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TIME-DEPENDENT INHIBITION OF PEA COTYLEDON DIAMINE OXIDASE BY SOME HYDRAZIDES

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The inhibition of diamine oxidase (EC 1.4.3.6) from pea cotyledons (PDAO) by some hydrazides has been studied. It was found that PDAO is inhibited in a time-dependent manner at pH = 7.0 by the hydrazides of acetic, benzoic, nicotinic, isonicotinic, picolinic and 3,4-dihydro-4-oxophtalazine-1-carboxylic acids, by 1-(carboxymethyl)trimethylammonium chloride hydrazide (Girard's reagent T), 1-(carboxymethyl)pyridinium chloride hydrazide (Girard's reagent P) and oxalic acid dihydrazide. The inhibition was partially reversible. Rate constants for enzyme inactivation were in the range 0.29–1.95 min^{-1}. The hydrazides give apparent noncompetitive inhibition at pH = 7.0, but for isonicotinic hydrazide, this changes to competitive inhibition at pH = 8.0. Apparent inhibition constants (K_{IAPP}) for the hydrazides with PDAO are in the range 0.005–1.5 mmol l^{-1}.

KEY WORDS: Diamine oxidase, time dependent inhibition, hydrazides.

INTRODUCTION

Plant and animal diamine oxidases (EC 1.4.3.6, diamine: O_2 oxidoreductase (deaminating) (copper containing) catalyse the oxidative deamination of aliphatic and aromatic amines and diamines.[1] They are inhibited by substrate analogs,[2] some alkaloids,[3,4,5] Cu^{2+} chelating agents[6,7] and carbonyl reagents.[8,9] Crabbe *et al.*[9] found that placental and pig kidney diamine oxidases are inhibited by hydrazine derivatives such as phenylhydrazine, *N,N*-dimethylhydrazine, isonicotinic acid hydrazide and Girard's reagent T (trimethyl acetylhydrazide ammonium chloride). The last two giving time-dependent inhibition. Similar results were found for human kidney diamine oxidase.[10] Time-dependent inhibition of bovine plasma and human placental diamine oxidase had previously been described for the hydrazides of pyrrol-1-ylbenzoic and pyrrol-1-ylphenylacetic acids,[11] and for bovine plasma diamine oxidase by some hydrazides of substituted phenylacetic acid[12] as well as PDAO by β-alanine hydrazide[13] and L-lysine hydrazide.[14]

Diamine oxidases contain Cu^{2+} and a organic cofactor tightly bound to protein at the active site. The nature of the organic cofactor has been debated for many years. Initially it was proposed that it was pyridoxal phosphate, and then later that it was pyrroquinoline quinone,[15,16] but these results are questionable nowadays.[17]

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The cofactor certainly contains a carbonyl group, which would explain the inhibition of diamine oxidases by carbonyl reagents.

The aim of this work was to investigate the inhibition of PDAO by some hydrazides since these are weaker nucleophilic agents than hydrazine and its derivatives, which are strong irreversible inhibitors[18] of the enzyme.

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MATERIAL AND METHODS

Enzymes and chemicals

Pea diamine oxidase (PDAO) was isolated from pea cotyledons, after 10 day growth in the dark, by the published method.[19] Protein content was determined by Lowry's method[20] (with bovine serum albumin as standard). Specific activity measured from oxygen consumption[21] was 347.5 nkat mg^{-1} (at pH = 7.0, 30°C, with 1,4-diaminobutane (putrescine) as substrate).

Pig kidney diamine oxidase (KDAO) was obtained from Sigma, USA. The lyophilized enzyme (0.5 g) was dissolved in 10 ml of distilled water. Specific activity of that enzyme solution measured from oxygen consumption (at pH = 9.0, 30°C, with putrescine as substrate) was 0.25 nkat mg^{-1} (of protein).

Catalase from bovine liver was obtained as a crystalline suspension from Reanal, Hungary with specific activity 2000 Bergmeyer's units/mg of protein.

