Studies on the Noradrenaline α-Receptor

I. Techniques of Receptor Isolation. The Distribution and Specificity of Action of *N*-(2-Bromoethyl)-*N*-ethyl-1-naphthylmethylamine, a Competitive Antagonist of Noradrenaline

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(Received July 14, 1966)

SUMMARY

Studies have been made of the potential usefulness of adrenergic blocking 2-halogenoethylamines for labeling the α-receptor. The uptake of ^{3}H-labeled *N*-(2-bromoethyl)-*N*-ethyl-*N*-1-naphthylmethylamine (^{3}H-SY.28) by rabbit vas deferens was shown to be nonspecific and the 2-halogenoethylamine apparently showed little specificity for the α-receptor. In an attempt to obtain greater specificity, rabbit aorta and vas deferens were pretreated with *N*,*N*-dimethyl-2-bromo-2-phenylethylamine, a short-lasting irreversible α-receptor antagonist, prior to treatment with ^{3}H-SY.28. Tissues pretreated with the short-lasting antagonist should take up less ^{3}H-SY.28 and the difference between this uptake and that of controls should reflect the concentration of α-receptor material. However, this treatment did not afford any significant protection against the uptake of ^{3}H-SY.28. The possible protective effects of various amines against labeling by ^{3}H-SY.28 were also investigated: no correlation could be found between the protective ability of these amines and their "direct" or "indirect" sympathomimetic activities. An investigation was also made of the finding that trypsin can reverse the blockade of ^{3}H-SY.28. It was concluded that the recovery of response following trypsin is probably unrelated to α-receptor regeneration.

The distribution of ^{3}H-SY.28 and its corresponding alcohol was studied in rats. Our results suggest that the prolonged tissue retention of radioactivity following ^{3}H-SY.28 is probably due to the high binding capacities of tissues for ^{3}H-SY.28 alcohol rather than to the presence of high concentrations of covalently bound ^{3}H-SY.28.

INTRODUCTION

The pharmacologist usually applies the term "receptor" with the meaning originally given by Langley (1), namely, the special cell constituent with which the drug molecule must intreact to produce the physiological response (2–4). Despite the relatively early origin of this definition, surprisingly little is known concerning receptor structure and function. However, because of the obvious importance of knowledge of such systems an increasing amount of work is being devoted to attempts to more rigorously analyze receptor structure.

There are certain analogies between this problem and the closely allied one of elucidating the structures of the active sites of enzymes (5–8). In the latter case, substantial progress has been made through the use of irreversibly acting substrate analogs which bind covalently to groups at the active centers of enzymes (9–11).

In theory similar methods are applicable

to the identification of binding sites at pharmacologic receptors. However, the study of active centers in enzymes is far simpler than in receptor systems, because the enzyme is often obtainable in a purified or partially purified form. Furthermore, the activities of enzymes can be followed *in vitro* during isolation and labeling procedures. To date this type of approach has not been possible in receptor systems, and it seems improbable that it will be applicable in the immediate future. The receptors for such agents as acetylcholine and noradrenaline are probably integral components of the cell membrane, and Trams (12) has pointed out that the activity of the receptor component may well depend upon the integrity of the cell membrane. Thus, it is difficult to envisage purification procedures for receptors which do not involve loss of their physiological activity.

A number of attempts have been made to isolate "receptor material," particularly the acetylcholine receptor (13–17), by studying the binding of reversibly acting neurotropic agents such as acetylcholine, decamethonium, (+)-tubocurarine etc., to various fractions of tissues known to contain the cholinergic receptor. Such approaches are unlikely to be useful because of the relative lack of specificity of such reversibly acting agents and the possibility of protein denaturation during the fractionation procedures. Furthermore, in such work it is impossible to correlate binding studies with the physiological responsiveness of the tissues.

A more profitable approach to receptor structure at the present time would appear, therefore, to involve the use of specific, irreversibly acting receptor antagonists which covalently bind to the receptor and which, with appropriate radioactive labeling techniques, may permit the isolation of labeled receptor material. An important prerequisite for the prosecution of this approach is that, during the receptor-labeling procedures, it should be possible to study simultaneously the uptake and binding of the antagonist with the physiological responsiveness of the tissue. Such a technique will be closely analogous to the procedures established for labeling the active sites of enzymes where enzymic activity is used as a criterion of the extent and specificity of the labeling procedure.

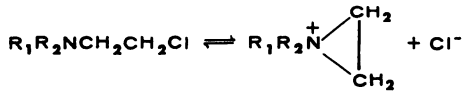
Several workers have attempted to apply such procedures to the isolation of receptor material. Takagi *et al.* (18) have reported the specific labeling of the acetylcholine receptor in the smooth muscle of the small intestine of the dog. In their experiments, smooth muscle was incubated with unlabeled *N,N*-dibenzyl-2-chloroethylamine (Dibenamine®) in the presence of atropine; after washout of the atropine ^{3}H-Dibenamine was added to label the exposed receptor. Control tissues were treated with unlabeled and ^{3}H-Dibenamine in the absence of atropine. From the results of Takagi *et al.*, two points emerge. First, despite treatment with unlabeled Dibenamine, tissues took up significant quantities of radioactivity on subsequent treatment with the same concentration of ^{3}H-Dibenamine. Clearly, extensive labeling of nonreceptor material by the ^{3}H-Dibenamine had occurred. It follows that in experiments where the tissue is protected with atropine and unlabeled Dibenamine, labeling with the ^{3}H-Dibenamine will also occur at these same nonspecific sites in addition to the labeling at the acetylcholine receptor. Takagi *et al.* (18) found approximately 10–30% less uptake of ^{3}H-Dibenamine in protected tissues, leaving a background labeling of 70–90%. Thus, even if it is assumed that atropine is completely specific for the acetylcholine receptor[1] only 10–30% of the tissue radioactivity is associated with the receptor and the problem of separating the labeled fractions into receptor and nonreceptor material still remains. The second point arising from Tagaki's work is that protection was observed in all fractions and ranged from 10% in the 105,000 *g* supernatant to 32%

