Amine Oxidases. XXI. A Rapid Method for the Determination of the Activity of Monoamine Oxidase and Monoamine Oxidase Inhibitors[1]

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A method for the measurement of monoamine oxidase (MAO) activity is described. It is based on the high degradation rate of *m*-iodobenzylamine and on the strong absorbance at 253 mμ of the *m*-iodobenzaldehyde formed by the action of this enzyme. The aldehyde (or the primary reaction product) appears not to undergo further oxidation by oxidoreductases. When "solubilized" homogenates are tested, well-defined reaction curves are obtained during the first minute of incubation. A linear relationship within a wide range is found between enzyme concentration and increase in absorbance and between the reciprocal values of substrate concentrations and reaction rates (Lineweaver–Burk relationship). With this procedure, the MAO levels in the liver and brain of several species are determined, and the effects of age, sex, and castration on the MAO levels of mouse liver are assessed. The method takes little time and is applicable to the study of rapid processes, such as analysis of the progressive inhibition of MAO during incubation of this enzyme with pargylamine and *o*-chloropargylamine.

Tabor, *et al.*,[2] were the first investigators who followed the action of an amine oxidase by a direct spectrophotometric method. They tested spermine oxidase (plasma amine oxidase) which attacks benzylamine among many other substrates. The product of enzymic action displays a very high extinction coefficient at 250 mμ, and can easily be detected spectrophotometrically. Since benzylamine is also attacked by monoamine oxidase [monoamine oxygen oxidoreductase (deaminating) EC 1.4.3.4], the same principle should be applicable to the determination of the activity of monoamine oxidases, a group of enzymes which is markedly different from spermine oxidases.[3] Before we could expect to produce a practical method, however, we first had to answer three questions: (1) can benzylamine, which in general is *not* a good substrate of MAO, be replaced by a better one, (2) is the aldehyde formed stable towards oxidation by one of the widely occurring aldehyde oxidoreductases, and (3) are the homogenates and mitochondrial suspensions translucent enough to permit absorbance determinations. Affirmative answers were found to these questions and a spectrophotometric method which was sensitive, rapid, and suitable for continuous recording was developed. This procedure turned out to be ready for the study of various facets of the chemistry and biology of MAO, in particular, of the evaluation of its inhibitors.

Experimental

Synthesis of *m*-Iodobenzylamine and *m*-Iodobenzaldehyde.—*m*-Iodotoluene (Eastman White Label Chemical) on photobromination[4] yielded *m*-iodobenzylbromide, m.p. 50°,[5] which was converted to *m*-iodobenzylamine, using hexamethylenetetramine.[6] *m*-Iodobenzylamine hydrochloride was purified by recrystallization from absolute ethanol: m.p. 193°, lit.[7] m.p. 193°. The ultraviolet spectrum showed a marked drop in absorbance between 250 and 280 mμ; at 253 mμ, the molar extinction coefficient in 0.067 *M* phosphate buffer pH 7.2 was 780 (for a 0.33×10^{-3} *M* solution the absorbance was 0.26).

Anal. Calcd. for C_7H_8ClIN: Cl, 13.15; N, 5.15. Found: Cl, 13.32; N, 5.05.

m-Iodobenzaldehyde was prepared from *m*-iodobenzyl bromide by the Sommelet reaction.[8] Hydrolysis of the bisulfite addition product and recrystallization from aqueous ethanol afforded the pure aldehyde with m.p. 57° (lit.[9] m.p. 57°.) and m.p. 226° for the semicarbazone (lit.[10] m.p. 225–226°). In phosphate buffer, the molar extinction coefficient of the aldehyde at 253 mμ was 9200. At this wave length, the absorbance is more than 10 times higher than that of the corresponding amine.

Treatment of Tissues with Detergent.—When tissues are homogenized in the presence of a suitable detergent, transparent solutions are obtained. These display the same MAO activity as the conventionally homogenized materials[11] and show much steadier light absorption; without such precaution, the reading is erratic for the first few minutes, because of settling of particles in the cuvette (as mentioned by Weissbach, *et al.*[12]). The recording is started usually from 10–20 sec. from the time the enzyme is pipetted into the cuvette. We measure the reaction velocity of the first minute or two from the start of the recording. In very active liver preparations, the reaction rate decreases and becomes nonlinear after this period.

