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LOCALIZATION, PURIFICATION AND SUBSTRATE SPECIFICITY OF MONOAMINE OXIDASE

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Summary

Bovine kidney monoamine oxidase (amine:oxygen oxidoreductase (deaminating) (flavin-containing), EC 1.4.3.4) has been purified to one band on disc electrophoresis, and is shown to be localized in the intra- and extramitochondrial membrane. Kinetic methods have been used to determine the effect of different substances on the enzyme activity. This enzyme shows a very high substrate specificity. It is suggested that phenol ring and one hydrogen atom each on the methylene and amine groups are responsible for the enzyme actity. *N*-methylbenzylamine exhibits a homotropic negative cooperative effect which is also supported by the n and R_s values. Benzylhydrazine is apparently a good substrate unlike phenylhydrazine, semicarbazide, harmaline and α- and β-naphthol which show an inhibitory effect on the enzyme activity. Methylamine has no effect. It is suggested that the enzyme may have different sites or different conformations for different substrates. The results of this communication demonstrate bovine kidney monoamine oxidase to be different from monoamine oxidase from other sources.

Localization, Purification and Substrate Specificity of Monoamine Oxidase

Since 1928, when monoamine oxidase (amine:oxygen oxidoreductase (deaminating) (flavin-containing), EC 1.4.3.4) was first described [1] various investigators have studied this enzyme, and the literature has also been reviewed [2—13]. Interest in this group of enzymes grew rapidly after the first report of an inhibitor of monoamine oxidase was published in 1952 [14, 14a]. To differentiate these enzymes from diamine oxidases Zeller et al. [15] proposed the name "monoamine oxidase". This enzyme has been considered as a single enzyme [16,17], but some authors report the presence of multiple

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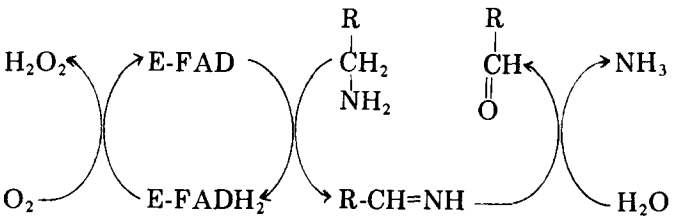
forms of monoamine oxidase [10,18—31]. This intracellular (mitochondrial) enzyme differs from the extracellular (plasma) monoamine oxidase in probably not requiring pyridoxal phosphate as a cofactor [6,32,33]. Though this enzyme has been "purified" from different sources [24,34—54], its intramitochondrial localization is still a subject of controversy [55—60]. Monoamine oxidase catalyses the oxidative deamination of monoamines with formation of corresponding aldehydes, hydrogen peroxide and ammonia [61—64] as shown below:



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

This enzyme apparently catalyzes the reaction according to a ping-pong mechanism [65—67]. The experimental data indicate that mitochondrial monoamine oxidase, like some other flavin enzymes, has a covalently bound flavin prosthetic group [68—74], but Tipton's results make it a subject controversy [75]. Several critical results and the different localization of this enzyme make it clear that much remains to be done to find the optimal extraction necessary for its purification. Procedures attempting to solubilize the enzyme have shown varying results [36,44,47,54,76—81]. In this paper a simple but tedious procedure is described, whereby the enzyme is extracted optimally and can be shown as single band electrophoretically.

Material and Methods

Potassium dihydrogen phosphate, dipotassium hydrogen phosphate, sucrose, Tris(hydroxymethyl)-aminomethane, glycine, Biuret solution, ammonium sulfate and benzylamine (redistilled) were obtained from E. Merck, Darmstadt. Bio-gel A-1.5 (200—400 mesh), acrylamide, *N,N,N,N*,-tetramethylenediamine, *N,N*-methylenebisacrylamide, ammonium persulfate and riboflavin were obtained from Bio-Rad Laboratories. Aluminum hydroxide-C γ-gel (pyrugen free and aged) and digitonin (p.a.) were obtained from Serva. Digitonin was recrystallized in hot absolute alcohol and dried over phosphorous pentoxide to give a clear solution. DEAE-Cellulose 52 was obtained from Whatman Biochemicals Ltd., England and Cleland's reagent (dithiothreitol), A grade, from Cal Bio Chem San Diego, California.

