

Subcellular localization of aldehyde reductase activities in ox brain

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The distribution of the two principal isoenzymes of aldehyde reductase (EC 1.1.1.2) has been studied in ox brain. The more active of these, which has been termed the high- K_m enzyme, has been shown to be located in the cytosol and the less abundant low- K_m form has a similar localization. *p*-Nitrobenzaldehyde, which has been used as a substrate in previous studies, causes the reduction of NADH in the presence of the mitochondrial fraction, but mixed substrate experiments with 1,3-dinitrobenzene and the effects of pH on the activity indicate that this is due to the presence of a nitro reductase activity which has been recently described [Köchli, Wermuth & von Wartburg (1980) *Biochim. Biophys. Acta* 616, 133–142] rather than to the low- K_m aldehyde reductase activity. Fractionation of the mitochondria indicated this activity to be largely confined to the mitochondrial inner membrane.

Two major forms of the NADPH-dependent aldehyde reductase (alcohol:NADP⁺ oxidoreductase, EC 1.1.1.2) have been detected in liver and brain from a number of animal species (see e.g. Turner & Tipton, 1972; Ris & von Wartburg, 1973; Tulsiani & Touster, 1977; Branlant & Biellmann, 1980). The predominant form, which has been called the high- K_m form (Turner & Tipton, 1972), is quite specific for NADPH and is sensitive to inhibition by anticonvulsants such as the barbiturates (see e.g. Turner & Tipton, 1972; Ris *et al.*, 1975) and sodium valproate (Whittle & Turner, 1978). The less abundant low- K_m enzyme is insensitive to these inhibitors and can use NADH as well as NADPH as the reducing cofactor. Although both these enzymes are capable of catalysing the reduction of the aldehydes derived from the oxidation of the catecholamines, kinetic studies have indicated that only the low- K_m enzyme is likely to play a significant role in this process under physiological conditions (Turner *et al.*, 1974; Anderson *et al.*, 1976). The function of the high- K_m enzyme is unclear, and the suggestion that it may play a significant role in the reduction of succinate semialdehyde (Kaufman *et al.*, 1979; Branlant & Biellmann, 1980) appears to be unlikely on the basis of kinetic studies (Rivett *et al.*, 1981).

Studies on the subcellular localization of these two enzymes have given conflicting results. In mouse liver both have been found to be present in the cytoplasmic fraction (Tulsiani & Touster, 1977) but

in rat brain the low- K_m enzyme activity has been reported to be confined to the mitochondria (Anderson *et al.*, 1976; von Wartburg *et al.*, 1977). A number of these studies have used *p*-nitrobenzaldehyde to determine the low- K_m aldehyde reductase activity in the presence of NADH, and the recent report of an NADH-dependent nitro reductase activity in rat brain that is active towards this substrate (Köchli *et al.*, 1980) casts doubt on the significance of the results obtained in this way.

In this paper we report the results of studies on the distribution of the aldehyde reductases in ox brain using pyridine-3-aldehyde, which has been shown to be a good substrate for both forms of the enzyme (Branlant & Biellmann, 1980) and *p*-carboxybenzaldehyde, which has been shown to be a better substrate for the high- K_m enzyme (Branlant & Biellmann, 1980). The activity of the low- K_m enzyme could be studied in the absence of interference from that of the high- K_m activity, since only the former enzyme has been shown to have detectable aldehyde reductase activity in the presence of 0.1 mM-NADH (Rivett *et al.*, 1981). The results, which indicated both forms of the enzymes to be cytoplasmic, are compared with those obtained when *p*-nitrobenzaldehyde was used as the substrate.

Materials and methods

Materials

NADH and NADPH were obtained from Boehringer. Pyridine-3-aldehyde and *p*-nitrobenzaldehyde were obtained from Sigma. 1,3-Dinitrobenzene was a kind gift from the Department of

Abbreviation used: Hepes, 4-(2-hydroxyethyl)-1-piperazine-ethanesulphonic acid.

Chemistry, Trinity College, Dublin, and pyrazole was from Eastman. All other chemicals were obtained from BDH.

p-Nitrobenzaldehyde and *p*-carboxybenzaldehyde were recrystallized from water before use. Stock solutions of *p*-nitrobenzaldehyde and 1,3-dinitrobenzene were made up in methanol/water (1:1, v/v) and methanol respectively. The concentrations of pyridine-3-aldehyde in solution were estimated enzymically by using aldehyde dehydrogenase (Racker, 1957).