[1] The work of Paton and Rang (19) has shown very clearly that the action of atropine in the guinea pig gut is not confined to the acetylcholine receptors. These authors found that atropine had at least three sites of binding, only one of which (the smallest) could be considered as a receptor binding site.

in the 10,000–50,000 *g* fraction. Either all the fractions contain receptor material or, and more probably, nonspecific protection is being observed in all fractions. The latter conclusion is in essential agreement with the work we shall report in this paper.

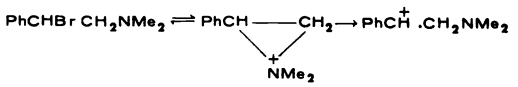
In similar experiments Sulman and associates (20) employed adrenaline to protect the α-receptor site in rabbit aorta against alkylation by Dibenamine. These workers extracted the phospholipid fraction, which they believe to be intimately associated with receptor function, and demonstrated protection, albeit as variable as that reported in the present study. Since these workers did not determine the degree of protection in other tissue fractions, it is difficult to evaluate the significance of their results. In any event, in the adrenergic receptor system it is clearly apparent that the protective action of noradrenaline and many other amines will be exerted at both the receptor and the amine uptake and storage sites. Since the latter may well constitute a greater fraction as compared to receptor sites, it is apparent that the results of any protection experiments as reported in this paper and by other workers (20, 21) will be subject to ambiguity of interpretation.

The present paper is concerned with an evaluation of the potential utility of *N*-(2 - bromoethyl) - *N* - ethyl - *N* - 1 - naphthylmethylamine (SY.28)[2] and *N*,*N*-dimethyl - 2 - bromo - 2 - phenylethylamine, which are representative of adrenergic α-blocking agents, as labels for the adrenergic α-receptor. It is now generally agreed that the mechanism of action of 2-halogenoethylamines involves the formation of the derived ethyleniminium ion which is the pharmacologically active species (22):



[2] Abbreviations used: SY.28, *N*-(2-bromoethyl)-*N*-ethyl-1-naphthylmethylamine; SY.28 alcohol, *N*-(2-hydroxyethyl)-*N*-ethyl-1-naphthylmethylamine; II, *N*,*N*-dimethyl-2-bromo-2-phenylethylamine.

In some instances, however, a carbonium ion may be a better representation of the alkylating species (23, 24) i.e.,



The mechanisms of action of 2-halogenoethylamines have been reviewed in detail recently (22, 25, 26). The most satisfying rationalization to date of their structure-activity relationship has been offered by Belleau (27), who analyzed the relative complementarities of the interactions of noradrenaline and 2-halogenoethylamines at the adrenergic α-receptor. Belleau concluded that the ammonium function of noradrenaline interacts at a complementary anionic site–probably a carboxylate or phosphate anion–and that this also represents the site alkylated by the 2-halogenoethylamines.

Pronounced differences in the duration of blockade produced by different 2-halogenoethylamines (22, 23, 27–30) were originally interpreted by Belleau (27) in terms of the relative ease of intramolecular hydrolysis of carboxylate or phosphate esters formed in the alkylation reaction (Fig. 1). We have provided quantitative evidence to support the intramolecular hydrolysis of a carboxylate ester as the best representation of α-receptor regeneration (28, 30). Thus, *N*,*N*-dimethyl-2-bromo-2-phenylethylamine (II) produces an irreversible block which shows complete recovery response to noradrenaline in 1.5–2 hr. In contrast, SY.28 produces an irreversible blockade of much longer duration with approximately 40% recovery of response in 24 hr.

These observations indicated an obvious approach to the problem of obtaining specific alkylation of the α-receptor, through the use of II to reduce nonspecific labeling by ^{3}H-SY.28. This projected series of papers will contain our analysis of the use of 2-halogenoethylamines in the determination of adrenergic receptor structure.

