Br. J. Pharmac. (1985), **84**, 417–424

The binding of doxepin to histamine H_1-receptors in guinea-pig and rat brain

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1 The affinity constant for doxepin obtained from inhibition of histamine-induced contraction of guinea-pig intestinal smooth muscle at 30°C was $2.6 \pm 0.18 \times 10^{10}\,M^{-1}$. The slope of a Schild plot was not significantly different from unity.

2 The affinity constant of doxepin did not vary markedly with temperature. At 37°C it was $3.75 \pm 0.02 \times 10^{10}\,M^{-1}$ and at 25°C $2.1 \times 10^{10}\,M^{-1}$.

3 Doxepin was a competitive inhibitor of [^{3}H]-mepyramine binding to guinea-pig cerebellar homogenates. The affinity constant derived for doxepin at 30°C was $1.12 \pm 0.45 \times 10^{10}\,M^{-1}$.

4 Hill coefficients for curves of doxepin or mepyramine inhibition of [^{3}H]-mepyramine binding in guinea-pig cerebellum, cerebral cortex and hippocampus did not differ significantly from unity.

5 The mean affinity of mepyramine for histamine H_1-receptors in rat brain homogenates at 30°C was $3.5 \times 10^8\,M^{-1}$. Hill coefficients for curves of doxepin or mepyramine inhibition of [^{3}H]-mepyramine binding to homogenates of rat cerebral cortex or rat whole brain were near unity.

6 These studies provide no evidence that doxepin binds preferentially to a sub-class of histamine H_1-receptors in rat brain.

Introduction

Doxepin, a tricyclic antidepressant with a very high affinity for the histamine H_1-receptor (Tran *et al.*, 1978; Figge *et al.*, 1979), has attracted attention as a possible ^{3}H-ligand for the H_1-receptor (Tran *et al.*, 1981; Taylor & Richelson, 1982) with potential advantages over [^{3}H]-mepyramine, the ^{3}H-ligand most commonly used (Schwartz *et al.*, 1980). However, in rat brain the number of sites with the character of H_1-receptors labelled by [^{3}H]-doxepin appears to be less than the number labelled by [^{3}H]-mepyramine (Tran *et al.*, 1981; Taylor & Richelson, 1982). Largely on the basis of this evidence the suggestion has been made that [^{3}H]-doxepin may bind preferentially to a subclass of H_1-receptors and thereby reveal a heterogeneity in the H_1-receptor population in rat brain (Taylor & Richelson, 1982).

This proposition is given some support by the observation that the Hill coefficient for the curve of doxepin inhibition of [^{3}H]-mepyramine binding in rat brain is 0.55 (Taylor & Richelson, 1982) and that other tricyclic antidepressants appear to be an order of magnitude more potent in displacing high-affinity [^{3}H]-doxepin binding than in displacing the binding of [^{3}H]-mepyramine (Taylor & Richelson, 1982). However, the binding of [^{3}H]-doxepin is complex and not all the data are consistent with the receptor heterogeneity explanation, as the authors note. Caution is also necessary in interpreting Hill coefficients < 1 obtained from inhibition of [^{3}H]-mepyramine binding, since even moderate concentrations of the ^{3}H-ligand appear to label secondary, non H_1-receptor sites in both guinea-pig and rat tissues (Hill & Young, 1980; 1981; Hadfield *et al.*, 1983). Evaluation of the studies of [^{3}H]-doxepin binding is further complicated by the wide range of values in the literature for the affinity constant for the interaction of doxepin with H_1-receptors. In guinea-pig brain, where [^{3}H]-doxepin appears to label the same number of sites as [^{3}H]-mepyramine, values reported range from 9.6×10^8 to $5 \times 10^{10}\,M^{-1}$ (Chang *et al.*, 1979; Figge *et al.*, 1979; Palacios *et al.*, 1979; Coupet & Szuchs-Myers, 1981; Tran *et al.*, 1981; Kanba & Richelson, 1983). A similar disagreement exists in rat brain, $1.4 \times 10^9\,M^{-1}$ to $5.0 \times 10^{10}\,M^{-1}$ (Tran *et al.*, 1978, 1981; Taylor &

Richelson, 1980; 1982), but in this species there is a further difficulty in that there is no reported affinity constant for doxepin derived from inhibition of a functional response mediated by H_1-receptors in an intact tissue preparation. In neither species has it been established with certainty that doxepin is purely a competitive ligand at the H_1-receptor.