The tissue or mitochondria were homogenized in a cold (approximately 4°) solution of 1.92 ml. of 0.067 *M* phosphate buffer pH 7.2 and 0.08 ml. of "Cutscum" (isooctylphenoxy-polyethoxyethanol, Fisher Scientific Co.)/100 mg. of tissue. Similarly, Gorkin, *et al.*,[13] solubilized mitochondria, but not homogenates, with the nonionic detergent OP-10. We homogenized the material with 50 up-and-down strokes in a Teflon pestle tissue grinder (Thomas, Philadelphia, Pa.) and centrifuged the product for 30 min. at 12,000*g*. The enzyme source was kept in an ice bath or at 4° at all times prior to the actual start of the reaction. The thin lipid layer which topped the liver preparation was gently pierced so that 1–2 ml. of the clear aqueous layer could be with-

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(3) E. A. Zeller in "The Enzymes," Vol. 8, P. D. Boyer, H. Lardy, and K. Myrbäck, Ed., Academic Press Inc., New York, N. Y., 1963, p. 313.

(4) J. F. Brewster, *J. Am. Chem. Soc.*, **40**, 406 (1918).

(5) All melting points were taken in a standard capillary tube melting point bath and are corrected for stem exposure.

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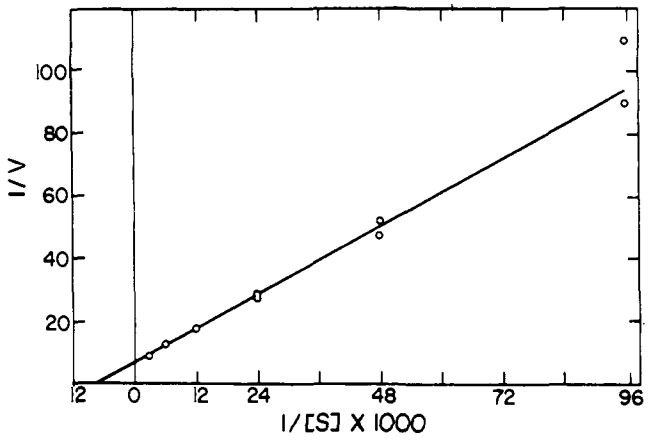

Figure 1.—Lineweaver-Burk plot for *m*-iodobenzylamine (substrate) and solubilized beef liver mitochondria (source of MAO).

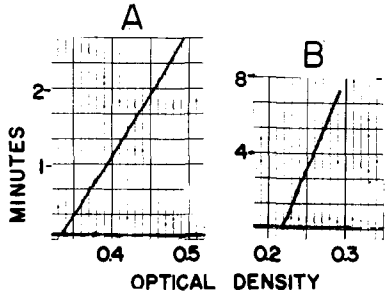

Figure 2.—Recording of monoamine oxidase activity. The curves represent increase in optical density at 253 mμ as a function of time: A, "solubilized" mouse liver homogenate (1.67 mg. of liver/ml.), run at a roller speed of 2.54 cm./min.; B, "solubilized" homogenate of human brain gray matter (3.33 mg./ml.), run at a roller speed of 6.2 mm./min.

drawn. This solution was diluted with an equal volume of cold phosphate buffer, whereas materials obtained from tissues with lower MAO activity, *e.g.*, brain, were used without further dilution. Since 253 mμ is not far away from the protein peak, one is limited in the amount of the enzyme source which can be used in the reaction mixture. For the quantities described above, large slit widths are required. These did not seem to interfere with the measurements (see Figure 1). Mitochondria were prepared according to Hogeboom, *et al.*,[14] and Cotzias and Dole,[11a] as described previously.[11b]

Choice of *m*-Iodobenzylamine as a Substrate.—Our studies on the occurrence of eutopic and dystopic complexes in the MAO reaction led to the recognition that with the exception of fluorine, the introduction of any substituent in the *meta* position led to much better substrates than benzylamine.[1b,15] By far the highest reaction rates, as expressed by maximum velocity (V), were observed with *m*-iodobenzylamine. This substrate was therefore selected for our further studies. In beef and mouse liver a value of V higher than that for tyramine, the classical substrate of MAO, was found.

