Pharmacology. The mouse intraperitoneal (ip) LD_{50} value for **2** at 24 h is 0.084 mg/kg, which is almost as toxic as **1** (LD_{50} = 0.060 mg/kg).[8] The poisoning symptoms[8] and toxicity of **2** are closely associated with its inhibition of the [^{35}S]TBPS binding site in the brains of mice 30 min after ip treatment (Figure 1A). Each assay involved a portion of the P_2 membrane fraction from the brains of two mice prepared in 0.32 M sucrose and washed three times with 1 mM EDTA by sedimentation, resuspension, and recentrifugation.[14] The final pellet was resuspended in 200 mM NaCl–50 mM sodium phosphate pH 7.4 assay buffer. Receptor assays involved various levels of the P_2 protein (50–300 μg, to ascertain the degree of linearity of binding) in 1 mL of assay buffer containing [^{35}S]TBPS (2 nM) alone or with unlabeled TBPS (2 μM) to correct for nonspecific binding (12% relative to the total binding). Following incubation for 30 min at 37 °C to achieve equilibrium between [^{35}S]TBPS and its binding site, the suspensions were subjected to rapid filtration on Whatman GF/C filters, three rinses with 2 mL of cold assay buffer, and liquid scintillation counting (LSC). The first symptoms of sedation and tremors by **2** appear at $\sim$30% inhibition and convulsions are evident at $\sim$70% inhibition of [^{35}S]TBPS binding. The behavior of **2** is the same as that of the polychlorocycloalkane insecticides in that the magnitude of inhibition at the [^{35}S]TBPS binding site correlates with the severity of the poisoning symptoms; however, **2** is at least 10-fold more potent as an ex vivo inhibitor than any of the polychlorocycloalkanes examined.[5,14] The slightly higher toxicity of **1** than **2** is not paralleled by their relative potencies in this ex vivo inhibition assay and the inhibition by **1** is not progressive with dose or consistently correlated with the poisoning symptoms. Perhaps the apparent anomalous behavior of **1** is due in part to partial dissociation from the binding site during membrane preparation and binding site assay.

Diazepam and phenobarbital administered ip at 10 and 100 mg/kg 15 and 5 min, respectively, before a 0.35 mg/kg dose of **2** greatly alleviate the poisoning symptoms at 30 min. However, these treatments do not significantly affect the ex vivo inhibition of the TBPS binding site 30 min after administering **2** with this regimen, i.e. (mean ± SD, n = 5–7) 68 ± 8% inhibition for **2** alone, 59 ± 15% inhibition for **2** with diazepam, and 61 ± 12% inhibition for **2** with phenobarbital.

The higher potency of **2** than **1** as an inhibitor of [^{35}S]TBPS binding evident ex vivo is also observed in vitro (Figure 1B). These assays were carried out as above but with EDTA/water-dialyzed brain P_2 membranes.[8] The inhibitors were introduced in 5 μL of dimethyl sulfoxide. Compound **2** with 50% inhibition at 0.25 nM is 10-fold more potent than **1** (2.5 nM) for in vitro inhibition of [^{35}S]TBPS binding.

The enhanced potency of **2** over **1** is also evident in inhibiting GABA-stimulated $^{36}Cl^-$ uptake by membrane vesicles from rat cerebral cortex.[7,15] The cerebral cortex homogenate in cold buffer (145 mM NaCl, 5 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 10 mM D-glucose, and 10 mM HEPES adjusted to pH 7.5 with Tris base) was centrifuged to obtain the 1000g pellet, which was washed by resuspension and recentrifugation. Various concentrations of **1** and **2** were preincubated with the brain vesicles (1.8 mg of protein) for 10 min at 30 °C and then GABA (100 μM final) and $^{36}Cl^-$ (0.1 μCi) were added and the uptake of $^{36}Cl^-$

was terminated 3 s later by addition of ice-cold buffer and rapid filtration, followed by LSC. GABA-stimulated $^{36}Cl^-$ uptake was calculated as the difference between basal uptake in the absence of GABA and total uptake in the presence of GABA. The enhanced potency of **2** over **1** in the [^{35}S]TBPS binding assay is also shown in the $^{36}Cl^-$ flux investigation, i.e., IC_{50} values of 25 and 40 nM, respectively (Figure 1C).

Because of its remarkable potency as a $GABA_A$ receptor antagonist, compound **2** is the best probe currently available for modeling the topography of the convulsant site of the chloride ionophore.