In view of the importance of the proposition that doxepin may bind selectively to a sub-class of H_1-receptors we have re-examined the interaction of doxepin with the H_1-receptor in the guinea-pig and the rat. To avoid the problems associated with the complex binding behaviour of [^{3}H]-doxepin our main line of approach has been via a study of doxepin inhibition of the binding of low concentrations of [^{3}H]-mepyramine. The results of this investigation are described here.

Methods

Measurement of histamine-induced contraction of intestinal smooth muscle

Strips of the longitudinal muscle from guinea-pig (Dunkin-Hartley strain, males) small intestine, prepared essentially as described by Rang (1964), were suspended in 10 ml Krebs-Henseleit solution (containing (mM): NaCl 116, KCl 4.7, $MgSO_4$ 1.2, KH_2PO_4 1.2, $NaHCO_3$ 25, $CaCl_2$ 2.5 and D-glucose 5.5) in a conventional organ bath and gassed continuously with 95% O_2: 5% CO_2. Contractions to histamine and carbachol were recorded isotonically. In experiments in which muscle strips showed appreciable spontaneous activity indomethacin (10^{-5} M final concentration) was added to the Krebs-Henseleit solution. This concentration of indomethacin had no significant effect on the dose-response curve to histamine. There was similarly no significant difference in the affinity constant for doxepin measured in the presence and absence of indomethacin. In the experiments at 25°C the muscle strips were first equilibrated at 30°C for at least 2 h before cooling to 25°C. Responses to carbachol were measured in all experiments as an indication of any changes of sensitivity of the tissue during the course of the experiment. Doxepin where present was a constituent of the Krebs-Henseleit solution in the reservoir.

The affinity constants of doxepin at 30°C and 37°C were obtained from the slope of a plot of (dose-ratio − 1) *versus* [doxepin]. At 25°C affinity constants were calculated from single shifts of the dose-response curve to histamine using the relationship dose-ratio $- 1 = K_a$.[doxepin].

The rate constant for recovery from blockade, k_{-1}, was calculated using the relationship $AR = AR_o \cdot \exp(-k_{-1} \cdot t)$, where AR is the occupancy of doxepin at time t and AR_o the occupancy at t = 0. The occupancy was obtained from the relationship (Paton, 1961) AR = (dose-ratio − 1)/dose-ratio.

Measurement of inhibition of [^{3}H]-mepyramine binding in brain tissues

Guinea-pigs (Dunkin-Hartley strain, males, or mixed race bred in the Centro de Investigacion) or rats (Wistar, both sexes) were killed by cervical dislocation and brain regions dissected out on ice. The tissues were either used immediately or stored frozen at −20°C in Krebs-Henseleit medium. There was no evidence of any loss of [^{3}H]-mepyramine binding even after several months of storage in this way. Brain regions were homogenised in 50 mM Na-K phosphate buffer (37.8 mM Na_2HPO_4, 12.2 mM KH_2PO_4), pH 7.5, using either a teflon-glass homogeniser with a motor-driven pestle or a polytron blender (setting 3, 3 × 15 s) and then centrifuged at 17,000 *g* for 30 min. The pellet was resuspended in buffer and recentrifuged at 17,000 *g*. The final pellet was suspended in the phosphate buffer (approximately 5 ml per g wet weight tissue), divided into smaller portions and stored at −20°C until required for use. Protein was determined by the method of Lowry *et al.*, (1951) using bovine serum albumin as standard.

