Journal of Bioenergetics and Biomembranes, Vol. 16, Nos. 5/6, 1984

Saturated Amines and Diamines as Substrates Which Inhibit Beef Liver Mitochondrial Monoamine Oxidase

Richard W. Von Korff[1] and Alan R. Wolfe[1]

Received April 11, 1984; revised June 5, 1984

Abstract

Monoamines and diamines of 8–12 carbon atoms initially serve as substrates for purified beef liver monoamine oxidase but then lead to inhibition. The inhibition is not solely the result of aldehyde formation as addition of decylaldehyde does not inhibit benzylamine oxidation. Furthermore, neither the addition of alcohol dehydrogenase and NADH nor of semicarbazide prevent the inhibition of diaminodecane oxidation. The formation of a Schiff base on the enzyme surface resulting in aggregation or occlusion of the enzyme may be a cause of the inhibition. When concentrated enzyme solutions ($\geq$1 mg/ml) are reduced by long-chain amines, 100% O_2 causes only partial return of the flavin peak at 450 nm while enzyme activity continues to decrease. Substantial recovery of activity occurs (over a 3–4 week period) when inhibited enzyme is sedimented and resuspended in fresh buffer. These observations are discussed and compared with inhibition observed by other investigators with the substrate phenylethylamine.

Key Words: Monoamine oxidase; MAO inhibitors; C_8–C_{12} amines; MAO and diamines.

Introduction

In over 50 years of research, David E. Green made phenomenal contributions both in terms of the nature and number of his investigations on cellular bioenergetics and on membrane structure and biochemistry. He was also responsible for intensive training of many persons who are now well known senior investigators. The senior author of this paper spent one of the most exciting and scientifically profitable years in David's laboratory (1951–1952).

We have been investigating some of the properties of the mitochondrial outer membrane enzyme, monoamine oxidase (E.C. 1.4.3.4). In a number of

[1]Michigan Molecular Institute, 1910 W. St. Andrews Road, Midland, Michigan 48640.

0145-479X/84/1200-0597$03.50/0

cases when solutions of MAO[2] (8–10 mg/ml) free of an absorbance peak at 412 nm were stored at 4°C a semiquinone peak appeared in the spectrum in that region (Von Korff and Wolfe, in preparation). Following dilution of these samples to scan the spectrum, the peak at 412 nm slowly disappeared. The samples were not stored under sterile conditions and we wondered whether diamines such as putrescine or cadaverine might be involved following formation by bacterial action. Since these compounds were not available in the stockroom, we tried 1,10-diaminodecane which proved to be an excellent substrate for 1–2 min but then led to total inhibition of the enzyme.

Blaschko (1952) reported that diamines with from 7 to 15 carbon atoms were substrates of MAO but made no mention of any inhibitory effects. We found that putrescine did not react with the enzyme but that cadaverine was a slowly reacting substrate and inhibitor. Neither compound resulted in the formation of a 412-nm peak. We found that monoamines of 8–12 carbon atoms were also substrates and inhibitors of varying degrees of reactivity. Alles and Heegaard (1943) reported on monoamines up to octylamine, but higher simple aliphatic amines were not studied. They noted that with aliphatic amines, the rate of oxygen utilization decreased slowly after about 15 min, but they did not report any marked inhibitions such as we have observed. Alles and Heegaard used various liver preparations of rather low activity.

We observed that when the inhibited enzyme was removed from the medium and dispersed in fresh buffer, there was a slow partial reversal of the inhibition by long-chain amines and diamines as substrates. Many aspects of the inhibitory actions of the long-chain amines and diamines are similar to the inhibition of MAO by phenylethylamine reported by Kinemuchi *et al.* (1982). This paper describes our observations with "long-chain" amine and diamines as inhibitory substrates.

Materials and Methods

Beef liver was obtained from the Marshall Packing Co., Bay City, Michigan. Fresh liver or fresh-frozen liver stored at −20°C was satisfactory for at least a year for MAO purification using a slight modification (Von Korff and Wolfe, in preparation) of the method of Salach (1978, 1979). L-α-dipalmitoyl phosphatidylcholine (synthetic), Triton X-100, dextran, polyethylene glycol, Ficoll, cobra venom, phospholipase C, and most of the amines

[2]Abbreviations: MAO, monoamine oxidase; DAD, 1,10-diaminodecane; DECA, 1-aminodecane (decylamine); ADH, alcohol dehydrogenase; PEA, phenylethylamine; NONA, nonylamine.

and diamines were obtained from the Sigma Chemical Co. Decylamine and decylaldehyde were obtained from Aldrich Chemical Co., Inc. Benzylamine and decylamine were converted to their hydrochlorides and recrystallized from alcohol.