Monoamine oxidase activity was measured spectrophotometrically at 37° C on a Beckman DB-G grating spectrophotometer using benzylamine as the substrate [51]. The reaction mixture contained in 3.0 ml: 150 μmol potassium phosphate buffer, pH 7.6, and 10 μmol of distilled benzylamine. All assays were carried out with freshly prepared solutions against a control lacking benzylamine. After equilibration of the assay system at 37° C, the reaction was started

by addition of monoamine oxidase and the benzylamine oxidation was measured by absorbance at 250 nm. One unit of enzyme activity is defined as the amount of enzyme catalysing a change in absorbance of 0.001 units/min at 250 nm in a cuvette with 1-cm light path. The specific activity is expressed as the units/mg protein measured by the Biuret method [82,83]. The degree of purification of the isolated monoamine oxidase was measured using the disc electrophoresis apparatus ISCO Model 1270 combined with Model 490. Disc gels were prepared according to the method of Maurer [84] in a Tris/glycine buffer pH 8.3. Optimal results were obtained at 5% acrylamide concentrations. Separation required about 120 min at 2 mA per gel column. The polyacrylamide gels were stained in 1% Amido Black dye solution in 7% acetic acid and destained in 7% acetic acid solution to locate the protein band. Bromophenol Blue was used as a front marker. Usually, up to 40 μg protein were traced per gel column to minimize errors resulting when smaller amounts of protein were used. Enzyme prepared according to the method described here showed a single band which has been used as a criterion for the purified enzyme (see Fig. 1). Results presented in this paper were carried out with the purified enzyme fraction.

Purification of monoamine oxidase

Preparation of high specific activity from bovine kidney mitochondria requires special handling. The procedures involved are therefore described in detail. All operations were carried out between 0 and 4° C.

A) Isolation of mitochondria. Bovine kidneys from freshly killed animals were obtained from the slaughterhouse and transported in ice. The cortical tissues were removed and placed in cold 0.25 M sucrose solution at 0° C. The mitochondria were prepared as described earlier [85]. The cortical tissues were homogenized for 1 min with 3 vols. of 0.25 M sucrose solution pH 7.6, in an Elvejhem Potter homogenizer. The homogenate was centrifuged for 10 min at 1000 × g. The supernatant fraction was transferred to a cooled vessel. The pellet was washed 3 times with 3 vols. of fresh sucrose solution, homogenized 0.5 min and centrifuged for 6 min at 1000 × *g*. The supernatant fractions were combined and centrifuged for 25 min at 11 000 × *g*. The pellet was washed with 0.25 M sucrose and centrifuged for 25 min at 11 000 × *g*. This sediment (mitochondria), if not used at once for monoamine oxidase extraction, can be transferred to cooled vessels and maintained at about —40° C for at least 3 months. The enzyme activity remains intact.

(B) Extraction of monoamine oxidase from isolated mitochondria. The optimal extraction of monoamine oxidase from isolated bovine kidney mitochon-

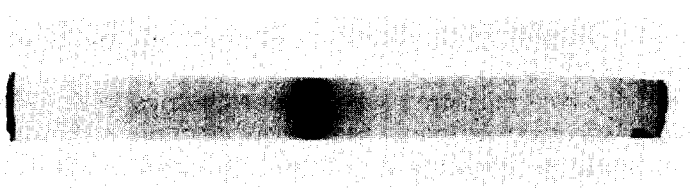


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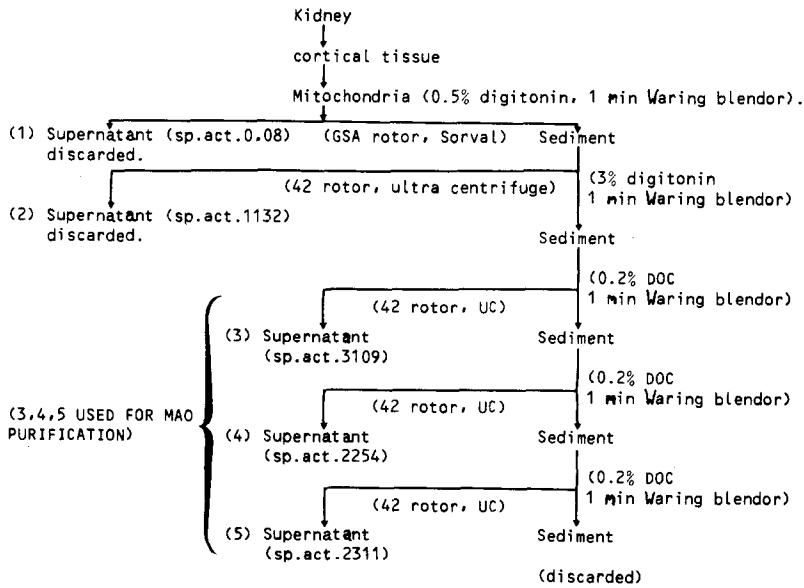
Fig. 1. Polyacrylamide gel disc electrophoresis of the purified bovine kidney monoamine oxidase (Specific activity 34 844). For details see Materials and Methods.

