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# The Catecholamine Carrier of Bovine Chromaffin Granules

## Form of the Bound Amine

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SUMMARY

The binding of [2-$^3$H]dihydrotetrabenazine (2-hydroxy-3-isobutyl-9,10-dimethoxy-1,2,3,-4,6,7-hexahydro-11bH-benzo [*a*]quinolizine), a tetrabenazine derivative which binds to the catecholamine carrier of chromaffin granule membranes, has been studied as a function of the pH. The number of binding sites was constant from pH 6.5 to pH 9.0, whereas the $K_D$ decreased to a minimal plateau value, obtained at pH values higher than 7.5, the drug p$K_a$. The pH dependency of the displacement of [$^3$H]dihydrotetrabenazine by noradrenaline was also investigated. Noradrenaline $K_D$ values derived from displacement experiments decreased logarithmically when the pH increased from 6.5 to 8.5, i.e., for pH values lower than the p$K_a$ of noradrenaline. These pharmacological data support our previous hypothesis based on kinetic data [Scherman and Henry, *Eur. J. Biochem.* **116**:535–539 (1981)] that the monoamine carrier of the chromaffin granule membrane binds and transports neutral amines, a form of low abundancy at physiological pH but for which it has a high affinity.

## INTRODUCTION

Catecholamines are taken up by the chromaffin granules of adrenal medulla by an active ATP-dependent process (1, 2). This process involves (a) an inwardly directed ATP-dependent $H^+$-translocase, which generates a proton electrochemical gradient (3–6), and (b) a reserpine- and tetrabenazine-sensitive specific carrier, which utilizes the proton electrochemical gradient to accumulate catecholamines (7–10). Kinetic (9–11) and thermodynamic (12) data have shown that catecholamine translocation is electrogenic, and it has been proposed that the carrier exchanges either a neutral amine for a proton or a protonated amine for two protons, both models being thermodynamically equivalent (8, 13). Discriminating between these two hypotheses requires determination of which form of the amine binds to the carrier. In a previous communication (14), we analyzed the pH dependency of noradrenaline uptake and we have showed that the $K_m$ for noradrenaline decreased logarithmically with the pH in the 6.0–8.5 pH range, whereas the $V_{max}$ varied only slightly. This observation suggested the transport of the less abundant neutral form, the concentration of which increased logarithmically with the pH at pH values lower than the p$K_a$. Nevertheless, this result was not fully conclusive since $K_m$ changes might have also been interpreted as reflecting a pH-dependent modification of the affinity of the carrier. In addition, similar (15–17) but also opposite (18) results have been reported, the latter leading to an opposite interpretation.

We have recently shown (19) that 2-hydroxy-3-isobutyl-9,10-dimethoxy-1,2,3,4,6,7-hexahydro-11bH-benzo[*a*] quinolizine (TBZOH[1]) bound to the monoamine carrier ($K_D$ = 2.5–5 nM, $B_{max}$ = 60 pmoles/mg of protein) and that noradrenaline displaced [$^3$H]TBZOH from its binding sites. Since TBZOH has a p$K_a$ of 7.5 (20), the relative abundance of its neutral form in the 6.5–9 pH range does not vary logarithmically with the pH, thus allowing comparison with noradrenaline. In the present communication, we have investigated the effect of pH on the characteristics of [$^3$H]TBZOH binding and on [$^3$H]TBZOH displacement by noradrenaline. The results give some new information on the nature of the bound form of the inhibitor TBZOH and of the substrate noradrenaline, and thus on the mechanism of catecholamine uptake.

## MATERIALS AND METHODS

*Chromaffin granule membrane preparation.* Bovine chromaffin granule membranes were prepared by osmotic lysis of granules isolated by centrifugation on a 1.6

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[1] The abbreviation used is: TBZOH, dihydrotetrabenazine.

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M sucrose layer (21, 22). Membranes were frozen in liquid nitrogen and were stored at −80°. They were rapidly thawed at 37°, centrifuged at 100,000 × *g* for 15 min, and resuspended in 5 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid/NaOH buffer (pH 7.5) containing 0.3 M sucrose.