MAO was assayed spectrophotometrically using a minor modification of the procedure described by Salach (1979) which in turn is a modification of the method of Tabor *et al.* (1954). The final reaction volume of 2.5 ml contained phosphate buffer, pH 7.5, 10 mM with 0.2% Triton X-100. The quartz cuvettes were placed in a thermostated (30°C) sample compartment of a Cary 118 spectrophotometer. After addition of a suitable amount of enzyme, benzylamine, 0.1 ml, pH 7.4, 0.083 M (final concentration 3.3 mM) was added and after mixing, the absorbance at 249 nm was recorded for several minutes. The activity was calculated as micromoles of benzaldehyde formed per minute per milliliter of enzyme (units/ml) using a molar extinction coefficient of 13,600 for benzaldehyde and using the linear portion of the recorded rate, in general from about 0.5 to 2 or 3 min. When enzyme units are specified, they are values obtained using the spectrophotometric assay with benzylamine unless specifically indicated as ΔO values.

MAO activity measurements based on oxygen utilization were performed with an Oxygraph (Gilson Medical Electronics Co.) using a Clark electrode (Yellow Springs Instrument Co.) with a 1.6-ml cell maintained at 30°C. Oxygen concentration was taken to be 223 μM or 446 nanoatoms/ml (714 nanoatoms/1.6 ml). Catalase was used so that 1 nanoatom of oxygen was consumed in the oxidation of 1 nanoatom of substrate to the corresponding aldehyde. The reaction mixture, final volume 1.60 ml, contained phosphate buffer, 10 mM, pH 7.5, and, as noted, bovine liver catalase (600 units). For benzylamine activities the substrate was 3.3 mM. The reaction was started by addition of enzyme and was usually linear for 1–3 min. Activity was calculated as μatom oxygen utilized/min/ml (U/ml) of enzyme. Specific activities were expressed as U/mg protein.

Protein was determined with a biuret procedure (Weichselbaum, 1946) for crude MAO and phospholipase, and with a modified Lowry procedure (Henry, 1964) for purified MAO.

Results

Figure 1A shows a typical polarographic trace of the rate of oxygen consumption when $\approx$1 mM DAD was added as the substrate for MAO in the presence of catalase. After a marked decrease in rate had occurred, neither the addition of horse liver ADH plus NADH (to remove aldehyde) nor of

