Table 1. Reactivity of MCR-(H-1), H-1 or MCR to NALL-1 cells tested by the indirect membrane immunofluorescent test

1st antibody	2nd antibody	3rd antibody	Full surface fluorescence
MCR-(H-1) Fraction 1	Rabbit anti-MCR	FITC-anti-rabbit 7S	+[a]
Fraction 2	Rabbit anti-MCR	FITC-anti-rabbit 7S	+[a]
Fraction 3	Rabbit anti-MCR	FITC-anti-rabbit 7S	−
MCR 100 μg/ml	Rabbit anti-MCR	FITC-anti-rabbit 7S	−
1 μg/ml	Rabbit anti-MCR	FITC-anti-rabbit 7S	−
H-1	Rabbit anti-MCR	FITC-anti-rabbit 7S	−
MCR-(H-1) Fraction 1	FITC-anti-mouse 7S		++[b]
Fraction 2	FITC-anti-mouse 7S		++[b]
Fraction 3	FITC-anti-mouse 7S		−
MCR	FITC-anti-mouse 7S		−
H-1	FITC-anti-mouse 7S		++[b]

[a] Approximately 100% of cells were positive, but fluorescence was weak.
[b] 100% of cells were strongly positive.

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The effect of solvent polarity upon rotational barriers in nikethamide

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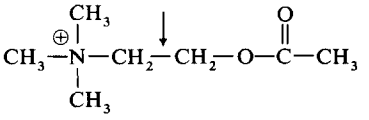
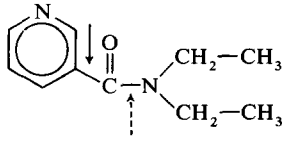
Stimulation of central muscarinic receptors, located in the medulla, causes respiratory stimulation [1]. Nikethamide is an analeptic reputed to have specific action on the medulla; however, it is known to cause widespread stimulation of the central nervous system [2]. There are a number of structural features in common between nikethamide and the endogenous neurotransmitter acetylcholine. Many quaternary ammonium compounds have been shown to interact with the acetylcholine receptor [3], and the pyridyl nitrogen of nikethamide and the quaternary nitrogen of acetylcholine may interact with the same anionic site of the receptor. In addition, the carbonyl oxygens of these molecules may participate in hydrogen bonding interactions with a target site on the receptor. We report on a chemical property of nikethamide which could account, in part, for its widespread action on the central nervous system: the hindered internal rotation of the diethylamide group.

Rotational barriers in cholinergic ligands can determine

whether a ligand is able to bind to a receptor at physiological temperature. From measurement of the energy barrier associated with rotation about the two carbon methylene chain (7.9 kcal/mole) [4], a measurement of the activation energy for acetylcholine binding to the acetylcholine receptor (5.5 kcal/mole) [5], and an estimate of the maximum available energy due to hydrogen bonding interactions between acetylcholine and the receptor (7 kcal/mole), Reed *et al.* [6] have suggested that energy barriers for conversion to preferred conformations in excess of 6 kcal/mole cannot be overcome by receptor–ligand interactions. Rotation about the two carbon methylene chain



will clearly affect the relative positions of the hydrogen bonding carbonyl oxygen and the quaternary nitrogen group. Rotation about the pyridyl ring carbon-carbonyl carbon bond (solid arrow) in nikethamide



will similarly affect the relative positions of the hydrogen bonding carbonyl oxygen and, in this case, the pyridyl nitrogen; however, rotation about the adjacent amide bond, affecting the rather bulky diethylamide group (in close juxtaposition to the important carbonyl function), would also be expected to affect the association of the drug with the receptor site. It is the rotatory event centred on the amide that forms the main focus of this communication.

The methods for calculating nuclear magnetic resonance linewidths associated with specific rate processes have been available for some time [7], and a complete discussion of the method for calculating the free energy of hindered internal motion in amides by Pople *et al.* [8]. The diethylamide group of nikethamide can be conveniently studied by this method. The methylene carbons exist in a plane with the amide nitrogen, the carbonyl carbon and oxygen, and a pyridyl ring carbon. Motion of the methylene carbons, resulting from motion about the amide bond, is a 180° flip exchanging their positions. As this rate of exchange increases, as can be experimentally induced by increasing the temperature of the nikethamide solution in the NMR sample tube, the Lorentzian line shapes of the magnetic resonance signals attributed to the methylene carbon atoms broaden. As evidenced in Fig. 1, further increases in the rate of rotation cause a downfield resonance, A, associated with the methylene carbon adjacent to the electron withdrawing oxygen, to coalesce at a centred frequency, C, with an upfield resonance, B. The free energy of this rotation can be determined from the temperature dependence of the nuclear magnetic resonance spectra.