dria is very difficult. On the basis of recent results [55,56,60] suggesting the double localization of monoamine oxidase in the outer and inner membrane, we also tried to extract this enzyme with digitonin from the outer membrane and then with deoxycholic acid from the inner membrane, obtaining the results presented in Scheme 1. Previous works on these extraction methods have been



Scheme 1. Extraction of monoamine oxidase (MAO) from the isolated mitochondria. DOC, deoxycholate; UC, ultracentrifuge.

published elsewhere [86]. Scheme 1 illustrates that the bulk of the enzyme activity is extracted with deoxycholate following two extractions with 3% digitonin. The findings support the results presented in the literature [55,56,60]. It would appear that optimal monoamine oxidase activity was extracted with deoxycholate, in the following way: 80 g isolated mitochondria were mixed with 0.5% digitonin in 0.25 M (600 ml) sucrose pH 7.6 and homogenised in a Waring blendor from General Electric (Model No. 5BA 60VL35A) for 1 min (care was taken to minimize foaming). This homogenate was centrifuged 60 min at 27 000 $\times g$ (Sorvall G.S.A. Rotor). The supernatant (600 ml), which contained practically no activity, was discarded.

The sediment was homogenized with 3% digitonin in 0.25 M sucrose, pH 7.6 (120 ml), for 1 min in a Waring blendor, centrifuged at 142 800 $\times g$ (Beckman Ultracentrifuge Rotor No. 42) for 60 min.

The yellow-brown supernatant, containing about 21% of the total activity (911 950 units/805 mg) was discarded. The pellet was homogenized in 120 ml 0.25 M sucrose containing 0.2% deoxycholate (final concentration) for 1 minute in a Waring blendor and centrifuged again at 142 800 $\times$ g for 60 min. The clear yellow supernatant (vol. 120 ml) was transferred to a precooled vessel kept in ice.

The sediment was subjected to two more extractions with 120 ml each of 0.25 M sucrose containing 0.2% deoxycholate (final concentration) in a Waring blendor.

The three supernatants were pooled (vol. 350 ml) and the final sediment containing little or no monoamine oxidase activity was discarded (see Scheme 1). The clear yellow supernatant was subjected to ammonium sulfate precipitation.

(C) Precipitation of the extracted monoamine oxidase activities with solid ammonium sulfate. To the supernatant fraction (350 ml), solid ammonium sulfate was added slowly to a final concentration of 25% (14.4 g solid ammonium sulfate/100 ml monoamine oxidase extract) with continous stirring. The pH was maintained at 7.6 by addition of crystalline K_2HPO_4 or 1 M phosphate buffer, pH 7.6.

When the ammonium sulfate was completely dissolved, the solution (with "yellow-brown crystals" or an amorphous precipitate) was allowed to stand at —20° C for 20 min. The precipitated protein was removed by centrifugation for 15 min at 48 000 × *g* (Sorval, Rotor No. SS34).

The supernatant (vol. 350 ml) was assayed for monoamine oxidase activity and discarded. The yellow-brown "honey-like" precipitate which settled at the bottom of the centrifuge tubes was dissolved in 0.001 M phosphate buffer pH 7.6 (final vol. 110 ml) and traced on sucrose solution for further purification.

(D) Removal of inactive protein by centrifugation with sucrose. The precipitate (0—25% ammonium sulfate fraction vol. 110 ml) was traced in centrifuge tubes containing 50 ml of 1.5 M sucrose solution, pH 7.6 (tubes for Rotor No. 42 for Beckman Ultracentrifuge). These tubes were placed in a No. 42 Rotor and centrifuged at 142 800 × *g* for 18 h at 5° C in the Beckman ultracentrifuge.

The clear yellow solution containing the activity which remains on top of the sucrose was removed and collected in a precooled vessel kept in ice (total volume 95 ml). The remainder was discarded.

