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Is monoamine oxidase activity in the outer mitochondrial membrane influenced by the mitochondrial respiratory state?

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Abstract

Monoamine oxidase activity was measured in isolated rat liver mitochondria using the radiochemical assay with [^{14}C]tyramine as substrate. With toluene as the extracting solvent the apparent activity in the resting state (State 4) was much higher than in the active state (State 3) in agreement with Smith and Reid (Smith, G.S. and Reid, R.A. (1978) Biochem. J. 176, 1011–1014). However, with ethyl acetate or diethyl ether as extracting solvents, the activity in both states was almost identical and several times higher than that measured with toluene. *p*-Hydroxyphenylacetaldehyde, *p*-hydroxyphenylacetalcohol and *p*-hydroxyphenylacetic acid were identified as final reaction products, the latter one being hardly extractable with toluene. It is concluded that monoamine oxidase activity is not influenced by the respiratory state of mitochondria and that differences found by Smith and Reid are due to different extractability of secondary reaction products. NADPH-dependent aldehyde reductase was tentatively identified in rat liver mitochondria, its specific activity amounting to about one fourth of that in the cytosol.

Keywords: Monoamine oxidase; Aldehyde reductase; Radiochemical assay; Outer membrane; Contact site; Mitochondrion

1. Introduction

Monoamine oxidase (EC 1.4.3.4), a flavoprotein enzyme catalyzing oxidative deamination of catecholamines and dietary monoamines, is located almost exclusively in the outer mitochondrial membrane [1] and often used as a marker for this membrane. It was therefore somewhat surprising when Smith and Reid [2] reported on a strong dependence of MAO activity in intact mitochondria on the respiratory state of these organelles, since such a dependence implies a functional link between the outer and the inner membranes. This opened a field for speculation on the role of contact sites between the two membranes [3] (for a review on the contact sites see Ref. [4]) and possible ways of interaction between them. However, by a stepwise removal of the outer membrane with digitonin in brain mitochondria it appeared that MAO was not located in the contacts [5,6].

The relation between MAO activity and the respiratory and energy states of mitochondria was not confirmed by Harris and Cooper [7] who tentatively ascribed the effects observed by Smith and Reid [2] to oxygen depletion. Nevertheless, the paper by Smith and Reid [2] is still referred to when possible interactions between the two mitochondrial membranes are discussed (e.g. Refs. [8,9]). This prompted us to re-examine possible relationships between mitochondrial respiration and energy status on one side and the activity of MAO on the other side. As result, we show that apparent differences in MAO activity depending on mitochondrial respiratory states are artifacts resulting from incomplete extraction by toluene of various products of tyramine oxidation.

2. Materials and methods

Liver mitochondria were isolated from fed male Wistar rats by conventional procedure using the homogenization and centrifugation medium composed of 225 mM mannitol, 75 mM sucrose, 1 mM EGTA and 5 mM Tris-HCl (pH 7.4). Mitochondria were washed twice and finally sus-

Abbreviations: MAO, monoamine oxidase.

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pended in the same medium without EGTA to form a suspension containing 50–60 mg protein/ml.

The assays for MAO were carried out at room temperature (20–22° C) essentially in the same medium as that used by Smith and Reid [2] which contained 120 mM sucrose, 6 mM $MgCl_2$, 12 mM phosphate, 5 mM succinate and 5 mM Hepes/NaOH (pH 7.4). The spectrophotometric assay was based on the original procedure of Weissbach et al. [10] using kynuramine as substrate. To avoid interference by mitochondrial turbidity, the reaction was followed in the dual-wavelength mode (Shimadzu Model UV-3000 spectrophotometer) at 329 nm (one of the two absorption maxima for the reaction product 4-hydroxyquinoline) versus 338 nm (the isosbestic point) in silica cuvettes of 1 cm light path. The reaction medium contained 0.2 mM kynuramine and about 1 mg mitochondrial protein in 2.5 ml total volume.