Subcellular fractionation

This was performed by a modification of the five-fraction procedure of de Duve *et al.* (1955). Samples of the midbrain, hypothalamus and cortex from fresh ox brains were washed in 0.32 M-sucrose, dried and weighed. About 30 g of material obtained from a single brain was used in each study. After homogenization in 10 vol. of 0.32 M-sucrose with a Dounce homogenizer the material was centrifuged at 500 *g* for 12.5 min. The pellet obtained was resuspended in about 5 vol. of 0.32 M-sucrose and was centrifuged as before. The pellet was retained as the 'nuclear' (N) fraction.

The supernatants from the two centrifugations were combined and centrifuged at 12000 *g* for 12.5 min and the pellet obtained was resuspended in about 5 vol. of 0.32 M-sucrose and centrifuged as before. The pellet was retained as the 'crude mitochondrial' (M) fraction. The combined supernatants were centrifuged at 27000 *g* for 11.5 min and the pellet was retained as the 'light mitochondrial' (L) fraction. The supernatant was centrifuged at 48750 *g* for 160 min, the pellet was retained as the 'microsomal' (P) fraction and the supernatant as the 'cytoplasmic' (S) fraction. All particulate fractions were suspended in 0.32 M-sucrose.

The crude mitochondrial fraction was further subfractionated by centrifugation on a discontinuous sucrose gradient by the method of Gray & Whittaker (1962) in order to separate it into myelin (My), synaptosomal (Sy) and mitochondrial (Mi) fractions. The mitochondrial fraction was suspended, by homogenization in a Dounce homogenizer, in 0.32 M-sucrose. All fractions were stored at 4°C and assayed within 2 days, or were frozen with liquid N₂ and stored at -20°C. No significant differences in the distributions of the enzymes assayed was found between these two methods of storage. Unless otherwise stated all fractions were made to 1% (w/v) with Triton X-100 before assay.

Submitochondrial fractionation

The distribution of enzyme activities within the mitochondrion was studied by investigating their release by digitonin (Schnaitman *et al.*, 1967; see also Greenawalt, 1974). A freshly-prepared mito-

chondrial (Mi) fraction was suspended in a medium containing 4.0% (w/v) mannitol, 2.4% (w/v) sucrose and 0.048% Hepes, adjusted to pH 7.4 with KOH, to give a final protein concentration of between 6 and 7 mg/ml. Aliquots of a freshly-prepared digitonin solution (5 mg/ml) were added to samples of the mitochondrial suspension to give the desired concentration and the mixtures were allowed to stand in ice for 30 min with occasional shaking. A small volume of the medium (see the legend to Fig. 3) was then added, samples of the mixtures were retained for assay and the remainder were centrifuged for 3 min in an O.Dich Minifuge. The pellets were resuspended in ice-cold medium that had been brought to 1% (w/v) with Triton X-100 and these and the supernatants were stored at 4°C and used within 24 h.

Samples of freshly-prepared mitochondrial (Mi) fractions were also subfractionated by the method of Craven *et al.* (1969) to yield outer-membrane, inter-membrane matrix, inner-membrane and inner matrix fractions. All these fractions were made to 1% (w/v) with Triton X-100 before assay.

Assay methods

RNA was determined by the method of Fleck & Munro (1962), DNA by the method of Burton (1956) modified as described by Nimmo & Tipton (1979), and protein concentration was determined by the Lowry method calibrated with bovine serum albumin. All enzyme activities were determined at 30°C. Catalase and succinate dehydrogenase activities were determined as described previously (Nimmo & Tipton, 1979). Monoamine oxidase activity was assayed radiochemically (Otsuka & Kobayashi, 1964) by using [1-¹⁴C]tyramine as the substrate. Lactate dehydrogenase was assayed spectrophotometrically at 340 nm in a mixture containing 90 mM-glycine/NaOH buffer, pH 10.0, 0.34 mM-NAD⁺, 33 mM-sodium lactate and the sample. Free and occluded enzyme activities were determined as described by Marchbanks (1967). Glutamate dehydrogenase was determined spectrophotometrically at 340 nm in a mixture containing 50 mM-sodium phosphate buffer, pH 7.4, 0.16 mM-NADH, 5 mM-2-oxoglutarate, 50 mM-(NH₄)₂SO₄ and the sample. Sulphite-cytochrome *c* reductase was determined by following the increase in A₅₅₀ in a mixture containing, in a total volume of 2.7 ml, 74 mM-Tris/HCl buffer, pH 8.5, 185 mM-sucrose, 0.46 mM-KCN, 0.09 mM-EDTA, 0.37 mM-sodium sulphite, 0.56 mg of cytochrome *c* and the sample.

