Absence of diamine oxidase activity from rabbit and rat lungs

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To study the presence of diamine oxidase (DAO) activity in any tissue with putrescine as the substrate, it is necessary to use inhibitors to block all pathways that could further metabolize γ -aminobutyraldehyde, which is the product of enzyme reaction. It is also necessary to inhibit any enzyme that may convert putrescine into higher polyamines. By this approach it was observed that lung tissue of both rat and rabbit exhibited no DAO activity. DAO activity was observed in the rat and rabbit intestine, the former showing 3 times as much activity as the latter. The other potential pathways of putrescine metabolism are of no consequence in the rat and rabbit intestine and lungs.

INTRODUCTION

As a precursor of the polyamines spermidine and spermine, putrescine plays an important role in cell physiology (Cohen, 1971; Tabor & Tabor, 1972; Bachrach, 1973). Its formation in mammalian tissues has been extensively studied, but its catabolism is less well known (Seiler & Al Therib, 1974; Perin *et al.*, 1983). Putrescine is a typical substrate of DAO (Kapeller-Adler, 1970), and its oxidative deamination by this enzyme was presumed to be the first step of putrescine catabolism (Tabor & Tabor, 1964). Seiler *et al.* (1985) suggested that polyamines and their natural derivatives were degraded in vertebrates by two types of enzymes, namely polyamine oxidase, involved in the interconversion of polyamines, and copper-containing amine oxidases that are capable of oxidatively deaminating diamines or polyamines and their natural monoacetyl derivatives.

DAO activity is generally determined with [14 C]putrescine as the substrate, by measuring the amount of radioactive Δ^1 -pyrroline and its polymers formed during the reaction (Okuyama & Kobayashi, 1961). This method for DAO assay has been used by many investigators (Kim *et al.*, 1969; Kusche *et al.*, 1978; Fogel *et al.*, 1981), and has been reported to be quite reliable, provided that inhibitors are used to block all other pathways (Perin *et al.*, 1983).

The action of DAO on putrescine results in the formation of γ -aminobutyraldehyde (Fogel *et al.*, 1978), which can be further metabolized as shown in Scheme 1. Putrescine can also be converted into spermidine and spermine (Scheme 1) (Pegg & McCann, 1982). To obtain the true value of DAO activity in tissues by the method of Okuyama & Kobayashi (1961), it is necessary to use inhibitors to block any further metabolism of γ -aminobutyraldehyde, as suggested by Fogel *et al.* (1978). There are no reports on the DAO activity in lung tissue by workers using this approach.

Pulmonary uptake of polyamines has been demonstrated in rat lung slices incubated *in vitro* (Smith, 1982). Perfusion experiments using isolated ventilated rabbit and rat lungs indicated significant pulmonary uptake of polyamines (S. B. Rao & H. M. Mehendale, unpublished

Scheme 1. Possible pathways of putrescine metabolism (Fogel *et al.*, 1978; Pegg & McCann, 1982)

Inhibitors of the respective enzymes, and the concentrations used in the assay system, are indicated. (1) DAO (diamine oxidase); aminoguanidine (1 mM). (2) ALDR (aldehyde reductase); phenobarbital (1 mM). (3) ADH (alcohol dehydrogenase); pyrazole (1 mM). (4) ALDH (aldehyde dehydrogenase); chloral hydrate (20 mM). (5) MAO (monoamine oxidase); pargyline (10 μ M). (6) Spermidine synthase; dicyclohexylammonium sulphate (1 mM).

work). This stimulated interest to investigate possible metabolism of polyamine by the lungs. Hence the present study was undertaken to investigate the DAO activity in rat and rabbit lungs, and intestinal enzyme preparations were used for comparison.

MATERIALS AND METHODS

Chemicals

Putrescine dihydrochloride, bovine albumin (Cohn fraction V) and the inhibitors dicyclohexylammonium

sulphate, pargyline, chloral hydrate, phenobarbital, aminoguanidine and pyrazole were all purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). [1,4-¹⁴C]Putrescine dihydrochloride (sp. radioactivity 104.6 Ci/mol) and Aquasol were from New England Nuclear (Boston, MA, U.S.A.). All other chemicals used were of analytical grade. Only double-glass-distilled water was used.

Animals

Male New Zealand White rabbits (2–3 kg) were purchased from a local supplier, and male Sprague-Dawley rats (200–300 g) were from Charles River Laboratories (Millis, MA, U.S.A.). All animals were maintained in our central animal facilities on commercial diet and water, on a 12 h-dark/12 h-light cycle, away from any known inducers, at 21 ± 1 °C and 50–80% relative humidity.