Methods

Carbon-13 Fourier transform NMR spectra were obtained on a Bruker WM-250 spectrometer operating at 5.8 Tesla (corresponding to a ^{13}C resonance frequency of 62 MHz). The internal thermocouple was calibrated using the temperature-dependent chemical shifts of both methanol and ethylene glycol. A calibration curve was constructed by a least-squares analysis. All spectra were proton decoupled, and the chemical shifts of the methylene carbons were recorded relative to the carbonyl carbon.

Spectra were calculated by the complete bandshape method utilizing the DNMR program of Kleier and Binsch [9] implemented on a VAX 11/780 computer. Inputting the frequencies of the resonances, the linewidths, and the

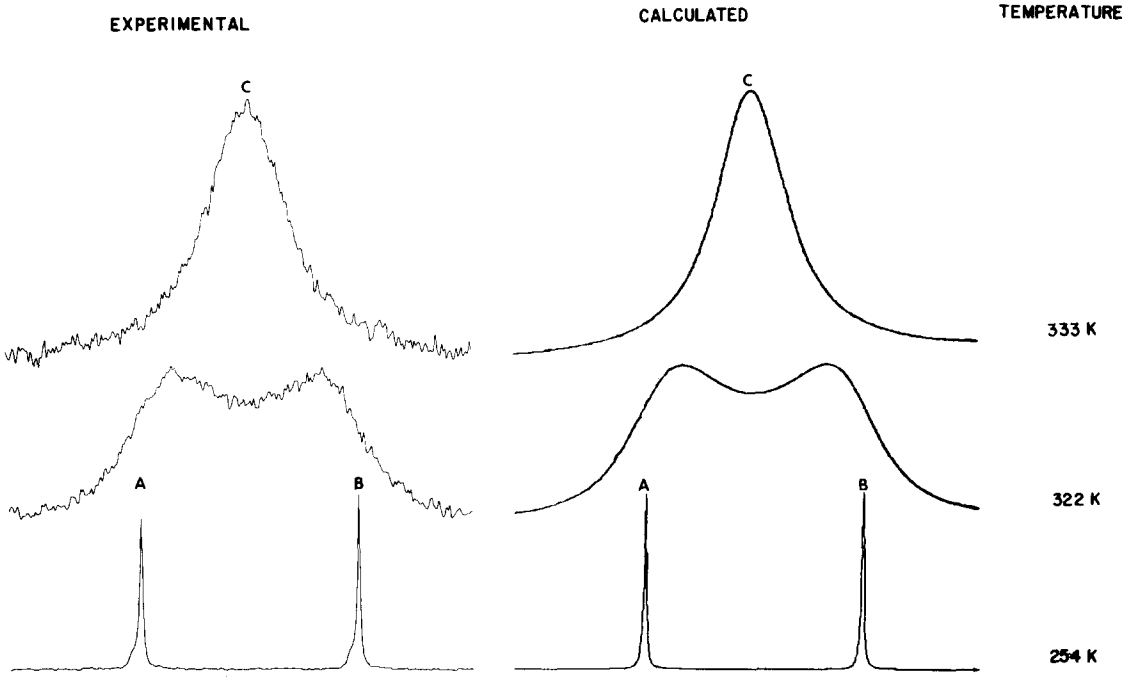


Fig. 1. Experimental and calculated nuclear magnetic resonance spectra of the methylene carbons of nikethamide at three temperatures. These experimental spectra were taken of 10.0 moles/100 ml nikethmide solvated in deuterated chloroform, and transients were taken at 333°K, 322°K, and 254°K. The rate constants used to calculate these fitted spectra were 1150.0 sec^{-1}, 410.0 sec^{-1} and 2.0 sec^{-1} respectively.

projected rate constant yielded a contracted Lorentzian line shape function. Multiplication of a line shape function over the experimental frequency range yielded a calculated spectrum whose rate constant and transverse relaxation time could be adjusted to fit the experimental spectrum.

