Protection of Sympathetic α-Receptors *In Vivo*

By F. P. LUDUENA and M. J. BRANIN

An intravenous dose of 100 mcg./Kg. of phenoxybenzamine in rats completely blocked the lethal effect of 1 mg./Kg. of epinephrine injected intravenously 3 hr. later. Alpha-sympathetic receptors were protected against the effect of phenoxybenzamine by the simultaneous injection of competitive α-adrenolytics. If the α-receptor protection was maximal, a 100 per cent mortality followed the injection of the 1 mg./Kg. dose of epinephrine. Graded degrees of receptor protection were obtained by the administration of graded doses of α-adrenolytics. A rank correlation coefficient of 0.902 was obtained between the α-receptor protective $dose_{50}$ and the adrenolytic $dose_{50}$ of 17 compounds tested including *dextro*-isoproterenol and *dextro*-hyoscyamine.

BETA-HALOALKYLAMINES produce a long-lasting inactivation of the sympathetic α-receptors (1). In 1949 Seed and McKay (2) demonstrated that previous administration of piperoxan in the anesthetized dog prevented the development of the adrenolytic action of *N*-(2-chloroethyl)dibenzylamine.[1] Nickerson and Gump (3) reported that the injection of epinephrine in anesthetized cats reduced the developing of the α-receptor blocking effect of β-haloalkylamines suggesting a certain degree of α-receptor protection. Clear evidence of α-receptor protection *in vitro* by both agonists and antagonists was provided by the results reported by Furchgott (4).

The experiments described below were carried out in order to quantitate α-receptor protection activity *in vivo* and to determine the relationship between this activity and adrenolysis.

METHODS

All injections were made in the tail vein of male white rats weighing approximately 100 Gm. Doses were expressed in terms of the bases.

Adrenolytic Activity—The technique, reported previously (5), consists of injecting intravenously a dose of epinephrine (200 mcg./Kg.) simultaneously (procedure *A*) with one of three or four graded doses of the experimental compound and plotting per cent survival against the dose on probit-log paper; in some experiments the compound was injected approximately 5 min. before epinephrine administration (procedure *B*).

Sympathetic α-Receptor Protection Test—Alpha-receptors were protected by adrenolytics against the long-lasting inactivation produced by phenoxybenzamine (0.1 mg./Kg.) injected intravenously. The "protectors" were injected in the same solution (procedure *C*) or 5 min. before phenoxybenzamine (procedure *D*). After an interval of 3 hr. a dose of epinephrine of 1 mg./Kg. was injected intravenously. If the sympathetic α-receptor had been protected and the adrenolytic effect of the "protector" had worn off, epinephrine would reach the unchanged α-receptor causing all the rats to die. With graded doses of the protector, the higher the α-receptor protective effect the higher the mortality. The ED_{50} was obtained by plotting mortality against dose on probit-log paper (Fig. 1).

RESULTS

In an effort to develop a test to measure the ability of certain compounds to protect sympathetic α-receptors from the inactivating action of phenoxybenzamine, the following variables were studied: dose of phenoxybenzamine, dose of epinephrine, dose of the α-receptor "protector," and the time intervals between the administration of the "protector" and phenoxybenzamine and between the administration of phenoxybenzamine and epinephrine. The results obtained using phentolamine as the "protector" are summarized in Table I.

Experiment *1* showed that the 20-min. interval between injections of phenoxybenzamine and epinephrine was too short, as 80% mortality resulted. When the interval was increased to 40 min. in experiment *2*, only 30% of the animals died, indicating that a higher proportion of α-receptors had been inactivated at the time of epinephrine injection. Using the time interval of 40 min. between the injection of graded doses of phenoxybenzamine and a dose of 0.20 mg./Kg. of epinephrine ($2.7 - 5 \times LD_{50}$), the adrenolytic ED_{50} of phenoxybenzamine was determined to be 0.014 ± 0.003 mg./Kg.[2] Further experiments with increasing doses of epinephrine (experiments *3–7*) indicated that once the adrenolytic ED_{100} of phenoxybenzamine had been reached further increases in the dose of epinephrine could not surmount the blockade of α-receptors.