*[$^3$H]TBZOH binding studies.* Membranes (10–15 μg of protein per milliliter, corresponding to 0.6–0.9 nM TBZOH binding sites) were incubated in 1 ml of 0.3 M sucrose/20 mM Tris-phosphate buffer at the indicated pH with various concentrations of [$^3$H]TBZOH at maximal specific activity ($K_D$* determination) or 1 nM [$^3$H]TBZOH and various unlabeled TBZOH concentrations ($K_D$ determination) or 1 nM [$^3$H]TBZOH, 2.5 mM ATP, 1.25 mM $MgSO_4$, and various noradrenaline concentrations (displacement experiments). After a 1-hr incubation at 25°, the medium was diluted by the addition of 4 ml of ice-cold sucrose buffered at the same pH and containing 100 μM tetrabenazine. The mixture was immediately filtered on HAWP Millipore filters previously washed by the same buffer. The filters were then washed twice with 4 ml of the same buffer and their radioactivity was measured by liquid scintillation in Aqualuma (Lumac). Nonspecific binding was determined by the addition of 2 μM TBZOH to the incubation mixture. It was proportional to free [$^3$H]TBZOH and it represented less than 20% of bound [$^3$H]TBZOH and less than 2% of total [$^3$H]TBZOH. Control experiments indicated that nonspecific binding was mainly due to adsorption of the drug on the filters and that it was pH-independent. Duplicate measurements of total nonspecific binding were performed, relative differences between duplicates being less than 5%. Calculation of the free and specifically bound drug concentrations, determination of the regression line describing the Scatchard plots, and calculation of $K_D$ and $B_{max}$ were performed with an HP 9825 computer. Where indicated, results are given as means ± standard error.

*Materials.* TBZOH was obtained by reduction of tetrabenazine (Fluka) by $NaBH_4$ in methanol (23). [$^3$H]TBZOH (12 Ci/mmole) was prepared as described (24).

## RESULTS

*pH dependency of [$^3$H]TBZOH binding.* The binding of [$^3$H]TBZOH was studied as a function of pH at a constant ligand concentration. Bound [$^3$H]TBZOH increased with the pH and reached a plateau value at pH 8.0 (Fig. 1). Because of the low concentration of ligand used, the increase in the binding might have originated from either an increase in the $B_{max}$ or a decrease in the $K_D$*, the equilibrium dissociation constant of [$^3$H]TBZOH. We therefore investigated systematically the effect of pH on binding isotherms (Table 1). Increasing the pH from 6.5 to 8.0 decreased $K_D$* from 11.0 to 4.7 and had a less marked effect on $B_{max}$. The increased binding shown in Fig. 1 was thus mainly due to a change in $K_D$*. It should be noted that at each pH value the Scatchard plots were linear, as shown by the high value of the correlation coefficients (Table 1). It can thus be concluded that in the pH range studied chromaffin granule membranes had only one class of binding sites, the concentration of which remained roughly constant.

The pH dependency of TBZOH binding was also investigated by measuring the effect of the pH on the displacement of [$^3$H]TBZOH bound to the membrane by unlabeled TBZOH (Table 1). At each pH the dissociation equilibrium constant ($K_D$) derived from the $EC_{50}$ was not different from $K_D$*.

The variation with pH of TBZOH binding allowed determination of the active form of the drug. Relative $K_D$ and $K_D$* [defined as (100 × $K_{D(min)}$)/$K_D$ and (100 ×