$\Delta G\ddagger$ (the free energy of activation for rotation about the carbon–nitrogen partial double bond) values were calculated from the rate constants of the fitted spectra using the Eyring absolute rate equation [10]:

$$k = K\,(K_b\frac{T}{h})\exp\,(\frac{\Delta G\ddagger}{RT})$$

Solutions of nikethamide were made to 1.0 mole/100 ml in D_2O, D_2O with 1.0 wt/100 ml Triton X-100 (a non-ionic detergent frequently used to solubilize membrane bound proteins [11]), CH_3OD, CH_3CH_2OD, and $CH_3(CH_2)_3OD$, and to 10.0 mole/100 ml in $CDCl_3$. Samples were freeze-thawed to remove dissolved oxygen and then sealed *in vacuo* in the NMR tube. All chemicals were reagent grade products obtained from the Sigma Chemical Co. (St. Louis, MO).

Results and discussion

Figure 1 shows both experimental and calculated nuclear magnetic resonance spectra of the methylene carbons of nikethamide at three temperatures. An increase in temperature concomitantly increased the rate of motion about the amide bond, as illustrated by the progressive line broadening and coalescence with increased temperature. Other spectra were recorded at 259°K, 263°K, 268°K, 273°K, 277°K, 282°K, 286°K, 291°K, and 301°K and then assigned rate constants by the complete bandshaping method [9]. $\Delta G\ddagger$ was calculated for each fitted spectrum. The mean $\Delta G\ddagger$ value for nikethamide dissolved in $CDCl_3$ was equal to 14.565 ± 0.043 kcal/mole.

Figure 2 shows the effect of solvent polarity on methylene carbon motion. It is clearly apparent that there exists an

