see Methods) of inhibition data from 15 concentrations of mepyramine in each experiment.

[[^{3}H]Mepyramine]	IC_{50}	
	4°	30°
nM	*nM*	
0.5	0.47 ± 0.05	1.18 ± 0.17
3	0.40 ± 0.05	4.19 ± 0.30

on the experimental protocol employed. At low temperature, if nonradiolabeled mepyramine is first incubated with the homogenate and [^{3}H]mepyramine is added subsequently, no true equilibration occurs in the usual period allowed for binding of the ^{3}H-ligand (60 min). This is illustrated by the experimental data in Table 2.

At 4° the IC_{50} values are independent of the concentration of [^{3}H]mepyramine used, whereas at 30° equilibration occurs and the inhibition curves are shifted to the right along the concentration axis to an extent dependent on the ^{3}H-ligand concentration. In a separate experiment at 30° in which nonradioactive mepyramine and 1.85 nM [^{3}H]mepyramine were added together, the IC_{50} was 2.4 ± 0.4 nM, giving an affinity constant of 1.8×10^9 M^{-1}, consistent (within experimental error) with the values determined from the data at 30° in Table 2. At 4° the mean affinity for mepyramine ($K_a \equiv 1/IC_{50}$) was 2.2×10^9 M^{-1}. This small change in affinity is consistent with the values obtained from the ratio k_1/k_{-1} (Table 1).

Temperature dependence of the interaction of other antagonists with the H_1 receptor. Quantitative measurement of the dissociation of nonradiolabeled antagonists is much less easily made than for ^{3}H-labeled compounds, but it is possible to obtain a qualitative measure of whether the rate of dissociation is very slow or relatively fast. Antagonist is allowed to bind to the receptor and then diluted into buffer at the required temperature. After a set time the incubation mixture is cooled to 4° and the free receptor fraction is measured with [^{3}H] mepyramine. If the dissociation of the unlabeled antagonist at 4° is appreciable, then this approach can yield only the information that it is relatively fast at this and higher temperatures. However, if the dissociation of the unlabeled antagonist is very slow at 4°, then a measure can be obtained of the extent of dissociation at higher temperatures without the complication of competition between the antagonist and [^{3}H]mepyramine during the final incubation period. The approach is illustrated by the results from three series of experiments (Table 3). The high level of nonspecific binding reflects the necessity of filtering a relatively large volume of undiluted [^{3}H]mepyramine (1 nM) and compares with approximately 10–15% promethazine-insensitive binding in experiments in which the incubation mixture was diluted 5-fold with ice-cold mepyramine-containing buffer prior to filtration (see legend to Table 2).

The data for mepyramine (Table 3) are consistent with the temperature sensitivity of the dissociation of the [^{3}H] mepyramine-receptor complex described above. However, the interpretation of the dissociation data for the other antagonists requires some knowledge of the equilibrium constant at low temperature; otherwise the expected occupancy cannot be calculated. A measure of the affinity at 4° for the antagonists studied was obtained from inhibition of [^{3}H]mepyramine binding using a protocol similar to that used for mepyramine; i.e., the antagonist was first equilibrated with the cerebellar homogenate before addition of the ^{3}H-ligand (see legend to Table 2). However, correct calculation of the affinity constant from the IC_{50} value requires a knowledge of the dissociation kinetics, so that the two sets of experiments, to obtain a measure of dissociation and to determine the affinity, are interdependent. The possible error in the affinity constant is reduced by using a reasonably low concentration of [^{3}H]mepyramine, since the values calculated from the IC_{50} assuming no equilibration with [^{3}H] mepyramine or complete equilibration differ by a factor of [[^{3}H]mepyramine]: $K_{mep} + 1$. For 0.5 nM [^{3}H]mepyramine at 4° the factor is 2.1, so that even in the worst case there should not be a major misinterpretation of the dissociation data in Table 3. The values obtained for the

TABLE 3

Effect of temperature on the dissociation of antagonists from the H_1 receptor

Cerebellar homogenate (0.28–0.37 mg of protein per milliliter) and antagonist were incubated in 50 mM phosphate buffer, pH 7.5 (200 μl), at room temperature (approximately 22°) for 60 min. Four aliquots (40 μl each) were then diluted in buffer (3.96 ml) at 4°, 15°, or 30° and allowed to stand for 60 min before cooling to 4° (if at 15° or 30°) and addition of [^{3}H]mepyramine (final concentration 1 nM). After incubation for an additional 60 min, 40 μl of 0.1 mM mepyramine (final concentration 1 μM) were added to each tube, and the bound [^{3}H] mepyramine was separated by filtration, as described under Methods. Nonspecific binding was assessed in incubations containing 5 μM promethazine (50 nM after dilution, giving an occupancy at 4° of 98%).

