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Characteristics of Rat Skull Benzylamine Oxidase (41515)

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Abstract. Benzylamine oxidase (BzAO) is an amine oxidase with a widespread distribution in mammalian tissues. The present study has characterized certain properties of BzAO using homogenate of rat skull as the enzyme source. BzAO activity was assayed after inactivation of monoamine oxidases with pargyline and was shown to be distinct from diamine oxidase, polyamine oxidase, lysyl oxidase, and ceruloplasmin. Only aromatic amines with a primary amino group were found to interact with BzAO. 2-Phenylethylamine, tryptamine, *p*-tyramine, and dopamine acted as substrates but were deaminated less rapidly than benzylamine, which showed an apparent K_m value of 2.8 μM and a V_{max} value of 220 pmole deaminated mg·protein^{-1}·min^{-1}. (+)-α-Methylphenylethylamine (amphetamine) acted as a noncompetitive inhibitor of benzylamine deamination. Overall, the K_m and K_i values of the amines for BzAO increased with increasing polarity. It is concluded that the nonprotonated form of benzylamine acts as substrate for the enzyme and that the catalytic mechanism of skull BzAO appears consistent with a double displacement or "ping-pong" reaction. Compared with brain monoamine oxidase type B, benzylamine is 50 times more avid for BzAO, which, in turn, appears to exhibit a lower K_m for oxygen than monoamine oxidase type B.

Benzylamine oxidase (BzAO) is an amine oxidase present in several species including man but, to date, its physiological function is unknown. Although benzylamine is oxidatively deaminated by BzAO it is also a substrate for monoamine oxidase (MAO; EC 1.4.3.4., monoamine: O_2 oxidoreductase deaminating), particularly the B-type (1–4). Unlike MAO, however, which is inhibited by pargyline, clorgyline, and deprenyl, BzAO is inhibited by carbonyl-trapping agents such as semicarbazide. Thus, differential assay of BzAO and MAO is possible utilizing the appropriate inhibitors (2, 3, 5, 6).

BzAO was first discovered in plasma (7) and since then the plasma enzyme has been purified and characterized (1, 8–11). More recently, BzAO activity has been found to be widespread in the peripheral organs of rat and man (2–6) but this BzAO has not received the detailed attention afforded the plasma enzyme. For instance, the substrate profile of the enzyme in cellular tissues has not been defined fully and differentiation of BzAO from amine oxidases other than MAO, and which also deaminate benzylamine, has not been complete. We have shown recently that bones of the rat skull contain good BzAO activity (6). Therefore, the present study was undertaken to define and characterize skull BzAO with regard to substrate specificity, kinetic constants, pH influences, and oxygen dependence. In the latter experiments, BzAO was compared with MAO type B obtained from rat brain.

Materials and Methods. Male Sprague–Dawley rats (TIMCO Breeding Laboratories, Houston, Tex.) weighing 150–250 g were used. The rats were given free access to food and water and were housed in air-conditioned quarters on a 12-hr light–dark cycle.

Preparation of tissues. Rats were killed by decapitation. The skull (carefully scraped free of all adhering tissue) and brain were rinsed thoroughly in saline (0.9% NaCl, w/v), blotted dry, and weighed. The skull was ground up with a mortar and pestle while the brain was minced with scissors. The tissues were then homogenized (Ultra Turrex, Model SDT, for 1 min at a setting of 4.5) using a tissue: buffer ratio of 1:10 in 1 mM phosphate buffer, pH 7.8. The ho-

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mogenates were centrifuged at 700g for 10 min and supernatant fractions of 1 or 2 ml were frozen at −25° in glass vials for subsequent assay. All preparative procedures were done at 4°.