The effect of the time interval between phenoxybenzamine and epinephrine administrations is shown in the results of experiments *8–11*. When the doses of phenoxybenzamine were 0.1 mg./Kg., high doses of epinephrine partially surmounted the blockade 7 hr. after phenoxybenzamine (experiment *10*) and killed all the rats 11 hr. later (experiment *11*). While the time parameter remained constant (18 hr.) in experiments *11*, *12*, and *13*, the dose of epinephrine was varied. Both 5.0 mg./Kg. (experiment *11*) and 1 mg./Kg. (experiment *12*) of epinephrine resulted in 100% mortality. However, at a still lower dose of epinephrine (0.20 mg./Kg., experiment *13*) none of the treated rats died, demonstrating that even at this prolonged time interval there was still some α-receptor blockade produced by phenoxybenzamine. Experiments *14*, *15*, and *16* showed that the adrenolytic effect of phentolamine (5 mg./Kg.), a competitive adrenolytic, did not last

Received February 9, 1967, from the Sterling-Winthrop Research Institute, Rensselaer, NY 12144
Accepted for publication May 17, 1967.

[1] Dibenamine.

[2] Only one of the four doses of phenoxybenzamine used to calculate the ED_{50} is presented in Table I.

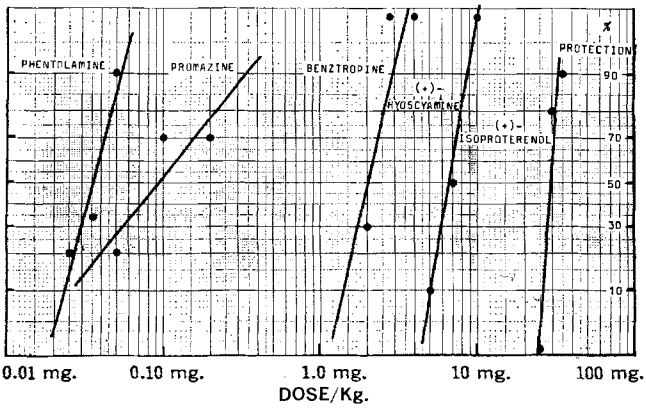


Fig. 1—Dose-effect curves of adrenolytics. Protection of α-sympathetic receptors in rats against the inactivation produced by intravenous phenoxybenzamine (0.1 mg./Kg.). Per cent protection = the per cent mortality following an intravenous high dose of epinephrine (1 mg./Kg.) injected 3 hr. after phenoxybenzamine.

TABLE I—INFLUENCE OF PHENTOLAMINE ON THE ADRENOLYTIC ACTIVITY OF PHENOXYBENZAMINE (PH) IN RATS[a]

Expt.	PH Dose, mg./Kg.	Phentolamine Dose, mg./Kg.	Epinephrine (E) Dose, mg./Kg.	Interval Between PH and E	No. of Rats	No. of Rats Killed	% Mortality
1	0.025	...	0.2	20 min.	10	8	80
2	0.025	...	0.2	40 min.	10	3	30
3	0.20	...	1.0	40 min.	10	0	0
4	0.20	...	5.0	40 min.	10	0	0
5	0.10	...	10.0	40 min.	6	0	0
6	0.10	...	20.0	40 min.	5	0	0
7	0.05	...	20.0	40 min.	5	1	20
8	0.10	...	5.0	3 hr.	5	0	0
9	0.10	...	5.0	5 hr.	4	0	0
10	0.10	...	5.0	7 hr.	10	3	30
11	0.10	...	5.0	18 hr.	5	5	100
12	0.10	...	1.0	18 hr.	5	5	100
13	0.10	...	0.2	18 hr.	5	0	0
14	...	5 (5 min.)[b]	5.0	...	5	3	60
15	...	5 (1 hr.)[b]	5.0	...	5	4	80
16	...	5 (3 hr.)[b]	5.0	...	10	10	100
17	0.10	5[c]	5.0	5 hr.	10	10	100
18	0.10	5	5.0	5 hr.	10	10	100
19	0.10	1	5.0	5 hr.	5	5	100
20	0.10	0.2	5.0	5 hr.	7	7	100
21	0.10	0.05	5.0	5 hr.	10	10	100
22	0.10	0.025	5.0	5 hr.	10	7	70
23	0.10	0.050	1.0	3 hr.	10	9	90
24	0.10	0.035	1.0	3 hr.	10	3	30
25	0.10	0.025	1.0	3 hr.	10	2	20

[a] Adrenolytic ED_{50} of phenoxybenzamine, 40 min. before epinephrine: 0.014 ± 0.003 mg./Kg. [b] Time interval between phentolamine and epinephrine. [c] Phentolamine was injected 15 min. before phenoxybenzamine; in experiments *18* to *25* the two drugs were injected in the same solution.

