INTERACTION OF LIVER ALCOHOL DEHYDROGENASE WITH PROTOBERBERINE ALKALOIDS

S.PAVELKA[a] and J.KOVÁŘ[b]

[a] *Institute of Medical Chemistry, Purkyně University, 662 43 Brno and*
[b] *Department of Biochemistry, Purkyně University, 611 37 Brno*

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Dedicated to Professor F. Šantavý on the occasion of his 60th birthday.

Kinetic and fluorometric equilibrium studies were carried out of the interaction of liver alcohol dehydrogenase with berberine derivatives and with a series of related alkaloids of the protoberberine group. The inhibitory power of the compounds tested was correlated with their structure and with some properties following from this structure, such as polarity, energy of their internal charge-transfer transition, and with the character of the individual substituents on their protoberberine backbone. The most effective inhibitor of liver alcohol dehydrogenase of the compounds tested is 13-ethylberberine which is bound more firmly to the enzyme at pH 10 than NAD and NADH. It follows from the results presented that a charge transfer may take place during the interaction of liver alcohol dehydrogenase with the compounds tested and that simultaneously may participate on this process a hydrophobic group or region of the enzyme which is located in the immediate neighborhood of the binding site for the alkaloid.

Berberine (*III*) and related alkaloids of the protoberberine group are inhibitors of various strength of horse liver alcohol dehydrogenase[1]. These compounds form fluorescent complexes with liver alcohol dehydrogenase; of these complexes the one formed by the enzyme and berberine has been examined in detail. The binding of berberine to liver alcohol dehydrogenase is paralleled by a considerable increase in fluorescence intensity compared to free berberine and by a shift of the fluorescence maximum toward lower wavelengths. Analogous changes in the emission spectrum of berberine are observed during its dissolving in solvents less polar than water. This characteristic feature makes berberine a "hydrophobic probe"[2].

The aim of this study is to characterize the series of berberine derivatives and of some related compounds of the protoberberine group with respect to their ability to inhibit liver alcohol dehydrogenase and to document their interaction with the enzyme with changes in their fluorescence. We have also made an effort to find a relation between the ability of these compounds to interact with liver alcohol dehydrogenase and their structure and some properties following from this structure. We have focused our attention especially on berberine derivatives substituted at carbon C(13) since we observed earlier[1] that corysamine (*XI*) (13-methylcoptisine) is a considerably stronger inhibitor of liver alcohol dehydrogenase than berberine.

EXPERIMENTAL

Materials

Horse liver alcohol dehydrogenase, isolated by a modified method of Theorell and coworkers[3], was used in the experiments. The purity of the enzyme used, determined from its absorption coefficient[4] at 280 nm (0·455 mg^{-1} cm^2), was 60–70%. The concentration of the enzyme was determined from kinetic measurements of its activity[5]. It was 0·5–0·6 μN and 0·03–0·1 μN for spectrometric and fluorometric measurements respectively. Coenzymes NAD and NADH were from Serva (quality degree III). NAD concentration was determined by enzymically catalyzed reduction using yeast alcohol dehydrogenase[6]. The measurements were made at 23·5°C in sodium phosphate buffer at pH 7 and ionic strength *I* 0·1 and in glycine buffer at pH 10. The remaining chemicals and solvents used were of commercial analytical purity grade.

The compounds tested were partly synthetized[7] (*VI*, *VII*, *XII*, *XIII*, *XIV*, *XV*, *XIX*, *XXIII*, and *XXIV*), small samples of the remaining alkaloids were gifts. Their purity was checked by the determination of melting points, by thin-layer chromatography, by ultraviolet and visible spectrometry, and also by nuclear magnetic resonance spectrometry. All these compounds share in common the basic protoberberine backbone (Table I). The compounds studied were used in the form of quaternary salts, chlorides, alternatively iodides (type a berberines), tetrahydro derivatives were used in the form of tertiary bases and N-methyltetrahydro derivatives also in the form of quaternary salts (type b berbines). Protoberberine (*I*), berberine (*III*), and 13-methylberberine (*XII*) were tested as salts of various acids (chloride, iodide, sulfate); almost the same results were obtained as regards the inhibitory power of different salts of the same alkaloid. Therefore only the trivial names of the alkaloids are mostly given below, regardless of the salt. The structure of the compounds tested is shown in Table I. Their concentrations were determined spectrophotometrically from the measured absorption coefficients[7].

Methods

The spectrophotometric kinetic measurements were carried out in Beckman DU Spectrophotometer in standard equipment with ERA recording accessory and Honeywell recorder. For fluorometric measurements the same spectrophotometer with a fluorescence accessory was used; the latter consisted of a low-pressure mercury lamp with Schott UG 11 filter as excited radiation source. The absorption spectra were measured in Specord UV-VIS Spectrophotometer (Zeiss, Jena) and the fluorescence spectra in a recording spectrofluorometer; the latter was built by Dr Smékal and consisted of two Zeiss monochromators, a 500 W xenon lamp (XBO 500) as radiation source, and EMI 9558 QB photomultiplier as detector of emitted radiation.

