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DISTRIBUTION AND PROPERTIES OF HUMAN INTESTINAL DIAMINE OXIDASE AND ITS RELEVANCE FOR THE HISTAMINE CATABOLISM

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High activities of diamine oxidase (EC 1.4.3.6) were measured in the intestinal tract of human subjects and of several mammalian species. The enzyme was localized in the mucosa and was distributed primarily in the cytoplasm; the only exception being the guinea-pig where it was located in the particulate fraction. Despite its instability the enzyme from human colonic mucosa was purified 80-fold. During the purification a soluble monoamine oxidase (EC 1.4.3.4) was separated from diamine oxidase. The pH optima of diamine oxidase for putrescine and histamine were 6.6–7.0 and 6.4–6.6, respectively. Short-chain aliphatic diamines were deaminated with the highest reaction velocity, but histamine and *N*⁷-methylhistamine were also excellent substrates. The K_m for putrescine was $8.3 \cdot 10^{-5}$ M, for histamine $1.9 \cdot 10^{-5}$ M and for *N*⁷-methylhistamine $9.7 \cdot 10^{-5}$ M. Typical substrates of monoamine oxidase were not deaminated by the enzyme. Aminoguanidine strongly inhibited human intestinal diamine oxidase ($IC_{50} = 1.1 \cdot 10^{-8}$ M). Because of its properties the intestinal diamine oxidase is considered to play a protective role against histamine in diseases such as ischaemic bowel syndrome, mesenteric infarction and ulcerative colitis.

Introduction

Previous attempts to demonstrate the role of histamine as a mediator in the pathophysiology of human disease have chiefly concentrated on protecting the organism from this substance. For example, histamine formation has been reduced by inhibiting histidine decarboxylase (EC 4.1.1.22) with glucocorticosteroids [1] and histamine receptors have been blocked by H_1 - and H_2 -receptor antagonists [2–4]. Enhancement of histamine catabolism, thus diminishing the effects of the amine, has been demonstrated for histamine methyltransferase (EC 2.1.1.8) in gastric secretion [5,6]

and for diamine oxidase (EC 1.4.3.6) in anaphylactic shock [7,8]. If, however, the inactivation of histamine by diamine oxidase was inhibited, following superior mesenteric artery occlusion a severe aggravation of shock was observed [9–12]. Hitherto these extensive studies in rabbits, dogs and pigs have provided the most convincing evidence for a physiological role of intestinal diamine oxidase as a protective enzyme.

In human subjects the protective effect of diamine oxidase may be required in diseases such as ischaemic bowel syndrome ('stress ulceration'), ulcerative colitis [13] and mesenteric infarction [2]. It is, however, unethical to design studies in man to establish this hypothesis.

To study this problem the content and activity of diamine oxidase in human intestinal mucosa

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was investigated. The properties of this enzyme were then compared with those from animal models.

Experimental Procedure

Materials

Preparation of the enzyme. Human intestinal tissues for analytical and preparative procedures were obtained from 18 patients with colorectal cancer (both sexes, age range 43–78 years) during abdominal operations. Anaesthesia included thiopentone, suxamethonium, alcuronium, ethrane and nitrous oxide. Immediately after resection parts of the colon and proximal sigma which were macroscopically not infiltrated and not required for histopathological examination were separated. The gut was rinsed with ice-cold saline, and the colonic mucosa was scraped off with a scalpel and then stored at -20°C .

Tissue samples from pigs and dogs were obtained from the terminal ileum. For rabbits and other rodents the distal half of the small intestine was used as the source of the enzyme. The porcine samples (five landrace pigs, female, approx. 150 kg, approx. 1 year old) were obtained from the slaughterhouse 10–20 min after death by exsanguination. Dogs (five mongrels, both sexes, 10–15 kg, approx. 1–7 years old) and rabbits (five German Giant animals marked with English dapples, male, 2.5–3.5 kg, 9 months old) were starved for 24 h with tap water ad libitum, then anaesthetized with pentobarbitone (15–30 mg/kg intravenously). The intestinal mucosa was thereafter processed in the same way as the human samples. Guinea-pigs (Pirbright, female, 600–700 g), rats (Sprague-Dawley Hannover, female, 200–250 g) and mice (NMRI Hannover, both sexes, 25–30 g) were anaesthetized with diethyl ether, after exsanguination the gut was removed immediately, rinsed with ice-cold saline and the whole wall of the ileum frozen and stored at -20°C .

