SELECTIVE AND REVERSIBLE INHIBITION OF DIAMINE OXIDASE BY 1,5-DIAMINO-3-PENTANONE*

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The synthesis of 1,5-diamino-3-pentanone, an effective inhibitor of diamine oxidase from pea epicotyl and from pig kidney is described. The type of inhibition is substrate-competitive. The new compound is 14 times more effective than 1,4-diamino-2-butanone toward the plant enzyme but 18 less effective toward the animal enzyme. After prolonged incubation of the two amino ketones with diamine oxidase, the enzyme activity is fully restored. The duration of inhibition depends on the amount of enzyme which suggests an enzymic degradation of the inhibitor. Reactivation was also achieved by the method of gel filtration. None of the inhibitors inhibited purified mitochondrial monoamine oxidase from pig kidney.

Some compounds of the series of aliphatic amino ketones exhibit inhibitory effects toward plant and animal diamine oxidase (diamine: O_2 – oxidoreductase, deaminating). The most potent of these had been the keto derivative of putrescine, 1,4-diamino-2-butanone[1–3]. Comparison with the effect of structurally related compounds led to the conclusion[3] that a greater inhibitory role is played by the β-aminoketo grouping in the diamino ketone molecule than by the α-amino-keto grouping. Direct proof was lacking since no suitable compound was available. The newly prepared 1,5-diamino-3-pentanone (3-keto-cadaverine) is even more potent than 1,4-diamino-2-butanone toward the diamine oxidase from pea epicotyl but not toward the homologous enzyme from pig kidneys. The preparation and inhibitory properties of the compound are described here.

EXPERIMENTAL

Material. 1,4-Diamino-2-butanone dihydrochloride was synthesized according to Michalský and coworkers[4]. The dihydrochlorides of 1,4-diaminobutane and 1,5-diaminopentane and hydrochlorides of isoamylamine and benzylamine were prepared from commercial bases (Koch-Light) and recrystallized from aqueous ethanol by adding ether. Tryptamine hydrochloride was from Loba-Chemie, Vienna. Pyridoxal 5-phosphate monohydrate was a preparation of Fluka.

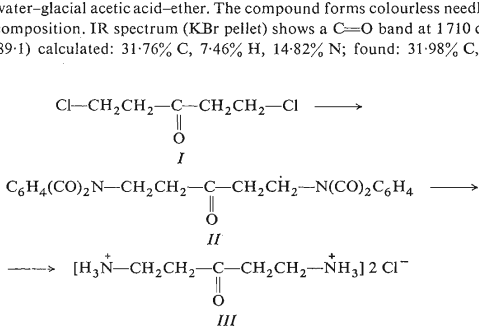
Pea diamine oxidase was isolated from the epicotyls of pea plants cultivated for 10 days in the

* The paper is dedicated to my teacher, Prof. A. Okáč, on the occasion of his 70th birthday.

dark. A modified procedure described previously was used for the isolation[3]. The specific activity of the enzyme was 18 units/mg protein. Diamine oxidase from pig kidney of a specific activity of 46 milliunits/mg protein was prepared as described before[3]. Solubilized monoamine oxidase was obtained from pig-kidney mitochondria by the digitonin procedure according to Erwin and Hellerman[5]. All the amine oxidases were kept at −60°C. Crystalline catalase from beef liver was obtained from Reanal, Budapest, and had a specific activity of 3000 Bergmeyer units/mg.

Synthesis of 1,5-*diphthalimido*-3-*pentanone* (II). To a solution of 50 mmol (7·75 g) 1,5-dichloro--3-pentanone[6] (*I*) in 100 ml dimethylformamide 0·1 mol (18·6 g) finely powdered potassium phthalimide was stepwise added under intense stirring, over a period of 20 min, the temperature of the suspension being kept at 30°C by external cooling. The reaction was terminated by 2-h heating to 100°C. After cooling, the reaction mixture was poured into 4 volumes of cold water and the precipitate was collected by filtration. The yield was 18·2 g (96·7%). Two-fold recrystallization from boiling glacial acetic acid yielded colourless needles, melting at 231—231·5°C. For $C_{21}H_{16}N_2O_5$ (376·4) calculated: 67·01% C, 4·28% H, 7·44% N; found: 67·21% C, 4·16% H, 7·54% N.

