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# The mechanism of action of 2-halogenoethylamines at the adrenergic α-receptor and a further investigation of the "spare receptor" hypothesis*

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The evidence for "spare-receptors" has been examined in the adrenergic α-receptor system of the rat vas deferens using *N*-(2-bromoethyl)-*N*-ethyl-1-naphthylmethylamine (SY.28) and *NN*-dimethyl-2-phenylethylamine hydrobromides as the antagonist species. It is clearly demonstrated that "spare-receptors" are not present in this tissue and that the observed parallel shift of the dose-response curve of noradrenaline produced by SY.28 is due to a competitive reversible phase of action of the corresponding ethyleneimine species rather than to the presence of "spare-receptors". Additionally, these experiments have revealed the existence of a significant non-competitive reversible phase of action of SY.28. From radiochemical studies and the observed first order recovery of tissue response following blockade by *NN*-dimethyl-2-bromo-2-phenylethylamine, it is concluded that the α-receptors in the rat vas deferens are indistinguishable from the α-receptors in the rabbit aorta in their behaviour towards this agent. From these results and those of others, it may be concluded that there is no existing evidence for "spare receptors" in adrenergic α-receptor systems.

In his original treatment of drug-receptor interactions Clark (1933, 1937) assumed that tissue response is proportional to the concentration of drug-receptor complex and that maximum response occurs with complete occupation of the receptors. During the last decade these assumptions have been subjected to extensive challenge and it now appears to be widely accepted that maximum tissue response does not necessitate 100% occupation of the receptors and that a substantial receptor "reserve" ("spare-receptors") may exist (Furchgott, 1954; Nickerson, 1956; Stephenson, 1956; Ariëns, Rossum & Koopman, 1960; Paton, 1961; Rossum & Ariëns, 1962; Burgen, 1966; Mackay, 1966a, b; Rossum, 1966). Estimates of the percentage of receptors occupied at maximum response have ranged from 1% to 0·0001% (Nickerson, 1956; Paton & Rang, 1966; Schild, 1962).

The concept of "spare-receptors" appears to be supported principally by experiments involving the use of 2-halogenoethylamines which interact irreversibly at adrenergic, cholinergic and histaminergic receptor sites. In many instances it is observed that treatment of the tissue with such agents produces a shift of the dose-response curve of the agonist to the right and that this shift precedes any decrease in the

* Previous paper May, Moran & others (1967).

maximum response obtainable. Such shifts would normally be regarded as representative of competitive reversible antagonism but for the one fact that 2-halogenoethylamines are known to be alkylating agents which can bind covalently to various tissue sites. It has, therefore, been assumed quite generally (Nickerson, 1956; Ariëns, and others, 1960; Rossum, 1966; Furchgott, 1967) that such shifts of the agonist dose-response curve without depression in the maximum response indicate the presence of a receptor "reserve" which must be irreversibly inactivated before a reduction in the tissue response can be obtained. However, from theoretical considerations of the mechanism of action of 2-halogenoethylamines we have proposed (Triggle, 1965a) that the portion of the dose-response curves considered to depict receptor "reserve" is in fact best attributed to a *competitive reversible* binding of the ethyleniminium ion at the receptor.

Recent work from our laboratory (Moran, May & others, 1967; May, Moran & others, 1967) concerned with the adrenergic $\alpha$-receptor system of the rabbit aorta and utilizing a different experimental approach to that outlined above, has provided strong evidence that "spare-receptors" do not exist in this system. In view of these results and because of the importance of the "spare-receptor" concept both to quantitative pharmacology and to problems of receptor isolation, we have initiated a program to re-examine the experimental evidence upon which the "spare-receptor" hypothesis is founded and to provide a mechanistic explanation for the observed shifts in dose-response curves.

## METHODS

*Pharmacology.* Pairs of rat vas deferens were suspended in water-jacketed baths containing 20 ml of modified Krebs-bicarbonate solution (Huković, 1961) maintained at 37° and bubbled with 5% carbon dioxide in oxygen. Cumulative dose-response curves were constructed from recording of isotonic contractions obtained by means of an ink-writing lever on a kymograph. Two control dose-response curves for noradrenaline were always determined on each tissue before treatment of one tissue with blocking agent: the paired tissue was not treated with blocking agent and served as a control.

Solutions of the 2-bromoethylamines were made up in neutral saline and allowed to stand at room temperature for 30 min before placing on ice. This insured that formation of the derived ethyleniminium ions was essentially complete.

