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Oxidation of *N*-alkyl and *C*-alkylputrescines by diamine oxidases

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N-Methyl-, *N*-ethyl-, *N*-propyl- and *N*-butylputrescine were assayed as substrates of diamine oxidases from pea seedling and pig kidney. With the exception of *N*-methylputrescine they were found to be oxidized to the corresponding aminoaldehydes. 1-Methyl-, 2-methyl-, 1-ethyl- and 1-propylputrescine were oxidized by the oxidases at lower rates than the *N*-alkylderivatives. 1,3-Dimethylputrescine had negligible oxidation rates while 1,4-dimethylputrescine (2,5-diaminohexane) was not a substrate. The oxidation of putrescine by the kidney oxidase was inhibited by 1,4-dimethylputrescine, while the pea oxidase was strongly inhibited by the former as well as by 2-methylputrescine and 1,3-dimethylputrescine. Serum amine oxidase did not oxidize the substituted putrescines although several of the latter inhibited spermidine oxidation by this oxidase.

N-Alkylputrescine; *C*-Alkylputrescine; Polyamine; Diamine oxidase; Inhibitor

1. INTRODUCTION

The naturally occurring polyamines putrescine, spermidine and spermine play an important role in controlling growth and differentiation processes [1–5]. The first metabolite of the polyamine biosynthetic pathway in mammals is putrescine (1,4-diaminobutane) which is formed by the decarboxylation of ornithine in a reaction catalyzed by L-ornithine decarboxylase (ODC). The potential relevance of inhibiting ODC activity as a way of affecting cell replication was the aim of many research efforts [6,7]. A series of *N*-alkyl and *C*-alkyl substituted putrescines has recently been synthesized [8,9] and assayed as inhibitors of ODC from rat liver and *E. coli* [10], and as promotors of cell growth and protein synthesis of a polyamine deficient strains of *E. coli* [11]. Further studies on the inhibitory effect of the alkyl-putrescines on the ODC activity of a hepatoma cell line indicated that there are marked differences between the in vitro and in vivo inhibitory effects. Thus, while 2-methylputrescine (fig.1) was a better inhibitor in vitro than in vivo of ODC activity, 1-methylputrescine showed the opposite behaviour (Ruiz, O. et al., unpublished).

Since it is well established that diamine oxidases play an important role in regulating the cellular levels of the natural polyamines [12] it is conceivable that they also act on the synthetic alkylputrescines affecting their in vivo cellular content. The effect of the synthetic alkylputrescines on the enzymatic oxidation of polyamines will also affect the cellular levels of the latter and therefore their regulatory influence as modulators of both

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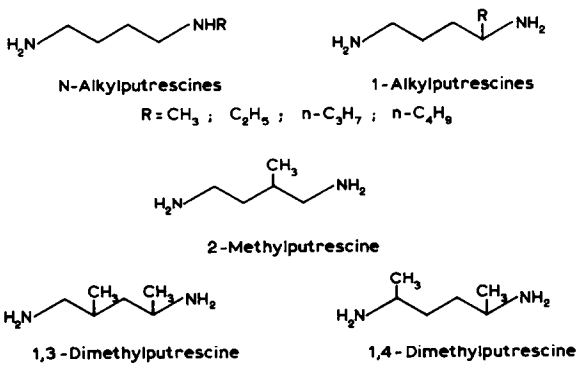

Fig.1. *N*-alkyl and *C*-alkylputrescines used in this study.

normal and pathological cell growth.

In this report we discuss the enzymatic oxidation of *N*-methyl-, *N*-ethyl-, *N*-propyl- and *N*-butylputrescine, as well as 1-methyl-, 1-ethyl-, 1-propyl-, 2-methyl-, 1,3-dimethyl- and 1,4-dimethylputrescine (fig.1) by diamine oxidase from pea seedlings and pig kidney, and the effect of these compounds on the activity of serum amine oxidase (SAO). The effects of the aforementioned diamines on the enzymatic oxidation of putrescine and spermidine by the oxidases was also examined.