Synthesis of 1,5-*diamino*-3-*pentanone dihydrochloride* (III). 1,5-Diphthalimido-3-pentanone (*II*, 20 g) was refluxed for 30 h in a mixture of 100 ml 37% HCl and 100 ml glacial acetic acid until all the compound dissolved. After cooling, the main fraction of the precipitated phthalic acid was removed and the filtrate was concentrated *in vacuo* to 10 ml. The residue of phthalic acid was filtered again, the filtrate was treated with charcoal and diluted with 5 volumes of glacial acetic acid. Addition of ether led to 8·9 g (88·6%) dihydrochloride *III* which was recrystallized from a mixture of water–glacial acetic acid–ether. The compound forms colourless needles melting at 198°C under decomposition. IR spectrum (KBr pellet) shows a C=O band at 1710 cm^{-1}. For $C_5H_{14}Cl_2N_2O$ (189·1) calculated: 31·76% C, 7·46% H, 14·82% N; found: 31·98% C, 7·26% H, 14·74% N.



Diamine oxidase activity was determined spectrophotometrically by the *o*-aminobenzaldehyde method[7,8], using 1,4-diaminobutane or 1,5-diaminopentane as substrate in the presence of 20 μg catalase. Activity of the pea enzyme was assayed at 25°C in 70 mM Sørensen phosphate buffer at pH 7·0 with 10 mM substrate; the pig kidney enzyme activity was assayed at 38°C in a 70 mM phosphate buffer of pH 7·4 with 1 mM substrate. Incubation time was 10 min (up to 10 min the change of absorbance is practically linear[2]). In inhibition experiments, the inhibitor was present in the reaction mixture in a final volume of 5 ml together with the substrate. The reaction was started by adding diamine oxidase and terminated by 1 ml of 15% trichloroacetic acid. The amount of enzyme for each experiment is given in international units, one unit (U) catalyzing under the above conditions the formation of 1 μmol Δ^1-pyrroline/min.

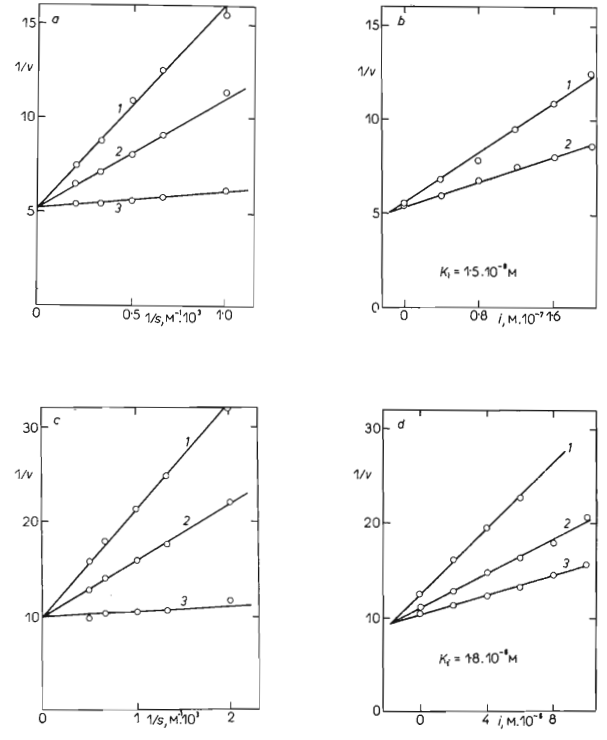


FIG. 1

Kinetics of Inhibition of Diamine Oxidase by 1,5-Diamino-3-pentanone

Initial rate v is expressed in μmol Δ^1-pyrroline per min, substrate s was 1,4-diaminobutane. *a* Lineweaver-Burk plot for the pea enzyme; 0·19 U, pH 7·1, 25°C, 0·16 μM inhibitor *1*, 0·08 μM inhibitor *2*, no inhibitor *3*. *b* Dixon plot for the pea enzyme; 0·19 U, pH 7·1, 25°C, substrate concentration 2 mM *1* and 4 mM *2*. *c* Lineweaver-Burk plot for the pig kidney enzyme; 0·10 U, pH 7·4, 38°C, 12 μM inhibitor *1*, 6 μM inhibitor *2* and no inhibitor *3*. *d* Dixon plot for the kidney enzyme; 0·10 U, pH 7·4, 38°C, substrate concentration 0·5 mM *1*, 1 mM *2* and 2 mM *3*.