In the radiochemical assay the procedure followed that of Smith and Reid [2]. The incubation mixture contained 1.0 ml of the medium to which 25 μl of 25 mM L-tyramine containing about $5 \cdot 10^5$ dpm [^{14}C]tyramine was added (final tyramine concentration was 0.6 mM). For State 3 conditions the mixture was supplemented with 2 mM ADP, 10 mM glucose and 3 units of hexokinase. The reaction was started by the addition of 50 μl of the mitochondrial suspension (corresponding to 2.5–3.0 mg protein) and carried in open test-tubes under vigorous shaking at room temperature (20–22° C). It was stopped after 3 min by addition of 0.25 ml of 4 M HCl. Preliminary experiments showed that the reaction was linear for at least 10 min under the conditions applied. For zero time control (blank) HCl was added before mitochondria.

For extraction of the reaction products 4 ml of the organic solvent was added to each test-tube, the tubes were stoppered and shaken vigorously twice for 1 min. In the case of toluene, anisole and ethyl acetate the tubes were centrifuged for 5 min at $200 \times g$ for better separation of the phases. In case of diethyl ether phase separation occurred after 5–10 min standing of the tubes without centrifugation. 3-ml aliquots of the organic phase were pipetted into scintillation vials containing 10 ml of scintillation cocktail (7 g 2,4-diphenyloxazole in 700 ml toluene + 300 ml ethanol) and counted for radioactivity. Apparent enzymic activity was calculated from the amount of the product extracted and the specific radioactivity of the substrate (determined from total counts in the zero time control). The recovery of the counts from the combined aqueous and organic phases was within 95 and 102%, indicating no appreciable loss of the material during the procedure and no formation of volatile oxidation products.

Paper chromatography of the reaction products was performed on Whatman No. 3 filter paper in the ascending system in the mixture of isopropyl alcohol/ammonia/water (8:1:1, v/v) according to Wurtman and Axelrode [11]. The chromatograms were dried overnight on air and autoradiographed with X-ray films. Chemical identification of the spots was performed by spraying the chromatograms with a solution of 2,4-dinitrophenylhydrazine (for aldehydes) and with universal pH indicator (No. 4080, British Drug Houses, Poole, UK) for acids.

Aldehyde-dependent NADPH oxidation was measured spectrophotometrically [12] in the dual-wavelength mode at 340 versus 400 nm at room temperature (20–22° C) in 1 cm light-path cuvettes containing 2.5 ml of 100 mM sodium phosphate (pH 7.0), 5 μM rotenone and 0.16 mM NADPH. The reaction was started by addition of 6 mM methylglyoxal (final concentration). The readings were corrected for blank reaction in the absence of substrate. Only those activities were taken as meaningful when addition of the substrate at least doubled the rate of NADPH oxidation.

For measurement of lactate dehydrogenase (EC 1.1.1.27) the cuvettes contained 100 mM sodium phosphate, 2 μM rotenone and 0.26 mM NADH and the reaction was started with 2 mM pyruvate.

The extinction coefficient for NADH and NADPH at the wavelength pair used was estimated as 5.6 mM^{-1} cm^{-1}.

[^{14}C]Tyramine hydrochloride was obtained from Amersham International (Amersham, UK; labelled in C-7) or New England Nuclear (Boston, MA, USA; labelled in C-1). No difference in the behaviour of these two compounds in our experimental system was found. *p*-Hydroxyphenylacetic acid was from Sigma (St. Louis, MO, USA).

3. Results

3.1. Determination of MAO activity using various extracting solvents

Applying experimental conditions of Smith and Reid [2], we were able to reproduce their results of a substantial apparent decrease of MAO activity under State 3 conditions (for definition of mitochondrial respiratory states see Ref. [13]). In our hands the activity in State 3 was, in most cases, about 50% of that in State 4 (Tables 1 and 2), although occasionally it amounted to as little as 10%, the value reported by the aforementioned authors. Of interest is the effect of rotenone, which increased the apparent activity in State 3 but decreased it in State 4, so that the differences between the two states almost disappeared (Table 1). Omission of succinate produced, in general, a decrease of the apparent MAO activity (not shown), although this result was not always reproducible. This is also consistent with the results of Smith and Reid [2], who observed a steady increase of MAO activity in State 1 (without respiratory substrate) from a very low rate up to the value comparable with that of State 4.

Using the spectrophotometric assay with kynuramine, no differences in MAO activity could be observed under transition from State 4 to State 3 (addition of ADP) and back to State 4 (addition of oligomycin) (Fig. 1).