MATERIALS AND METHODS

Pharmacology. Rabbit aortic strips were set up to record isotonic contractions ac-

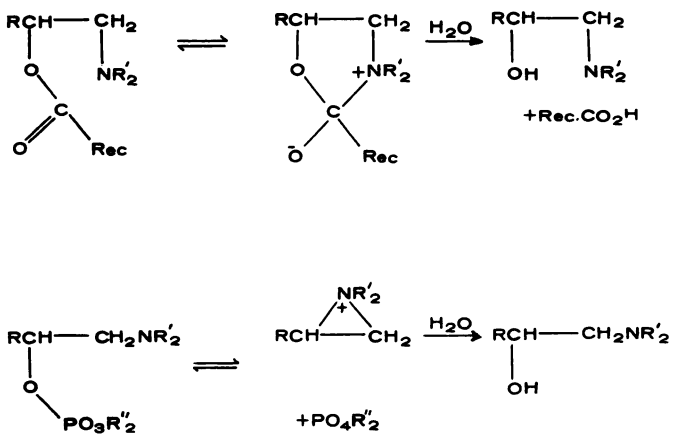


FIG. 1. *Hydrolysis reactions*

cording to the procedure described by Furchgott and Bhadrakom (31). Strips were suspended in a modified Krebs-bicarbonate solution (32) containing 10^{-5} M ethylenediamine tetraacetic acid (33) at 37 ± 0.5° and aerated with a gas mixture of 95% O_2 and 5% CO_2. All solutions were prepared with glass-distilled water. The responses of these strips to noradrenaline and blocking agents were determined as previously described (28, 30): further details of experiments are given in the appropriate tables.

In vivo studies. Male Holtzmann rats, weighing 100–125 g, were injected with freshly prepared solutions of SY.28 or the corresponding alcohol into the tail vein. At appropriate intervals the animals were decapitated, a sample of blood was collected in a heparinized tube, and the tissues were prepared immediately or stored at −15° in individual glass vials.

Preparation of tissues for counting. Brain, heart, lung, kidney, and spleen were homogenized in 5 volumes of 0.25 M sucrose, and aliquots, equivalent to 166 mg of tissue, were lyophilized in glass counting vials. Samples of other tissues (50–100 mg) and blood (100 μl) were lyophilized directly. All tissue samples were solubilized in 1–2 ml of Hyamine® hydroxide and treated according to the method of Herberg (34) which involves bleaching with 0.1 ml of hydrogen peroxide (30%), addition of scintillator, and final neutralization with concentrated hydrochloric acid. Aqueous samples were counted directly in the scintillation fluid which had the following composition: 900 ml dioxane, 100 ml toluene, 60 g naphthalene, 10 g PPO, and 0.5 g dimethyl POPOP.

Radioactivity measurements. Samples were placed in the counting chamber of a Packard Tri Carb® liquid scintillation spectrometer, model 3224, maintained at +7°. Despite neutralization with hydrochloric acid, tissue samples treated with Hyamine exhibited considerable phosphorescence, and it was necessary to allow this to decay before counting. Therefore, samples remained in the sample changer for 24 hr prior to counting. A minimum of 10,000 counts was collected for each sample, and appropriate backgrounds were determined for each tissue. The absolute disintegration rate was determined by internal standardization, which was found to be considerably more accurate than either channels' ratio or automatic external standardization methods (35).

Partition studies. The alcohol corresponding to ^{3}H-SY.28 (6×10^{-8} mole) was added to a mixture of 5 ml $CHCl_3$ and 5 ml H_2O and allowed to equilibrate with shaking for 24 hr. Aliquots from the two phases were counted and the ratio of the disintegration rates determined.

Materials. Trypsin (10,000 BAEE units/mg), chymotrypsin (10,000 ATEE units/mg), subtilisin (8–10 units/mg), and phos-

phodiesterase (types I and II) were obtained from Sigma Chemical Company. The unlabeled SY.28 and *N,N*-dimethyl-2-bromo-2-phenylethylamine were synthesized by the procedures given below and in part II of this series. All other chemicals, unless otherwise specified, were of reagent or analytical grade. Concentrations of the various drugs (g/ml) refer to the salts: noradrenaline bitartrate, SY.28 hydrobromide, and *N,N*-dimethyl-2-bromo-2-phenylethylamine hydrobromide.

Synthesis of N-(2-bromoethyl)-N-ethyl-N-1-naphthyl(^3H)methylamine hydrobromide, 1-$C_{10}H_7CH^3HN(Et)CH_2CH_2Br,HBr$. 1-Naphthaldehyde (1.6 g, 0.01 mole) dissolved in methanol (12 ml) was added dropwise to a solution of tritiated sodium borohydride (110 mg, specific activity 250 mC/mmole from New England Nuclear Corporation) in 2 ml of 5% sodium hydroxide. After 2 hours of stirring a further 30 mg of sodium borohydride was added and most of the solvent was removed. The remaining oil was extracted into chloroform and the extract was dried over anhydrous magnesium sulfate.

To the chloroform solution of 1-naphthylmethanol prepared above was added pyridine (0.85 g), and the solution was cooled to 0°. Thionyl chloride (1.5 g) was added in one portion and the reaction brought to and maintained at 35° for 1 hour. Water (10 ml) was added and the organic phase was separated and dried with magnesium sulfate. Evaporation gave a viscous oil whose infrared spectrum was identical with that of an authentic sample of 1-naphthylmethyl chloride.

The 1-naphthylmethylchloride (1.4 g), *N*-ethylethanolamine (0.7 g) potassium carbonate (1.4 g) and butan-1-one were refluxed and stirred for 6 hours. Water (10 ml) was added, the mixture extracted with ether (2 × 10 ml), and the ether extracts were dried. Removal of the solvent gave an oil whose infrared spectrum was almost identical with that of an authentic sample of *N*-(2-hydroxyethyl)-*N*-ethyl-*N*-1-naphthylmethylamine.