Aldehyde reductase activity was assayed at 340 nm in the presence of 100 mM-sodium phosphate buffer, pH 7.0, 0.67 mM-sodium azide, 2 mM-pyrazole, 10 μM-*p*-carboxybenzaldehyde and 0.1 mM-NADPH. The low-*K_m* enzyme activity was deter-

mined with 0.28 mM-pyridine-3-aldehyde and 0.133 mM-NADH in the same buffer/azide/pyrazole mixture. *p*-Nitrobenzaldehyde reducing activity was determined in a mixture containing 100 mM-sodium phosphate buffer, pH 6.5 or 7.0, 0.67 mM-sodium azide, 2 mM-pyrazole, 333 μ M-*p*-nitrobenzaldehyde, 133 μ M-NADH and the sample.

The distributions of enzymes and nucleic acids in subcellular and submitochondrial fractions are expressed as relative specific activities (de Duve *et al.*, 1955). Mixed substrate experiments were determined by comparing the activities of substrates assayed individually at their K_m concentrations and as a mixture of two, each at the same concentration (Dixon & Webb, 1979).

Results and discussion

The distribution of the reducing activities towards *p*-nitrobenzaldehyde in subcellular fractions is compared with that of a number of markers of known

subcellular localization in Fig. 1. Significant enrichment of activity occurred in the crude mitochondrial and cytoplasmic fractions when activity was determined at pH 7.0, but only in the cytoplasmic fraction when assayed at pH 6.5. This pH-dependence would be more consistent with the activity being due to the nitro reductase, which has an alkaline pH optimum (Köchli *et al.*, 1980) rather than to the low- K_m aldehyde reductase which has an acid pH optimum with this substrate (Turner & Tipton, 1972). When 0.1 mM-NADPH replaced NADH in the assay at pH 7.0 the initial rate in the mitochondrial fraction was decreased by approx. 52%. This would not be consistent with the low- K_m aldehyde reductase being involved, since NADPH would be expected to be a better substrate than NADH under these conditions (Rivett *et al.*, 1981).

The results of further subfractionation of the crude mitochondrial fraction, shown in Fig. 2, indicated that the activity towards *p*-nitrobenzaldehyde in the presence of NADH was mainly concentrated in the mitochondrial fraction.

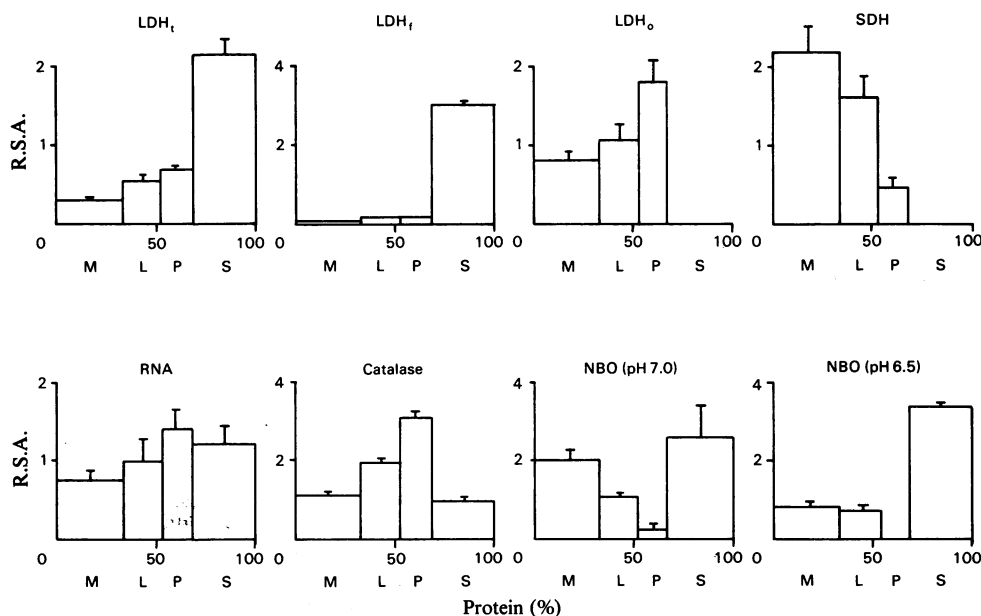


Fig. 1. Distributions of components in subcellular fractions derived from ox brain