Table 1
Apparent activity of monoamine oxidase depending on the mitochondrial respiratory state and the presence of rotenone; extraction with toluene

Respiratory state	Apparent activity (% of State 4)	
	no rotenone	plus rotenone
State 4	100	72 ± 4
State 3	53 ± 3	76 ± 9

Apparent monoamine oxidase activity is expressed as percentage of that in State 4 (without rotenone) which amounted to 1.26 ± 0.43 nmol/min per mg protein (mean values ± S.D. for 4 experiments). Rotenone concentration was 5 μM.

The picture which emerges from these results is inconsistent with the postulation [2] that the respiratory state of mitochondria influences the activity of MAO. This prompted us to investigate whether the differences between MAO activities in States 3 and 4 might reflect experimental artifacts resulting from accumulation of reaction products which are not readily extractable by toluene. To check this assumption we examined the efficacy of various organic solvents to extract labelled reaction products of tyramine oxidation. It appeared (Table 2) that the highest extraction was achieved with ethyl acetate, and the lowest values were obtained with toluene. Also the differences in apparent activity between States 3 and 4 almost disappeared when ethyl acetate or diethyl ether were used as extractants. No differences between the two states were visible either when samples extracted with toluene were re-extracted with diethyl ether and the two extracts were combined (Table 3). Multiple extractions with the same solvent showed (data not presented) that 90% or more of extractable radioactivity was recovered in the first extraction with ethyl acetate, whereas this value was 75–80% for the extraction with diethyl ether. As already shown by Otsuka and Kobayashi [14], a single extraction with toluene recovered about 35% of the total amount of tyramine oxidation product(s).

It was also found (data not shown) that, with ethyl acetate and diethyl ether as extractants, no difference in the apparent activity could be observed whether succinate was present or not.

The four solvents used differed by their extractability of the reaction substrate, tyramine, giving various blank values (Table 2). The lowest values were obtained with the most nonpolar solvent, toluene, and the highest with ethyl acetate. The latter result is in agreement with the observations of other authors [15,16] who recommended washing of the organic phase with water. Confirming the results of others [17,18], we found a mixture of toluene and ethyl acetate (1:1, v/v) as the extractant of choice, giving extraction efficiency comparable to that of ethyl acetate alone and no significant differences between States 3 and 4, but low blank values, similar to those obtained with toluene alone (not shown).

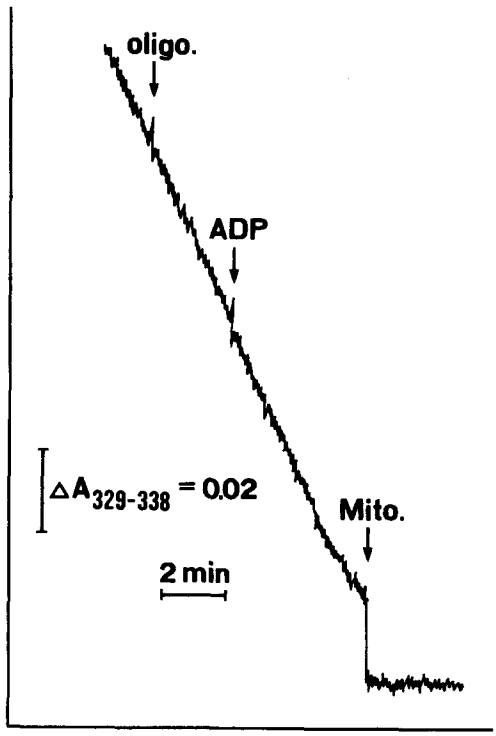

Fig. 1. Spectrophotometric measurement of MAO with kynuramine as substrate in mitochondria under States 4 and 3. The following additions were made where indicated: mitochondria (Mito.), 1.2 mg protein; ADP, 1.5 mM; and oligomycin (oligo.), 10 μg. Other conditions of the assay are described under Materials and methods. The time is running from right to left.