The oil from above (1.75 g) was dissolved in chloroform (15 ml) and stirred and cooled to 0°. Phosphorus tribromide (2.3 g) was added and the mixture was refluxed for 6 hr. The solvent was removed and the residue was extracted with boiling ethanol (2 × 6 ml) and filtered. On cooling the filtrate gave *N*-(2-bromoethyl)-*N*-ethyl-*N*-1-naphthyl-(^{3}H)-methylamine hydrobromide with m.p. and mixed m.p. 166–68°. Yield 2.2 g (59% overall). Before use, this material was recrystallized to constant specific activity; reverse isotope dilution demonstrated the product to be radiochemically pure. The specific activity was 110.7 mC/mmole.

RESULTS

The Uptake of ^{3}H-Labeled N-(2-Bromoethyl)-N-ethyl-N-1-naphthylmethylamine (^{3}H-SY.28)

Our preliminary experiments involved the use of tritium-labeled *N*-(2-bromoethyl)-*N*-ethyl-*N*-1-naphthylmethylamine (^{3}H-SY.28), an agent that has been widely employed as an adrenergic α-blocking agent (36). Experiments were carried out to investigate the effect of SY.28 concentration on the labeling of rabbit vas deferens, a tissue known to contain α-receptors (37). The results are shown in Fig. 2, where it can be seen that the tissue uptake of ^{3}H-SY.28 is proportional to the medium concentration over the 100-fold concentration range studied which included the minimum concentration (2.4×10^{-9} g/ml) required for 100% blockade. In order to reduce the importance of this nonspecific uptake, all subsequent experiments (unless otherwise indicated) employed the minimum concentration of SY.28 necessary for 100% blockade. (This concentration was always determined in parallel pharmacologic experiments.)

Previous work from this laboratory has shown that *N,N*-dimethyl-2-bromo-2-phenylethylamine (II) produces an irreversible block in rabbit aortic strips and vas deferens which shows complete recovery to noradrenaline response in 1.5–2.0 hr (28). Therefore, strips of rabbit aorta and vas deferens were incubated with varying concentrations of II and were then washed at

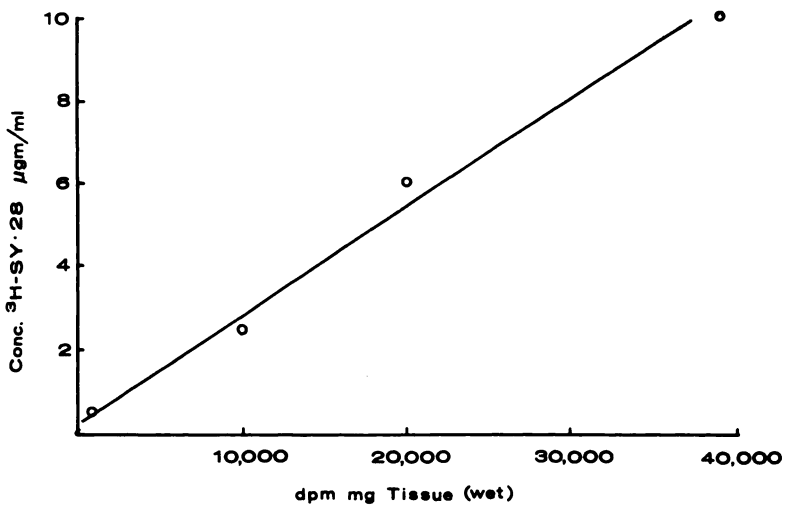


FIG. 2. *Uptake of ³H-SY.28 by rabbit vas deferens as a function of the concentration of ³H-SY.28*

Rabbit vas deferens (30–50 mg wet weight) was incubated in Krebs solution for 10 min with various concentrations of ³H-SY.28, after which they were washed 3 times with 10-ml portions of Krebs solution over a period of 1½ hr. Tissues were counted as described in Materials and Methods.

frequent intervals for 2 hr. Simultaneous pharmacologic experiments on these tissues demonstrated that at the lower concentrations of II ($<5 \times 10^{-5}$ g/ml for 5 min), α-receptor response had been completely restored within 2 hr. The tissues were then treated with ³H-SY.28 (3×10^{-6} g/ml for 10 min) to label the regenerated α-receptor: this amount of ³H-SY.28 produced 100% blockade of the response to noradrenaline. The results of a typical experiment are summarized in Table 1. It is apparent that, in general, the pretreatment procedure did not afford significant protection against the uptake of ³H-SY.28. Furthermore, no correlation is apparent between the concentration of II used as protecting agent and the uptake of ³H-SY.28.

TABLE 1

Effect of N,N-dimethyl-2-bromo-2-phenylethylamine in protection against uptake of ³H-SY.28.

Aortic strips (10–20 mg wet weight) or vas deferens (30–50 mg wet weight) were incubated in Krebs solution for 10 min with various concentrations of *N,N*-dimethyl-2-bromo-2-phenylethylamine; they were then washed 3 times with 10-ml portions of Krebs solution. Tissues were then incubated with 3×10^{-6} g/ml of SY.28 for 10 min followed by 3 washes with 20 ml Krebs solution over a period of 3 hr.