Relationship between Oxidation and Deamination.—The use of *m*-iodobenzaldehyde as a basis for activity determinations rests on the assumption that the product formed by dehydrogenation of the amine does not undergo further oxidation. If the ratio of equivalents of oxygen taken up to molecules of NH_3 released by an MAO system is greater than one ($O/NH_3 > 1$), it is an indication that the primary product of the enzyme reaction undergoes further oxidation. We found that *m*-iodobenzylamine is resistant toward this kind of oxidation. Using five concentrations of this substrate ranging from 2 to 10 mM with liver mitochondria of seven species, and running the incubation for 30 min., we observed that the O/NH_3 was never more

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(15) E. A. Zeller, *Ann. N. Y. Acad. Sci.*, **107**, 811 (1963).

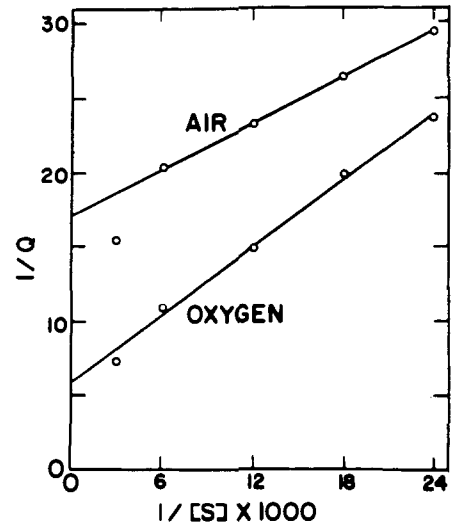

Figure 3.—Lineweaver-Burk plots obtained for two oxygen concentrations. Solubilized beef liver mitochondria are used as a source of MAO and *m*-iodobenzylamine as a substrate. The reaction rate, Q, expresses the increase in optical density at 253 mμ/mg./min.

than 10% away from unity. The same was true for liver homogenates. For tyramine we found ratios as high as 3 and 4 (cat and dog liver). Furthermore, the synthetic *m*-iodobenzaldehyde does not undergo any oxidation perceptible to manometric and photometric measurements under the conditions of our experimental procedure. In this respect, *m*-iodobenzylamine seems to be an ideal substrate.

Assay Procedure.—The standard test solutions consist of 0.2 ml. of enzyme solution and 2.8 ml. of 0.36×10^{-3} M *m*-iodobenzylamine in phosphate buffer, 0.067 M, pH 7.2 (giving a final concentration of 0.33×10^{-3} M), or 0.2 ml. of enzyme in phosphate buffer only. Vessels containing substrate and buffer solutions are shaken under oxygen at 38° for at least 10 min. and are then sealed with Parafilm (manufactured by Marathon, American Can Co., Menosha, Wis.); a separate erlenmeyer flask is used for each sample to avoid excessive oxygen dilution after breaking the seal. The enzyme solution, however, is kept in an ice bath and is not flushed with oxygen. The change in absorbance is measured at a wave length of 253 mμ in a Model 14 Cary recording spectrophotometer at a compartment temperature of 38°. An example of actual recordings is given in Figure 2. The liver samples, 1.67 mg./ml., are run for approximately 2 min. at a roller speed of 2.54 cm./min., while the brain sample, 3.3 mg./ml., are measured for 4 min. at a speed of 6.2 mm./min. Rates are expressed as differences in optical density/g. of enzyme/min. In contrast to evaluations of manometric experiments, where oxygen uptake is calculated for the total amount of enzyme present, all photometric data reported here pertain to milligrams or grams of enzyme present in 1 ml. The Beckman ultraviolet spectrophotometer and Gilford four-channel photometer have also been found to be suitable for these measurements. Oxygen consumption is determined under similar conditions as photometric changes with regard to buffer, temperature, and oxygen concentration, as described previously.[11b,16]

Effect of Oxygen Concentration on the Reaction Rate.—Earlier observations[17] regarding the effect of oxygen concentration on MAO activity were confirmed by our study. As shown in Figure 3, the rate of degradation of *m*-iodobenzylamine was much slower in presence of air than of pure oxygen. At 0.33×10^{-3} M concentration, the ratio of the two rates was 0.51. For this reason, the reaction system was saturated with oxygen (see preceding paragraph).