3.2. Identification of the reaction products

Data presented in the preceding section strongly suggest that (i) more than one reaction products accumulate during

Table 2
Comparison of the efficiency of the extraction of monoamine oxidase products with various organic solvents

Solvent	products extracted			Blank (% of total radioactivity)
	State 4 (nmol/min per mg)	State 3	State 3 (% of State 4)	
Toluene (7)	1.6 ± 0.2	0.8 ± 0.2	46 ± 8	0.02
Anisole (2)	5.7	4.4	77	0.04
Diethyl ether (3)	5.6 ± 1.8	4.9 ± 1.4	89 ± 6	0.10
Ethyl acetate (3)	7.9 ± 0.7	6.7 ± 0.7	85 ± 7	0.5

Values refer to the means ± S.D. for the number of different mitochondrial preparations given in parentheses or are mean values for two experiments.

Table 3
Recovery of the reaction products of monoamine oxidase with toluene and diethyl ether in States 4 and 3

Respiratory state	Extraction with:	
	toluene	toluene followed by diethyl ether
State 4	14000	34000
State 3	5400	30000

The incubation mixture contained 2.5 mg protein of mitochondria. The last column presents the sum of radioactivity extracted first with toluene and subsequently with diethyl ether. The activity is expressed in dpm per the total extraction sample. The results are from one representative experiment.

enzymatic oxidation of tyramine by liver mitochondria, (ii) their proportion varies depending on incubation conditions (energy state of mitochondria, presence or absence of respiratory substrate and respiratory inhibitors), and (iii) the various reaction products may be differently extracted by various solvents. To check these assumptions we examined reaction products by paper chromatography.

Three major radioactive spots with R_F values of 0.98, 0.65–0.75 and 0.40 (designated as A, B and C, respectively) were found when oxidation products of [^{14}C]tyramine were separated by paper chromatography with isopropyl alcohol/ammonia/water as solvent (Fig. 2). After evaporating the solvent, spot A was stained yellow when the chromatogram was sprayed with 2,4-dinitrophenylhydrazine, suggesting its aldehyde nature, and was therefore identified as *p*-hydroxyphenylacetaldehyde. Spot C was stained with a pH indicator, indicating its acidic character. It was identified as *p*-hydroxyphenylacetic acid on the basis of the same R_F value as the authentic compound (not shown). Spot B was not stained with either reagent and was supposed to correspond to *p*-hydroxyphenylacetalcohol. As clearly visible in Fig. 2, proportions