Gel filtration of inhibited diamine oxidase was done on 1·1 cm . 32 cm columns of Sephadex G-25 equilibrated with 0·1M phosphate buffer of pH 7·0. An 0·5 ml volume of the reaction mixture in the same buffer containing 2·3 units of pea diamine oxidase and 4 mM inhibitor together with a drop of 5% potassium chromate and a drop of 2% blue dextran was placed on the first column. Elution was done with the equilibration buffer at a rate of 0·5 ml/min. As soon as the zone of blue dextran reached the column bottom 2-ml fractions began to be collected. The control reaction mixture without inhibitor was processed in the same manner on another column of the same size. Fractions were analyzed for diamine oxidase and the inhibitor zone was localized by a simple spot test (prevention of orange colour after adding a drop of the sample to a mixture of diamine oxidase, 5 mM *o*-aminobenzaldehyde and 10 mM 1,5-diaminopentane of pH 7·0). It was shown by preliminary experiments that the two indicators, chromate and blue dextran, do not affect diamine oxidase activity or 1,5-diamino-3-pentanone stability. In the experiment with 1,4-diamino-2-butanone, chromate had to be omitted. Diamine oxidase was found to be eluted together with blue dextran (fractions 1, 2), diaminoketones with fractions 6—8, and chromate, which indicated complete elution of the column, with fractions 9—11.

Oxygen consumption was measured polarographically in 3 ml of the reaction mixture with the aid of an oxygen electrode (electrode system Au—Ag/AgCl, polyethylene membrane), attached to a recording analyzer of dissolved oxygen[9]. The absorption spectra were recorded in the Optica Milano CF 4 spectrophotometer. The melting points were determined in a Kofler block and have been corrected.

RESULTS

Specificity and Kinetics of Diamine Oxidase Inhibition

Oxygen consumption showed 1,5-diamino-3-pentanone to inhibit the activity of diamine oxidase from pea and from kidney in a way similar to that of 1,4-diamino-2-butanone when a diamine was used as substrate. At a concentration of 1 μM, the compounds blocked fully the oxidation of tryptamine, catalyzed by pea diamine oxidase in 67 mM phosphate buffer of pH 8·0. On the other hand, using solubilized mitochondrial monoamine oxidase from pig kidney, we could not detect any inhibition even after 15 min of preincubating the enzyme with as much as 3 mM inhibitor at 37°C, provided that the enzyme activity was measured in the presence of catalase in a phosphate buffer of pH 7·6 with 3 mM benzylamine or isoamylamine as substrate.

If diamine oxidase from pea epicotyl or from pig kidney was added to a reaction mixture containing both 1,5-diamino-3-pentanone and 1,4-diaminobutane, the enzyme inhibition observed decreased with increasing substrate concentration. The kinetic data were evaluated graphically according to Lineweaver and Burk and according to Dixon (Fig. 1*a*—*d*), indicating a competition between 1,5-diamino-3-pentanone and 1,4-diaminobutane. With 1,5-diaminopentane as substrate the inhibition of both enzymes had a rather mixed-type character. The difference may be due to a secondary reaction associated with diamine oxidase inactivation by 1,5-diaminopentane and peroxidase.[10] A slight peroxidase activity could be demonstrated even in the used purified preparations of pea diamine oxidase.

Dixon's plot (Fig. 1*b*, *d*) yielded the following apparent inhibition constants: pea diamine oxidase $K_i = 1{\cdot}5 \cdot 10^{-8}$M, pig kidney diamine oxidase K_i $1{\cdot}8 \cdot 10^{-6}$M (or $1{\cdot}4 \cdot 10^{-6}$M when using 1,5-diaminopentane as substrate). A comparison with analogous data for 1,4-diamino-2-butanone as inhibitor (K_i $2{\cdot}1 \cdot 10^{-7}$M for the pea

TABLE I

Effect of Enzyme Concentration on Reactivation of Diamine Oxidase During its Incubation with 1,5-Diamino-3-pentanone

Diamine oxidase was incubated at three different concentrations in 5 ml 50 mM phosphate buffer with 0·1 mg catalase and inhibitor. At the time intervals shown, enzyme activity was estimated in a 1 ml aliquot. Incubation conditions: for pea enzyme 25°C pH 7·0, 1 μM inhibitor; for pig kidney enzyme 38°C, pH 7·4, 100 μM inhibitor.