Incubations in 50 mM Na-K phosphate buffer, pH 7.5, (final volume normally 1.08–1.09 ml) contained [^{3}H]-mepyramine, doxepin (where appropriate) and 0.34–0.36 mg homogenate. Where a protein concentration lower than 0.36 mg ml$^-$ was required the incubation volume was increased such that the total amount of protein present remained 0.36 mg. Equilibration was for 60 min at 30°C and was terminated by addition of 4 ml ice-cold buffer containing mepyramine 1 μM. The mixture was filtered immediately through a Whatman GF/B glass fibre filter and the membrane-bound tritium trapped on the filter determined by liquid scintillation counting after allowing the filter to soak overnight in Brays solution (dioxan : naphthalene : PPO : POPOP, 100 : 15 : 0.8 : 0.1, v/w/w/w) or in toluene : Emulsifier Mix No 1 (Fisons) : water : BuPBD (70 : 30 : 10 : 0.6, v/v/v/w). Replicate determinations (5–8) were made at each inhibitor concentration. The inhibition given by promethazine 2 μM was measured in each experiment. The concentration of [^{3}H]-mepyramine was determined by scintillation counting.

Analysis of curves of inhibition of [^{3}H]-mepyramine binding

Curves of percentage of uninhibited binding of [^{3}H]-mepyramine *versus* concentration of inhibitor were

fitted to a Hill equation

% of uninhibited binding of [^{3}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 inhibitor-insensitive binding. The best-fit values ± estimated standard error of n, IC_{50} and NS were obtained by non-linear regression analysis using a modified Marquardt procedure as implemented in the Harwell Library routine VB01A on the Cambridge IBM 3081. Each point was weighted by the reciprocal of the variance associated with it.

Where the data were insufficient for treatment in this way (the experiments represented in Figure 1) the IC_{50} was measured as the concentration of inhibitor giving 50% inhibition of the binding sensitive to promethazine 2 μM.

The affinity constant for mepyramine, K_{mep}, was calculated from the relationship $K_{mep} = 1/(IC_{50} - [^3H\text{-mepyramine}])$, which assumes that the substitution of an atom of tritium for an atom of hydrogen has no significant effect on the affinity constant. The affinity of doxepin, K_a, was calculated from the relationship $K_a = ([[^3H]\text{-mepyramine}].K_{mep} + 1)/IC_{50}$. K_{mep} in guinea-pig brain was taken to be 1.6×10^9 M^{-1} on the basis of previous determinations (Hill *et al.*, 1981) and the values obtained in this study. In rat brain K_{mep} was taken to be 3.5×10^8 M^{-1}, the mean of the 4 determinations made (Table 4).

Drugs

[^{3}H]-mepyramine, 27.3 and 28 Ci mmol^{-1}, was obtained from New England Nuclear and Amersham International. Carbamylcholine chloride (carbachol) and histamine dihydrochloride were purchased from Sigma and mepyramine maleate and promethazine hydrochloride from May & Baker. Doxepin hydrochloride (batch 3-3760, 82% *trans*- and 18% *cis*-isomer) was a kind gift from Pfizer Ltd.

Results

Doxepin inhibition of histamine-induced contraction of guinea-pig intestinal smooth muscle

Doxepin produced parallel shifts of the dose-response curve for the histamine-induced contraction of guinea-pig intestinal smooth muscle. The affinity constant for the H_1-receptor determined from these shifts showed no great change between 25°C and 37°C (Table 1). The slope of the Schild plot did not differ significantly from unity at either 30°C or 37°C, the two temperatures at which the data were sufficiently extensive for this type of analysis (Table 1).