3 hr. against a high dose of epinephrine (5 mg./Kg.). The injection of phentolamine together with phenoxybenzamine abolished the long-acting adrenolytic action of the latter (experiments *18–25;* experiment *9 versus* experiments *18–20*). It was clear that at the doses used there was a "protection" of the sympathetic α-receptors. Experiments *22–25* showed that graded doses of phentolamine produced graded degrees of receptor protection and that an ED_{50} value could be obtained.

On the basis of the results obtained with phentolamine, the procedure previously described was adopted to test the α-receptor protective activity of a series of adrenolytics. Compounds were chosen which had weak, moderate, and strong adrenolytic activity.

The doses of phenoxybenzamine and epinephrine (0.1 and 1 mg./Kg., respectively) and the 3-hr. interval between the two injections, which the authors adopted to test the 17 compounds in the correlation study, may not be the most satisfactory. For example, although the adrenolytic effect of phentolamine disappears within 3 hr., the effect of trifluoperazine and tolazoline lingers beyond the 3-hr. interval giving false α-receptor "protection" readings. For this reason these two compounds could not be tested by the method described above. Another difficulty was the result of differences in onset of action between some adrenolytics and phenoxybenzamine. In order to demonstrate "protective" activity compounds I and III had to be injected 5 min. before phenoxybenzamine (Table II, procedure *D*). The time of onset of action might have been reduced by increasing the dose of the "protector." However, in the case of compounds I and III the increase in dosage prolonged

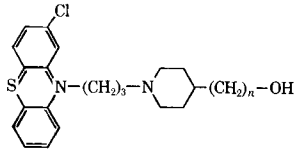
TABLE II—RELATIONSHIP BETWEEN ADRENOLYTIC AND SYMPATHETIC α-RECEPTOR PROTECTIVE ACTIVITIES IN RATS

Compd.	Form	Adrenolytic Activity ED_{50}, mg./Kg.	Sympathetic α-Receptor Protective Activity ED_{50}, mg./Kg.
I	HCl	0.020 ± 0.002[a]	0.032 ± 0.006[a]
Phentolamine	HCl	0.022 ± 0.004	0.035 ± 0.003
II	HCl	0.024 ± 0.012	0.090 ± 0.042
III	Maleate	0.033 ± 0.006[a]	0.037 ± 0.010[a]
Perphenazine	Base	0.036 ± 0.007	0.255 ± 0.053
IV	Base	0.040 ± 0.008	0.034 ± 0.007
Promazine	HCl	0.041 ± 0.010	0.094 ± 0.025
V	Base	0.048 ± 0.009	0.053 ± 0.012
VI	Base	0.062 ± 0.011	0.039 ± 0.013
Phenindamine	Hydrogen tartrate	0.080 ± 0.028	0.350 ± 0.083
Piperoxan	HCl	0.230 ± 0.054	3.85 ± 0.57
Yohimbine	HCl	0.60 ± 0.041	3.35 ± 0.47
Benztropine	Methanesulfonate	1.14 ± 0.21	2.07 ± 0.26
VII	HCl	8.3 ± 1.3	7.3 ± 1.3
(+)-Hyoscyamine	1,2-Oxobornanesulfonate	8.5 ± 1.5	6.75 ± 0.45
VIII	HCl	10.4 ± 1.9	9.2 ± 1.3
(+)-Isoproterenol	*d*-Bitartrate	12.2 ± 2.4	29.5 ± 0.95

[a] These compounds were administered approximately 5 min. before epinephrine (procedure *B*, adrenolytic test) and phenoxybenzamine (procedure *D*, sympathetic α-receptor protective test).

