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COMPARISON OF COUPLING SUBSITES AND INHIBITION EFFECTS OF PIPERIDINE ALKALOIDS AND AMINOKETONES ON PLANT AMINE OXIDASES

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In the present work we compare the binding subsites of inhibitors from a series of alkaloids and aminoketones on pea and sainfoin diamine oxidase (EC 1.4.3.6; DAO) by the graphical method. As standard competitive inhibitors we have chosen oxoanalogs of the substrates, namely, 1,4-diamino-2-butanone and 1,5-diamino-3-pentanone, which were compared with the alkaloids (+)-sedamine, (−)-norallosedamine, (−)-norsedamine, L-lobeline, cinchonine and aromatic analogs of aliphatic aminoketones such as l-amino-3-phenyl-3-propanone and l-amino-3-phenyl-2-propanone. In the case of pea DAO all inhibitors compete for the same subsites with 1,4-diamino-2-butanone and 1,5-diamino-3-pentanone ($\alpha = \infty$). In the case of sainfoin enzyme they are bound to other subsites and the interaction constants ($0 < \alpha < 1$) point to a positive attraction between these two types of inhibitors. With sainfoin DAO, l-amino-3-phenyl-3-propanone is bound into the same subsite as 1,4-diamino-2-butanone. Cinchonine and l-amino-3-phenyl-3-propanone are bound to two different subsites and the value of the interaction constant ($1 < \alpha < \infty$) shows repulsion between the inhibitors.

KEY WORDS: Diamine oxidase, competitive inhibitors, alkaloids, examining overlapping subsites.

INTRODUCTION

Diamine oxidase (DAO; EC 1.4.3.6; diamine: O_2-oxidoreductase/deaminating; copper containing/) has been isolated from many plants[1], especially from Fabaceae. It contains copper at the active site and pyrroloquinoline quinone as a cofactor[2] and plays an important role in metabolism of polyamines and in the biosynthesis of alkaloids[1].

DAO is inhibited by chelating agents of copper[3], by compounds reacting with the carbonyl group of the cofactor[4], by a series of substrate analogs[5,6] and by alkaloids[7] from Cinchona succirubra and piperidine alkaloids[8] from Lobelia inflata and Sedum acre. Substrate analogs and alkaloids inhibit diamine oxidase competitively. Simultaneously it was evident that diamine oxidase participants in the metabolism of these alkaloids[9].

In the present work we have investigated the inhibition effect of certain other piperidine alkaloids and aromatic aminoketones and aminoalcohols, which inhibit DAO competitively and compared the binding site for inhibition by these inhibitors with those from a series of substrate analogs using the Yonetani–Theorell method[10]

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for examining overlapping subsites of the enzyme active site. For comparison purposes we have studied two plant enzymes–DAO from pea[11] (Pisum sativum) and from sainfoin[12] (Onobrychis viciifolia), with regard to their kinetic properties and active sites.

MATERIALS AND METHODS

Enzymes and Chemicals

Diamine oxidase (EC 1.4.3.6) from the cotyledons of pea (Pisum sativum) was isolated by a five step procedure and purification method[11]. The protein content was determined according to the method of Bradford[13]-20 mg.ml^{-1}, and the specific activity by the guaiacol method[14]-95.2 nkat.mg prot.$^{-1}$.

Diamine oxidase from the roots and shoots of sainfoin (Onobrychis viciifolia) was isolated by gel chromatography[12]. The protein content was found to be 4.2 mg.ml^{-1} and the specific activity 348 nkat.mg prot.$^{-1}$.

Peroxidase (EC 1.11.1.7) from horse radish (Armoracia rusticana) was a salt-free lyophylisate from Reanal Budapest, Hungary with specific activity 8 350 nkat.mg prot.$^{-1}$.

Catalase (EC 1.11.1.6) from beef liver was a crystalline suspension from Reanal Budapest, Hungary with specific activity 2 000 units per mg protein. (Unit Definition: One unit will decompose 1 μmole of H_2O_2 per min at pH 7.0 at 25° C, while the H_2O_2 concentration falls from 10.3 to 9.2 mmol.l^{-1}.).