2. MATERIALS AND METHODS

2.1. *Materials*

N-Alkyl- and 1-alkylputrescines were obtained by synthesis [8,9]. 2-Methylputrescine, 1,3-dimethyl- and 1,4-dimethylputrescine were obtained from the substituted pyrroles by ring opening with hydroxylamine and reduction of the dioximes following the described procedures [9]. All the diamines were obtained as their dihydrochlorides and their purity was checked by TLC and by derivatization to the corresponding bis-(benzyloxycarbonyl) derivatives. *o*-Aminobenzaldehyde, *N*-methyl-2-benzothiazolone hydrazone and 5-amino-2,3-dihydro-1,4-phthalazinedione (luminol) were from Sigma.

2.2. *Enzymes*

Pea seedling diamine oxidase was purified by the method described by Hill [13] (spec. act. 10 units/ml, 100 units/mg; 1 unit catalyzes the oxidation of 1 μmol of putrescine/h). Porcine diamine oxidase (0.1 unit/mg) was purchased from Sigma (St. Louis, MO). Bovine serum amine oxidase was purified as described [14] and its activity was assayed according to Tabor et al. [15], using benzylamine as substrate (110 units/ml, 50 units/mg).

2.3. *Assays of enzymatic activity*

The activity of the diamine oxidases was determined by two procedures. When the oxidation products were Δ'-pyrroline or its *C*-alkylated derivatives, the latter were trapped with *o*-aminobenzaldehyde and the resulting quinazolium salt was estimated [16]. Diamine, 20 μl (0.5 μmol), was added to 100 μl enzyme and 100 μl of 0.2 M phosphate buffer (pH 7.4). The reaction mixture was incubated at 37°C with 100 μl of 0.1% *o*-aminobenzaldehyde solution in a final volume of 350 μl. The absorbance was recorded in a Spectronic 1010 spectrophotometer at 435 nm after dilution of the incubation mixture to 1 ml with phosphate buffer. An $\epsilon_{435} = 1.85 \times 10^3\ l \cdot mol^{-1} \cdot cm^{-1}$ was used. When the inhibitory effects of the synthetic diamines were measured, the incubation mixture contained in a final volume of 350 μl: putrescine (0.5 μmol), inhibitor (0.5 or 1.0 μmol), enzyme, buffer and *o*-aminobenzaldehyde at the concentrations described above. The incubations were run at 37°C at the indicated times.

The activities of the diamine oxidases and of serum oxidase were also determined by trapping the oxidation products (aminoaldehydes) with *N*-methyl-2-benzothiazolone hydrazone and by measuring the absorbance of the formed bis-hydrazone at 660 nm ($\epsilon_{660} = 6.25 \times 10^3\ l \cdot mol^{-1} \cdot cm^{-1}$) [17]. The reaction mixture contained, in a final volume of 240 μl: 100 μl of 0.1 M Tris-HCl buffer (pH 7.2), 50 μl of the pea or 100 μl of the kidney enzyme, and 0.2 μmol substrate. Incubations were carried out at 37°C for 30 min unless otherwise stated. After this incubation, 0.5 ml of a 0.4% aqueous *N*-methyl-2-benzothiazolone hydrazone hydrochloride solution was added and the tubes were kept at 25°C for 30 min. Finally, 2.5 ml of a 0.2% ferric chloride solution was added to each tube and absorbances were recorded after another 25 min at 25°C.