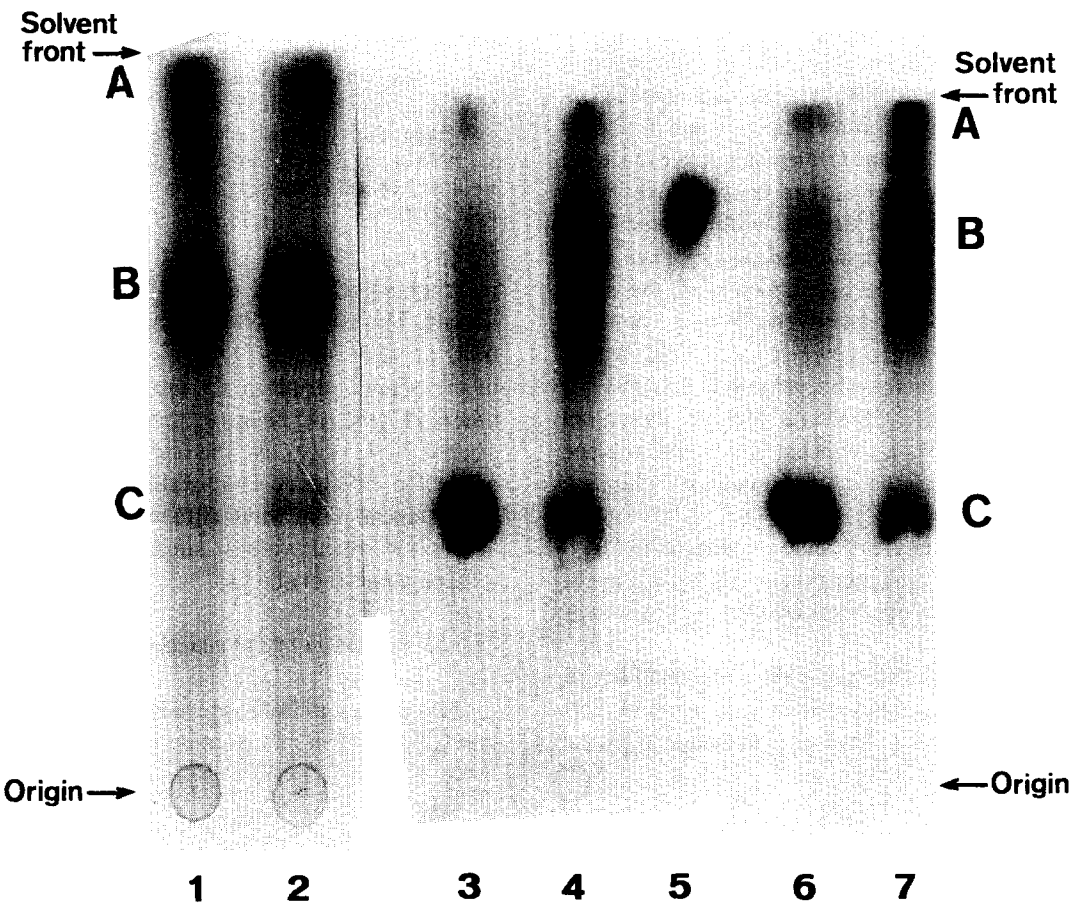

Fig. 2. Chromatography of oxidation products of [^{14}C]tyramine after extraction with toluene, diethyl ether and ethyl acetate. Incubation conditions were as described under Materials and methods except that the samples contained $2 \cdot 10^6$ dpm/ml [^{14}C]tyramine. After incubation, acidification with HCl and extraction, the organic phases were evaporated under nitrogen. Thereafter, the dry material was dissolved in 200 μl diethyl ether and applied on chromatography filter paper (20 cm long strips). Chromatograms were developed for about 3 h. After evaporation of the solvent overnight, the chromatograms were exposed to X-ray film for 3 days. The autoradiograms correspond to the following samples (extracting solvent indicated in parentheses): lane 1, State 4 (toluene); lane 2, State 3 (toluene); lane 3, State 3 (diethyl ether); lane 4, State 4 (diethyl ether); lane 5, [^{14}C]tyramine; lane 6, State 3 (ethyl acetate); lane 7, State 4 (ethyl acetate). The spots were identified as follows: A, *p*-hydroxyphenylacetaldehyde; B, *p*-hydroxyphenylacetalcohol; C, *p*-hydroxyphenylacetic acid.

Table 4
Distribution of NADPH-dependent aldehyde reductase in rat liver subcellular fractions

Fraction	Lactate dehydrogenase		Aldehyde reductase	
	specific activity [a]	% of the cytosol	specific activity [a]	% of the cytosol
Cytosol	70607		32	
"Intact" mitochondria	31	0.4	3.0	9
Frozen and thawed mitochondria	31	0.4	7.3	23

Aldehyde reductase and lactate dehydrogenase were measured as described under Materials and methods. The mitochondrial fraction was additionally washed once with 100 mM KCl + 10 mM Tris-HCl to further reduce contamination by cytosolic proteins. A representative experiment out of three similar ones is shown.

[a] Specific activity is expressed in nmol/min per mg protein.

between the three spots differed depending on the extraction solvent and on mitochondrial respiratory state. In general, spot C was stronger in State 3 than in State 4, whereas the opposite was true for spots A and B. On the other hand, spot C was strong when the oxidation products were extracted with either diethyl ether or ethyl acetate (Fig. 2, lanes 3, 4, 6 and 7) but was scarcely visible among reaction products extracted with toluene (lanes 1 and 2). Relative amounts of the three major reaction products were determined by cutting off the spots and counting their radioactivity. It appeared that the distribution of radioactivity was essentially the same using diethyl ether and ethyl acetate as extractants and amounted to about 15%, 65% and 20% for spots A, B and C, respectively, in State 4 mitochondria and to about 10%, 20% and 70%, respectively, under State 3 conditions (mean values for three experiments).