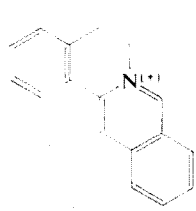
The inhibitory power of the compounds tested (% of inhibition and $K_{0.5}$-values) were established by spectrophotometric determination of the enzyme activity at pH 10 according to Dalziel[5]. The inhibition constants were determined by fluorometric measurements of the reaction rate[8]. The measurements were made in quartz cyllindrical Wycor cells; the total volume of the reaction mixture was 4·0 ml and the fluorescence increase at 460 nm was recorded. Since the inhibitors tested strongly absorbed in the wavelength range of the excited radiation (c. 365 nm), a correction was made for the change in radiation intensity emitted by coenzyme NADH (resulting from enzymically catalyzed NAD reduction), caused by the presence of the inhibitors. The intensity of fluorescence of a known quantity of NADH in solutions containing different inhibitor concentrations was therefore measured. Calibration curves were obtained by plotting the ratios of NADH fluorescence values at a certain inhibitor concentration and the NADH fluorescence values at zero inhibitor concentration *versus* the inhibitor concentrations used. From these curves

TABLE I

Structure of Compounds Tested

(*a*) Basic Backbone of Compounds of Type *a*, (*b*) of Compounds of Type *b*.
Designation of rings of protoberberine backbone and numbering of carbon atoms see next page.
Substitution of compound *XX* on C (1), by OCH_3 compound *XXI* on C (1), by OCH_3 and compound *XXIII* on C (8) by CH_3.

Number	Type	Name	Substituents on						
			C (2)	C (3)	C (9)	C (10)	C (11)	C (13)	
I	*a*	protoberberine	H	H	H	H	H	H	
II	*a*	coptisine	O—CH_2—O		O—CH_2—O		H	H	
III	*a*	berberine	O—CH_2—O		OCH_3	OCH_3	H	H	
IV	*a*	palmatine	OCH_3	OCH_3	OCH_3	OCH_3	H	H	
V	*a*	jatrorrhizine	OCH_3	OH	OCH_3	OCH_3	H	H	
VI	*a*	berberrubine	O—CH_2—O		OH	OCH_3	H	H	
VII	*a*	ethylberberrubine	O—CH_2—O		OC_2H_5	OCH_3	H	H	
VIII	*a*	ψ-coptisine	O—CH_2—O		H	O—CH_2—O		H	
IX	*a*	ψ-epiberberine	OCH_3	OCH_3	H	O—CH_2—O		H	
X	*a*	ψ-palmatine	OCH_3	OCH_3	H	OCH_3	OCH_3	H	
XI	*a*	corysamine	O—CH_2—O		O—CH_2—O		H	CH_3	
XII	*a*	13-methylberberine	O—CH_2—O		OCH_3	OCH_3	H	CH_3	
XIII	*a*	13-ethylberberine	O—CH_2—O		OCH_3	OCH_3	H	C_2H_5	
XIV	*a*	13-methoxyberberine	O—CH_2—O		OCH_3	OCH_3	H	OCH_3	
XV	*a*	13-ethoxyberberine	O—CH_2—O		OCH_3	OCH_3	H	OC_2H_5	
XVI	*b*	stylopine	O—CH_2—O		O—CH_2—O		H	H	
XVII	*b*	canadine	O—CH_2—O		OCH_3	OCH_3	H	H	
XVIII	*b*	thalictricavine	O—CH_2—O		OCH_3	OCH_3	H	CH_3	
XIX	*b*	N-methylcanadinium chloride	O—CH_2—O		OCH_3	OCH_3	H	H	
XX	*b*	N-methyl-13,14-didehydro-1-methoxycanadinium chloride	O—CH_2—O		OCH_3	OCH_3	H	H	OCH_3
XXI	*a*	1-methoxyberberine	O—CH_2—O		OCH_3	OCH_3	H	H	OCH_3
XXII		5,8-dihydro-9,10-dimethoxy-13-methyl-6*H*-benzo[*g*]-1,3-dioxolo-[7,8]benzopyrano[3,4,5-*ija*]-quinolizinium chloride							
XXIII	*a*	coralyne	OCH_3	OCH_3	H	OCH_3	OCH_3	H	CH_3
XXIV	*a*	norcoralyne	OCH_3	OCH_3	H	OCH_3	OCH_3	H	



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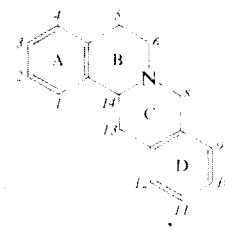

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TABLE II

Results of Kinetic Measurements

Measured spectrophotometrically in glycine buffer at pH 10 and 23·5°C.