Enzyme assays. BzAO and MAO-B activities were determined radiochemically by the method of McCaman *et al.* (12) as modified by Callingham and Laverty (13). BzAO activity was measured after preincubating 25 μl homogenate with 25 μl pargyline (4×10^{-4} M for 20 min at 37°) to inhibit MAO activity. Semicarbazide (4×10^{-4} M) was used in place of pargyline to measure MAO activity. Following the appropriate preincubation, 50 μl of the radiolabeled amine (freshly prepared in 0.2 M phosphate buffer, pH 7.8) was added and each reaction was run, in triplicate, for 15 min at 37°. Enzyme activity was stopped by cooling the tubes on ice followed by the addition of 10 μl 3 N HCl. Blank values were obtained by adding the acid just prior to the addition of the labeled amine and represented less than 3% of the test values. Deaminated products were extracted in 600 μl of toluene:ethylacetate (1:1, v/v, saturated with water) and a 400-μl aliquot taken for liquid scintillation counting with quench correction. The efficiency of extraction for the various deaminated metabolites ranged from 80% (dopamine) to 98% (benzylamine) and all results are corrected for recovery.

Potential substrates and inhibitors. Various amines with a potential for substrate capability were added to the assay tubes at the same time as benzylamine (K_m concentration) whereas potential inhibitors were preincubated for 20 min at 37° prior to the addition of substrate. Control tubes contained appropriate amounts of water in place of the added amine or inhibitor and the enzymatic reaction was run for 15 min, as described above. In order to maximize inhibition of benzylamine deamination all compounds were tested at a concentration of 1 mM. None of the added substances interfered with the extraction of the deaminated metabolites.

Kinetic studies. The initial velocity rates were measured after a 5-min incubation at 37°. With BzAO, the final concentrations of the substrates were: 1, 1.25, 1.67, 2.5, 5, 10, and 20 μM for benzylamine; 3, 4.35, 6.67, 13.33, 40, and 166.67 μM for both 2-phenylethylamine and dopamine; 16.7, 25, 33.33, 50, 66.67, and 100 μM for both tryptamine and p-tyramine. With MAO, 100, 167, 250, 500, and 800 μM benzylamine was required. The apparent K_m and V_{max} values were calculated by computer program according to the method of Wilkinson (14).

K_i values were determined by adding the unlabeled amine at the same time as labeled benzylamine. The final concentrations of the unlabeled amines were: 7.5, 15, and 30 μM for 2-phenylethylamine and 25, 50, and 100 μM for both tryptamine and (+)-amphetamine.

Oxygen tensions were altered in some kinetic experiments by bubbling the assay tubes for 15 sec at 4° with either nitrogen or oxygen and quickly sealing the tubes with rubber stoppers.

Effect of pH. The pH of the final assay mixture was varied between 5 and 10 by adjusting the pH of the 0.2 M phosphate buffer.

Protein. The protein content of homogenates was measured by the microbiuret method of Goa (15) with bovine serum albumin (Fraction V) as the standard.

Chemicals. The following radiolabeled compounds were used: [^{14}C]benzylamine HCl (12.5 mCi·mmole^{-1}); [^{3}H]tryptamine HCl (0.71 Ci·mmole^{-1}); [^{14}C]dopamine HCl (44.8 mCi·mmole^{-1}); 2-[^{14}C]phenylethylamine HCl (52.0 mCi·mmole^{-1}) and [^{14}C]-tyramine HCl (50.7 mCi·mmole^{-1}). The specific activities and concentrations of these compounds were adjusted using the nonlabeled hydrochloride salts of the respective amines. All other compounds and chemicals were of analytical grade where possible.

Results. Table I shows the effect of various compounds on the deamination of benzylamine in homogenate of rat skull. Monoamine oxidases were inactivated previously by preincubation with pargyline (see Materials and Methods). Overall, 23 different compounds were tested including derivatives of 2-phenylethylamine, indoleamines, diamines, polyamines and

TABLE I. EFFECT OF VARIOUS COMPOUNDS ON THE DEAMINATION OF BENZYLAMINE BY BENZYLAMINE OXIDASE[a]

Compound	Percentage control activity	Compound	Percentage control activity
Benzylamine	1.2	Histamine	55.0
2-Phenylethylamine	2.6	Lysine vasopressin	82.1
Phenylethanolamine	3.0	*N*-Methylphenyl-ethanolamine	86.5
(+)-Amphetamine	6.4	*N*-Methylphenyl-ethylamine	97.1
N-(1-naphthyl)ethylene-diamine	6.9	Putrescine	102.0
Tyramine	10.1	Cadaverine	108.0
Tryptamine	13.1	KCN	109.0
Dopamine	17.2	(±)-Penicillamine	110.0
5-Hydroxytryptamine	25.4	Spermine	115.0
Normetanephrine	28.8	Spermidine	115.0
N,N-dimethyl-*p*-phenylenediamine	29.0	γ-Aminobutyric acid	118.0
Norepinephrine	36.1		