Putrescine (1,4-diaminobutane) from Koch Light, England, was converted to the dihydrochloride. Dehydroputrescine (1,4-diamino-2-butene) dihydrochloride was prepared from 1,4-dibromo-2-butene.[22]

The hydrazide of isonicotinic acid (**I**), *N*,*N*′-Diacetylhydrazine (**V**), Girard's reagent T (1-(carboxymethyl)trimethylammonium chloride hydrazide) (**VII**) and oxalic acid dihydrazide (**XI**) were from Merck, Germany; Girard's reagent P (1-(carboxymethyl)pyridinium chloride hydrazide) (**VIII**) was from Lachema Brno, Czechoslovakia. Hydrazides of nicotinic acid (**II**), picolinic acid (**III**) and benzoic acid (**VI**) were prepared from the corresponding ethyl esters (Merck, Germany) by reaction with hydrazine hydrate. Acetic acid hydrazide (**IV**) was prepared from acetamide (Carlo Erba, Italy) by reaction with hydrazine hydrate. The hydrazide of 3,4-dihydro-4-oxophtalazin-1-carboxylic acid (**IX**)[20] and 1,2,3,4-tetrahydrophtal-azine-1,4-dione (**X**)[23] were prepared by literature methods.

The purity of the hydrazides was confirmed (absence of hydrazine) by paper chromatography[24] (chromatographic paper Whatman No. 4, solvent diethylether: methanol:H_2O:HCl (50:30:15:4), with detection by ninhydrin reagent). Structures of the hydrazides are shown in Figure 1.

Ninhydrin reagent[26] was prepared by dissolving 750 mg of ninhydrin and 112.8 mg of hydrindantine in 60 ml of hot acetic acid followed by the addition of 12 ml of phosphoric acid. Ehrlich's reagent was prepared according to Macholán *et al.*[25]

Diamine oxidase activity determination

(*1*) *Dehydroputrescine method.*[25] The reaction mixture (5.0 ml) containing 2.5 ml of phosphate buffer (0.2 mol l^{-1}) pH = 7.0 or 8.0 (for PDAO) or Tris buffer (0.2 mol l^{-1}) pH = 8.0 or 9.0 (for KDAO), 0.1 ml of catalase solution (50 Bergmeyer's units), 0.1 ml of ethanol and 0.1 ml of diamine oxidase (5 nkat) and

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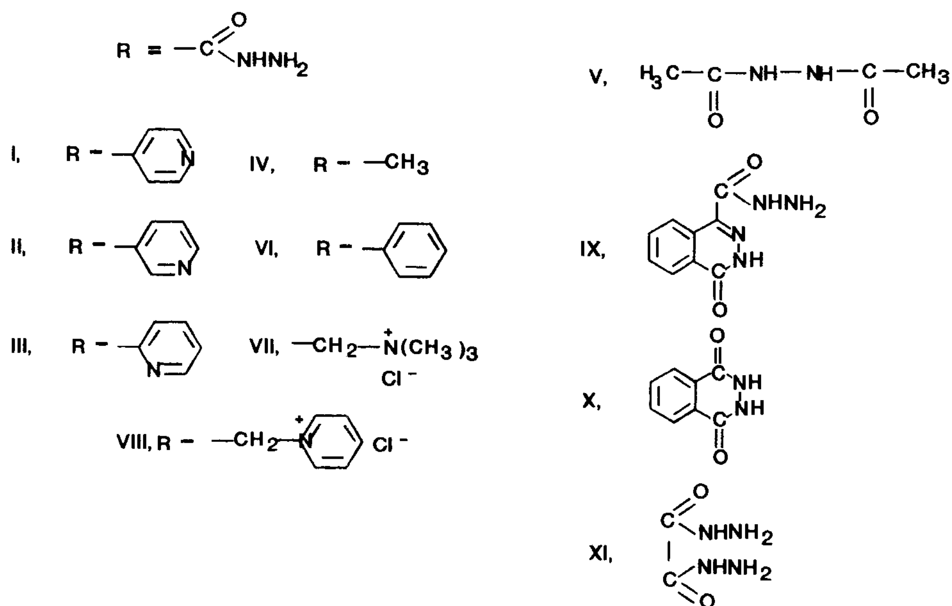

