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STUDIES OF MONOAMINE OXIDASES

EFFECT OF TRITON X-100 AND BILE SALTS ON MONOAMINE OXIDASE IN BRAIN MITOCHONDRIA*

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Abstract—Triton X-100 and the bile salts, cholate and deoxycholate, detergents often used in the solubilization of monoamine oxidase (MAO) from mitochondria, have been found to cause an inhibition of the enzyme activity. With beef brain mitochondria, it was found that there was a differential effect of Triton X-100 on the putative MAO types A and B, with MAO-A being more susceptible to inhibition by Triton X-100. This was indicated by the greater loss of serotonin-deaminating than of phenyl ethylamine-deaminating activity in the presence of Triton X-100. Although the bile salts also caused substantial inactivation at concentrations above 0.1%, no differentiation between MAO types could be made. Kinetic studies of the inhibition by Triton X-100 indicated two different mechanisms were occurring with the two MAO types. The inhibition was competitive for MAO-A, but uncompetitive for MAO-B. Removal of Triton X-100 by co-polymer beads restored some, but not all of the activity for both MAO-A and MAO-B types. This suggests that the activity loss may have been due in part to inactivation when the enzyme was separated from the mitochondrial membrane.

Monoamine oxidase (MAO, monoamine:O_2 oxidoreductase, EC 1.4.3.4) is located in the outer membrane of mitochondria [1]. It is considered to occur as two functional forms, A and B, with distinct substrate specificities and inhibitor sensitivities [2, 3], although specific information on the physical relationship of the A and B forms is lacking. Numerous investigators have sought to purify the enzyme, and preparations of considerable purity have been obtained [4–8]. A striking feature of many purified preparations from tissue sources initially containing both A and B forms has been a low recovery of the A-type activity as indicated by the deamination of serotonin. Most purification procedures have used detergents to solubilize the enzyme which is tightly bound to the mitochondrial membrane. The detergent most commonly used is Triton X-100. The bile salts, cholate and deoxycholate, have also been used in conjunction with Triton X-100. Very few investigations report on the possible adverse effects of the detergents on the enzyme in terms of A and B forms. This study reports on the differential effects of Triton X-100 and the bile detergents on the A and B activities of MAO in beef brain mitochondria.

MATERIALS AND METHODS

Materials. All radiochemicals ([2-^{14}C]serotonin binoxalate, 51.5 mCi/mmole; [1-^{14}C] phenylethylamine hydrochloride, 48.25 mCi/mmole; and [1-^{14}C]tyramine hydrochloride, 50 mCi/mmole) and liquid scintillation chemicals were obtained from the New England Nuclear Corp., Boston, MA. The cation resins, AG50W-X8 (100–200 mesh) and Amberlite CG-50 (100–200 mesh) were obtained from the Bio-Rad Corp., Richmond, CA, and the A. H. Thomas Co., Philadelphia, PA, respectively. The co-polymer beads, Bio-Beads SM-2, were also obtained from the Bio-Rad Corp. Sodium cholate and sodium deoxycholate were obtained from the CalBiochem-Behring Corp., La Jolla, CA. Triton X-100 was from the Rohm and Haas Co., Philadelphia, PA.

Methods. Mitochondrial fractions from beef brain cortex were prepared, and MAO activity was assayed, as described previously [9, 10]. Radiometric assays were used to measure the deamination of ^{14}C-labeled serotonin (5-HT, 0.5 mM), β-phenyethylamine (PEA, 0.5 mM) and tyramine (TYR, 1.0 mM). The radioactive substrates were diluted with appropriate concentrations of cold compounds to a final specific radioactivity of 5 mCi/mmole. The substrate concentrations used were those determined to be optimal for each substrate with beef brain mitochondria under the conditions of assay (0.05 M potassium phosphate buffer, pH 7.4, 20 min, 37°, unless otherwise indicated). Protein concentrations used in the assays ranged between 50 and 200 μg per assay. The enzyme reaction with all substrates was found to be linear at the concentrations used and for times at least up to 30 min. Mitochondrial suspensions were preincubated in the presence or absence of detergents for 10 min at 37° prior to the addition of the substrate to start the enzyme reaction. To ensure that the presence of the detergents did not interfere with the separation of products from