Crude enzyme preparations for studying the distribution and stability of diamine oxidase were obtained by homogenizing 0.5 g frozen tissue with 24.5 ml of ice-cold 200 mM sodium potassium phosphate buffer (pH 7.6) using an Ultra-Turrax homogenizer (microshaft TP 10N). After centrifu-

gation in a Christ Minifuge (Heraeus, Osterode) ($0-4^{\circ}\text{C}$, $900 \times g$ for 10 min), in a Sorvall RC2B centrifuge ($0-4^{\circ}\text{C}$, $13\,000 \times g$ for 30 min) and in an ultracentrifuge (Beckman Spinco L2-75B, $0-4^{\circ}\text{C}$, $78\,000 \times g$ for 60 min) the supernatants were used as source of the enzyme.

For purification of human intestinal diamine oxidase the pooled mucosa samples were homogenized after thawing with 1 vol. of 10 mM Tris-HCl buffer (pH 7.6). During all further purification steps the temperature did not exceed 4°C . The homogenate was centrifuged for 30 min at $40\,000 \times g$ in the Sorvall centrifuge. The supernatants were collected and the pellets were rehomogenized with 1 vol. buffer and centrifuged as described for the first step. Thereafter both supernatants were combined and used as the starting material.

Ion-exchange chromatography using DEAE-cellulose formed the first purification step for the enzyme from human colonic mucosa. The supernatant was applied to four parallel running columns of DEAE-cellulose (2.5×55 cm, Serva) equilibrated with 10 mM Tris-HCl buffer (pH 7.6). After washing the columns, the enzyme was eluted by a discontinuous gradient with 0.4 M NaCl dissolved in Tris-HCl buffer as described above. Fractions of about 10 ml were collected and tested both for diamine oxidase and monoamine oxidase (EC 1.4.3.1) activity, since a soluble fraction of the latter enzyme was always found in preparations of intestinal mucosa. The fractions containing the higher diamine oxidase activity were pooled (105 ml) and used directly for affinity chromatography.

A Con-A Sepharose column (1.5×10 cm) was equilibrated with 10 mM Tris-HCl buffer (pH 7.6) containing 0.4 M NaCl. The enzyme preparation was applied to this column and washed. Thereafter proteins bound to Con-A Sepharose were eluted with 200 ml of a solution containing 1 M α -methyl-D-glucoside, 10 mM Tris-HCl buffer (pH 7.6) and 0.4 M NaCl. Again, the fractions with the highest diamine oxidase activity were pooled (72 ml) and used for further purification.

In many species molecular sieve chromatography has provided a complete separation of mono- and diamine oxidase activities from intestinal mucosal preparations [14–16]. However, for this procedure the volume of the eluate had to be rapidly reduced. Ultrafiltration was performed in

an Amicon chamber, model 202 equipped with a Diaflo PM 30 membrane for about 2 h at 4°C under a constant filtration pressure of 30 lb/inch² and magnetic stirring. A 4.8-fold concentration of the eluate was achieved in combination with some increase in purity, and a considerable elimination of monoamine oxidase activity (Table I). In previous studies the soluble monoamine oxidase of intestinal mucosa exhibited a higher molecular weight than the diamine oxidase. Thus, this elimination was explained by inactivation of the enzyme [15,16].

The last step of purification was the gel filtration on Sephacryl S-200 (Table I). Three 5-ml portions of the concentrated eluate were applied consecutively to a column (2.5 × 55 cm) equilibrated with 20 mM sodium/potassium phosphate buffer (pH 7.6). Those fractions with a high diamine oxidase activity were pooled and stored at 4°C for further experiments which had to be performed as quickly as possible.

Reagents. For the isotope and the coupled NADH test of diamine oxidase activity the same reagents were used as described by Kusche et al. [17], and for measuring monoamine oxidase activity the same reagents as described by Wurtman and Axelrod [18]. [*side chain*-2-¹⁴C]Tryptamine, specific radioactivity 1.84 GBq/mmol, was purchased from New England Nuclear; 5.92 kBq were added to each incubation sample.

