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SUBCELLULAR DISTRIBUTION OF MONOAMINE OXIDASE ACTIVITY IN RAT LIVER AND VAS DEFERENS

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Abstract—After differential centrifugation 24 per cent of the total MAO activity of rat liver homogenates and 42 per cent of that in vas deferens was recovered in a microsomal fraction, the remainder being recovered in a mitochondrial pellet. The distribution of enzyme activities known to be associated with the outer membranes of mitochondria (kynurenine hydroxylase and $NADH_2$-cytochrome *c* reductase) was similar to that of MAO, but succinic dehydrogenase activity was almost entirely mitochondrial. When vas deferens homogenates were subjected to density gradient centrifugation, a small particle fraction containing MAO and $NADH_2$-cytochrome *c* reductase activities could be clearly separated from a fraction containing noradrenaline storage particles. It is concluded that MAO activity is not specifically associated with noradrenaline storage particles, and that the MAO activity recovered in microsomal fractions may arise as an artefact when mitochondria are disrupted during tissue homogenization.

STUDIES of the subcellular localization of monoamine oxidase (MAO) (EC.1.4.3.4.) in liver and other tissues have shown that MAO is predominantly a mitochondrial enzyme.[1–5] Sub-mitochondrial fractionation techniques have further indicated that MAO in rat liver mitochondria is largely associated with the outer membranes of the mitochondrion.[6, 7] Although MAO is thus generally considered to be a mitochondrial enzyme, variable amounts of MAO activity have been reported to occur in microsomal fractions on differential centrifugation of tissue homogenates.[2–4] Ostwald and Strittmatter,[2] and Baudhuin *et al.*,[3] for example, found that approximately 20 per cent of the total MAO activity of rat liver homogenates was recovered in a microsomal fraction. In these and other studies[5] the subcellular distribution of MAO did not exactly parallel that of succinic dehydrogenase (SDH) activity, generally considered to be a marker enzyme activity for mitochondria;[8] the amount of SDH recovered in microsomal fractions was considerably less than 20 per cent of the total activity. Roth and Stjärne[9] recently reported that about a third of the MAO activity in homogenates of bovine splenic nerve was recovered in a pellet obtained by high speed centrifugation (50,000 *g* for 30 min). Since this fraction was rich in noradrenaline (NA) storage particles, and since it appeared to be devoid of mitochondria by morphological criteria, these authors have suggested that part of the MAO in bovine splenic nerves may be located in NA storage granules.[9, 10] We have tested this hypothesis by examining the subcellular distribution of MAO and other mitochondrial enzymes in the rat vas deferens, a smooth muscle tissue with a rich sympathetic innervation. Parallel studies using rat liver, an organ in which the greater part of the MAO activity is not considered to be associated with the sympathetic innervation, were included for comparison.

METHODS

Subcellular fractionation

Vas deferens and liver were removed from adult male Wistar rats (200–250 g) and homogenized in an all-glass hand homogenizer with 4–10 vol. of ice-cold 0·25 M sucrose. For differential centrifugation studies the homogenates were centrifuged for 2 min at 1000 *g* to remove large fragments of unbroken tissue and other debris. The supernatants were centrifuged at 12,000 *g* for 10 min to yield a mitochondrial pellet (P1), and the resulting supernatant was centrifuged at 100,000 *g* for 60 min to yield a microsomal pellet (P2) and a clear supernatant (S). Pellets P1 and P2 were resuspended in 2 ml of 0·25 M sucrose. Fractions were maintained at or near 0° throughout the preparation procedures. P1, P2 and S fractions were frozen and stored at −20° prior to assay. For continuous density gradient centrifugation, sucrose gradients were prepared as described by Potter and Axelrod.[11] Two types of gradient were used; both were prepared in polycarbonate tubes of 2·5 ml capacity of the MSE Superspeed 50 ultracentrifuge. Type I gradients (gradient volume = 1·8 ml) ranged from 1·75 M at the bottom of the tube to 0·61 M at the top of the gradient (Fig. 1). Type II (gradient volume = 1·2 ml) ranged from 0·75 M at tube bottom to 0·40 at the top of the gradient. Gradients were stored at 4° for at least 1 hr before use. For type I gradients, 0·3 ml of liver or vas deferens homogenates, prepared as described above, were layered on to the gradient and the tubes were centrifuged at 100,000 *g* for 60 min. For type II gradients, vas deferens homogenates were first centrifuged at 12,000 *g* for 10 min and

