INTERACTION BETWEEN HORSE LIVER ALCOHOL DEHYDROGENASE AND APORPHINE ALKALOIDS*

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Dedicated to Professor J. Tomko on the occasion of his 60th birthday.

The interaction between horse liver alcohol dehydrogenase (ADH) and aporphine alkaloids has been studied by kinetic and fluorescence methods. The aporphine alkaloids inhibit ADH. The inhibitory effect depends on the position and type of the substituents in the aporphine nucleus. Aporheine shows the strongest binding to the enzyme, and that irrespective of the configuration of the molecule. The dissociation constant of the complex enzyme-aporpheine is 20—25 μmol l^{-1}. The results indicate that the aporphine alkaloids bind to the active center of alcohol dehydrogenase with stechiometry 1 : 1.

Isoquinoline alkaloids include a series of biologically active compounds which have found practical application[1-3]. One of the ways how to clarify the fundamental mechanism of their biological activity is to study the interactions of these alkaloids with the biopolymers. The purpose of our work has been to study the interaction between aporphine alkaloids[4] and horse liver alcohol dehydrogenase (ADH). Our data were compared with those obtained from studies of the binding of berberine alkaloids[5-7]. Evidence has been provided that berberine alkaloids bind to the extensive hydrophobic domain of the active center of the enzyme, they have an inhibitory effect on its activity, and during binding to the biopolymer they change their optical properties, especially the fluorescence[7].

EXPERIMENTAL

Materials. Horse liver ADH was isolated and characterized as previously described[8]. A part of this enzyme was obtained from Boehringer. The commercial enzyme was dialyzed against a sodium phosphate buffer of pH 7, *I* 0·1 prior to use. The enzyme concentration has been given

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as the molar concentration of the active centers. The coenzyme NAD and NADH were supplied by Boehringer, ADP by Reanal, *o*-phenanthroline by Lachema, chloroprothixene by Farmakon, and ethylberberine was prepared and characterized as previously described[8,9]. The aporphine alkaloids were made available from the collections of the Institute of Med. Chemistry (Olomouc).

Spectrometric and kinetic measurements were carried out on a Cary 118 spectrophotometer in a sodium phosphate buffer of pH 7, *I* 0·1 at 25°C. The inhibition was estimated with the help of double reciprocal plots (reciprocal reaction velocity *vs* reciprocal NAD or ethanol concentrations), the effect of the other inhibitors was determined according to Yonetani and Theorell[10].

Fluorescence and fluorescence-polarizing measurements were carried out on an Aminco--Bowman spectrofluorometer (equipped with a polarizing adapter). The polarization (*P*) was calculated according to the relation:

$$P = \frac{I_{VV} - GI_{VH}}{I_{VV} + GI_{VH}}, \quad G = \frac{I_{HV}}{I_{HH}},$$

where *I* is the intensity of the light, the indexes V and H are symbols for the positions of the excitation and the emission polarizer (vertical and horizontal, respectively), and *G* stands for the correction factor. The relative quantity of the bound ligand to the enzyme ($\bar{x}$) was calculated according to the relation[11]:

$$\bar{x} = 1 + \frac{(P/P_b - 1)\, F/F_0}{1 - P_0/P_b},$$

TABLE I

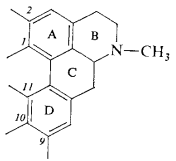
Inhibition of alcohol dehydrogenase with aporphine alkaloids. Measured in sodium-phosphate buffer at pH 6·0 (*I* 0·05), c_{CH_3CHO} 2 mmol l^{-1}, c_{NADH} 0·1 mmol l^{-1}, c_{ADH} 0·1 μmol l^{-1}. $I_{0.5}$ designates the concentration of the inhibitor which causes a decrease in the initial rate to one half