3.3. Demonstration of NADPH-dependent aldehyde reductase activity in mitochondria

Rat liver mitochondrial fraction exhibited a substantial oxidation of NADPH with methylglyoxal as electron acceptor. Since NADPH-dependent aldehyde reductase (alcohol:$NADP^+$ oxidoreductase, EC 1.1.1.2) has been found so far in the cytosolic fraction [19,20] and because intact mitochondria are impermeable to externally added nicotinamide nucleotides, it was essential to examine whether the observed activity was due to cytoplasmic contamination or was also intrinsic to mitochondria.

Lactate dehydrogenase (EC 1.1.1.27) was used as cytoplasmic marker. To diminish adsorption of this enzyme, and presumably of other cytoplasmic proteins, to mitochondria, an additional washing of the mitochondrial fraction with 100 mM KCl + 10 mM Tris-HCl was performed. After such treatment, specific activity of lactate dehydrogenase in the mitochondrial fraction amounted to less than 0.5% of that in the cytosol. Aldehyde reduction assayed in the sucrose medium was negligible in the mitochondrial fraction (not shown) and could be totally accounted for by cytosolic contamination. However, in 100 mM phosphate, conditions which make the inner mitochondrial membrane leaky to nicotinamide nucleotides [21], this activity in the mitochondrial fraction was one-tenth of that in the cytosol. It increased to one fourth when mitochondria were disrupted be repeated freezing and thawing prior to the assay (Table 4).

4. Discussion

Two different forms of MAO, designated A and B, have been identified on the basis of different substrate specificity and sensitivity to inhibitors [22]. Both forms are present in rat liver mitochondria in comparable amounts and both oxidize tyramine [22]. Therefore, the conclusions discussed below may apply to both forms.

4.1. Reaction products of tyramine oxidation in liver mitochondria

Oxidative deamination of primary amines by MAO yields corresponding aldehydes. The latter compounds can, however, be further metabolized by other enzymes to acidic or alcoholic derivatives [16,23] whose extraction efficiency may be different with various solvents [16,18]. Therefore, all assay methods based on measurements of the organic reaction product(s) of MAO activity should determine not only the immediate product, i.e., the aldehyde, but also its metabolites which can eventually arise. Chromatography of radioactive products which appear after incubation of [^{14}C]tyramine with intact liver mitochondria revealed three spots (Fig. 2) identified as *p*-hydroxyphenylacetaldehyde, *p*-hydroxyphenylacetic acid and *p*-hydroxyphenylacetalcohol (the latter one identified tentatively). Thus, the following reactions are expected to occur when tyramine is oxidized by liver mitochondria:

$$\text{tyramine} + O_2 \Rightarrow p\text{-hydroxyphenylacetaldehyde} + NH_3 + H_2O_2 \quad (1)$$

$$p\text{-hydroxyphenylacetaldehyde} + NAD^+ \rightleftarrows p\text{-hydroxyphenylacetic acid} + NADH + H^+ \quad (2)$$

$$p\text{-hydroxyphenylacetaldehyde} + NADPH + H^+ \Rightarrow p\text{-hydroxyphenylacetalcohol} + NADP^+ \quad (3)$$

Reaction 1 is catalyzed by MAO and proceeds in the outer mitochondrial membrane. Reaction 2 is catalyzed by aldehyde dehydrogenase (EC 1.2.1.3) present within the inner membrane-matrix compartment [24]. This reaction is reversible and its direction depends on the redox state of mitochondrial NAD. Reaction 3 can be catalyzed by aldehyde reductase, the occurrence of which in liver mitochondria has been tentatively demonstrated in the present investigation. It requires NADPH as source of reducing equivalents [19,25] and is practically irreversible because of highly negative redox potential of this coenzyme. Using ethyl acetate or diethyl ether (most likely also anisole, although this was not examined in detail), all three reaction products could be quantitatively extracted and their sum appeared the same irrespectively of the mitochondrial respiratory state (Table 2), reflecting the real MAO activity, although the proportions between the three compounds differed substantially between States 3 and 4.