Results

Effect of Enzyme Concentration on the Reaction Rate.—As shown in Figure 4, the reaction rate is a

(16) E. A. Zeller and S. Sarkar, *J. Biol. Chem.*, **237**, 2333 (1962).

(17) (a) F. J. Philpot, *Biochem. J.*, **31**, 856 (1937); (b) H. I. Kohn, *ibid.*, **31**, 1693 (1937).

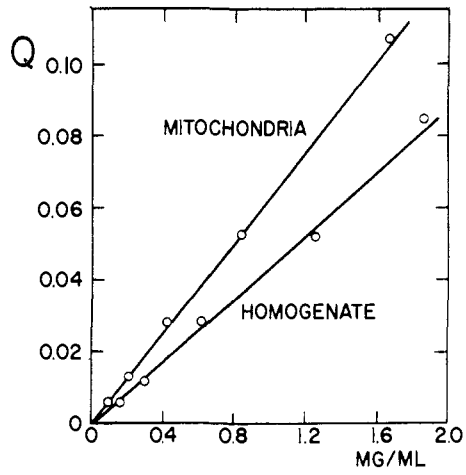

Figure 4.—Relationship between enzyme concentration (mg./ml.) and reaction rate. Homogenates and mitochondria were prepared from beef liver. Q values represent increase in optical density at 253 mμ/mg./min. with 0.33×10^{-3} M *m*-iodobenzylamine.

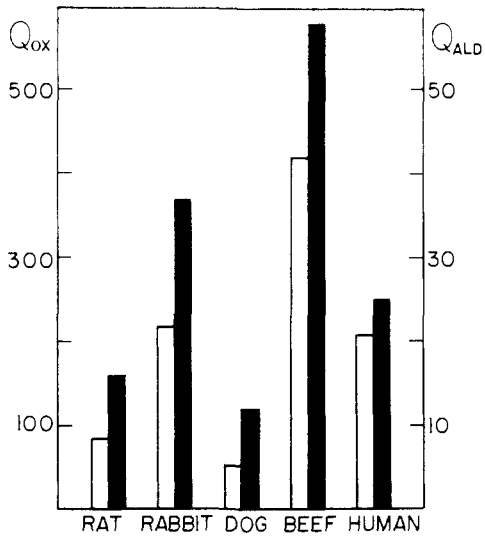

Figure 5.—Comparison of photometric and manometric determinations of monoamine oxidase. For the photometric procedure (black columns) 0.33×10^{-3} M and for the manometric (white columns) 5×10^{-3} M *m*-iodobenzylamine is used. The other conditions regarding buffer, pH, temperature, and oxygen concentration are the same for both methods. The results are given as Q_{ox} and Q_{ALD} values, indicating μequiv. of oxygen/g./hr. and difference in optical density/g./hr. (initial velocities).

linear function of the enzyme quantity added in the form of homogenate or mitochondria within a wide range.

Effect of Substrate Concentration on the Reaction Rate.—To find out whether our method could be applied for kinetic studies, we investigated the effect of substrate concentration on the reaction velocity and obtained Lineweaver–Burk diagrams of the type shown in Figure 1. These indicate that the Michaelis–Menten relationship appears to be satisfied within a wide range of concentrations (see also Figure 3).

Comparison between Manometric and Photometric Determinations.—The results of the photometric and the manometric methods were compared with each other for a number of different materials. Since the discovery of this enzyme by Hare,[18] the latter procedure has been extensively used in enzymological and pharmacological studies. The two sets of data, as listed in Figure 5, display fair agreement in spite of the difference in substrate and enzyme concentrations.

(18) M. L. C. Hare, *Biochem. J.*, **22**, 968 (1928).

Table I

Monoamine Oxidase Activities in Various Organs[a]

Species (sex)	Liver homogenate	Brain homogenate
Mouse (m)	26	2.6 (whole brain)
Rat (m)	16	
Guinea pig (f)	16	4.3 (central gray matter)
Rabbit (m)	28	2.5 (central gray matter)
Dog (f)	25	1.4 (central gray matter)
Cattle (f)	56	
Man (m)	19	3.8 (central gray matter)

[a] The given values represent differences in optical density/g./min. The determinations are carried out with homogenates under standard conditions (see Assay Procedure).