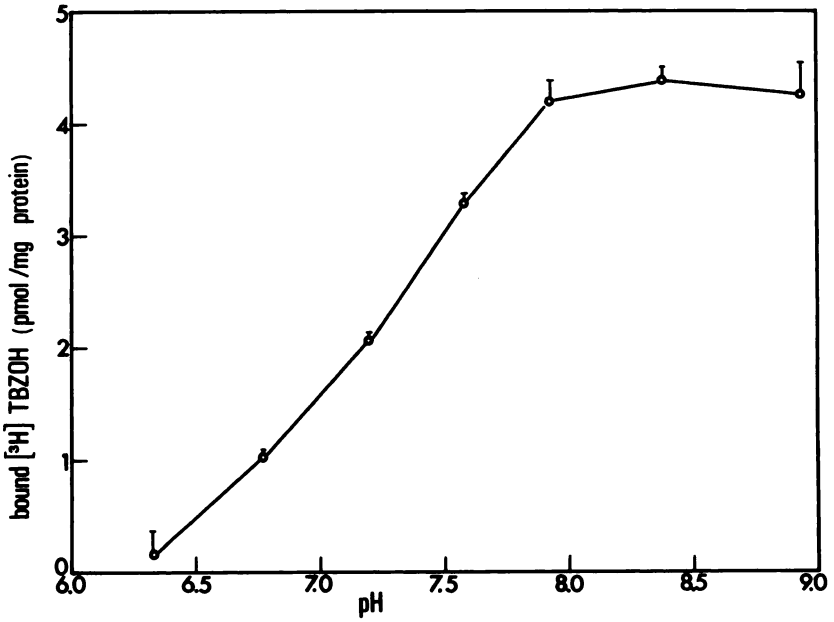



FIG. 1. *[$^3$H]TBZOH binding as a function of the pH*

Specific [$^3$H]TBZOH binding was measured as a function of the pH at a drug concentration of 1 nM and at a membrane concentration of 12 μg of protein per milliliter. Triplicate experiments were performed; *bars* indicate standard error.

TABLE 1

*pH dependency of TBZOH binding*

[$^3$H]TBZOH binding and [$^3$H]TBZOH displacement by TBZOH were performed as indicated under Materials and Methods. In the former case, 10 [$^3$H]TBZOH concentrations were used which were in the 2–50 nM range for pH 6.0 and 6.5 experiments and in the 0.5–25 nM range for other pH values. For [$^3$H]TBZOH displacement experiments, 6–8 TBZOH concentrations were used and triplicate determinations were made. Maximal binding in the absence of TBZOH represented less than 12% of the total radioactivity and had a pH profile identical with that of Fig. 1. Nonspecific binding (less than 10% of bound radioactivity) was not affected by the displacer concentration and was subtracted. Energization by ATP did not change the binding constants.

| pH | [$^3$H]TBZOH binding | | | [$^3$H]TBZOH displacement by TBZOH | |
|---|---|---|---|---|---|
| | $K_D$*[a] | $B_{max}$[a] | Correlation coefficient[a] | $EC_{50}$[b] | $K_D$[c] |
| | *nM* | *pmoles/mg protein* | | *nM* | *μM* |
| 6.0 | 13.9 | 30.2 | 0.98 | 21.0 | 19.6 |
| 6.5 | 11.0 | 48.0 | 0.98 | 9.5 | 8.7 |
| 7.0 | 6.9 | 53.0 | 0.97 | 7.2 | 6.4 |
| 7.5 | 5.45 ± 0.05 | 58.5 ± 1.0 | 1.0 | 6.0 | 5.1 |
| 8.0 | 4.7 | 61.5 | 0.96 | 5.5 | 4.6 |
| 8.5 | 4.9 | 64.0 | 1.0 | 5.5 | 4.6 |
| 9.0 | 4.8 | 60.8 | 0.99 | 6.2 | 5.2 |

[a] Determined from Scatchard plots of the data.

[b] Determined from the linear part of semilog plots (Hill coefficient, 1.0)

[c] Derived from the $EC_{50}$ by the formula: $K_D = EC_{50}/(1 + S^*/K_D^*)$ (ref. 24), with $S^* = 1$ nM.

$K_D^*{}_{(min)})/K_D^*$ where $K_{D(min)}$ and $K_D^*{}_{(min)}$ were minimal values observed at alkaline pH] were plotted as a function of pH (Fig. 2). The experimental data fitted reasonably well the theoretical line describing the relative abundance of the neutral form of the drug. This experiment thus indicates that binding involves the deprotonated form of the molecule and not the cationic one, the abundance of which varies with pH in a different (symmetrical) way. The variation with pH of the relative $K_I$ [defined as $(100 \times K_{I(min)}/K_I$] derived from previously measured values of the inhibition constant of noradrenaline uptake by TBZOH (20) is also indicated in Fig. 2. The similarity between kinetic and binding data supports the view that TBZOH binding occurs on the monoamine carrier.

