INHIBITION OF DIAMINE OXIDASE BY CINCHONA ALKALOIDS AND LOBELINE

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Purified diamine oxidase from pea seedlings and hog kidney is inhibited *in vitro* by cinchona alkaloids and lobeline. The plant enzyme is inhibited competitively in all cases and the inhibition constants lie in the range 0·15—3·8 mM. The inhibitory effect decreases in the series lobeline, cinchonine, cinchonidine, quinidine, quinidinone, and cinchonidinone. Quinine shows a weak effect only. The inhibition of the animal enzyme by cinchona alkaloids is noncompetitive, the inhibition by lobeline is competitive. Quinidinone is the most potent inhibitor (K_i = 0·5 mM) and an inhibitory effect shows also quinine (K_i = 3·2 mM). The relationship between the structure and the inhibitory effect of the alkaloids as well as the possible connection between the inhibition and the physiological side effects of the alkaloids are discussed.

Diamine oxidases (histaminases, EC 1.4.3.6) of plant and animal origin are inhibited by chelating agents complexing copper, by compounds reacting with the carbonyl group and by a series of substrate analogs[1-5]. Reports on the interaction of the alkaloids with diamine oxidase from hog kidney[6,7] and human placenta[7] are scarce. These findings together with the fact that plants often synthetize the alkaloids from products of the diamine oxidase reaction[8] have led us to a more systematic examination of the interactions of plant and animal diamine oxidase with these naturally occurring bases. We have investigated 27 different substances and found that significant inhibitory effects show lobeline and cinchona alkaloids; the effect of these was studied quantitatively.

EXPERIMENTAL

Material and Methods

Lobeline, quinine, and quinidine hydrochlorides and cinchonine base were commercial preparations. The content of crystal water of quinine and quinidine hydrochloride determined thermogravimetrically was 1·5 mol. Quinidinone (m.p. 106—8°C) and cinchonidinone (m.p. 126—8°C) were prepared according to Woodward and coworkers[9] by Oppenauer oxidation of quinine

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and cinchonine, respectively, with benzophenone catalyzed by potassium tert. butylate. Both ketones showed in their IR spectrum a characteristic carbonyl vibration band at 1692 cm^{-1} and 1700 cm^{-1}, respectively. All free bases were dissolved in an equivalent quantity of 0·1M-HCl before use.

Pea diamine oxidase was partially purified from etiolated pea seedlings following an described method[10]; according to the activity determination (specific activity 14 U/mg protein) the preparation was 32%. Diamine oxidase from hog kidney cortex of specific activity 21 mU/mg of protein was prepared according to Bardsley and cowokers[11] with the omission of the last two purification steps. Crystalline bovine liver catalase (2% suspension in 0·5% aqueous thymol solution, activity 3000 Bergmeyer units per mg) was from Reanal, Budapest.

The activity of plant diamine oxidase was determined at 430 nm by the *o*-aminobenzaldehyde method[12] at 30°C in 5 ml of 0·1M potassium phosphate buffer, pH 7, in the presence of 20 μg of catalase; 0·4–2 mM 1,4-diaminobutane was used and the activity units were calculated by using a molar extinction coefficient of 2370M^{-1} cm^{-1} (ref.[13]). When quinidinone was tested, whose yellow color in the solution interferes with the reaction product of 1,4-diaminobutane, 1,5-diaminopentane[13] was used as substrate and the absorbance was measured at 470 nm. The activity of animal diamine oxidase was determined spectrophotometrically[14] at 563 nm and 38°C in 3 ml of 0·1M potassium phosphate buffer at pH 8·1 in the presence of 50 μg of catalase; 0·33–1·66 mM 1,4-diamino-2-butene was used as substrate. In both cases the enzymes were preincubated 10 min with the corresponding alkaloid at appropriate concentration, the reaction was started by the substrate and stopped after 5 min of the enzymic reaction (the dependence of absorbance on time is at least 5 min linear). The initial reaction rate v is given in all cases in μmol of product formed in 1 min under the conditions given.

RESULTS

The results of screening tests with 0·5–1% solutions of all the alkaloid types available showed that pea diamine oxidase is selectively sensitive to lobeline and almost to all cinchona bases. Atropine, scopolamine, arecoline, colchicine, narcotine, papaverine, strychnine, brucine, mescaline, yohimbine, solanine T, caffeine, nicotine, bulbocapnine, domestine, isocorydine, and isothebaine were ineffective. The enzyme obtained from hog kidney was inhibited by cinchona alkaloids, by alkaloids with an aporfine backbone (bulbocapnine, domestine, isocorydine, and isothebaine), and by lobeline. The interaction of both diamine oxidases with lobeline and alkaloids from cinchona bark was investigated by kinetic methods. It was demonstrated that it is necessary to incubate the alkaloid with the enzyme 10 min before the addition of substrate in order that maximum inhibition be achieved. Kinetic data obtained under these conditions at fixed oxygen concentration (air) were analyzed graphically according to Lineweaver and Burk ($1/v$ *versus* $1/s$), according to Dixon ($1/v$ *versus* i), and according to Hunter and Downs ($i.v_i/v - v_i$ *versus* s) or by plotting $v/v - v_i$ *versus* $1/i$ (ref.[15]).