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unreacted substrates, detergent controls were carried out in which detergent was added to control assay systems after the reaction had been stopped. The amount of product was the same as with normal controls. Amberlite CG-50 resin, washed by the method of Pisano [11], was used to separate the deamination products from unreacted 5-HT and PEA, whereas AG50W-X8 resin was used for TYR. Samples were counted in a Searle Analytic Delta 300 Liquid Scintillation Counting System.

Turbidity of the mitochondrial suspensions was determined on a Gilford model 2000 spectrophotometer, measuring the optical density at 550 nm. Mitochondria were suspended in pH 7.4-buffered 0.25 M sucrose. Appropriate concentrations of detergent were added to the suspension and optical density readings were made within 1 min after mixing.

For the enzyme solubilization studies, mitochondria were mixed with various concentrations of Triton X-100 and allowed to stand at 4° for 30 min before centrifugation of the sample for 1 hr at 106,000 *g*. The resultant precipitate was resuspended in 0.05 M potassium phosphate buffer (pH 7.4) and both the resuspended precipitate and supernatant fractions were assayed for MAO activity and protein concentration.

The removal of Triton X-100 from enzyme samples was accomplished through the use of co-polymer beads (Bio-Beads, SM-2) as described by Holloway [12], using a column procedure. Following treatment with 0.5% Triton X-100 and centrifugation as described above, 2-ml aliquots of the supernatant fraction were passed through 0.7 × 7.5 cm columns containing washed co-polymer beads equilibrated with 0.05 M potassium phosphate buffer, pH 7.4. The column was washed with the same buffer, and fractions were collected. The removal of the detergent was indicated by the marked decrease in optical absorbance at 275 nm.

Protein concentration was determined by the method of Lowry *et al.* [13] as modified by Peterson [14].

Kinetic studies were carried out using the standard assay procedures described above. The mitochondria were preincubated in the absence or presence of Triton X-100 for 10 min at 37° before starting the enzyme reaction by addition of substrate. The substrate concentrations ranged from 2.5×10^{-5} M to 1×10^{-3} M.

RESULTS

Effect of detergent on MAO activity and mitochondrial turbidity. Figures 1, 2 and 3 show the effects of various concentrations of Triton X-100, sodium cholate and sodium deoxycholate, respectively, on the relative MAO activity and relative turbidity of the brain mitochondrial suspensions. In accordance with generally accepted criteria [2, 3], the deamination of 5-HT was taken as an indication of MAO-A activity whereas the deamination of PEA was used to indicate MAO-B activity. Tyramine deamination reflects both A- and B-type activity. There may be, however, some overlap with regard to specificity. From previous inhibition studies with clorgyline and deprenyl [15], it is estimated that beef brain cortex mitochondria contains approximately 60–70% MAO-A and 30–40% MAO-B.

The turbidity measurements indicate clearly that all three detergents were effective in bringing about a disruption of the intact mitochondria, with Triton X-100 being the most effective of the three at low concentrations. Concomitant with the decrease in turbidity, however, MAO activity also decreased. At 0.1%, there was little effect of cholate, either with regard to disruption of the mitochondria or to MAO activity. At the same concentration of deoxycholate, the relative turbidity was decreased by 50 per cent, as was the activity with the substrates, 5-HT and TYR. MAO-B activity, as indicated by the deamination of PEA, was slightly more stable, being reduced by only 40 per cent but, in general, it was not possible to differentiate between the loss of MAO-A, indicated by 5-HT deamination, or of MAO-B with either cholate or deoxycholate. In contrast, differentiation was obtained with the use of Triton X-100. With 0.1% Triton X-100, the turbidity was decreased by 80 per cent, while the activities