Concentration of *N,N*-dimethyl-2-bromo-2-phenylethylamine (g/ml)	Dpm/mg tissue wet weight	
	Vas deferens	Aortic strips
10^{-2}	1435	5210
10^{-3}	1854	4899
10^{-4}	1773	4814
10^{-5}	1729	3919
10^{-6}	2050	4829
10^{-7}	1535	4431
Control	1722	4805

Other experiments were carried out in an attempt to correlate the uptake of ³H-SY.28 with the state of physiological responsiveness of the tissue. The responses of aortic strips to 10^{-8} g/ml of noradrenaline were determined, and the tissues were divided into two groups. One group was pretreated with 10^{-5} g/ml of *N,N*-dimethyl-2-bromo-2-phenylethylamine (II) and the other with 3×10^{-6} g/ml of SY.28. After 2 hr the strips pretreated with II gave the original (pre-block) response to noradrenaline but no response was obtained with the strips pretreated with SY.28. Both groups of strips were then exposed to ³H-SY.28 (3×10^{-6} g/ml for 10 min) which produced or maintained complete block to noradrenaline. The strips were then washed thor-

oughly and counted: strips pretreated with II averaged 3788 dpm whereas strips pretreated with SY.28 averaged 3977 dpm/mg tissue (wet weight). Since tissues pretreated with unlabeled SY.28 should be at least *partially* resistant to further uptake of ^{3}H-SY.28, it seemed probable that this technique would not afford a useful method of obtaining specifically labeled α-receptor material. In further experiments, strips were completely blocked with II (5×10^{-6} g/ml for 5 min) and were washed at intervals until the pre-block response to noradrenaline was regained. These strips, together with nontreated, noradrenaline-responsive control strips, were blocked with ^{3}H-SY.28 (2.5×10^{-6} g/ml for 10 min) and then washed and counted. Strips pretreated with II were protected to some extent in 4 out of 8 experiments; however, the results were extremely variable, as may be judged from the mean counts $\pm$ standard deviation which were 8180 ± 1440 in strips pretreated with II and 8570 ± 2060 dpm/mg tissue dry weight in the controls.

TABLE 2

Effect of pretreatment with various alkylating agents on the uptake of ^{3}H-SY.28 in rabbit aortic strips

Tissues were incubated in Krebs solution for 15 min with the unlabeled alkylating agent followed by 3 washings with 10 ml of Krebs solution over a period of 2 hr. After this pretreatment, tissues were incubated in 6×10^{-6} M ^{3}H-SY.28 for 10 min after which they were washed 3 times in 10 ml of Krebs solution over a period of 1½ hr, dried, and counted in the usual manner.

Pretreatment	Concentration (M)	Dpm/mg tissue dry weight, as % of control
N,N-Dimethyl-2-bromo-2-phenylethylamine	10^{-5}	118
	10^{-4}	109
	10^{-4}	89
N,N-bis(2-Chloroethyl)amine	10^{-5}	107
	10^{-4}	106
	10^{-4}	62
N,N-Dimethyl-2-bromo-2-phenylethylamine + *N,N*-bis(2-chloroethyl)amine	10^{-4}	101
	10^{-4}	—

In connection with the above experiments, it appeared of interest to determine the effect of ^{3}H-SY.28 uptake by pretreatment with a 2-halogenoethylamine devoid of adrenergic blocking activity. For this purpose *N,N*-bis(2-chloroethyl)amine was employed. Several experiments using this agent alone and in combination with *N,N*-dimethyl-2-bromo-2-phenylethylamine (II) were carried out and the results are summarized in Table 2. It is apparent that pretreatment of the tissues with *N,N*-bis(2-chloroethyl)amine alone or in combination with II does not produce any significant overall reduction in the uptake of ^{3}H-SY.28 as compared with nontreated control strips.

Effect of Various Enzymes on the Blockade Produced by SY.28

Several experiments were carried out in an attempt to determine the relative amounts of ^{3}H-SY.28 bound to receptor and nonreceptor sites. These experiments were based upon a recent report by Graham (38) that trypsin, but not chymotrypsin, reverses the blockade produced by SY.28 in guinea pig vas deferens. On this basis it appeared possible to measure the amount of enzymically released receptor-bound ^{3}H-SY.28. After establishing the response to noradrenaline, vas deferens was blocked to the extent of 60–80% with SY.28 (10^{-8} g/ml) and then incubated with trypsin (100 units/20 ml muscle bath) until an increased response to noradrenaline could be observed. It was possible to observe increases of 20% as compared to the original response; however, control experiments showed that the response in nonblocked preparations also increased by 20%. The nonspecific protease, subtilisin, and phosphodiesterase gave similar results and, as with trypsin, caused substantial relaxation of the tissue. In contrast, chymotrypsin treatment was ineffective in this apparent reversal of blockade, nor did it cause tissue relaxation.