Christopher J. Palmer,* Loretta M. Cole
John E. Casida

Pesticide Chemistry and Toxicology Laboratory
Department of Entomological Sciences
University of California
Berkeley, California 94720
Received December 7, 1987

Novel Antihypertensives Targeted at Dopamine β-Monooxygenase: Turnover-Dependent Cofactor Depletion by Phenyl Aminoethyl Selenide

Sir:

We and others have recognized the attractiveness of dopamine β-monooxygenase (DBM; EC 1.14.17.1) as a target point for modulation of adrenergic activity and thus the pharmacological potential of substrate analogues and inhibitors for this important enzyme of catecholamine metabolism.[1-7] While the biological role of DBM is the benzylic hydroxylation of dopamine (DA) to norepinephrine (NE), we have previously demonstrated several new kinetically facile monooxygenase activities for DBM—stereoselective sulfoxidation,[7] oxygenative keton-

(14) Cole, L. M.; Casida, J. E. *Life Sci.* 1986, 39, 1855–1862.
(15) We thank Henry I. Yamamura and Takaaki Obata of the Department of Pharmacology, Arizona Health Sciences Center, Tucson, AZ, for generously carrying out the $^{36}Cl^-$ flux assays.

(1) Padgette, S. R.; Herman, H. H.; Han, J. H.; Pollock, S. H.; May, S. W. *J. Med. Chem.* 1984, 27, 1354–1357.
(2) (a) Herman, H. H.; Pollock, S. H.; Padgette, S. R.; Lange, J. R.; Han, J. H.; May, S. W. *J. Cardiovasc. Pharmacol.* 1983, 5, 725–730. (b) Herman, H. H.; Pollock, S. R.; Fowler, L. C.; May, S. W. *J. Cardiovasc. Pharmacol.*, in press.
(3) May, S. W.; Herman, H. H.; Padgette, S. R.; Roberts, S. F.; Wimalasena, K. *Acta Pharm. Suec., Suppl.* 1985, 2, 302–313.
(4) (a) May, S. W.; Phillips, S. R.; Mueller, P. W.; Herman, H. H. *J. Biol. Chem.* 1981, 256, 8470–8475. (b) May, S. W.; Phillips, S. R.; Mueller, P. W.; Herman, H. H. *J. Biol. Chem.* 1981, 256, 2258–2261.
(5) (a) Kruse, L. I.; Kaiser, C.; DeWolf, W. E., Jr.; Frazee, J. S.; Erickson, R. W.; Ezekiel, M.; Ohlstein, E. H.; Ruffolo, R. R., Jr.; Berkowitz, B. A. *J. Med. Chem.* 1986, 29, 887–889. (b) Ross, S. T.; Kruse, L. I.; Ohlstein, E. H.; Erickson, R. W.; Ezekiel, M.; Flaim, K. E.; Sawyer, J. L.; Berkowitz, B. A. *J. Med. Chem.* 1987, 30, 1309–1313.
(6) Bargar, T. M.; Broersma, R. J.; Creemer, L. C.; McCarthy, R. J.; Hornsperger, J. M.; Palfreyman, M. G.; Wagner, J.; Jung, M. J. *J. Med. Chem.* 1986, 29, 315–317.
(7) May, S. W.; Phillips, S. R. *J. Am. Chem. Soc.* 1980, 102, 5981–5983.
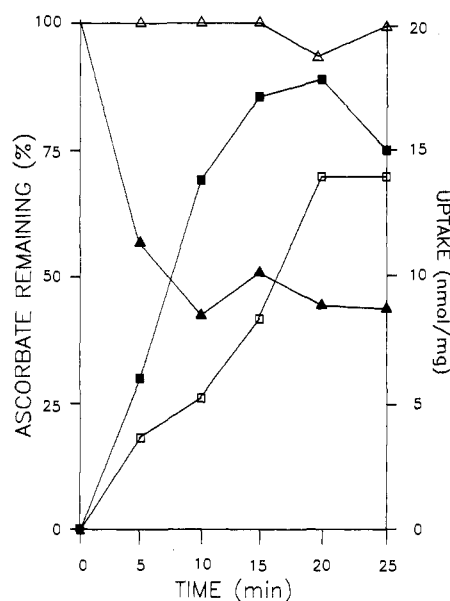
Figure 1. Time courses of PAES or PAESe uptake and ascorbate levels in resealed chromaffin granule ghosts under turnover conditions. Ascorbate-washed ghosts were resealed in a medium containing 100 mM KCl, 150 mM sucrose, 10 mM fumarate, 10 mM ascorbate, 100 µg/mL catalase, and 10 mM Tris–phosphate (pH 7.0) and purified in a discontinuous Ficoll gradient. Resealed ghosts (0.3 mg) were preincubated at 30 °C, pH 7.0, in a medium containing 4.5 mM ATP, 4.5 mM MgSO$_4$, 4.5 mM glucoascorbate, 100 µg/mL catalase, 10 mM HEPES, and 0.3 M sucrose (total volume 2.5 mL) for 10 min. The incubation was initiated by the addition of either PAES or PAESe to a final concentration of 1.0 mM. Three hundred microliter aliquots of the incubation mixture were withdrawn at the indicated time intervals and diluted into 5 mL of ice-cooled 0.4 M sucrose/10 mM HEPES, pH 7.0. At the end of the incubation period, samples were centrifuged at 36000g for 25 min at 4 °C, and washed pellets were lysed with 100 µL of 0.1 M HClO$_4$. Internal ascorbate levels were detected and quantitated by reversed-phase HPLC–EC (for details, see ref 14). PAES and PAESe uptake experiments were performed under identical conditions except that glucoascorbate was replaced with 4.5 mM ascorbate and final concentrations of PAES or PAESe were 0.9 mM. Both PAES and PAESe levels were quantitated by reversed-phase HPLC–UV. (△) Internal ascorbate levels in PAES incubations, (▲) internal ascorbate levels in PAESe incubations, (□) PAES uptake, (■) PAESe uptake.

