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# $H_2$ Histaminic Receptors in Rat Cerebral Cortex. 1. Binding of [$^3$H]Histamine†

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ABSTRACT: Saturable binding of [$^3$H]histamine in equilibrium with homogenates of rat cerebral cortex reveals Hill coefficients between 0.4 and 1.0, depending upon the conditions. Data from individual experiments are well described assuming one or two classes of sites. Only the sites of higher affinity ($K_{P1}$ = 3.9 ± 0.5 nM) are observed when binding is measured by isotopic dilution at a low concentration of the radioligand (<1.5 nM) in the presence of magnesium or by varying the concentration of the radioligand. The sites of lower affinity ($K_{P2}$ = 221 ± 26 nM) appear during isotopic dilution at higher concentrations of the radioligand or at lower concentrations either upon the addition of guanylyl imidodiphosphate (GMP-PNP) or upon the removal of magnesium. Estimates of the second- and first-order rate constants for association and dissociation of [$^3$H]histamine agree well with $K_{P1}$. Apparent capacities corresponding to $K_{P1}$ and $K_{P2}$ are of the order of 100 ($[R_1]_t$) and 1300 pmol/g of protein ($[R_2]_t$), respectively. Simple interconversion cannot account for the changes in binding that occur upon adding GMP-PNP or removing magnesium, since the increase in $[R_2]_t$ exceeds the decrease in $[R_1]_t$. Moreover, the apparent amount of high-affinity complex exhibits a biphasic dependence on the concentration of [$^3$H]histamine; an increase at low concentrations is offset by a decrease that occurs at higher concentrations. The latter appears to be positively cooperative and concomitant with formation of the low-affinity complex. These and other observations indicate that the binding of histamine is inconsistent with models commonly invoked to rationalize the binding of agonists to neurohumoral receptors. GMP-PNP and magnesium reciprocally alter capacity at the sites of higher affinity, however, and the reduction caused by GMP-PNP reflects a substantial increase in the rate constant for dissociation at the sites that appear to be lost. The sites labeled by [$^3$H]histamine thus reveal the properties of neurohumoral receptors linked to a nucleotide-specific G/F protein.

Saturable binding of [$^3$H]histamine first was reported by Palacios et al. (1978) in homogenates prepared from various regions of rat brain. Their observations subsequently were confirmed by ourselves (Kandel et al., 1980) and extended by Barbin et al. (1980). Apart from the report of Singh & McGeer (1979), there has been general agreement that the sites in mammalian brain bind histamine with an apparent dissociation constant of 7–9 nM and a capacity of the order of 100 pmol/g of protein. Comparisons of pharmacological specificity (Barbin et al., 1980; Kandel et al., 1980) and capacity (Kandel et al., 1980) have established that they are distinct from $H_1$ receptors labeled by [$^3$H]mepyramine (Hill et al., 1977; Tran et al., 1978) and from an imidazole-specific site labeled by [$^3$H]cimetidine (Burkard, 1978; Smith et al.,

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1980). Barbin et al. (1980) have presented evidence that the sites are not associated with storage or transport systems or related to histamine *N*-methyltransferase. They further have demonstrated that the regional and subcellular distribution, postnatal changes, the sensitivity to kainic acid and to lesion of the medial forebrain bundle, and the sensitivity to GTP all tend to identify the sites as postsynaptic receptors for histamine. All drugs that inhibit the specific binding of [$^3$H]-histamine ultimately have been reported to do so with Hill coefficients indistinguishable from one (Barbin et al., 1980), although the preliminary report of Palacios et al. (1978) implied that values were somewhat lower for $H_1$ and $H_2$ antagonists. While apparent affinities reported for a small number of $H_2$ agonists bear some resemblance to their $H_2$ pharmacological potencies, agreement has been poor for the $H_2$ antagonists cimetidine and metiamide. In the absence of a recognized, pharmacological specificity, the identity of the sites has remained a mystery. The present report describes a further examination of the saturable binding of [$^3$H]histamine in homogenates of rat cerebral cortex, with particular attention to the behavior of histamine itself. The results indicate that the sites interact with a G/F protein in the manner characteristic of cyclase-linked receptors and suggest that the binding of histamine is a cooperative process. Evidence presented in the following papers identifies the sites as $H_2$ histaminic receptors (Steinberg et al., 1985a,b). A preliminary report of this work has appeared elsewhere (Wells et al., 1985).

## Materials and Methods

*Histaminic Drugs and Other Chemicals.* [$^3$H]Histamine was obtained from Amersham Corp. (40–54 Ci/mmol) and New England Nuclear (32.2 Ci/mmol) and unlabeled histamine from Sigma. Early samples of the unlabeled material were purified by sublimation, but the inhibitory activity of the compound was unchanged, and the practice was discontinued. Ranitidine was kindly donated by Glaxo Canada Ltd., Toronto, and dimaprit by Smith Kline and French Research Ltd., Welwyn Garden City, U.K. Guanylyl imidodiphosphate (GMP-PNP)[1] and AMP-PNP were purchased from Boehringer-Mannheim. Tris was obtained as the free base from Sigma and EDTA as the free acid from British Drug Houses; buffer solutions were adjusted to the appropriate pH with sulfuric acid. All other chemicals were reagent grade or better and were used without further purification.

*Preparation of Tissue.* Male Wistar rats were obtained from Charles River Canada Inc. and starved overnight prior to decapitation. Cerebral cortices were removed and homogenized in ice-cold sucrose (0.32 M, 6 mL/cortex) using a Potter-Elvehjem tissue blender. The homogenate was centrifuged at 900*g* and 4 °C for 10 min; the supernatant fraction in turn was centrifuged at 113000*g* and 4 °C for 30 min to yield a pellet that was stored at −80 °C until required. The crude material was washed extensively prior to use in the binding assays, as this increased the fraction of total binding inhibitable by unlabeled histamine and seemed to improve the reproducibility of the data. Thawed pellets were suspended in buffer (50 mM Tris and 10 mM $MgCl_2$, pH 7.48; 20 mg of protein/mL), incubated for 30 min at 37 °C, and diluted to about 2 mg of protein/mL with ice-cold buffer (50 mM Tris and 1 mM EDTA, pH 7.48) prior to centrifugation at 125000*g* and 4 °C for 20 min. The pellets from this step then were washed 6 times by resuspension in ice-cold buffer (50 mM Tris and 1 mM EDTA, pH 7.48; 2 mg of protein/mL) and centrifugation at 125000*g* and 4 °C for 20 min. Washed pellets were stored at −80 °C until required for the binding assays.

*Binding Assays.* Washed pellets were suspended at pH 7.48 in buffer (100 mM Tris) containing 1 mM EDTA and, when required, 10 mM $MgCl_2$. The final concentration of protein was 0.5–1.0 mg/mL in most experiments but was as high as 2.0 mg/mL in some; protein was measured according to Lowry et al. (1951) with bovine serum albumin as the standard. Homogenates were kept in an ice bath until required for the binding assay. All measurements were carried out at 30 °C, and homogenates were preincubated at that temperature for 15 min prior to the addition of histamine or other ligands. The initial concentrations of all ligands exceeded the final concentrations in the reaction mixture by at least 100-fold, thereby ensuring that changes in the concentration of protein were negligible.

For kinetic studies on the stability or time dependence of binding, reaction mixtures were prepared in glass containers as described in the legend to the appropriate figure. Quadruplicate aliquots (0.5 or 1.0 mL) then were removed from time to time, transferred to polypropylene tubes (0.5- or 1.5-mL capacity), centrifuged for 2 min at about 9000*g* (Beckman microfuge B), and processed further as described below. The duration of centrifugation was chosen as the minimum that would yield a firm pellet; periods exceeding 4 min appeared to permit the net movement of radioligand between the pellet and the supernatant fraction when binding was measured prior to the attainment of equilibrium. Estimates of $k_{off}$ were about 20% larger, for example, after centrifugation for 5 min rather than for 2. Nonspecific binding in kinetic studies was estimated in a parallel experiment prepared from the same homogenate and solution of [$^3$H]histamine but in which unlabeled histamine was added at the same time as the radioligand to yield a final concentration of 1.0 mM. The total concentration of the radioligand was measured in 100-$\mu$L aliquots taken from both reaction mixtures. Observed levels of nonspecific binding then were corrected for any difference assuming a linear relationship between nonspecific binding and the total concentration of [$^3$H]histamine.

For studies at equilibrium, aliquots (0.5 or 1.0 mL) of the preincubated homogenate were added to polypropylene centrifuge tubes containing [$^3$H]histamine and any other ligands dissolved in deionized water (5 or 10 $\mu$L). There has been no evidence for any interaction between the radioligand and the plastic. The suspension then was mixed on a vortex mixer, incubated at 30 °C for 45 min, and centrifuged for 5 min (Beckman microfuge B). The duration of centrifugation was without effect on total binding when the reaction was at equilibrium. Following centrifugation, pellets were superficially washed 3 times with 0.1 M NaCl and dissolved overnight at 40 °C in a commercial solubilizer (NCS, Amersham). All measurements were performed in quintuplicate. Each experiment included an estimate of nonspecific binding, taken as the level of binding in the presence of 1.0 mM unlabeled histamine.

*Assay of Radioactivity.* Radioactivity was assayed in a nonaqueous cocktail (Ready-Solv NA, Beckman) to which glacial acetic acid had been added (4 mL/L of cocktail) in order to prevent chemiluminescence. All samples were counted twice for 5 min in a liquid scintillation spectrometer (Beckman Model LS9100, LS7500, or LS7800), and individual rates of disintegration were determined by using an external standard. Replicate samples and duplicate counts then were averaged

[1] Abbreviations: GMP-PNP, guanylyl imidodiphosphate; AMP-PNP, adenylyl imidodiphosphate; Tris, tris(hydroxymethyl)aminomethane; EDTA, ethylenediaminetetraacetic acid.

to obtain the mean value used in subsequent analyses. Standard errors of the mean for four or five replicates counted twice generally were less than 1% and thus fall within the area of the symbols in subsequent figures.

*Analysis of Data.* All analyses were performed with total binding per unit volume of reaction mixture taken as the dependent variable. Bound radioligand generally is presented as picomoles per gram of protein or as a percentage, and only that component inhibitable by saturating concentrations of unlabeled histamine is illustrated in most figures. Absolute levels reported for inhibitable, or specific, binding have been corrected where necessary for the small difference between nonspecific binding in the absence of an unlabeled ligand and total binding in the presence of a saturating concentration of an unlabeled ligand (Seeman et al., 1984). This correction is implicit with equations in which capacity is an explicit parameter.

The time dependence of binding was analyzed by assuming a reversible interaction between the radioligand (P) and the receptor (R) as shown below; $k_{off}$ and $k_{on}$ represent the first- and second-order rate constants for the reverse and forward reactions, respectively.

$$P + R \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} PR$$

The integrated expression for the increase in total binding ($B_{obsd}$) with time ($t$) when the initial concentration of the complex (PR) is zero is given by eq 1 (Moelwyn-Hughes,

$$B_{obsd} = \frac{[P]_t[R]_t}{\frac{1}{2}([P]_t + [R]_t + K_P) + \beta k_{on} t} + C([P]_t - [PR]) \quad (1)$$

1971); $C$ represents the fraction of free radioligand that appears as nonspecific binding and was found to be independent of time, $K_P = k_{off}/k_{on}$, and $\beta^2 = {}^1/_4([P]_t + [R]_t + K_P)^2 - [P]_t[R]_t$. In this and other expressions, the subscript $t$ is used to indicate that the total, as opposed to the free, concentration of ligand or receptor is the relevant quantity with respect to the model. Decreases in total binding with respect to time have been described by eq 2, in which $B_{max}$ and $B_{min}$ are the values

$$B_{obsd} = (B_{max} - B_{min})e^{-kt} + B_{min} \quad (2)$$

of $B_{obsd}$ at $t = 0$ and infinite time, respectively, and $k$ is the first-order rate constant. The quantity $B_{max} - B_{min}$ generally was a negligible fraction of the total radioligand.