Substrates: 1,2-diaminoethane (pro. synth., Riedel de Haen); 1,3-diaminopropane (puriss); 1,4-diaminobutane dihydrochloride (putrescine)

(purum); 1,5-diaminopentane dihydrochloride (cadaverine) (puriss); 1,6-diaminohexane (purum); 1,7-diaminoheptane (purum); 1,8-diaminooctane (purum); 1,9-diaminononane (purum); (all obtained from Fluka).

Histamine and its derivatives: histamine dihydrochloride (puriss, Fluka); *N*⁷-methylhistamine and *N*^π-methylhistamine (GR, Calbiochem); 5-methylhistamine, 2-methylhistamine, *N*^α-methylhistamine and *N*^α,*N*^α-dimethylhistamine (gift from Smith Kline and French Laboratories Ltd., Welwyn Garden City); *N*^α-5-dimethylhistamine and 5-ethylhistamine (gift from Professor W. Schunack, Department of Pharmacy, University of Mainz). 5-Hydroxytryptamine hydrogen oxalate (serotonin) (puriss); spermidine trihydrochloride (puriss); spermine tetrahydrochloride (puriss); tryptamine hydrochloride (purum); benzylamine (puriss); (all from Fluka).

Other reagents: aminoguanidine sulphate (monohydrate) (puriss, Schuchardt, München); potassium hydrogen phosphate (GR); disodium hydrogen phosphate dihydrate (GR) and Tris (GR); (all from Merck); DEAE-cellulose (GR) (capacity 0.7 mequiv./g, mesh 50–200, Serva); Sephacryl S-200 superfine and concanavalin A Sepharose (Pharmacia); α-methyl-D-glucoside (Sigma), pentobarbitone (Abbott), diethyl ether (Merck).

Methods

Determination of diamine oxidase activity. In this study the enzymic activity was measured by

TABLE I
PURIFICATION OF HUMAN INTESTINAL DIAMINE OXIDASE

Purification step	Total volume (ml)	Total protein (mg)	Total activity (nmol/min)	Diamine oxidase			Monoamine oxidase total activity (cpm/min)
				Yield (%)	Specific activity (nmol/min per mg protein)	Purification (-fold)	
1. Supernatant	690	19606	3055	100	0.15	1	2592000
2. DEAE-cellulose	105	2659	1416	46	0.53	3	65300
3. Con A-Sepharose	72	210	1298	42	6.17	40	7300
4. Ultrafiltration (Amicon PM-30)	15	135	1264	41	9.36	60	630
5. Sephacryl S-200	76	48	590	19	12.29	80	0

two different methods. In experiments on distribution and stability of the enzyme the modified isotope assay [17] of Okuyama and Kobayashi [19,20] was chosen with [1,4-¹⁴C]putrescine and unlabelled putrescine as substrates. For experiments on the subcellular distribution of the enzyme, the final substrate concentration was reduced from 1 to 0.3 mM; this produced a higher specific radioactivity as had been demonstrated with diluted enzyme solutions [13]. After three periods of incubation (20, 40, 60 min) Δ_1 -pyrroline in combination with its spontaneously formed polymers was extracted into toluene and was measured by liquid scintillation counting.

Chloral hydrate ($2 \cdot 10^{-2}$ M) was added to a second series of incubation mixtures, in order to investigate a possible interference in the assay that could occur by the oxidization of γ -aminobutyraldehyde prior to its cyclization to Δ_1 -pyrroline using endogenous aldehyde dehydrogenase (EC 1.2.1.3). However, no significant increase in radioactivity was observed in samples with addition of the inhibitor. The enzymic activity is expressed in nmol/(min per g fresh wt. or mg protein).

The coupled NADH test [22] with ammonia as a second product measured was used for assessing substrate specificity of diamine oxidase activity. None of the amines tested interfered with the glutamate dehydrogenase (EC 1.4.1.3) indicator system. The reaction velocity was measured at 366 nm in an Eppendorf spectrum-line photometer with an automatic change of cuvettes.