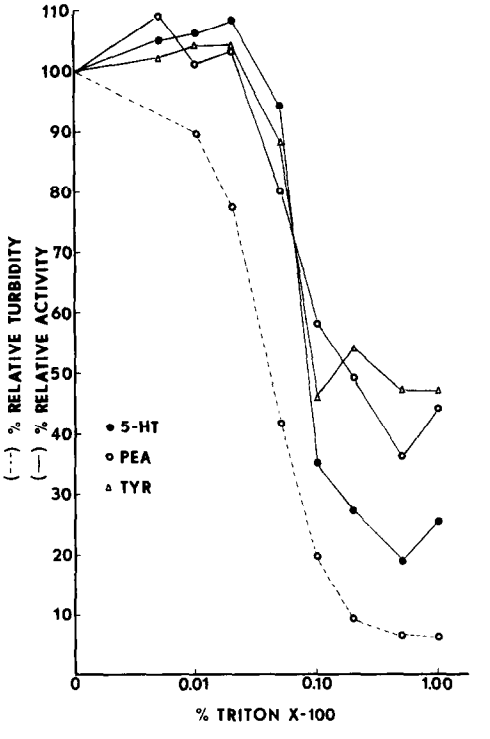

Fig. 1. Effect of Triton X-100 on MAO activity and mitochondrial turbidity. MAO activity (—) was determined in the presence of Triton X-100 using 5-HT (●), PEA (○) and TYR (△) as substrates. Assays were performed in 50 mM phosphate buffer (pH 7.4), incubating at 37° for 20 min and using approximately 75 μg protein/reaction mixture of 0.20 ml. Activity values were expressed relative to control samples containing no detergent assayed under the same conditions. The turbidity of the mitochondrial suspension (---) was measured as the $O.D._{550}$, and values for detergent-containing samples were expressed relative to a mitochondrial suspension without detergent. Relative activity and turbidity values are plotted against the per cent detergent concentration on a logarithmic scale.

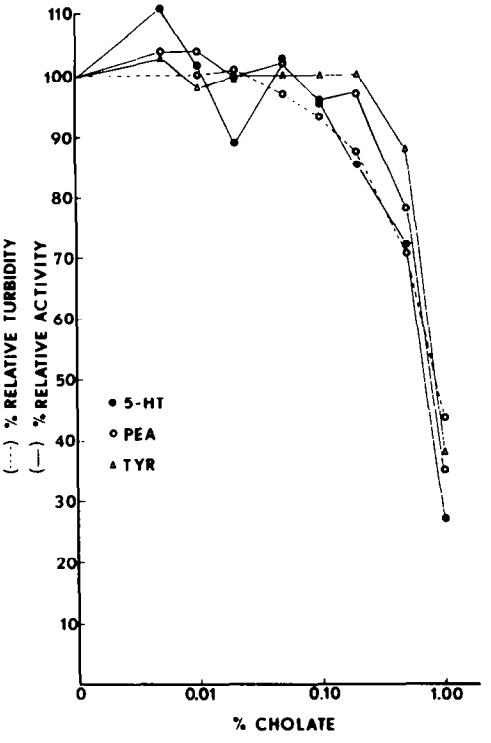

Fig. 2. Effect of cholate on MAO activity and mitochondrial turbidity. Conditions for the determination of MAO activity (—) and mitochondrial turbidity (---) in the presence of sodium cholate are described in the legend of Fig. 1.