Equation 3 has been used to provide empirical descriptions

$$B_{obsd} = (B_{max} - B_{min}) \sum_{S=1}^{n} \left( \frac{F_S K_S^{n_{HS}}}{K_S^{n_{HS}} + [A]^{n_{HS}}} \right) + B_{min} \quad (3)$$

of data from experiments in which binding at equilibrium was measured over a range of concentrations of an unlabeled drug ($A$). $B_{max}$ and $B_{min}$ represent total binding of the radioligand at [A] = 0 and [A] $\rightarrow \infty$, respectively. Inhibitable binding ($B_{max} - B_{min}$) consists of $n$ components, each constituting a fraction $F_S$ where $S = 1, 2, ..., n$; $K_S$ and $n_{HS}$ represent the inhibitory potency (i.e., $IC_{50}$) and the Hill coefficient of A with respect to component $S$. Equation 4 describes the special case of eq 3 in which $n_H$ is one for all components.

$$B_{obsd} = (B_{max} - B_{min}) \sum_{S=1}^{n} \left( \frac{F_S K_S}{K_S + [A]} \right) + B_{min} \quad (4)$$

Data acquired at equilibrium also were analyzed in terms of a model in which the radioligand (P) and the unlabeled drug (A) bind to a mixture of noninterconverting sites as follows:

$$PR_S \underset{[P]/K_{PS}}{\overset{}{\rightleftharpoons}} R_S \underset{}{\overset{[A]/K_{AS}}{\rightleftharpoons}} AR_S$$

Sites of type $S$ ($S = 1, 2, ..., n$) bind P and A with dissociation constants $K_{PS}$ and $K_{AS}$, respectively, and constitute the fraction $F_S$ of all sites. Total binding of the radioligand ($B_{obsd}$) is described by eq 5, in which $C$ is the fraction of free radioligand

$$B_{obsd} = [R]_t \sum_{S=1}^{n} \left[ \frac{F_S}{1 + \frac{K_{PS}}{[P]_t - [PR]_t} + \frac{K_{PS}([A]_t - [AR]_t)}{K_{AS}([P]_t - [PR]_t)}} \right] + C([P]_t - [PR]_t) \quad (5)$$

that appears as nonspecific binding and where $[R]_t = \sum_{S=1}^{n}[R_S]_t$, $F_S = [R_S]_t/[R]_t$, $[PR_S] = [R_S]_t[P]/([P] + K_{PS})$, $[AR_S] = [R_S]_t[A]/([A] + K_{AS})$, $[PR]_t = \sum_{S=1}^{n}[PR_S]$, $[AR]_t = \sum_{S=1}^{n}[AR_S]$, $[P]_t = [P] + [PR]_t$, and $[A]_t = [A] + [AR]_t$. A special case of eq 5 arises when P and A are the radiolabeled and unlabeled analogues of the same compound. $K_{PS}$ then can be taken as equal to $K_{AS}$, and total binding is described by eq 6. Equations 5 and 6 were solved numerically.

$$B_{obsd} = [R]_t \sum_{S=1}^{n} \left[ \frac{F_S}{1 + \frac{K_{PS}}{[P]_t - [PR]_t} + \frac{[A]_t - [AR]_t}{[P]_t - [PR]_t}} \right] + C([P]_t - [PR]_t) \quad (6)$$

*Statistical Procedures.* All functions were fitted to experimental data by using an iterative procedure based on the nonlinear, least-squares algorithm of Marquardt (1963). The data were weighted according to the nature of the experiment. For kinetic studies, and for studies at equilibrium on the inhibition of [$^3$H]histamine by unlabeled ligands, standard errors were an essentially constant percentage of the mean, and the data were weighted accordingly. This approximation is not valid, however, at the lowest concentrations of [$^3$H]histamine in experiments where the concentration of the radioligand is varied. Since all samples were counted to a constant time, and not to a constant error, the counting error is relatively high and predominates under such conditions. The measured error on replicate determinations thus tends to be a higher percentage of the mean value than at higher concentrations of the radioligand. Data from experiments involving concentrations of [$^3$H]histamine below 0.5 nM therefore were weighted according to the measured error on each point.

Statistical variation is indicated throughout by the standard error. Where the results of two or more experiments are involved, the data from each generally were analyzed independently according to the particular equation; the mean values of each parameter are shown, and the standard errors thus reflect variability from experiment to experiment. Where only a single experiment or a single analysis of more than one experiment is involved, parametric errors reflect the fit of the model to the data.

Equations 3 and 4 generally were fitted by allowing the program to select for optimal values of $n_{HS}$ (eq 3 only), $K_S$, $F_S$, $B_{max}$, and $B_{min}$; variable parameters in eq 5 and 6 were $K_{AS}$ (eq 5 only), $K_{PS}$, $F_S$, $[R]_t$, and $C$. In most cases, the values obtained for $B_{max}$ and $B_{min}$ are not presented per se but have been used to calculate levels of specific binding. The results of different experiments have been compared in some instances by simultaneous analysis of two or more sets of data, with some

parameters common to more than one experiment. Except where indicated otherwise, individual sets of data were assigned separate values for $B_{max}$ and $B_{min}$ (eq 3 and 4) or for $[R]_t$ and $C$ (eq 5 and 6). Further details regarding the selection of variable parameters are described where appropriate.

The fit of individual expressions has been assessed by testing the significance of the $t$ statistic for the correlation coefficient of neighboring residuals (Reich et al., 1972). The fits of different models, or of the same model with different sets of parameters, were compared by testing the significance of the $F$ statistic for the difference in the variance of residuals (Snedecor & Cochran, 1967). All such tests were performed on weighted residuals. Levels of significance were calculated according to reported algorithms for the $t$ statistic (Adams, 1969; Hill, 1970) and for the $F$ statistic (Hill & Joyce, 1967; Bruning & Kintz, 1977). Values of $P$ for certain tests are distinguished by subscripts as follows: $P_0$, the difference between two means ($t$ statistic); $P_1$, the correlation of neighboring residuals for the best fit of eq 4 ($n = 1$) or of eq 3 ($n = 1$) with $n_{H1}$ fixed at 1 ($t$ statistic); $P_2$, the difference in the variance of residuals between best fits of eq 3 ($n = 1$) with $n_{H1}$ fixed at 1 and $n_{H1}$ variable ($F$ statistic); $P_3$, the difference in the variance of residuals between best fits of eq 4 with $n = 1$ and $n = 2$ ($F$ statistic); $P_4$, the difference in the variance of residuals between best fits of eq 4 to replicate experiments taken individually and to all replicates taken together with one or more parameters in common ($F$ statistic); $P_5$, the difference in the variance of residuals between best fits of eq 4 ($n = 1$) with five variable parameters ($K_1$, $K_2$, $F_2$, $B_{max}$, and $B_{min}$) and with four variable parameters, the fifth ($K_1$, $K_2$, or $F_2$) having been fixed at an arbitrary value. Other tests are described where appropriate in the text.

## Results

### *Kinetic Studies*

Total binding of 1.3 nM [$^3$H]histamine to homogenates of rat cerebral cortex is maximal within about 30 min (Figure 1) and remains unchanged for up to 6 h thereafter; total binding in the presence of 1 mM unlabeled histamine is independent of time over the same period. For the example illustrated in Figure 1, the data can be described assuming that the inhibitable component of binding arises from a reversible, bimolecular reaction between the radioligand and a single population of sites (eq 1). The mean value of $k_{on}$ from three experiments is 0.89 ± 0.15 $\mu M^{-1}$ $s^{-1}$ for an equilibrium dissociation constant ($K_P$) of 3.9 nM; the latter value was determined independently as described below. Agreement between the data and the model was not uniformly comparable to that in Figure 1, however, suggesting that the bimolecular process implicit in eq 1 may be overly simplistic.

The long-term stability observed in the presence of [$^3$H]-histamine is not found when the homogenate is incubated in the absence of the radioligand. As illustrated in Figure 1 (inset), the capability to bind [$^3$H]histamine falls off with time, and the decrease derives entirely from that component inhibitable by the unlabeled ligand. The decrease is slow, however, and characterized by a half-time of about 600 min if one assumes an exponential process (eq 2) that ultimately reduces specific binding to zero. Less than 2% of the specific binding is lost during the 15-min period of incubation that precedes the addition of histaminic ligands in most experiments.

The net dissociation of [$^3$H]histamine from the particulate fraction is well described by a single exponential (eq 2) for the first 20 min following the addition of excess, unlabeled histamine to suspensions previously equilibrated with low

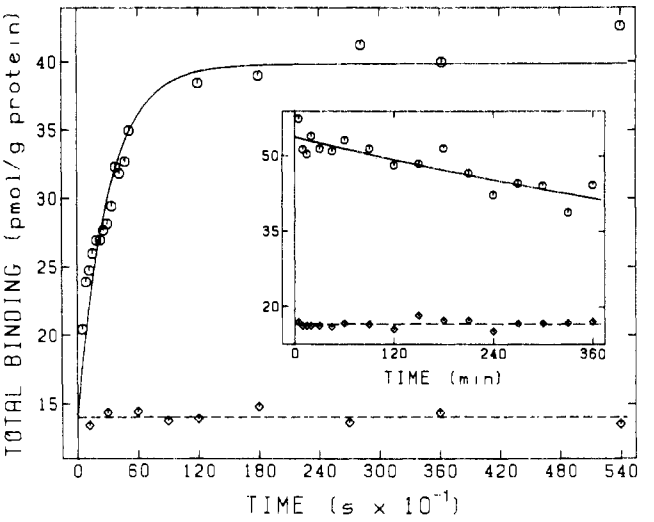


FIGURE 1: Time dependence of the binding of [$^3$H]histamine. Homogenized tissue (0.87 mg of protein/mL) was incubated at 30 °C for 15 min and then added to [$^3$H]histamine either alone (○) or together with unlabeled histamine (◇); final concentrations of the radioligand and the unlabeled analogue were 1.27 nM and 1.0 mM, respectively. Total binding was measured in aliquots removed at the times shown on the abscissa and represented less than 3% of the total radioligand in all samples. The solid line represents the best fit of eq 1 to the data acquired in the absence of unlabeled histamine. $K_P$ was set to 3.9 nM, and $C$ was set to yield an intercept of 14.0 pmol/g of protein (see below); parametric values obtained by regression are as follows: $k_{on} = 0.62 \pm 0.06$ $\mu M^{-1}$ $s^{-1}$; $[R]_t = 132 \pm 5$ pmol/g of protein. (Inset) Homogenates were transferred from an ice bath to a water bath at 30 °C; aliquots then were removed at the times shown on the abscissa and added to [$^3$H]histamine either alone (○) or together with unlabeled histamine (◇) to yield final concentrations of 1.40 nM and 1.0 mM, respectively. Total binding was measured after incubation for 45 min at 30 °C. The solid line represents the best fit of eq 2 to the data acquired in the absence of unlabeled histamine, with $B_{min}$ set to 16.5 pmol/g of protein (see below); parametric values obtained by regression are as follows: $B_{max} = 53.8 \pm 1.0$ pmol/g of protein; $k = 0.0011 \pm 0.0002$ $min^{-1}$. The dashed line in each experiment represents the best fit of a straight line with a slope of zero to the data for nonspecific binding and corresponds to 14.0 ± 0.1 (outer frame) or 16.5 ± 0.2 pmol/g of protein (inset). In each case, the assumption of a finite slope yielded a value indistinguishable from zero and a negligible decrease ($P > 0.60$) in the variance of residuals.

concentrations (<1.4 nM) of the radioligand (Figure 2). Moreover, the mean value of 0.0046 ± 0.0004 $s^{-1}$ obtained for $k_{off}$ (Table I) compares favorably with the value of 0.0035 $s^{-1}$ calculated from the independent estimates of $k_{on}$ (0.89 $\mu M^{-1}$ $s^{-1}$) and $K_P$ (3.9 nM). In each experiment, however, the asymptotic level of binding predicted by the best fit of eq 2 always exceeded the level attained when the radioligand and excess, unlabeled histamine were added simultaneously to an aliquot of the same suspension. The discrepancy corresponds to 10–35% of inhibitable binding and is illustrated further by the increase in the variance of residuals ($P < 0.0005$) that occurs when the data are refitted with the asymptote ($B_{min}$) fixed at the value obtained upon simultaneous addition of labeled and unlabeled histamine. It therefore appears that the rate constant of dissociation is much less than 0.0046 $s^{-1}$ at a minor fraction of the sites labeled by the radioligand.