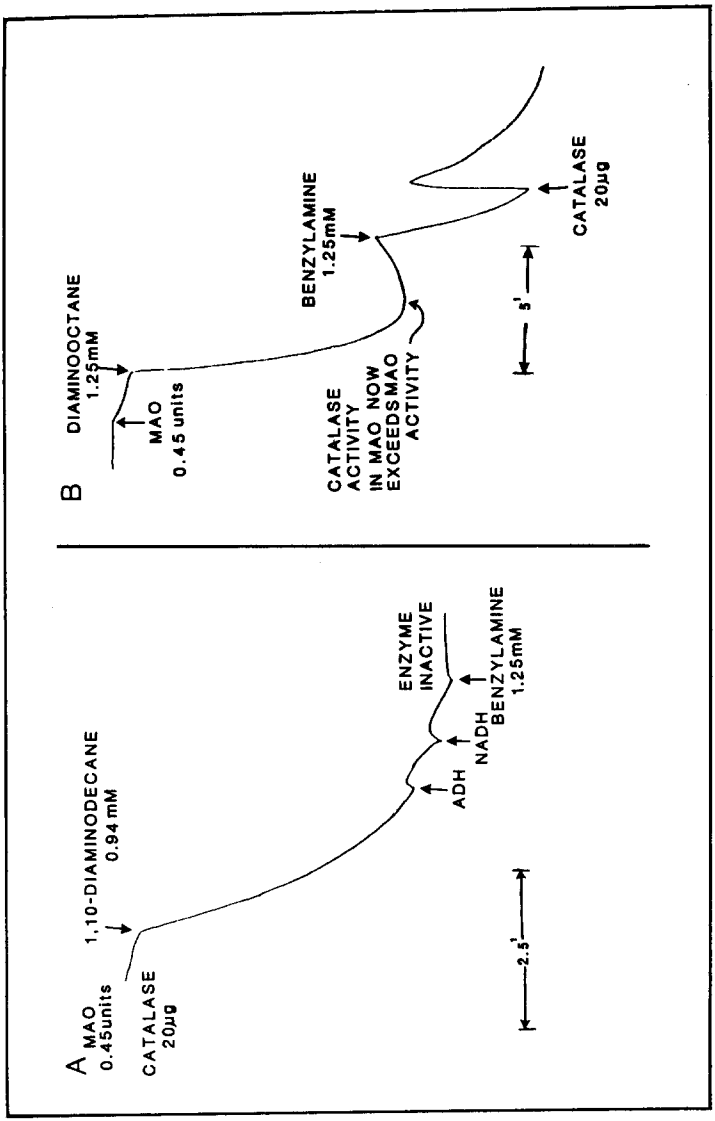

Fig. 1. Oxygen electrode traces showing diamines as substrates and inhibitors of MAO. (A) Diaminodecane as substrate: MAO, 0.45 units, 2.4 U/mg, plus 20 μg of catalase were added to the cell followed by other additions as noted in the figure. Total $\Delta O_2 = -88$ μM, rate = 55.8 μM O_2/min. (B) Diaminooctane as substrate: MAO and later addition of catalase as for (A); other additions as noted. Total ΔO_2 prior to point where $-\Delta O = +\Delta O$ (prior to BZA addition) = 79 μM, rate = 73.9 μM O_2/min. Since excess catalase was not present, ΔO (μatoms)/Δamine (μmole) = is <2 and >1. In this and subsequent figure legends, rate refers to the linear portions of the traces.

benzylamine elicited any activity. In an experiment not shown here, semicarbazide failed to block the inhibition of MAO by diaminodecane.

Figure 1B shows an experiment for which 1,8-diaminooctane was the substrate. Catalase was not added initially. As the MAO activity decreased,