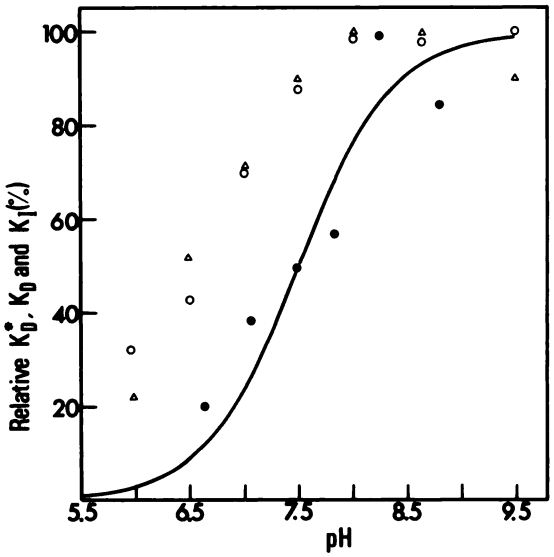



FIG. 2. *Relationship between the activity and binding of TBZOH and the relative abundance of its neutral form*

The percentage of the neutral form (*solid line*) was calculated assuming a $pK_a$ of 7.5 for TBZOH. Relative $K_D^*$ (○) and $K_D$ (△) were defined as indicated in the text and were derived from the data of Table 1, with $K_D^*{}_{(min)} = 4.7$ nM and $K_{D(min)} = 4.6$ nM. Relative $K_I$ values (●) were taken from Footnote 2, with $K_{I(min)} = 4.6$ nM.

*Displacement of bound [$^3$H]TBZOH by noradrenaline.* It has previously been shown that noradrenaline displaced [$^3$H]TBZOH from its binding sites on chromaffin granule membranes (19). This experiment was performed at pH 7.5 under conditions which induced catecholamine accumulation, i.e., in the presence of ATP and $MgSO_4$, allowing direct comparison with $K_m$ measurements (14). Noradrenaline also displaced [$^3$H]TBZOH with similar characteristics in the absence of ATP-$MgSO_4$. Under these conditions, the effect of noradrenaline was of the competitive type (Fig. 3). A $K_I$ of 1.6 mM (at pH 7.5) was derived from the data (Fig. 3, *inset*).

The experiment of displacement of TBZOH by noradrenaline was repeated at various pH values (Fig. 4). This parameter had a marked effect on the $EC_{50}$, without affecting the shape of the curve characterized by a Hill coefficient of 0.9–1.0. An increase in the pH resulted in a decrease in the $EC_{50}$ (Fig. 4). The dissociation constant of noradrenaline was calculated from the $EC_{50}$ (Table 2). The value at pH 7.5 ($K_D$ = 1.7 mM) did not differ from the $K_I$ derived from the data of Fig. 3 (1.6 mM), thus showing that displacement experiments had been conducted correctly. The $K_D$ of noradrenaline varied dramatically with the pH and in this respect differed from that of [$^3$H]TBZOH or TBZOH.

## DISCUSSION

The pH dependency of the properties of the catecholamine carrier has been summarized in Fig. 5. In addition to the pharmacological results described in this communication, Fig. 5 also includes previously reported kinetic data (14).

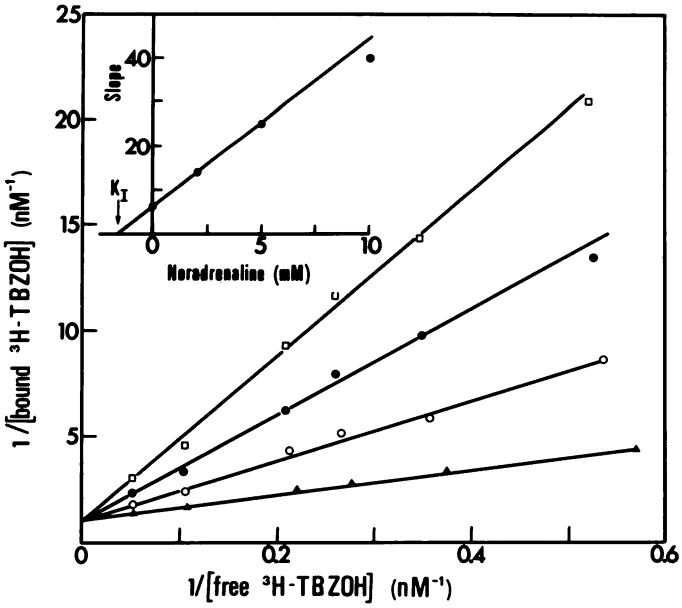



FIG. 3. *Competition between [³H]TBZOH and noradrenaline binding*

Membranes (15.5 μg of protein per milliliter) were incubated for 40 min at 25° in the presence of [³H]TBZOH, 2.5 mM ATP, 1.25 mM $MgSO_4$, and various concentrations of noradrenaline: ▲, 0 mM; ○, 2 mM; ●, 5 mM; □, 10 mM. Duplicate measurements were performed. Results were plotted according to Lineweaver and Burk. *Inset*, Determination of the $K_I$ for noradrenaline. $K_I$ = 1.6 mM.