The plot of reciprocal initial rate of the enzymic reaction (v, in the presence of inhibitor v_i) *versus* reciprocal substrate concentration (s) or alkaloid concentration (i) for cinchonine and pea diamine oxidase are shown in Fig. 1. The intersection points of the lines indicate that the character of the inhibition is competitive. Similar dia-

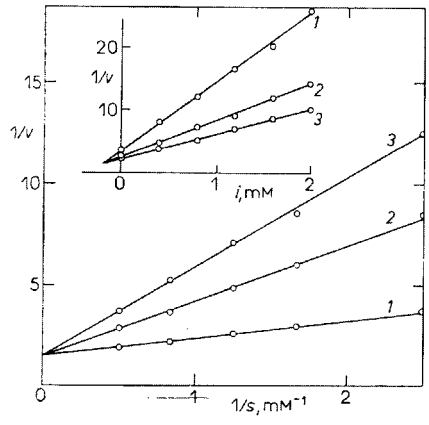


FIG. 1

Competitive Inhibition of Pea Diamine Oxidase by Cinchonine

Initial rate v is given in μmol of Δ^1-pyrroline formed in 1 min; s, substrate (1,4-diaminobutane). The Lineweaver–Burk plot is shown in the main graph; 0·66 U of enzyme, pH 7·0 and 30°C. *1* in the absence of inhibitor, *2* 0·4 mM, *3* 0·8 mM cinchonine. The insert shows the Dixon plot for the following substrate concentrations: *1* 0·4 mM, *2* 0·8 mM, and *3* 1·2 mM; concentration of cinchonine (i) as shown.

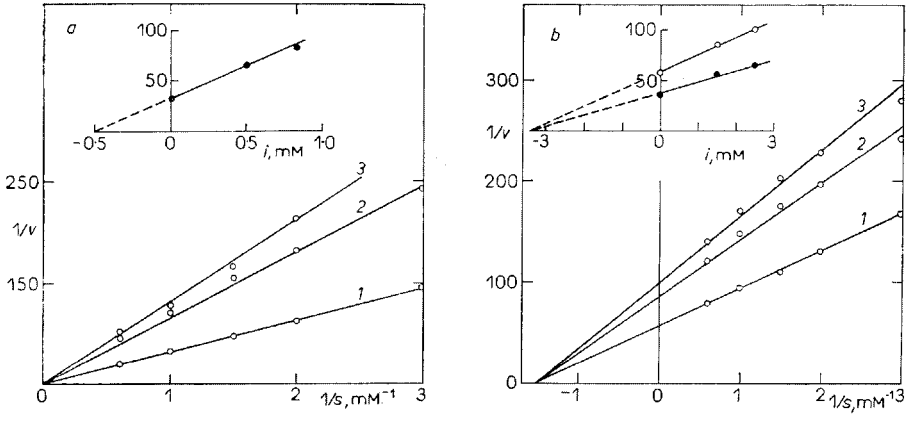


FIG. 2

Kinetics of Inhibition of Hog Kidney Diamine Oxidase by Lobeline and Quinine

Initial rate v is given in μmol of pyrrol formed in 1 min; s substrate (1,4-diamino-2-butene). *a* Double reciprocal plot of competitive inhibition by lobeline; 20 mU of enzyme, pH 8·1, 38°C. *1* in the absence of inhibitor, *2* 0·5 mM, *3* 0·83 mM lobeline. The insert shows the replot of slopes as function of lobeline concentration (i) and the method of reading the $K_{i\ slope}$-value. *b* Double reciprocal plot showing noncompetitive inhibition by quinine; 18 mU of enzyme, pH 8·1 and 38°C; *1* in the absence of inhibitor, *2* 1·5 mM, and *3* 2·5 mM quinine. The insert shows the replots of slopes (●) and intercepts (○) as functions of quinine concentration (i), yielding identical values of $K_{i\ slope}$ and $K_{i\ intercept}$.

grams were obtained with plant enzyme and quinidine, cinchonidine, quinidinone, cinchonidinone, and lobeline. The inhibition of hog diamine oxidase by lobeline and quinine is shown in Fig. 2. Whereas the former compound is a competitive inhibitor with respect to the substrate, quinine and the remaining cinchona bases tested show a different mechanism of inhibition. The intercepts of the $1/v$ *versus* $1/s$ plot on the vertical axis indicate a noncompetitive type of interaction and this is evidenced by the fact that the intercepts of these plots lie at one point on the horizontal axis.