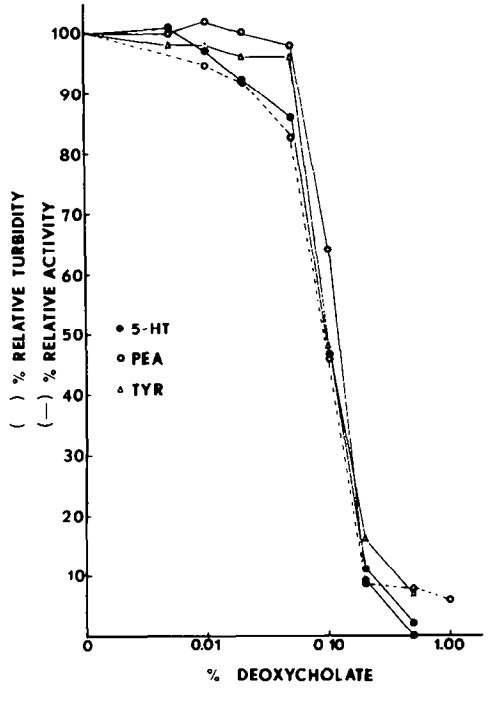

Fig. 3. Effect of deoxycholate on MAO activity and mitochondrial turbidity. Conditions for the determination of MAO activity (—) and mitochondrial turbidity (---) in the presence of sodium deoxycholate are described in the legend of Fig. 1.

remaining, as measured with 5-HT, TYR and PEA, were 35, 46 and 58 per cent respectively.

Solubilization of MAO-A and MAO-B by Triton X-100. Following treatment of the brain mitochondria with various concentrations of Triton X-100, the activity and protein content were assessed in the resultant supernatant and particulate fractions separated by centrifugations. In Fig. 4, the distribution of protein between supernatant and precipitate fractions as affected by the different concentrations of detergent is shown. Approximately 50% of the protein appeared in soluble form when exposed to 0.1% Triton X-100. The percentage of soluble protein appeared to be leveling off at approximately 75% total protein at a detergent concentration of approximately 0.5%. At every concentration of detergent tested, almost all of the protein was recovered.

In contrast to the protein recovery, activity measurements indicated that a considerable amount of enzyme activity was lost in the detergent treatment (Figs. 5 and 6). MAO-A and -B activities in the precipitate fractions decreased markedly with increasing concentrations of Triton X-100, as might be expected when enzyme is solubilized from a particulate membrane through the action of detergent.

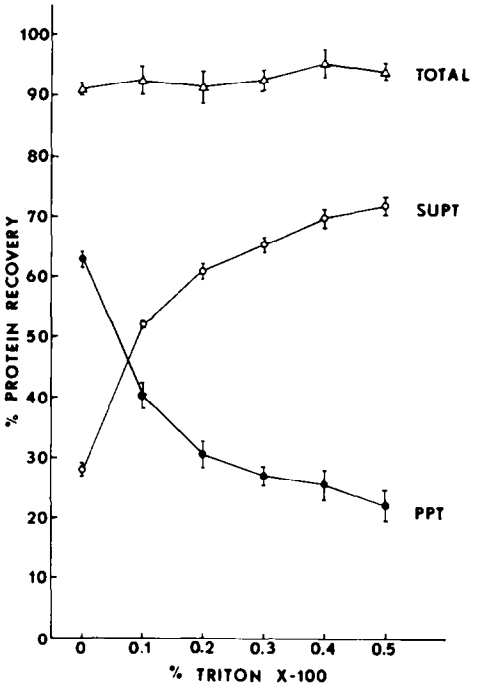

Fig. 4. The distribution of mitochondrial protein after treatment with Triton X-100. Mitochondrial suspensions were treated with Triton X-100, followed by centrifugation to obtain supernatant (○) and precipitate (●) fractions as described in Methods. The amount of protein recovered is expressed as a percentage of the protein in the original mitochondrial suspension (approximate concentration was 2.4 mg/ml). The total protein recovered (△) represents the sum of the protein in the supernatant and precipitate fractions at the given Triton X-100 concentration. Each value is the mean of four separate recoveries ± S.E.M.