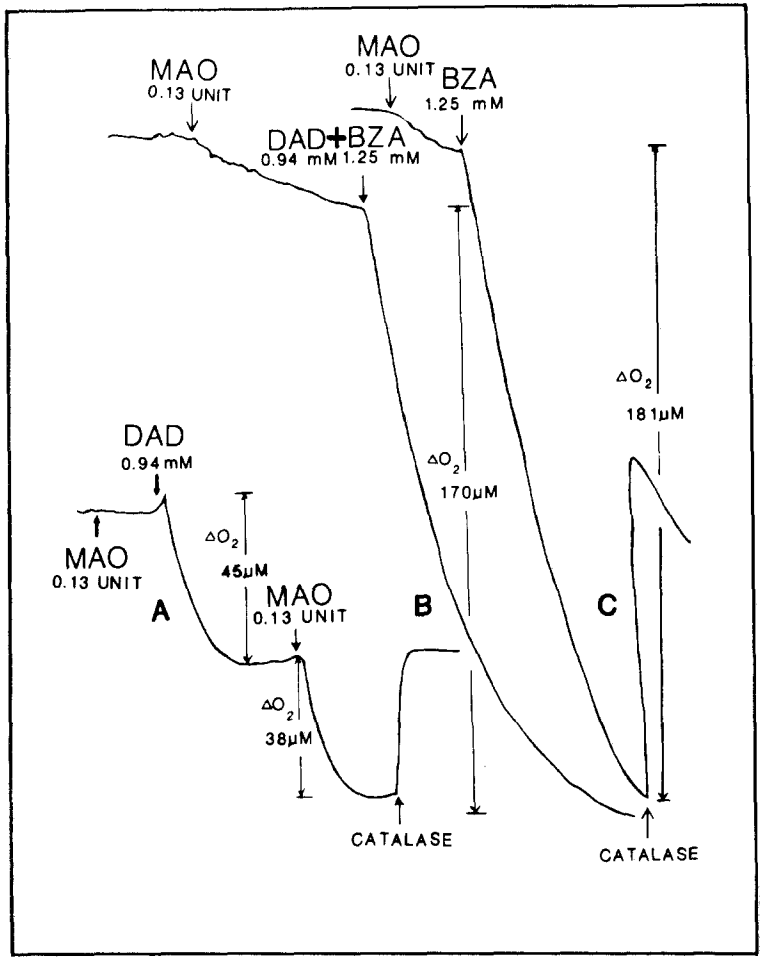

Fig. 2. Oxygen electrode traces illustrating partial protection by benzylamine of MAO inhibition by diaminodecane. (A) MAO, 0.13 unit, 3.4 U/mg, DAD added to 0.94 mM. When activity ceased, a second addition of MAO was made. Following inhibition of the second addition of MAO, catalase released O_2 but did not restore MAO activity. Total $\Delta O_2 = -86$ μM, rates = 33.5, 35.2 μM O_2/min. (B) As for (A) but benzylamine (BZA), 1.25 mM, was added in addition to DAD; 170 μM O_2 was consumed before activity nearly ceased. Total $\Delta O_2 = -170$ μM, rate = 38 μMO_2/min. (C) as for (B) but benzylamine was the sole substrate. Catalase was added after 181 μM O_2 was consumed. Addition of catalase liberated O_2, and MAO activity was again evident. Total $\Delta O_2 = -181$ μM, rate = 62.2 μM O_2/min.

oxygen was liberated from the accumlated H_2O_2 (due to traces of catalase activity in the MAO) at a rate exceeding that at which MAO consumed oxygen. MAO activity was still evident after addition of benzylamine and then of catalase.

Figure 2 illustrates a substantial protection of MAO activity by benzylamine against inactivation by DAD (Trace B compared with trace A). A control with benzylamine alone shows that the protection is incomplete (trace B compared to trace C).

Figure 3A shows that DECA, although initially a good substrate, inactivated MAO completely. As in the experiment of Fig. 1B, benzylamine was then no longer oxidized. In the absence of DECA (Fig. 3B), benzylamine oxidation continued at a rapid rate.

We have often been troubled by an apparent endogenous substrate oxidation on adding MAO to the polarographic cell following an experiment

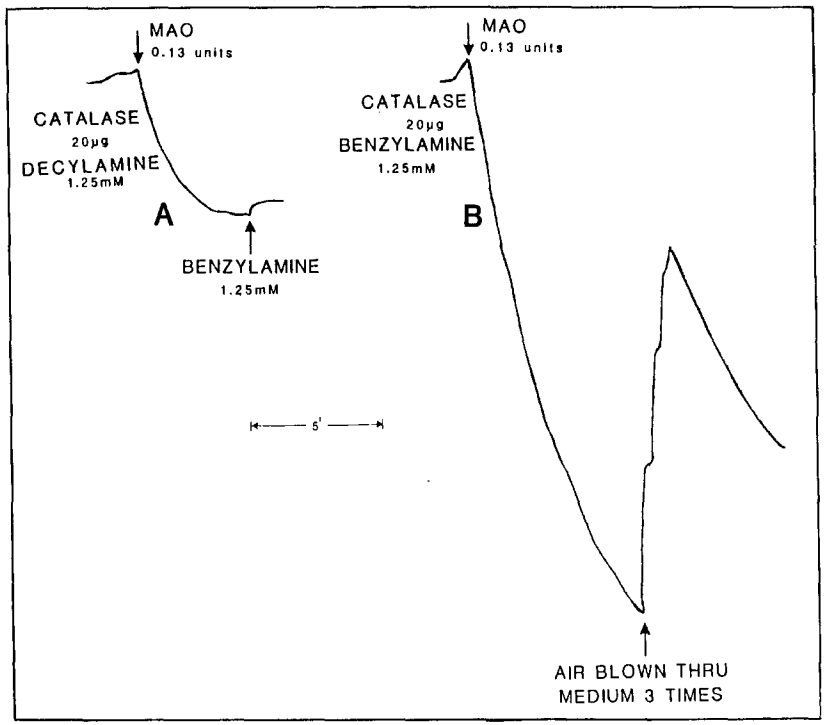

Fig. 3. Inactivation of MAO with decylamine. (A) curve showing DECA (1.25 mM), initially a good substrate, rapidly inactivated MAO. At this point, benzylamine was unreactive. MAO used, 0.13 U, 3.3 U/mg; catalase 20 µg, DECA, 1.25 mM. Total $\Delta O_2 = -39$ µM, rate = 24.6 µM O_2/min. (B) As for (A) but benzylamine was the sole substrate. ΔO_2 prior to air addition = 141 µM. Total $\Delta O_2 = -235$ µM, rate = 35.2 µM O_2/min.