Tissue preparations

Animals were anaesthetized with pentobarbital sodium (50 mg/kg, intravenously), which was mixed with heparin (1000 units/kg). To remove the lungs, the trachea was exposed by a longitudinal ventral incision on the neck and cannulated with a suitable length of polyethylene tubing. The diaphragm and rib cage were incised to expose the heart, after which the lungs were carefully removed intact. The pulmonary artery was cannulated either after (rabbit) or before (rat) removal of the lungs. The heart was cut across the ventricles, and the lungs were perfused with warm 0.9% NaCl to remove the blood from the vascular system. The lungs were then cut from extra-pulmonary tissue, blotted and weighed. The intestinal tissues, 1–2 cm from the stomach to about 20–30 cm in length, were cut, and the luminal side was washed with 0.9% NaCl to clean off the food matter, and then blotted and weighed.

Enzyme preparation and assay

The weighed tissues were homogenized (1:3, w/v) in KH₂PO₄/Na₂HPO₄ buffer (pH 7.6, 0.2 M) in a Brinkmann tissue homogenizer and the homogenate was centrifuged for 30 min at 48000 *g* in a Sorvall RC-5B refrigerated centrifuge at 4 °C. The supernatants were used as the enzyme source. The method for the assay of DAO was similar to that described by Kusche *et al.* (1973). The required quantities of the inhibitors were dissolved in phosphate buffer (pH 7.6, 0.2 M) such that the addition of 0.5 ml of this solution gave the final concentration of the inhibitor required in the assay system. The enzyme reaction was terminated with 14% (v/v) HClO₄. Two different blanks were used for each assay, as described in the procedure by Kusche *et al.* (1973).

Recovery of [¹⁴C]putrescine and products

To account for recovery of the radioactivity added for DAO assays, the contents of the tube used for enzyme assay were centrifuged at 4000 *g* for 30 min, for the separation of proteins, and the water layer was decanted into another tube. The proteins were washed with 0.5 ml of phosphate buffer twice, and the centrifugation and decantation steps were repeated. The supernatant and all washings were collected, made alkaline and treated with toluene. The samples were continuously mixed by inversion for a fixed time of 4 min, and the toluene-extractable radioactivity was measured. After removal of

Table 1. Diamine oxidase activity of rat and rabbit lung and intestine, showing the recovery of ¹⁴C of substrate and products formed

The final composition of incubation mixture (37 °C) was 0.2 M-phosphate buffer (pH 7.6), 4–5 mg of protein, 1 mM-[¹⁴C]putrescine and unlabelled putrescine. The reaction was stopped after 60 min with 14% HClO₄ and the fractions were radioassayed in 5 ml of 0.35% 2,5-phenyloxazole in toluene. Proteins were solubilized in alkali before radioassay. Blank 1 = 14% HClO₄ + enzyme; Blank 2 = no enzyme. Results are means ± s.e.m. for four animals.

Animal species	Organ	Radioactivity in different fractions (c.p.m.)				Total radiolabel recovered (c.p.m.)	Recovery %
		Toluene	Proteins	Water			
Rat	Lung	Test	450 ± 116	4910 ± 1713	125966 ± 5315	131291 ± 3529	99.2 ± 0.2
		Blank 1	843 ± 45	5097 ± 1250	124526 ± 5213	130467 ± 3951	99.2 ± 0.9
		Blank 2	1042 ± 15	—	126349 ± 2183	127391 ± 2174	96.6 ± 1.3
	Intestine (control)	Test	23806 ± 1335	6445 ± 2198	95339 ± 8916	125589 ± 7361	95.1 ± 3.3
		Blank 1	824 ± 119	6501 ± 1298	125144 ± 4593	132468 ± 3900	100.2 ± 1.0
		Blank 2	879 ± 57	—	120836 ± 7249	121714 ± 7302	91.9 ± 3.5
Rabbit	Lung	Test	956 ± 57	5234 ± 1412	119777 ± 4574	126383 ± 2776	98.4 ± 1.5
		Blank 1	1084 ± 47	6200 ± 1466	119324 ± 3843	126607 ± 2352	98.8 ± 1.6
		Blank 2	1017 ± 14	—	118665 ± 5754	119683 ± 5764	93.3 ± 3.4
	Intestine (control)	Test	10018 ± 573	4646 ± 1635	108982 ± 3721	123646 ± 5167	96.0 ± 3.5
		Blank 1	870 ± 43	5115 ± 628	123335 ± 1877	129320 ± 1744	100.7 ± 1.8
		Blank 2	913 ± 40	—	119030 ± 5868	119943 ± 5830	93.4 ± 3.3

the toluene layer, 0.2 ml of the water layer was counted separately for radioactivity with Aquasol as the scintillation fluid. The proteins were dissolved by incubation at 37 °C for 2 h in 5 ml of 2% (w/v) Na₂CO₃ in 0.1 M-NaOH. A 0.2 ml portion of this solution was used for radioassay, with Aquasol as the scintillation fluid. The sum total of radioactivity in the toluene layer, water layer and proteins accounted for the total radioactivity recovered after the experiment. Protein in the enzyme preparation was measured by the method of Lowry *et al.* (1951), with bovine albumin (Cohn fraction V) as standard.