ization,[4,8] epoxidation,[9] alkyne oxidation,[9] and oxidative N-dealkylation.[10]

We wish to illustrate a novel strategy for designing modulators targeted at an enzyme such as DBM. The strategy entails design of an alternate substrate such that it will be readily converted by the target enzyme to a product, which is then capable of causing local depletion of an essential cofactor for the target enzyme itself. Such a strategy of *turnover-dependent cofactor depletion* is conceptually quite distinct from outright inhibition of the enzyme of interest and might represent an especially useful approach to modulating compartmentalized cofactor-dependent enzymes for therapeutic purposes.

Table I. Substrate and Product Stoichiometries for Oxygenation of PAES and PAESe by DBM

	PAES[a]		PAESe[a]	
	expected	found	expected	found
O$_2$ consumed, mM	0.25	0.24	0.25	0.57
ascorbate consumed, mM	0.50	0.40	0.50	1.00
product formed, mM	0.25	0.24	0.25	b
change in O$_2$ after ascorbate readdition,[c] mM		0.00		0.63

[a] Initial [substrate], 0.25 mM. Reaction mixtures contained 1.03 mM ascorbate and other components of the standard DBM assay mixture (ref 11) and had been preequilibrated with 100% O$_2$ (ca. 1.1 mM) for 10 min. Each reaction was initiated by addition of substrate to the final concentration indicated and was then followed by monitoring the dissolved oxygen concentration in the reaction mixture. Upon termination of enzymatic oxygen consumption, aliquots of each reaction mix were withdrawn and analyzed by HPLC in order to quantitate substrate, product, and ascorbate. [b] Due to the very low concentration of product in the reaction mix, the product peak was barely visible and was poorly resolved by HPLC. [c] After samples were withdrawn for quantitation of substrate, product, and ascorbate, the reaction mixtures were reequilibrated with 100% O$_2$, and ascorbate was again added to a final concentration of 1.0 mM.

Biochemistry. We have previously reported that, consistent with its broad oxygenation competence, DBM readily oxygenates the prototypical selenide substrate, phenyl 2-aminoethyl selenide (PAESe), to the corre-

PAES: X = S
PAESe: X = Se
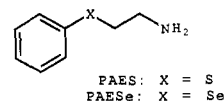
sponding selenoxide (PAESeO) via the normal, ascorbate-dependent reductive oxygenation pathway of DBM catalysis.[11] Under standard in vitro assay conditions, PAESeO is nonenzymatically reduced back to PAESe with the concomitant and stoichiometric oxidation of reduced ascorbate (ASCH$_2$); as a consequence, DBM-catalyzed turnover of PAESe is prematurely terminated due to depletion of the essential reductant, ASCH$_2$, from the assay solution. While these findings illustrate novel biochemistry for the selenide substrate, a meaningful rationale for drug design can only be established by demonstrating such ASCH$_2$ depletion even in the presence of the physiological ASCH$_2$ recycling system.