The interaction of doxepin with the H_1-receptor was reversible, but recovery on washout was very slow. In one experiment at 30°C in which this was tested the dose-ratio of 62 produced by 2 nM doxepin declined to 8.4 at 120 min and 2.4 at 280 min after washing out the antagonist. Assuming exponential kinetics this corresponds to an apparent rate constant for dissociation of 1.4×10^{-3} min^{-1} ($t_{0.5}$ *circa* 500 min). The dose-response curve to carbachol, measured concurrently in all these experiments, showed no significant change in position except in that at 37°C in the presence of 100 nM doxepin. This shift gave an approximate K_a of doxepin for the muscarinic receptor of 2×10^7 M^{-1}, in reasonable agreement with the literature value on the guinea-pig ileum of 1×10^7 M^{-1} (Figge *et al.*, 1979).

Doxepin and mepyramine inhibition of [^{3}H]-mepyramine binding to guinea-pig brain

To confirm that doxepin is a competitive antagonist of [^{3}H]-mepyramine binding to the histamine H_1-receptor a series of experiments was carried out in which the IC_{50} for doxepin inhibition of the promethazine-sensitive binding of [^{3}H]-mepyramine to homogenates of

Table 1 Doxepin inhibition of histamine-induced contraction of guinea-pig intestinal smooth muscle

Temperature (°C)	K_a (M^{-1})	*Slope of Schild plot*
37	$3.75 \pm 0.02 \times 10^{10}$	1.05 ± 0.03 (5)
30	$2.61 \pm 0.18 \pm 10^{10}$	1.10 ± 0.06 (9)
25	2.1 ± 10^{10}	—

Measurements on longitudinal muscle strips from guinea-pig small intestine were made as described under Methods. Affinity constants, K_a, were obtained from the best-fit slope, determined by linear regression analysis, of a plot of (dose-ratio − 1) *versus* [doxepin]. The value at 30°C is derived from the combined results from 3 experiments and that at 37°C from a single experiment. The figures in parentheses indicate the number of dose-ratios measured at each temperature. The value of K_a at 25°C is the mean of 2 determinations (1.9×10^{10} and 2.3×10^{10} M^{-1}) from independent experiments in each of which a single shift of the dose-response curve to histamine was measured 2 h after equilibration with 0.4 nM doxepin. Indomethacin (10 μM) was present in both experiments at 25°C, in one of the experiments at 30°C and in the experiment at 37°C.

guinea-pig cerebellum was measured at various concentrations of [^{3}H]-mepyramine. In some of these experiments, in which the number and spread of the data points was adequate, the level of doxepin-insensitive binding of [^{3}H]-mepyramine was determined by non-linear regression analysis (see Methods). In no case did the value obtained differ significantly from the percentage insensitive to 2 μM promethazine.

The variation of IC_{50} with [^{3}H-mepyramine] was linear with a positive slope (Figure 1), indicating a competitive interaction. The affinity constant of doxepin, obtained from 1/intercept, was $1.12 \pm 0.45 \times 10^{10}$ M^{-1} and that for [^{3}H]-mepyramine, from slope/intercept, $1.60 \pm 0.63 \times 10^9$ M^{-1}.

There was no indication in this series of experiments that the binding of doxepin was other than to a single population of sites. To confirm this, Hill coefficients were determined for doxepin inhibition of the binding of low concentrations of [^{3}H]-mepyramine in a series of measurements using tissues from 3 regions of guinea-pig brain. To reduce the appreciable experimental error encountered in the experiments using very low concentrations of protein (cf. legend to Figure 1), the concentration present was increased to 0.34 mg ml^{-1}. Parallel measurements were also made of mepyramine inhibition of [^{3}H]-mepyramine binding.