The dissociation of [$^3$H]histamine apparently is not controlled by the histaminic drug used to block the forward reaction. As indicated in Table I, the rate constant is essentially the same irrespective of whether the unlabeled drug is histamine, the $H_2$ agonist dimaprit, or the $H_2$ antagonist ranitidine. In contrast, there is a 3.5-fold increase ($P_0 = 0.00063$) in the apparent rate constant when unlabeled histamine is added together with excess GMP-PNP (Table I and Figure 2). The nucleotide-mediated loss of binding reported initially by Barbin

Table I: Comparison of First-Order Rate Constants for the Net Dissociation of [$^3$H]Histamine[a]

| condition | unlabeled ligand(s) added with [$^3$H]histamine | unlabeled ligand(s) added after equilibration for 45 min | $k_{off}$ ($s^{-1}$) | $P_0$ for comparison with condition 5 | 4 | 3 | 2 |
|---|---|---|---|---|---|---|---|
| 1 | | 1 mM histamine | 0.0046 ± 0.0004 (4) | 0.47 | 0.00063 | 0.24 | 0.33 |
| 2 | | 1 mM ranitidine | 0.0052 ± 0.0005 (6) | 0.90 | 0.00031 | 0.070 | |
| 3 | | 1 mM dimaprit | 0.0056 ± 0.0006 (4) | 0.82 | 0.0013 | | |
| 4 | | 1 mM histamine + 0.1 mM GMP-PNP | 0.016 ± 0.002 (4) | 0.0066 | | | |
| 5 | 0.1 mM GMP-PNP | 1 mM histamine | 0.0060 ± 0.0018 (4) | | | | |

[a] Suspensions (0.56–0.97 mg of protein/mL) preincubated for 15 min at 30 °C were mixed with [$^3$H]histamine and, when required, with GMP-PNP to yield final concentrations of 1.0–1.4 nM and 0.1 mM for the radioligand and the nucleotide, respectively. The incubation was continued for 45 min, and net dissociation of the radioligand then was initiated by the inclusion of unlabeled ligand(s) at the concentrations shown. Aliquots subsequently were removed at different times to assay for binding, and eq 2 was fitted to the data to obtain values for $k_{off}$. Further details are given in the legend to Figure 2. The number of independent experiments is indicated in parentheses.

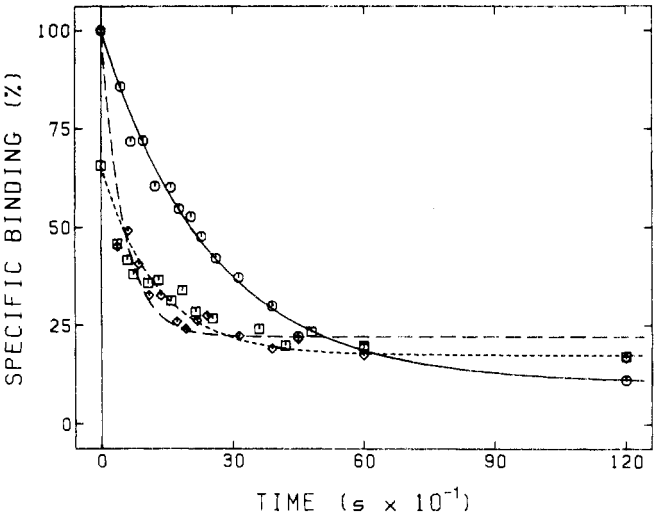


FIGURE 2: Dissociation of [$^3$H]histamine in the absence and presence of GMP-PNP. Homogenized tissue (0.86–0.91 mg of protein/mL) was incubated at 30 °C for 15 min; [$^3$H]histamine then was added either alone (○, ◇) or together with 10 mM GMP-PNP (□) to yield final concentrations of 1.27–1.35 nM for the radioligand and 0.1 mM for the nucleotide. Following incubation for 45 min, aliquots were removed to assay for binding, and unlabeled histamine was added either alone (○, □) or together with GMP-PNP (◇) to yield final concentrations of 1.0 mM for histamine and 0.1 mM for the nucleotide. Total binding subsequently was measured in aliquots removed at the times shown on the abscissa. The lines represent best fits of eq 2 to the experimental data; $B_{max}$ was fixed in each case at the value measured immediately prior to the addition of unlabeled histamine. Parametric values obtained by regression are as follows: in the absence of GMP-PNP (—○—), $k_{off}$ = 0.0040 ± 0.0002 $s^{-1}$ and $B_{min}$ = 22.6 ± 0.3 pmol/g of protein; for GMP-PNP added with unlabeled histamine (--◇--), $k_{off}$ = 0.020 ± 0.002 $s^{-1}$ and $B_{min}$ = 21.5 ± 0.3 pmol/g of protein; for GMP-PNP added with the radioligand (···□···), $k_{off}$ = 0.0080 ± 0.0013 $s^{-1}$ and $B_{min}$ = 21.6 ± 0.3 pmol/g of protein. Zero percent on the ordinate is taken as the level of binding in control experiments containing 1.0 mM unlabeled histamine added at the same time as the radioligand; absolute values are 18.9 (○), 17.8 (◇), and 16.5 pmol/g of protein (□). One hundred percent on the ordinate is taken as $B_{max}$ for the experiment without GMP-PNP (54.3 pmol/g of protein) and for the experiment in which GMP-PNP was added with unlabeled histamine (35.4 pmol/g of protein). For the experiment in which GMP-PNP was added with the radioligand, 100% is taken as the level of binding after 45 min (45.3 pmol/g of protein) in a control sample from which the nucleotide was omitted; $B_{max}$ in the presence of the nucleotide was 35.2 pmol/g of protein.

et al. (1980) thus arises from a noncompetitive effect on the rate of dissociation. GMP-PNP reduces but does not eliminate binding at equilibrium (Barbin et al., 1980); it thus is possible to add the nucleotide with the radioligand rather than with unlabeled histamine and thereby to measure the rate of dissociation for binding that coexists with the nucleotide. The rate constant determined in this manner is not significantly different from that determined in the absence of GMP-PNP ($P_0$ = 0.47; Table I), suggesting that the nucleotide acts selectively on a subpopulation of the sites. Moreover, the data are almost superimposable with those obtained when unlabeled histamine and GMP-PNP are added simultaneously (Figure 2). The agreement further suggests that the apparent increase in $k_{off}$ from 0.0046 to 0.016 $s^{-1}$ at all sites in fact derives entirely from a much larger increase at a nucleotide-sensitive subpopulation.

### *Studies at Equilibrium*

*Binding of Histamine.* Total binding of [$^3$H]histamine at various concentrations of the radioligand is illustrated in Figure 3A. A portion of the binding can be inhibited by 1 mM unlabeled histamine to reveal a nonspecific component that increases linearly with the total concentration of radioligand. The inhibitable, or specific, component is illustrated in Figure 3B and can be described assuming that [$^3$H]histamine binds to a uniform population of mutually independent sites (eq 6, $n$ = 1). Estimates of the capacity from four experiments vary from 65 to 217 pmol/g of protein, with a mean value of 114 ± 35 pmol/g of protein; the mean dissociation constant is 4.0 ± 0.6 nM (log $K_{P1}$ = −8.40 ± 0.07, Table II). Similar results are obtained when a low concentration of the radioligand is diluted isotopically with increasing concentrations of the unlabeled analogue (Figure 4). Among seven experiments performed at 1.0–1.5 nM [$^3$H]histamine, only one revealed a significant correlation of neighboring residuals ($P_1$ < 0.05) for the best fit of eq 3 ($n$ = 1) with $n_{H1}$ fixed at 1 (Table II). Moreover, individual estimates of the Hill coefficient generally are indistinguishable from 1, as indicated by the absence of a significant decrease in the variance of residuals ($P_2$ > 0.15) when the value of $n_{H1}$ is determined by regression. The mean Hill coefficient is 0.96 ± 0.03 (Table II), and the data thus are well described assuming a single population of sites. The mean value of $K_{P1}$ obtained from eq 6 ($n$ = 1) is 3.8 ± 0.8 nM (log $K_{P1}$ = −8.42 ± 0.09; Table II). The affinity of histamine determined by isotopic dilution thus is virtually identical with that determined by varying the concentration of the radioligand. A combination of results from the 11 experiments summarized in Table II yields a mean value for $K_{P1}$ of 3.90 ± 0.53 nM (log $K_{P1}$ = −8.409 ± 0.059). Equation 6 also can be used to estimate the capacity from data obtained by isotopic dilution, and values range from 40 to 207 pmol/g of protein for the seven experiments summarized in Table II. Owing to the low concentrations of [$^3$H]histamine relative to $K_{P1}$, however, $[R_1]_t$ and $K_{P1}$ are highly correlated in individual experiments. This dependence has been avoided by estimating $[R_1]_t$ according to eq 5 ($n$ = 1) with $K_{P1}$ fixed at the mean value of 3.9 nM. Capacities estimated in this way tend to show