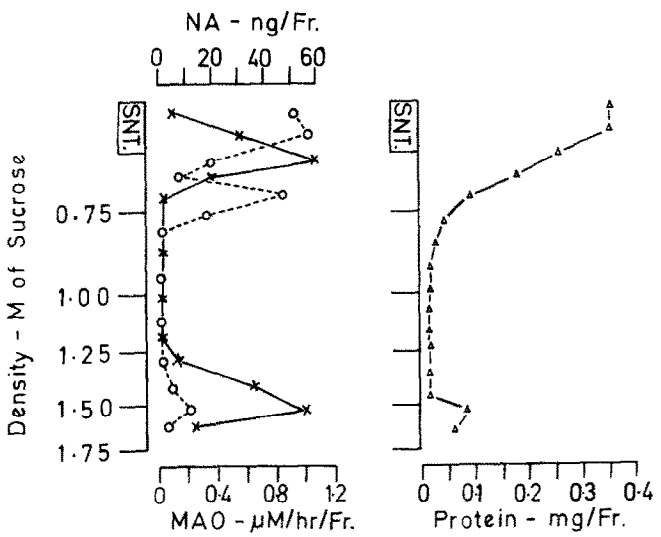

FIG. 1. Distribution of protein (△—△—△—△), MAO activity (×—×—×—×) and noradrenaline (○ - - ○ - - ○ - - ○) on a sucrose density gradient (Type 1) after centrifugation of vas deferens homogenate. MAO activity, protein and noradrenaline were assayed on aliquots of the fractions from a single gradient. Similar results were obtained in two other experiments. SNT = supernatant fraction. Fr. = fraction. MAO activity is expressed as μmole H^3 -tyramine deaminated/hr.

0·6 ml of the resulting supernatant fractions were then layered on to the gradients. These were then centrifuged at 120,000 *g* for 60 min. Gradients were sampled either by piercing the bottom of the tube and collecting 10 drop fractions, or more commonly by piercing the bottom of the tube and displacing the contents upwards by pumping

in a dense sucrose solution (2·0 M) at a constant rate and timed fractions were then collected from the top of the tube.

Enzyme assays

MAO was assayed by the method of McCaman, McCaman, Hunt and Smith[12] using H^3-tyramine (generally labelled; Sp. act. = 2·1 c/m-mole: New England Nuclear Corpn., Boston, Mass.) or H^3-5-hydroxytryptamine (5-HT) (generally labelled; Sp. act. = 300 mc/m-mole; Radiochemical Centre, Amersham, England).

L-Kynurenine-3-hydroxylase activity was assayed by the method of Okamoto, Yamamoto, Nozaki and Hayaishi.[13]

$NADH_2$-Cytochrome *c* reductase was measured by a modification of the method of Nason and Vasington.[14] The rate of disappearance of $NADH_2$ fluorescence was measured in a Farrand spectrofluorometer at 335/455 mμ.

Succinic dehydrogenase activity was measured by the method of Green, Mii and Kohout.[15]

Protein was estimated by the method of Lowry, Rosebrough, Farr and Randall.[16]

Noradrenaline in P1, P2 and S fractions of vas deferens was estimated by the fluorometric method of Euler and Lishajko[17] after extraction by ion-exchange chromatography.[18] The noradrenaline content of density gradient fractions from vas deferens was estimated by a modification of the radiochemical enzymic method of Saelens, Schoen and Kovacsics[19] which allows accurate determination of as little as 1 ng of noradrenaline.[20] With this method it proved possible to determine noradrenaline in 10 μl aliquots of the density gradient fractions, so that parallel enzyme assays and protein determinations could be performed on the same samples.

RESULTS

(1) *Differential centrifugation of liver and vas deferens homogenates*

Protein, cytochrome *c* reductase, succinic dehydrogenase and MAO activities were estimated in the fractions P1 (mitochondrial), P2 (microsomal), and S (supernatant) derived by differential centrifugation of liver and vas deferens homogenates. In addition kynurenine-3-hydroxylase activity was measured in the subcellular fractions of liver; this enzyme could not be detected in vas deferens homogenates. Noradrenaline was measured in the subcellular fractions of vas deferens. The results, (Table 1) confirm that an appreciable proportion of the total MAO activity in both tissues is recovered in a microsomal fraction (P2). The results were closely similar when either tyramine or 5-HT were used as substrates for the MAO assay. In agreement with previous findings,[2, 5] the proportion of the total SDH activity in either tissue recovered in the microsomal fraction, was significantly smaller than the recovery of MAO in this fraction. However, the subcellular distribution of two other enzymes, known to be localised in mitochondrial outer membranes (kynurenine-3-hydroxylase and $NADH_2$ cytochrome-*c* reductase[13, 21]) was similar to that of MAO. When results from vas deferens were compared with liver, a significantly higher proportion of all the measured enzyme activities was found in the microsomal fraction of the vas deferens (Table 1).