The activity of serum amine oxidase was also determined by measuring the amount of hydrogen peroxide formed during the oxidation using a luminescence based method [18]. The diamine or

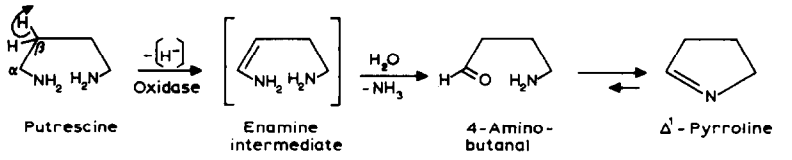

Fig.2. Hypothetical mechanism of putrescine oxidation.

spermidine, 100 μl (0.2–1.0 nmol dissolved in 0.2 M borate buffer, pH 8.6, containing 1 M NaCl), was added to 250 μl of 67 mM glycine buffer (pH 8.6) in a disposable 4 ml polystyrene test tube (Lumacuvette, Lumac BV, The Netherlands). To each tube 200 μl of the luminescence reagent (Luminol, 11.7 μg/ml) and peroxidase (30 μg/ml) in a 1:1 ratio were added. The tubes were kept in the dark for 15 min and the background was measured in a Lumac model 2010 biocounter. The activity of the oxidase was determined by injecting 100 μl of the former and by recording the luminescence for 3 min using a 60 s integrator.

2.4. *Protein determination*

Protein concentration was estimated by the procedure of Bradford [19] using bovine serum albumin as the standard.

3. RESULTS

3.1. *Oxidation of* N-*alkyl and* C-*alkylputrescines by diamine oxidases from pea seedlings and porcine kidney*

Enzymatic oxidation of putrescine produces 4-aminobutanal which is in equilibrium with the cyclic Δ′-pyrroline (fig.2) [12]. This enzymatic reaction can be measured by condensation of the latter with *o*-aminobenzaldehyde which results in the formation of a quinazolium salt [16], or by condensation of the aminoaldehyde with benzothiazolone hydrazone which gives a bishydrazone cation. If the *N*-alkylputrescines were oxidized by diamine oxidases the expected *N*-alkylaminobutanals will be in equilibrium with the cyclic *N*-alkyl-Δ′-pyrrolines. The latter are quaternary salts which will not cyclize with *o*-amino-

Table 1

Relative oxidation rates of *N*-alkyl and *C*-alkylputrescines by pea seedling and pig kidney diamino oxidases

Substrate	Pea seedling diamine oxidase		Pig kidney diamine oxidase	
	Activity (nmol/30 min)	Relative rate (%)	Activity (nmol/30 min)	Relative rate (%)
Putrescine	180	100.0	120	100.0
N-Methylputrescine	2	1.0	2	2.0
N-Ethylputrescine	125	69.0	58	48.0
N-Propylputrescine	105	59.0	30	25.0
N-Butylputrescine	137	76.0	50	42.0
1-Methylputrescine	32	18.0	7	6.0
1-Ethylputrescine	28	16.0	4	4.0
1-Propylputrescine	45	25.0	4	4.0
2-Methylputrescine	15	8.0	13	11.0
1,3-Dimethylputrescine	1	1.0	1	1.0
1,4-Dimethylputrescine	0	0	0	0

The incubation mixture and conditions were described in section 2. The activity was estimated by aldehyde formation using *N*-methyl-2-benzothiazolone hydrazone. The values are the mean of three determinations

benzaldehyde to give the quinazolium salt. Therefore the enzymatic oxidation of *N*-alkylputrescines was measured by reaction with the benzothiazolone hydrazone, a method which was also used to estimate the enzymatic oxidation of the *C*-alkylputrescines. Although the latter give Δ'-pyrrolines by oxidation, which condense with *o*-aminobenzaldehyde, the bishydrazone formation was used for both types of diamines for comparative purposes (table 1 and fig.3). *N*-Ethyl-, *N*-propyl- and *N*-butylputrescine were enzymatically oxidized at fair rates although smaller than that of putrescine, by both the plant and the mammalian diamine oxidases. The very low oxidation rate of *N*-methylputrescine was somewhat surprising since *N*-methylaminobutanal is a natural product in tobacco leaves formed by the enzymatic oxidation of *N*-methylputrescine in a reaction catalyzed by an oxidase [20].