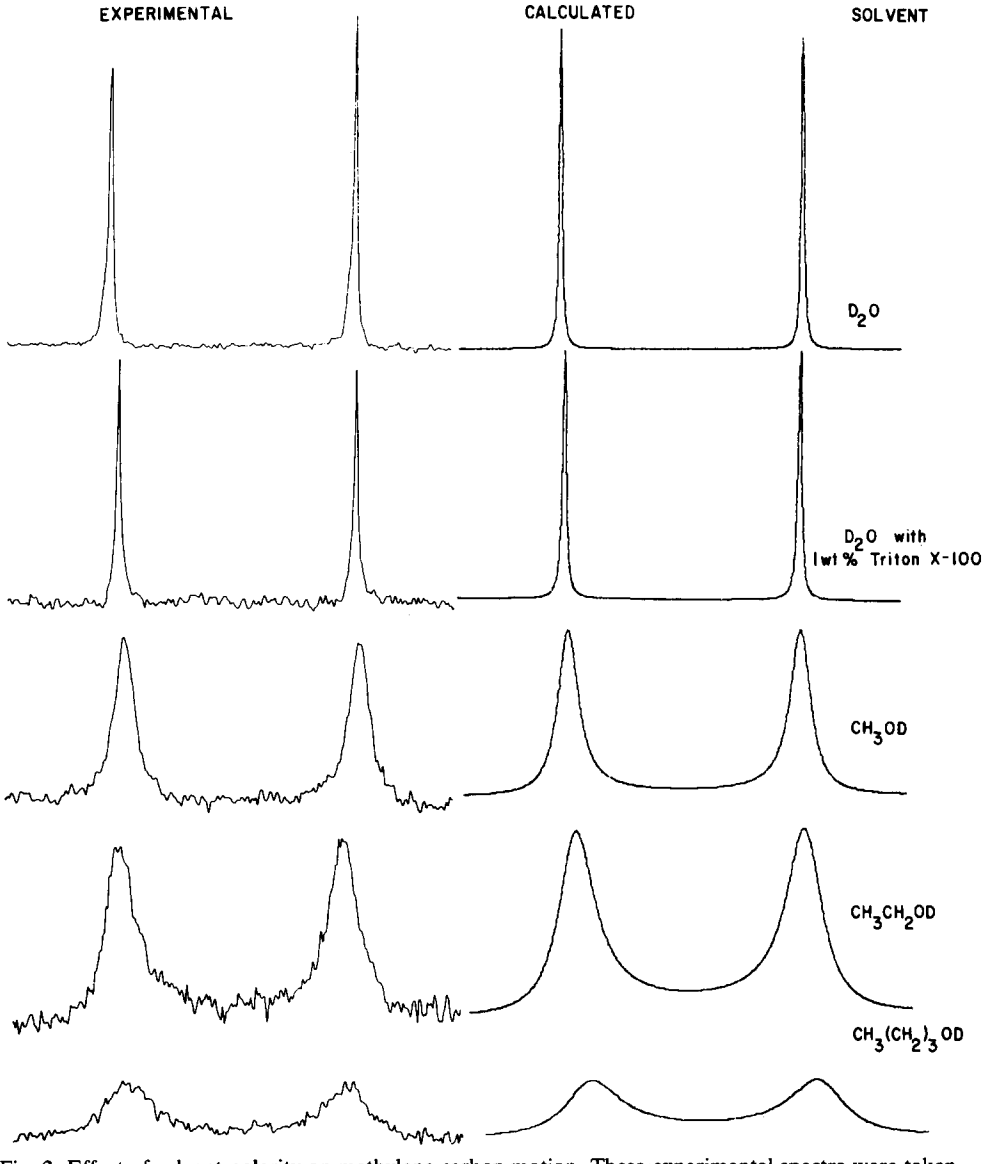


Fig. 2. Effect of solvent polarity on methylene carbon motion. These experimental spectra were taken of 1.0 mole/100 ml nikethamide dissolved in a series of solvents of decreasing polarity. All spectra were recorded at 310°K. The rate constants increased from 1.0 sec^{-1} in D_2O to 5.0 sec^{-1}, 73.0 sec^{-1}, 135.0 sec^{-1}, and 205.0 sec^{-1} in D_2O with 1.0 wt/100 ml Triton X-100, CH_3OD, CH_3CH_2OD, and $CH_3(CH_2)_3OD$ respectively.

inverse relation between methylene group motion and solvent polarity. This effect can be explained, at least in part, by the increasing probability of hydrogen bond formation between nikethamide and the solvent as solvent polarity is increased. Since viscosity effects were normalized in all of the calculated spectra, the $\Delta G\ddagger$ values, summarized in Table 1, indicate the degree to which movement about the amide bond was hindered by its partial double bond character as well as by hydrogen bonding to the solvent. The free energy of rotation of nikethamide in $CDCl_3$, a nearly nonpolar solvent, reflects the energy of rotation of the bond alone. The greater energies required for rotation in the more polar solvents reflect the increased degree of hydrogen bonding.

Table 1. $\Delta G\ddagger$ values calculated from the rate constants of line shapes fit to experimental spectra*

Solvent	ΔG (kcal/mol)
D_2O	18.16 ± 0.13
D_2O with 1.0wt% Triton X-100	17.17 ± 0.07
CH_3OD	15.52 ± 0.05
CH_3CH_2OD	15.14 ± 0.06
$CH_3(CH_2)_3OD$	14.89 ± 0.06
$CDCl_3$	14.56 ± 0.04

* The free energy for rotation about the amide bond decreased with decreasing solvent polarity.

The resulting increase in rotatory motion of the rather bulky diethylamide group in the less polar solvents is fully consistent with the anticipated greater mobility of non-polar moieties in non-polar solvents. Increasing rotatory motion accompanying decreasing solvent polarity is also expected from a consideration of hydrogen bonding potential: hydrogen bond interactions between nikethamide and solvent molecules would be adversely affected by rapid rotation of the bulky diethylamide group (i.e. rapid rotation would be disruptive to the extensive hydrogen bond network formed between drug and solvent molecules).

Diethylamide group motion at physiological temperature under conditions of varying solvent polarity is depicted in Fig. 2. The spectrum of nikethamide in D_2O is reflective of rigid molecule with an energy barrier to rotation of 18.16 ± 0.13 kcal/mole, while the spectrum of nikethamide in butanol reflects a considerable increase in the degree of rotatory freedom, with $\Delta G\ddagger$ = 14.89 ± 0.06 kcal/mole. The difference between the two energy barriers reflects the energy required to increase the rate of rotation from 1.0 sec^{-1} (in D_2O) to 205.0 sec^{-1} (in $CH_3(CH_2)_3OD$).