Alkaloid	Substitution					$I_{0.5}$ μmol l^{-1}
	$C_{(1)}$	$C_{(2)}$	$C_{(9)}$	$C_{(10)}$	$C_{(11)}$	
(+)-Aporheine (*I*)	OCH_2O		H	H	H	95
(−)-Aporheine (*I*)						95
(−)-Nucipherine (*II*)	OCH_3	OCH_3	H	H	H	400
(+)-Domestine (*III*)	OCH_3	OCH_3	OCH_2O		H	300
(+)-Glaucine (*IV*)	OCH_3	OCH_3	OCH_3	OCH_3	H	680
(−)-Glaucine (*IV*)						650
(+)-Bulbocapnine (*V*)	OCH_2O		H	OCH_3	OH	850
(+)-Isocorydine (*VI*)	OCH_3	OCH_3	H	OCH_3	OH	>1 000
(+)-Mecambroline (*VII*)	OCH_2O		H	OH	H	>1 000
(+)-Isothebaine (*VIII*)	OCH_3	OCH_3	H	H	OCH_3	>1 000

where P and F are the values of the polarization and the fluorescence of the ligand in the presence of the enzyme. P_b is the polarization of the ligand completely bound to the enzyme, P_0 and F_0 are the values of the polarization and the fluorescence of the free ligand. The polarization titration data were processed according to Scatchard[12].

RESULTS AND DISCUSSION

The aporphine alkaloids inhibit ADH weakly up to fairly strongly, some of them do not inhibit the activity of the enzyme practically at all (Table I). If compared with the effect of the berberine alkaloids on this enzyme[9], their effect is somewhat weaker. As in the berberine alkaloids, the inhibitory effect depends on the position and type of the substituents in the aporphine nucleus. A considerable increase in the affinity to the enzyme can be seen in compounds having a methylenedioxy group on ring A *vs* compounds with methoxyl groups (aporheine (*I*) *vs* nucipherine (*II*), bulbocapnine (*V*) *vs* isocorydine (*VI*)) or in those having the methylenedioxy group on ring D (domestine (*III*) *vs* glaucine (*IV*) and nucipherine (*II*)). The firmer binding



I–VIII

to the enzyme can be accounted for by an enlargement of the planar portion of the molecule (rings A and D), and by an increase in the dipole moment of the molecule of the ligand, respectively. The presence of a hydroxyl group on ring D decreases the inhibitory activity if compared with that of the unsubstituted ring D (mecambroline (*VII*) *vs* aporheine (*I*)), which may be due to an unrequired ionization of the hydroxyl group. It appears that substitution at $C_{(11)}$ of the ring D is unfavourable for the interaction with ADH.

The ADH inhibition depends on the conformation of the aporphine system. According to the UV, ^{1}H-NMR spectra and the optical rotatory data, the alkaloids substituted in the positions 11 or 10, 11 possess a rigid conformation which compared to derivatives with an unsubstituted ring D or with substituents in the positions 9, 10 is characterized by a greater deflection of the aporphine system from planarity[13]. Thus, we explain the weakening of the interactions with the enzyme in bulbocapnine (*V*), isocorydine (*VI*) and isothebaine (*VIII*). The configuration of the aporphine system has practically no effect on the inhibitory power (Table I). The binding ADH

site for aporphines is obviously non-planar but it is flexible enough to allow the binding of both non-planar enantiomers.

The strongest ADH inhibitor of the tested aporphine alkaloids is aporheine (*I*). The inhibitory power of aporheine (*I*) depends on the pH of the medium; it is stronger in the acidic region (under pH 5·5, the values $I_{0.5}$ are *c.* 70 μmol l^{-1}) and weaker in the alkaline region (above pH 7, the $I_{0.5}$ reaches values of *c.* 170 μmol l^{-1}). This finding is in agreement with the p*K* value of aporheine[14] (6·12) and it indicates a firmer binding of the cationic form of the alkaloid to ADH. A similar phenomenon was observed in connection with the binding of other cationic ADH inhibitors, *e.g.* chloroprothixene[15] and acridine orange[8].