(E) Adsorption on aluminum hydroxide gel Cγ. To the clear yellow fraction (95 ml) aluminum hydroxide gel Cγ was added slowly with continual stirring to the final ratio of 2 mg gel/mg protein (608 mg gel/304 mg protein). The pH value during this step was kept constant at 7.6. This suspension was stirred for 60 min in ice, centrifuged at 15 000 × *g* for 5 min. The sediment was discarded. The clear yellow supernatant (vol. 120 ml) was dialysed twice against 10 l of 0.02 M phosphate buffer, pH 7.6, for altogether 18 h. The first volume (10 l) was changed after 4—5 hours. This dialysed enzyme (vol. 165 ml) was traced on a DEAE-cellulose column for further purification.

(F) DEAE-cellulose (DE52) chromatography. The dialysed supernatant (165 ml) was applied to a DEAE-cellulose (DE 52) column (5 × 25 cm) which had been equilibrated with 0.02 M phosphate buffer pH 7.6. The column was washed with 150 ml of 0.02 M phosphate buffer but no activity was found in the eluate. Then 200 ml of 0.04 M phosphate buffer pH 7.6 were passed through and 7-ml fractions were collected (Beckman automatic fraction collector). Little activity (0.01% of the total activity) was found in these fractions.

800 ml of the third buffer, 0.04 M phosphate buffer pH 7.6, containing 1% (final concentration) digitonin was applied to the column to elute the majority of the enzyme activity. 7-ml fractions of the effluent were collected. The enzyme was eluted in two peaks and the tubes containing highest specific activity (second peak) were pooled, (vol. 270 ml) and traced on a hydroxyapatite column.

TABLE I

Relative rates of oxidation of various substrates by bovine kidney monoamine oxidase. Enzyme activity was measured with 2.5 and 5 mM substrate concentrations in 0.1 M phosphate buffer pH 7.6 at 37°C. The value obtained with benzylamine is considered as 100%.

Fraction	Total protein (mg)	Total activity (units)	Specific activity (units/mg)	Yield (%)
Mitochondrial extract	1197	3 268 800	2 730	100
0—25% Ammonium sulfate	770	3 322 000	4 314	101.6
Sucrose centrifugtion	304	1 873 400	6 162	57.3
Alumina gel Cγ treatment	264	1 812 000	6 863	55.4
DEAE-cellulose column	81	891 250	11 900	27.2
Hydroxyapatite column	30	520 800	18 000	15.9
Biogel-A				
Fraction 1	4.5	156 800	34 844	4.8
Fraction 2	2.3	59 400	25 826	1.8

(G) Hydroxyapatite column chromatography. 80 ml suspension of hydroxyapatite was washed and stirred twice in 2 l of 0.02 M phosphate buffer pH 7.6 for 30 min, then allowed to settle for 30 min. The supernatants were discarded. The slurry was poured into a column (3 × 10 cm) and allowed to settle overnight. The column was then equilibrated with 0.04 M phosphate buffer, pH 7.6. The enzyme solution (270 ml) obtained after step F (DEAE-cellulose column chromatography) was applied to this column (3 × 10 cm) and the major activity was eluted with 1 M phosphate buffer, pH 7.6. The eluting buffers used for the hydroxyapatite column were 100 ml 0.08 M and 100 ml 0.25 M phosphate buffer pH 7.6, 200 ml 0.75 M phosphate buffer containing 1% digitonin and 200 ml 1 M phosphate buffer containing 1% digitonin. The fractions eluted with 1 M phosphate buffer pH 7.6 containing 1% digitonin were pooled and dialysed against 12 l 0.04 M phosphate buffer pH 7.6 containing 0.2 mM dithiothreitol.

These 120 ml were then traced on a Bio-Gel column for furher purification.

(H) Gel filtration with Bio-Gel (200—400 mesh). Bio-Gel A-1.5 (200—400 mesh) was washed 3 times in about 6 l of 0.04 M phosphate buffer pH 7.6 and finally equilibrated with 3 l of the same buffer containing 2 mM dithiothreitol. Excess buffer was decanted and the gel was poured into a column (5 × 90 cm), allowed to stand overnight, and then washed with about 2 l of phosphate buffer pH 7.6 containing 2 mM dithiothreitol. Elution was carried out with the dithiothreitol containing 0.04 M phosphate buffer, pH 7.6, and 7-ml fractions were collected in a broad peak. Fractions having the highest specific activity were collected. These results are summarized in Table I. The enzyme thus purified showed one band on the disc electrophoresis.