Antagonist (concentration before dilution)	% Uninhibited binding of [^{3}H]mepyramine after dilution at		
	4°	15°	30°
Mepyramine (10 nM)	58 ± 2 (59)[a]	76 ± 3	98 ± 4 (92)[b]
Promethazine (20 nM)	60 ± 3 (59)[a]	68 ± 2	88 ± 2 (91)[b]
Promethazine (5 μM)	52 ± 2	64 ± 2	57 ± 5
Chlorpromazine (5 nM)	89 ± 3 (69)[a]	—	100 ± 2 (98)[b]
Chlorpromazine (10 nM)	82 ± 2 (65)[a]	—	96 ± 2 (96)[b]
Chlorpromazine (50 nM)	70 ± 3 (62)[a]	—	75 ± 2 (79)[b]
Promethazine (5 μM)	61 ± 3	—	44 ± 2
Tripelennamine (10 nM)	96 ± 3	98 ± 4	109 ± 3
Tripelennamine (20 nM)	93 ± 3	98 ± 4	105 ± 4
Tripelennamine (50 nM)	90 ± 3	98 ± 3	106 ± 3
Promethazine (5 μM)	72 ± 2	50 ± 2	53 ± 2

[a] The figure in parentheses is the value expected if the dissociation from the receptor at 4° is very slow and assumes that the affinity at 22° can be obtained from the values at 4° and 30° by linear interpolation.

[b] Expected value if equilibration with receptor is fast at 30° and very slow at 4°.

TABLE 4

Binding affinities of H_1 antagonists at 4° and 30°

Affinity constants are derived from IC_{50} values for the inhibition of the binding of [^{3}H]mepyramine. Cerebellar homogenate (0.28–0.37 mg of protein per milliliter) and antagonist in 50 mM phosphate buffer, pH 7.5 (1 ml), were incubated on ice for at least 2 hr to allow equilibration. [^{3}H]Mepyramine (0.5 nM final concentration) was then added, and incubation was continued for 60 min. The incubation was terminated by the addition of 4 ml of ice-cold buffer containing 1 μM mepyramine; the bound [^{3}H]mepyramine then was separated by filtration as described under Methods. Affinity constants at the low temperature were calculated assuming very slow dissociation of the antagonist from the receptor, whereas those at 30° were derived assuming equilibration between the antagonist and [^{3}H]mepyramine.

Antagonist	K_a	
	4°	30°
	M^{-1}	
Chlorpromazine	$(9.5 \times 10^8)^a$	7.6×10^8
Mepyramine	2.2×10^9	1.7×10^9
Mequitazine	2.9×10^8	1.7×10^9
Promethazine	1.3×10^9	7.8×10^8
Tripelennamine	$(2.3 \times 10^9)^b$	4.2×10^8

[a] May be an underestimate, since chlorpromazine appears to undergo some dissociation from the receptor over 60 min at 4° (see Table 3 and text).

[b] Appears to equilibrate with the receptor much faster than mepyramine at 4°. The affinity is calculated assuming equilibration.

affinity constants at 4° and 30° are set out in Table 4. The values at 30° are in good agreement with previous determinations or measurements on the guinea pig ileum (8, 10). (K_a for mequitazine from inhibition of histamine-induced contraction of guinea pig ileum, 1.0×10^9 M^{-1}).

It is evident that the properties of promethazine binding are similar to those of mepyramine (Tables 3 and 4). At 4° dissociation is very slow, but it is relativley fast at 30°. The limited dissociation at 15° suggests that the rate constant is even less than that for mepyramine. In contrast, tripelennamine has largely or completely dissociated from the receptor in the course of the incubation at 4°. This complicates calculation of the expected occupancy of [^{3}H]mepyramine (see Discussion). Chlorpromazine also appears to undergo significant dissociation at 4°, and similar results were obtained in a second experiment. Dissociation at 30° appeared to be complete, as with all of the other antagonists studied. Mequitazine did not dissociate from the receptor to any significant extent at 4° (data not shown), but—alone of the antagonists—the affinity constant at this temperature appears to be appreciably lower than at 30° (Table 4). Measurement of the time course of the development of the inhibition produced by 1 nM and 3 nM mequitazine demonstrated that this was not an artifact of incomplete equilibration, and a second determination of the affinity yielded a value similar to that in Table 4.