FIGURE 1 Structures of hydrazides: (**I**)—Isonicotinic acid hydrazide. (**II**)—Nicotinic acid hydrazide. (**III**)—Picolinic acid hydrazide. (**IV**)—Acetic acid hydrazide. (**V**)—*N*,*N*′-Diacetylhydrazine. (**VI**)—Benzoic acid hydrazide. (**VII**)—1-(carboxymethyl)trimethylammonium chloride hydrazide (Girard's reagent T). (**VII**)—1-(carboxymethyl)pyridinium chloride hydrazide (Girard's reagent P). (**IX**)—3,4-Dihydro-4-oxophtalazine-1-carboxylic acid hydrazide. (**X**)—1,2,3,4-Tetrahydrophtalazine-1,4-dione and (**XI**)—oxalic acid dihydrazide.

water (with or without inhibitor) was incubated at 30°C for 15 min and the reaction commenced by addition of 0.1 ml of dehydroputrescine (final concentration, 0.1–0.25 mol l^{-1}). After 5 min (for PDAO), and 10 min (for KDAO) the reaction was stopped by the addition of 2 ml of Ehrlich's reagent, and the mixture heated in a boiling water bath for 30 min and then cooled (ice). After cooling the absorption at 563 nm, due to the production of pyrrole ($\varepsilon = 63000$ l mol^{-1} cm^{-1}) from oxidized dehydroputrescine was measured.

(*2*) *Ninhydrin method.*[27] The reaction mixture and time of reaction were the same as in the dehydroputrescine method, but reaction was started by addition of 0.1–0.5 mol l^{-1} (final concentration) of putrescine as substrate and the reaction stopped by addition of 2 ml of ninhydrin reagent. The reaction mixture was then heated in a boiling water bath for 30 min, cooled (ice), and the absorption at 440 nm due to oxidized putrescine ($\varepsilon = 18786$ l mol^{-1} cm^{-1}) was measured.

In both spectrophotometric methods it was necessary to centrifuge (3000× G, 5 min) the reaction mixtures with KDAO to remove precipitated protein before measuring absorption. The dehydroputrescine method was used for all inhibitors: the ninhydrin method was used only for comparison of inhibition of both enzymes by isonicotinic hydrazide (**I**).

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(*3*) *Oxygen consumption.*[21] A Clark electrode (E5047 pO_2 electrode, Radiometer Copenhagen) was used for the screening of inhibitors, determination of the activity of the prepared enzymes and for measuring the time-dependency of the inhibition. The reaction mixture (3.5 ml) containing phosphate buffer (0.1 mol l^{-1}), contained 25 μg of catalase (50 Bergmeyer's units), 0.1 ml of ethanol, 0.1 ml of diamine oxidase solution and water (with or without inhibitor). The mixture was placed in a thermostated cell (30°C) with magnetic stirring and Clark oxygen electrode and air bubbled through to saturate with oxygen (0.772 μmol l^{-1} O_2 at 30°C and 760 Torr). Then the reaction was started by addition of putrescine (final concentration 5.7 mol l^{-1}) and the initial rate of oxygen consumption was measured.

Measurement of time-dependent inhibition

The reaction mixture (2 ml) containing phosphate buffer (0.1 mol l^{-1}) pH = 7.0 and diamine oxidase (140 nkat) was incubated at 30°C in a thermostated flask and the inhibitor added. At appropriate intervals over 30 min, 0.1 ml of the mixture was assayed for remaining diamine oxidase activity by its oxygen consumption as described above.