Compound	Inhibition, %							$K_{0.5}$, μM
Concentration, μM	20	40	50	100	200	300		
Protoberberine *I*			15	24	30	35		800
Coptisine *II*	26	42	45	57				70
Berberine *III*	17	26	30	43				120
Palmatine *IV*		8	12	25				450
Jatrorrhizine *V*		23	26	35				300
Berberrubine *VI*		18	22	32	41			360
Ethylberberrubine *VII*			23	40				≈160
ψ-Coptisine *VIII*				35				>300
ψ-Epiberberine *IX*			12	20				>800
ψ-Palmatine *X*				10				>1 000
Concentration, μM	0·5	1	2	5	10	20	40	
Corysamine *XI*			14	31	47	62	75	11·5
13-Methylberberine *XII*			15	33	50	67		10
13-Ethylberberine *XIII*	25	43	63	82	90			1·3
13-Methoxyberberine *XIV*				23	34	48	64	22
13-Ethoxyberberine *XV*				30	46	63	76	12
Concentration, μM	10	20	40	50	100	200	300	
Stylopine *XVI*[a]	0							—
Canadine *XVII*[a]	0							—
Thalictricavine *XVIII*[a]	0							—
Compound *XIX*				0	0	0	0	—
Compound *XX*	27	41	56	62	75			30
1-Methoxyberberine *XXI*		21	38	43	59			70
Compound *XXII*		31	47	54	66			45
Coralyne *XXIII*				0	0	0	0	—
Norcoralyne *XXIV*				0	0	0	0	—

[a] Saturated solutions (approximate concentration 10 μM).

the correction factors corresponding to the given inhibitor concentrations were read. The measured values of reciprocal initial reaction rates were multiplied by these factors; the K_i-values were then determined from the Lineweaver–Burke graphical plot of the values thus corrected. The dissociation constants of the enzyme–inhibitor complex K_{EI} were established by fluorometric titration of the enzyme solution by the corresponding inhibitor, as described earlier[1].

RESULTS AND DISCUSSION

The results of kinetic inhibition measurements of enzyme activity as regards alcohol oxidation[5] are given in Table II. Per cent of inhibition at the given inhibitor concentration is given side by side with $K_{0.5}$-values, *i.e.* such inhibitor concentrations at which the measured rate of the reaction catalyzed by liver alcohol dehydrogenase equals half the rate of the same reaction measured under identical conditions and at zero inhibitor concentration. These values were obtained in the case of strong inhibitors directly from the graphical plot of the degree of inhibition *versus* the concentration of the corresponding inhibitor; in the case of weak inhibitors the values were extrapolated from the corresponding graphs. The results given in Table II will serve as a basis of all considerations which follow.

Effect of Hydrophobic Character of Berberines on their Inhibitory Power

We used parameter log P (where P is the partition coefficient for octanol–water) as a measure of the effect of hydrophobic character of the compounds studied on their association with the enzyme (or better, on the log of reciprocal dissociation constant or reciprocal $K_{0.5}$). The log P values were calculated from the data for berberine (log $P = -2{\cdot}12$) as a sum of this value and of the contributions of the individual substituents to the change of log P for berberine[9].

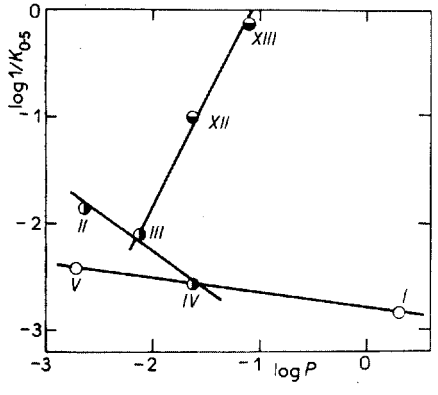


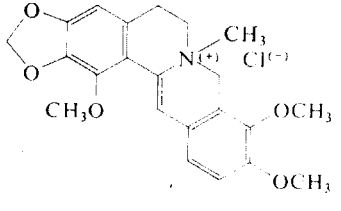
FIG. 1

Dependence of Inhibitory Power of Berberines on their Polarity

The polarity is expressed by means of parameter log P, where P is the partition coefficient for octanol–water and the compound given. The inhibitory power is expressed by log $1/K_{0.5}$, where $K_{0.5}$ stands for such an inhibitor concentration at which the measured rate of the enzymic reaction is equal half the rate of the same reaction measured under identical conditions in the absence of the inhibitor.

It follows from the graphical plot of log $1/K_{0.5}$ *versus* log P, shown for the most important compounds tested in Fig. 1, that the decrease of hydrophobic character of the inhibitors caused by the substitution of hydrogen atoms of aromatic rings A and D of protoberberine by methoxyl groups (sequence protoberberine (*I*), palmatine (*IV*), jatrorrhizine (*V*)) and/or by the replacement of the pair of the *ortho*-methoxyl groups by a methylenedioxy group (sequence palmatine (*IV*), berberine (*III*), coptisine (*II*)) results in an increase of their inhibitory power. On the other hand, the increase of the hydrophobic character of berberine derivatives caused by labelling carbon C(13) with an alkyl also increases the inhibitory power of these derivatives compared to unsubstituted berberine, even more intensively than in the first case (sequence berberine (*III*), 13-methylberberine (*XII*), 13-ethyberberine (*XIII*), *cf.* Fig. 1).