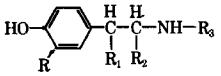
TABLE III—CHEMICAL STRUCTURE

Phenothiazines



Compd.	*n*	Compd.	*n*
I	3	IV	1
II	2	V	0
III	6	VI	4

Hydroxyphenyl Amines



		R	R_1	R_2	R_3	Salt
VII	(±)-	OH	OH	H	$-CH_2-CH_2-CH_2-CH_3$	HCl
VIII		OH	H	H	$-CH(CH_3)_2$	HCl
IX	(+)-	OH	OH	H	$-CH_2-CH_3$	HCl
X	(+)-	H	OH	H	$-CH(CH_3)_2$	*l*-Bitartrate
XI	(±)-	OH	OH	H	$-CH(CH_3)-CH_3$	Methanesulfonate
XII	(−)-	OH	OH	H	$-CH(CH_3)-CH_3$	*d*-Camphorsulfonate
XIII	(±)-	OH	OH	Et	$-CH(CH_3)_2$	HCl

their adrenolytic effect beyond the 3-hr. interval. The adrenolytic activity of these two compounds was also determined in the same manner; *i.e.*, by injecting the compound 5 min. before epinephrine. It is interesting to note that the adrenolytic ED_{50} values obtained in this manner (procedure *B*, see under *Methods*) were lower than those obtained by the standard method (procedure *A*). The values in mg./Kg. were for compound I: 0.020 ± 0.002 (*B*) *versus* 0.032 ± 0.008 (*A*); and for compound

TABLE IV—EFFECT OF (−) AND (+)-EPINEPHRINE MIXED WITH PHENOXYBENZAMINE

First Injection			2nd Injection 3 hr. Later		
Phenoxybenzamine Dose, mg./Kg.	Agonist	Dose, mg./Kg.	(−)-Epinephrine Dose, mg./Kg.	No. of Rats Inj.	No. Killed
0.2	(−)-Epinephrine	0.05	0.2	10	0
0.1	(−)-Epinephrine	0.05	5.0	10	0
0.1[a]	(−)-Epinephrine	0.2	5.0	8	0
0.2	(+)-Epinephrine	1.0	5.0	10	0
0.1	(+)-Epinephrine	4.0	5.0	10	0
0.1[a]	(+)-Epinephrine	12.0	5.0	4	0

[a] Several rats died following the first injection.

III: 0.033 ± 0.006 (*B*) *versus* 0.11 ± 0.022 (*A*). It is obvious that the full effect of these adrenolytics could not develop when injected simultaneously with epinephrine.

Correlation Study—The degree of association between adrenolytic activity (ED_{50}) and sympathetic α-receptor protective activity (ED_{50}) of 17 compounds was determined using the Spearman rank correlation method (6). The rank correlation coefficient found was 0.902. The ED_{50} values obtained are listed in Table II and the chemical structures of some of the compounds are shown in Table III.

In addition to some standard adrenolytics, a few weak adrenolytics were tested in order to widen the activity range of the groups of compounds studied. Among the latter (+)-hyoscyamine, which is responsible for the adrenolytic activity of atropine (7), and (+)-isoproterenol (8, 9) were found to be weak α-receptor protectors.

Other (+)-isomers of sympathomimetic amines were tested, but since the two ED_{50} values could not be obtained, they were not included in the correlation study. The *dextro*-isomer of *N*-ethyl norepinephrine (compound IX, Table III) was found to have a very weak adrenolytic activity (17.5 ± 8.9 mg./Kg.). Partial protection (37.5% mortality) of α-receptors was obtained at a dose of 80 mg./Kg. Higher doses were not tested. The other compound (X, Table III), the *dextro*-isomer of the *meta*-desoxy analog of isoproterenol, protected α-receptors (ED_{50}: 40 ± 3.2 mg./Kg.), but its adrenolytic ED_{50} could not be determined by the standard method. However, in rats receiving 40 mg./Kg. in the same intravenous injection with epinephrine, the LD_{50} of epinephrine was increased to 0.1 ± 0.011 mg./Kg. from a control value of 0.05 ± 0.008 mg./Kg.

Effect of Agonists on the Adrenolytic Activity of Phenoxybenzamine—Adrenergic agonists in high concentrations protect sympathetic α-receptors from the inactivating action of *N*-(2-chloroethyl)-dibenzylamine *in vitro* (4). The authors tested the effect of (−) and (+)-epinephrine mixed with another haloalkylamine, phenoxybenzamine, to determine whether agonists could occupy a sufficiently large proportion of receptors to allow epinephrine injected 3 hr. later to produce its lethal effect (Table IV).