The greater the lipophilicity of a receptor binding site environment, the greater the degree of allowed rotatory motion for the diethylamide group. The acetylcholine receptor binding site environment is believed to contain both a lipophilic region [12] and a hydrophilic region [13] (i.e. an anionic site to accommodate the complementary cationic group of cholinergic ligands and a hydrogen bond donor to stabilize the carbonyl function of the ligands). Thus, the environment of the acetylcholine receptor site is neither entirely lipophilic nor entirely hydrophobic but, rather, a combination of these two extremes. At physiological temperature in a non-polar environment, a rotamer lifetime of approximately 0.005 sec is compatible with known physiological relaxation–excitation processes [14]; however, a more polar environment would be expected to increase rotamer lifetimes and thereby further enhance the kinetics of nikethamide association with the receptor site.

In investigations concerning the respiratory-analeptical effect of nikethamide analogues, Sattler *et al.* [15] found *N,N*-pyrimidyl 5-methyl nicotinamide to cause respiratory convulsions in rabbits, *N,N*-pyridyl 5-methyl nicotinamide to cause less pronounced spasms, and *N,N*-diethyl nicotinamide (nikethamide) to cause a respiratory increase. Pyrimidyl and pyridyl groups would rotate slower (because of their increased bulk) than the diethyl group and preclude its widespread binding to central nervous system receptors in the medulla. At the same time, the diethylamide rotation is not so fast that it will not affect a respiratory response. Nikethamide, for instance, slightly increases the contraction of rabbit ileum caused by administration of acetylcholine, whereas nicotinamide does not affect the contraction [16].

Summary

In summary, dynamic nuclear magnetic resonance techniques were used to study the hindered internal rotation of the amide bond of the analeptic nikethamide. The rotatory motion of this bond was studied in a series of solvents of increasing polarity: $CDCl_3$, $CH_3(CH_2)_3OD$, CH_3CH_2OD, CH_3OD and D_2O. Motion about the amide bond was increasingly hindered in direct proportion to solvent polarity, correlating with enhanced hydrogen bond formation between nikethamide and the more polar solvent molecules. Diethylamide group motion would be expected to affect binding of the carbonyl oxygen to cholinergic receptor sites. The degree to which association to a receptor site can be affected by this rotatory motion may vary from 0 to 4 kcal/mole, the variability being entirely dependent upon the polarity of the binding site. An increase in rotamer lifetime, corresponding to a more polar environment, would be expected to enhance the kinetics of nikethamide association to the receptor site.

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Inhibition of bovine plasma amine oxidase by lead

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A variety of symptoms are noted in patients suffering from acute lead poisoning. These include anemia [1], impaired motor function [2] and encephalopathy characterized by seizures [3, 4]. The biochemical defects giving rise to these symptoms are beginning to be defined. As a result of lead intoxication, blood lead levels rise. Lead may interact with a variety of serum and cellular enzymes to either enhance or inhibit their activity [5]. Furthermore, after lead crosses the blood–brain barrier, it has the potential to alter the metabolism of various compounds of the central nervous system. Studies using animals indicate that, after exposure to lead, the concentrations of several biogenic amines are altered in the brain [6, 7].

Recently, work in this laboratory has demonstrated that inorganic lead can inhibit two enzymes involved in norepinephrine metabolism, namely phenylethanolamine-*N*-methyltransferase [8] and dopamine-β-hydroxylase* *in vitro*. We wished to extend our studies regarding the effects of lead of amine metabolism to include a degradative enzyme. Because blood lead levels rise dramatically as a result of lead intoxication, we chose serum amine oxidase (benzylamine oxidase or amine:oxygen oxidoreductase, deaminating, E.C. 1.4.3.6). This enzyme catalyzes the deamination of biogenic amines according to the reaction:

$$RCH_2NH_2 + O_2 + H_2O \rightarrow RCHO + NH_3 + H_2O_2$$

The serum enzyme, unlike mitochondrial monoamine oxidases, is soluble and contains copper [9]. The exact nature of the active site group(s) is uncertain. Sulfhydryl [10] or imidazolium groups [9] have been implicated. Either of these functional groups could be affected by heavy metals. Dooley and Coolbaugh [11] report that micromolar concentrations of Cu^{2+} can inhibit this enzyme *in vitro*.