Cinchonine was obtained from Léčiva, Prague and aminoguanidine and 8-hydroxychinoline from Lachema, Brno as analytical grade. Hydrochloride of lobeline was obtained from Sigma, USA. The dihydrochloride of putrescine (1,4-diaminobutane) was obtianed from Koch Light, England. Hydrochlorides of (+)-sedamine[15], (−)-norsedamine[15], (−)-norallosedamine[15], l-amino-3-3-phenyl-3-propanone[16], l-amino-3-phenyl-2-propanone[17], l-amino-3-phenyl-2-propanol[18] and dihydrochlorides of 1,5-diamino-3-pentanone[6], 1,4-diamino-2-butanone[19] and E-1,4-diamino-2-butene[20] were prepared.

Enzyme Activity Determination

Spectrophotometric determination of enzyme activities [14] was performed in a 2 cm cell in a light-tight chamber of an EK 5 adapter of a Specol 10 spectrophotometer (Carl Zeiss Jena, GDR), thermostated to 30° C. The reaction mixture with total volume of 3.4 ml contained 0.1 mol.l^{-1} potassium phosphate buffer (pH 7.0), guaiacol in a final concentration of 0.5 mmol.l^{-1}, peroxidase (20 nkat), diamine oxidase (0.5–1.0 nkat) and the inhibitor or inhibitors. The reaction was started by an injection of 0.1 ml putrescine (final concentration 0.03–0.3 mmol.l^{-1}) directly into the cell. Time-dependent increase in absorption at 436 nm was recorded on a line recorder.

The second method for determinatin of enzyme activities used as substrate E-1, 4-diamino-2-butene[20]. The reaction mixture with a total volume 3 ml contained 0.1 mol.l^{-1} potassium phosphate buffer (pH 7.0), catalase (80 units), diamine oxidase (0.5–1.0 nkat) and the inhibitor or inhibitors. Reaction was started by an addition of substrate (final concentration 0.2–2 mmol.l^{-1}). It was thermostated at 30° C and then the reaction stopped by addition of 2 ml of modificated Ehrlich's reagent after 6 min followed by incubation at 50° C and after 30 min the reaction mixture was cooled by

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ice. The absorbance (red colouration) was measured spectrophotometrically at 563 nm.

The enzyme was always preincubated for ten min with the inhibitor or inhibitors in a reaction mixture and then the reaction was started by addition of substrate. When the time-dependence of inhibition was measured, the enzyme was incubated at 30° C with inhibitor in potassium phosphate buffer (0.1 mol.l^{-1}), enzyme activity was determined in appropriate aliquots at suitable time intervals.

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RESULTS

The character of the inhibition by single inhibitors was studied for both enzymes by kinetic methods. The kinetic data obtained were processed on a PMD 85-1 computer with our own software. With pea diamine oxidase all the measurements were performed by a method using E-1,4-diamino-2-butene as a substrate[20], and some with the substrate 1,4-diaminobutane with detection of hydrogen peroxide by the guaiacol method. All the measurements with sainfoin diamine oxidase were performed only by the guaiacol method with 1,4-diaminobutane as a substrate. It is know that L-lobelline[8], cinchonine[7] and 1,4-diamino-2-butanone[6] inhibit pea diamine oxidase competitively with inhibition constants of 0.17, 0.2, and 0.00042 mmol.l^{-1}.

We found that the pea diamine oxidase was inhibited by (+)-sedamine, (−)-norallosedamine, (−)-norsedamine, 1-amino-3-phenyl-3-propanone and 1-amino-3-phenyl-2-propanone competitively whereas 1-amino-3-phenyl-2-propanol was without an inhibition effect.

Sainfoin diamine oxidase was competitively inhibited by L-lobelline, (−)-norallosedamine, cinchonine, 1-amino-3-phenyl-3-propanone and 1,4-diamino-2-butanone. 8-Hydroxyquinoline inhibited non-competitively. Time-dependent inhibition was found with aminoguanidine where the activity decreased with time. The

TABLE I
Type of inhibition and inhibition constants* for pea and sainfoin diamine oxidases.

inhibitor	type of inhibition	K_i/mmol.l^{-1}/
pea diamine oxidase		
(+)-sedamine	competitive	0.9
(−)-norallosedamine	competitive	0.062
(−)-norsedamine	competitive	0.03
1-amino-3-phenyl-3-propanone	competitive	0.58
1-amino-3-phenyl-2-propanone	competitive	0.37
1-amino-3-phenyl-2-propanol	no inhibition	----
sainfoin diamine oxidase		
L-lobeline	competitive	0.185
1-amino-3-phenyl-3-propanone	competitive	0.92
(−)-norallosedamine	competitive	0.72
1,4-diamino-2-butanone	competitive	0.00063
aminoguanidine	time-dependent, irreversible	----
8-hydroxyquinoline	noncompetitive	0.48
cinchonine	competitive	0.17

*Activities of pea diamine oxidase were determined by the method using E-1,4-diamino-2-butene as substrate. Activities of sainfoin diamine oxidase were determined by the guaiacol method.