DISCUSSION

The results presented here make clear that the binding of [^{3}H]mepyramine to the histamine H_1 receptor is strongly temperature-dependent. The change in the binding affinity between 4° and 30° is modest, but there is a great change in the magnitude of the rate constants for association and dissociation. It is also clear from the limited survey made of other H_1 antagonists that these properties are not restricted to mepyramine. Similarly, evidence in the literature indicates that they are not peculiar to the histamine H_1 receptor. A closely similar temperature dependence of the interaction between estradiol and the uterine cytosol receptor was reported over 10 years ago (11), and more recently the same marked effect on the rate constants has been noted for the digitonin-solubilized muscarinic acetylcholine receptor from brain (12, 13). The binding affinities of muscarinic antagonists are little altered by changes in temperature (14). However, how exact an analogy can be drawn between the H_1 and muscarinic receptors is uncertain. Evidence has been presented (summarized in ref. 15) that the equilibrium between antagonists and the muscarinic receptor cannot be described adequately by the equation $A + R \rightleftharpoons AR$, and it has been suggested that the initial antagonist-receptor complex may undergo a conformational change. The evidence is based on the poor agreement between affinity constants determined from equilibrium measurements and from the ratio of the association and dissociation rate constants (e.g., see ref. 12) and on the nonlinearity of plots of the observed rate constant for complex formation, k_{on}, against the concentration of [^{3}H]antagonist (16).

We have attempted to test whether the simple model describes adequately the interaction of [^{3}H]mepyramine with the H_1 receptor. The smaller number of H_1 receptors and the propensity of [^{3}H]mepyramine to bind to non-receptor sites at high concentrations make it difficult to achieve the same accuracy as for the muscarinic receptor or to test for linearity of plots over as wide a range of antagonist concentrations. Nonetheless, the ratio k_1/k_{-1} (Table 1) gives a good estimate of the value of K_a measured at "equilibrium" (Table 4). Similarly, plots of k_{on} against the concentration of [^{3}H]mepyramine approximate well to linearity within the accuracy of our measurements. However, any firm judgment of the acceptability of the simple model must be deferred until more exact measurements can be made. This applies also to direct measurement of the dissociation rate constant below 15°. At 4° we have little indication of any dissociation of [^{3}H]mepyramine at all, and it seems to be slower than would have been predicted from extrapolation of the Arrhenius plot (Fig. 2) or from the on-rate measurements (Fig. 4). However, at temperatures where a direct comparison could be made between the values of k_{-1} determined from measurement of dissociation and from measurement of the over-all on-rate we were unable to detect any significant difference. It may be revealing when it becomes possible to make accurate comparison at lower temperatures.

The limitations of our measurements of the temperature dependence of the interaction of nonradiolabeled ligands with the H_1 receptor are also apparent. However, it is clear that promethazine behaves much like mepyramine, whereas tripelennamine, which differs structurally from mepyramine only in the lack of a p—OCH_3 group on the benzyl residue, dissociates much more readily at low temperature. This is sufficient to provide a simple kinetic explanation for the observation (2) that promethazine inhibition of histamine-induced contraction of guinea pig ileum appears to become noncompeti-

tive at temperatures below 20°, while tripelennamine remains competitive.

Even allowing for the kinetic complexities it is clear that no simple generalization can be made on the effect of temperature on the affinity constant. Whereas the affinities of mepyramine, promethazine, chlorpromazine, and tripelennamine increased (modestly in the case of chlorpromazine) as the temperature fell, that of mequitazine, a very hydrophobic phenothiazine derivative (17), appeared to fall. A much more extensive investigation will be necessary to try to establish the molecular basis for this effect, but, as with the temperature sensitivity of the rate constants, it seems very likely that hydrophobic interactions between drug and receptor will prove to play a key role.

The kinetic consequences of very slow equilibration of drug and receptor and the complications introduced into the interpretation of inhibition curves have been elaborated very clearly by Rodbard and his colleagues in the estradiol study (11). Their conclusions have recently been confirmed and extended (18, 19). If equilibration is neither negligible nor complete, exact analysis is complex, and this would apply in particular to the tripelennamine/[^{3}H]mepyramine experiments in Table 4. The situations in which no equilibration or full equilibration occurs are relatively straightforward to evaluate and are exemplified by mepyramine inhibition of [^{3}H]mepyramine binding (Table 2). The important point is that "equilibrium" experiments can only be interpreted correctly when the approximate magnitude of the rate constants is known.

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