Determination of monoamine oxidase activity. This was determined by the method of Wurtman and Axelrod [18]. The products of [*side chain*-2-¹⁴C]tryptamine were extracted into toluene and measured by liquid scintillation counting.

Protein determination. During the purification procedure the method of Lowry et al. [23] was used.

Results

Distribution of human diamine oxidase compared to that in other mammals

High activities of diamine oxidase were found in the intestines of human subjects and of all other species investigated (Table II). These high activi-

TABLE II

DIAMINE OXIDASE ACTIVITY IN THE INTESTINE OF MAN, DOG, PIG, RABBIT, GUINEA-PIG, RAT AND MOUSE FOLLOWING THREE STEPS OF CENTRIFUGATION

Mean \pm S.D., $n = 5$ individuals per species. Centrifugation steps were not performed successively, but with three aliquots of the same homogenate at the same time (variable instability of the enzyme in different species). For further conditions see Materials and Methods.

Species	Diamine oxidase activity (nmol/min per g) in the supernatants following centrifugation at		
	900 \times g	13000 \times g	78000 \times g
Man	27 \pm 14	29 \pm 15	20 \pm 9
Dog	26 \pm 16	27 \pm 15	22 \pm 13
Pig	113 \pm 36	114 \pm 26	101 \pm 33
Rabbit	27 \pm 11	25 \pm 10	25 \pm 12
Guinea-pig	12 \pm 9	2 \pm 1	1 \pm 1
Rat	69 \pm 39	64 \pm 27	50 \pm 28
Mouse	66 \pm 23	44 \pm 18	47 \pm 20

ties had not been found in other tissues, e.g., kidney, liver and lung and so are consistent with the hypothesis that the enzyme has a special physiological function in the intestine.

When human colonic mucosa and muscular layer were prepared separately and used for enzyme determinations, over 95% of the total diamine oxidase activity was found in the mucosa.

With regard to species specificity the subcellular distribution of diamine oxidase activity was not uniform in mucosal cells. In pigs, rabbits and dogs the enzyme was chiefly located in the cytoplasm, in human subjects, rats and mice mainly in the cytoplasm, but to a considerable extent also in the mitochondrial fraction whereas in guinea-pigs virtually all enzymic activity was found in the particulate fraction (Table II).

Properties of human intestinal diamine oxidase

Stability of human intestinal diamine oxidase. In rabbits [14,15], dogs [14,16] and pigs [14] the intestinal diamine oxidase was found to be stable for several weeks when stored at -20°C . In the human intestinal mucosa less than 10% of the enzymic activity was lost per week when the tissue

samples were stored at -20°C . However, in contrast to the three species mentioned above, the purified human enzyme preparation lost more than 90% of its activity within 24 h when stored under the same circumstances.

pH and ionic strength optima. Human intestinal diamine oxidase both in crude enzyme extracts and in most purified preparations showed a pH optimum for putrescine between 6.6 and 7.0 (Fig. 1) and for histamine a pH optimum between 6.4 and 6.6 (Fig. 1). In addition, the enzymic activity depended on the ionic strength of the phosphate buffer. The optimum molarity of the sodium/potassium phosphate buffer at the optimum pH of 7.0 was 100 mM with 1 mM putrescine as substrate. At lower and higher buffer concentrations the reaction velocity of the enzyme was reduced.

Substrate specificity and Michaelis constants. According to the criteria of Zeller [24], a typical diamine oxidase prefers short-chain aliphatic diamines as substrates. The enzyme from human