The *C*-alkylputrescines were poorer substrates of the diamine oxidases than the *N*-alkyl derivatives (table 1 and fig.3). 1-Propylputrescine (1,4-diaminoheptane) was a good substrate of the pea oxidase while it was a very poor substrate of the kidney oxidase. While 1-methylputrescine (1,4-diaminepentane) was a better substrate for the pea oxidase, 2-methylputrescine was a better substrate of the kidney oxidase, and its oxidation rate was enhanced at higher substrate concentrations and longer incubation times (fig.3A,B). Similar results were obtained for both oxidases when the oxidation of the *C*-alkylputrescines were measured by estimating the formation of Δ'-pyrroline derivatives (not shown).

1,4-Dimethylputrescine (2,5-diaminehexane) was not oxidized by either oxidase. Therefore, it is very likely that secondary amino groups cannot be oxidized to keto groups by diamine oxidases.

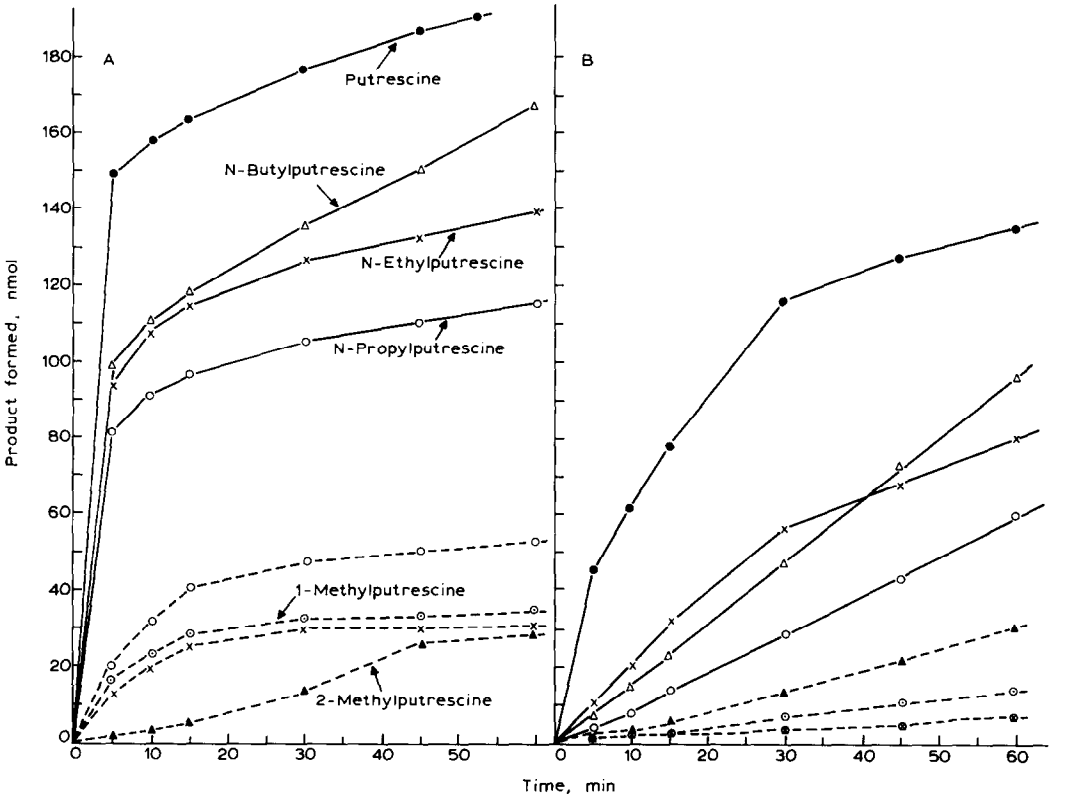

Fig.3. Oxidation rates of putrescine, *N*-alkyl-, and *C*-alkylputrescines by the (A) pea seedling diamine oxidase, and (B) pig kidney diamine oxidase. The oxidadtion products were estimated as their bishydrazone derivatives; (——) *N*-alkylputrescines; (– – –) *C*-alkylputrescines. (●) Putrescine; (×) *N*-ethyl and *C*-ethylputrescine; (○) *N*-propyl and *C*-propylputrescine; (△) *N*-butylputrescine; (▲) 2-methylputrescine; (⊙) 1-methylputrescine.