Agonist Protection

In a representative series of experiments strips of rabbit vas deferens were incubated

with various amines prior to and during exposure to ^{3}H-SY.28. The tissues were then thoroughly washed and counted and the results are presented in Table 3. Many similar experiments were carried out with other tissues (aortic strips and uterus), but the results are not presented here because of their close similarity to those in Table 3. These results show that the degree of protection afforded by a given agent is rather variable. From Table 3 it is obvious that there is no relation between the direct ^{3}H-SY.28 and the effect of II on this distribution was carried out. In protection experiments, rats received II 1 hr prior to the administration of ^{3}H-SY.28. The animals were sacrificed 18 hr later, and tissues were removed and counted. The results are presented in Table 4. All tissues from animals that were pretreated with II possessed significantly less radioactivity than the controls. The data of Table 4 suggest that nonspecific protection and/or protection against binding of hydrolysis products of

TABLE 3

Effect of various amines on the uptake of ^{3}H-SY.28 in rabbit vas deferens

Tissues weighing 10–15 mg (dry weight) were incubated in the presence of amine for 15 min before addition of ^{3}H-SY.28. After 10 min exposure to the radioactive alkylating agent, the pieces of tissue were washed 3 times over a period of 1½ hr, dried, weighed, and counted. All incubations were done in Krebs solution, and the first wash fluid contained the amine.

^{3}H-SY.28 concentration:	Per cent protection[a]					
	5×10^{-6} M		4.5×10^{-6} M		5.6×10^{-6} M	
Amine concentration:	2×10^{-4} M	2×10^{-5} M	10^{-4} M	10^{-4} M	10^{-4} M	Uptake[b] ID_{50} (M)
Methamphetamine	—	—	40	44	37	6.7×10^{-7}
D-Amphetamine	5	0	10	57	31	1.8×10^{-7}
Histamine	6	—	7	0	—	—
Benzylamine	63	45	—	—	0	—
Tyramine	37	47	40	9	27	4.5×10^{-7}
Dopamine	41	25	24	8	44	1.7×10^{-7}
D-Noradrenaline	31	0,33,33	34	0	39	2.7×10^{-7}
DL-Isopropylnoradrenaline	11	13	15	4	39	2.5×10^{-5}

[a] $\%\ \text{Protection} = 100 - \left(\frac{\text{dpm/mg tissue wet weight amine treated}}{\text{dpm/mg tissue wet weight control}} \times 100\right)$.

[b] Data from Iverson (41). Figures represent the drug concentration producing 50% inhibition of noradrenaline uptake$_1$.

sympathomimetic effects (39, 40) of these amines and the protection they afford against uptake of ^{3}H-SY.28. However, many of the amines listed have considerable affinities for the noradrenaline uptake-storage sites (41) (the ID_{50} values listed refer to the concentrations of the amines required to prevent 50% uptake of noradrenaline).

In Vivo Studies with ^{3}H-SY.28

Since we were interested in determining whether it was possible to obtain protection *in vivo* against the uptake of ^{3}H-SY.28, an investigation of the tissue distribution of ^{3}H-SY.28 are significant contributing factors. SY.28 is known to hydrolyze fairly rapidly at physiological pH to the corresponding alcohol. In solubility measurements (see Materials and Methods), it was found that SY.28 alcohol partitions between $CHCl_3$:H_2O in the ratio 9.6:0.4 and it was of interest to determine the distribution of this lipophilic compound.

Rats were treated with ^{3}H-SY.28 or the corresponding alcohol and were sacrificed 18 hr later. The tissue levels of radioactivity are given in Table 5. The distribution of both compounds is very similar with the exception of levels in brain, heart, and

TABLE 4

Effect of pretreatment with N,N-dimethyl-2-bromo-2-phenylethylamine on the uptake of ^{3}H-SY.28 in the rat in vivo

Three animals received 1.2 mg/kg body weight *N,N*-dimethyl-2-bromo-2-phenylethylamine 1 hr prior to receiving 1.14 mg/kg ^{3}H-SY.28. Controls received saline instead of the alkylating agent. All injections were into the tail vein. Values represent the average of duplicate determinations from 3 animals killed 18 hours after receiving the radioactive compound.

Tissue	Dpm/mg tissue wet weight	
	Control	Pretreated
Liver	280	211
Brain	191	131
Spleen	399	258
Lung	857	398
Kidney	487	235
Body fat	179	135
Testes	175	93
Muscle	448	181
Heart	1,525	1,104
Blood (100 μl)	83,935	46,060

skeletal muscle. To determine the retention of ^{3}H-SY.28 alcohol in tissues, animals were given ^{3}H-SY.28 alcohol and sacrificed at 1, 3, and 8 days. The results in Table 6

TABLE 5

Distribution of SY.28 and the corresponding alcohol

Rats received 1.89 mg/kg body weight of either, ^{3}H-SY.28 or the corresponding ^{3}H-alcohol, *N*-(2-hydroxyethyl)-*N*-ethyl-*N*-1-naphthylmethylamine, by intravenous injection and were sacrificed 18 hours later.

Tissue	Dpm/mg wet weight	
	SY.28	Alcohol
Liver	401	447
Brain	541	94
Spleen	404	481
Lung	1,383	1,590
Kidney	668	777
Body fat	457	277
Testes	137	280
Muscle	701	96
Heart	2,762	707
Blood (100 μl)	123,000	121,500

TABLE 6

Retention of N-(2-hydroxyethyl)-N-ethyl-N-1-naphthylmethylamine in rats

Rats received 1.7 mg/kg body weight of the ^{3}H-alcohol by intravenous injection and were sacrificed at the times indicated.