Tissues were blocked with *NN*-dimethyl-2-bromo-2-phenylethylamine and cumulative dose-response curves of noradrenaline were obtained during the recovery of the response at the stated time intervals. Repetition of dose-response curves at intervals of less than 30 min occasionally resulted in erratic responses so that in general only two curves, determined at 30 min intervals, were obtained from the same tissue; the paired tissue being used as a control. The first-order rates of recovery of tissue response were determined as previously described (May & others, 1967).

Other tissues were blocked to varying degrees with SY.28, [*N*-(2-bromoethyl)-*N*-ethyl-1-naphthylmethylamine hydrobromide], followed by determination of the dose-response curves in a cumulative manner. In some cases, this was followed by treatment with $10^{-3}$ M sodium thiosulphate containing an ED50 (determined from control curve) of noradrenaline for 4 min followed by a 15 min washout period after which the dose-response curve was redetermined.

*Radioactivity measurements.* The rate of tritium loss from tissue blocked with [$^3$H]*NN*-dimethyl-2-bromo-2-phenylethylamine was determined by taking aliquots of the bath fluid at 5 min intervals. Disintegration rates were determined by liquid scintillation counting using internal standards for quench correction (Rogers & Moran, 1966). In all cases a minimum of 10,000 counts was collected. The first order rate of tritium loss was obtained by plotting this data according to Rose (1964) as previously described (May & others, 1967).

*Partition experiments.* [$^3$H]*NN*-Dimethyl-2-hydroxy-2-phenylethylamine (May & others, 1967) was added to a mixture of 5 ml $CHCl_3$ and 5 ml $H_2O$ and allowed to equilibrate with shaking for 24 h. Aliquots from the two phases were counted and the ratio of the disintegration rates determined.

## RESULTS

Fig. 1 shows the dose-response curves of noradrenaline determined on the rat vas deferens before and after exposure to SY.28 at a concentration of $2{\cdot}5 \times 10^{-7}$M for 1 min followed by washout of excess SY.28. It is apparent that, after this treatment with SY.28, the dose-response curve of noradrenaline is shifted to the right with no decrease in maximum response, a finding which accords with previously published data of other workers (Ariëns & others, 1960; Ariëns, Simonis & Rossum, 1964) and which is normally interpreted as evidence for a receptor "reserve" despite the fact that this phenomenon is typical of competitive antagonism. After the blockade by SY. 28, sodium thiosulphate ($10^{-3}$ M) and noradrenaline ($3 \times 10^{-6}$ M, ED50) were added for 15 min, washed out, and the dose-response curve to noradrenaline redetermined. It can be seen (Fig. 1) that the dose-response curve is shifted back to the left and is essentially super-imposable upon the control curve at response levels of greater than 60%. The inset in Fig. 1 shows that sodium thiosulphate alone has no significant effect.

Some tissues were blocked with SY. 28 to a degree sufficient to cause a decrease in maximum response and were then treated with sodium thiosulphate in the presence of

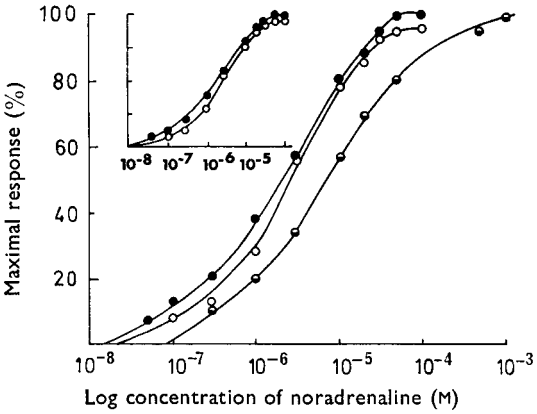


FIG. 1. Effect of sodium thiosulphate on blockade mediated by SY. 28. Rat vas deferens was blocked with SY. 28 ($2{\cdot}5 \times 10^{-7}$ M) for 1 min and the dose-response curve for noradrenaline determined after washout (◒). Tissues were then treated with $10^{-3}$ thiosulphate for 15 min and the dose-response curve redetermined (○). Controls were treated with saline (●). Inset shows the effect of $10^{-3}$ M sodium thiosulphate alone (○) as compared to controls (●). Dose-response curves are the average of 9 experiments.