Results

Fig. 2 shows the inhibition of bovine kidney monoamine oxidase with phenylhydrazine. Phenylhydrazine was shown to inhibit pig plasma [50] and beef plasma [87] monoamine oxidases. With pig plasma monoamine oxidase

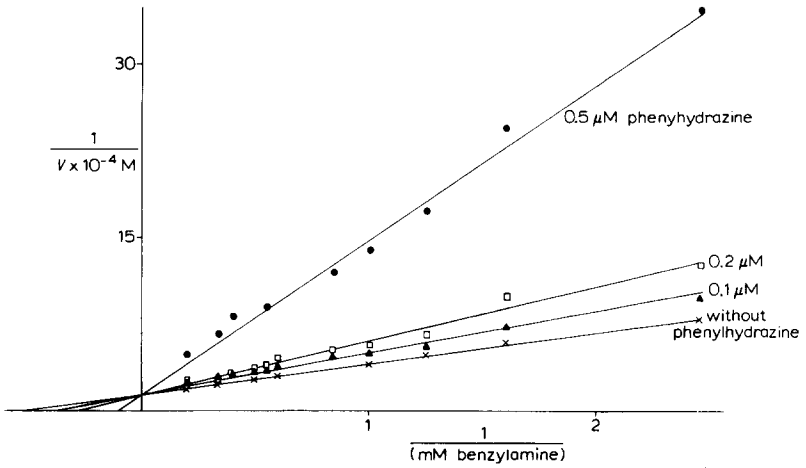


Fig. 2. Lineweaver-Burk plot of the effect of phenylhydrazine concentrations on the oxidation of benzylamine during the reaction catalyzed by bovine kidney monoamine oxidase. (Preincubation time: 1 min). For assay see Material and Methods. It demonstrates a kinetic pattern typical of competitive inhibition.

the inhibition was found to be noncompetitive. The enzyme was incubated at 23° C with phenylhydrazine in the presence of 0.1 M phosphate buffer pH 7.6 with varied concentrations of inhibitor for a specific time. Time required for 50% inhibition ($t_{0,5}$) was found to be 18.5 minutes at 23° C with 1 μM phenylhydrazine final concentration. The pH value after the reaction was found to be 7.6 (optimum). The control runs were carried out withut phenylhydrazine to elucidate any temperature effect.

Michaelis-Menten kinetics of the enzyme with or without phenylhydrazine are seen in Fig. 2. It demonstrates a kinetic pattern typical of competitive inhibition in the sense that the V value in contrast to K_m values remains unchanged, showing the effect of the inhibitor on the enzyme substrate affinity.

Fig. 3 shows the inhibitory effect of semicarbazide on bovine kidney mono-

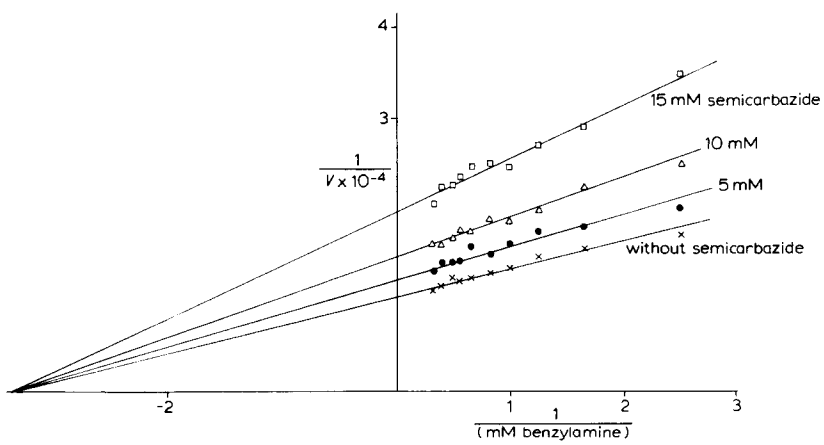


Fig. 3. Lineweaver-Burk plot of semicarbazide inhibition. The enzyme was preincubated for 70 min at 37° C with semicarbazide. The reciprocal of the change in absorbance at 250 mμ (V) is plotted against the reciprocal of the benzylamine concentrations. It demonstrates a kinetic pattern typical of non-competitive inhibition.