The interpretation of the results of kinetic studies of the inhibition of aporheine (*I*) is difficult in view of the complexity of the mechanism of the reaction catalyzed by ADH. At low ethanol concentrations, the ADH is governed by the Theorell–Chance mechanism[16] (the formation of the ADH–ethanol complex is kinetically negligible). Since in our measurements comparatively high ethanol concentrations have been used (Table II, where K_m for ethanol[17] equals 590 μmol l^{-1}), there are not fulfilled the conditions for the validity of the Theorell–Chance mechanism but more likely for the mechanism of "preferred pathway"[16,18]. For the relatively high NAD concentrations, the inhibitory constants calculated from double reciprocal plots for variable ethanol concentrations can be interpreted in such a manner that K_{ii} fairly corresponds to the dissociation constant for the dissociation of the inhibitor from the complex enzyme–NADH–inhibitor, whereas K_{is} roughly reflects the dissociation constant of the enzyme–NAD–inhibitor complex. At a variable NAD concentration, the value K_{ii} reflects the interaction of the inhibitor with the enzyme–

TABLE II

Inhibitory behaviour of aporheine (*I*) to alcohol dehydrogenase at pH 7·0. $c_{Aporheine}$ 0, 30, and 100 μmol l^{-1}, c_{ADH} 0·05 μmol l^{-1}. K_{ii} and K_{is} are mean values of the inhibitory constants of the intercept and the slope determined on the basis of the double reciprocal plots

Variable concentration mmol l^{-1}	Fixed concentration mmol l^{-1}	Type of inhibition	K_{ii} μmol l^{-1}	K_{is} μmol l^{-1}
Ethanol (0·5—50)	NAD (0·31)	competitive--noncompetitive	54	21
NAD (0·02—04)	ethanol (3)	noncompetitive	111	111
NAD (0·02—04)	ethanol (10)	acompetitive	55	→∞

–NADH complex. The K_{is} constant can be interpreted in dependence on the fixed ethanol concentration. At a high ethanol concentration, K_{is} characterizes particularly the binding of the inhibitor to the enzyme-ethanol complex, at a low ethanol concentration, the value K_{is} also significantly reflects the ability of the binding of the inhibitor to the free enzyme.

The results of the kinetics of ADH inhibition with aporheine (*I*) show (Table II) that in the presence of NAD the binding of aporheine to ADH is stabilized. This finding is supported by the fact that the K_{is} value is low for the variable ethanol concentration if compared with the K_{ii} values which characterize the binding of aporheine to the ADH–NADH complex. On the other hand, the binding of ethanol to the enzyme inhibits the interaction of aporheine with ADH as is shown by the K_{is} value at high fixed ethanol concentrations for the variable NAD concentration ($K_{is} \to \infty$). The competitive behaviour of aporheine *vs* ethanol is consistent with the fact that aporheine acts as a total inhibitor. Since for ethanol in a concentration of 3 mmol l^{-1} and for the variable NAD concentration, the K_{is} value can be determined (Table II), it is obvious that a binary ADH-aporheine complex is also formed. However, this value cannot be interpreted as a dissociation constant of this binary complex because the interference of the ADH–ethanol complex is too strong.

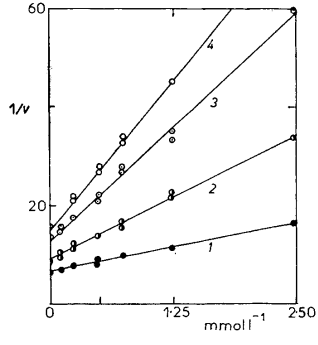


FIG. 1

ADP Effect (mmol l^{-1}) on ADH inhibition with aporheine. Sodium-phosphate buffer of pH 7·0 (*I* 0·1), c_{CH_3CHO} 0·1 mmol l^{-1}, c_{NADH} 0·01 mmol l^{-1}. *v* initial reaction rate in relative units; $c_{aporheine}$ *1* 0, *2* 30, *3* 100, and *4* 200 μmol l^{-1}

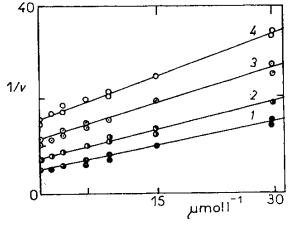


FIG. 2

Effect of *o*-phenanthroline (μmol l^{-1}) on ADH inhibition with aporheine. The experimental conditions are identical with those in Fig. 1

For a closer characterization of the interaction between aporphine alkaloids and ADH, the effect of other known inhibitors of this enzyme on the aporheine binding (*I*) has been studied. The interaction between aporheine and the enzyme is stabilized by the presence of ADP (Fig. 1). Thus, the assumption is confirmed that the aporphine alkaloids do not bind to the binding site for the coenzyme and its fragments and that the coenzyme and its fragments favourably influence the interaction between aporheine and ADH. The aporphine alkaloids do not bind directly to zinc in the active ADH center but evidently not too far from it. This results from the mutual