Obviously, it is not only the hydrophobic character of the substituents which plays a role in the observed effects of substitution of the protoberberine backbone on the inhibitory power of the corresponding derivatives: this is evidenced by the fact that the slopes of the profiles shown in Fig. 1 are completely different. Likewise, the different effect of substitution of the methoxyl group by a hydroxyl in case of jatrorrhizine and berberrubine on the inhibitory power of these compounds shows that the polarity or nonpolarity itself of the inhibitor is not decisive for its inhibitory power. Jatrorrhizine (*V*) can be derived from palmatine (*IV*) by replacing the methoxyl group by a hydroxyl on carbon C(3), berberrubine (*VI*) by the same substitution on carbon C(9) from berberine (*III*). Whereas jatrorrhizine is – in accordance with its higher polarity – a stronger inhibitor than palmatine, the inhibitory power of berber-



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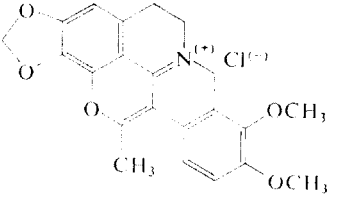
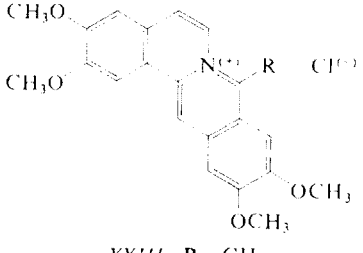


XXII



XXIII, R = CH_3
XXIV, R = H

rubine is lower than that of berberine (Table II). One of the possible explanations of the different effect of the substitution of the methoxyl group on the A or D ring, respectively by a hydroxyl can lie in the different ability of these phenolic protoberberine alkaloids to donate a proton and thus to form the corresponding ampho ions of different inhibitory power.

Relations between Internal Charge Transfer (CT) in Berberines and their Inhibitory Power

Compounds of the protoberberine type are capable of internal charge transfer which is reflected by a little intensive maximum of their absorption spectrum in the highest wavelength range[10]. The energy of the excited state of these compounds is emitted in the form of fluorescent radiation. Since berberine behaves as a hydrophobic probe, whose characteristic feature is an increase of the dipole moment in excited state[2], these compounds have been examined also from this viewpoint. Their CT absorption and emission energies were measured in water, ethanol, and dioxane (medium of different dielectric constant) and the approximate value of change of their dipole moment during the transition from ground to excited state was calculated according to the equation derived by Lippert[11]:

$$\tilde{\nu}_A - \tilde{\nu}_F \approx \frac{2}{hc_0}\left(\frac{\varepsilon - 1}{2\varepsilon + 1} - \frac{n - 1}{2n^2 + 1}\right) \cdot \left(\frac{(\mu_e - \mu_g)^2}{a^3}\right) + \text{const.}$$

In this equation $\tilde{\nu}_A$ is the wave number of the last absorption maximum, $\tilde{\nu}_F$ the wave number of the fluorescence maximum, h Planck's constant, c_0 the velocity of light *in vacuo*, ε the static dielectric constant of the solvent, n the refractive index of the solvent, μ_e the dipole moment of the first singlet state of the solute, μ_g the dipole moment of the ground state of the solute, and a the radius of Onsagers cavity of the molecule of the solute.

The results of these measurements are summarized in Table III. The dipole moment values are calculated with a considerable error caused above all by the inexactly determined radius of Onsager's cavity (an approximate value $a \approx 5$ Å was taken for protoberberine and $a \approx 6$ Å for the remaining compounds); therefore the resulting values are only compared to each other and from the qualitative viewpoint only. The differences in dipole moments of the compounds measured are given as absolute values in Table III. As shown elsewhere[11], the dipole moment is higher in the excited state than in the ground state ($\mu_e > \mu_g$) on condition that *1*) the spectral shifts resulting from solvent changes are smaller for the last absorption maximum than the corresponding shifts of the fluorescence maximum, *2*) the emission energy drops during the transition from a solvent with a lower dielectric constant to a solvent with a higher

dielectric constant. The quantum fluorescence yield is with typical hydrophobic probes usually considerably lower in a more polar solvent than in a nonpolar solvent.

It is obvious from Table III that *1*) berberine (*III*) only is a typical hydrophobic

TABLE III

Absorption and Fluorescence Characteristics of Some Compounds Studied and Approximate Values of Changes in their Dipole Moments During Excitation

The compound solutions were excited at the wavelength of the last absorption maximum of the corresponding compound. The relative fluorescence intensities are those of aqueous and dioxane solutions of these compounds at $5 . 10^{-5}$M concentration.