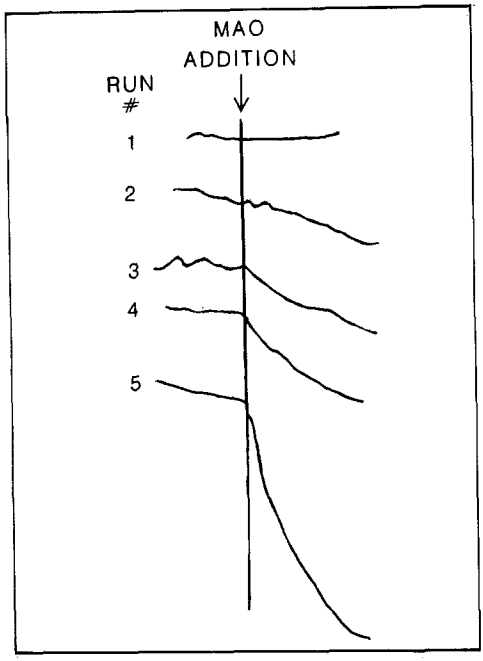

Fig. 4. Evidence for amine deposition on the polarographic cell surface. Prior to each trace shown, the rate of O_2 utilization was measured with a substrate as listed below. The cell was then thoroughly washed; fresh buffer (10 mM P_i) and then MAO were added. The "endogenous substrate" oxidation rate was then measured prior to the next addition of substrate. The substrate (1.25 mM) used for the experiment previous to trace 1 was tyramine; that for traces 2, 3, DAD; for trace 4, DAD and benzylamine; and for trace 5, DECA.

with "long-chain" amines (see Figs. 1B, 2B, and 2C). Figure 4 shows how an apparent endogenous substrate oxidation is a function of the nature of the substrate used for the previous run. Recently we have confirmed these observations with [^{14}C]nonylamine showing tenacious retention of ^{14}C in the cell and using fluorescamine to demonstrate that long-chain amines are only slowly removed from the glass surfaces following reaction of the higher amine homologs with MAO. Turbidity is often apparent as the inhibition develops, suggesting the deposition of difficulty soluble products.

Table I shows the relative initial oxygen utilization observed when various substrates (each at 1.25 mM) were reacted with the same MAO sample. The minimum rate was at a chain length of 9 carbon atoms (nonylamine).

Table I. Relative Rates of Oxygen Utilization with Several Amines as Substrates for Purified Beef Liver MAO[a]

Amine	Relative rates (ΔO)
Benzyl	1.00
Amyl	1.00
Hexyl	1.19
Heptyl	0.87
Octyl	0.79
Nonyl	0.36
Decyl	0.65
Dodecyl	0.68

[a]The buffer was 10 mM P_i, pH 7.5, 30°C. Catalase, 20 μg, and MAO were added. Substrate was then added to a final concentration of 1.25 mM. After several minutes the cell was washed three times with water and the next experiment started.