Table 2

Inhibitory effects of synthetic *N*- and *C*-alkylputrescines on the oxidation of putrescine by pea seedling and pig kidney diamine oxidases

Addition	Pea seedling diamine oxidase		Pig kidney diamine oxidase	
	Activity (nmol/30 min)	Inhibition (%)	Activity (nmol/30 min)	Inhibition (%)
–	407		167	
N-Methylputrescine	406		166	
N-Ethylputrescine	367	10 (10)	167	
N-Propylputrescine	360	12 (0)	167	
N-Butylputrescine	340	17 (12)	167	
1-Methylputrescine	259	36 (24)	157	6 (0)
1-Ethylputrescine	308	24 (7)	167	
1-Propylputrescine	340	17 (0)	167	
2-Methylputrescine	108	73 (77)	151	10 (0)
1,3-Dimethylputrescine	224	45 (30)	170	
1,4-Dimethylputrescine	183	55 (40)	61	64 (47)

The incubation mixture contained in a final volume of 350 μl, 0.5 μmol of putrescine and 1 μmol of the inhibitor. Oxidation of putrescine was determined by quinazolium cation formation. In parentheses are the inhibitions of putrescine oxidation after 90 min incubation. The values are the mean of three determinations

1,3-Dimethylputrescine (1,4-diamine-3-methylpentane) was a very poor substrate of the oxidases (table 1). This could suggest that the oxidation of putrescine to form aminobutanal proceeds through an enamine intermediate formed by subtraction of a hydride at C-2 (fig.2). The formation of such an intermediate in the case of 1,3-dimethylputrescine will be hindered by the presence of the bulky methyl group at C-3 (fig.1).

3.2. *Inhibition of the enzymatic oxidation of putrescine by* N- *and* C-*alkylputrescines*

Since putrescine is the metabolic precursor of spermidine and spermine, the depletion of cellular pools of putrescine by oxidation will strongly influence the polyamine content of the cells. Therefore the effect of the synthetic alkylputrescines on the oxidation of putrescine by diamine oxidases was examined (table 2 and fig.4). The oxidation of putrescine was measured by estimation of the quinazolium cation formed (see section 2) since: (i) the *N*-alkyl Δ'-pyrrolines do not condense with *o*-aminobenzaldehyde; (ii) although *C*-alkylputrescines form Δ'-pyrrolines, their oxidation rate is low enough to allow the

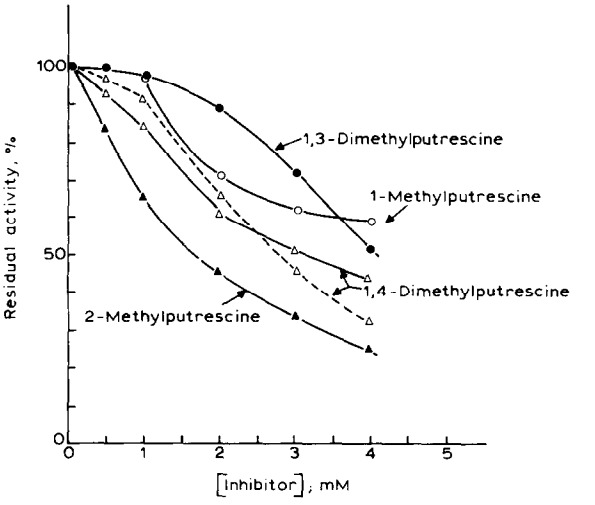

Fig.4. Inhibition of putrescine oxidation by increasing concentrations of *C*-alkylputrescines. (——) Pea seedling diamine oxidase and (– – –) pig kidney diamine oxidase. Putrescine oxidation was estimated using quinazolium cation formation.

estimation of putrescine oxidation by this method.