None of the enzyme activities studied showed any activity in the supernatant fraction in either tissue. This is in agreement with most previous studies which have found only negligible MAO activity in the supernatant fraction. Roth and Stjärne,[9]

however, reported considerable MAO activity in the supernatant fraction of bovine splenic nerve homogenates. In their enzyme assay procedure, however, no allowance was made for the possibility that non-enzymic destruction of the substrate (H^3-dopamine) might occur during the incubation period, and this result is, therefore, equivocal.

TABLE 1. DISTRIBUTION OF MAO, PROTEIN, NORADRENALINE AND OTHER ENZYME ACTIVITIES IN FRACTIONS OBTAINED BY DIFFERENTIAL CENTRIFUGATION OF RAT LIVER AND VAS DEFERENS HOMOGENATES (For details see text)

	Total Recovered (P1 + P2 + S) per g wet wt.	Percentage of total recovered activity		
		P1 (mitochondria)	P2 (microsomes)	S (supernatant)
Liver				
Protein	150·4 ± 8·92 mg	34 ± 1·4	21 ± 1·8	45 ± 3·6
MAO (5-HT)	24·5 ± 0·37 μmole/hr	76 ± 2·3	24 ± 0·7	nil
MAO (TM)	43·2 ± 1·19 μmole/hr	76 ± 4·2	24 ± 1·3	nil
$NADH_2$-Cyt.*c* reductase	17·5 ± 0·57 μmole/min	66 ± 3·8	34 ± 2·6	nil
Kynurenine 3-hydroxylase	65·5 ± 3·74 μmole/min	77 ± 8·6	23 ± 2·6	nil
Succinate dehydrogenase	44·7 ± 3·21 μmole/hr	93 ± 7·2	7 ± 0·2	nil
Vas deferens				
Protein	57·7 ± 4·60 mg	22 ± 0·5	14 ± 1·1*	64 ± 7·3
MAO (5-HT)	3·6 ± 0·04 μmole/hr	58 ± 1·6	42 ± 0·8†	nil
MAO (TM)	5·0 ± 0·05 μmole/hr	58 ± 1·3	42 ± 0·6†	nil
$NADH_2$-Cyt *c* reductase	21·4 ± 0·82 μmole/hr	54 ± 3·6	46 ± 4·1 n.s.	nil
Succinate dehydrogenase	5·9 ± 0·58 μmole/hr	86 ± 8·4	14 ± 2·2*	nil
Endogenous noradrenaline	4·22 ± 0·213 μg	19 ± 2·9	52 ± 5·4	29 ± 6·7

MAO activity was measured with H^3-5-hydroxytryptamine (5-HT) and H^3-tyramine (TM) as substrates. Results are mean values ± S.E. Means for 4 experiments.
* = $P < 0.05$; † = $P < 0.001$; n.s. = $P > 0.05$ when compared to corresponding results from liver P2 fractions

(2) *Density gradient centrifugation of vas deferens homogenates*

The distribution of MAO (tyramine substrate), protein and endogenous noradrenaline on Type I gradients prepared from vas deferens homogenates is shown in Fig. 1. In three experiments of this type, MAO activity was recovered in two regions of the gradient: in a layer or pellet at the bottom of the tube, and in a cream coloured "fluffy" layer immediately below the clear brown supernatant. In confirmation of previous studies, noradrenaline was found to be localised in the supernatant layer and in a particulate layer slightly below the supernatant. The position of the particle bound noradrenaline peak was generally slightly below that of the upper layer of the MAO activity, as illustrated in Fig. 1. This finding was confirmed when vas deferens homogenates were centrifuged on Type II gradients, which were designed to examine the distribution of components of the microsomal and supernatant fractions in more detail. In two experiments of this type the distributions of MAO (tyramine substrate) and endogenous noradrenaline were clearly different (Fig. 2). The "fluffy" particle layer containing most of the MAO activity in these gradients was again situated

immediately below the supernatant. This layer also contained most of the $NADH_2$-cytochrome *c* reductase activity. (Fig. 2).

In one experiment rat vas deferens was obtained 14 days after surgical sympathectomy.[22] When this tissue was homogenized and centrifuged on a Type I gradient, MAO activity could again be recovered in both heavy and light layers, with a distribution similar to that seen in normal tissues. Fluorometric assays of vas deferens obtained from similarly treated animals showed that the endogenous noradrenaline content was reduced by more than 95 per cent by this procedure. The noradrenaline content of the density gradient fractions was too low for reliable assay.