The results in Table IV show that the isomers of epinephrine could not protect the α-receptors. If the dose of the agonist injected together with phenoxybenzamine was increased, some of the animals died. From these experiments it may be concluded that if the proportions of α-receptors occupied by the agonists in competition with phenoxybenzamine are sufficiently large, the rats die, and that in the animals that survive (Table IV) an insufficient number of receptors were protected from the action of phenoxybenzamine.

Effect of Catecholamines with No α-Receptor Activity—Since in experiments with the *dextro*-isomers of catecholamines, very large doses had been used, it was of interest to test as controls, compounds which have strong β-receptor agonist activity and no action on α-receptors. Compound XII, the *levo* form, is one of the most potent β-receptor agonists. At a dose of 20 mg./Kg. it had no adrenolytic activity when injected together with epinephrine (0.2 mg./Kg.). Injected with phenoxybenzamine (20 mg./Kg.) it had no α-receptor protective activity. On the other hand, the racemic form (compound XI) had an approximate adrenolytic ED_{50} of 15 mg./Kg. and an α-receptor protective ED_{50} of 17.8 ± 2.4 mg./Kg., indicating that in both tests the *dextro*-isomer was responsible for the observed activities. Compound XIII at 50 mg./Kg. had neither adrenolytic activity nor any protective effect on the receptor. Since XIII is the racemic form of the compound, it may be concluded that α-ethyl substitution in the molecule of (+)-isoproterenol abolishes its α-receptor blocking activity.

DISCUSSION

The results reported above have demonstrated that complete protection of the sympathetic α-receptor *in vivo* can be obtained by the injection of adrenolytics in intravenous doses as low as 0.1 mg. (or less)/Kg. The correlation study indicated a close relationship between adrenolytic and receptor protective activity. This strongly suggests that the degree of affinity of the competitive adrenolytic for the sympathetic α-receptor is involved whether the compound blocks the access of epinephrine (adrenolytic test) or phenoxybenzamine (α-receptor protective test) to the receptors.

The results obtained with the α-receptor protective test confirm the findings indicating that (+)-isoproterenol is a sympathetic α-receptor blocker (8, 9) against the negative results reported by Levy and Ahlquist (10). It has been reported (11) that in the series of *N*-alkyl analogs of norepinephrine the ethyl, propyl, and isopropyl analogs have intrinsic activity between 1 and 0 (partial agonism) and that the same was true for the *N*-*tert*-butyl and the *N*-propyl analogs of octopamine. When the adrenolytic activity of (+)-isoproterenol was discovered, it was suggested (8) that the partial agonism of isoproterenol shown by the racemic form was due to the mixture of an agonist (*levo*-isomer)

and an antagonist (*dextro*-isomer). The results obtained with (+)-isoproterenol and compounds IX, XI, XII, and XIII strongly suggest that the same explanation applies to all the compounds in the sympathomimetic amine series reported as partial agonists by Ariëns and Simonis (11).

CONCLUSIONS

Phentolamine, trifluoperazine, promazine, phenindamine, benztropine, and other competitive sympathetic α-receptor blockers injected intravenously with phenoxybenzamine in rats blocked the long-acting adrenolytic effect of the latter. A highly significant correlation was found between the sympathetic α-receptor protective and adrenolytic activities of 17 compounds.

REFERENCES

(1) Nickerson, M., *Pharmacol. Rev.*, **11**, 27(1949).
(2) Seed, J. C., and McKay, E. A., *Proc. Soc. Exptl. Biol. Med.*, **70**, 724(1949).
(3) Nickerson, M., and Gump, W. J., *J. Pharmacol. Exptl. Therap.*, **97**, 25(1949).
(4) Furchgott, R. F., *ibid.*, **111**, 265(1954).
(5) Luduena, F. P., O'Malley, E., and Oyen, I. H., *Arch. Intern. Pharmacodyn.*, **122**, 111(1959).
(6) Siegel, S., "Nonparametric Statistics," McGraw-Hill Book Co., Inc., New York, N. Y., 1956, p. 202.
(7) Luduena, F. P., and Branin, M. J., *J. Pharm. Sci.*, **55**, 280(1966).
(8) Ariëns, E. J., *Biochem. Pharmacol.*, **8**, 115(1961).
(9) Luduena, F. P., *Arch. Intern. Pharmacodyn.*, **137**, 155(1962).
(10) Levy, B., and Ahlquist, R. P., *J. Pharmacol. Exptl. Therap.*, **133**, 202(1961).
(11) Ariëns, E. J., and Simonis, A. M., *Arch. Intern. Pharmacodyn.*, **127**, 479(1960).