TABLE II

PURIFICATION OF BOVINE KIDNEY MONOAMINE OXIDASE

Substrate (5 mM)	Enzyme activity (%)
Benzylamine	100
α-methylbenzylamine	10.3
N-methylbenzylamine	103
N-dimethylbenzylamine	1.3
Phenethylamine	10.3
Phenylhydrazine	1.2
Benzylhydrazine	57.0
Histamine	5.17
Methylamine	3.5
Allylamine	1.6
Diethylamine	1.5
Triethylamine	1.6
Ethylamine	5.1
Hexylamine	5.1
Heptylamine	5.2
Penicillamine	6.9
Dimethylglycine	3.5
Cysteamine	3.4
Sarcosine	5.1
Methionine	5.1
Aspargine	5.8
Glutamine	5.1

amine oxidase. The enzyme was incubated at 37° C for 35 min and also for 70 min with different concentrations of semicarbazide. The enzyme activity was measured before and after the experiment in controls without semicarbazide. The K_i value for semicarbazide was calculated according to the procedure of Dixon [88] and was found to be 16.6 mM at 37° C after 70 min of incubation. As Fig. 3 shows, the inhibition of semicarbizide was found to be of noncompetitive type, in which the K_m value remains constant and the values of V vary with the concentrations.

Bearing in mind the results on the substrate specificity of monoamine oxidase from different sources [26,51,87,89—93], one can say that this enzyme from bovine kidney exhibits a different behaviour towards various substrates and thus differentiates itself from the monoamine oxidase activity obtained from other sources. This enzyme from bovine kidney reacts fairly well with kynuramine, tyramine and tryptamine [69]. As Table II shows, with the exception of benzylamine, benzylhydrazine and *N*-methylbenzylamine the enzyme activity was less than 10% with all other compounds tested at 2.5 or 5 mM concentrations. Concentration variables with the latter compounds did not affect the activity. It appears as if at least one phenyl group, one hydrogen atom from a methylene group and one from an amine group are required for the reaction catalysed by the enzyme from bovine kidney. Benzylamine, a good substrate for this enzyme, does not for instance react with rat brain or guinea pig liver

monoamine oxidase [26,90]. The K_m value differs from those obtained for monoamine oxidase from other sources. Benzylhydrazine, an inhibitor for beef liver monoamine oxidase [47], reacts fairly well with the enzyme from bovine kidney showing about 60% of the benzylamine activity and a K_m value of $4.1 \cdot 10^{-4}$ M. The kinetics obtained with benzylhydrazine follow normal Michaelis-Menten kinetics like those obtained with benzylamine, but are different than the negative cooperative effect measured with *N*-methylbenzylamine (Fig. 4). These unique results were obtained repeatedly with pure redistilled *N*-methylbenzylamine (containing no benzylamine). As shown in the inset to Fig. 4 an apparent hyperbolic curve is obtained by plotting the reaction velocity against *N*-methylbenzylamine concentrations. When a double reciprocal plot of activity versus *N*-methylbenzylamine concentration was made, the enzyme showed a pronounced deviation from classical Michaelis-Menten kinetics (two K_m values, $K_{m_1} = 1.2 \cdot 10^{-4}$ M, $K_{m_2} = 2.7 \cdot 10^{-4}$). These results indicate that this apparent homotropic negative cooperative effect which has been suggested for several other enzymes can be applied in this case at least with respect to *N*-methylbenzylamine. R_s value calculated according to the procedure of Koshland et al. [94,95]. was found to be 140 and the n value from the Hill plots of the initial velocity data was 0.7. The enzyme from bovine kidney thus differs strongly from that reported by other authors.

After the interesting, but opposite inhibitory results of phenylhydrazine and semicarbazide, it was desirable to obtain potent inhibitors for monoamine oxidase [96]. The harmala alkaloids are considered to be the most active inhibitors. Fig. 5 describes the inhibitory action of harmaline on bovine kidney monoamine oxidase. No preincubation was needed for this inhibition. Plotting the inhibitory action of different harmaline concentrations at varying concen-