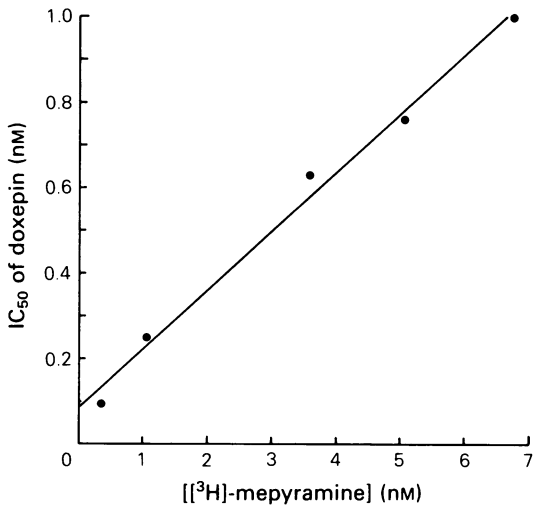

Figure 1 Variation of IC_{50} for doxepin inhibition of the promethazine-sensitive binding of [^{3}H]-mepyramine with the concentration of [^{3}H]-mepyramine. Each point was derived from an inhibition curve with 6–11 concentrations of doxepin (5–7 replicate determinations at each concentration), except that the value of the IC_{50} at the lowest concentration of [^{3}H]-mepyramine, 0.35 nM, is the mean of 5 independent measurements at a protein concentration of 0.035–0.060 mg ml^{-1}. The experiment with 1.06 nM [^{3}H]-mepyramine contained 0.12 mg protein ml^{-1} and the others 0.36 mg protein ml^{-1}. The percentage inhibition given by 2 μM promethazine was measured in each experiment. The line drawn was obtained from linear regression analysis.

Table 2 Hill coefficients of curves of doxepin and mepyramine inhibition of [^{3}H]-mepyramine binding in guinea-pig brain

	Hill coefficient	
Region	*Mepyramine*	*Doxepin*
Cerebellum	1.02 ± 0.09 (15)*	1.07 ± 0.04 (13)
Cerebral cortex	0.96 ± 0.05 (9)	1.10 ± 0.08 (11)
Hippocampus	0.93 ± 0.04 (13)	0.98 ± 0.05 (15)

Hill coefficients are best-fit values ± estimated s.e. mean obtained from non-linear regression analysis of curves of doxepin or mepyramine inhibition of the binding of 0.21–0.43 nM [^{3}H]-mepyramine. The number of points on each curve is shown in parentheses. All experiments contained 0.34 mg protein.

*Taken from Wallace (1983).

For neither mepyramine nor doxepin in any of the regions was the Hill coefficient, n_H, significantly different from unity (Table 2). However, the curve for mepyramine inhibition of [^{3}H]-mepyramine binding in hippocampus, overall n_H 0.93 ± 0.04, showed evidence of a tail on the end of the curve and was fitted well, assuming binding of [^{3}H]-mepyramine to two independent sites with 78 ± 2% associated with the high-affinity site, 6 ± 2% with a secondary site (K_a $1.6 \pm 1.8 \times 10^7$ M^{-1}) and 16 ± 3% mepyramine-insensitive binding. The K_a for the high-affinity site, $1.0 \pm 0.1 \times 10^9$ M^{-1} (Table 3) is reasonably close to the usual value for binding to H_1-receptors (cf. Table 3 and 1.6×10^9 M^{-1} from Figure 1). A second experiment with the same preparation of hippocampal homogenate, but using doxepin as inhibitor, showed clear evidence of a tail on the curve and the propor-

Table 3 Affinity constants for doxepin and mepyramine binding to guinea-pig brain

	K_a (M^{-1})	
Region	*Mepyramine*	*Doxepin*
Cerebellum	$1.5 \pm 0.1 \times 10^9$	$8.5 \pm 0.1 \times 10^9$
Cerebral cortex	$1.4 \pm 0.1 \times 10^9$	$1.7 \pm 0.2 \times 10^{10}$
Hippocampus	$1.0 \pm 0.1 \times 10^9$	$8.4 \pm 0.1 \times 10^9$

Affinity constants were calculated from best-fit values of the IC_{50} as described under Methods. The value for mepyramine in hippocampus is the high-affinity component from a two-site fit to the inhibition curve (cf. text). All experiments were carried out at 30°C and contained 0.34 mg protein.