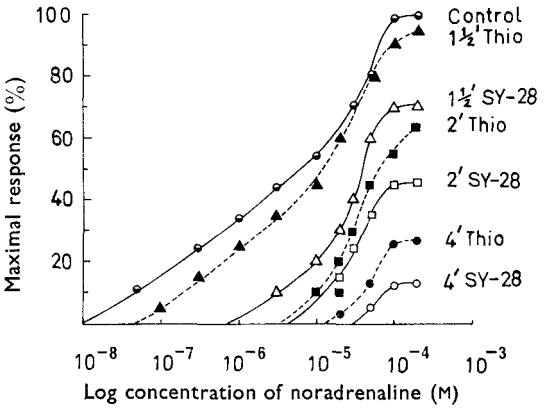


FIG. 2. Effect of sodium thiosulphate on the response of vas deferens blocked to varying degrees with SY. 28. Dose-response curves for noradrenaline were determined on control tissues (◒) and compared to the curves obtained from tissues blocked with SY. 28 ($2{\cdot}5 \times 10^{-7}$) for the designated times before (——) and after (- - - -) treatment with $10^{-3}$ M sodium thiosulphate.

an ED50 of noradrenaline ($3 \times 10^{-6}$ M). From Fig. 2 it can be observed that thiosulphate relieves a portion of the inhibition as measured by the maximum response, e.g. 70% response after SY.28 block increases to 95% response after treatment with sodium thiosulphate. Furthermore, there is an apparent shift of the dose-response curves from tissues treated with thiosulphate back towards the control curve similar to that observed in Fig. 1. This shift is more pronounced in those tissues treated for shorter periods of time with SY.28. Tissues which were blocked to the extent of 100% with SY.28 showed no reversal of inhibition when treated with noradrenaline and thiosulphate under identical conditions; this result would be expected if all receptors were alkylated.

It was not possible to make similar experiments with sodium thiosulphate using *NN*-dimethyl-2-bromo-2-phenylethylamine as the α-blocking agent since it was impossible to obtain a shift to the right of the dose-response curve of noradrenaline

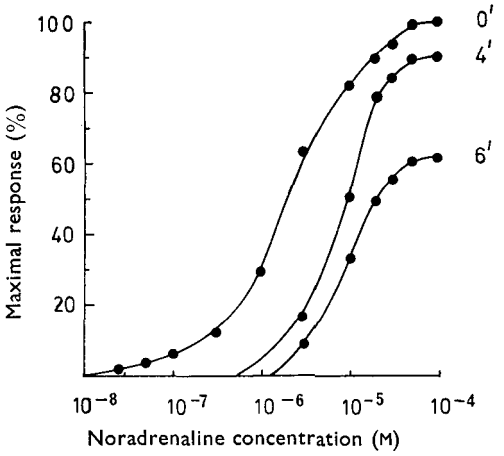


FIG. 3. Dose-response curves of noradrenaline following exposure of vas deferens to *NN*-dimethyl-2-bromo-2-phenylethylamine. Tissues were treated with *NN*-dimethyl-2-bromo-2-phenylethylamine ($1{\cdot}3 \times 10^{-6}$ M) for the times indicated followed by washout and determination of the dose-response relation. Dose-response curves represent the average of 3 experiments.

without a simultaneous decrease in the maximum response (Fig. 3). This is probably due to the greater reactivity of the *NN*-dimethyl-2-phenylethyleniminium ion (Triggle, 1964, 1965a). The situation is further complicated by the fact that *NN*-dimethyl-2-bromo-2-phenylethylamine produces an irreversible blockade of rather short duration (Triggle, 1964, 1965a; Kimelberg & Triggle, 1965; May & others, 1967) and significant recovery occurs via intramolecular hydrolysis during washout and determination of one dose-response relation.

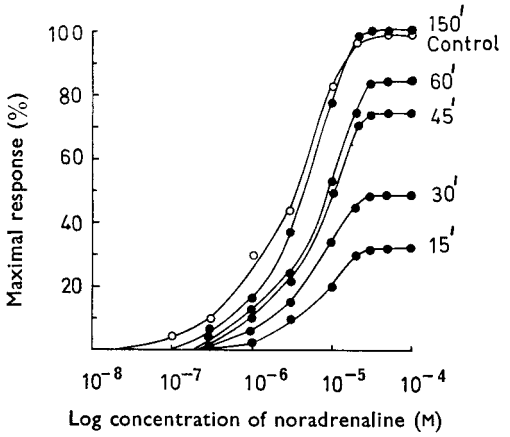


FIG. 4. Recovery of response of vas deferens blocked with *NN*-dimethyl-2-bromo-2-phenylethylamine. After complete blockade of the noradrenaline response of rat vas deferens by *NN*-dimethyl-2-bromo-2-phenylethylamine ($1{\cdot}3 \times 10^{-5}$ M for 5 min) tissue response was determined at the times indicated.