The fractions 'crude mitochondria' (M), 'light mitochondria' (L), 'microsomes' (P) and 'cytoplasm' (S) were prepared and assayed as described in the text. It was not possible to obtain accurate estimates of enzyme activities in the 'nuclear' (N) fraction and relative specific activity (R.S.A.) values are expressed in terms of the activities determined in the combined supernatants from this fraction. R.S.A. is defined (de Duve *et al.*, 1955) as (percentage of component in a given fraction/percentage of protein in that fraction). The mean values from five separate determinations are shown in each case, with the error bars representing S.E.M., except for catalase activity, where two separate determinations were made, and the NADH oxidizing activity in the presence of *p*-nitrobenzaldehyde, where three determinations were made at pH 7.0 and two at pH 6.5. Over 90% of the DNA was recovered in the N fraction. The abbreviations used are: LDH_t, total lactate dehydrogenase; LDH_f, free lactate dehydrogenase; LDH_o, occluded lactate dehydrogenase; SDH, succinate dehydrogenase; NBO, NADH oxidizing activity in the presence of *p*-nitrobenzaldehyde.

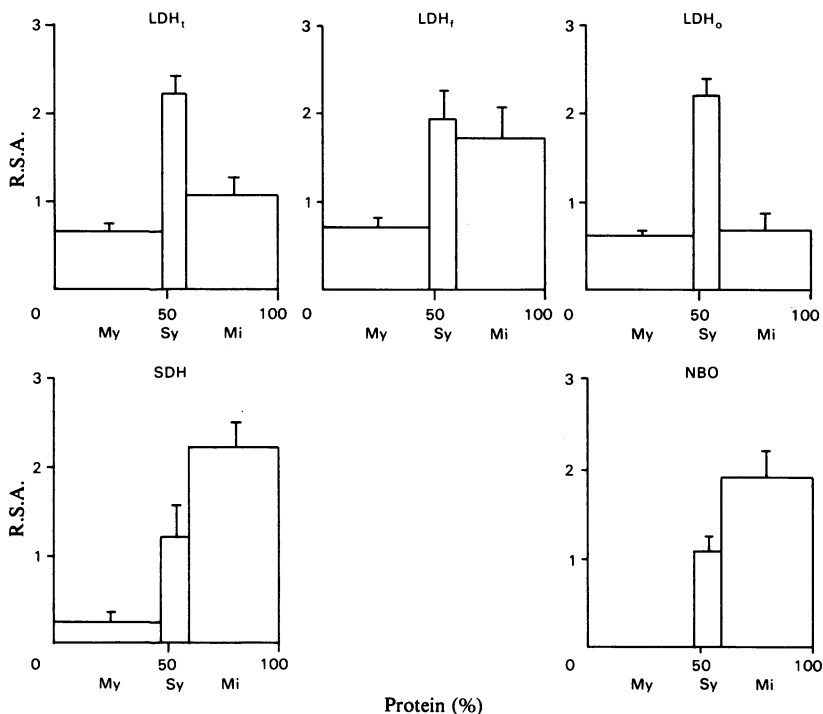


Fig. 2. Distributions of components in fractions derived from the crude mitochondrial fraction from ox brain. The method described in the text was used to fractionate the M fraction into myelin (My), synaptosomal (Sy) and mitochondrial (Mi) fraction. Relative specific activity (R.S.A.) values were calculated from the activity in the M fraction. Other details were as described in Fig. 1. Activity towards *p*-nitrobenzaldehyde (NBO) was assayed at pH7.0.

The purified mitochondrial fraction was used to determine the K_m values for *p*-nitrobenzaldehyde and 1,3-dinitrobenzene at pH7.0 and in the presence of $133\ \mu\text{M}$ -NADH. These were found from double-reciprocal plots to be $833\ \mu\text{M}$ and $2.2\ \text{mM}$ respectively.