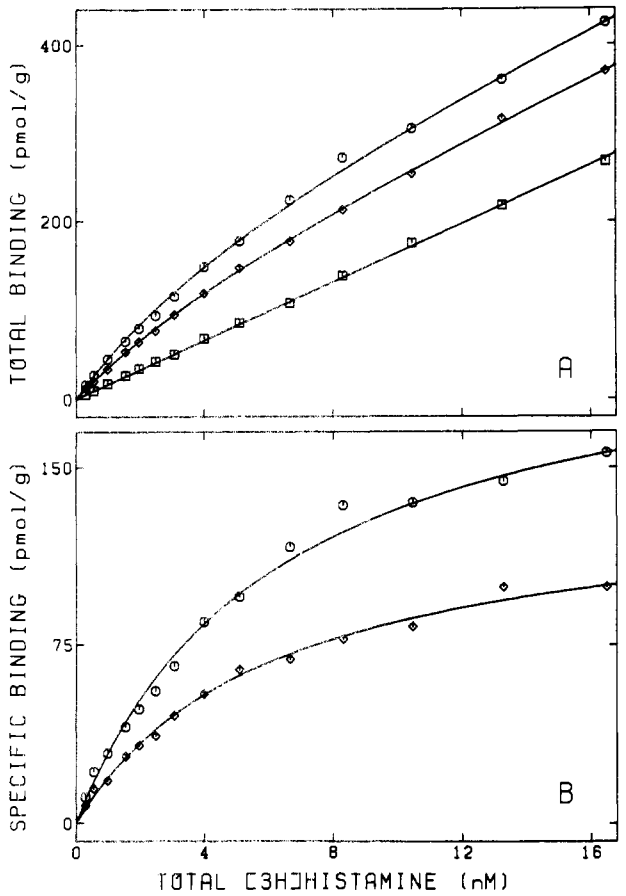


FIGURE 3: Binding of [$^3$H]histamine at different concentrations of the radioligand with and without GMP-PNP. Total binding (A) was measured following equilibration of the suspension (0.90 mg of protein/mL) with [$^3$H]histamine alone (○), in the presence of 0.1 mM GMP-PNP (◊), or in the presence of 1.0 mM unlabeled histamine (□). The lines represent the best fit of eq 6 ($n$ = 1) to the experimental data taken together, with a common value of $K_{P1}$ and separate values of $[R_1]_t$ for data in the absence and presence of GMP-PNP; parametric values obtained by regression are as follows: $K_{P1}$ = 6.12 ± 0.06 nM, $[R_1]_t$ (without GMP-PNP) = 214 ± 11 pmol/g of protein, $[R_1]_t$ (with GMP-PNP) = 137 ± 8 pmol/g of protein, and $C$ = 16.4 ± 0.2 pmol g$^{-1}$ nM$^{-1}$. The data also were fitted assuming separate values for both $K_{P1}$ and $[R_1]_t$ (fit not shown), with little change in the variance of residuals ($P$ = 0.58); parametric values obtained by regression are as follows: without GMP-PNP, $K_{P1}$ = 6.3 ± 0.7 nM and $[R_1]_t$ = 217 ± 13 pmol/g of protein; with GMP-PNP, $K_{P1}$ = 5.7 ± 0.9 nM and $[R_1]_t$ = 132 ± 12 pmol/g of protein; $C$ = 16.4 ± 0.2 pmol g$^{-1}$ nM$^{-1}$. Specific binding (B) represents total binding less the fitted estimate of nonspecific binding and is corrected for the small increase in the latter that occurs in the presence of excess, unlabeled histamine (Seeman et al., 1984).

less variability; for the data summarized in Table II, the range is from 66 to 188 pmol/g of protein, and the mean value is 104 ± 16 pmol/g of protein. The mean capacity from the 11 experiments listed in Table II is 107 ± 15 pmol/g of protein at the sites corresponding to $K_{P1}$.

The consistent results obtained from two approaches to the estimation of $K_{P1}$, together with the good agreement between fitted curves and the experimental data, suggest that binding is to a uniform population of mutually independent sites. This consistency is lost, however, when isotopic dilution is carried out at concentrations of the radioligand exceeding 1.5 nM. The data illustrated in Figure 4 are representative of the results from eight experiments performed at four concentrations of [$^3$H]histamine. The Hill coefficient decreases from 0.95 to about 0.46 (Table III), and the binding patterns become distinctly biphasic, as the concentration of radioligand increases from below 1.5 to 10 nM. At 3.3 nM [$^3$H]histamine and

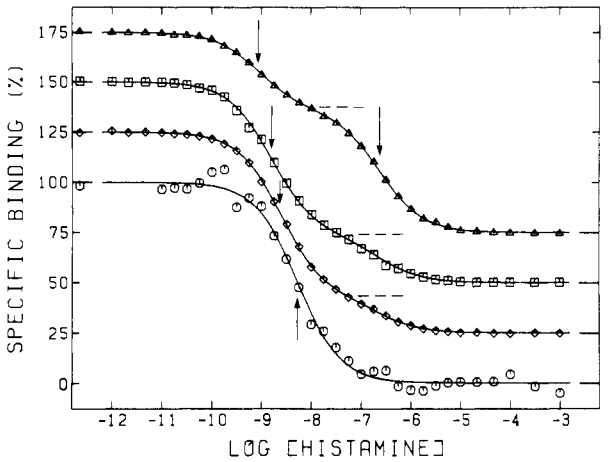


FIGURE 4: Inhibition of [$^3$H]histamine by unlabeled histamine at four concentrations of the radioligand. Total binding was measured following incubation of the suspension with 1.22 (○), 3.49 (◊), 5.84 (□), or 10.1 nM (△) [$^3$H]histamine together with unlabeled histamine at the concentrations shown on the abscissa. The lines represent the best fit of eq 4 ($n$ = 2) to the four sets of data taken together, with a common value of $K_2$ and separate values of $K_1$, $F_2$, $B_{max}$, and $B_{min}$. $F_2$ was set to zero for the data at 1.22 nM [$^3$H]histamine. Parametric values obtained by regression are as follows: log $K_2$ for all curves, −6.63 ± 0.03; (○) log $K_1$ = −8.27 ± 0.02; (◊) log $K_1$ = −8.61 ± 0.02 and $F_2$ = 0.18 ± 0.01; (□) log $K_1$ = −8.80 ± 0.03 and $F_2$ = 0.24 ± 0.01; (△) log $K_1$ = −9.05 ± 0.05 and $F_2$ = 0.61 ± 0.01. Values plotted on the ordinate are normalized at 100% and 0% to the values of $B_{max}$ and $B_{min}$, respectively, obtained from eq 4; successive curves are offset by 25% for clarity. Points at the lower limit on the abscissa represent binding in the absence of unlabeled histamine. log $K_1$ and log $K_2$ are indicated by the arrows and $F_2$ by the dashed lines.

above, good but empirical descriptions of the data are obtained with eq 4 assuming two classes of sites differing in affinity for the unlabeled drug. There is excellent agreement between duplicate experiments at each concentration of the radioligand: parametric values are virtually identical (Table III), and the data are superimposable when normalized on the ordinate to the fitted values of $B_{max}$ and $B_{min}$ (not shown). Moreover, the increase in the variance of residuals at each concentration of [$^3$H]histamine is negligible ($P_4 > 0.5$) when eq 4 is fitted to the two sets of data taken together rather than separately and assuming common rather than separate values of $K_1$, $K_2$, and $F_2$.

The fitted curves in Figure 4 are obtained from simultaneous analysis of the four sets of data assuming a single value of $K_2$ common to the three curves wherein it appears but separate values of $K_1$, $F_2$, $B_{max}$, and $B_{min}$; the global variance of residuals is not increased significantly ($P$ = 0.30) over that obtained assuming separate values for all parameters, indicating that $K_2$ is independent of the concentration of [$^3$H]histamine for the data shown in the figure. A similar result is obtained for all eight experiments summarized in Table III. Simultaneous analysis assuming a single value of $K_2$ and four values of $K_1$ and $F_2$, one for the pair of curves at each concentration of [$^3$H]histamine, yields a variance of residuals not significantly greater ($P$ = 0.57) than that obtained assuming three values of $K_2$. If the inhibition corresponding to $F_2$ is competitive, the absence of any change in $K_2$ implies that the concentration of radioligand is well below its equilibrium dissociation constant ($K_{PS}$) for the sites under consideration. For the two experiments at about 10.5 nM [$^3$H]histamine, the highest concentration used, a mean value of 226 nM can be calculated for $K_{P2}$ (log $K_{P2}$ = −6.65 ± 0.01) from the expression $K_{P2} = K_2$ − [free [$^3$H]histamine], which assumes that the labeled and unlabeled forms of the drug bind with the same affinity. A similar calculation yields a mean value of 247 nM (log $K_{P2}$

Table II: Mean Parametric Values for the Specific Binding of Histamine with and without Magnesium and GMP-PNP[a]

| | | vary [$^3$H]histamine[b] | | | vary unlabeled histamine[c] | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | | eq 6[d] | | | | | | eq 6[e] | | eq 5[e,f] | | |
| [$MgCl_2$] (mM) | [GMP-PNP] (mM) | $N$ | $-\log K_{P1}$ | $[R_1]_t$ (pmol/g) | $N$ | eq 3[d] $n_{H1}$ | eq 4[e] $F_2$ | $-\log K_{P1}$ | $-\log K_{P2}$ | $[R_1]_t$ (pmol/g) | $[R_2]_t$ (pmol/g) |
| 10 | 0 | 4 | 8.40 ± 0.07 | 65–217 | 7[h] | 0.96 ± 0.03 | $g$ | 8.42 ± 0.09 | $g$ | 66–188 | $g$ |
| 10 | 0.1 | 2 | 8.33 ± 0.09 | 30–132 | 5[i] | 0.66 ± 0.05 | 0.25 ± 0.06 | 8.52 ± 0.10 | 6.57 ± 0.10 | 17–68 | 432–1340 |
| 0 | 0 | 2 | 8.35 ± 0.17 | 54–75 | 2[j] | 0.64 ± 0.04 | 0.48 ± 0.02 | 8.38 ± 0.04 | 6.83 ± 0.15 | 26–48 | 1090–1900 |

[a] Values listed in the table reflect best fits of each equation to the data from independent experiments; the number ($N$) of independent experiments is indicated. The reaction mixture in all experiments contained 100 mM Tris and 1.0 mM EDTA. [b] Total binding was measured at various concentrations of the radioligand with and without 1.0 mM unlabeled histamine (e.g., Figure 3). [c] Total binding was measured at a single concentration of the radioligand (1.0–1.5 nM) and various concentrations of unlabeled histamine (e.g., Figure 6). [d] The expression was fitted assuming a single class of sites ($n = 1$). [e] The expression was fitted assuming two classes of sites ($n = 2$) except where indicated otherwise. [f] Values of log $K_{P1}$ and log $K_{P2}$ were fixed at −8.409 and −6.655, respectively, as described in the text. [g] $F_2$ was fixed at zero. [h] Values of $P_1$: >0.2 (6), 0.023; $P_2$: >0.15 (6), 0.016. [i] Values of $P_1$: >0.1 (2), <0.003 (3); $P_2$: 0.0072, <0.0008 (4); $P_3$: <0.0006 (5). [j] Values of $P_1$: 0.18, <0.00001; $P_2$: 0.0014, <0.00001; $P_3$: 0.0016, <0.00001.