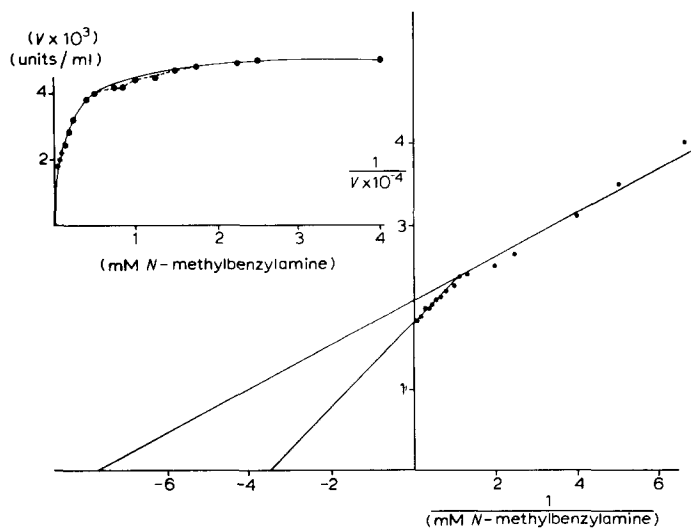


Fig. 4. Lineweaver-Burk plot of the effect of *N*-methylbenzylamine on bovine kidney monoamine oxidase. V, the change in absorbance at 250 mμ (enzyme activity) is plotted against the reciprocal of the millimolar concentration of *N*-methylbenzylamine. The inset represents the relation with the reaction velocity plotted as a function of the substrate, *N*-methylbenzylamine. The other experimental conditions were similar to those described in Materials and Methods. For further details see text.

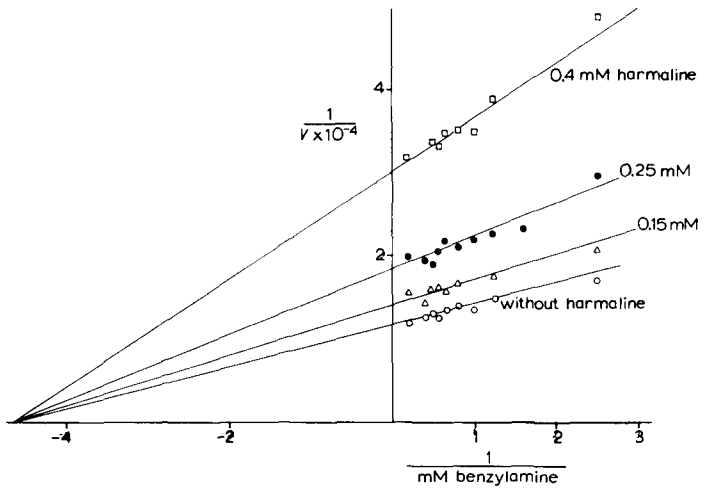


Fig. 5. Lineweaver-Burk plot of the data obtained with benzylamine in presence and absence of harmaline. The inhibitory action of different harmaline concentrations at varying concentrations of the substrate benzylamine shows a noncompetitive type of inhibition. No preincubation with harmaline was needed.

trations of the substrate benzylamine showed a non competitive type of inhibition. The interpretation given for the semicarbazide, is also valid for harmaline.

The pH optimum at 37° C and at 20° C was found to be between 7.6 and 8.0. At both these temperatures the pH optimum and the shape of the curve remained the same, but the value for V is less than that at 37° C demonstrating the temperature dependence of the enzyme. Competitive inhibition of the enzyme was demonstrated with α- and β-naphthol. Both decrease the enzyme substrate affinity 4—5 times respectively, changing the K_m value for benzylamine from $2.5 \cdot 10^{-4}$ M to $8.0 \cdot 10^{-4}$ M (α-naphthol) and $11.6 \cdot 10^{-4}$ (β-naphthol). We found the same kinetics with naphthol for this enzyme as obtained by Erwin and Hellerman [69].

Discussion

Although monoamine oxidase has been isolated from different organs and work has been presented using different specific activities, the localization and the kinetic behavior is still controversial. Taking these arguments under consideration, we have presented similarities and differences of this membrane-bound enzyme from bovine kidney mitochondria with those from other sources. As Scheme 1 shows, the enzyme is strongly bound to the mitochondrial membrane. The extraction of this enzyme in a Waring blendor with 0.5% digitoning showed practically no enzymic activity in the supernatant after subjection to 142 800 $\times g$ centrifugation for 60 min. The second and third extraction, under similar conditions, represented only about 24% of the total activity. Therefore, during the extraction of the enzyme at high speeds for further purification, we used 0.2% deoxycholate after the first two extractions with digitonin. Additional extractions with digitonin, with 0.5% and 3% digitonin respectively, did not increase enzyme activity in the supernatants obtained

after 142 800 × *g*-centrifugation, and may be interpreted as follows: First, that the enzyme is localized in both inner and outer membranes and that with digitonin, only the enzyme activity of the outer membrane is extracted. Second, the enzyme is bound differently to the outer membrane (due to the irregular distribution of lipids in the membrane) and therefore a strong detergent like deoxycholate is required to extract the enzyme. Third, a very simple explanation which could be postulated is that there being different forms of the enzyme, one is loosely bound (extracted with digitonin) and the other tightly bound (extracted with deoxycholate). The extraction of the enzyme from the mitochondria alone with deoxycholate showed low specific activity varying between 300 and 550 units/mg. Therefore this tedious extraction procedure (Scheme 1), using different detergents, was applied.