To date, no reports have appeared regarding the effects of inorganic lead on serum amine oxidase. In the present study we describe the kinetics of lead inhibition of the bovine plasma amine oxidase *in vitro*.†

Materials and methods

Materials. DL-Penicillamine, 1,3-dithiothreitol, benzylamine, tranylcypromine,‡ serotonin, kyuramine, pargyline and amine oxidase (partially purified from bovine plasma) were obtained from the Sigma Chemical Co., St. Louis, MO. 5-Hydroxy[side chain-2-^{14}C]tryptamine creatine sulfate (50–60 mCi/mmole) was purchased from the Amersham Corp., Arlington Heights, IL.

Assay methods. The assay for plasma amine oxidase activity was based on the method of McEwen and Cohen [12] with several modifications. Unless otherwise noted, the enzyme was exposed to $Pb(NO_3)_2$ for 10 min at 37° prior to addition of the reaction mixture. In experiments in which a chelator was also added, the enzyme was incubated with $Pb(NO_3)_2$ after which chelator was added and the samples were incubated at 37° for 30 min prior to addition of reaction mixture. The assay mixture contained 5 μmoles benzylamine and 40 μmoles Tris–HCl (pH 7.2) in a final volume of 1 ml unless otherwise noted. Controls were run in the absence of enzyme or benzylamine. The mixture was incubated for 90 min at 37°. The reaction was stopped by addition of 100 μl of 60% perchloric acid. Cyclohexane (1 ml) was added, and the tubes were vortexed and allowed to stand at room temperature for 15 min. After a second mixing they were centrifuged in an IEC Clinical Centrifuge for 10 min. The absorbance of the cyclohexane layer was determined at 242 nm using a Gilford model 2220 spectrophotometer. The data represent the means of duplicate experiments that differed by less than 10%.

One unit of enzyme activity is defined as the amount of enzyme catalyzing an absorbance increase of 0.001/min at 242 nm and 37°. This corresponds to 75 pmoles benzylaldehyde formed per min per ml.

In assays using kynuramine as substrate, the method of Harada *et al.* [13] was followed except that 75 mM Tris–HCl (pH 7.4) was used and the final volume was 1 ml. With serotonin, the only modification of the method of Honecker *et al.* [14] was the use of 0.1 M Tris–HCl (pH 7.4) and a final volume of 250 μl.

Results and discussion

Prior to the $Pb(NO_3)_2$ inhibition studies, the conditions of enzyme assay were verified. The oxidation of benzylamine was linear over the 90 min assay, and the rate of oxidation was proportional to the concentration of enzyme. Addition of 0.16 mM tranylcypromine to 7 μg protein abolished 84% of amine oxidase activity. Pargyline at a final concentration of 0.3 mM did not inhibit enzyme activity. This is consistent with data on the pig plasma enzyme [15].

The inhibition of plasma amine oxidase by $Pb(NO_3)_2$ was independent of the exposure time. When 28 μg protein was exposed to 0.3 μmole (0.3 mM) $Pb(NO_3)_2$ for intervals between 0 and 30 min prior to addition of reaction mixture, the enzyme activity was inhibited 86% in all cases. Samples incubated under identical conditions, but without $Pb(NO_3)_2$, showed no loss of enzyme activity. Therefore, we routinely incubated the enzyme with $Pb(NO_3)_2$ for 10 min prior to addition of reaction mixture. Under these conditions, amine oxidase activity was inhibited completely with 0.4 μmole (0.4 mM) $Pb(NO_3)_2$ (Fig. 1).

It was of interest to determine whether the Pb^{2+} inhibition of amine oxidase was reversible. When the concentration of $Pb(NO_3)_2$ was held constant at 0.3 mM and the amount of enzyme was varied, the resulting graph intercepted the origin (Fig. 2). This suggests that Pb^{2+} was acting as a reversible inhibitor [16]. To confirm this, we exposed the lead-treated enzyme to various compounds known to bind Pb^{2+} very tightly. When 11 units of enzyme activity were exposed to 0.3 μmole Pb^{2+} for 30 min, the

* S. Barsoum and M. L. Caspers, manuscript in preparation.

† Part of this work was presented to the American Society of Biological Chemists, June 5–9, 1983, San Francisco, CA, U.S.A. [*Fedn. Proc.* **42**, 2077 (1983)].

‡ Abbreviations: tranylcypromine, *trans*-2-phenylcyclopropylamine; serotonin, 5-hydroxytryptamine; and pargyline, *N*-methyl-*N*-2-propynylbenzylamine.