The slopes and intercepts read from double reciprocal plots were analyzed in the sense of classical Cleland's methods[16]. The replots of intercepts or slopes of the lines *versus* the alkaloid concentration were linear in all cases. The intercept on the ordinate yielded the $-K_i$-value. All alkaloids investigated in this study and the types of inhibition which they cause are summarized in Table I. The values of inhibitor constants

TABLE I

Type of Inhibition, Inhibitor Constants (K_i, mM) and Change of Gibbs Energy ($-G°$, kJ mol^{-1}) of Diamine Oxidase–Alkaloid Interaction

Inhibitor	Enzyme[a]	Inhibition[b]	Lineweaver – Burk plot				Dixon plot[c] K_i
			slope effect		intercept effect		
			K_i	$-G°$	K_i	$-G°$	
Lobeline (*I*)	P	C	0·15	22·2	∞	—	0·19
	K	C	0·48	19·8	∞	—	0·52
Cinchonidine (*IIa*)	P	C	1·1	17·2	∞	—	—
	K	—	—	—	—	—	—
Quinine (*IIb*)	P	C	[d]	—	—	—	—
	K	NC	3·45	14·7	3·4	14·7	3·2
Cinchonine (*IIIa*)	P	C	0·2	21·5	∞	—	0·2
	K	NC	3·15	14·9	3·16	14·9	3·45
Quinidine (*IIIb*)	P	C	1·1	17·2	∞	—	1·7
	K	NC	3·25	14·8	3·2	14·8	2·3
Cinchonidinone (*IV*)	P	C	2·7	14·9	∞	—	3·85
	K	NC	4·1	14·2	4·1	14·2	4·3
Quinidinone (*V*)	P	C	1·45	16·5	∞	—	2·87
	K	NC	0·5	19·6	0·5	19·6	0·49

[a] P diamine oxidase from pea seedlings, K diamine oxidase from hog kidney cortex. [b] C competitive inhibition, NC noncompetitive inhibition. [c] Similar K_i values were obtained also by the graphical plot of $i.v_i/v-v_i$ *versus* s and of $v/v-v_i$ *versus* $1/i$, respectively. [d] High value.

K_i are also given. The change of the Gibbs energy ΔG° for the interaction E + I ⇄ EI was calculated from the relation $-\Delta G^\circ = 2{\cdot}303.RT \log K_i$. It is evident from the values measured that pea diamine oxidase is most sensitive to lobeline, next follow cinchonine, cinchonidine, quinidine, quinidinone, and cinchonidinone. Quinine shows a minor effect only. The strongest inhibitor of animal diamine oxidase is also lobeline. Cinchona alkaloids in general were weaker inhibitors and (including quinine) did not differ substantially in their effect.

DISCUSSION

The reciprocal initial rate of a two-substrate enzymic reaction in the presence of inhibitor is given according to Cleland[16]

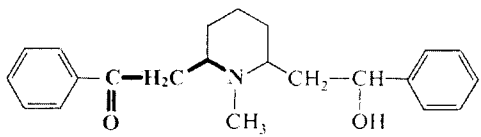
$$1/v_i = (K_a/V[A])(1 + i/K_{i\ slope}) + 1/V(1 + i/K_{i\ intercept}),$$

where K_a is the Michaelis constant for substrate A at fixed concentration of the second substrate (oxygen) and $K_{i\ slope}$ and $K_{i\ intercept}$ are inhibitor constants which may be of complex nature. The true values of the dissociation constant of the enzyme–inhibitor reaction are given by the intercept on the *abscissa* in the replot obtained by plotting slopes and intercepts of the primary double reciprocal graph *versus* inhibitor concentration *i*. In competitive inhibitions where a variable substrate competes with the inhibitor for the same binding site in the active center of the enzyme, slope effects only can be analyzed and $K_{i\ intercept}$ is very high. Non-competitive inhibition is characterized both by slope and intercept effects.