4.2. Choice of proper extractants

The solvents most commonly used for extraction of the reaction product(s) of MAO activity in the radiochemical assay are toluene [11], anisole [14], ethyl acetate [15] and diethyl ether [16], or their mixtures [17,18]. Toluene was found to be suitable in case of tryptamine as substrate, efficiently extracting both the immediate oxidation product, indoleacetaldehyde, and the final product, indoleacetic acid [11]. It appeared, however, rather poor extractant when other amines were used as MAO substrates [14,16] and was practically ineffective in extracting *p*-hydroxyphenylacetic acid, the final product of tyramine oxidation [16]. Nevertheless, it has been applied by several authors, probably because of the advantage that, being highly nonpolar, it extracts negligible amounts of the non-reacted substrate, giving blank values close to zero (Table 2). It has to be mentioned here, however, that the radiochemical assays for MAO give significantly lower values than the polarographic procedure and assays coupled to alcohol dehydrogenase [26]. The reason for this discrepancy is not clear.

4.3. Dependence of the final products of tyramine oxidation on incubation conditions and energy state of mitochondria

Under State 4 conditions with succinate as the respiratory substrate but in the absence of rotenone, mitochondrial nicotinamide nucleotides are maximally reduced due to reversed electron flow (NADH) and operation of the energy-dependent transhydrogenase (NADPH). Under such conditions the equilibrium of Reaction 2 is shifted to the left and the aldehyde is likely to be reduced to the corresponding alcohol (Reaction 3). As result, the reaction products consist of *p*-hydroxyphenylacetaldehyde and *p*-hydroxyphenylacetalcohol, with only a small amount of *p*-hydroxyphenylacetic acid. In contrast, under State 3 the mitochondrial $NADH/NAD^+$ ratio is decreased and therefore more *p*-hydroxyphenylacetic acid can accumulate (Reaction 2 shifted to the right). Moreover, because of the competition for the protonmotive force between the ATP-synthesizing system and energy-dependent nicotinamide nucleotide transhydrogenase, the $NADPH/NADP^+$ ratio is also decreased, resulting in a decrease of the amount of *p*-hydroxyphenylacetalcohol. These predictions are fully confirmed by comparison of relative amounts of tyramine oxidation products presented in Fig. 2 (compare lanes 3 and 6 with lanes 4 and 7 for States 3 and 4, respectively). In the absence of succinate the $NADH/NAD^+$ ratio is low because of the absence of reversed electron flow, and hence *p*-hydroxyphenylacetaldehyde can be further oxidized to its corresponding acid, giving low apparent MAO activity with toluene as extracting solvent. In the presence of rotenone the $NADH/NAD^+$ ratio becomes independent of the mitochondrial energy state because of inhibition of both the reversed electron transfer and NADH oxidation. Hence, the apparent MAO activity determined using toluene as the extracting solvent was approximately the same in States 4 and 3 in the presence of this inhibitor (Table 1).

In conclusion, apparent differences in MAO activity between various energy states of mitochondria, as observed by Smith and Reid [2], are artifacts resulting from poor extraction of *p*-hydroxyphenylacetic acid by toluene. Since in our experiments the samples were vigorously shaken during incubation, the observed differences could not be ascribed to oxygen depletion, as suggested by Harris and Cooper [7].

4.4. Location of MAO in the contact sites?

The present paper clearly shows that there is no evidence for an influence of the energy state of the inner mitochondrial membrane on the activity of monoamine oxidase in the outer membrane. Thus, the results of Smith and Reid [2] can no longer be used as an argument for the location of MAO in contact sites. In fact, in experiments with brain mitochondria using gradual extraction of the outer membrane by digitonin, MAO was localised in outer membrane domains outside rather than inside the contact sites [5,6].

4.5. The presence of aldehyde reductase in liver mitochondria

The occurrence of aldehyde reductase in various animal tissues has been known for more than three decades [27,28]. Its absolute specificity towards NADPH has been demonstrated [20,25]. Using pig brain, Turner and Tipton [20] found the enzyme almost exclusively located in the cytosol. The results presented here demonstrate, to our knowledge for the first time (Table 4), that NADPH

oxidation by aldehydes in rat liver also occurs in mitochondria, though at a lower rate than in the cytosolic fraction. This finding may suggest that NADPH-dependent aldehyde reductase, at least in liver, is located partly in mitochondria and could be responsible for the reduction of *p*-hydroxyphenylacetaldehyde to *p*-hydroxyphenylacetalcohol in the radiochemical assay for MAO.

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