Table II

Monoamine Oxidase Activities in Mouse Liver[a]

Animals	Females	Males
Immature animals	30 ± 4.0	28 ± 6.6
Mature animals	34 ± 6.8	26 ± 4.9
Mature, castrated	32 ± 3.7	37 ± 6.6

[a] These values, obtained from 6 animals each, represent mean and standard deviations and give difference in optical density/g./min. The determinations are carried out with homogenates under standard conditions.

Measurement of Monoamine Oxidase Activity in Various Tissues.—As a test for the applicability of the method, we determined levels of MAO activity in liver and brain of various species (Table I), including the liver of immature, adult, and castrated mice (Table II).[19] For the latter, standard deviations are given as an indicator of the scattering range of the data. In all instances duplicate determinations were carried out. In a set of six livers, the average difference between two observations was slightly less than 5%.

Analysis of Inhibition Reactions.—For several reasons the photometric procedure can advantageously be used for the analysis of inhibition reactions. Large amounts of homogenates, as required for manometric and related methods, inactivate most of the iproniazid. The pI_{50} values, which range from 4.9 to 5.1 for beef liver mitochondrial MAO, drop to 3 in the presence of homogenates.[21] With the small amounts of homogenates required for our procedure, the pI_{50} was 4.8, and with solubilized mitochondria 5.5 (Figure 6).

For a second example we determined the rate of inactivation of MAO under the influence of pargylamine[22] and its *o*-chloro derivative. It has been previously observed that within the first minutes of incubation of the enzyme with iproniazid and other hydrazine derivatives,[21,23] with phenylcyclopropylamine,[16] and with

(19) Some of these data have been presented by E. A. Zeller, J. S. Schweppe, and R. E. Lane, at the 145th National Meeting of the American Chemical Society, New York, N. Y., Sept. 1963. They confirm and extend previous observations [J. S. Schweppe, E. A. Zeller, and G. M. Higgins, *Proc. Staff Meetings Mayo Clinic*, **26**, 371 (1951) and ref. 20a] regarding the effect of age and sex on liver MAO levels.

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(21) (a) E. A. Zeller, J. Barsky, and E. R. Berman, *J. Biol. Chem.*, **214**, 267 (1955); (b) A. N. Davison, *Biochem. J.*, **67**, 316 (1957).

(22) N-Benzyl-N-methylpropynylamine (Abbott Laboratories, North Chicago, Ill.).

(23) A. L. Green, *Biochem. J.*, **84**, 217 (1962).

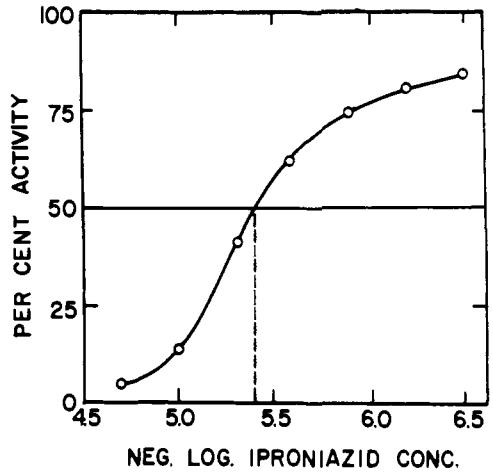

Figure 6.—Determination of pI_{50} (negative logarithm of concentration producing 50% inhibition) for iproniazid and solubilized beef liver mitochondria under standard conditions. Preincubation of enzyme with iproniazid lasted 15 min.