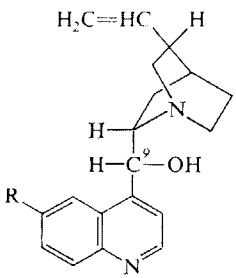
The character of inhibition of pea and hog kidney diamine oxidase by lobeline (*I*) is competitive. We assume that this is caused by binding of the compound to the active center of the enzyme through an amino ketone grouping (marked in the formula *I*) since various amino ketones are relatively potent competitive inhibitors of both diamine oxidases[3]. The lower affinity of lobeline for the enzyme (following from a higher K_i-values) compared to, *e.g.* the value reported for 1-amino-3-butanone (K_i = 0·06 mM, pea diamine oxidase, 25°C) (ref.[2]) can be ascribed to steric effects connected with the substitution at the nitrogen atom and with a higher complexity of the lobeline molecule.

A competition with the substrate show also all cinchona alkaloids tested when interacting with pea diamine oxidase. The ketone derivatives cinchonidinone (*IV*) and quinidinone (*V*) are, however, weaker inhibitors than the corresponding fundamental bases cinchonidine (*IIa*), cinchonine (*IIIa*), and quinidine (*IIIb*). The amino ketone group on the quinuclidine ring does no longer play the key role in the binding of these bulky molecules to the active center of the enzyme. A comparison of the

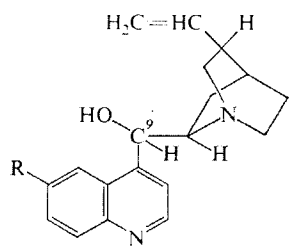
K_i-values shows that cinchonidine (*IIa*) is more effective than quinine (*IIb*) (K_i--value not given because of low effectiveness) and cinchonine (*IIIa*) than quinidine (*IIIb*). On the other hand, cinchonine (*IIIa*) is more effective than cinchonidine (*IIa*) and quinidine (*IIIb*) than quinine (*IIb*). This shows that a role in the inhibition of pea diamine oxidase plays the absence of the methoxyl on the quinoline ring and the configuration of S on $C_{(9)}$. Cinchonine having both these structural features gives the lowest $K_i$value.



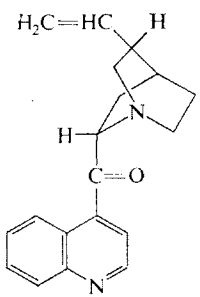
I



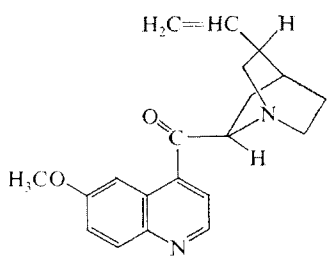
IIa, R = H *IIb*, R = OCH_3



IIIa, R = H *IIIb*, R = OCH_3



IV



V

In the case of hog kidney diamine oxidase, which is non-competitively inhibited by cinchona alkaloids, the K_i-values correspond to a concentration of alkaloid which brings about a 50% inhibition. The K_i-values vary with all the alkaloids tested (except for quinidinone) around 3–4 mM and therefore no unambiguous conclusions

can be derived from the results. The linear replots of intercepts or slopes of double reciprocal graphs *versus* alkaloid concentration exclude partial inhibition (hyperbolic dependence) or the binding of two alkaloid molecules (parabolic dependence).

Non-competitive inhibition has been observed with human placental diamine oxidase treated with harmine, protoveratrine, and diamorfine[7]. Harmine caused non-competitive inhibition[7] in experiments with the enzyme from hog kidney whereas bulbocapnine, an aporfine alkaloid, caused competitive inhibition[6]. The different type of inhibition of hog and plant diamine oxidase by cinchona alkaloids and the fact that hog enzyme is inhibited also by aporfine alkaloids point to a more complex interaction whose elucidation would require a greater number of alkaloids or model compounds to be examined. The inhibitory activity was determined qualitatively also with quinotoxine and cinchotoxine.

We assume that the inhibition of diamine oxidase can be of importance in human physiology and in the toxicology of the alkaloids tested. The enzyme is localized mainly in the digestive tract, kidney and placenta, and its level markedly increases in pregnancy. The enzyme obviously plays a role[17] in the protection against histamine and other biogenic amines produced by the animal organism, intestinal microflora, or contained in food. Since human diamine oxidase resembles in substrate specificity classical hog kidney diamine oxidase[18], it is almost sure that antimalaric doses of quinine can interfere with this protective system: the inhibition cannot be suppressed by the amines acumulated because of its noncompetitiveness. High doses of quinine administered orally produce gastralgia, vomiting, and diarrhea[19]; the effects of quinidine, cinchonine, and cinchonidine are similar. It cannot be excluded that the abortive effects of these bases are connected with the inhibition of diamine oxidase. Interest deserves the fact that even lobeline whose oral application used to be recommended against tobacco habituation and against respiratory affections, shows undesired effects on the digestive tract[19].

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