However, it was possible to determine dose-response curves for noradrenaline during the period when the response of the vas deferens was recovering from exposure to *NN*-dimethyl-2-bromo-2-phenylethylamine. Dose-response curves were determined every 15 or 30 min following complete blockade by *NN*-dimethyl-2-bromo-2-phenylethylamine ($1{\cdot}3 \times 10^{-5}$ M for 5 min). Normally, only 2 curves were determined on each tissue at 30 min intervals; the paired tissue served as a control. The results of these experiments are shown in Fig. 4 from which it is apparent that the maximum response is always obtained at essentially the same concentration of noradrenaline. Furthermore, it is important to note that there is no dose-response curve showing full recovery of maximum response with a simultaneous shift to the right relative to the control curve.

The results from Fig. 4 have been plotted as previously described in order to determine the $t_{\frac{1}{2}}$ for recovery of tissue response to noradrenaline after blockade by *NN*-dimethyl-2-bromo-2-phenylethylamine. From Fig. 5, the $t_{\frac{1}{2}}$ was found to be 22 min. In duplicate experiments, using [$^3$H]*NN*-dimethyl-2-bromo-2-phenylethylamine, the rate of radioactivity appearing in the bath fluid during the recovery process was determined and found to have a $t_{\frac{1}{2}}$ of 24 min.

In the partition experiments it was found that *NN*-dimethyl-2-hydroxy-2-phenylethylamine partitions between $CHCl_3$–$H_2O$ in a ratio of 51 : 49 as compared to a ratio of 96 : 4 for *N*-ethyl-*N*-(2-hydroxyethyl)-1-naphthylmethylamine (Moran & others, 1967).

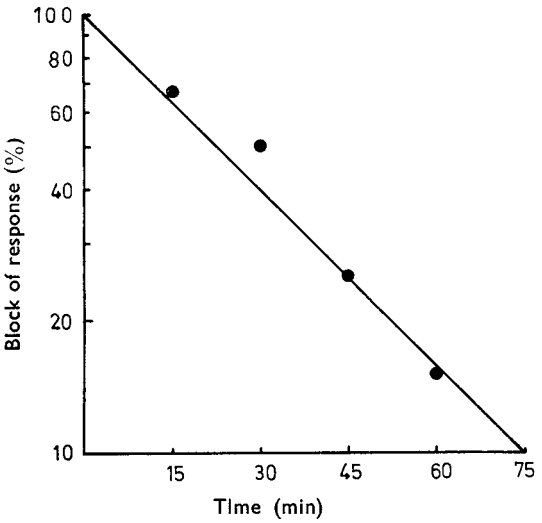


FIG. 5. Plot of first order rate of recovery of response of vas deferens to noradrenaline following blockade by *NN*-dimethyl-2-bromo-2-phenylethylamine. Data from Fig. 4 were plotted as previously described (Moran & others, 1967) to determine first order rate constants.

## DISCUSSION

Sodium thiosulphate is known to react rapidly with ethyleniminium ions to form Bunte salts (Bunte, 1874; Fruton, Stein & Bergmann, 1946) and to prevent, *but not reverse*, the irreversible blockade of adrenergic α-receptors produced by 2-halogenoethylamines (Graham, 1962). However, sodium thiosulphate should reverse any blockade of α-receptors caused by reversible binding of ethyleniminium ions. This effect will be most readily demonstrable in the presence of an agonist which, through competition with the ethyleniminium ions, should make the latter more accessible for reaction with sodium thiosulphate. This effect is shown clearly in Fig. 1. Brief treatment of the vas deferens with SY.28 produces a shift to the right of the noradrenaline dose-response curve, unaccompanied by any decrease in the maximum height of response. This shift is completely reversed by subsequent treatment with sodium thiosulphate and can, therefore, be attributed to a reversible antagonism produced by SY.28, rather than to the presence of "spare-receptors". However, the shift in the dose-response curve of noradrenaline shown in Fig. 1 is not very large, a finding in accord with the work of Ariëns & others (1960, 1964) who have reported only a small "reserve" or absence of "spare-receptors" in rat vas deferens.