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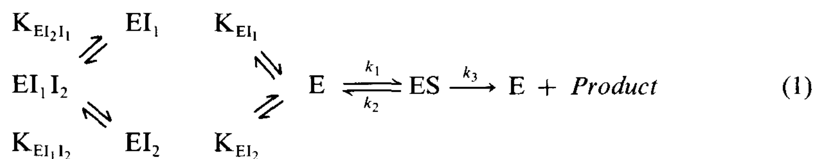
sample of inhibited enzyme formed using aminoguanidine 0.0015 mmol.l^{-1} was dialysed 20 h against distilled water. The enzyme activity was not restored and therefore the inhibition can be considered as irreversible.

The results for the single inhibitors and their inhibition constants is given in Table I.

It is known that aliphatic aminoketones which competitively inhibit are continuously degraded by diamine oxidase[6] until the original activity of enzyme is restored. It is possible that aromatic aminoketones with free amino group are degraded too and therefore we have performed measurements of time-dependence of the inhibition by l-amino-3-phenyl-3-propanone of pea and sainfoin diamine oxidase and as a comparison the inhibition by 1,4-diamino-2-butanone of sainfoin diamine oxidase. It was found that aromatic aminoketones are also degraded, but more slowly than the aliphatic compounds. Inhibitors from the alkaloid group exhibited time-stable inhibition for both enzymes because they do not have free amino group which could be degraded.

To compare the binding sites of inhibitors we have used the Yonetani-Theorell method for examining overlapping subsites of enzyme active sites[10]. The method is based upon the steady state kinetic equations:



(1)

When $K_{EI_1I_2} = \alpha.K_{EI_2}$ is introduced, then $K_{EI_2I_1} = \alpha.K_{EI_1}$ and for the rate of reaction with two inhibitors it follows that:

$$v_i = V_m / \left(1 + \frac{K_m}{[S]}\right) . \left(1 + \frac{[I_1]}{K_{EI_1}} + \frac{[I_2]}{K_{EI_2}} + \frac{[I_1].[I_2]}{\alpha.K_{EI_1}.K_{EI_2}}\right) \quad (2)$$

on rearrangement we obtain the equation of the straightline $1/v_i = A + B.[I_1]$ when $[I_2]$ and [S] are constants.

$$\frac{1}{v_i} = \frac{1}{V_m} + \frac{K_m}{[S].V_m} . \left(1 + \frac{[I_2]}{K_{EI_2}}\right) + \frac{K_m}{[S].V_m.K_{EI_1}} . \left(1 + \frac{[I_2]}{\alpha.K_{EI_1}}\right).[I_1] \quad (3)$$

When $\alpha = \infty$, then B = const. even for various $[I_2]$ for which then a beam of parallel straightlines is obtained, if $\infty > \alpha > 0$, then a beam of straightlines intersect at one point from whose cooridinates the interaction constant α is obtained. When $\alpha = \infty$, inhibitors compete for the same subsite of the enzyme active site. When $\infty > \alpha > 0$, inhibitors interact with the different subsites. When the interactions of I_1 and I_2 with enzyme are strictly independent of each other, then $\alpha = 1$. When a positive attraction occurs between I_1 and I_2 in the EI_1I_2 complex, then $1 > \alpha > 0$. When I_1 and I_2 interact repulsively in the EI_1I_2 complex, thus $\infty > \alpha > 1$.

We have compared the binding site for single types competitive inhibitors – lobeline, sedamine and cinchonine alkaloids and aromatic aminoketones with that for 1,4-diamino-2-butanone and 1,5-diamino-3-pentanone as typical inhibitors from a series of substrate analogs.