tions of the three components, 75 ± 1% for the high-affinity site (K_a 9.5 × 10^9 M^{-1}), 13 ± 2% for the secondary site and 12 ± 1% doxepin-insensitive, were similar to those in the experiment with mepyramine. However, the secondary site is apparently not invariably present in hippocampal homogenates. A second experiment with doxepin using a different homogenate yielded a simple hyperbolic curve, as mirrored by the Hill coefficient of 0.98 ± 0.05 (Table 2). The percentage of doxepin-insensitive and 2 μM promethazine-insensitive binding of [^{3}H]-mepyramine, usually *circa* 16%, were closely similar for the hippocampal homogenates, as was also the case in all experiments with cerebellar and cerebral cortical homogenates. The levels of non-specific binding in the latter tissues were approximately 8% and 20–30%, respectively.

The affinity constants determined for mepyramine in cerebellum and cerebral cortex (Table 3) were in good agreement with the value, 1.6 ± 0.6 × 10^9 M^{-1}, deduced from the series of experiments represented in Figure 1. The values for doxepin in cerebellum and hippocampus are lower than that from the IC_{50} v. [[^{3}H]-mepyramine] plot and may reflect some depletion of the free ligand concentration.

Doxepin and mepyramine inhibition of [^{3}H]-mepyramine binding to rat brain

Measurements of mepyramine and doxepin inhibition of [^{3}H]-mepyramine binding to homogenates of rat cerebral cortex and rat whole brain were made under the same experimental conditions as in guinea-pig brain. The best fit values of the Hill coefficient and the affinity constant are set out in Table 4. The errors were greater in these experiments than in the corresponding series with guinea-pig tissues, partly because of the very low H_1-receptor occupancy of 0.44–0.57 nM [^{3}H]-mepyramine in rat brain (13–17%, taking K_a for [^{3}H]-mepyramine to be 3.5 × 10^8 M^{-1}) and the consequent low level of inhibitor-sensitive binding (350–450 d.p.m.), representing 41–50% of the total amount of [^{3}H]-mepyramine bound.

In none of the experiments with either doxepin or mepyramine was there any indication of binding to more than a single site (Table 4). The curve for doxepin inhibition of [^{3}H]-mepyramine binding to whole brain homogenate is shown in Figure 2. In one of the experiments in cerebral cortex with mepyramine the Hill coefficient was significantly greater than unity (Table 4), but this was not reproducible. The lack of evidence for any secondary sites contrasts with our earlier observations (Hill & Young, 1980), but this is apparently not solely due to the lower concentration of [^{3}H]-mepyramine employed here, since even when in one experiment the concentration was increased to 2.4 nM, the Hill coefficient was still near unity (Table 4).

The affinity constant for mepyramine binding to H_1-receptors in rat brain, 3.5 ± 0.4 × 10^8 M^{-1} (mean ± s.e. of the 4 determinations in Table 4) is clearly lower than in guinea-pig brain (Tables 1 and 3), in confirmation of previous reports (Chang *et al.*, 1979; Palacios *et al.*, 1979; Hill & Young, 1980). In contrast the values obtained for doxepin (Table 4) are similar to those obtained in guinea-pig brain using the same experimental protocol (Table 3).

Discussion

The results presented above provide no indication that the binding of doxepin to the H_1-receptor in either

Table 4 Parameters of mepyramine and doxepin inhibition of [^{3}H]-mepyramine binding to rat cerebral cortex and whole brain

Inhibitor	*[[^{3}H]-mepyramine]*	n	n_H	K_a (M^{-1})
Cerebral cortex				
Mepyramine	0.45 nM	17	1.57 ± 0.15	3.0 ± 0.3 × 10^8
	0.49 nM	15	0.94 ± 0.10	3.7 ± 0.6 × 10^8
	2.44 nM	14	1.16 ± 0.23	2.7 ± 0.9 × 10^8
Doxepin	0.44 nM	17	1.00 ± 0.13	7.2 ± 1.0 × 10^9
	0.57 nM	15	1.07 ± 0.17	8.1 ± 1.4 × 10^9
Whole brain				
Mepyramine	0.50 nM	15	1.51 ± 0.31	4.6 ± 0.6 × 10^8
Doxepin	0.43 nM	14	1.04 ± 0.14	6.3 ± 0.7 × 10^9