[a] Homogenate was preincubated for 30 min at 37° with pargyline to inactivate monoamine oxidase. KCN and (±)-penicillamine were then added and preincubated for a further 30 min at 37°. Other compounds were added at the same time as [^{14}C]benzylamine (3 μM) and the reaction was run for 15 min at 37° using triplicate determinations. All test compounds were used at 1 mM.

other miscellaneous substances. Only amines containing a primary amine group caused marked inhibition of benzylamine deamination. Thus, 2-phenylethylamine was found to be a very good inhibitor whereas *N*-methyl-phenylethylamine was inactive. In general, inhibitory activity of the phenylethylamines decreased as polarity increased. The aliphatic straight chain diamines (cadaverine and putrescine) and the aliphatic straight chain polyamines (spermine and spermidine) were inactive at inhibiting benzylamine deamination. Peptidyl-bound lysine (lysine vasopressin) failed to give marked inhibition and it is noteworthy that the enzymatic activity was insensitive to (±)-penicillamine and potassium cyanide.

Certain aromatic monoamines were selected to test for substrate capability and kinetic analysis. Table II shows that 2-phenylethylamine, certain of its derivatives, and the indoleamine, tryptamine, acted as substrates for rat skull BzAO. However, it is clear that benzylamine is the preferred substrate. Ring hydroxylation, as with *p*-tyramine and dopamine, gave increased K_m values. 2-Phenylethylamine and

TABLE II. KINETIC CONSTANTS OF VARIOUS AMINES FOR BENZYLAMINE OXIDASE[a]

Amine	K_m (μM ± SE)	V_{max} (pmole deaminated·mg protein^{-1}·min^{-1} ± SE)	Interaction with benzylamine	K_i (μM)
Benzylamine	2.8 ± 0.2	220.2 ± 0.1	—	—
2-Phenylethylamine	14.5 ± 1.1	47.8 ± 1.8	Competitive	14.2
Tryptamine	54.3 ± 7.4	57.2 ± 4.2	Competitive	55.0
p-Tyramine	66.8 ± 7.6	36.6 ± 2.3	—	—
Dopamine	269.7 ± 10.1	80.7 ± 2.0	—	—
(+)-Amphetamine	—	—	Noncompetitive	35.5

[a] All experiments were done at pH 7.8 under an atmosphere of air, using triplicate determinations at each substrate concentration used. The K_m and V_{max} values for dopamine must be regarded as approximations since the concentrations of dopamine employed were below those required for valid estimates (see Materials and Methods). The K_i value for (+)-amphetamine is likely to represent a hybrid value resulting from an interaction with both the oxidized and reduced forms of BzAO (see Discussion).

tryptamine were shown to act as competitive inhibitors of benzylamine deamination and the resulting K_i values agree well with the K_m determinations. (+)-Amphetamine (alpha-methyl-phenylethylamine) acted as a noncompetitive inhibitor with an inhibition constant consistent with its low polarity.

The effect of varying pH on the kinetic constants for benzylamine was determined at pH 6.9, 7.3, 7.8, and 8.2. Full double reciprocal plots were done at each pH and the K_m and V_{max} values computed. The results are illustrated in Fig. 1. Over the pH range studied, the apparent K_m values declined almost linearly as pH increased. It was noted, however, that calculation of the pH-independent K_m values (the K_m in terms of the concentration of nonprotonated benzylamine) showed little deviation from 0.05 μM (Fig. 1). The pH-independent K_m values were computed from McEwen's (16) modification of the Henderson–Hasselback equation by substituting in the experimentally derived K_m values and using 9.37 as the pK_a for benzylamine. The V_{max} values at pH 6.9 and 7.3 were comparable but diminished rates were obtained at the two higher pH values. In another experiment (Fig. 2), the deamination of a single concentration of benzylamine (50 μM) was studied. Maximal deamination was found at pH 7.4.