Compound	Solvent	$\tilde{\nu}_A$ 10^{-3} cm^{-1}	$\tilde{\nu}_F$ 10^{-3} cm^{-1}	$\mu_e - \mu_g$ D	Relative intensity
Protoberberine *I*	water	27·50	23·90	5	13 200
	ethanol	27·43	23·70		480
Coptisine *II*	water	21·67	18·35	≈0	930
	ethanol	21·40	17·95		≈2 800
Berberine *III*	water	23·60	18·0	27	43
	ethanol	23·14	18·6		≈520
Palmatine *IV*	water	23·60	18·7	12	27
	ethanol	23·08	18·37		330
Jatrorrhizine *V*	water	23·54	19·6	5	23
	ethanol	22·75	18·95		340
Berberrubine *VI*	water	22·82	19·25	—	37
	ethanol	22·0	17·95		370
ψ-Epiberberine *IX*	water	27·15	20·0	—	100
	ethanol	26·35	18·7		920
Corysamine *XI*	water	22·2	18·85	0	220
	ethanol	22·0	18·65		1 530
13-Methylberberine *XII*	water	24·07	18·85	24	24
	ethanol	23·45	19·05		460
13-Ethylberberine *XIII*	water	23·95	19·25	13	32
	ethanol	23·54	19·10		140
13-Methoxyberberine *XIV*	water	23·50	19·05	—	91
	ethanol	23·10	18·35		410
13-Ethoxyberberine *XV*	water	23·50	18·6	14	66
	ethanol	23·16	18·55		105
1-Methoxyberberine *XXI*	water	23·64	19·2	17	31
	ethanol	23·10	19·1		270
Coralyne *XXIII*	water	23·52	20·75	20	≈15 300
	ethanol	23·30	21·15		≈3 200
Norcoralyne *XXIV*	water	24·05	20·7	23	≈5 000
	ethanol	23·6	21·05		≈3 800

probe because its dipole moment is higher in excited state than in the ground state and because the intensity of its fluorescence in dioxane is many times higher than in water. This behavior of berberine, atypical compared to that of other compounds of the berberine group, might be explained by asymmetric substitution on aromatic rings A and D: an important role here plays the different electron donor character of groups containing oxygen atoms with free electron pairs. It can be postulated that two methoxy groups in *ortho* position bound to aromatic ring D do not affect its basicity to such a degree as the methylenedioxy group bound in the same position since their conjugation effects are opposite. By contrast, there is a cooperation of the two oxygen atoms in the case of the methylenedioxy group since the nonbonding electrons of the second oxygen atom can transfer *via* the methylene bridge thus making it considerably more easier for electrons of the methylenedioxy group to join the conjugated π-electron system of the aromatic rings[10]. In the case of phenolic protoberberine alkaloids jatrorrhizine (*V*) and berberrubine (*VI*) with asymmetric substitution on A and D rings, still another factor plays a role, namely the ability of their phenolic hydroxyl to donate a proton and to form ampho ions of different properties. This characteristic feature of jatrorrhizine and berberrubine has been discussed above in connection with the effect of the polarity of these compounds on their inhibitory power. The effect of asymmetric substitution on A and D rings during the substitution of berberine on C(13) is obviously overlapped by the effects of substituents on C(13).

The action of substituents on the protoberberine backbone of the compounds studied can be explained by assuming that the interaction of the free electron pair with the conjugated π-electron system stabilizes the excited state of the molecule more than in the case of the unsubstituted molecule. Hence, the energy of transition from the ground to the excited state of molecule decreases after the replacement of the hydrogen atom by an electron donor group.

2) There is obviously no correlation between the change of dipole moment of the compounds studied during their excitation and their inhibitory power. The effect of the magnitude of the dipole moment of berberine derivatives in ground state on the inhibitory power of these derivatives will be discussed below in connection with the correlation of the sigma constants of substituents bound to C(13).

3) All the compounds studied, except for protoberberine (*I*), coralyne (*XXIII*), and norcoralyne (*XXIV*), show a more intensive fluorescence in less polar solvents than in more polar solvents. This could be caused by a better separation of energy levels of the singlet and triplet excited state in a less polar solvent or by smaller losses of energy of excited state by distortion motions of the molecule rings. The probability of the second possibility is suggested by the fact that the co-planarity of ring A with the remaining rings as a result of dehydrogenation of ring B (coralyne (*XXIII*), norcoralyne (*XXIV*)) and thus the practically impossible distortion of the rings increase many times the intensity of fluorescence.

4) There is no relation between the fluorescence intensity or change of fluorescence intensity, respectively, in various solvents and the inhibitory power of the berberines.

5) There is a certain, at least qualitative relation between the inhibitory power of the compounds studied and the energy of their CT transition. Compounds which do not show the latter (*XVI*, *XVII*, *XVIII*, *XIX*, *XXIII*, *XXIV*) do not inhibit at all. Compounds which show it in the short wavelength range, *i.e.* at a high excitation energy where CT is difficult (protoberberine (*I*), ψ-berberines (*VIII*, *IX*, *X*)) are weak inhibitors. On the other hand, coptisine (*II*) showing a low excitation energy is the strongest inhibitor of all protoberberine alkaloids studied if we disregard derivatives substituted on C(1) and C(13).

Appearingly the CT interaction can play a role in the interaction of these compounds with liver alcohol dehydrogenase. The region of C and D rings of the alkaloid molecule could act as an electron acceptor for an electron donor group of the enzyme during this interaction.