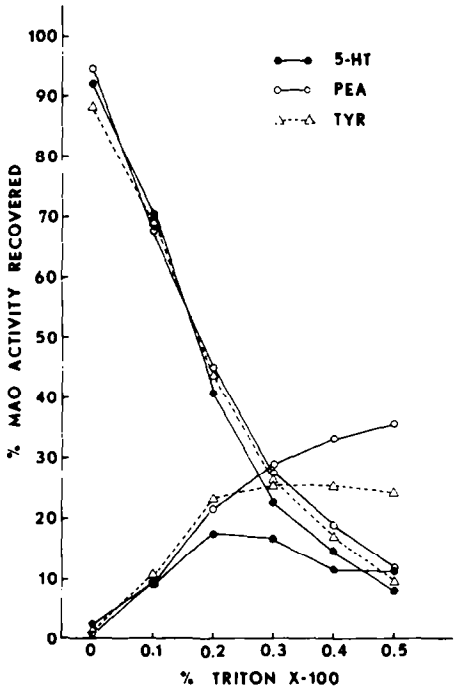

Fig. 5. Distribution of MAO activity after treatment with Triton X-100. MAO activity was determined using 5-HT (●), PEA (○) and TYR (△) as substrates in the supernatant (lower curves) and precipitate fractions (upper curves) obtained after treatment of mitochondrial suspensions with Triton X-100 and centrifugation as described in Methods. Assays were performed in 50 mM phosphate buffer (pH 7.4), incubating at 37° for 20 min. The amount of protein used per reaction mixture of 0.2 ml ranged from 60 to 180 μg depending on the fraction being assayed. Assay conditions were the same for all three substrates. Activity is expressed as a percentage of the total activity found in untreated mitochondrial fractions assayed under the same conditions.

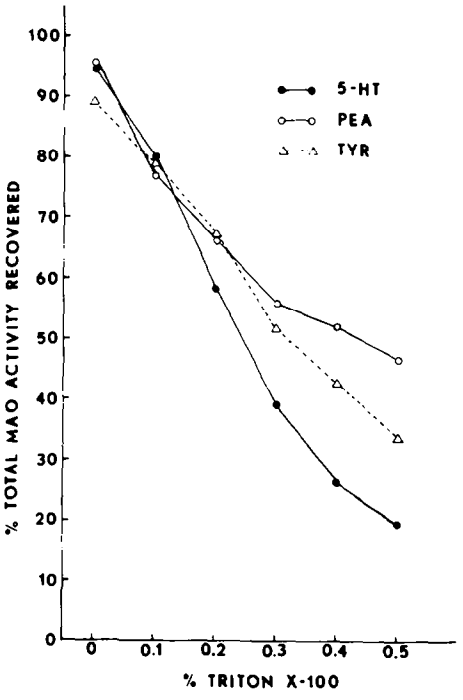

Fig. 6. Recovery of MAO activity after treatment with Triton X-100. Values represent the sum of the MAO activities determined in the supernatant and precipitate fractions described for Fig. 5, expressed as a percentage of the activity found in untreated mitochondrial fractions.

Although there was a gradual increase of MAO-B activity (PEA deamination) in the soluble fraction with increasing concentration of Triton X-100, this increase did not parallel the deacrease in the particulate fraction. Measurements with 5-HT also showed an initial increase in activity in the supernatant fraction with increasing concentrations of Triton X-100, up to 0.2% Triton X-100, but at higher concentrations, activity in the supernatant fraction decreased. The results with TYR were consistent with it being a substrate for both MAO-A and MAO-B.