The results of altered oxygen tensions on the deamination of benzylamine by BzAO are shown in Fig. 3 and Table III. For comparison, a similar experiment was done with MAO from rat brain. We have shown previously that the deamination of benzylamine by rat brain homogenate is due entirely to MAO type B (6). Under an at-

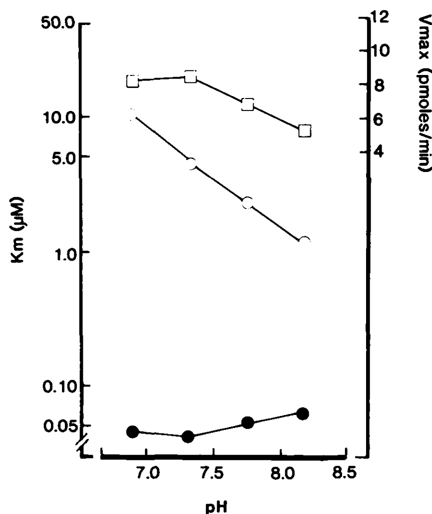

FIG. 1. Effect of pH on the Michaelis–Menten constants of benzylamine for benzylamine oxidase. Ordinate, left-hand side: experimentally derived K_m values (○) and calculated pH-independent K_m values (●) on a log scale. Ordinate, right-hand side: V_{max} values (□). Abscissa: pH of the reaction mixture. The values were derived from full double reciprocal plots using triplicate determinations at each benzylamine concentration. The enzyme reaction was run for 5 min after inactivation of monoamine oxidase with pargyline. The pH-independent K_m values (the K_m in terms of the concentration of nonprotonated benzylamine) were computed from McEwen's (17) modification of the Henderson–Hasselback equation using the experimentally derived K_m values and 9.37 as the pK_a for benzylamine.

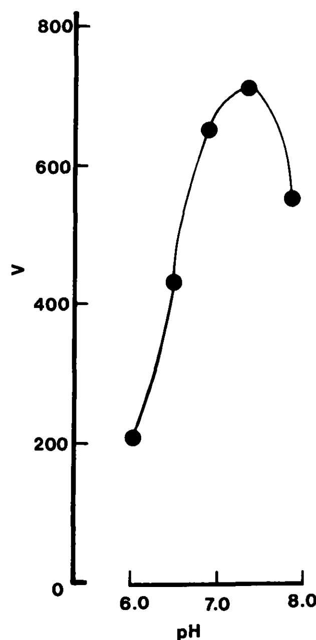

FIG. 2. pH optima curve for benzylamine deamination by benzylamine oxidase. Ordinate: v, velocity of the enzyme reaction. Abscissa: pH of the reaction mixture. Benzylamine oxidase activity was assayed using 50 μM benzylamine for 15 min at 37° after inactivation of monoamine oxidase with pargyline. Each point is the mean of triplicate determinations.

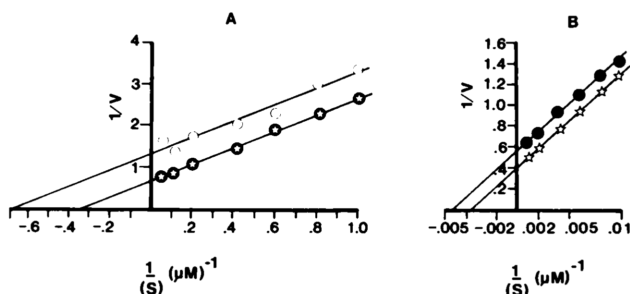

FIG. 3. Double reciprocal plots for benzylamine deamination by benzylamine oxidase (A) and monoamine oxidase type B (B) in the presence of differing oxygen tensions. Ordinate: reciprocal of velocity (v) of the enzyme reaction in arbitrary units. Abscissa: reciprocal of the benzylamine concentration. The enzyme reactions were run for 5 min and each point is the mean of triplicate determinations done under an atmosphere of air (●), oxygen (☆), or nitrogen (○) at pH 7.8. Note that the double reciprocal plots for benzylamine oxidase, whether assayed under air or oxygen, are identical. For kinetic constants, see Table III and for further experimental details, see Materials and Methods.

mosphere of oxygen the double reciprocal plot for BzAO was identical to that obtained under air. In contrast, a parallel, uncompetitive shift was obtained for MAO type B. For BzAO, oxygen dependence only became evident after flushing the assay tubes with nitrogen. The apparent K_m and V_{max} values were then decreased by 50% (Table III). It is clear from Table III that BzAO appears as a high-affinity, low-capacity enzyme for benzylamine deamination, compared with MAO type B.