RESULTS

It was found that hydrazides (**I**–**IV**), (**VI**–**IX**) and (**XI**) are time-dependent inhibitors of PDAO. The scheme[28] for first order reaction of a time-dependent inhibitor is:

$$E + I \underset{k_{-1}}{\overset{k_{+1}}{\rightleftharpoons}} EI \overset{k_{+2}}{\rightarrow} EI^* \quad (1)$$

where k_{+1}, k_{-1} and k_{+2} are rate constants. EI is the enzyme-inhibitor complex and EI* is the inactive enzyme. When $K_I = k_{-1}/k_{+1}$ is introduced the equation becomes:

$$\ln([EI^*]/[E_0]) = -k_{+2} \cdot t/(1 + K_I/[I]) \quad (2)$$

where [EI*] and $[E_0]$ are the concentrations of the inactive and initial active enzyme respectively, *t* is time. Where $[I] \gg [E_0]$, the apparent first-order rate constant (k_{app}) is given by

$$k_{app} = k_{+2}/(1 + K_I/[I]) \quad (3)$$

where k_{app} is the slope of the plot of $\ln([EI^*]/[E_0])$ (or log of remaining activity) against time.

Plots of time-dependencies of log(% remaining diamine oxidase activity) versus time for some hydrazides are shown in Figures 2 and 3. From secondary graphs (not shown) of the reciprocal slopes from these dependencies ($1/k_{app}$) against reciprocal concentration of inhibitor ($1/[I]$) the constants K_I and k_{+2} were determined in account with equation (4),

$$1/k_{app} = 1/k_{+2} + K_I/k_{+2} \cdot 1/[I] \quad (4)$$

where $1/k_{+2}$ is given by the intercept on the axis and K_I/k_{+2} by the slope. The values for K_I and k_{+2} are shown in Table I.

Inhibition of PDAO by the hydrazides used here appeared to be partially reversible since on dialysis of the inhibited enzyme (incubated for 24 h with inhibitor) using

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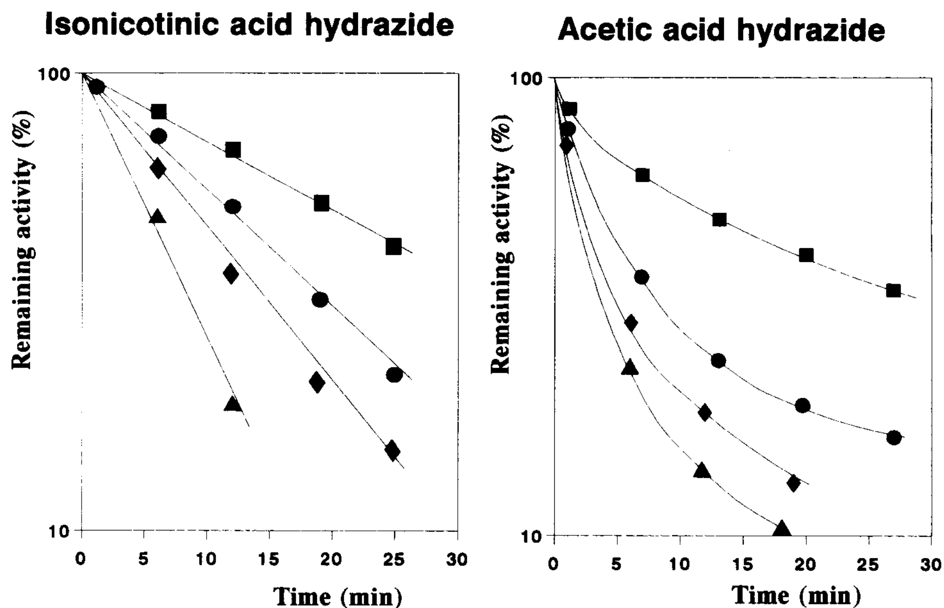

FIGURE 2 Time-dependency of PDAO inhibitions by isonicotinic acid hydrazide (■, 1 mmol l^{-1}; ●, 2 mmol l^{-1}; ◆, 3 mmol l^{-1}; ▲, 5 mmol l^{-1}) and acetic acid hydrazide (■, 0.01 mmol l^{-1}; ●, 0.03 mmol l^{-1}; ◆, 0.05 mmol l^{-1}; ▲, 0.06 mmol l^{-1}).