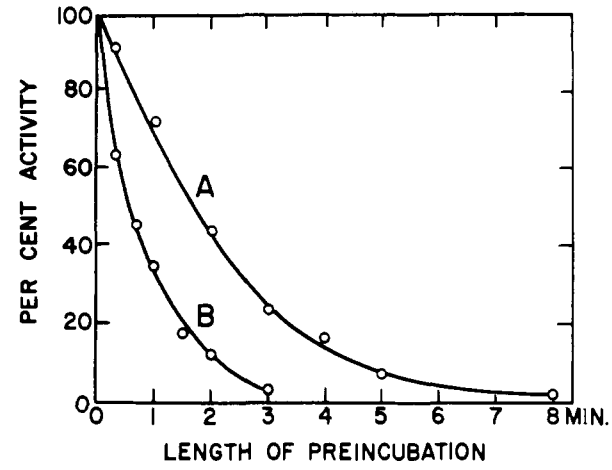

Figure 7.—Inhibition of beef liver monoamine oxidase by (A) pargylamine and (B) *o*-chloropargylamine. Solutions of the two inhibitors (2 μM) were incubated with solubilized beef liver mitochondria for various lengths of time before the addition of *m*-iodobenzylamine.

pargylamine[24] the degree of inhibition increases rapidly. The new photometric procedure, requiring very short reading periods, seems to be suited for the analysis of these fast processes and thus for the screening of MAO inhibitors. In Figure 7 the inactivation of MAO by 2 μM pargylamine and *o*-chloropargylamine is demonstrated; with the latter compound, substantial inhibition was noted after 10 sec.

Discussion

For the assessment of monoamine oxidase (MAO) activity there is only one available method which is more sensitive than the one described here. In this procedure the measurement is carried out with the help of isotopically labeled substrates[20b,c] and yields discrete points of the reaction curve only. The same is true for the method based on the disappearance of serotonin[25] or on the formation of indoleacetic acid.[26] We are mainly interested in initial velocities, which can be determined most easily with manometric and photometric measurements. Only the latter two shall be discussed henceforth. Since the original investigations on MAO,[18] the conventional manometric procedures have been extensively used for the study of this enzyme. They require large amounts of homogenates, 20–200 mg./ml. or more or the equivalent amounts of mitochondria and microsomes, as compared with the 0.5–4 mg./ml. needed for the present method. This feature of the Warburg technique makes it almost impossible to analyze the distribution of MAO in organs of low enzymic activity obtained from small laboratory animals, such as mouse brain, and to avoid inactivation of inhibitors by high tissue concentrations (see section on Inhibition Reactions). With preparations of low MAO activity an apparent or true lag phase is often observed and erratic readings are encountered, so that not too rarely it becomes difficult to determine the initial velocity precisely. In addition, the oxygen uptake may not only be caused by the MAO reaction *per se*, but also by oxygen consumption due to other reactions with endogenous substrates. Although it is possible to avoid this extraneous oxygen consumption by adding cyanide and semicarbazides to the measuring system,[17b,27] these agents may complicate the experimental situation considerably. As an example we mention the strong effect of cyanide on the inhibition of MAO by hydrazine derivatives.[21b,28] In spite of these shortcomings, the manometric procedure is still a very valuable tool in the investigation of MAO, in particular of substrate patterns. *m*-Iodobenzylamine appears to be a suitable substrate not only for photometric, but also for manometric determinations.

Other photometric procedures of MAO measurements are based on the oxidation of benzylamine[13] and kynuramine.[12] That benzylamine is often not a suitable substrate has been mentioned above. Kynuramine is deaminated and the resulting aldehyde condenses intramolecularly to form 4-hydroxyquinoline. The latter compound has a much lower molar extinction coefficient at 360 mμ than the starting product. The difference in molar optical density between the starting and end product is approximately 5000, as compared with 8400 for the pair, *m*-iodobenzylamine and *m*-iodobenzaldehyde. The initial optical readings with 0.1 mM kynuramine are relatively high, so that small differences in optical density may be of doubtful value. One could avoid this difficulty in part by following the increase in optical density between 310 and 335 mμ which accompanies the formation of 4-hydroxyquinoline. But even in this case, the molar extinction coefficient of the substrate ranges from 1000 to 3000. No experimental data for this procedure, however, are given. The sensitivity of the two methods may be best compared by indicating the differences in molar optical density induced by the same material. For the rat, the values are 2.5 (Figure 4 in ref. 12) and 16 (Table II of this paper).

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(28) T. E. Smith, H. Weissbach, and S. Udenfriend, *Biochemistry*, **2**, 746 (1963).