Since it is well documented that adrenal chromaffin granules, a physiological locus of DBM catalysis, possess a cytochrome b_{561} dependent ASCH$_2$ recycling system,[12–14] we examined the effect of PAESe turnover on local ASCH$_2$ levels in chromaffin granule ghost preparations, where efficient recycling might overcome any PAESeO-promulgated ASCH$_2$ depletion. Initial experiments[14] confirmed that our ghost preparations exhibit active DBM-catalyzed oxygenation of DA to NE and b_{561}-mediated regeneration of ASCH$_2$. Figure 1 illustrates time courses for uptake and ascorbate levels under turnover conditions for both PAESe and its sulfur cognate, PAES, an active DBM substrate undergoing facile enzymatic heteroatom oxygenation.[4,7,10] It is evident that ATP- and time-dependent uptake of both PAESe and PAES into the ghosts occurs readily, and a time-dependent depletion of internal ASCH$_2$ concomitant

(8) May, S. W.; Philips, R. S.; Herman, H. H.; Mueller, P. W. *Biochem. Biophys. Res. Commun.* **1982**, *104*, 38–44.

(9) (a) May, S. W.; Mueller, P. W.; Padgette, S. R.; Herman, H. H.; Phillips, S. R. *Biochem. Biophys. Res. Commun.* **1983**, *110*, 161–168. (b) Padgette, S. R.; Wimalasena, K.; Herman, H. H.; Sirimanne, S. R.; May, S. W. *Biochemistry* **1985**, *24*, 5826–5839.

(10) (a) Wimalasena, K.; May, S. W. *J. Am. Chem. Soc.* **1987**, *109*, 4036–4046. (b) Sirimannne, S. R.; Herman, H. H.; May, S. W. *Biochem. J.* **1987**, *242*, 227–233.

(11) May, S. W.; Herman, H. H.; Roberts, S. F.; Ciccarello, M. C. *Biochemistry* **1987**, *26*, 1626–1632.

(12) Diliberto, E. J., Jr.; Allen, P. L. *J. Biol. Chem.* **1981**, *256*, 3385–3395.

(13) Njus, D.; Knoth, J.; Cook, C.; Kelly, P. M. *J. Biol. Chem.* **1983**, *258*, 27–30.

(14) Herman, H. H.; Wimalasena, K.; Fowler, L. C.; Beard, C. A.; May, S. W. *J. Biol. Chem.* **1988**, *263*, 666–672.
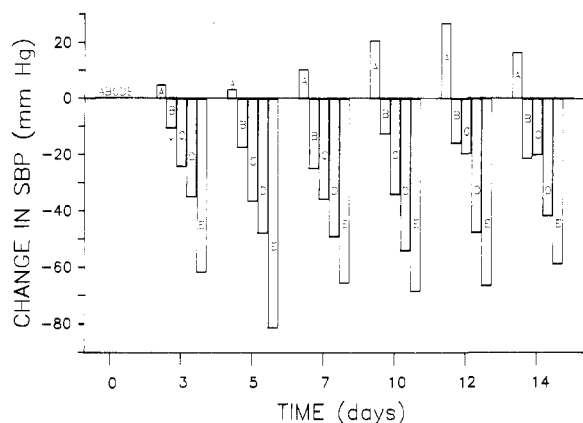
Figure 2. Chronic effect of PAESe on systolic blood pressure in spontaneously hypertensive rats. Male spontaneously hypertensive rats (SHR/NCr1BR, Charles River Breeding Laboratories) identical in age and weighing 250 g at the inception of the experiment were divided into groups of five and dosed ip, b.i.d. with either normal saline or the dose of PAESe indicated below for 2 weeks. Systolic blood pressure was measured on the days indicated by using an indirect tail-cuff blood pressure monitoring technique. A bars: SHR dosed with 0.9% saline (2 mL/kg). B bars: SHR dosed with 25 mg/kg PAESe. C bars: SHR dosed with 37.5 mg/kg PAESe. D bars: SHR dosed with 50 mg/kg PAESe. E bars: SHR dosed with 62.5 mg/kg PAESe. Data are presented as mean responses for each group. All mean values for PAESe-treated SHR were statistically different ($p < 0.05$) from control values on the same day *except* those marked with an asterisk.

with PAESe—but not PAES—turnover is clearly evident. In addition, time-dependent buildup of sulfoxide product is readily seen in the HPLC chromatographs of the PAES experiments, but there is no observable buildup of the recyclable selenoxide product, PAESeO. This contrasting effect of sulfide and selenide on $ASCH_2$ levels and the buildup of PAESO but not PAESeO clearly imply that the sulfoxide product does not undergo back-reaction with $ASCH_2$.