Relation between Inhibitory Power of C(13) Berberine Derivatives and Character of Substituents on C(13)

We examined some berberine derivatives substituted on C(13) in more detail since they showed the greatest inhibitory power. We determined the corresponding kinetic inhibition constants K_i for ethanol oxidation and the dissociation constants of the

TABLE IV

Comparison of Constants $K_{0.5}$, K_i, and K_{EI}, Characterizing Ability of Berberine and its Derivatives Substituted on C (13) to Bind to Liver Alcohol Dehydrogenase

Given are the ratios of fluorescence intensity of 30 μM solutions of the compounds in dioxane to fluorescence intensity of 30 μM solutions in water and the ratios of fluorescence intensity of 30 μM solutions of the compound in buffer at pH 7 after the addition of the enzyme (final enzyme concentration in cell 2 μN) to fluorescence intensity of these solutions without enzyme. The samples were subjected to excitation by radiation at 365 nm, the fluorescence was detected in the region of the corresponding emission maximums.

Compound	$K_{0.5}$(μM)	K_i(μM)		K_{EI}(μM)		$\frac{F_{diox.}}{F_{water}}$	$\frac{F(E+I)}{F(I)}$
	pH 10	pH 10	pH 7	pH 10	pH 7		
Berberine	120	115	40	90; 150	36	23	2·4
13-Methylberberine	10	8	13	8·2	11·5	24	3·7
13-Ethylberberine	1·3	0·7	0·9	1·5	2·0	35	9·6
13-Methoxyberberine	22	37	—	—	—	6	1·8
13-Ethoxyberberine	12	—	—	—	—	5·4	1·6

enzyme–inhibitor complex K_{EI} (Table IV). The $K_{0.5}$, K_i, and K_{EI}-values are similar for each compound given in all cases mentioned; we used therefore only the $K_{0.5}$--values which are the easiest to be obtained experimentally.

Table IV also lists changes in the intensity of fluorescence maximums of the compounds studied which take place during the transition of these compounds from an aqueous medium to a less polar solvent (dioxane) and analogous changes occuring during the interaction of these compounds with the enzyme. These changes serve as a basis for fluorometric titrations of the enzymes by inhibitors; these titrations, by which the K_{EI}-constants were determined, can be also used for a more detailed characterization of interaction of these compounds with liver alcohol dehydrogenase[12]. The increase of fluorescence of the inhibitor in the presence of the enzyme is a function of both $K_{0.5}$ and of the ratio of fluorescence intensity in dioxane to fluorescence intensity in water. Analogous relations can be found also for the remaining compounds (not listed in Table IV); however, the changes of fluorescence of these compounds during their interaction with the enzyme are mostly smaller, mainly because of the lower affinity of these compounds for the enzyme.

Fig. 2 shows the dependence of the inhibitory power of berberine derivatives on the hydrophobic character of substituents bound to C(13). As a measure of the hydrophobic character of the substituent serves the magnitude of its contribution to the change of log P of berberine; the inhibitory power of the corresponding derivatives is expressed by log $1/K_{0.5}$. The correlation of these two magnitudes is not too good. The parameters of the regression curve are $\log 1/K_{0.5} = 0{\cdot}88 + 0{\cdot}93 \log P$ (s 0·59).

We have made an effort to express the relation between Hammett constants of substituents on C(13) and the inhibitory power of the corresponding berberine derivatives. The σ_m constants correspond mostly to the induction effect of the substituents, the σ_p constants roughly express the sum of their induction and conjugation effect. The difference $(\sigma_p - \sigma_m)$ reflects the conjugation effect of substituents. The

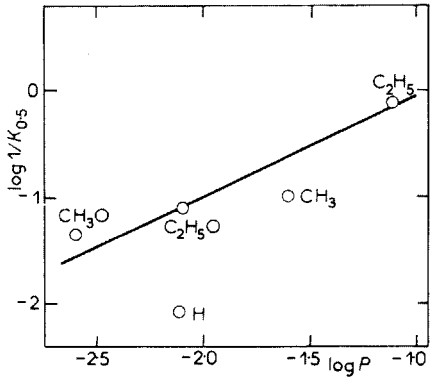


FIG. 2

Dependence of Inhibitory Power of Berberine and its C(13) Derivatives on Polarity of Substituents on C(13)

The symbols are the same as those given in the legend to Fig. 1.

correlation of each of these three parameters with the inhibitory power of the corresponding derivatives, however, is bad. The standard deviation of the extrapolated regression curves is higher than 0·7 in all cases. Thus, *e.g.* we obtained the following regression line for parameter σ_p : $\log 1/K_{0.5} = -1{\cdot}12 + 0{\cdot}02\sigma_p$ (s 0·72).