It appears to be generally accepted that the progressive decline in the response to an agonist of a tissue which has been treated with SY.28 or other related 2-halogenoethylamines is due to an irreversible inactivation of an increasing proportion of the receptor sites. The data presented in Fig. 2 show that the decline in the response to noradrenaline of rat vas deferens pretreated with SY.28 can be *partially, but not completely, reversed* by treatment of the tissue with sodium thiosulphate. It is highly improbable that the partial reversal of blockade can be attributed to any reaction of sodium thiosulphate with the alkylated receptor, a conclusion which is strengthened by the finding that complete blockade of the α-receptors by SY.28 is unaffected by sodium thiosulphate. It is, therefore, probable that the progressive depression by SY.28 of the maximum height of the response to noradrenaline shown in Fig. 2 may be attributed

to a dual mode of action of SY.28—an irreversible phase unaffected by sodium thiosulphate and a reversible non-competitive phase which is abolished by incubation of the tissue with sodium thiosulphate*. The presence of the latter component of action of 2-halogenoethylamines active at the adrenergic α-receptor does not appear to have been explicitly recognized hitherto, but its existence is in good agreement with our previous studies involving the use of [$^3$H]SY.28 which indicated clearly the lack of specificity of this agent (Moran & others, 1967).

The previous discussion indicates that the existence of a significant reversible phase of action of an (ultimately) irreversibly acting agent is sufficient to produce dose-response curves which have been interpreted as evidence for the existence of "spare-receptors". This interpretation is rendered invalid, at least for the α-receptors of the rat vas deferens, by the finding that sodium thiosulphate can reverse the parallel shift of the dose-response curves produced by SY.28. A valuable further test of the "spare-receptor" hypothesis would be provided by the use of an irreversibly acting agent that has no significant phase of reversible interaction at the receptor. The dose-response curves for noradrenaline from vas deferens treated with *NN*-dimethyl-2-bromo-2-phenylethylamine (Fig. 4) do not show parallel shifts unless accompanied by a decline in maximum response (compare Fig. 1), suggesting the absence of a reversible phase of action. However, the use of this agent in progressively inactivating the α-receptor system is complicated by its short duration of action ($t_{\frac{1}{2}}$ for recovery, 22 min), so that during washout procedures a significant recovery of response occurs via intramolecular hydrolysis (Triggle, 1965a, b; Kimelberg & Triggle, 1965). This difficulty was avoided by a procedure in which the tissue was completely blocked to noradrenaline by *NN*-dimethyl-2-bromo-2-phenylethylamine and washed thoroughly under conditions demonstrated to remove completely [$^3$H]*NN*-dimethyl-2-hydroxy-2-phenylethylamine from the tissue (May & others, 1967). The dose-response curves of noradrenaline obtained during the recovery of tissue response we believe to be uncomplicated by the presence of reversibly bound antagonist. The curves shown in Fig. 4 demonstrate that the maximum response to noradrenaline at any level of recovery of tissue response occurs at the same concentration of noradrenaline and, furthermore, when full recovery of response is obtained there is no dose-response curve showing a maximum response with a simultaneous shift to the right relative to the control curve.

We have previously demonstrated the absence of "spare-receptors" in the rabbit aorta α-receptor system (May & others, 1967). Since this conclusion was based on a different experimental approach to those described here and because Rossum (1965; Rossum & Mujić, 1965) has suggested that the α-receptors in different tissues may have different properties, it was desirable to determine whether the characteristics of irreversible α-receptor blockade were the same in both the rabbit aorta and the rat vas deferens. From Fig. 4 it is possible to obtain a first-order plot (Fig. 5) for recovery of tissue response with a $t_{\frac{1}{2}}$ of 22 min essentially identical with our previously determined

* The terms, competitive and non-competitive, are used with regard to the site of action of the 2-halogenoethylamines whereas the terms, reversible and irreversible, refer to the chemical mechanism of action (Kimelberg, Moran & Triggle, 1965). Experimentally, it is difficult to distinguish competitive irreversible inhibition from non-competitive reversible or irreversible inhibition. In the present situation, thiosulphate will distinguish between reversible and irreversible inhibition produced by SY.28 but *a priori* it cannot be said that the decrease in maximum response observed after treatment with SY. 28 and sodium thiosulphate is due to competitive or non-competitive irreversible blockade. In agreement with Waud (1962), we have shown that agonists can protect non-specific sites as well as the receptor sites (Moran & others, 1967; May & others, 1967).