Reversibility of Triton X-100 inhibition. Approximately 90–95 per cent of the Triton X-100 was removed by co-polymer bead treatment of the supernatant fractions obtained after each treatment of mitochondrial suspensions with 0.5% detergent. Assay of the detergent-freed supernatant fraction

Table 1. Recovery of MAO activity after Triton X-100 removal*

Substrate	Relative specific activity +Triton X-100	Relative specific activity −Triton X-100	Relative increase in activity
5-HT	0.26 ± 0.04 (9)	1.17 ± 0.34 (4)	4.50
TYR	0.36 ± 0.02 (9)	0.78 ± 0.05 (4)	2.17
PEA	0.49 ± 0.02 (9)	0.63 ± 0.07 (4)	1.29

* Activity was measured in supernatant fractions obtained from the treatment of mitochondria with 0.5% Triton X-100, before and after removal of the detergent by SM-2 Bio-Beads as described in Methods. Assays were performed in 50 mM phosphate buffer (pH 7.4) at 37°. The protein concentrations used were 150–170 μg for samples before Triton X-100 removal and 65–75 μg for samples after Triton X-100 removal per reaction mixture of 0.20 ml. Except for the substrates, conditions for all assays were identical. The activities are expressed relative to specific activity found in intact, untreated mitochondria which was taken as 1.00. The relative increase compares the relative specific activity for each substrate before and after Triton X-100 removal. Results are the mean values for the number of experiments given in parentheses ± S.E.M.

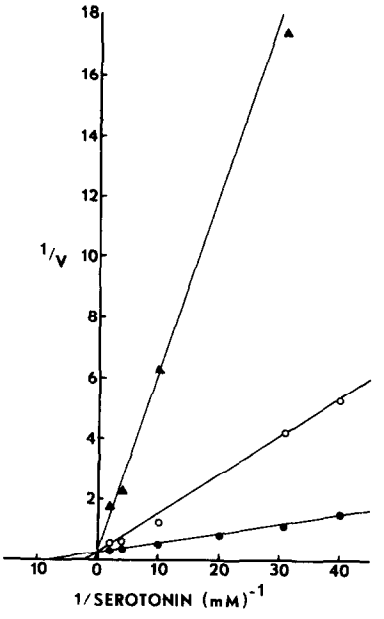

Fig. 7. Lineweaver–Burk plot of Triton X-100 inhibition of 5-HT deamination. The deamination of 5-HT was determined in the absence (●—●) or presence of Triton X-100 at 0.05% (○—○) and 0.10% (▲—▲) concentrations. Mitochondria were preincubated ± Triton X-100 for 10 min before starting the enzyme reaction by addition of 0.5 mM [^{14}C]-5-HT. Assays were performed in 50 mM phosphate buffer (pH 7.4), incubating for 20 min at 37° and using approximately 100 μg protein/reaction mixture of 0.2 ml. Units of velocity (v) are nmoles substrate oxidized·(mg protein)$^{-1}$·min^{-1}.

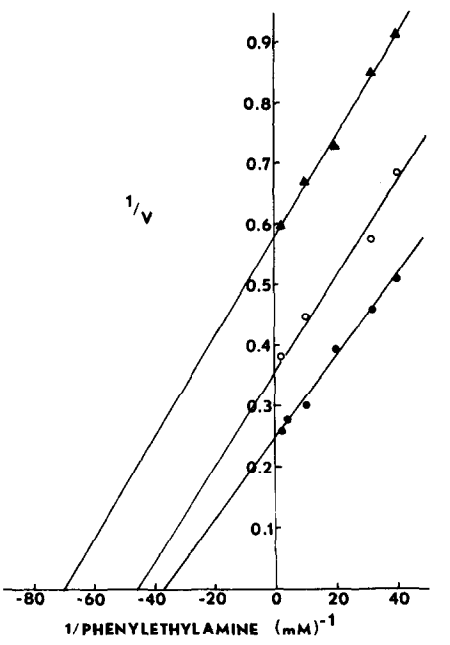

Fig. 8. Lineweaver–Burk plot of Triton X-100 inhibitio of PEA deamination. The deamination of PEA was deter mined in the absence (●—●) or presence of Triton X-10 at 0.05% (○—○) and 0.10% (▲—▲) concentrations Mitochondria were preincubated ± Triton X-100 for 10 mi before starting the enzyme reaction by addition of 0.5 mM [^{14}C]PEA. Assays were performed in 50 mM phosphat buffer (pH 7.4), incubating for 20 min at 37° and usin approximately 100 μg protein/reaction mixture of 0.2 ml Velocity (v) is in nmoles substrate oxidized·(m protein)$^{-1}$·min^{-1}.