The method of mixed substrates (Dixon & Webb, 1979) was used to assess whether the activity towards these two substrates was due to a single enzyme. The initial rate obtained when both substrates were present at their K_m concentrations was found to be $71.1 \pm 3.9\%$ (mean \pm range from two separate experiments) of the sum of the rates obtained when each substrate was assayed separately at the same concentration. A value of 67% would be expected if a single enzyme were responsible for both these activities (Dixon & Webb, 1979) and since 1,3-dinitrobenzene has been shown to be a good substrate for the nitro reductase activity in rat brain mitochondria (Köchli *et al.*, 1980) the present results would be consistent with this being responsible for the activity observed towards both these substrates.

Fractionation of brain mitochondria with digi-

tonin required higher detergent:protein ratios than those used in the fractionation of liver mitochondria (Greenawalt, 1974) and gave less clearcut results, but the results shown in Fig. 3 are consistent with the activity towards *p*-nitrobenzaldehyde having a similar location as succinate dehydrogenase within the mitochondrion. This was confirmed by the results of the submitochondrial fractionation experiments shown in Table 1, which indicated this activity to be predominantly confined to the inner membrane fraction. This localization is similar to that recently reported for the nitro reductase activity in rat brain (Köchli *et al.*, 1980).

The distribution of the total aldehyde reductase activity, assayed using *p*-carboxybenzaldehyde and NADPH as the substrates, is shown in Fig. 4. These results clearly show the enzyme activity to be cytoplasmic, in agreement with previous results obtained with the high- K_m enzyme (Anderson *et al.*, 1976; von Wartburg *et al.*, 1977; Tulsiani & Touster, 1977; Branlant & Biellmann, 1980). The distribution of the low- K_m activity, assayed with pyridine-3-aldehyde and NADH as the substrates, is shown in Fig. 4. No significant activity could be

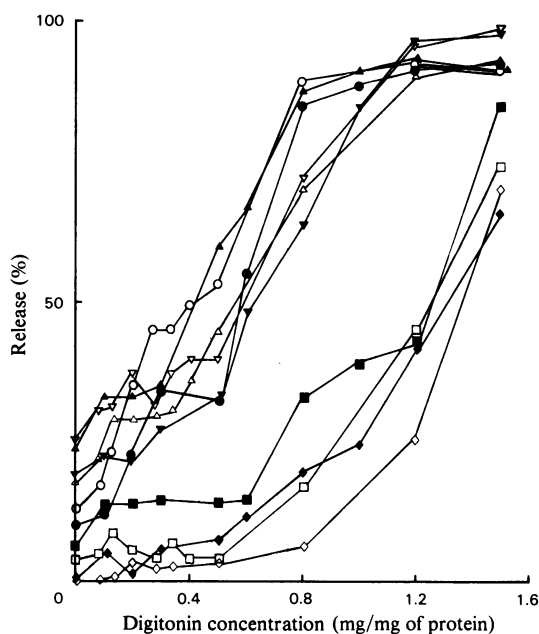


Fig. 3. Release of enzyme activities from the mitochondrial (Mi) fraction following treatment with digitonin

The Mi fraction was treated with digitonin and centrifuged before the supernatant was assayed as described in the text. The results of two separate experiments are given. In one experiment (open symbols) the protein concentration was 6.22 mg/ml, incubation was carried out in a volume of 2.5 ml and 1.0 ml of the mannitol/sucrose/Hepes medium (see the text) was added before centrifugation. In the other experiment (closed symbols) the protein concentration was 6.93 mg/ml, incubation was carried out in a volume of 3.0 ml and 0.5 ml of the medium was added before centrifugation. The results are expressed as the percentage of the total activity (pellet plus supernatant) released into each supernatant. The activities assayed were Δ , \blacktriangle sulphite-cytochrome *c* reductase; \circ , \bullet glutamate dehydrogenase; ∇ , \blacktriangledown monoamine oxidase; \diamond , \blacklozenge succinate dehydrogenase; \square , \blacksquare NADH oxidizing activity in the presence of *p*-nitrobenzaldehyde determined at pH 7.0. The experimental points have been connected by lines for clarity.

detected in the crude mitochondrial fraction and the greatest enrichment was found in the cytoplasmic fraction. This result contrasts with those of Anderson *et al.* (1976) and von Wartburg *et al.* (1977) who reported a predominantly mitochondrial localization for the low- K_m aldehyde reductase. As shown here these results may have been due, at least in part, to the use of *p*-nitrobenzaldehyde as a