Incubation min	Inhibition, %					
	pea diamine oxidase mU/ml			kidney diamine oxidase mU/ml		
	80	160	240	50	100	150
0	55·8	56·8	55·3	41·7	39·2	40·2
15	54·5	51·5	47·6	—	—	—
30	49·5	43·3	32·5	39·1	32·9	29·5
60	42·1	6·2	1·6	32·6	21·2	11·0
90	—	—	—	28·6	3·9	0

TABLE II

Effect of Preincubation of Pea Diamine Oxidase with Diamino Ketone on Its Reactivation on a Sephadex G-25 Column

Preincubation was done at 25°C, at pH 7·0, with 4 mM inhibitor.

Preincubation min	Enzyme activity (in U) after preincubation and elution					
	no inhibitor	1,5-diamino--3-pentanone	reactivation %	no inhibitor	1,4-diamino--2-butanone	reactivation %
2	2·05	2·03	99·0	2·01	1·94	96·5
90	2·24	2·07	92·4	2·06	1·77	85·9
165	2·29	2·03	88·6	2·14	0·83	38·8
255	—	—	—	2·12	0·44	20·7
300	2·30	2·15	93·4	—	—	—
420	—	—	—	2·22	0·22	9·9
1 440	1·98	1·77	89·4	2·28	0·4	1·7

enzyme and K_i $1{\cdot}0 \cdot 10^{-7}$M for the pig kidney enzyme) shows 1,5-diamino-3-pentanone to be a 14 times more potent inhibitor of the pea enzyme but a 18 times weaker inhibitor of the kidney enzyme than 1,4-diamino-2-butanone.

Time Course of Inhibition and Reactivation by Gel Filtration

When incubating diamine oxidase from pea epicotyls or from pig kidney in 50 mM phosphate buffers of pH 7·0 and 7·4 respectively with catalase and low concentration of 1,5-diamino-3-pentanone it was observed that with increasing incubation time the inhibition tends to decrease until the original enzyme activity is fully restored. The curve of inhibition dependence on time has a sigmoid shape. The higher the inhibitor concentration used, the longer time required for disappearance of inhibition (Fig. 2). At a constant concentration of inhibitor the inhibition period was reduced as the diamine oxidase concentration was increased (Table I). Similarly, during prolonged incubation with 1,4-diamino-2-butanone, a gradual reactivation of diamine oxidase of plant and animal origin took place but the shape of the inhibition curves was different. During the first 10 min of incubation, a transient slight increase of inhibition was observed (Fig. 3).

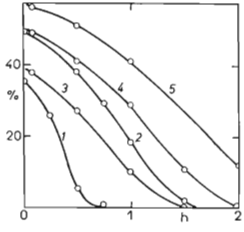


FIG. 2

Time Dependence of Inhibition of Diamine Oxidase by 1,5-Diamino-3-pentanone

The diamine oxidase was incubated with the inhibitor in a 50 mM phosphate buffer in the presence of catalase (40 μg/ml), enzyme activity being estimated in 1 ml aliquots at suitable time intervals. Incubation conditions: for the pea enzyme 0·18 U/ml, 25°C, pH 7·1, inhibitor 0·5 μM *1* and 1·2 μM *2*; for the pig kidney enzyme 0·14 U/ml, 38°C, pH 7·4, inhibitor 80 μM *3*, 120 μM *4* and 160 μM *5*.

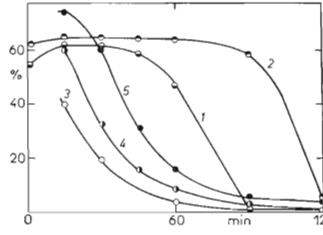


FIG. 3

Time Dependence of Inhibition of Diamine Oxidase by 1,4-Diamino-2-butanone

The experiment was performed as in Fig. 2. Incubation conditions: for the pea enzyme (◒, ◓) 0·18 U/ml, 25°C, pH 7·0, inhibitor 0·8 μM *1* and 1·2 μM *2*; for the kidney enzyme (○, ◑, ●) 0·14 U, 38°C, pH 7·4, inhibitor 0·5 μM *3*, 0·8 μM *4* and 1·2 μM *5*.

Diamine oxidase that has been fully inhibited by excess inhibitor could be reactivated also on a column of Sephadex G-25. If the inhibitor was in contact with pea diamine oxidase at 25°C for several min, full restoration of enzyme activity was achieved during passage of the mixture of enzyme and inhibitor through the column. With increasing preincubation time the amount of reactivated enzyme decreased, in particular in experiments with 1,4-diamino-2-butanone (Table II). Similar results have been obtained with pig kidney diamine oxidase.