value of 23 $\pm$ 6 min for the rabbit aortic strip system. Furthermore, in experiments using [$^3$H]*NN*-dimethyl-2-bromo-2-phenylethylamine it was found that the $t_{\frac{1}{2}}$ for the process of tritium loss from the recovering tissue was 24 min, in good agreement with the above figures and with our previously determined value (20·7 + 4·1 min) for the rabbit aortic strip system (May & others, 1967). The conclusion is thus permissible that the $\alpha$-receptors in the rat vas deferens and rabbit aorta exhibit an identical behaviour towards *NN*-dimethyl-2-bromo-2-phenylethylamine.

The ability of an ethyleniminium ion to produce reversible, rather than irreversible, blockade probably depends upon a combination of factors determined by the reactivity and lipophilic character of the ion. Clearly a low reactivity towards nucleophiles will result in a slow rate of reaction with the receptor grouping providing a long-lasting reversible phase of action. This represents the behaviour exhibited by the *N*-ethyl-*N*-1-naphthylmethylethyleniminium ion (from SY.28) which is of lower reactivity and greater lipophilic character* than the *NN*-dimethylphenylethyleniminium ion with which it has not proved possible to obtain dose-response curves indicating any significant reversible phase of action (Fig. 3). Furthermore, it might be expected that the binding of antagonists having a high lipophilic character, such as SY.28, to non-receptor lipophilic sites could produce the non-competitive blockade observed in the present study (Fig. 2).

Previous studies (Moran & others, 1967) have demonstrated that it is not possible to effectively wash tissues free of the alcohol corresponding to SY.28. Thus, despite washout times of 10–20 min, sufficient ethyleniminium ion corresponding to SY.28 is probably left in the tissues to cause both reversible competitive and non-competitive blockade.

The data reported in this paper provide clear evidence that "spare-receptor" do not exist in the rat vas deferens $\alpha$-receptor system thus substantiating the conclusion drawn earlier from unrelated studies on the rabbit aortic strip $\alpha$-receptor system (May & others, 1967). This is in agreement with the data of Lewis & Miller (1966) on the rat seminal vesicle preparation. It is noteworthy that early experiments of other workers (Chen & Russell, 1950; Graham & Lewis, 1953), demonstrated that, with low doses of 2-halogenoethylamines, the inhibition was competitive (reversible), whereas with larger doses, the inhibition became non-competitive (irreversible or non-competitive reversible).† It seems probable that, at least for the adrenergic $\alpha$-receptor system, "spare-receptors" are of little or no significance. The work reported in the present paper also demonstrates that the use of irreversibly acting antagonists in quantitative pharmacology may yield results which are subject to misinterpretation unless the mechanisms of action of the antagonist are carefully examined. It is appropriate to note that SY.28 and several related 2-halogenoethylamines are also irreversible antagonists at the cholinergic receptor and similar shifts are noted which have been interpreted as indicative of a receptor reserve. However, relatively high concentrations and prolonged incubation times are necessary to produce irreversible blockade. Apparently, the reactivities of the derived ethyleniminium ions towards the

* See footnote to p. 44.

† Since it is not possible to obtain partition data for the ethyleniminium ions we have taken the partition ratios between $CHCl_3$ and $H_2O$ of *N*-ethyl-*N*-(2-hydroxyethyl)-1-naphthylmethylamine and *NN*-dimethyl-2-hydroxy-2-phenylethylamine as giving a measure of the relative lipophilicities of the corresponding ethyleniminium ions. Evidence was presented in a previous paper in this series for the pronounced ability of *N*-ethyl-*N*-(2-hydroxyethyl)-1-naphthylmethylamine to bind to tissue fractions.

alkylatable site at the cholinergic receptor are relatively low and consequently a pronounced phase of reversible antagonism might be expected. A similar phenomenon at the active site of acetylcholinesterase is discussed by Belleau & Tani (1966). We have, therefore, re-examined the mechanisms of action of such compounds in cholinergic systems. The complex results of this investigation will be published in a separate paper.

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