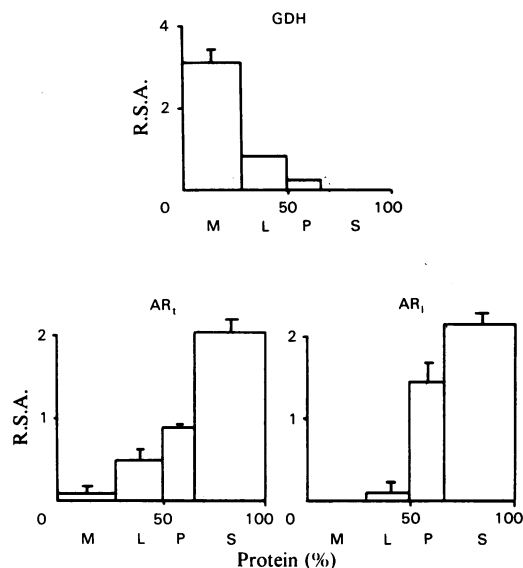


Fig. 4. Subcellular distribution of aldehyde reductase activities and glutamate dehydrogenase in ox brain. Details of the methods used were as described in the text and legend to Fig. 1. Relative specific activity (R.S.A.) values were calculated from the sum of the recovered activities in each case (de Duve *et al.* 1955). The values given are the means \pm s.e.m. from four experiments in which the activity of the low- K_m aldehyde reductase (AR_1) was determined. For glutamate dehydrogenase (GDH) and total aldehyde reductase activity assayed with *p*-carboxybenzaldehyde and NADPH (AR_2) two and three separate determinations were made respectively. The distributions of other components were found to be similar to those shown in Fig. 1.

Table 1. Distribution of components in submitochondrial fractions from ox brain

The mitochondrial (Mi) fraction was further fractionated as described in the text. Results are expressed as the percentage of total activity recovered in each fraction and as the means \pm s.e.m. from three separate experiments. NBO represents the NADH oxidizing activity in the presence of *p*-nitrobenzaldehyde at pH 7.0.

Enzyme	Recovery (%) in:			
	Outer membrane	Intermembrane matrix	Inner membrane	Inner matrix
Glutamate dehydrogenase	19 \pm 2.8	49.8 \pm 9.3	18.7 \pm 7.3	21.8 \pm 9
Succinate dehydrogenase	21.4 \pm 4	1 \pm 1	77 \pm 5.3	0
Monoamine oxidase	30.9 \pm 8.1	8.3 \pm 2.4	57.8 \pm 11	2.9 \pm 0.5
Sulphite-cytochrome <i>c</i> reductase	11.9 \pm 3.9	75.3 \pm 2.3	0	12.2 \pm 1.6
NBO	13.6 \pm 1.3	9 \pm 5.3	71.6 \pm 6.8	5.8 \pm 4.9

substrate in the earlier studies. The nitro reductase activity that is responsible for this confusion is, as yet, poorly characterized and further work is required to determine its properties and function.

Further fractionation of the crude mitochondrial fraction showed the aldehyde reductase activity assayed with *p*-carboxybenzaldehyde and NADPH to be enriched only in the synaptosomal fraction. The activity determined with pyridine-3-aldehyde and NADH was too low in the crude mitochondrial fraction to allow an accurate assessment of its possible synaptosomal localization.

The results in Fig. 4 indicate some enrichment of aldehyde reductase in the microsomal fraction. von Wartburg *et al.* (1977), who observed a similar phenomenon, have suggested that there may be a specific microsomal aldehyde reductase different from those previously characterized. More detailed studies on the properties of this microsomal activity will, however, be necessary before this can be concluded to be the case.

The subcellular locations of the enzymes involved in the metabolism of the catecholamines and 5-hydroxytryptamine are different. Monoamine oxidase has been shown to be present in the mitochondrial outer membrane (Schnaitman *et al.*, 1967; Tipton, 1967), the aldehyde dehydrogenase associated with the metabolism of these substrates is localized in the mitochondrial matrix (Tank & Weiner, 1977; Tipton *et al.*, 1981), catechol *O*-methyltransferase is, at least largely, cytoplasmic (see e.g. Guldberg & Marsden, 1975; Goldberg & Tipton, 1978) and the present work has shown both aldehyde reductase activities to be cytoplasmic. Further work will be necessary to assess whether these different enzyme locations affect the alternative pathways of the metabolism of these amines.

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