Tissue	Dpm/mg tissue wet weight			Per cent 24 hr value at 8 days
	1 day	3 days	8 days	
Liver	781	143	61	8
Brain	69	23	10	19
Spleen	686	270	70	12
Lung	760	681	208	27
Kidney	849	451	118	13
Fat	305	86	18	7
Testes	170	28	11	6
Muscle	135	57	27	20
Heart	1,518	806	281	21
Blood (100 μl)	116,900	19,600	18,100	13

show considerable tissue levels of radioactivity after 8 days. It would appear, therefore, that the delayed clearance of ^{3}H-SY.28 could result from prolonged retention of the derived alcohol. In a further investigation of this point, homogenates were centrifuged at 12,000 *g* for 1 hr, and the radioactivity in the supernatant fraction was determined and compared with the radioactivity of the total homogenate. From Table 7, it is apparent that the supernatant fraction from animals receiving ^{3}H-SY.28 alcohol contained more

TABLE 7

Distribution of SY.28 and its corresponding alcohol in tissue homogenates

Homogenates of tissues from animals in which distribution studies were made were centrifuged at 12,000 *g* for 1 hr, and the radioactivity in the supernatant and whole homogenate was determined.

Tissue	Compound injected	Dpm/ml tissue		Per cent tritium in supernatant
		Homogenate	Supernatant	
Brain	Alcohol	15,614	9,103	58
	SY.28	89,861	35,238	39
Liver	Alcohol	74,291	56,300	75
	SY.28	66,608	40,382	60

radioactivity than the supernatant from ^{3}H-SY.28-treated animals. The supernatant fractions from these experiments were then dialyzed against phosphate buffer (pH 6.0) or against tap water after the protein had been precipitated in the dialysis tubing with 10% trichloroacetic acid. The results (Table 8) demonstrate that approximately the same amount of radioactivity was removed from the supernatant fraction regardless of whether the animal had received ^{3}H-SY.28 or ^{3}H-SY.28 alcohol.

TABLE 8

Dialysis of supernatants from tissue homogenates to remove loosely bound material

The supernatant fractions (Table 7) were dialyzed against pH 6.0 phosphate buffer or, after precipitation of protein with 10% trichloroacetic acid (TCA) against tap water; 1 ml of supernatant material was dialyzed against two changes of 2 liters at 4° over a 48-hr period.

Tissue	Compound injected	Per cent lost on dialysis	
		pH 6.0	TCA
Brain	Alcohol	33	21
	SY.28	9	15
Liver	Alcohol	52	55
	SY.28	55	55

DISCUSSION

A key requirement for the effective application of irreversibly acting antagonists to the problem of receptor isolation is the procurement of *specifically* labeled tissues. It must be emphasized that specificity of action (in a chemical sense) must be demonstrated before one can equate radioactive labeling data with the desired material. This criterion has not been established in published work claiming the isolation of receptor material. It was, therefore, necessary to investigate this aspect of the problem in the case of the adrenergic receptor. The complete absence of any break in the uptake curve for SY.28 (Fig. 2) at concentrations greater than that necessary for 100% blockade of the α-receptors suggests that there are one or more secondary sites which are alkylated at least as readily as the α-receptor and which are present in great excess. These observations are analogous to those made by Trams (12), who, working with protein fractions from the electric organ of the electric eel, demonstrated that the amount of dimethyltubocurarine bound by the "receptor protein" increased with increasing concentration of the drug although the percentage of dimethyltubocurarine bound with increased concentration steadily decreased. The nonlinear uptake curve observed by Trams led him to suggest that there were a number of titratable sites, other than the receptor, which gradually became saturated with the drug. From Tram's results, it is apparent that dimethyltubocurarine showed some discrimination between the various binding sites proposed. Our results with ^{3}H-SY.28 suggest also the existence of binding sites other than the α-receptor in the rabbit vas deferens; however, the linear nature of the uptake curve (Fig. 2) indicates that SY.28 shows little discrimination between these binding sites.

The data in Tables 1 and 2 demonstrate that pretreatment of tissues with unlabeled alkylating agents is ineffective in preventing, even partially, subsequent alkylation by labeled agent. It seems probable that the absence of any consistency in the uptake figures of Table 1 is due to the dominance of the nonspecific alkylation reactions of SY.28—a conclusion in accord with the previous experiment. In addition the possibility must be considered that nonspecific alkylation could cause partial denaturation of protein and lipoprotein constituents resulting in the exposure of nucleophilic groups normally unavailable for alkylation reactions. Similar results were also obtained in experiments designed to correlate the uptake of ^{3}H-SY.28 with the physiological responsiveness of the tissue. Although the α-receptor was blocked completely with unlabeled SY.28, further treatment with ^{3}H-SY.28 resulted in uptake similar in quantity to untreated controls. Pretreatment of tissues with *N,N*-dimethyl-2-bromo-2-phenylethylamine should alkylate available nucleophilic sites including the α-receptor; the latter will completely

regenerate by the intramolecular hydrolytic mechanism within 2 hr leaving, in theory, a tissue where nucleophilic sites other than the α-receptor are still alkylated. Treatment of the tissue with ^{3}H-SY.28 should then give a preparation in which only the α-receptors bear the tritium label. However, it must be recognized that nonreceptor carboxyl groups which are alkylated will also regenerate by this mechanism and that this may complicate the results. However, it would be anticipated that tissues pretreated with II should be at least partially protected and take up less ^{3}H-SY.28. Although protection was observed in four out of eight experiments, the degree of protection was not significant and certainly would not meet the criteria necessary for isolation procedures.