DISCUSSION

1,5-Diamino-3-pentanone is another substrate analogue which inactivates reversibly *in vitro* diamine oxidases from different sources. The substrate-competitive type of inhibition indicates binding at the active site of the enzyme. By catalytic hydrogenation the compound loses its inhibitory properties, much like 1,4-diamino -2-butanone, the diaminoalkanol formed acting as substrate for both the pea and the kidney diamine oxidase[11]. The keto group in the diamine molecule is thus essential for inhibition to take place and it cannot be replaced by a hydroxyl. In the enzyme active centre there appear to be a specific binding site for it, probably near the hydrophobic region localized between the binding sites for the substrate amino groups[12] (Fig. 4A).

The newly described substance differs from 1,4-diamino-2-butanone in the degree of its inhibition of pea and kidney diamine oxidase. It is known that the length of the carbon chain is of importance for enzymic oxidizability of diamines. The different

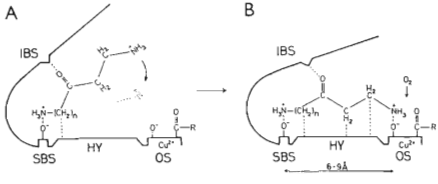


FIG. 4

Schematic Representation of the Active Centre of Diamine Oxidase Showing the Binding of Diamino Ketone during Inhibitory Action A and during Its Oxidative Degradation B

SBS binding site for the substrate amino group (apparently COO^-), HY hydrophobic binding site, IBS inhibitor binding site, OS oxidative site. For kidney diamine oxidase R = H, $n = 1$, for pea diamine oxidase R is not known, $n = 2$. The binding interactions of the inhibitor are shown in dashed lines.

behaviour of the two homologous diamino ketones however, cannot be accounted for by the length of the carbon chain since the parent compounds from which the ketones are derived, *i.e.* 1,4-diaminobutane and 1,5-diaminopentane, represent the best substrates for both diamine oxidases, the values of their apparent K'_m constants being rather close[2,12]. The mutual position of the keto group and the amino groups must play an equally important role during the formation of the enzyme-inhibitor complex. With pea diamine oxidase, the β-amino grouping is bound better than the α-amino grouping since even other β-amino ketones (1-amino-3-butanone and 1-amino-4-bromo-3-butanone) act as potent competitive inhibitors of the enzyme[2,3]. With 1,4-diamino-2-butanone which is simultaneously an α-amino and a β-amino ketone, there is less likelihood that it will be bound by its β-amino group as compared with the symmetrical molecule of 1,5-diamino-3-pentanone. This may account for the higher inhibitory effect of the latter compound. On the other hand, its weaker effect on diamine oxidase from the pig kidney cortex is associated with different structural requirements of this enzyme. Here it is likely that the α-amino-keto grouping is preferred since the enzyme is powerfully inhibited by 1,3-diamino-2-propanone. Likewise, aminoacetone is more potent than the above 1-amino-3-butanones[3] in spite of its short carbon chain.

Reactivation of diamine oxidase at low concentrations of 1,5-diamino-3-pentanone which take place spontaneously is undoubtedly caused by an enzymic degradation of the inhibitor. This is supported by the fact that the drop of inhibition with time is accelerated with increasing enzyme concentration, further by the sigmoid shape of the curves which is typical of enzymic destruction of an inhibitor[13] and by the fact that no spontaneous decomposition of the compound has been observed. A possible way of degradation of the inhibitor based on the present views of the diamine oxidase active centre[12,14] is depicted in Fig. 4. Similarly, in the case of 1,4-diamino-2-butanone, enzymic oxidation is the most likely explanation for the loss of inhibition in spite of the fact that the inhibitor is less stable in a buffered medium[1,2]. The cause of the incomplete reactivation of diamine oxidase on a Sephadex column after extended incubation of the enzyme with excess inhibitor may be due to a modification of the carbonyl cofactor. The absorption band of pyridoxal-5-phosphate (388 nm) which is a coenzyme of diamine oxidase from pig kidney, gradually disappears on adding 1,5-diamino-3-pentanone at pH 7·4 and a new maximum appears at 286 nm, similarly to the case of 1,4-diamino-2-butanone[2]. Further study is required to elucidate this problem.

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