Best-fit values of the Hill coefficient, n_H, and the IC_{50} were obtained by non-linear regression analysis and affinity constants, K_a, calculated from IC_{50} as described under Methods. *n* is the number of points on each curve. All experiments were carried out at 30°C and all incubations contained 0.34 mg protein.

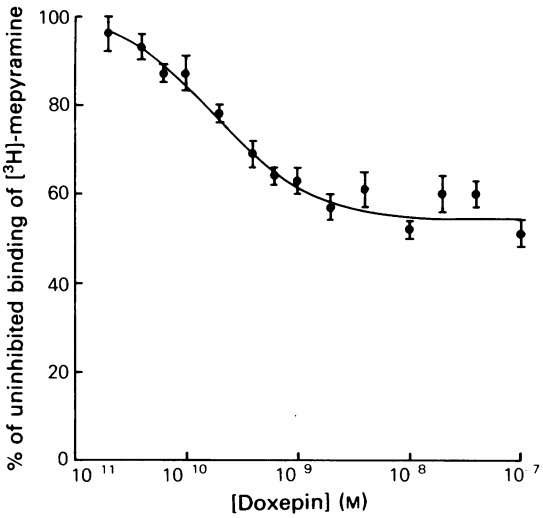

Figure 2 Inhibition by doxepin of the binding of 0.43 nM [^{3}H]-mepyramine to rat whole brain homogenate. The experimental conditions were as described under Methods. Error bars represent the approximate s.e. of the ratio × 100 of the binding in the presence of doxepin (5 replicates) to that with no doxepin present (20 replicates). The curve drawn was obtained from weighted non-linear regression analysis (see Methods).

guinea-pig or rat brain is anything but a simple equilibrium with a uniform population of binding sites. The same is in general true for mepyramine. Only in one homogenate of guinea-pig hippocampus was there any evidence for secondary binding sites. The apparent discrepancy between these observations and those from earlier studies in which Hill coefficients < 1 were reported for mepyramine inhibition of [^{3}H]-mepyramine binding (Hill *et al.*, 1978; Coupet & Szuchs-Myers, 1981; Hill & Young, 1981) could be due to differences between tissues, to the lower concentration of [^{3}H]-mepyramine used in the present study, as the work of Hadfield *et al.* (1983) would suggest, or to a modification of the protocol of the binding assay, in which excess non-radioactive mepyramine is present in the ice-cold buffer added to terminate the equilibration (Daum *et al.*, 1982; Wallace & Young, 1983). Whatever the reason may be, it is clear that under the conditions used the interpretation of doxepin inhibition curves is not complicated by non-uniform binding of [^{3}H]-mepyramine. Hill coefficients near unity also make it unlikely that the lower affinity of mepyramine in rat brain compared with guinea-pig brain is an artifact associated with high levels of non-specific binding, as has been suggested (Carswell & Nahorski, 1982). The mean value determined in the present study, $3.5 \times 10^8\ M^{-1}$ is the same as the value recently obtained, $3.5 \times 10^8\ M^{-1}$, for mepyramine inhibition of histamine-induced accumulation of inositol 1-phosphate in lithium-treated slices of rat cerebral cortex (Brown *et al.*, 1984) and compares well with the K_a values, $5.3 \times 10^8\ M^{-1}$ and $3.4 \times 10^8\ M^{-1}$ obtained from mepyramine inhibition of the histamine and 2-pyridylethylamine induced fall in coronary perfusion pressure in the rat isolated heart (Aker *et al.*, 1984).