As shown in table 2, *N*-alkylputrescines inhibited very little putrescine oxidation by the plant enzyme and had no effect on the kidney enzyme. The latter was only inhibited by 1,4-dimethylputrescine, although this inhibition decreased with incubation time (table 2, parentheses). The pea seedling diamine oxidase was strongly inhibited by 2-methylputrescine and this inhibition did not decrease with longer incubation times. The inhibitory effects of the other *C*-methylputrescines decreased at longer incubation times (table 2).

When the inhibitory effect of the *C*-methylputrescines was measured as a function of their concentration (fig.4) it was found that 2-methylputrescine was the strongest inhibitor of the plant oxidase (50% inhibition when used at the same concentration of the substrate).

3.3. *Interaction of serum amine oxidase with* N- *and* C-*alkylputrescines*

Serum amine oxidase (SAO) from ruminants has a high activity toward spermine and spermidine and degrades them to aminoaldehydes [21]. The synthetic *N*- and *C*-alkylputrescines were not found to be oxidized by this oxidase when assayed either by the bishydrazone method or by the very sensitive luminometric method (where the oxidation of 50 pmol of spermidine could be detected). When the diamines were assayed as inhibitors of spermidine oxidation, *N*-butylputrescine and 1-propylputrescine were found to inhibit more than 50% the oxidation of spermidine (table 3).

Table 3

Inhibitory effects of *N*-alkyl and *C*-alkylputrescines on the enzymatic oxidation of spermidine by serum amine oxidase

Inhibitor	Concentration (mM)	Activity (nmol of spermidine oxidized/h)	Inhibition (%)
–		158	–
N-Methylputrescine	1.6	160	–
N-Ethylputrescine	1.6	136	14
N-Propylputrescine	0.8	114	28
	1.6	98	38
N-Butylputrescine	0.8	74	53
	1.6	51	68
1-Methylputrescine	0.8	138	13
	1.6	115	28
1-Ethylputrescine	0.8	112	29
	1.6	104	34
1-Propylputrescine	0.8	95	40
	1.6	66	58
2-Methylputrescine	1.6	130	18
1,3-Dimethylputrescine	0.8	115	28
	1.6	91	42
1,4-Dimethylputrescine	1.6	148	–

The incubation mixture and conditions were described in section 2. Spermidine was used at a concentration of 0.8 mM. Oxidation of spermidine was estimated using *N*-methyl-2-benzothiazolone hydrazone

Noteworthy is the total lack of inhibition of this oxidase by 1,4-dimethylputrescine and the low inhibitions exerted by 1-methyl- and 2-methylputrescines.

4. DISCUSSION

Different substrate specificities of diamine oxidases from pea seedling and porcine kidney were found when their activities were assayed with the *C*-alkylputrescines. 1-Propylputrescine and 2-methylputrescine were oxidized at very different rates by the plant and mammalian oxidases (fig.3A,B), indicating that although both enzymes have similar catalytic functions, they might differ in their molecular structures. The difference between both oxidases is also evident from the lack of inhibition of the kidney enzyme by almost all the synthetic diamines which nevertheless inhibit the plant enzyme (table 2).

2-Methylputrescine was found to be oxidized more efficiently than 1-methylputrescine by the kidney diamine oxidase (fig.3B), a fact which could explain its decreased in vivo inhibitory effect on ornithine decarboxylase activity as compared with that of 1-methylputrescine (see section 1). The inhibitory effect of 2-methylputrescine on the oxidation of putrescine by the plant enzyme (table 2) will contribute to maintain high cellular polyamine levels. Therefore, although 2-methylputrescine is an excellent in vitro inhibitor of ornithine decarboxylase its usefulness as an in vivo inhibitor will be limited by its behaviour toward the diamine oxidase system in different tissues.

It can be concluded that the search for inhibitors of ornithine decarboxylase must also take into account their interactions with the oxidase systems present in the cells.

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