Table III: Parametric Values for the Inhibition of [$^3$H]Histamine by Unlabeled Histamine at Different, Fixed Concentrations of the Radioligand[a]

| | | eq 4 | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| [[$^3$H]histamine] (nM) | eq 3 $n_{H1}$ | $-\log K_1$ | $-\log K_2$ | $F_2$ | $[PR_1]$[b] (pmol/g of protein) | $[PR_2]$[c], (pmol/g of protein) | $[R_2]_t$[d] (pmol/g of protein) | goodness of fit $P_1$ | $P_2$ | $P_3$ |
| 1.02 | 0.95 ± 0.09 | 8.23 ± 0.01 | | $e$ | 17.7 | | | 0.89 | 0.60 | 0.56 |
| 1.22 | 0.94 ± 0.08 | 8.27 ± 0.01 | | $e$ | 22.1 | | | 0.22 | 0.50 | 0.28 |
| 3.30 | 0.67 ± 0.02 | 8.61 ± 0.01 | 6.62 ± 0.03 | 0.181 ± 0.003 | 93.4 | 20.6 | 1399 | <0.00001 | <0.00001 | <0.00001 |
| 3.49 | 0.68 ± 0.03 | 8.61 ± 0.01 | 6.59 ± 0.02 | 0.178 ± 0.003 | 81.1 | 17.6 | 1131 | <0.00001 | <0.00001 | <0.00001 |
| 5.84 | 0.57 ± 0.03 | 8.83 ± 0.01 | 6.74 ± 0.04 | 0.257 ± 0.061 | 85.6 | 29.6 | 1152 | <0.00001 | <0.00001 | <0.00001 |
| 5.84 | 0.57 ± 0.03 | 8.83 ± 0.01 | 6.76 ± 0.03 | 0.255 ± 0.006 | 85.8 | 29.4 | 1142 | <0.00001 | <0.00001 | <0.00001 |
| 10.1 | 0.43 ± 0.03 | 9.04 ± 0.01 | 6.61 ± 0.03 | 0.604 ± 0.003 | 48.6 | 74.1 | 1696 | <0.00001 | <0.00001 | <0.00001 |
| 10.8 | 0.48 ± 0.06 | 9.08 ± 0.18 | 6.64 ± 0.08 | 0.644 ± 0.032 | 35.8 | 64.8 | 1393 | <0.00001 | <0.00001 | <0.00001 |

[a] Values listed in the table reflect the best fits of eq 3 ($n = 1$) and 4 ($n = 1$ or 2) to the data from each experiment; the data are illustrated in Figure 4. [b] The quantity of specifically bound [$^3$H]histamine corresponding to $F_1$. [c] The quantity of specifically bound [$^3$H]histamine corresponding to $F_2$. [d] The apparent capacity corresponding to $F_2$, calculated as described in the text. [e] $F_2$ was fixed at zero.

= −6.61 ± 0.02) for the data at 3.4 nM [$^3$H]histamine, the lowest concentration that reveals a biphasic curve. The free concentration of radioligand thus is only 4.6% of $K_{P2}$ at 10.5 nM [$^3$H]histamine and only 1.4% at 3.4 nM.

The mean value of $K_{P2}$ is 214 ± 15 nM (log $K_{P2}$ = −6.67 ± 0.03) for the six experiments listed in Table III. Binding of similar affinity is found under other conditions, as described below, and the mean value of $K_{P2}$ from all experiments is 221 ± 26 nM (log $K_{P2}$ = −6.655 ± 0.051). The latter value has been used to calculate the capacity corresponding to $F_2$ ($[R_2]_t$) according to the expression $[R_2]_t = [PR_2](K_{P2} + [P])/[P]$, where $[PR_2]$ represents $F_2$ in absolute units (Table III). Although the extrapolation is long, capacities estimated in this manner compare favorably from experiment to experiment at the same and different concentrations of the radioligand. The binding of lower affinity thus appears consistent with the notion that labeled and unlabeled histamine compete for a single population of sites. The mean capacity of 1320 ± 90 pmol/g of protein from the data in Table III is 6–20-fold greater than that found for the sites of higher affinity (Table II), but not unreasonably large for a neurohumoral receptor. Capacities as high as 2000 pmol/g of protein have been reported, for example, for muscarinic receptors in similar preparations of tissue (Hulme et al., 1978).

In contrast to the binding associated with $K_2$, that associated with $K_1$ cannot be rationalized in terms of simple competition. Since $K_1 = K_{P1} + [P]$ for competitive inhibition, an increase in the free concentration of radioligand from 1.1 to 10.5 nM would be expected *to increase* $K_1$ from 5.0 to 14.4 nM if $K_{P1}$ is 3.9 nM. The values listed in Table III indicate, however, that $K_1$ *decreases* 6-fold from 5.5 nM (log $K_1$ = −8.26) at about 1.1 nM [$^3$H]histamine to 0.87 nM (log $K_1$ = −9.06) at about 10.5 nM [$^3$H]histamine. Simultaneous analysis of the data indicates that the decrease is significant even for small changes in the concentration of radioligand. Comparing 5.8 and 10.5 nM [$^3$H]histamine, for example, the four sets of data can be fitted (eq 4, $n = 2$) assuming one value for $K_2$ and two values for $K_1$ and $F_2$, one for each concentration of the radioligand. If the data then are refitted assuming one value each for $K_1$ and $K_2$, and two values for $F_2$, the total number of parameters is reduced by 1 to yield a significant ($P$ = 0.00030) increase in the variance of residuals. Similar increases occur when comparing 3.4 with 5.8 nM ($P < 0.00001$), and when comparing 1.1 with 3.4 nM ($P < 0.00001$). A further discrepancy with competitive models is found in the observation that, at 3.3 nM [$^3$H]histamine and above, the concentration of the radioligand exceeds the value of $K_1$. The significance of $K_1$ thus is unclear at higher concentrations of the radioligand.

Uncertainty over the significance of $K_1$ precludes calculation of the corresponding capacity, since the basis for extrapolation is unknown. It is of interest, however, to compare the number of occupied sites corresponding to $F_2$ ($[PR_2]$) with that corresponding to $F_1$ ($[PR_1]$). The data in Table III indicate that the former increases in a regular manner from about 19 pmol/g of protein at 3.4 nM [$^3$H]histamine to about 69 pmol/g of protein at 10.5 nM [$^3$H]histamine; in contrast, the latter increases from 20 to 86 pmol/g of protein, and then falls to about 42 pmol/g of protein, at increasing concentrations of the radioligand. If the binding patterns reflect two populations of unrelated sites, both quantities are expected to increase in a monotonic manner describable by a rectangular hyperbola. For the sites corresponding to $F_1$, predicted occupancy is 20, 42, 53, and 65 pmol/g of protein at 1.1, 3.4, 5.8, and 10 nM [$^3$H]histamine, respectively, assuming that $K_{P1}$ is 3.9 nM and taking 20 pmol/g of protein as the basis for

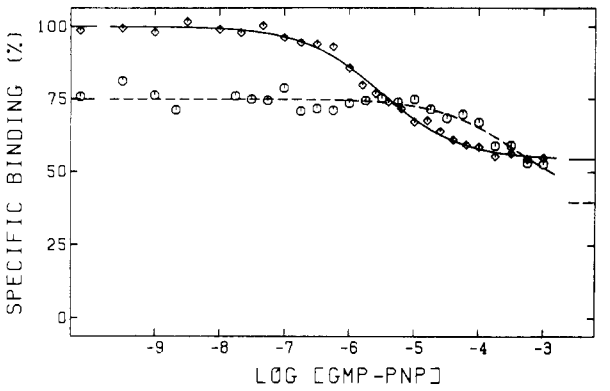


FIGURE 5: Effect of GMP-PNP on the binding of [$^3$H]histamine with (◊) and without (○) magnesium. Total binding was measured following incubation of the suspension, both in the presence (◊) and in the absence (○) of 10 mM $MgCl_2$, with [$^3$H]histamine and with GMP-PNP at the concentrations shows on the abscissa. The concentration of protein was 1.7 (◊) and 1.2 mg/mL (○); that of [$^3$H]histamine was 1.70 and 1.29 nM, respectively. The lines represent best fits of eq 3 ($n$ = 1) to the experimental data, with $n_{H1}$ in the absence of magnesium fixed at the corresponding value obtained in the presence. Parametric values obtained by regression are as follows: with magnesium, log $IC_{50}$ = −5.52 ± 0.05, $n_{H1}$ = 0.71 ± 0.05, $B_{max}$ = 23.3 ± 0.1 pmol/g of protein, and $B_{min}$ = 15.5 ± 0.2 pmol/g of protein; without magnesium, log $IC_{50}$ = −3.42 ± 0.29, $B_{max}$ = 29.2 ± 0.2 pmol/g of protein, and $B_{min}$ = 21.9 ± 1.7 pmol/g of protein. The two sets of data were acquired in separate experiments and have been normalized as follows. Each experiment included a measurement of specific binding in the absence of GMP-PNP, but under the alternate condition with respect to magnesium; since divalent cations alter the size of the pellet, nonspecific binding in the presence of 0.1 mM unlabeled histamine was measured for each condition. Specific binding in the absence of magnesium then was normalized to that in the presence according to the mean ratio from the two comparative tests. Values plotted on the ordinate are normalized at 100% to the asymptotic value of eq 3 at low concentrations of GMP-PNP in the presence of magnesium and at 0% to total binding measured in the presence of 1.0 mM histamine either in the presence (9.1 pmol/g of protein) or in the absence (16.0 pmol/g of protein) of magnesium.

comparison. This discrepancy between the calculated values of [$PR_1$] and those listed in Table III further indicates that the data cannot be rationalized in terms of a mixture of sites. Such a comparison is valid, however, only if the variation in capacity is small from preparation to preparation. In seven experiments performed below 1.5 nM [$^3$H]histamine (Table II), levels of binding varied over the comparatively narrow range of 16–29 pmol/g of protein, with a mean value of 23 ± 2 pmol/g of protein. Moreover, there is good agreement between the duplicate experiments at each concentration of [$^3$H]histamine (Table III). It therefore seems likely that differences in the capacity of the tissue are not the prime determinant of observed differences in the absolute levels of binding at different concentrations of the radioligand.

*Effect of GMP-PNP and Magnesium on the Binding of Histamine.* The specific binding of [$^3$H]histamine at equilibrium is reduced but not eliminated in the presence of GMP-PNP or in the absence of magnesium. As illustrated in Figure 5, the half-maximal effect of GMP-PNP in the presence of magnesium is observed at 3.0 μM, and maximal inhibition is achieved by about 0.1 mM. AMP-PNP is without effect at concentrations up to 1.0 mM (data not shown). The partial inhibition observed at saturating concentrations of GMP-PNP could arise at equilibrium from a major decrease in the affinity of [$^3$H]histamine for some of the sites or from a smaller decrease in the affinity for all of the sites. The former alternative is favored by the similarity in $k_{off}$ between values measured following equilibration with the radioligand alone and following equilibration with the radioligand in the presence of GMP-PNP (Table I). The large increase observed in $k_{off}$ when GMP-PNP is added with unlabeled histamine following equilibration with the radioligand alone (Table I) indicates that the effect of the nucleotide is noncompetitive at the susceptible sites. A Hill coefficient of 0.71 is obtained for GMP-PNP from the data illustrated in Figure 5, and there is a substantial increase in the variance of residuals ($P_2$ = 0.00014) when the data are refitted with $n_{H1}$ fixed at 1. The deviation from 1 suggests that several factors are involved in mediating the effect of the nucleotide on the binding of the radioligand. In the absence of magnesium, the specific binding of [$^3$H]histamine is reduced and GMP-PNP is without effect at concentrations up to about 30 μM. The small decrease that occurs at higher concentrations is not sufficiently well defined to be compared in detail with that found in the presence of magnesium. It perhaps is noteworthy, however, that maximal inhibition is similar under both conditions if both curves are assumed to have the same Hill coefficient. The similarity suggests that magnesium increases the apparent affinity of both [$^3$H]histamine and GMP-PNP and that its removal does not preclude an action of the nucleotide on residual, high-affinity binding of the radioligand.

The reduction in specific binding is proportionately the same at all concentrations of [$^3$H]histamine either in the presence of 0.1 mM GMP-PNP (Figure 3) or in the absence of magnesium (data not shown). Good descriptions of the data are obtained with eq 6 ($n$ = 1), and the mean, parametric values summarized in Table II indicate that a 40–60% decrease in apparent capacity is accompanied by little if any change in $K_{P1}$. This is illustrated further by the data in Figure 3, where measurements in the absence and presence of nucleotide were carried out on the same day with aliquots from the same suspension of tissue. The variance of residuals is not increased significantly ($P$ = 0.58) when the two sets of data are fitted assuming a common value for $K_{P1}$ and separate values for $[R_1]_t$, rather than separate values for both parameters; in contrast, there is a substantial increase ($P$ = 0.00007) when the data are fitted assuming a common value for $[R_1]_t$ and separate values for $K_{P1}$.