Since the correlation of nonpolarity of substituents on C(13) and of their Hammett constants with the inhibitory power of the corresponding berberine derivatives were bad, we tried to link together both correlations and to bring the inhibitory power into relation with both parameters simultaneously[13]:

$$\log 1/K_{0.5} = a + b \log P + c\sigma .$$

The results of these regressions are given in Table V and they can be summarized in the following fundamental points:

1) The correlation is good if σ_m and also the difference $(\sigma_p - \sigma_m)$ are used together with $\log P$ as parameters. The best correlation was obtained when parameters $\log P$ and σ_p (s 0·14) were used. In spite of that, the results should be judged with reservation

TABLE V

Comparison of Values of $\log (K_{0.5})_o/K_{0.5}$, Determined from Kinetic Inhibition Measurements with Values Calculated from Correlation Equations with Parameters $\log P$ and σ for Berberine (*III*) and its Derivatives Substituted on C (13).

To make the calculation easier, $\log P$ for berberine (*III*) was taken equal zero. The inhibitory power of the compounds is expressed in $\log (K_{0.5})_o/K_{0.5}$, where $(K_{0.5})_o$ stands for $K_{0.5}$ for berberine (120 μM).

Correlation Equations

1) $\log (K_{0.5})_o/K_{0.5} = 0{\cdot}35 + 2{\cdot}16 \log P + 8{\cdot}6\,\sigma_m$ (s 0·31)

2) $\log (K_{0.5})_o/K_{0.5} = -0{\cdot}04 + 1{\cdot}59 \log P - 3{\cdot}65\,(\sigma_p - \sigma_m)$ (s 0·24)

3) $\log (K_{0.5})_o/K_{0.5} = 0{\cdot}02 + 1{\cdot}08 \log P - 4{\cdot}36\,\sigma_p$ (s 0·14)

Compound	Subst.	$\log P$	σ_m	$\sigma_p - \sigma_m$	σ_p	Measured	$\log (K_{0.5})_o/K_{0.5}$ calc. from Eq.		
							(*1*)	(*2*)	(*3*)
III	H	0	0	0	0	0·00	0·35	−0·04	0·02
XII	CH_3	0·5	−0·07	−0·10	−0·17	1·08	0·84	1·11	1·30
XIII	C_2H_5	1·0	−0·07	−0·09	−0·16	1·96	1·92	1·56	1·80
XIV	OCH_3	−0·48	0·12	−0·38	−0·26	0·74	0·38	0·49	0·65
XV	OC_2H_5	0·02	0·10	−0·34	−0·24	1·00	1·25	1·01	1·08

since only a small series of compounds were examined and the values of sigma constants of the corresponding substituents do not differ much from each other.

2) A decrease of the polarity of the substituent on C(13) unambiguously increases the inhibitory power of the corresponding derivative in all the regressions given: b is larger than zero in all cases. It can be assumed therefore that the binding site of the enzyme for berberine is at least partly of hydrophobic character and that this hydrophobic group of the enzyme comes into the neighborhood of carbon C(13) during the interaction of the enzyme with the inhibitor.

3) A fact deserving interest is that the inhibitory power of the derivatives decreases with the increasing values of σ_p and $(\sigma_p - \sigma_m)$ of the substituents $(c < 0)$ and on the contrary increases with the increasing values of σ_m of the substituents $(c > 0)$. This fact can be explained perhaps by a different action of the induction and conjugation effects of substituents bound to carbon C(13) on the magnitude of the positive charge in the region of C and D rings and on the size of the conjugated system.

The substituents on C(13) with an -I effect remove electrons from the C and D rings and thus increase the positive charge in this part of the molecule. This enhances the electron acceptor nature of this system during the postulated CT interaction with the enzyme. The +M effect of the substituents on C(13) is reflected by the participation of mobile electrons of these substituents (free electron pairs of oxygen or electron contribution of alkyls by hyperconjugation) in conjugation with π-electrons of C and D rings; this leads to an extension of the conjugated system, similarly to the case of electron donor substitution on carbons C (9, 10). This extension of the conjugated system can facilitate the interaction of berberines molecules with the electron donor parts of the enzyme. (The most probable electron donors for a CT interaction in the enzyme molecule are the side chains of aromatic amino acids, especially the indole ring of tryptophan). It is essential that the −I and +M effects of the substituents act simultaneously. This follows from the fact that the correlation of the inhibitory power of berberine derivatives with their parameters log P and σ_p is the best of all correlations (s 0·14).

CONCLUSIONS

Structure Necessary for Inhibition

The necessary structure which the compounds must have in order to be able to exert an effect on the reaction catalyzed by liver alcohol dehydrogenase can be defined as follows:

A positive charge on nitrogen; there is a planar arrangement $=\overset{(+)}{\text{N}}\!\!<$, in all these compounds except for N-methyl-13,14-didehydro-1-methoxycanadinium chloride (XX) which has a quaternary nitrogen $\overset{|}{\underset{/|\backslash}{\text{N}^{(+)}}}$.

A sp^3 hybrid state of carbons C(5) and C(6) in B ring of the protoberberine structure of these compounds.

A sp^2 hybrid state of carbons C(13) and C(14) in C ring. Appearingly, the aromatic character of the C ring of the protoberberine backbone of the compounds examined is not entirely necessary for their inhibitory ability since compounds (*XX*) and (*XXII*) also inhibit. The carbons in question are both sp^2 in compound (*XXII*), yet they are not linked by a double bond.