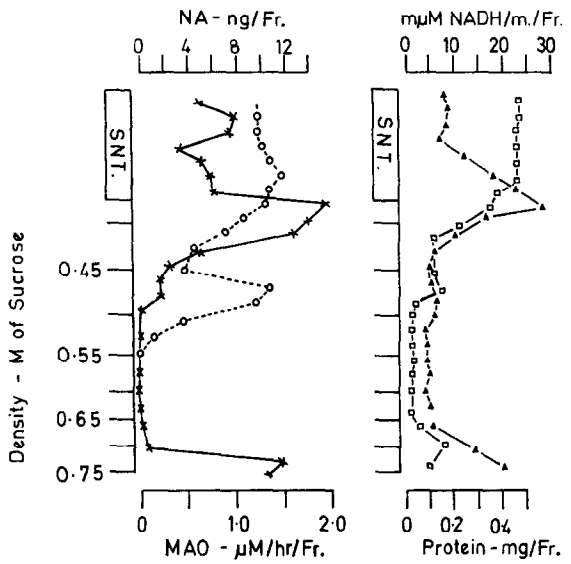

FIG. 2. Distribution of protein (□—□—□—□), noradrenaline (○ - - ○ - - ○ - - ○), MAO (×—×—×—×) and $NADH_2$-cytochrome *c* reductase activity (△—△—△—△) on a sucrose density gradient (Type II) after centrifugation of vas deferens low speed supernatant. All parameters were assayed in aliquots of the fractions from a single gradient. Results are representative of two similar experiments. MAO activity is expressed as μmole H^3-tyramine deaminated per hour; $NADH_2$-cytochrome *c* reductase activity as mμmole $NADH_2$ oxidized/min. SNT = supernatant fraction. Fr. = fraction.

DISCUSSION

The present results confirm that a considerable proportion of the total MAO activity of rat liver homogenates can be recovered in a "microsomal" fraction after differential centrifugation. An even higher proportion of the total MAO activity of vas deferens homogenates was similarly recovered in a "microsomal" fraction, which also contained a large proportion of the total endogenous noradrenaline content. Only a small proportion of the total SDH activity in either liver or vas deferens was recovered in the "microsomal" fraction, which would, thus, appear to be relatively uncontaminated with intact mitochondria. Our results, therefore, agree with those of Roth and Stjärne[9] who found that much of the MAO activity in splenic nerve homogenates was recovered in a high speed pellet which contained noradrenaline storage particles, and appeared to be devoid of mitochondria. However, our interpretation of these findings differs from that of Stjärne,[10] who suggested "that

MAO is actually contained in the NA storage granules themselves". Density gradient centrifugation of vas deferens homogenates showed that the "microsomal" MAO activity was present in small particles of low density, which could be separated quite clearly from the more dense NA storage granules. Furthermore, the MAO containing particles could also be demonstrated in sympathetically denervated vas deferens, in which the presence of NA-storage particles could no longer be detected. It thus seems unlikely that the NA storage particles in vas deferens, or in other regions of the sympathetic nervous system have any appreciable MAO activity associated with them. This conclusion is in keeping with the findings of Potter and Axelrod[23] who reported that NA storage particles isolated from rat heart homogenates had little or no MAO activity.

There remains the question of the origin of the "microsomal" MAO activity in vas deferens and liver homogenates. We believe that the most likely explanation is that "microsomal" MAO arises as an artefact during homogenization, and that the enzyme recovered in this fraction is present in fragments of mitochondrial outer membranes. In disrupted liver mitochondria, MAO, $NADH_2$-cyt.*c* reductase and kynurenine hydroxylase activities are associated with the outer membranes of the mitochondrion, which can be separated from the heavier fragments of inner membrane containing SDH and the various cytochrome components of the respiratory chain.[6, 13, 24] The low recovery of SDH in "microsomal" fractions in the present experiments, therefore, does not necessarily imply an absence of mitochondrial contamination, since this fraction may contain fragments of outer mitochondrial membrane arising as an artefact during the homogenization procedure. In support of this view, it was found that the distribution of other outer mitochondrial membrane enzyme activities (kynurenine hydroxylase and $NADH_2$-cytochrome *c* reductase) was similar to that of MAO in both vas deferens and liver homogenates. Furthermore, in vas deferens, where the fibrous nature of the tissue entails considerably longer periods of homogenization than in the liver, higher proportions of MAO and $NADH_2$-cyt.*c* reductase activity and of SDH activity were recovered in the "microsomal" fraction than in the liver. This would be expected since harsh mechanical treatments are known to cause extensive damage to mitochondrial structure.[25] Similar considerations presumably apply to studies of bovine splenic nerves, in which harsh mechanical disruption methods (Ultra–Turrax) are frequently employed.

An alternative possibility is that "microsomal" MAO arises from enzyme activity present in the membranes of the endoplasmic reticulum, which would thus represent a truly microsomal portion of the total enzyme activity. It has been suggested that the membranes of the endoplasmic reticulum are continuous with the outer membranes of mitochondria,[24] and the present results can be explained equally well on this basis.

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