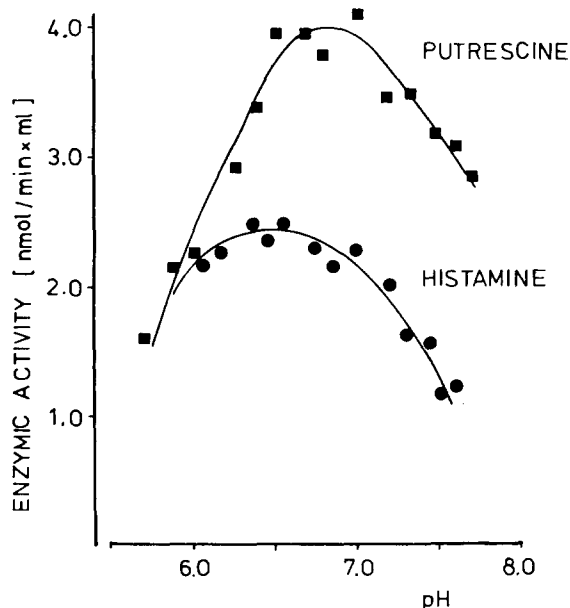


Fig. 1. pH optimum of purified human intestinal diamine oxidase. The enzymic activity was measured with histamine (0.1 mM) or putrescine (1 mM) as substrates. With histamine the coupled NADH test (mean from two to three experiments), with putrescine the coupled NADH test and the $[^{14}\text{C}]$ putrescine test (Mean from six experiments) were used.

intestinal mucosa deaminated these compounds with the highest reaction velocity (Table III). However, it proved important to study several substrate concentrations in comparing the relative reaction velocities, since substrate inhibition of the intestinal diamine oxidases from rabbits and dogs was observed at supra-optimum concentrations [14,15].

The highest reaction velocity was observed with 1,3-diaminopropane, as had been previously noted with porcine intestinal diamine oxidase [14].

Histamine was at best deaminated with a velocity of 32% of that with putrescine as substrate. However, in comparison with other diamine oxidases isolated from pig kidney [25] or pea seedlings [22], this relative activity against histamine was rather high.

In addition, the human enzyme also oxidized metabolites of histamine and several histamine

TABLE III

DEAMINATION OF ALIPHATIC DIAMINES, HISTAMINE AND HISTAMINE DERIVATES BY THE HUMAN INTESTINAL OXIDASE

The reaction velocities were compared to that with 1 mM putrescine (diaminobutane) = 100%. This value corresponded to 3.8 nmol oxidized putrescine/min per ml incubation mixture. Each value was the mean of two determinations with the coupled NADH test.

Amine	Final concentration		
	5 mM	1 mM	0.1 mM
1,2-Diaminoethane	3	0	0
1,3-Diaminopropane	148	87	27
1,4-Diaminobutane	73	100	89
1,5-Diaminopentane	84	119	96
1,6-Diaminohexane	41	57	13
1,7-Diaminoheptane	63	41	9
1,8-Diaminooctane	36	15	0
1,9-Diaminononane	35	26	2
Histamine	30	38	53
<i>N</i> ⁷ -Methylhistamine	85	101	93
2-Methylhistamine	57	74	108
<i>N</i> ^α -Methylhistamine	16	18	20
5-Methylhistamine	13	20	28
5-Ethylhistamine	0	42	30
<i>N</i> ^α -Methylhistamine	0	0	0
<i>N</i> ^α -5-Dimethylhistamine	0	0	0
<i>N</i> ^α , <i>N</i> ^α -Dimethylhistamine	0	0	0

analogues. *N*⁷-Methylhistamine, the most important product of histamine methylation by histamine methyltransferase (EC 2.1.1.8), was more rapidly deaminated than histamine itself. Side-chain methylated histamines, however, were not substrates for human intestinal diamine oxidase.

Typical substrates of monoamine oxidase (tyramine, serotonin, benzylamine) were not deaminated by diamine oxidase nor was spermine or spermidine degraded by this enzyme.

For three substrates, putrescine, histamine and *N*⁷-methylhistamine, the Michaelis constants were determined. The corresponding values were $8.3 \cdot 10^{-5}$, $1.9 \cdot 10^{-5}$ and $9.7 \cdot 10^{-5}$ M. In all cases a strong substrate inhibition at supra-optimum concentrations was observed.

Inhibition by aminoguanidine. Aminoguanidine is known to be both a specific and potent inhibitor of mammalian diamine oxidases *in vitro* and *in vivo*. With putrescine (1 mM) and histamine (0.1 mM) as substrates a concentration of $1.1 \cdot 10^{-8}$ M inhibited the enzyme activity by 50%

Discussion

For diamine oxidase to exhibit a general physiological function in the intestinal tract by protecting the organism from histamine, a number of conditions must be fulfilled [2]. High activities of the enzyme should be rather specifically localized in the intestinal mucosa. The properties of the enzyme should enable it to be effective under physiological and pathological conditions. Activation, release or inhibition of the enzyme *in vivo* should alter the effects of histamine, and histamine H₁- and H₂-receptor antagonists should reverse the effects of an inhibition of diamine oxidase as has already been demonstrated in animal experiments [2,9,11].