As shown in Fig. 2, phenylhydrazine shows competitive inhibition, compared to irreversible inhibition with plasma monoamine oxidase [50]. These results show that phenylhydrazine (structurally similar to the substrate) competes with benzylamine for the enzyme active center, as has also been reported by other authors [87,97,98] indicating phenyl group specificity of the enzyme active center. The same competitive type of inhibition was also observed with α- and β-naphthol.

Conserving the -NH-NH_2 group and removing the phenyl group produced a noncompetitive type of inhibition by semicarbazide (Fig. 3). Contradictory results have been published concerning the inhibitory effect of semicarbazide [35,48,51,89,90]. With bovine kidney monoamine oxidase we observed a noncompetitive inhibition, which is probably due to the structural differences in the substrate- and phenyl group-specificity of this enzyme. Another explanation for the noncompetitive type of inhibition could be that at different concentrations of the inhibitor a certain amount of the enzyme is inactivated, resulting in a lower V but the same K_m value, because the remainder of the enzyme continues to work with the same affinity for the substrate. This enzyme differs more in its behaviour towards semicarbazide than either human plasma [35,35a,89] pig liver [48] beef plasma [51] or guinea pig liver or kidney [90] monoamine oxidase.

Comparing the inhibitory action of semicarbazide with the action of benzylhydrazine on the enzyme show once again the important role of the phenyl group and one hydrogen atom both on the methylene and on the amino group of the benzylhydrazine. Benzylhydrazine thus changes the inhibitory effect of phenylhydrazine into enzyme-substrate kinetics with a K_m value of $4.1 \cdot 10^{-4}$ M. Conversely, benzylhydrazine is a strong inhibitor of beef liver monoamine oxidase [47]. Because of the substrate-specific kinetics of the enzyme we decided to investigate the effect of *N*-methylbenzylamine on monoamine oxidase from bovine kidney. We found an apparent negative cooperative effect [85] of *N*-methylbenzylamine on this enzyme. Strange types of kinetics with different compounds for this enzyme from other sources have been published by several authors [30,38,47,100] and involvement of allosteric effects and/or several binding sites with cooperative effects have been discussed.

The biphasic curve (Fig. 4) can lead to the interpretation that the enzyme has two different active centers with different affinities for *N*-methylbenzylamine, one of them working at a lower substrate concentration and the other

at a higher one, thus showing two different apparent K_m and V values.

A different interpretation of these results would be that bovine kidney monoamine oxidase might have different subunit structures with different affinities for *N*-methylbezylamine. Yet another possible interpretation could be that there are different forms of the enzyme with different affinities for the substrate, and even at low substrate concentrations the enzyme form with a high K_m value for the substrate is also contributing, thus leading to the biphasic curve with two different apparent K_m values. *N*-Methylbenzylamine does not react with beef plasma monoamine oxidase [51], but was shown to react with beef liver monoamine oxidase [101].

The enzyme from bovine kidney is different in this respect from monoamine oxidase from other sources. The n- and the R_s values of 0.7 and 140 respectively and the two K_m and two V values strongly indicate a negative cooperative effect of *N*-methylbenzylamine on monoamine oxidase from bovine kidney. We can further support our proposal of the strong substrate specificity of this enzyme with the help of different substrates summarised in Table II. Out of these substrates only benzylamine, methylbenzylamine and benzylhydrazine reacted fairly well with the enzyme.

As shown in Fig. 5, a non-competitive type of inhibition was found with harmaline as with semicarbazide. The interpretation given above for this type of inhibition can also be applied to the inhibitory effect of harmaline or monoamine oxidase from bovine kidney. Once again the different behavior of bovine kidney monoamine oxidase in comparison to those of rat liver [102] and pig liver [103] is clear. From the results documented in this communication, this enzyme appears the most complex form of monoamine oxidase studied so far.

Acknowledgement

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