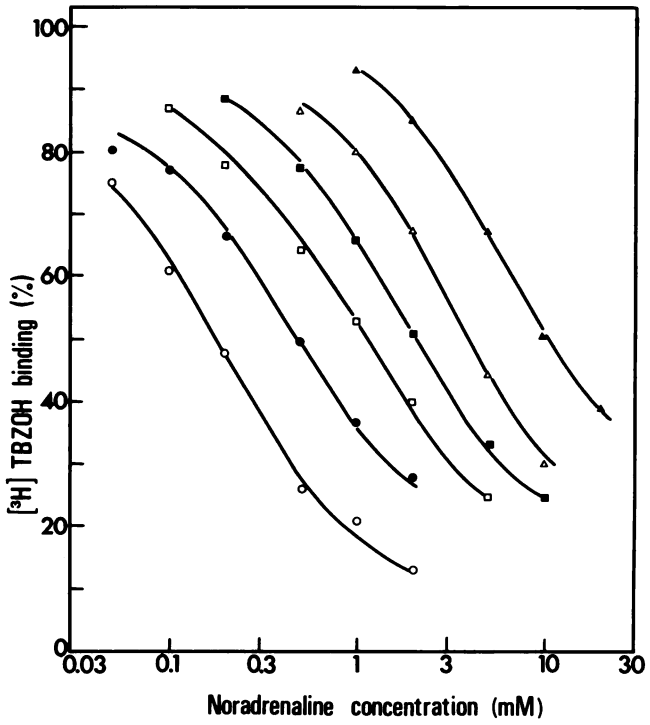



FIG. 4. *Displacement of [³H]TBZOH by noradrenaline at various pH values*

Membranes (12 μg of protein per milliliter) were incubated with [³H]TBZOH, ATP, $MgSO_4$, and noradrenaline, as indicated under Materious and Methods. [³H]TBZOH binding in presence of noradrenaline (duplicate measurement) was expressed as a percentage of controls performed without noradrenaline and at the same pH (triplicate measurements). Nonspecific binding (less than 10% of bound radioactivity) was not affected by noradrenaline concentration and was subtracted. The pH profile of the controls was identical with that shown in Fig. 1. The pH of the medium was as follows: ▲, 6.64; △, 7.07; ■, 7.45; □, 7.82; ●, 8.26; ○, 8.78. At pH 8.8, the addition to the incubation medium of bovine superoxide dismutase, which blocks noradrenaline autoxidation (14), had no effect on the $K_D$ value.

Figure 5A describes the behavior of parameters related to the number of active carrier molecules, $B_{max}$ for [³H]TBZOH binding and $V_{max}$ for noradrenaline uptake. Both are independent of the pH in the 6.5–8.0 pH range, thus showing that the monoamine carrier has no ionizable group with a $pK_a$ in the investigated pH range. This contention is supported by the linearity of the different Scatchard plots describing the saturation isotherms of [³H]TBZOH binding, since the existence of two forms of the carrier having different affinity and in acido-basic equilibrium would imply nonlinear Scatchard plots.

Figure 5B summarizes the information on the inhibitor TBZOH. The pH affected in the same way the equilibrium dissociation constant (measured as $K_D^*$ for [³H]TBZOH or $K_D$ for TBZOH) and the inhibition constant $K_I$ for noradrenaline uptake (20). From the data of Fig. 2, it is concluded that the monoamine carrier binds only to the neutral form of the drug (for further discussion, see ref. 20). According to this interpretation, at pH lower than the $pK_a$ of the drug ($pK_a$ = 7.5 for TBZOH), only apparent $K_D$, $K_D^*$, and $K_I$ were measured, which were dependent upon the relative concentration of neutral amine. True values were measured at pH higher than 7.5 and were $K_{D(min)}$, $K_D^{*}{}_{(min)}$, and $K_{I(min)}$ used in Fig. 2.