Several attempts were made to hydrolyze the receptor-SY.28 complex through the use of various enzymes. In agreement with the work of Graham (38) an apparent partial reactivation of the blocked receptor was observed with trypsin, but not with chymotrypsin. However, a similar effect was also noted with subtilisin and phosphodiesterase. Since it appears improbable that trypsin, subtilisin, and phosphodiesterase would all be equally effective in hydrolyzing the drug-receptor complex, it seems possible that damage of the tissue, resulting in relaxation, may serve as an explanation of the apparent reversal of blockade.

It has been amply demonstrated that agonists and competitive antagonists acting at the α-receptor afford protection against the irreversible blocking actions of 2-halogenoethylamines (25, 42–47). The evidence that 2-halogenoethylamines can produce reasonably specific pharmacologic actions stands in contrast to our results on the apparent nonspecificity of these agents as indicated by studies of the uptake of tritium-labeled SY.28. It, therefore, appeared desirable to determine the effects of sympathomimetic and other amines on the uptake of SY.28. From the results in Table 3 it seems probable that the effectiveness of the various amines in preventing the uptake of ^{3}H-SY.28 is due to their protection of the uptake and storage sites as well as to their protection of the α-receptors. Thus, methamphetamine which is devoid of significant agonistic and antagonistic activities at the α-receptors is at least as effective as noradrenaline in preventing the uptake of ^{3}H-SY.28. The variability in these experiments is similar to that observed by Dikstein and Sulman (20), who carried out similar experiments using labeled Dibenamine, and found protection by adrenaline in 10 out of 14 experiments with a rather wide spread (12–100%) in the degree of protection.

Although it is possible to block preferentially the storage sites with *N,N*-dimethyl-2-bromo-2-phenylethylamine (II) (28), combination of this technique with agonist protection was not attempted because previous experiments showed that II does not afford protection against nonspecific uptake of ^{3}H-SY.28, although it is effective in pharmacologic protection of the receptors.

In Vivo Studies with ^{3}H-SY.28

In our studies we have found that *N,N*-dimethyl-2-bromo-2-phenylethylamine (II) can protect *in vivo* against the uptake of ^{3}H-SY.28 or its metabolites in rat tissues (Table 4): this stands in contrast to the *in vitro* work (see page 19), although the species and experimental conditions are quite different. However, the *in vivo* protection observed in rats is probably not of great significance because, according to Graham (48), the pharmacologic blockade produced by this dose of ^{3}H-SY.28 is completely reversed after 18 hr. In addition protection was observed in blood which is devoid of receptor material.

During the course of this investigation Graham reported (48) on the distribution of ^{14}C-SY.28 in rats. He observed that this compound (or a derivative) remained in the tissues long after the block had reversed. Although his results are not strictly comparable to ours since he was using a dose level of 10 mg/kg as compared to 1–2 mg/kg in the present experiments, our data (Tables 5–8) suggest that this delayed clearance may be due to prolonged retention of SY.28 alcohol (or other break-

down products) in tissues. We have found, in agreement with Graham (48), high levels of radioactivity in tissues after the adrenergic blockade htas reversed. These results do not support the earlier contentions of Axelrod (49) and Brodie (50) that storage in fat plays a significant role in determining the duration of action of 2-halogenoethylamines. From our results, it would appear that a significant proportion of this slowly cleared radioactivity is due to hydrolysis or metabolic products of ^{3}H-SY.28 rather than to covalently bound ^{3}H-SY.28.

It appears that while it is possible to obtain receptor protection and specificity of action with irreversibly acting antagonists at a pharmacologic level, it is not possible to obtain specificity of action at the chemical level with the agents currently available. This is not surprising, since it is well known that 2-halogenoethylamines can react with a variety of nucleophilic groups (26, 51, 52), although this broad spectrum of reactivity does not preclude their use in receptor isolation experiments provided that the 2-halogenoethylamine group forms part of a suitably specific carrier molecule or that effective protection experiments are designed. In the adrenergic receptor system it is clear that the protective action of noradrenaline and many other amines will be exerted at both the receptor and the amine uptake and storage sites. Since the latter may well constitute a greater fraction as compared to receptor sites, it is apparent that the results of any protection experiments as reported in this paper and by other workers (20, 21) will be subject to ambiguity of interpretation. Further work on the isolation of receptor material must, therefore, be attempted with irreversibly acting antagonists that are more specific than those currently available, or techniques must be employed that are designed to circumvent the problem of nonspecific irreversible action. We are actively exploring both possibilities and are currently using a double label procedure to that used by Fox and Kennedy (53) in their isolation of the "M" protein associated with β-galactoside transport in *Escherichia coli*.

ACKNOWLEDGMENTS

The authors wish to thank Miss Marlene Calderone for her technical assistance.

This work was supported by Grant GM 11603 from the United States Public Health Service.

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