Discussion. Recently we reported BzAO activity in the bones of rat skull (6) and the present study has investigated certain properties of this amine oxidase.

TABLE III. APPARENT KINETIC CONSTANTS FOR BENZYLAMINE DEAMINATION BY BENZYLAMINE OXIDASE (BzAO) AND MONOAMINE OXIDASE TYPE B (MAO-B) UNDER DIFFERING OXYGEN TENSIONS[a]

Tissue and condition	K_m (μM)	V_{max} (pmole deaminated · mg protein^{-1} · min^{-1})
BzAO (skull)		
Oxygen	3.0	223
Air	3.0	223
Nitrogen	1.5	113
MAO-B (brain)		
Oxygen	223.5	3630
Air	158.5	2620

[a] For double reciprocal plots, see Fig. 2.

The results shown in Table I give important information concerning the characteristics and definition of BzAO. The enzyme displays characteristics typical of pyridoxal-dependent amine oxidases (1) in that a primary amine group is required for interaction. The inability of the aliphatic diamines (putrescine and cadaverine) and the aliphatic polyamines (spermine and spermidine) to inhibit benzylamine deamination clearly distinguish BzAO from diamine oxidase and polyamine oxidase (spermine oxidase). An indication for substrate overlap with diamine oxidase is suggested from the inhibitory action of histamine (45%) and with ceruloplasmin from the marked inhibition with *N,N*-dimethyl-*p*-phenylenediamine (71%). However, the involvement of ceruloplasmin is ruled out by insensitivity to potassium cyanide (17). BzAO is also quite distinct from lysyl oxidases since it is resistant to inhibition by (±)-penicillamine and was only slightly affected by lysyl vasopressin. In addition, according to Shieh *et al.* (18), benzylamine is not a substrate for lysyl oxidase. Thus, overall, there is little doubt that BzAO of rat skull represents a separate and distinct activity.

Of the amines tested for substrate capability, it is obvious that benzylamine acts as the preferred substrate (Table II). Skull BzAO, however, is not without catalytic activity upon the indoleamine and

phenylethylamine structure with tryptamine and 2-phenylethylamine interacting competitively toward benzylamine. Ring hydroxylation of 2-phenylethylamine, as with *p*-tyramine and dopamine, resulted in higher K_m values indicating a reduced affinity for BzAO. Thus, BzAO resembles MAO type B which also shows a distinct preference for relatively nonpolar amines (benzylamine and 2-phenylethylamine). However, benzylamine possesses a much lower K_m value for BzAO than MAO type B. Under an atmosphere of air, the respective apparent K_m values were about 3 and 158 μM at pH 7.8 (Table III). This is not the case for 2-phenylethylamine which exhibits an apparent K_m value of 3 μM for MAO type B (19) versus 14.5 μM for BzAO (Table I).

The low apparent K_m value of benzylamine for BzAO suggests that this amine might act as the endogenous substrate for the enzyme. Certainly, low concentrations of benzylamine would be deaminated preferentially by BzAO rather than by MAO type B. So far, however, benzylamine has not been detected in the tissues of animals or man, nor is it present in the atmosphere. On the other hand, the V_{max} values for the endogenously occurring amines (Table II) are so low that deamination by BzAO would be predicted to be negligible compared with that resulting from MAO, an enzyme which consistently exhibits V_{max} values in the nanomolar per minute range, even with benzylamine as substrate (Table III). It is not known whether this difference reflects turnover rates of the respective enzymes or merely differences in enzyme concentration. Also, with intact cells, *in vivo*, it is possible that the access of amines to BzAO versus MAO may differ, resulting in a relatively greater metabolic role of BzAO than would be predicted from *in vitro* studies. Recently, BzAO has been reported to be located in the plasma membrane of cells (20) and as such it would be much more readily available to plasma-borne substrates than mitochondrial MAO. In fact, in the isolated perfused rabbit lung, Roth and Gillis (21) found that 30% of 2-phenylethylamine deamination proceeded via BzAO using a perfusion concentration (1·1 μM) which should favor metabolism by MAO. It remains to be established, however, whether the rabbit lung enzyme possesses the same properties as those reported here for rat skull BzAO.