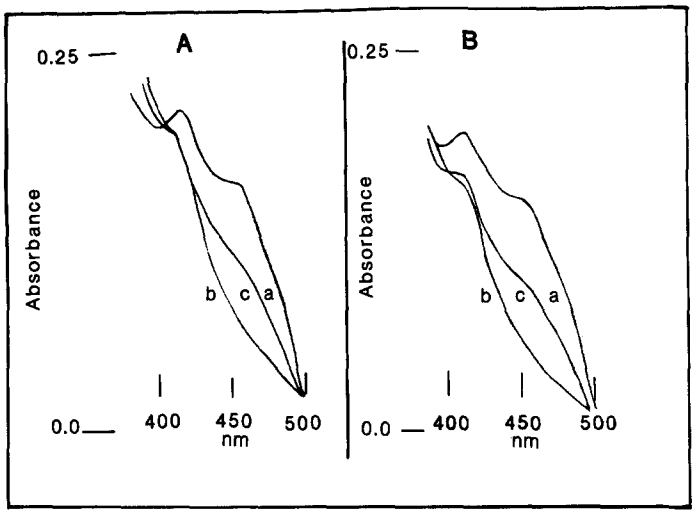

Fig. 5. Bleaching of the flavin absorbance at 450 nm by long-chain amines and partial return of 450-nm absorbance on oxygenation. To 0.9 unit of MAO, specific activity 3.3 units/mg in 0.5 ml imidazole buffer, 20 mM, pH 7.5, containing 0.2% Triton X-100 (spectra A-a, B-a), was added diaminodecane (1 mM) to yield spectrum A-b and nonylamine (1 mM) to yield spectrum B-b. Treatment with 100% O_2 resulted in only partial return (A-c) and (B-c) of the oxidized spectra while enzyme activity continued to decrease (see Table II). A similar experiment with benzylamine (not shown) led to reduction but a return to the oxidized spectrum after oxygenation. Furthermore, there was no loss of activity. The intervals a-b and b-c were only as long as required to make the additions, mix, and read the spectrum (about 1 min).

Table II. MAO Activity Before and After Treatment with Diaminodecane or Nonylamine and After Reoxygeneration with 100% O_2[a]

Conditions	Diaminodecane		Nonylamine
	ΔO	ΔA_{249} (U/ml)	ΔA_{249} (U/ml)
Before long-chain amine	2.4	4.0	4.5
After long-chain amine	—	2.1	2.7
After 100% O_2	1.2	1.7	2.0
Overnight after 100% O_2	0.6	0.9	—

[a]These data are from the experiments of Fig. 5A and 5B. Aliquots were withdrawn for assay in aerobic media using benzylamine as substrate and observing $\Delta A_{249\,nm}$.

The effect of long-chain amines and diamines on the spectrum of MAO is shown in Fig. 5. The addition of DAD (Fig. 5A) or NONA (Fig. 5B) caused bleaching of the 450-nm flavin absorbance peak. Although the solution initially was aerobic, the reaction depleted the available oxygen. Samples withdrawn for assay in aerobic medium showed extensive loss of activity with benzylamine as substrate (Table II). Although aeration with 100% O_2 resulted in a partial return of the 450-nm absorbance peak (Fig. 5A and B curve c), the activity with benzylamine continued to decrease (Table II). The flavin could be completely reoxidized if the protein was denatured with sodium dodecyl sulfate (see Discussion).

A comparison was made of the rates at which MAO was inactivated by amines of chain length C_6 to C_9 (Fig. 6). In this series, sufficient enzyme (1 mg/ml) was used to obtain a spectrum prior to and following substrate addition (1 mM). Figure 6 shows that the rate and extent of inhibition increased with an increase in chain length of the amine from C_6 to C_9. The reduction of flavin was most rapid with hexylamine (almost immediate) and slowest with nonylamine (13 min).

To determine if inhibition was reversible, nonylamine-inhibited enzyme was centrifuged, and the enzyme pellet dispersed in fresh imidazole buffer and assayed. The sample was stored at 4°C and assayed at intervals as indicated in Fig. 7 (2, 3, 5, etc. days). There was a slow return of activity over a period of 20 days.

A similar experiment was done with cadaverine. The inhibition in that case was nearly complete after 60 min incubation in 1 mM cadaverine. The total activity of the inhibited enzyme increased from 0.06 to 1.46 units after 30 days in fresh buffer.

Discussion

The behavior of C_8 to C_{12} mono- and diamines as substrates and inhibitors resembles that of phenylethylamine as reported by Kinemuchi *et al.*