Confirmation of the important distinction between the enzymology of PAES and PAESe is presented in Table I, which compares results of solution experiments carried out under substrate-limiting conditions. It is evident from the tabulation of stoichiometries that product recycling indeed occurs with PAESe, since the reaction continues beyond the point that would represent stoichiometric oxygenation of all substrate initially present and terminates only when all the $ASCH_2$ is depleted. Enzymatic turnover can then be reinitiated by addition of fresh $ASCH_2$. In contrast, with PAES, enzymatic turnover stops as soon as all the substrate is oxygenated even though about half the initial $ASCH_2$ still remains in the reaction solution; addition of fresh $ASCH_2$ does *not* reinitiate turnover. Moreover, in the PAES experiment, HPLC analysis revealed complete conversion of sulfide to stable sulfoxide product at the termination point.

The observed depletion of $ASCH_2$ due to PAESe turnover in chromaffin granule ghosts containing b_{561} $ASCH_2$-regeneration system is consistent with the redox chemistry of these species. It is now well-established that semidehydroascorbate is the immediate product of DBM turnover, and it is this species that is recycled by b_{561} at the expense of extravesicular ascorbate.[12-19] In contrast,

we find that no trappable semidehydroascorbate is formed during the nonenzymatic reaction of PAESeO and $ASCH_2$.[11] Similarly, the oxidation of catechols by selenoxides is apparently a two-electron oxidation process.[20,21] Thus, if the PAESeO/$ASCH_2$ back-reaction is indeed a two-electron process in a kinetic sense (i.e., occurs without significant buildup of semidehydroascorbate), then formation of fully oxidized ascorbate would occur as selenide is processed in the vesicle. Since fully oxidized ascorbate cannot be re-reduced by b_{561}, the net result of selenide oxygenation is the effective depletion of an essential cofactor for NE production.

Pharmacology. The effect of PAESe[22] on systolic blood pressure in vivo was examined in chronic dosing experiments over a 2-week period. Age- and weight-matched male SHR in groups of five were dosed ip, b.i.d. with either normal saline or with PAESe. The results (Figure 2) clearly demonstrate that PAESe causes a marked, dose-dependent antihypertensive effect, with as much as a 50% reduction in blood pressure beginning ca. 48 h after the first dose and extending over the entire 2-week period. It is noteworthy that concomitant, dose-dependent, marked reductions in heart rate were also observed, consistent with a mechanism of decreased NE availability for sympathetic release.

The possibility of developing specific, enzyme-targeted molecules with clinical potential is the primary goal of much current effort directed toward rational design of enzyme inhibitors. The usual initial design strategy underlying such efforts focuses on potent binding of inhibitor (or reaction transients in the case of k_{cat} inhibitors) at the active site of a chosen target enzyme. While we certainly recognize that additional in vivo mechanisms may well be operative with PAESe, the results reported here do illustrate a novel initial approach for design of pharmacologically active, enzyme-targeted compounds with possible clinical potential.

(15) Wakefield, L. M.; Cass, E. G.; Radda, G. K. *J. Biol. Chem.* 1981, *261*, 9739-9745.

(16) Ljones, T.; Skotland, T. *FEBS Lett.* 1979, *108*, 25-27.
(17) Skotland, T.; Ljones, T. *Biochim. Biophys. Acta* 1980, *630*, 30-35.
(18) Diliberto, E. J., Jr.; Allen, P. L. *J. Biol. Chem.* 1981, *256*, 3385-3393.
(19) Diliberto, E. J., Jr.; Allen, P. L. *Mol. Pharmacol.* 1980, *17*, 421-426.
(20) Balenovic, K.; Bregant, N.; Perina, I. *Synthesis* 1973, 172.
(21) Marino, J. P.; Schwartz, A. *Tetrahedron Lett.* 1979, *35*, 3253-3256.
(22) The chronic antihypertensive activities of PAES and its derivatives are reported elsewhere (Herman, H. H.; Pollock, S. H.; Fowler, L. C.; May, S. W. *J. Cardiovasc. Pharmacol.*, in press.)

Sheldon W. May,* Kandatege Wimalasena
Heath H. Herman, Lydia C. Fowler
Melanie C. Ciccarello, Stanley H. Pollock

School of Chemistry
Georgia Institute of Technology
and Mercer University Southern School of Pharmacy
Atlanta, Georgia 30332

Received February 5, 1988