The first claim could be fulfilled in many aspects. In human intestinal mucosa high activities of the enzyme were demonstrated first by Werle [26] and thereafter by many other authors [13,17,21,27–31]. The absolute values obtained by Baylin et al. [27] appear to be rather low, but in the assay with β -[³H]histamine the substrate concentration was only $7 \cdot 10^{-8}$ M which was far below the substrate optimum of about 10^{-4} M. Low absolute activities corresponded well to high val-

ues when compared directly with the [¹⁴C]putrescine assay [32]. High activities of diamine oxidase were also discovered in the intestinal tract of other mammals such as Rhesus monkeys [33], landrace and miniature pigs [10,34,35], cattle, horses and sheep [35] and dogs [36–41], cats [36–38,42], rabbits [15,34–37,43,44], rats [12,32–34,36–38,43–49] and mice [36,37,43].

Only in guinea-pigs have variable activities of diamine oxidase been described. Relatively high activities were found by Cotzias and Cole [43] and Zeller [45], very low activities by Waton [36], Kim et al. [37] and Backus et al. [38] and no activity at all by Taylor and Lieber [33] and Valette et al. [34]. In our study, after centrifugation of the homogenate, high activities of diamine oxidase were only found in the $900 \times g$ supernatant.

Another point in favour of a specific role for diamine oxidase was its distribution in the different layers of the intestinal wall; not only in man but also in rats [50] the enzyme was found predominantly in the mucosa.

Finally, the subcellular distribution of the intestinal diamine oxidase was consistent with its proposed function of histamine degradation. The enzyme was predominantly a soluble cytoplasmic protein [38] as had also been found in many other tissues [51]. A small proportion of diamine oxidase activity was found bound to mitochondrial or microsomal membranes, but at present the possibility cannot be excluded that some of this binding occurred as an artefact after homogenization. Only one exception occurred: in guinea-pigs the enzyme seemed to be insoluble and chiefly bound to cellular structures. Further work is required to examine the properties of the guinea-pig enzyme.

The second condition, that the properties of the diamine oxidase activity should be enable it to be effective under physiological and pathological conditions, was also fulfilled. The substrate specificity of the human enzyme corresponded to that of intestinal diamine oxidases from other mammals [14,16]. With respect to the reaction velocity, the aliphatic diamines containing three to six methylene groups were the best substrates for the human intestinal enzyme, similar to results obtained with the enzyme from pig kidney [52–54], human placenta [55], and dog and pig intestine [14,16]. Histamine was catabolized by the human intesti-

nal enzyme with 32% of the reaction velocity of putrescine which was in the range of 28–85% observed with other mammalian diamine oxidases from various tissues [14,16,52,54–58]. This percentage was significantly higher than that for diamine oxidase from pea seedlings [22] (only 5%!). In addition, the K_m of human intestinal diamine oxidase for histamine was within the range of $6 \cdot 10^{-6}$ – $7 \cdot 10^{-4}$ M which has been determined in various tissues and species [14–16,33,52,54–58].

A physiological role for human intestinal diamine oxidase in histamine catabolism was further suggested by its catalytic activity against N^7 -methylhistamine which is the product of the most important pathway of histamine inactivation in man. The human enzyme greatly exceeded the relative reaction velocity observed with diamine oxidases from other sources [54,59–61].

Since N^7 -methylhistamine caused a strong product inhibition of histamine methyltransferase [62], the human intestinal diamine oxidase has probably two roles in the histamine catabolism: (1) direct inactivation of the substrate and (2) elimination of the strongest inhibitor of the second main enzyme in histamine inactivation.

The transfer of findings in animal experiments to human physiology and pathology can be established only with caution. However, the uniformity of the severe cardiovascular reactions to diamine oxidase inhibition in mesenteric artery occlusion in three mammalian species [11] and the similarity in distribution and properties of intestinal diamine oxidase in human subjects and these species support the hypothesis that intestinal diamine oxidase is a protective enzyme in the human intestinal tract.

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