revealed an increase in MAO activity, as indicated in Table 1. The order of increase in activity paralleled the order of loss of activity observed in the original supernatant fraction with a higher recovery of activity with 5-HT as substrate than with PEA or TYR. The resultant relative activities upon removal of Triton X-100, however, were not the same for all three substrates as one might have expected. More 5-HT deaminating activity was recovered than PEA or TYR deaminating activity.

Kinetic study of Triton X-100 inhibition. The differences observed with respect to the effect of Triton X-100 on the extent of inhibition and the recovery of MAO-A type and MAO-B type activities were reinforced by kinetic analyses. Different mechanisms were revealed by double-reciprocal plots for the three prototype substrates used (Figs. 7–9). With 5-HT (Fig. 7), the inhibition was competitive, whereas with PEA as substrate the inhibition was uncompetitive (Fig. 8). The inhibition with TYR was mixed competitive (Fig. 9).

DISCUSSION

The effectiveness of Triton X-100 and the bile salts in breaking down membranes and rendering membrane proteins soluble is now well established [16]. It is generally assumed that, because of their mild action of causing minimal change in protein

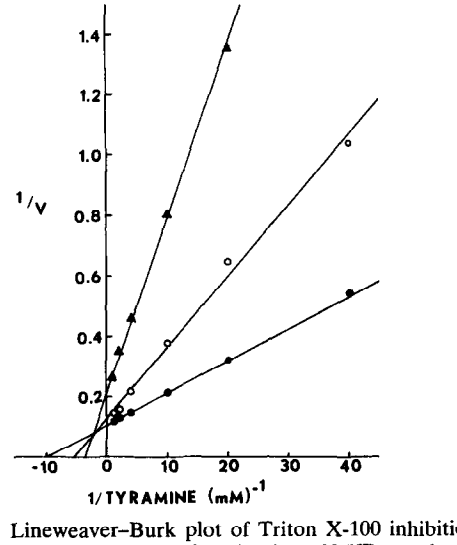

Fig. 9. Lineweaver–Burk plot of Triton X-100 inhibitio of TYR deamination. The deamination of TYR was deter mined in the absence (●—●) or presence of Triton X-10 at 0.05% (○—○) and 0.10% (▲—▲) concentrations Mitochondria were preincubated ± Triton X-100 for 10 mi before starting the enzyme reaction by addition of 1.0 mM [^{14}C]TYR. Assays were performed in 50 mM phosphat buffer (pH 7.4), incubating for 20 min at 37° and usin approximately 100 μg protein/reaction mixture of 0.2 ml Velocity (v) is in nmoles substrate oxidized·(m protein)$^{-1}$·min^{-1}.

conformation, these detergents do not affect enzyme activity. This is not, however, always the case. As noted here for MAO and reported for other membrane enzymes [17–19], inhibition of enzyme activity by detergent can occur. This inhibition is not totally related to the solubilization process.

The experiments on the effect of detergents on the turbidity of the mitochondrial suspensions indicated that considerable solubilization of protein occurred with relatively low concentrations of detergents. With Triton X-100, however, there was a difference between the effects on the enzyme activities of MAO-A and MAO-B. It was quite evident that MAO-A was much more sensitive than MAO-B to Triton X-100 treatment. What was not evident was whether this was due to a differential release of A and B forms from the mitochondrial membrane or due to a greater inhibitory effect of Triton X-100 on MAO-A. Study of the distributions of enzyme activity and protein between supernatant and precipitate fractions following treatment with Triton X-100 suggested that the latter was more likely. If differential release were the case, one might have expected greater variation among the three substrates in the activities that occurred in the precipitate fractions, to coincide with the differences found in the supernatant fractions. The mitochondrial membrane lipid environment has been said to be an important influence on the expression of MAO activity in terms of A and B characteristics [3, 20], although this is still a subject of controversy. The assumption here is that, if membrane lipid is important for MAO activity, the released enzyme would be less active than the enzyme still remaining particulate bound.