With pea diamine oxidase the measurements of activity were performed using E-1,4-diamino-2-butene as substrate and 1,5-diamino-3-pentanone as reference in-

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TABLE II
Comparison of subsites of the enzyme active site for inhibitor and aliphatic diaminoketone binding.*

inhibitor	type of interaction	interaction constant
pea diamine oxidase		
L-lobeline	compete for the same subsite	∞
(−)-norallosedamine	compete for the same subsite	∞
l-amino-3-phenyl-3-propanone	compete for the same subsite	∞
l-amino-3-phenyl-2-propanone	compete for the same subsite	∞
cinchonine	compete for the same subsite	∞
sainfoin diamine oxidase		
L-lobeline	compete for different subsites	0.057
(−)-norallosedamine	compete for different subsites	0.256
l-amino-3-phenyl-3-propanone	compete for the same subsite	∞
cinchonine	compete for different subsites	0.214

*Inhibition of pea diamine oxidase was determined by the method using E-1,4-diamino-2-butene as a substrate and subsite binding was compared with 1,5-diamino-3-pentanone except for cinchonine. Inhibition of pea DAO by cinchonine and inhibition of sainfoin DAO were determined by the guaiacol method and subsites binding was compared with 1,4-diamino-2-butanone. Inhibition of pea DAO by L-lobeline was determined by both methods which gave identical results.

hibitor; selected measurements were checked by the guaiacol method with 1,4-diamino-2-butanone as reference inhibitor. With sainfoin diamine oxidase all the measurements were performed only by guaiacol method. The data obtained were processed by a computer for Yonetani-Theorell plots and the results were calculated. With pea

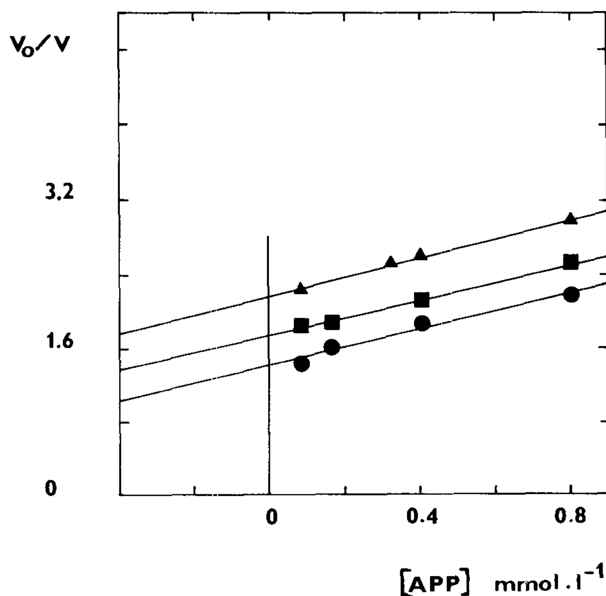

FIGURE 1 Yonetani–Theorell plot of inhibition of pea DAO by l-amino-3-phenyl-2-propanone (APP) and by 1,5-diamino-3-pentanone in concentrations of: ●–without 1,5-diamino-3-pentanone, ■–0.2 and, ▲–0.4 μmol.l^{-1}. Inhibitors compete for the same subsites of the enzyme ($\alpha = \infty$).

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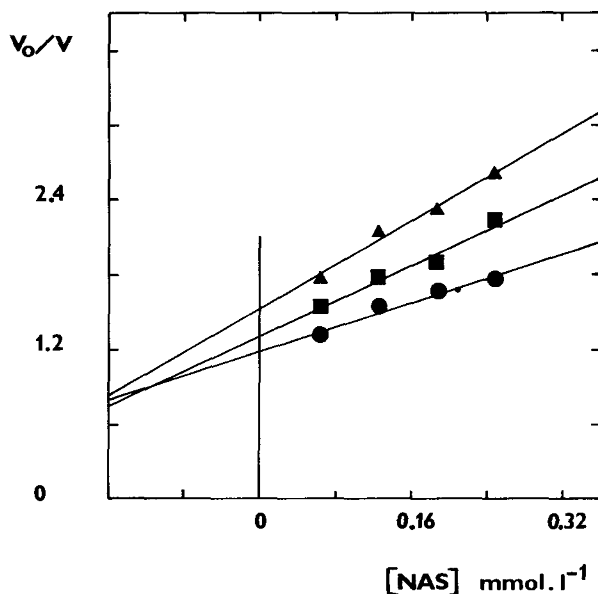