The suggestion that GMP-PNP and magnesium act uniquely to reduce capacity is not confirmed, however, by studies on the inhibition of [$^3$H]histamine by unlabeled histamine. Five experiments performed at or below 1.4 nM [$^3$H]histamine and in the presence of 0.1 mM GMP-PNP yield a mean Hill coefficient of 0.66 ± 0.05 (Table II); individual values differ significantly from one, as indicated by the increase in the variance of residuals and by the correlation of neighboring residuals when the data are refitted with $n_{H1}$ fixed at 1. A similar pattern is observed in the absence of magnesium, where the mean Hill coefficient from two experiments is 0.64 ± 0.04 (Table II). For each experiment with GMP-PNP or without magnesium, the data can be described empirically by eq 4 assuming two classes of sites as illustrated in Figure 6; substantial reductions in the variance of residuals ($P_3$ <0.0016; Table II) indicate the fit to be significantly better than that obtained assuming only one class of sites.

The data presented in Figure 6A are taken from an experiment in which measurements in the absence and presence of 0.1 mM GMP-PNP were made on the same day with aliquots from the same suspension of tissue. Data in the absence of nucleotide reveal a Hill coefficient of 0.96 ± 0.03 and are well described by eq 6 assuming a single class of sites. In the presence of nucleotide, the Hill coefficient is 0.78 ± 0.05 and two classes of sites are required for eq 6, as indicated both by the correlation of neighboring residuals with one class ($P$ =

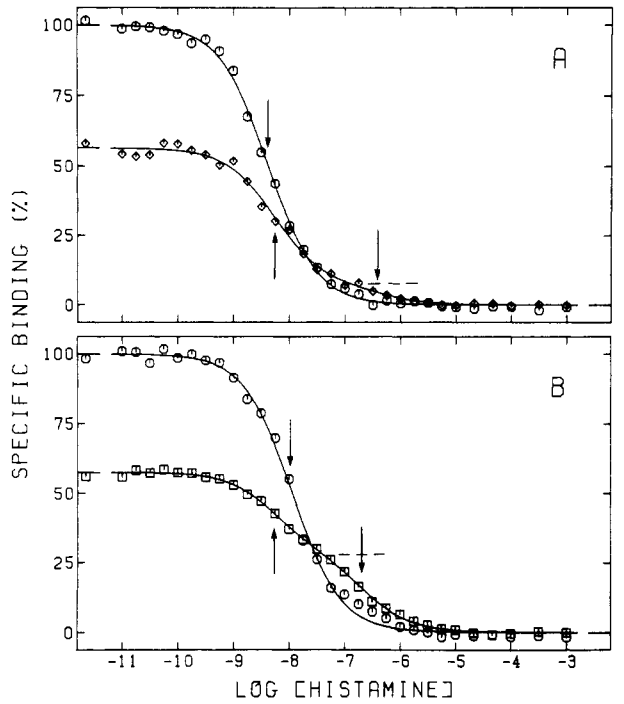


FIGURE 6: Effect of GMP-PNP and magnesium on the inhibition of [$^3$H]histamine by unlabeled histamine. Total binding was measured following incubation of the suspension with [$^3$H]histamine [(A) 1.21 nM; (B) 1.49 nM] and unlabeled histamine under three sets of conditions: in the presence of 10 mM $MgCl_2$ [A and B (○)], in the presence of 10 mM $MgCl_2$ plus 0.1 mM GMP-PNP [A (◇)], and in the absence of both $MgCl_2$ and GMP-PNP [B (□)]. For both (A) and (B), the two sets of data were acquired on the same day using aliquots from the same preparation of tissue. The lines represent the best fit of eq 6 to the experimental data assuming either one (○) or two (◇, □) classes of sites; parametric values obtained by regression are as follows: [A (○)] log $K_{P1}$ = −8.53 ± 0.02 and $[R]_t$ = 111 ± 4 pmol/g of protein; [A (◇)] log $K_{P1}$ = −8.41 ± 0.06, log $K_{P2}$ = −6.40 ± 0.27, $F_2$ = 0.92 ± 0.03, and $[R]_t$ = 790 ± 373 pmol/g of protein; [B (○)] log $K_{P1}$ = −8.01 ± 0.03 and $[R]_t$ = 190 ± 10 pmol/g of protein; [B (□)] log $K_{P1}$ = −8.41 ± 0.07, log $K_{P2}$ = −6.67 ± 0.05, $F_2$ = 0.976 ± 0.003, and $[R]_t$ = 1080 ± 90 pmol/g of protein. Values plotted on the ordinate are normalized at 100% and 0% to the asymptotic values of eq 6. Points at the lower limit on the abscissa indicate binding in the absence of unlabeled histamine. The arrows and dashed lines indicate the values of log $K_S$ and $F_2$, respectively, derived from eq 4; the fitted curves from eq 4 and 6 are superimposable.

0.0026), and by the decrease in the variance of residuals ($P$ <0.00001) upon addition of the second. The value of −8.53 ± 0.02 obtained for log $K_{P1}$ in the absence of nucleotide agrees closely with that of −8.41 ± 0.06 obtained in the presence, while the corresponding capacity decreases from 111 ± 4 to 62 pmol/g of protein. GMP-PNP thus appears to reduce binding through a reduction in the capacity associated with $K_{P1}$. This interpretation is supported by simultaneous analysis of the two sets of data assuming that a single value of $K_{P1}$ is common to both; the corresponding variance of residuals is not significantly greater ($P$ = 0.11) than that obtained with two values of $K_{P1}$. In addition, however, GMP-PNP appears to generate a second class of sites of much lower affinity (log $K_{P2}$ = −6.40 ± 0.27) and much higher capacity ($[R_2]_t$ = 729 pmol/g of protein). Both sets of data were obtained with aliquots from the same homogenate, and the capacities therefore are directly comparable. Since a loss of 49 pmol/g of protein corresponding to $F_1$ cannot account for the appearance of 729 pmol/g of protein corresponding to $F_2$, the effect of GMP-PNP cannot reflect a simple interconversion of sites from one state of affinity to the other. This paradox would be resolved were the nucleotide not only to reduce the affinity of a portion of the sites otherwise of higher affinity ($K_{P1}$) but also to increase the affinity of sites otherwise of much lower affinity ($K_P \gg 1$ μM) and not observable at any practicable concentration of radioligand. Alternatively, the model underlying eq 6 may be inappropriate.

The effect of GMP-PNP on the inhibition of [$^3$H]histamine by unlabeled histamine is mimicked by the removal of magnesium (Figure 6B). Data acquired on the same day with aliquots from the same suspension of tissue reveal Hill coefficients of 0.93 ± 0.05 and 0.60 ± 0.02 in the presence and absence of magnesium, respectively; good agreement with eq 6 is obtained assuming one class of sites for the former and two classes for the latter. Values of log $K_{P1}$ are similar in the presence (−8.01 ± 0.03) and absence (−8.41 ± 0.07) of magnesium, while the corresponding capacity decreases from 190 ± 10 to 26 pmol/g of protein. This is accompanied by the appearance of a much larger capacity (1050 pmol/g of protein) of much lower affinity (log $K_{P2}$ = −6.67 ± 0.05).

A comparison of the mean, parametric values listed in Table II indicates that no estimate of $K_{P1}$ differs significantly from any other ($P_0$ > 0.2). This parameter thus is not sensitive to GMP-PNP or magnesium and is independent of whether the experimental procedure involves isotopic dilution or different concentrations of the radioligand. The similarity suggests that $K_{P1}$ reflects binding to the same sites under all conditions. The observation that the corresponding capacity is increased upon the addition of magnesium, and then decreased upon the addition of GMP-PNP, suggests that magnesium promotes the formation of a state that is not favored in the presence of the nucleotide. It appears, however, that a finite number of sites can exist in that state either in the absence of magnesium or in the presence of GMP-PNP. The possibility that the manipulations with magnesium and GNP-PNP lead to the same end is supported by the similarity in the mean values of $K_{P2}$ and the corresponding capacities (Table II). Also, the increase in the variance of residuals is negligible ($P$ = 0.47) when the data acquired in the presence of GMP-PNP (Figure 6A) and in the absence of magnesium (Figure 6B) are analyzed simultaneously assuming common rather than separate values of $K_{P1}$ and $K_{P2}$ (eq 6) for the two curves.

Estimates of $K_{P2}$ obtained by isotopic dilution below 1.5 nM [$^3$H]histamine, either with GMP-PNP or without magnesium (Table II), are indistinguishable from those obtained by isotopic dilution at higher concentrations of the radioligand (Table III); similar agreement is found for the apparent capacity. The mean value of log $K_{P2}$ from the 13 experiments in which it is observed is −6.655 ± 0.051 ($K_{P2}$ = 221 ± 26 nM). Its apparent absence from the binding patterns obtained by varying the concentration of radioligand (Figure 3) may reflect the relatively low contribution to specific binding below 10 nM [$^3$H]histamine (Table III) and, in the presence of GMP-PNP or in the absence of magnesium, the relatively low ratio of specific to total binding at all concentrations of the radioligand.

## Discussion

Homogenates of washed tissue from rat cerebral cortex bind [$^3$H]histamine in a novel and complex manner not reported previously. At least two affinities apparently are involved. The higher affinity is observed under all conditions examined; in contrast, the lower appears only upon isotopic dilution either at higher concentrations of the radioligand (>1.5 nM) or at lower concentrations upon the removal of magnesium or the addition of GMP-PNP. The data show good, internal consistency when the binding ostensibly of higher affinity is characterized under conditions that apparently suppress that of lower affinity. Experiments performed at what appears to be thermodynamic equilibrium reveal a single class of sites

with the same affinity and comparable capacity irrespective of whether the variable ligand is labeled or unlabeled histamine. Estimates of the dissociation constant ($K_{P_1}$) thus are independent of whether the calculation is based solely on the concentration of the radioligand or primarily on that of the unlabeled analogue. Moreover, parametric values determined at equilibrium are generally consistent with those determined kinetically, at least for most of the sites, although there is evidence from the latter data that a minor fraction of the bound radioligand dissociates more slowly than might be expected. The overall pattern of binding revealed by the present data under a narrow range of conditions thus recalls that reported by previous investigators for [$^3$H]histamine in rat cerebral cortex (Palacios et al., 1978; Barbin et al., 1980; Kandel et al., 1980); indeed, the present values of $K_{P_1}$ (3.9 nM), $k_{on}$ (0.89 $\mu M^{-1}$ $s^{-1}$), and $k_{off}$ (0.0046 $s^{-1}$) are in excellent agreement with the corresponding values reported by Barbin et al. (1980) ($K_P$ = 7.8 nM, $k_{on}$ = 0.63 $\mu M^{-1}$ $s^{-1}$, and $k_{off}$ = 0.0038 $s^{-1}$). A further similarity with the observations of Barbin et al. (1980) lies in the action of guanylyl nucleotides on the binding pattern defined by a range of concentrations of the radioligand. They have reported that 0.1 mM GTP reduces apparent capacity by 37% without disturbing the rectangular hyperbolic nature of the binding and with little or no effect on apparent affinity. It therefore seems likely that the same sites have been labeled by [$^3$H]histamine in both investigations.