The substrate profile of skull BzAO seems similar to that of peripheral rat blood vessels (2, 22) and the plasma BzAO of several species, including man (1, 7–9, 23). However, it is unlikely that the activity in skull results from contamination with blood since rat serum (2) and whole blood (21) are virtually devoid of BzAO activity. It is of interest to note that in the organs of man, BzAO does not act upon 2-phenylethylamine and dopamine (2). Furthermore, the apparent K_m value for benzylamine is about 40 times higher than that reported here (2). Thus, the rat enzyme may not serve as a reliable model for the BzAO of man.

Evidence exists that pig (11) and human (16) plasma BzAO acts upon the nonprotonated form of the amine substrate although contradictory conclusions have been drawn in other species (25, 26). In the present study, kinetic experiments done with benzylamine between pH 6.9 and 8.2 gave apparent K_m values ranging from 11.0 to 1.1 μM. However, when these experimentally derived K_m values were calculated with respect to the concentration of nonprotonated benzylamine, a relatively constant, pH-independent value of 0.05 μM was obtained (Fig. 1). This result suggests strongly that the nonprotonated form of benzylamine acts as the substrate for rat skull BzAO and that ionizable binding sites on the enzyme are not altered radically between pH 6.9 and 8.2. For comparison, the pH-independent K_m values for 2-phenylethylamine and tryptamine were calculated to be 0.15 and 0.22 μM, respectively. Thus, benzylamine remains as the most highly preferred substrate, although on a pH-independent basis, its K_m value is now only three times less than that of 2-phenylethylamine (compare with Table II).

From the present experiments, it is not clear whether the effect of pH on product formation (Figs. 1 and 2) is due to a direct

influence on catalytic activity and/or whether it results from effects on substrate ionization. Progressive saturation of BzAO up to the pH optimum of 7.4, followed by substrate inhibition, might explain the data. That BzAO is subject to substrate inhibition has been noted previously (2, 6). This interpretation, however, although attractive, remains to be proven.

From our kinetic studies (Fig. 3) it appears that BzAO possesses a lower K_m for oxygen than brain MAO-B. Whereas an increase in the oxygen tension of the reaction mixture produced a parallel, uncompetitive shift in the double reciprocal plot for MAO-B, it was without effect on BzAO. Oxygen dependence for BzAO only became evident after lowering the oxygen tension by flushing with nitrogen. Thus, it may be argued that the oxygen concentration of the reaction mixture under an atmosphere of air is near to or even at saturation for BzAO but not MAO-B. Following flushing with nitrogen, the K_m and V_{max} values decreased by 50% due to an uncompetitive shift in the double reciprocal plot. This result indicates, but does not prove, a double displacement or "ping-pong" mechanism of catalysis. The same catalytic mechanism has been reported for plasma BzAO (10, 11, 27, 28), rat heart BzAO (29), and MAO (30). However, the precise nature of the presumed "ping-pong" reaction for BzAO may differ from that with MAO since (+)-amphetamine was found to be a noncompetitive inhibitor of the reaction (Table II) whereas it acts competitively toward MAO (31). Noncompetitive inhibition in a "ping-pong reaction occurs when an inhibitor combines with both forms of the enzyme. Therefore, the K_i constant for (+)-amphetamine (Table II) is likely to represent a hybrid value resulting from an interaction with both the oxidized and reduced forms of BzAO.

While the present study provides new information concerning certain enzymatic properties of BzAO, the physiological function of this enzyme remains an enigma (23). Studies in man show that the activity of plasma and serum BzAO is reduced in patients suffering from cancer or severe burns (32) while increased activity in plasma has been associated with fibrotic liver disease (9). Whether the BzAO of bone, muscle cells, and other cellular elements is similarly affected is, at present, unknown. The characterization and definition of the enzyme, as described here, should better enable and encourage investigations involving the cellular tissues of rats.

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