RESULTS

The net recovery of radioactivity from the different fractions of the assay system in all cases was nearly 100% (Table 1), indicating that all the radioactivity was accounted for and that there were no volatile products formed in the incubations. The DAO activity of rat and rabbit intestine was 4795 ± 201 and 1629 ± 88 c.p.m./h per mg of protein respectively. DAO activity was undetectable in the lungs of both rat and rabbit.

No significant effect of the inhibitors was evident on the DAO activity, except for aminoguanidine, which showed 100% inhibition of rat and rabbit intestinal DAO. The mixture of inhibitors which contained aminoguanidine also showed similar inhibition to that with aminoguanidine alone. The use of inhibitors confirms the absence of DAO activity from the rat and rabbit lungs.

Varying the concentration of enzyme gave a linear curve for products formed from rat and rabbit intestinal DAO, but there was no effect with lung DAO, confirming the absence of this activity from lungs of both species (results not shown).

DISCUSSION

DAO activity was undetectable in the lung tissue of both rat and rabbit. However, assay for DAO activity done with intestinal tissue as a positive control indicated significant DAO activity, with rat intestine showing 3 times as much activity as that of rabbit. These values are significantly higher than those reported by Kim *et al.* (1969) for rat and rabbit intestines. They analysed the intestine by broadly dividing it into three segments, proximal, middle and distal. Only a small portion of the proximal region was analysed in the present work. It has been reported that different areas of the intestine show variable DAO activity (Kusche *et al.*, 1978).

The recovery of total radioactivity in our experiments was nearly 100%, indicating that no radioactive volatile products were formed during the enzyme assay. Further, this also indicated that non-specific binding of the substrate to tissue proteins did not occur, permitting the substrate to be freely available for enzyme reaction. Formation of polyamine-peptide conjugates has been reported by Rennert *et al.* (1980).

Tsuji & Nakajima (1978), measuring γ -aminobutyric acid as the end product of the enzyme reaction in their studies *in vitro*, reported the presence of an enzyme in the lungs of rats which they suggested to be DAO. However, the enzyme activity was very low in the lungs as compared with the activity in small intestine. Further, these workers were not confident whether this could be DAO, since they indicated that histamine was a better substrate than

putrescine for the enzyme. In the present study, the method for DAO assay is applicable with only putrescine or cadaverine as substrate, and not histamine (Okuyama & Kobayashi, 1961), enabling us to determine the presence of any putrescine-specific enzyme. Since none was detected in the lung tissues by this assay, it is unlikely that the activity reported by Tsuji & Nakajima (1978) is of DAO.

The presence of γ -aminobutyrate in the lungs of rats (Tsuji & Nakajima, 1978) and mice (Fogel *et al.*, 1981) was reported, after these animals were injected with radioactive putrescine. However this cannot be taken as conclusive evidence for the formation of γ -aminobutyrate in this organ, as it could have been transported from other sites of synthesis. Moreover, our studies with inhibitors to block the metabolic pathway for the conversion of putrescine or γ -aminobutyraldehyde into γ -aminobutyrate did not change the value of DAO activity significantly in rat and rabbit intestine and lung. Thus our observations suggest the absence from lungs and intestine of the enzymes necessary to form γ -aminobutyrate. Since the use of inhibitors failed to increase Δ^1 -pyrroline, the product of DAO activity, the presence of enzymic reactions other than DAO (Scheme 1) seems to be of little significance in rat and rabbit intestine. When these inhibitors were used with lung homogenate, the results did not reveal any DAO activity, which further confirmed the absence of DAO from this tissue.

Interestingly, it has been reported by Baylin *et al.* (1975) that, in patients with small-cell carcinoma of the lung, high DAO and histaminase activities were found when these enzymes were assayed separately with putrescine and histamine as substrates respectively. The normal lungs from the same patients had no DAO or histaminase activity. Our results with normal rabbit and rat lungs clearly indicate the absence of DAO activity with putrescine as substrate. This raises the question of whether cancerous growth could cause an induction of the enzyme, and hence could it be a good marker for detecting lung cancer. Studies by Perin *et al.* (1983) were able to demonstrate the induction of DAO, with synthesis of new enzyme molecules. This was also shown in regenerating rat liver (Sessa *et al.*, 1982), and in rat kidney during compensatory hypertrophy (Desiderio *et al.*, 1982). Hence induction of DAO activity with certain types of tumours in the lungs is a distinct possibility, which has clinical implications.

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