Figure 5C summarizes the data available on noradrenaline, a substrate of the carrier. Considering the structural analogy existing bewteen noradrenaline and TBZOH, the substrate and the inhibitor were anticipated to present similarities in their binding to the carrier. This hypothesis is supported by the data of Fig. 3, showing that [³H]TBZOH and noreadrenaline bound competitively. Nevertheless, the noradrenaline equilibrium dissociation constant, $K_D$ (derived from [³H]TBZOH displacement experiments), and the Michaelis constant, $K_m$, of noradrenaline (14) have the same pH dependency, which differs from that of the constants of TBZOH shown in Fig. 5B. The log of noradrenaline constants decreased linearly with the pH with a slope of 0.9 (Fig. 5C). The different pH profile of the equilibrium dissocia-

TABLE 2

*pH dependency of $K_D$ for noradrenaline*

Noradrenaline equilibrium dissociation constants ($K_D$) were derived from the [³H]TBZOH displacement experiments of Fig. 4, by using the formula of Table 1 with $S^*$ = 1 nM and $K_D^*$ for [³H]TBZOH extrapolated from Table 1.

| pH | $K_D$ |
|---|---|
| | μM |
| 6.64 | 9300 |
| 7.07 | 3500 |
| 7.45 | 1730 |
| 7.82 | 930 |
| 8.26 | 410 |
| 8.78 | 145 |

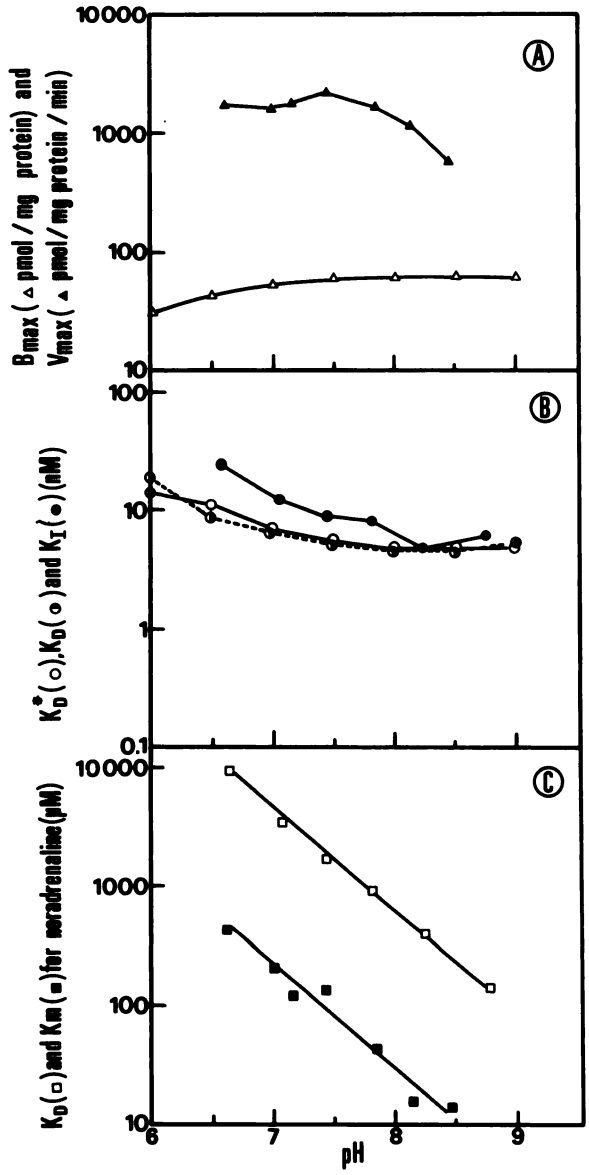



FIG. 5. *pH dependency of the properties of the catecholamine carrier*

Pharmacological (*open symbols*) and kinetic (*closed symbols*) results are expressed on a log scale as a function of the pH.

A. $B_{max}$ (△), number of [$^3$H]TBZOH binding sites derived from Table 1; $V_{max}$ (▲), maximal velocity of noradrenaline uptake derived from ref. 14.

B. $K_D^*$ (○) and $K_D$ (◑), equilibrium dissociation constants for [$^3$H]TBZOH and TBZOH, respectively, derived from Table 1; $K_I$ (●), inhibition constant of TBZOH for noradrenaline uptake, derived from ref. 20.