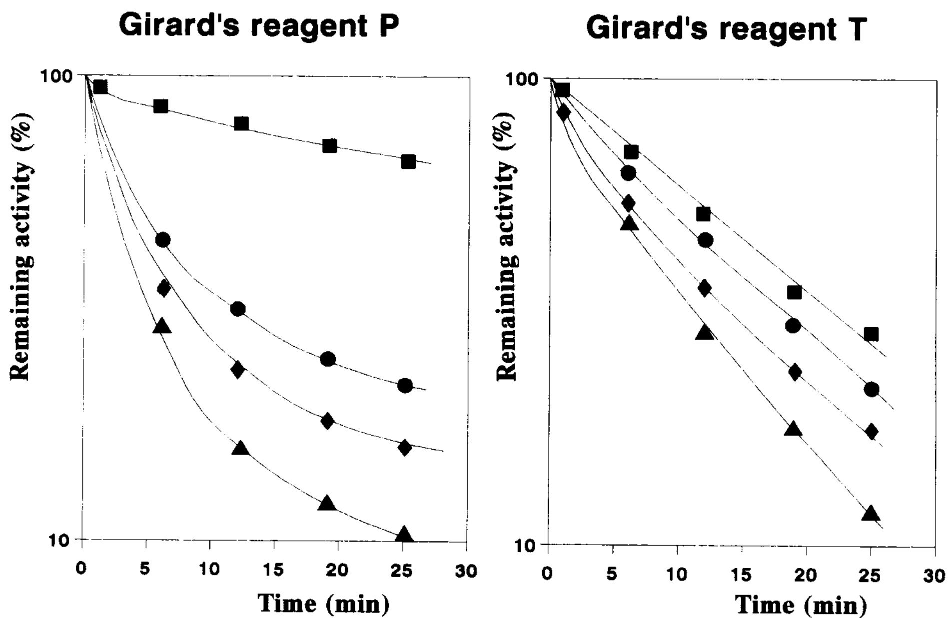

FIGURE 3 Time-dependency of PDAO inhibitions by Girard's reagent P (■, 0.01 mmol l^{-1}; ●, 0.02 mmol l^{-1}; ◆, 0.03 mmol l^{-1}; ▲, 0.05 mmol l^{-1}) and Girard's reagent T (■, 0.4 mmol l^{-1}; ●, 0.5 mmol l^{-1}; ◆, 0.6 mmol l^{-1}; ▲, 0.9 mmol l^{-1}).

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TABLE I
Dissociation constants of EI complexes (K_I) and rate constants (k_{+2}) for time-dependent inhibitors of PDAO

Hydrazide	k_{+2} (min^{-1})	K_I ($mmol\ l^{-1}$)
I	0.45	0.32
II	0.29	0.10
III	1.66	1.31
IV	1.25	1..07
V	not inhibited	
VI	1.95	1.40
VII	0.66	0.42
VIII	0.36	0.03
IX	0.50	0.48
X	not inhibited	
XI	0.50	0.09

TABLE II
Apparent character of the inhibition and inhibition constants for hydrazides as inhibitors of PDAO and KDAO (activity 5 nkat after 15 min of preincubation)