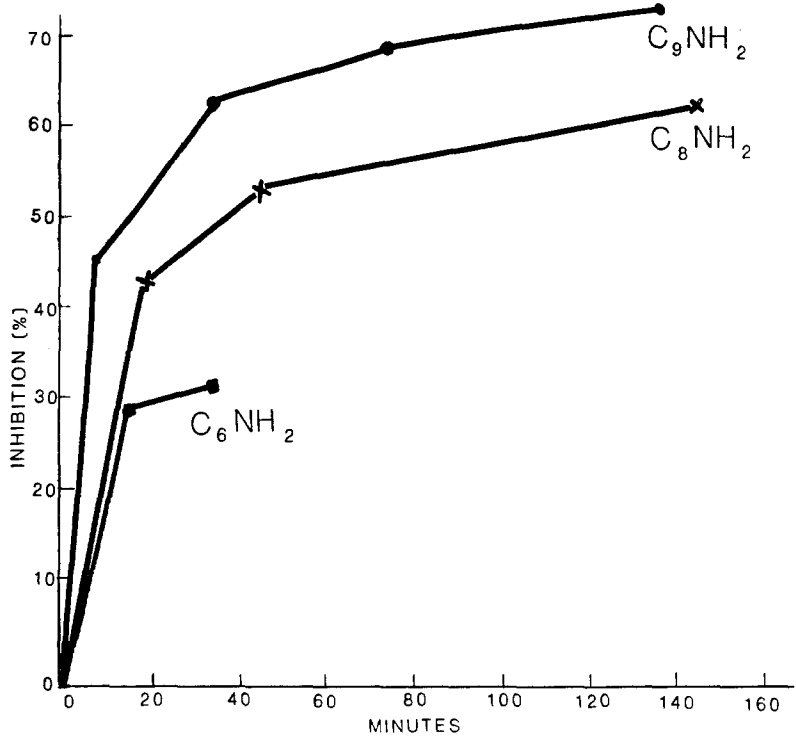

Fig. 6. Development of inhibition of MAO by several monamines (C_6 to C_9). Equal portions of the same enzyme were used for each test. MAO, 100 μl, 0.5 mg, 2.2 units/mg, was added to 0.40 ml imidazole buffer, 20 mM, pH 7.6, containing 20 μg catalase and 0.2% Triton X-100. The spectrum was scanned and 2–3 μl of sample was withdrawn for a spectrophotometric assay in 2.5 ml of aerobic phosphate buffer, 10 mM, pH 7.5, using 3.3 mM benzylamine as substrate. Amine, 5 μl of 0.10 M, was then added (final concentration 1 mM). At intervals as indicated in the figure, 2–3-μl aliquots were withdrawn for assay with benzylamine as described above.

(1982). These authors reported that inhibition of MAO by PEA was time dependent and not evident if initial rates were measured. Some important differences between their observations and ours are apparent: (1) Kinemuchi *et al.* used rat liver mitochondria and homogenates of human liver whereas we used purified MAO from beef liver. (2) Kinemuchi *et al.* were concerned with small degrees of inhibition by PEA compared to tyramine as substrate whereas we focused on the marked inhibition which occurs with ≥0.5 mM concentrations of "long-chain" amines or diamines compared to benzylamine at 3 mM. (3) With "long-chain" amines or diamines at 1 mM nearly complete inhibition usually develops within 5 min or less whereas in the studies of Kinemuchi *et al.* the inhibition at 1 mM PEA developed more slowly and to a lesser extent.

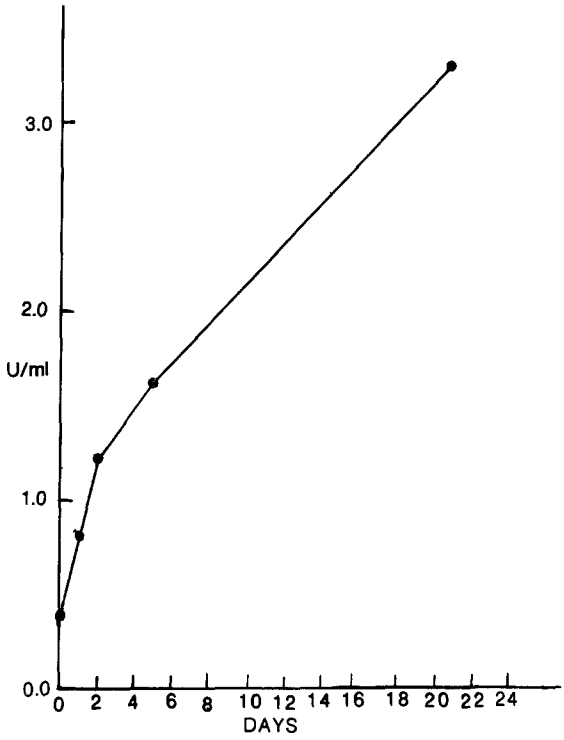

Fig. 7. Recovery of activity of a nonylamine-inhibited MAO following centrifugation and dispersion in fresh buffer. MAO, 1.44 mg, 3.1 units/mg in 1.0 ml imidazole buffer, 18 mM, pH 7.6, containing 0.1% Triton X-100, was treated with small portions of nonylamine (total 70 μl of 0.1 M); final concentration 7.0 mM. After 2 hr the enzyme was 94% inhibited as measured by assaying 5-μl portions in aerobic buffer with benzylamine as substrate. Under the assay conditions the nonylamine concentration was only 10 μM, thus eliminating competitive inhibition as a factor. The enzyme was recovered by centrifugation at 150,000 $\times$ g for 1 hr and the pellet dispersed in fresh imidazole buffer. The solution was assayed for MAO activity immediately with benzylamine as substrate and at intervals as indicated in Fig. 7 during storage at 4°C.