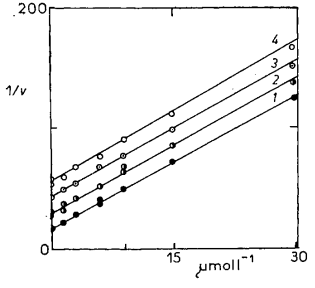


FIG. 3

Effect of 13-ethylberberine (μmol l^{-1}) on ADH inhibition with aporheine. The experimental conditions are identical with those in Fig. 1

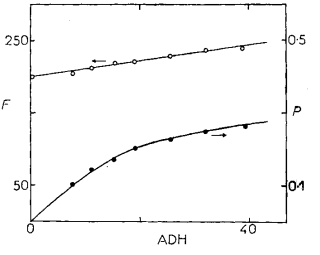
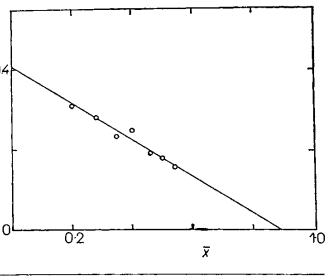


FIG. 4

Changes in the intensity of fluorescence (*F*, ○) and in the polarization of fluorescence (*P*, ●) during increasing ADH concentration (μmol l^{-1}) in the medium. Sodium-phosphate buffer of pH 7·0 (*I* 0·1), $c_{aporheine}$ 4·7 μmol l^{-1}; λ_{exc} 320 nm and λ_{em} 370 nm

FIG. 5

Processing of polarization titration data according to Scatchard. The experimental conditions are identical with those in Fig. 4. The relative quantity of aporheine bound to the enzyme ($\bar{x}$), M_f indicates the concentration of free enzyme subunits in μmol . l^{-1}; the value of the dissociation constant (negative reciprocal slope of the obtained curve) equals 21 μmol l^{-1}; the molar binding ratio of the active center of the enzyme to aporheine (the intercept of the axis $\bar{x}$) is equal to 0·89



effect of aporheine and *o*-phenanthroline (zinc chelating agent) (Fig. 2). During the binding of these two inhibitors to ADH there occurs a slight labilization of the corresponding complexes which contain both simultaneously bound compounds. Aporheine (*I*) and 13-ethylberberine are mutually competitive and the obtained straight lines take a parallel course (Fig. 3). A similar dependence is to be seen in the presence of chloroprothixene. The berberine alkaloids and the psychopharmaca of chloroprothixene type bind to the extensive hydrophobic ADH substrate site ("active site pocket") to occupy two very close binding sites[7,9,14]. Consequently, the aporheine alkaloids are in all probability bound to the same site of the enzyme.

The fluorescence measurements have shown that when aporheine (*I*) binds to ADH it comes to a slight increase in the intensity of the fluorescence of aporheine (Fig. 4). A similar phenomenon is observed in hydrophobic solvents. The binding site of ADH for aporphine alkaloids is in agreement with the assumed hydrophobic nature. On the basis of the graphical treatment of the data of polarizing titration (Scatchard's Diagram – Fig. 5, plot according to Eisenthal and Cornish-Bowden[18]), the value of the dissociation constant of the binary complex ADH-aporheine has been determined as $20-25\ \mu\text{mol}\ l^{-1}$, and that of the stechiometry of interaction between aporheine and the active center of the enzyme approaches the value 1·0. This is in agreement with the assumed existence of one binding site for aporphine alkaloids in the active ADH center. The value of the dissociation constants of the binary complex approaches values of the inhibition constants given in Table II.

The measurements of the polarization of fluorescence provide confirmatory evidence for the stabilization of the ADH–aporheine complex in the presence of the coenzyme. Addition of NAD to the mixture of ADH and aporheine in unsaturated concentrations of the enzyme brings about a further increase in polarization. A similar stabilizing NAD effect on the binding to the enzyme has also been observed in other ligands which are bound to the substrate ADH domain, *e.g.* in auramine O (ref.[19,20]) chloroprothixene[15], and acridine orange[8].

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