Hydrazide	Apparent character of inhibition	pH	K_{Iapp} ($mmol\ l^{-1}$)
PDAO			
I	competitive	7.0	3.4
	noncompetitive	8.0	4.4
II	noncompetitive	7.0	2.5
III	noncompetitive	7.0	0.08
IV	noncompetitive	7.0	0.005
V	not inhibited	7.0	—
VI	noncompetitive	7.0	0.24
VII	noncompetitive	7.0	0.73
VIII	noncompetitive	7.0	0.32
IX	noncompetitive (weak)	7.0	not determined
X	not inhibited	7.0	—
XI	noncompetitive	7.0	0.034
KDAO			
I	competitive	8.0	0.55
	noncompetitive	9.0	1.5

distilled water for 10 h restored the activity by 30–45% of that of the native enzyme. Consequently, inhibition rate constants described are apparent rate constants.

The type of PDAO inhibition was measured by the dehydroputrescine method using 5 nkat of enzyme and 15 min preincubation. The inhibition constants were calculated from double reciprocals plots of 1/v against 1/[S] and their secondary graphs of slopes against concentration of inhibitor [I]. Isonicotinic acid hydrazide, which is used as a tuberculostatic agent, was also examined against pig kidney diamine oxidase (KDAO). All the results are summarized in Table II.

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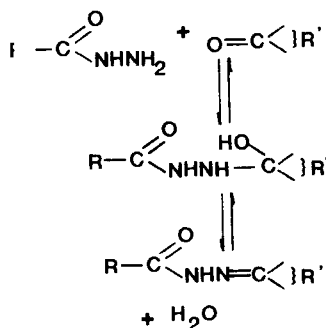

FIGURE 4 Interaction of hydrazide with carbonyl group in active site of diamine oxidase.

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DISCUSSION

Hydrazides are weaker nucleophilic agents than hydrazines as illustrated by the pK_a values of hydrazine[29] ($pK_a = 8.23$) and acetic acid hydrazide[30] ($pK_a = 3.24$). Hydrazides react slower with the carbonyl group in the active sites of PDAO and KDAO than hydrazines. It is very likely that hydrazide forms a carbinolamine at the active site (in equilibration with free hydrazide) which is slowly irreversible dehydrated to acylhydrazone (see Figure 4). Reactivity of hydrazides is similar to that of aminoguanidine, which is a time-dependent inhibitor[9] of pig kidney and human placental diamine oxidase.

Hydrazides based on a diacyl hydrazine (**V** and **X**) do not inhibit diamine oxidase. However (**IX**), which is structurally similar to (**X**) but has an additional free hydrazide group, inhibits diamine oxidase. These results indicate that possession of a free hydrazide group is essential for inhibition.

The most potent inhibitor studied is acetic acid hydrazide (**VI**) with $k_{+2} = 1.95\ min^{-1}$ whereas the weakest are nicotinic acid hydrazide (**II**) ($k_{+2} = 0.29\ min^{-1}$) and Girard's reagent P (**VIII**) ($k_{+2} = 0.36\ min^{-1}$).

The noncompetitive inhibition of PDAO by isonicotinic acid hydrazide at pH = 7.0 was found to change to competitive inhibition at pH = 8.0 and a similar effect was found with KDAO at pH $8 \rightarrow 9$ except that the type of inhibition was reversed relative to PDAO. The secondary graphs of double reciprocal plots of slopes against inhibitor concentration are not linear, but exponentially curved. It would seem that the type inhibition is dependent on the proportion of enzyme activity remaining and the inhibitor concentration since at higher enzyme activity the inhibition is noncompetitive, whereas at lower enzyme activity the inhibition was found to be competitive. At pH = 8.0 the activity of PDAO is less[7] than that at pH = 7.0 and this change may be responsible for the change in the type of inhibition. At the intermediate pH = 7.7 it was that a mixture of both types of inhibition operated.

Generally, hydrazides are time-dependent inhibitors and give nonlinear semilogarithmics plots of remaining PDAO activity against time (see Figures 2 and 3), but it is possible to find a range of inhibitor concentrations, which lead to linear plots,[9] as shown in Figure 2 for isonicotinic acid hydrazide.

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