Irreversible Enzyme Inhibitors XCII

Inhibition of Xanthine Oxidase by Some Purines and Pyr midines

By B. R. BAKER and J. L. HENDRICKSON

Twenty-six selected compounds were investigated as inhibitors of xanthine oxidase, the enzyme that can also detoxify 6-mercaptopurine; these compounds consisted of 19 purines, four pyrimidines, two 8-azapurines, and one imidazole. Among the inhibitors that complexed to xanthine oxidase as well or better than the substrate, hypoxanthine, were thioguanine, adenine, and 6,8-dihydroxy-2-methylthiopurine (XXVI). The larger 2-benzylthio-6,8-dihydroxypurine (XXVII) was synthesized and found to inhibit equally as well as XXVI. Then 2-benzylthiohypoxanthine (XXVIII) and 8-benzylthiohypoxanthine (XXIX) were synthesized; these two compounds were complexed elevenfold and threefold better, respectively, to the enzyme than the substrate. Thus the enzyme showed bulk tolerance for the benzylthio group of XXVII–XXIX; the benzylthio group is a logical group for placement of electrophilic groups to give candidate active-site-directed irreversible inhibitors of xanthine oxidase.

XANTHINE OXIDASE is a catabolic enzyme involved in degradation of purines in a cell; the enzyme normally oxidizes hypoxanthine (I) and xanthine (II) to uric acid (III) (1, 2); the latter is then either excreted or further catabolized depending upon the species. The enzyme can also oxidize 2-hydroxypurine (IV) or 8-hydroxypurine to uric acid (III) (3) and adenine (V) to 6-amino-2,8-dihydroxypurine (VI) (4). The enzyme slowly oxidizes 4-hydroxypyrazolo-[3,4-*d*]pyrimidine (IX) to the 4,6-dihydroxy derivative (X), both of which are good inhibitors of xanthine oxidase (5, 6); in fact, IX is now marketed[1] for the treatment of gout since IX slows formation of uric acid. Other known inhibitors of xanthine oxidase are an assortment of N^6-substituted 2-hydroxyadenines (XI) and S^6-substituted 2-hydroxy-6-mercaptopurines (XII) (7).

Of interest to cancer chemotherapy is the oxidation of the anticancer agent, 6-mercaptopurine (VII), to thiouric acid (VIII) (8–10) by xanthine oxidase in mammals, thus detoxifying the drug. It has been proposed (11) that the selective action on cells by 6-mercaptopurine (VII) may be due in part to selective detoxification; if a cell can activate 6-mercaptopurine (VII) to its lethal ribonucleotide (12–14), but due to a lack of sufficient xanthine oxidase cannot detoxify VII by oxidation to the nontoxic thiouric acid, then the cell will be killed. Those normal and cancer cell lines that can oxidize VII to VIII would be less affected by VII; thus some cancer cell lines that can activate 6-mercaptopurine (VII) to the ribonucleotide may not respond to the drug due to the rapid detoxification of VII. (Scheme I.)

Received February 27, 1967, from the Department of Chemistry, University of California, Santa Barbara, CA 93106
Accepted for publication April 24, 1967.
This work was generously supported by grant CA-08695 from the National Cancer Institute, U.S. Public Health Service, Bethesda, Md.
Taken from the senior thesis submitted by J. L. Hendrickson.
The technical assistance of Maureen Baker and Pepper Caseria with some of the assays in Tables I and II is acknowledged. See under *Experimental* for assay method.
Previous paper: Baker, B. R., and Coward, J. K., *J. Heterocyclic Chem.*, **4**, 202(1967).
[1] Trademarked as Allopurinol by Burroughs Wellcome Co.