The frequent development of turbidity and the adherence of amines to glass following the MAO reaction with these "long-chain" amines, coupled with the very slow return of activity when inhibited enzyme is recovered and suspended in fresh buffer, suggest that some type of aggregation may have occurred. The addition of decylaldehyde in alcohol in small amounts does not lead to inhibition of the benzylamine reaction although turbidity arises due to the insolubility of the aldehyde in buffer. Reaction of the enzyme with

products from amine is a possibility. Either amine per se or imine and aldehyde formed in the reaction may be involved. The inhibition may be due to the formation of Schiff base on the enzyme surface. However, addition of NADH + ADH, while preventing formation of visible turbidity, did not prevent the inhibition.

Although protein recovery following centrifugation was not determined in the nonylamine experiment, 54% of the original activity had returned after 20 days. In another experiment in which 93% inhibition was observed with 1 mM cadaverine, 58% of the original activity was found after 30 days without correction for protein loss on centrifugation or for any concomitant loss of activity due to the prolonged storage time. Both reversal of inhibition due to cadaverine and loss of activity due to storage may have occurred simultaneously.

Hellerman and Erwin (1968) reported observations somewhat similar to those of our Fig. 5 using pargyline-inhibited beef kidney MAO. They observed reduction of the flavin absorption at 460 nm and about 50% reoxidation in that area following treatment with oxygen although enzyme activity apparently did not return.

Reoxidation of the flavin on treatment with denaturing agents (sodium dodecyl sulfate or urea) following reduction by Parnate has been described by Paech *et al.* (1979). Inactivation of MAO by Schiff base formation at one time was suggested by Hellerman and Erwin (1968) for the mode of inactivation of MAO by Parnate. Paech *et al.* (1979) reported that this could be ruled out since treatment with borohydride did not prevent release of inhibitor by denaturing agents. Kinemuchi *et al.* (1982) reported data demonstrating both reversible and irreversible inhibition of MAO by PEA.

We have now synthesized [^{14}C]nonylamine to investigate how much isotope, if any, remains associated with the inhibited enzyme and, if so, what is the nature of the material. It is hoped that isotopic data may provide clues to the nature of the inhibition and of the slow recovery of activity when inhibited enzyme is stored in fresh buffer.

Acknowledgment

This work was supported in part by a grant from the National Institute of Mental Health, Grant No. 2R01 MH 33481-02.

References

Alles, G. A., and Heegaard, E. V. (1943). *J. Biol.* **147,** 487–503.
Blaschko, R. (1952). *Pharmacol. Rev.* **4,** 414–458.

Hellerman, L., and Erwin, V. G. (1968). *J Biol. Chem.* **243,** 5234–5243.
Henry, R. J. (1964). *Clinical Chemistry, Principles and Techniques,* 1st edn., Hofer Medical, Harper and Row, p. 190.
Kinemuchi, H., Arai, Y., Oreland, L., Tipton, K. F., and Fowler, C. J. (1982). *Biochem. Pharmacol.* **31,** 959–964.
Paech, C. Salach, J. I., and Singer, T. P. (1979). In *Monoamine Oxidase: Structure, Function, and Altered Functions* (Singer, Thomas, P., Von Korff, R. W., and Murphy, Dennis, L., eds.), Academic Press, pp. 39–50.
Salach, J. I. (1978) in *Methods in Enzymology* (S. Fleischer and L. Pacher, eds.), **53,** 495–501, Academic Press.
Salach, J. I. (1979). *Arch. Biochem. Biophys.* **192,** 128–137.
Tabor, C. W., Tabor, H., and Rosenthal, S. M. (1954). *J. Biol. Chem.* **208,** 645–661.
Weichselbaum, T. E. (1946). *Am. J. Clin. Path., Tech. Suppl.* **10,** 40–49.