A certain size of the angle between rings A and D (very roughly 20°, as can be estimated from models of molecules of compounds showing inhibitory ability). The dehydrogenation of the B ring of berberines, *i.e.* the planarization of the entire molecule, eliminates their inhibitory ability (*cf.* coralyne (*XXIII*) and norcoralyne (*XXIV*)). Similarly, the total hydrogenation of the C ring of berberines, which results, besides others, also in an increase of the angle between the A and D rings compared to the original compounds with an aromatic C ring, eliminates their inhibitory ability (*cf.* coptisine (*II*) *versus* stylopine (*XVI*), berberine (*III*) *versus* canadine (*XVII*), 13-methylberberine (*XII*) *versus* thalictricavine (*XVIII*)).

Effect of Substituents on A *and* D *Rings*

The substitution of hydrogens of aromatic rings A and D of the protoberberine backbone of the compounds studied by electron donor groups significantly increases the inhibitory ability of the corresponding berberines compared to unsubstituted original protoberberine.

The inhibitory power of the berberines depends on the character of these substituents on A and D rings: it increases in the sequence protoberberine (*I*), palmatine (*IV*), jatrorrhizine (*V*) after the replacement of H by OCH_3 or by OCH_3 and OH, respectively. It increases even more markedly in the sequence palmatine (*IV*), berberine (*III*), coptisine (*II*) after the replacement of one or two pairs of ortho methoxyl groups by a methylenedioxy group.

The inhibitory power of protoberberine alkaloids also depends on the position of electron donor substituents on ring D. Synthetic pseudo-berberines (substituents on carbons C(10, 11)) show – compared to the "genuine" berberines (substituents on carbons C(9, 10)) – a substantially lower inhibitory power (*cf.* ψ-coptisine (*VIII*) and ψ-palmatine (*X*) *versus* coptisine (*II*) and palmatine (*IV*)). The marked effect of differences in the position of the same substituents on ring D manifests itself in entirely different absorption spectra of pseudo-berberines compared to berberines[10].

Effect of Substituents on Carbon C(13)

The substitution of hydrogen on C(13) affects very significantly the inhibitory power of the berberines (*cf.* coptisine (*II*) *versus* corysamine (*XI*), berberine (*III*) *versus* C(13) derivatives of berberine (*XII*–*XV*)).

The inhibitory power of C(13) derivatives of berberine considerably depends on the nature of substituents on C(13) and is a function of the sum of polarity, induction, and conjugation effects of these substituents. An important part will also play the steric factor. The substituents on C(13) can namely cause a sterical hindrance during the distortion of the rings of the alkaloid molecule (possibility of nonbinding interactions between the substituent on C(13) and hydrogen on C(1)). The mechanism of fixation of the positions of rings toward each other in the molecule of the compound tested can also participate significantly on increasing the strength of the bond between the alkaloid and the enzyme in the complex.

C(13)-Alkylberberines are relatively strong inhibitors; 13-ethyberberine (*XIII*) is the strongest inhibitor of all compounds tested: it binds more firmly at pH 10 to the enzyme than its coenzymes, NAD and NADH (ref.[14]).

Effect of Other Substituents

The substitution of hydrogen on C(1) by a methoxyl group causes a marked increase of the inhibitory power of berberines (compare berberine (*III*) *versus* 1-methoxyberberine (*XXI*) and N-methyl-13, 14-didehydro-1-methoxycanadium chloride (*XX*)). This substitution substantially increases the possibility of nonbinding interactions between substituents on carbons C(1) and C(13). Other effects obviously play a role in addition to the steric effect of the methoxyl group on C(1) in the case of compound (*XX*).

Character of Interaction of Liver Alcohol Dehydrogenase with Protoberberine Alkaloids

The interaction of liver alcohol dehydrogenase with the protoberberine alkaloids tested most likely has a complex character: the molecules of the enzyme and of the compounds tested are too complicated and therefore it is impossible on the basis of the results presented here to localize unambiguously groups and parts of molecules responsible for their mutual interaction. Nevertheless, certain conlusions can be drawn from the results presented here.

There may exist a charge transfer between the enzyme and protoberberine alkaloids.

There is most likely a group of the enzyme serving as CT donor (*e.g.*, tryptophan and some other aromatic amino acid, or the sulfur of methionine or cysteine), which is responsible for binding these compounds. The region of C and D rings of the alkaloid molecule will then be the electron acceptor partner.

The participation is probable of some enzyme group bearing a negative charge, which may be important for coulombic interaction between the enzyme and the alkaloid (*e.g.* the carboxyl of aspartic or glutamic acid). A fact deserving mention is that there is no essential difference in the binding of the compounds tested at pH 7,

where the total net charge of the enzyme is positive, and at pH 10, where the total net charge of the protein is negative.

Some hydrophobic group or region of the enzyme, located in the immediate neighborhood of the binding site of the alkaloid, will most likely participate on the interaction.

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