The binding of lower affinity is selective in its appearance but reveals some of the properties expected for a uniform population of noninteracting sites. The apparent dissociation constant is essentially the same whenever the sites are observed; also, the apparent capacity varies within a relatively narrow range considering the long extrapolation and the small contribution of those sites to total binding. In each case, however, binding ostensibly of lower affinity is accompanied by some paradoxical feature of the data that precludes analysis in terms of the multisite model expressed in eq 6 and implied in eq 4. The effects of GMP-PNP and magnesium suggest that the two affinities reflect two states of the same sites, but those effects cannot be rationalized in terms of simple interconversion between the two states. A comparison of apparent capacities indicates that the sites of lower affinity created by addition of the nucleotide, or by removal of the cation, greatly outnumber those of higher affinity that are lost. Upon isotopic dilution at or above 3.3 nM [$^3$H]histamine, the binding of higher affinity reveals at least three features that are incompatible with the model. First, apparent occupany ($PR_1$) does not increase with the concentration of [$^3$H]histamine in the manner predicted by eq 6 for a probe with a dissociation constant of 3.9 nM. Second, the apparent dissociation constant ($K_1$, eq 4) decreases at higher concentrations of the radioligand, in contrast to the increase predicted by eq 6. Third, the apparent dissociation constant is numerically smaller than the total concentration of the radioligand. Several considerations suggest that some or all of these paradoxical relationships arise from a cooperative process.

Isotopic dilution at different concentrations of [$^3$H]histamine reveals a bell-shaped relationship between the absolute level of binding defined by $F_1$ ($PR_1$) and the concentration of the radioligand. Two opposing processes thus appear to control the formation of $PR_1$, but the observed dependence can be assessed only qualititatively since different preparations of tissue are involved. As noted above, absolute levels of binding have been sufficiently consistent throughout the investigation to suggest that the descending limb of the relationship does not reflect a major deficiency in the capacity of those preparations for the radioligand. Among the experiments summarized in Table III, however, the dependence of total specific binding (i.e., [$PR_1$] + [$PR_2$]) on the concentration of [$^3$H]-histamine is not well described by a rectangular hyperbola; in contrast, a rectangular hyperbola provides an excellent description of the data when points at all concentrations of [$^3$H]histamine are measured in the same preparation of tissue (Figure 3). The discrepancy presumably arises from small differences in capacity among the experiments summarized in Table III. A quantitative assessment of the data therefore requires that absolute levels of binding corresponding to $F_1$ and $F_2$ be normalized such that total specific binding increases with the concentration of [$^3$H]histamine in a hyperbolic manner. In order that the normalization reflect the situation observed experimentally, the dissociation constant ($K_P$) of the radioligand has been taken as 3.9 nM and the capacity ($[R]_t$) as 107 pmol/g of protein. Total specific binding has been calculated according to the relationship $[PR] = [R]_t[P]/(K_P + [P])$ for the concentrations of [$^3$H]histamine ([P]) listed in Table III; the quantity PR then can be divided into $PR_1$ and $PR_2$ according to the appropriate value of $F_2$. Values of $PR_1$ and $PR_2$ obtained in this manner are plotted as a function of the concentration of [$^3$H]histamine in Figure 7A. Both quantities reveal the general pattern found with the original data: $PR_1$ exhibits a bell-shaped dependence, while $PR_2$ increases in an apparently monotonic fashion. Three observations can be made from an inspection of the data. First, at least two processes control the formation of $PR_1$ in an opposing manner. Second, the ascending and descending limbs of the $PR_1$ dependency are not symmetrical; the two processes thus are characterized by different Hill coefficients. Third, the disappearance of $PR_1$ seems to be concomitant with the appearance of $PR_2$; the same process therefore may control both events.

Equation 7 represents an attempt to provide an empirical

$$B_{obsd} = B_{min} + (B_{max} - B_{min}) \left[ I_{\alpha a} + \frac{I_{\alpha b}[P]^{n_\alpha}}{[P]^{n_\alpha} + K_\alpha^{n_\alpha}} \right] \times \left[ I_{\beta a} + \frac{I_{\beta b}[P]^{n_\beta}}{[P]^{n_\beta} + K_\beta^{n_\beta}} \right] \quad (7)$$

framework within which to analyze changes in $PR_1$ and $PR_2$. $B_{obsd}$ reflects either $PR_1$ or $PR_2$, both of which are assumed to vary between a minimal value ($B_{min}$) and some maximal value ($B_{max}$); the former has been taken as zero, since no binding can occur in the absence of the radioligand. The four quantities designated as $I$ are set to 1, 0, or −1, depending upon the quantity represented by $B_{obsd}$. For $PR_1$, the increase is controlled by the first process ($\alpha$) and the decrease by the second ($\beta$); $I_{\alpha a}$ and $I_{\alpha b}$ thus are set to 0 and 1, respectively, while $I_{\beta a}$ and $I_{\beta b}$ are set to 1 and −1. For $PR_2$, the first process ($\alpha$) is without effect, and the increase is controlled by the second ($\beta$); $I_{\alpha a}$ and $I_{\alpha b}$ thus are set to 1 and 0, respectively, while $I_{\beta a}$ and $I_{\beta b}$ are set to 0 and 1. The solid lines in Figure 7A represent the best fit of eq 7 to the data for $PR_1$ and $PR_2$ taken together; both sets of data were assumed to share common values of $B_{max}$, $K_\beta$, and $n_\beta$. The restraint on $K_\beta$ and $n_\beta$ presupposes that the same process controls the decrease in $PR_1$ and the increase in $PR_2$; it therefore precludes changes that would give rise to unobserved shoulders or minima in the relationship between total specific binding and the concentration of [$^3$H]histamine. The dashed lines represent the independent contribution of each process to the formation of

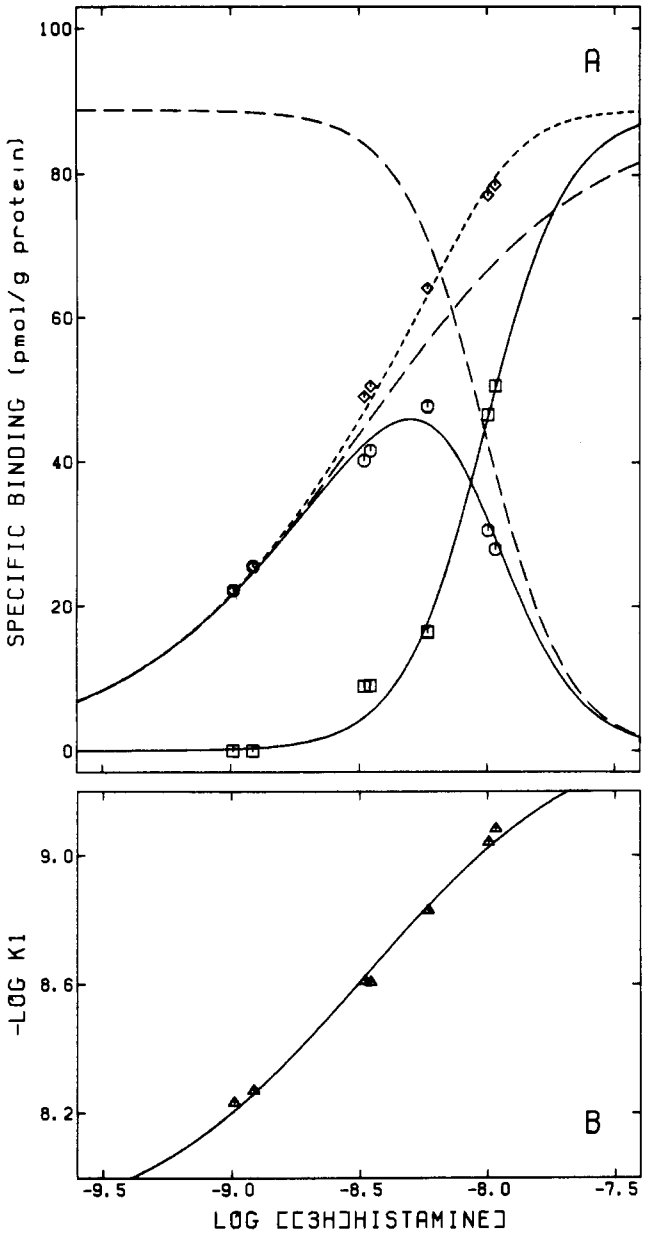


FIGURE 7: Concentration dependence for the effect of [$^3$H]histamine on the inhibitory behavior of unlabeled histamine. (A) Total specific binding of [$^3$H]histamine (◇) ($PR_1$ + $PR_2$) and the corresponding values of $PR_1$ (○) and $PR_2$ (□) were calculated as described in the text. The solid lines represent the best fit of eq 7 to the data for $PR_1$ and $PR_2$ taken together. $K_\alpha$ and $n_\alpha$ were unique to $PR_1$, while $K_\beta$, $n_\beta$, and $B_{max}$ were common to both $PR_1$ and $PR_2$; $I_{\alpha a}$, $I_{\alpha b}$, $I_{\beta a}$, and $I_{\beta b}$ were set as described in the text. Parametric values obtained by regression are as follows: log $K_\alpha$ = −8.49 ± 0.07, log $K_\beta$ = −8.01 ± 0.02, $n_\alpha$ = 0.97 ± 0.13, $n_\beta$ = 2.7 ± 0.2, and $B_{max}$ = 89 ± 5 pmol/g of protein. The dashed lines were calculated according to eq 7 by using the parametric values listed above and illustrate the individual processes ($\alpha$ and $\beta$) that control the level of $PR_1$. The dotted line was calculated according to eq 7 and illustrates the sum of $PR_1$ and $PR_2$. (B) Apparent affinities of unlabeled histamine (log $K_1$, eq 4) are taken from Table III. The line represents the best fit of eq 7 with $I_{\alpha a}$, $I_{\alpha b}$, $I_{\beta a}$, and $I_{\beta b}$ fixed at 0, 1, 1, and 0, respectively; log $K_\alpha$ was fixed at −8.49, and $n_\alpha$ at 0.97 (see above). Limiting values of −log $K_1$ ($B_{min}$ and $B_{max}$) obtained by regression are 7.81 and 9.43, respectively. All points in (A) and (B) were weighted equally during the fitting procedures.

$PR_1$ and were calculated from the parametric values obtained from the fitting procedure. Since the parameters for the second process are common to both $PR_1$ and $PR_2$, the descending dashed line is the mirror image of the solid line that describes the change in $PR_2$. The dotted line represents the sum of $PR_1$ and $PR_2$, or total specific binding.