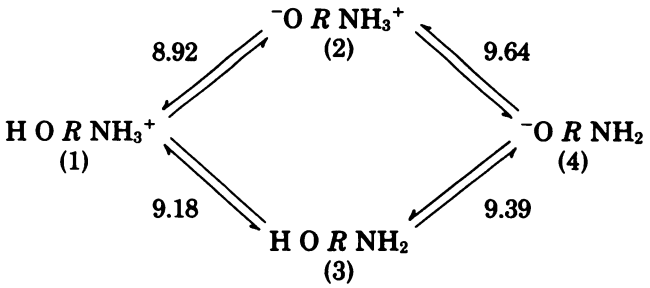
C. $K_D$ (□), noradrenaline equilibrium dissociation constant derived from Table 2. $K_m$ (■), Michaelis constant for noradrenaline uptake (ref. 14).

tion constants of the two competitive ligands, TBZOH and noradrenaline, rules out the possibility that the logarithmic variation with pH of the $K_m$ reflects a pH-dependent modification of the affinity of the carrier. The results of Figs. 5B and 5C can be easily reconciled by taking into account the high $pK_a$ values of noradrenaline (see below). In the case of the substrate, the carrier binds to a species in acido-basic equilibrium, which is of minor abundance in the investigated pH range. The constants of Fig. 5C are therefore only apparent constants, related to the true constants by the equation

$$\text{True } K_m \text{ or } K_D \sim (\text{apparent } K_m \text{ or } K_D) \times 10^{-pK_a}/[H^+]$$

This observation explains, at least partially, the inefficiency of the substrates (noradrenaline and serotonin) in displacing bound [$^3$H]TBZOH at pH 7.5 (19).

According to Pratesi and Grana (26), catecholamine acido-basic equilibria are described by the following scheme in which the $pK_a$ values of noradrenaline have been indicated:




From these data, the log of the reciprocal of the relative concentration of the various species has been calculated, and its variation with the pH is shown in Fig. 6. This scheme can be used to determine the species which binds to the carrier (active species), since the apparent $K_D$ and $K_m$ are inversely proportional to the relative concentration of the bound species. The zwitterion (*2*) and the neutral molecule (*3*) are the only species which fit the data of Fig. 5C. Our data do not allow a discrimination between these two species, but we favor the hypothesis of the transport of the neutral amine since (a) phospholipid bilayers should be more easily penetrated by the

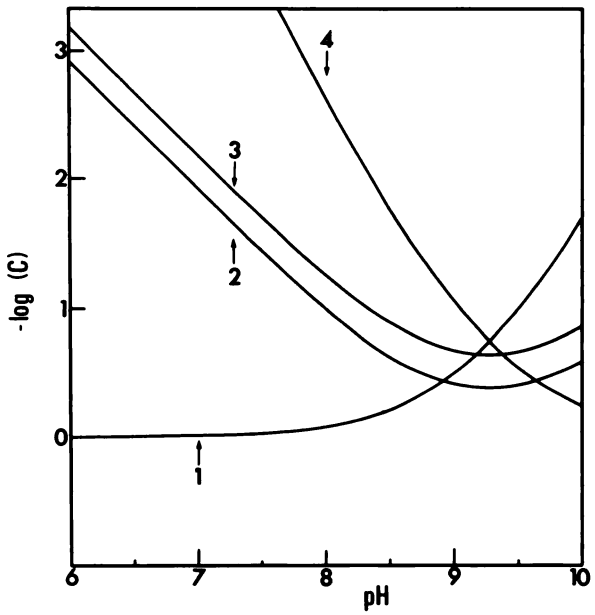



FIG. 6. *Relative abundance of the various noradrenaline species*

Curves were calculated from the data of Pratesi and Grana (26). *1*, cation; *2*, zwitterion; *3*, neutral form; *4*, anion.

uncharged than the charged species and (b) the *o*-methylated derivative TBZOH binds efficiently to the carrier. Our data allow us to conclude that the monoamine carrier does not transport the cationic form (*1*) of the amine as proposed by Knoth *et al.* (18), but that it binds and transports a form of low abundancy at physiological pH, for which it has a high affinity.

## ACKNOWLEDGMENTS

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