The greater inhibitory effect of Triton X-100 on MAO-A activity was demonstrated by the large increase in MAO-A activity following removal of the detergent from the supernatant fractions. Inhibition by the presence of Triton X-100, however, was not the only factor contributing to the loss of activity upon extraction since the amounts of activity recovered with the three substrates were not comparable. The activity recovered with PEA as substrate was less than that with 5-HT, which could be an indication that MAO-B is perhaps more dependent than MAO-A upon the membrane lipid environment.

The kinetic studies of the action of Triton X-100 towards MAO offer additional evidence of the differences between MAO-A and MAO-B. The inhibition of 5-HT deamination (MAO-A) was competitive, indicated by an increase in the apparent K_m with increasing concentrations of detergent (Fig. 7). The action towards MAO-B (PEA deamination) was that of uncompetitive inhibition. In the case of uncompetitive inhibition, the inhibitor binds to the ES complex, rather than competing with substrate for binding or in some other way affecting the binding of substrate to enzyme [21]. Tyramine, being a substrate of both MAO-A and MAO-B, thus might be expected to show characteristics of both. Indeed, a mixed-type inhibition was observed with TYR, indicating that, in addition to the inhibitor preventing the breakdown of the active complex, there was also interference with the binding of substrate as well [22], a mixture of the MAO type A and MAO type B inhibition mechanisms. These results differ somewhat from another recent report on the effect of Triton X-100 on MAO in rat liver mitochondrial membrane vesicles [23]. In that study, mixed competitive inhibition was reported for all substrates—5-HT, TYR, PEA and benzylamine. This difference in results could be due either to enzyme source (rat liver vs beef brain), since considerable species variation has been noted for MAO, or to different experimental conditions. Although no conditions or actual data were given for the rat liver kinetic studies, it was also reported that Triton X-100 inhibition was irreversible if the enzyme was preincubated at 37° for 30 min. These longer heating conditions also reduced the substrate specificity effects noted with less drastic conditions.

There has been recent evidence indicating that MAO-A and MAO-B are separate and distinct protein entities [24]. Previous speculations have been that the A and B types represented lipid modifications of the same protein or perhaps different catalytic sites on the same molecule. The fact that there had been previously no conclusive denial of either possibility has been due primarily to an inability to purify the two enzyme activities separately from each other. There have been numerous reports on the purification of MAO from mitochondria, some citing a fair degree of success in obtaining a relatively, homogenous preparation [4–8]. Many of the earlier purifications were reported prior to an awareness of the A and B types. Although subsequent purifications have taken the two activities into account, no purified preparation from a tissue source containing A and B demonstrated a purified MAO-A fraction. A review of reports on purified MAO reveals that MAO-A activity, as demonstrated by 5-HT deamination, has been lost upon purification, or that the investigators used only a B-type substrate to characterize the purified preparation. A common characteristic of most of the purification procedures has been the use of Triton X-100 as the extracting agent, often in conjunction with cholate or deoxycholate. After the initial extraction, the detergent is often incorporated in the buffer systems used throughout the purification procedures, presumably to keep the enzyme as a soluble protein. The implications of the present study are that Triton X-100, while necessary for solubilizing MAO from the mitochondrial membrane, is detrimental to MAO activity. If continually present in purification buffers, it can serve as an endogenous inhibitor of the enzyme, acting, in particular, more effectively on MAO-A. Removal of the detergent allows only a partial recovery of activity, indicating that some other constituent may be necessary to maintain the enzyme in soluble form or to keep the enzyme active. This other constituent may be a lipid factor.

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