FIGURE 2 Yonetani–Theorell plot of inhibition of sainfoin DAO by (−)-norallosedamine (NAS) and by 1,4-diamino-2-butanone in concentrations of: ●–without 1,4-diamino-2-butanone, ■–0.82 and, ▲–1.64 μmol.l^{-1}. Inhibitors compete for different subsites of the enzyme with positive attraction (α = 0.26).

diamine oxidase for all inhibitors the interaction constant $\alpha = \infty$ was found, i.e. they are bound into the same subsite as 1,4-diamino-2-butanone and 1,5-diamino-3-pentanone. With sainfoin diamine oxidase l-amino-3-phenyl-3-propanone is bound into the subsite, but lobeline, sedamine and cinchonine are bound a subsite different to that for 1,4-diamino-2-butanone, $\infty > \alpha > 0$. The results obtained are given in Table II. Typical Yonetani-Theorell plots are shown in Figure 1 where l-amino-3-phenyl-2-propanone competes for the same subsite as 1,5-diamino-3-pentanone in the pea enzymes while in Figure 2, (−)-norallosedamine and 1,4-diamino-2-butanone compete for different subsites in the sainfoin diamine oxidase.

DISCUSSION

Diamine oxidases from the cotyledons of pea and from the roots and shoots of sainfoin are competitively inhibited by a number of substrate analogs such as 1,4-diamino-2-butanone and 1,5-diamino-3-pentanone as well as by alkaloids, i.e. products of biosynthesis in which diamine oxidase takes part. The compounds are structurally very different and therefore a comparison of their binding subsites was undertaken. Using Yonetani–Theorell graphical method for examining overlapping subsites of an enzyme active site we have used 1,4-diamino-2-butanone and 1,5-diamino-3-pentanone as standard competitive inhibitors of the substrate analogs type for comparative studies. Both have been described by Macholán[5,21–23] with inhibition constants for pea enzyme of $K_i = 2.1.10^{-7}$ mol.l^{-1} and $K_i = 1.5.10^{-8}$ mol.l^{-1} respective-

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ly. For the sainfoin enzyme the constants were measured previously by us[12] and they are, $K_i = 6.3.10^{-7}$ and $1.6.10^{-7}$ mol.l^{-1}. For the pea DAO we have compared their binding sites with the alkaloids L-lobeline (−)-norsedamine, cinchonine and the aromatic aminoketones l-amino-3-phenyl-3-propanone and l-amino-3-phenyl-2-propanone. In all cases we obtained interaction constants where $\alpha = \infty$ which indicates that all these compounds are bound into the same subsite of the active site of the enzyme. The oxo group is essential for inhibition effectively of both aliphatic aminoketones.

An inhibition constant about an order lower for 1,5-diamino-3-pentanone as compared with 1,4-diamino-2-butanone indicates a more suitable bonding arrangement of β-aminoketone to the enzyme. The oxidation at 1,5-diamino-3-pentanone proceeds more quickly too. As is seen from Table I, alkaloids and aromatic aminoketones possess similar inhibition constants. Alkaloids are not oxidized by enzymes because they do not have a primary amino group whereas aromatic aminoketones have, however, the oxidation rate of aromatic aminoketones is essentially slower than for 1,5-diamino-3-pentanone and 1,4-diamino-2-butanone and is comparable for both enzymes.

From the fact that compounds so structually different are bound into the same subsite of the active site of the pea enzyme as aliphatic diaminoketones which are structural analogs of substrate it can be deduced that the active site of the enzyme is on the surface or in an exposed opened pocket.

The alkaloids L-lobelline, (−)-norallosedamine, aromatic aminoketone l-amino-3-phenyl-3-propanone and aliphatic diaminoketones 1,5-diamino-3-pentanone and 1,4-diamino-2-butanone exhibit a competitive character for inhibition of the sainfoin enzyme, and the inhibition constants are given in Table I.