The agreement between the fitted curves and the data illustrated in Figure 7A indicates that eq 7 provides at least a first approximation of the changes in $PR_1$ and $PR_2$. A value of 89 ± 5 pmol/g of protein is obtained for $B_{max}$, which thus compares favorably with the mean capacity of 107 ± 16 pmol/g of protein calculated from the results of several experiments and used to normalize data plotted on the ordinate. The values of $n_\alpha$ (0.97 ± 0.10) and $K_\alpha$ (3.3 ± 0.4 nM) for the process that controls the increase in $PR_1$ are almost identical with the Hill coefficient and the apparent dissociation constant ($K_{P1}$ = 3.9 ± 0.5 nM) for the interaction of histamine with the sites of higher affinity. This agreement reflects the relatively small amount of $PR_2$ observed at low concentrations of the radioligand, which in turn suggests that the sites are predominantly in a state of higher affinity for histamine under those conditions. Concentrations of histamine above about 3 nM seem to provoke a cooperative process in which 1 equiv of the ligand causes several sites to interconvert from the state of higher affinity to a state ostensibly of lower affinity. The model implies that this interconversion is independent of the formation of $PR_1$; that is, $PR_1$ does not lie in the mechanistic pathway that leads to $PR_2$. All sites are interconvertible, however, and the formation of $PR_2$ is expected ultimately to reduce the number of sites in the state of higher affinity to zero. Since the apparent affinity associated with $PR_2$ is 221 nM, histamine seems to bind in a negatively cooperative manner when all sites are considered; in contrast, the relative values of $n_\beta$ (2.7 ± 0.1) and $n_\alpha$ (0.97 ± 0.13) suggest that the interconversion itself is positively cooperative. This curious arrangement may relate to the uncertainty over the physical significance of all parameters derived from eq 4–6. The underlying model is clearly at variance with the data, and parametric values derived therefrom are suspect. It is not obvious, for example, that $K_{P1}$ and $K_{P2}$ (eq 6) represent the dissociation constants of histamine for the two states identified with $PR_1$ and $PR_2$, respectively, in Figure 7A. The significance of $K_{P2}$ is particularly uncertain in view of the paradoxical circumstances associated with its appearance. Also, the relationship between [$PR_2$] and the concentration of [$^3$H]histamine is not a rectangular hyperbola characterized by an apparent affinity of 221 nM, either for the data in Figure 7A or for similar data in which $PR_2$ is defined by $F_2$ for the $H_2$ agonist dimaprit (Steinberg et al., 1985b). The significance of $K_{P1}$ is perhaps less uncertain, at least under those conditions that seem to discourage the formation of $PR_2$. It is particularly noteworthy that the value of 3.9 nM obtained for $K_{P1}$ in the present investigation is almost identical with that of about 5.0 nM observed when the sites are solubilized in 1% digitonin (Wells et al., 1985). The solubilized preparation reveals none of the paradoxical effects found in suspension, and the binding of lower affinity is not observed.

A word is in order concerning the pattern of total specific binding predicted by eq 7. The calculated line agrees well with the present data, as illustrated in Figure 7A, but the expression is not a rectangular hyperbola. This implies that the hyperbolic pattern revealed by total specific binding at different concentrations of the radioligand arises fortuitously and is not dictated by the properties of the system. Such a coincidence cannot be ruled out, since high levels of nonspecific binding preclude measurements at saturating concentrations of [$^3$H]histamine. On the other hand, eq 7 is only an empirical descriptor of the data; an expression that reflects more accurately the molecular events involved may predict a hyperbolic dependence for total binding.

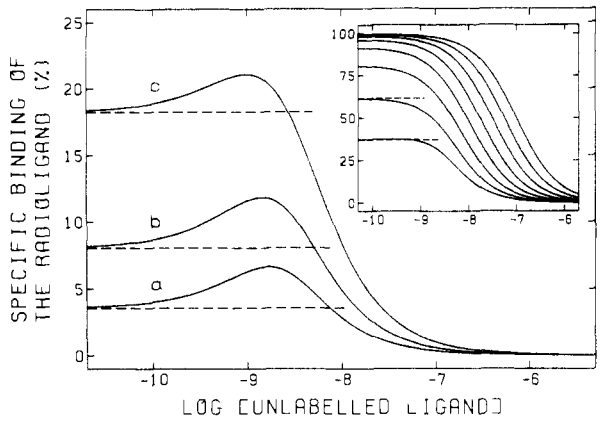


FIGURE 8: Simulated inhibition of a radioligand by its unlabeled analogue in a positively cooperative system. The curves were calculated according to a second-degree Adair equation for the binding of a radioligand (P) and an unlabeled drug (A) with identical affinities for a positively cooperative dimer ($\log K_1 = -7.81$; $\log K_2 = -9.43$). Values of log [P] are as follows: a, −9.50; b, −9.25; c, −9.00; d, −8.75; e, −8.50; f, −8.25; g, −8.00; h, −7.75; i, −7.50; j, −7.25; k, −7.00. Curves d–k are shown in the inset from left to right. The dashed lines indicate the level of binding in the absence of unlabeled drug.

The major cause of uncertainty over $K_{P1}$ is the decrease in $K_1$ (eq 4) that occurs at higher concentrations of the radioligand. The change is illustrated in Figure 7B, where the line reflects the best fit of eq 7 with $K_\alpha$ and $n_\alpha$ fixed at the values obtained from the analysis illustrated in Figure 7A. The agreement between the data and the fitted curve raises the possibility that the change in $K_1$ and the increase in $PR_1$ are controlled by the same process. It follows that the increase in $PR_1$ may arise in part from an increase in the affinity of histamine rather than simply as a consequence of the law of mass action. Although suggested by the data, positive cooperativity is difficult to rationalize in terms of models that reduce to the Adair equation. The curves shown in Figure 8 illustrate the binding patterns expected from isotopic dilution in a positively cooperative system with two identical subunits. At low concentrations of the radioligand, binding first increases through a maximum as the cooperativity is expressed at intermediate concentrations of the unlabeled drug and then decreases to zero through competitive inhibition. At higher concentrations of the radioligand (Figure 8, inset) the cooperativity is fully expressed in the absence of unlabeled drug and only the competitive limb of the curve is observed. The peak predicted by the Adair equation for low concentrations of radioligand is not observed experimentally: the data illustrated in Figures 4 and 6 are well described assuming a rectangular hyperbola for the inhibition corresponding to $F_1$. When the simulated curves define a rectangular hyperbola, the apparent binding constant of the unlabeled drug increases in a competitive manner with increasing concentrations of the radioligand; in contrast, the value of $K_1$ is found to decrease, as illustrated in Figures 4 and 7B.

The relationship between the concentration of [$^3$H]histamine and the inhibitory potency of the unlabeled analogue ($K_1$, eq 4) leads to the paradoxical situation in which the former exceeds the latter. Such a reversal is at variance with any model commonly used to describe neurohumoral receptors and implies that unlabeled histamine inhibits the radioligand through binding at sites that are not labeled by the latter. This could occur, for example, if the dissociation constant of the unlabeled drug were lower than that of the radioligand or if the unlabeled drug could bind at sites that were inaccessible to the radioligand. Neither possibility appears likely, however, since there is no evidence that the substitution of tritium for hydrogen changes the pharmacological specificity of histamine. A partial resolution of this paradox might be possible if the changes in $K_1$ and $F_1$ (eq 4) were controlled allosterically by a comparatively small population of sites that did not discriminate between isotopic analogues of histamine but contributed negligibly to specific binding.

The scheme represented by eq 7 may provide some insight into the effects of GMP-PNP and magnesium on the pattern of isotopic dilution at 1.0–1.5 nM [$^3$H]histamine. A decrease in the value of $K_\beta$ simultaneously would decrease $PR_1$ and increase $PR_2$, in qualitative agreement with the changes observed experimentally upon the addition of GMP-PNP or the removal of magnesium. It can be shown, however, that several parameters would have to change for eq 7 to agree quantitatively with the observed effects on $PR_1$ and $PR_2$ while continuing to predict a pattern of total binding that approximates a rectangular hyperbola.

Guanylyl nucleotides and magnesium are known to modulate the interaction between neurohumoral receptors and a G/F complex that mediates the action of the receptor on adenylate cyclase (Sternweis et al., 1980). The interaction between receptor and G/F complex, and the modulation of that interaction, is reflected in the binding of agonists in a characteristic manner (Bird & Maguire, 1978). Magnesium generally is seen to favor the interaction, thereby leading to a state of relatively higher affinity for the agonist; in contrast, guanylyl nucleotides disfavor or preclude the interaction, thereby leading to a state of lower affinity for the agonist. Barbin et al. (1980) noted that the reduction caused by GTP in the apparent capacity for [$^3$H]histamine recalls the action of the nucleotide in other neurohumoral systems; the present results demonstrate that the action of magesium also is consistent with its effect in other systems and suggest that the actions of GMP-PNP and magnesium are reciprocal. The increase brought about by GMP-PNP in the rate constant for dissociation of bound [$^3$H]histamine confirms that the action of the nucleotide is noncompetitive with respect to the binding of the radioligand. [$^3$H]Histamine thus labels a population of sites that behave in every respect as neurohumoral receptors that act via a G/F complex sensitive to guanylyl nucleotides. Their further characterization and identification as $H_2$ histaminic receptors is described in the following papers (Steinberg et al., 1985a,b). The complex behavior of histamine revealed by the present data thus may be relevant to the mechanistic events linking stimulus and response in $H_2$ systems.

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**Registry No.** GMP-PNP, 34273-04-6; Mg, 7439-95-4; histamine, 51-45-6.

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# $H_2$ Histaminic Receptors in Rat Cerebral Cortex. 2. Inhibition of [$^3$H]Histamine by $H_2$ Antagonists†

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ABSTRACT: Sites labeled by [$^3$H]histamine in homogenates of rat cerebral cortex reveal a pharmacological specificity typical of $H_2$ receptors. Fourteen $H_2$ antagonists inhibit the specific binding of the radioligand to the same level; Hill coefficients are near or equal to one for five compounds and markedly lower for nine. The binding patterns of individual antagonists (A) are well described by the empirical expression $Y = F_1K_1/(K_1 + [A]) + F_2K_2/(K_2 + [A])$, in which $F_1$ and $F_2$ sum to 1; $F_2$ is 0 for those drugs that reveal a Hill coefficient of 1. Concentrations of A that reduce specific binding by 50% ($IC_{50}$) correlate well ($r = 0.991$; $P < 0.00001$) and show good numerical agreement with potencies reported for inhibition of the response to histamine in $H_2$-mediated systems. The correlation is poorer when $IC_{50}$ is replaced by either $K_1$ ($r = 0.973$) or $K_2$ ($r = 0.921$) for those antagonists that reveal both; the antihistaminic activity of the drug thus appears not to be associated preferentially with one or other class of sites. Since $F_2$ varies from 0.16 to 0.60 among those antagonists that discern heterogeneity, the antagonist appears to determine the distribution of sites between the two classes. Moreover, a correlation among antagonists between values of $K_1$ and $K_2$ ($r = 0.975$; $P = 0.00001$) suggests that the apparent heterogeneity reflects different conformers within an otherwise homogeneous population. $H_2$ antagonists appear to be noncompetitive with respect to each other and to the radioligand: one antagonist has relatively little effect on the values of $K_1$, $K_2$, and $F_2$ revealed by another; also, estimates of $K_1$ and $K_2$ are independent of the concentration of [$^3$H]histamine between 1.3 and 10 nM, although the radioligand exhibits an apparent dissociation constant of 3.9 nM [Steinberg, G. H., Eppel, J. G., Kandel, M., Kandel, S. I., & Wells, J. W. (1985) *Biochemistry* (preceding paper in this issue)].

Evidence presented in the preceding report indicates that sites labeled by [$^3$H]histamine in homogenates of rat cerebral cortex respond to guanylyl nucleotides and magnesium in a manner that implies the presence of a nucleotide-specific G/F protein, and establishes the identity of the sites as neurohumoral receptors (Steinberg et al., 1985a). The binding of histamine is shown to be a complex process that cannot be understood in terms of conventional models for the interaction between neurohumoral agents and their receptors but appears to involve an interconversion of receptors between at least two states of affinity. Neurohumoral modulators acting via the G/F protein, and histamine itself, all appear to regulate the

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