The binding measurements represented in Figure 1 confirm that doxepin is a competitive inhibitor of [^{3}H]-mepyramine binding. This cannot be inferred with certainty from a slope of unity of a Schild plot derived from antagonism of agonist action on a tissue with a large receptor reserve, e.g. histamine-induced contraction of guinea-pig intestinal muscle strips, since an irreversible or pseudo-irreversible antagonist can produce the same result (Rang, 1966). The very high affinity of doxepin for the H_1-receptor makes it likely that it will have undergone only a very limited equilibration with histamine in the time that the agonist is present (20–40 s). The affinity constants determined for doxepin on guinea-pig tissues from inhibition of the functional response to histamine and from inhibition of [^{3}H]-mepyramine binding are in reasonable accord and are comparable with the value of $1.8 \times 10^{10}\ M^{-1}$ at 37°C obtained by Figge *et al.* (1979) from measurements on guinea-pig ileal segments. The value of the affinity constant for doxepin in rat brain appears to be of the same order of magnitude as in guinea-pig brain, although in the rat at present there is no value determined from inhibition of a functional response with which the binding values may be compared. The reasons for the rather low values of the affinity constant for doxepin reported from some of the binding studies in both guinea-pig and rat brain (Chang *et al.*, 1979; Tran *et al.*, 1978; Coupet & Szuchs-Myers, 1981) are not entirely clear. The temperature at which measurements were made does not seem to be a major factor and it is more likely that the low values are a result of the very low concentrations of doxepin needed for significant receptor occupancy (50% occupancy at approximately $5 \times 10^{-11}\ M^{-1}$) and the consequent ease with which depletion of the concentration of free doxepin through tissue binding can occur. This has been demonstrated directly for doxepin inhibition of [^{3}H]-mepyramine binding in rat brain (Taylor & Richelson, 1980). A small degree of depletion may also be responsible for the lower values of the affinity of doxepin in cerebellum and hippocampus in the experiments in Table 3, but the magnitude of the effect indicates that any increase in the Hill coefficient as a result of depletion, if the depletion were due solely to receptor binding, would be small (Hill, 1979). In the experiments carried out on rat brain homogenates the 'concentration' of receptor present, *circa* 0.05 nM, was less than the smallest concentration, 0.1 nM, used by Taylor & Richelson (1980) in their

study of the effect of increasing the amount of receptor present on the apparent affinity of doxepin.

The point of particular interest in the present measurements on rat cerebral cortex and rat whole brain is that the Hill coefficients of unity give no indication that doxepin binds to a sub-set of the receptors labelled by [^{3}H]-mepyramine. The errors inherent in the experiments (cf. Figure 2 and Table 4) make it difficult to be certain that a small fraction of the receptor population, certainly less than 10%, did not have a different affinity for doxepin than the majority. However, it would still be difficult to reconcile our observations, K_a 7×10^9 M^{-1}, with those from the earlier study with [^{3}H]-doxepin, where the bulk of the binding, presumably including the putative low-affinity [^{3}H]-mepyramine sites, was to a saturable site with K_a 2.8×10^8 M^{-1} (Taylor & Richelson, 1982). It seems more likely that the differences between the two studies arise from the difficulties of analysis of the complex binding of [^{3}H]-doxepin. In discussing the evidence for and against their proposal that doxepin may bind preferentially to a sub-class of histamine H_1-receptors in rat brain, Taylor & Richelson (1982) noted that the differences they observed between [^{3}H]-mepyramine and [^{3}H]-doxepin binding were 'somewhat contradictory and might not represent a true biological phenomenon'. The results of the present study suggest that this note of caution may be entirely justified.

Our thanks are due to the Medical Research Council for financial support. Part of this work was carried out during a visit of J.M.Y. to Mexico under the auspices of the Royal Society and the Academia de Investigacion of Mexico. We are grateful to Mr E. Addison of Pfizer Ltd for providing details of the *cis : trans* isomer ratio of doxepin.

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(*Received June 15, 1984.*
Revised September 12, 1984.
Accepted September 28, 1984.)