The character of the inhibition of sainfoin enzyme by aminoguanidine and 8-hydroxyquinoline was studied. The aminoguanidine exhibits an irreversible time-dependent inhibition similary to that for other amine oxidases with carbonyl cofactor. It is highly probably that it is bound to the oxo group in position 5 of pyrroloquinoline quinone which is a cofactor of sainfoin DAO[12]. A noncompetitive character of inhibition is exhibited by 8-hydroxyquinoline which probably chelates central Cu^{2+}. To compare binding subsites with 1,4-diamino-2-butanone, the compounds L-lobeline, (−)-norallosedamine, cinchonine and l-amino-3-phenyl-3-propanone were used. Mutuality of binding sites was also compared using cinchonine and l-amino-3-phenyl-3-propanone (Figure 3).

Larger and structurally more complex inhibitors, such as alkaloids, have interaction constants α within the range from 0.06 for L-lobeline through 0.21 for cinchonine to 0.26 for (−)-norallosedamine. This means that these inhibitors are bound to different subsites to those for aliphatic diaminoketones. The value of interaction constants indicate a positive attraction of inhibitors of the alkaloid type and 1,4-diamino-2-butanone. The aromatic analog l-amino-3-phenyl-3-propanone has an interaction constant $\alpha = \infty$ which means that it is bound into the same subsite as 1,4-diamino-2-butanone. This fact can be assigned to the structural similarity with aliphatic diaminoketones. When comparing binding subsites of cinchonine and l-amino-3-phenyl-3-propanone we have found an interaction constant $\alpha = 1.46$ which means repulsion of both inhibitors. Inhibition of pea and sainfoin DAOs by l-amino-3-phenyl-3-propanone and l-amino-3-phenyl-2-propanone decreases with time. The secondary aromatic alcohol, l-amino-3-phenyl-2-propanol which is obtained by reduction of the corresponding ketone, does not inhibit at all which agrees with the data

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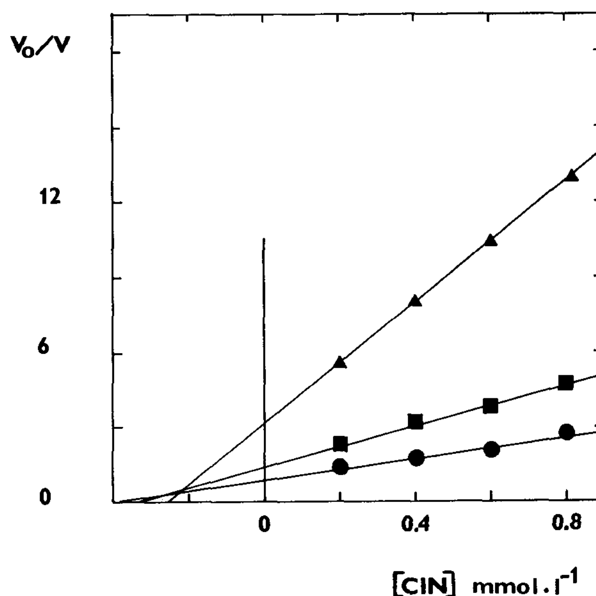

FIGURE 3 Yonetani-Theorell plot of inhibition of sainfoin DAO by cinchonine (CIN) and l-amino-3-phenyl-3-propanone in concentrations of: ●–without l-amino-3-phenyl-3-propanone, ■–1.12 and, ▲–2.24 mmol.l^{-1}. Inhibitors compete for different subsites of the enzyme with repulsion (α = 1.46).

given by Macholán[21] associated with interaction of the enzyme with hydroxyputrescine and hydroxycadaverine.

It can be concluded that both enzymes have, for competitive inhibitors, differently accessible inhibition subsites. In the active site of sainfoin DAO two inhibitors can simultaneously be bound for inhibitor subsites. In the case of the pair cinchonine and l-amino-3-phenyl-3-propanone they repulse each other while for other pairs of alkaloid–aliphatic aminoketone a positive attraction occurs. Therefore it is clear that the active site of sainfoin DAO is more accessible than that of pea DAO. Only one inhibitor can be bound into the active site of pea enzyme which points to the fact that it is less accessible and more closed than the site of the sainfoin enzyme. Regardless of a number of similar kinetic properties and substrate specificity both enzymes differ in molecular weight (sainfoin DAO, 25 000[12], pea DAO, 180 000[11]) and in binding subsites in the active site. Using the Yonetani-Theorell graphical method for examining overlapping subsites of enzyme active sites we have partially contributed to the mapping and comparison of the active sites of pea and sainfoin DAOs. A study of further properties of the active sites of both enzymes is being continued.

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