CHARACTERIZATION OF BINDING SITE OF HORSE LIVER ALCOHOL DEHYDROGENASE FOR BERBERINES AND AURAMINE O

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Spectrophotometric, fluorometric, and kinetic measurements show that the molecule of horse liver alcohol dehydrogenase contains two binding sites for these compounds. Berberines and auramine O bind most likely at the same site of the enzyme subunit, at a region referred to as the "active site pocket". The chief role in the binding play hydrophobic forces and the sterical arrangement of the active site; hydrogen bonds are also possible. Electrostatic forces do not practically participate on the binding. The interaction also involves a charge transfer to these ligands from some group of the enzyme, most likely from a phenylalanine residue.

Protoberberine alkaloids interact with horse liver alcohol dehydrogenase[1]. This interaction has been examined in detail in the case of berberine and it has been found that this compound inhibits the enzymatic activity and binds at the active site of the enzyme. The binding of berberine affects the affinity of the apoenzyme for the coenzyme and its fragments and simultaneously prevents ethanol and inhibitors competitive with ethanol from binding[2]. The binding of berberine to the enzyme is paralleled by changes in its absorption and fluorescence emission spectrum[1].

This study has been aimed at a more profound analysis of the binding of berberines to horse liver alcohol dehydrogenase, especially of 13-ethylberberine which of the protoberberine alkaloids tested so far is the strongest inhibitor of this enzyme[3].

From the methodical viewpoint this analysis is based predominantly on optical methods (absorption and fluorescence emission spectroscopy). An effort is made to deduce from the results obtained conclusions on the mode of binding of berberines to horse liver alcohol dehydrogenase and to characterize the binding site for these ligands in more detail.

EXPERIMENTAL

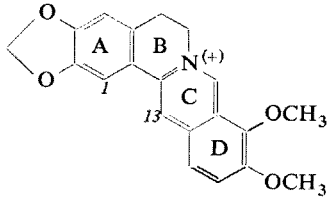
Material

Horse liver alcohol dehydrogenase was prepared according to Theorell and coworkers[4]. The activity of the enzyme was determined kinetically[5] in Beckman DU Spectrophotometer equipped

with a Honeywell recording millivoltmeter. The purity of the enzyme preparation determined spectrophotometrically[6] was 70–90%. Coenzyme NAD (grade III) was from Serva, FRG.

Berberinium chloride (*I*) (Merck, FRG) was recrystallized three times from water. Protoberberinium chloride was a gift[3]. Berberine derivatives substituted at position 13(13-ethylberberine, 13-methylberberine, and 13-ethoxyberberine) were iodides prepared semisynthetically[7]. Their purity was checked by thin-layer chromatography and spectroscopy. Auramine O (*II*) was recrystallized four times from acetonitrile[8,9] and its purity was checked spectrophotometrically. The remaining chemicals were of analytical purity.

All experiments unless stated otherwise were carried out at 23·5°C in sodium phosphate buffer at pH 7, ionic strength 0·1.



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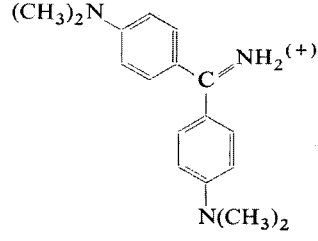


II

Methods

The absorption spectra were measured in Cary 118 Spectrophotometer. The spectrum of ethylberberine bound to the enzyme was obtained by summing up the spectrum of the free ligand and the difference spectrum of the mixture containing the enzyme and ethylberberine measured against both components in separate cells.

The Scatchard graphical treatment[10] of the spectrophotometric titration of the enzyme with ethylberberine was used for the determination of the number of binding sites of the enzyme for this ligand and for the determination of the dissociation constant of the enzyme–ligand complex:

$$r/X_f = K^{-1} \cdot (n - r), \qquad (1)$$

where K is the dissociation constant, n the number of binding sites, X_f the concentration of the free ligand, and r the binding ratio ($r = X_b/c_0$, X_b stands for the concentration of the bound ligand and c_0 for the total enzyme concentration). The concentration of bound ligand was established from the difference in absorbance of the mixture of the enzyme and the ligand and the absorbance of both isolated components at the wavelength where the difference in absorption coefficients of bound and free ethylberberine (350 nm) is maximal. The difference in absorbance is given by

$$\Delta A = \varepsilon_b \cdot X_b + \varepsilon_f \cdot X_f - \varepsilon_f \cdot X_0, \qquad (2)$$

where ε are the corresponding absorption coefficients and X_0 the total ligand concentration ($X_0 = X_f + X_b$). It holds for the concentration of the bound ligand that

$$X_b = \Delta A/(\varepsilon_b - \varepsilon_f). \qquad (3)$$

Fluorometric measurements were carried out in Beckman DU Spectrophotometer with a fluorometric adaptor consisting of a low-pressure mercury lamp and a Schott UG 11 filter. Fluorescence was recorded by a Honeywell recording millivoltmeter. Kinetic measurements of the increase in NADH concentration during the enzyme-catalyzed reaction[11] were performed with a quinine standard (c. 3 μM solution of quinine sulfate in water). A fluorescein standard (c. 0·1 μM aqueous solution) was used for the measurements in the range of 510—540 nm (titration of enzyme with berberines).

The determination of the dissociation constant of the enzyme–ligand complex was carried out by fluorometric titration of the enzyme (c. 0·1 μM) with an excess of the ligand (0—6 μM) of ethylberberine, 0—20 μM of methyl- and ethoxyberberine, and 0—50 μM of berberine). The measured fluorescence value was corrected for the fluorescence of the free ligand and in case of ligand concentrations higher than 20 μM also for the nonlinearity of the relation between fluorescence and concentration[1]. Provided that the corrected fluorescence is a linear function of concentration of the bound ligand, the dependence of corrected fluorescence on total ligand concentration has a hyperbolic character and can be linearized as follows:

$$1/F = 1/F_m + K/F_m \,.\, 1/X_0 \,, \tag{4}$$

where F_m stands for the fluorescence at complete saturation of the enzyme with the ligand. The reciprocal corrected fluorescence can be plotted *versus* reciprocal ligand concentration in a graph. This graphical treatment of experimental data is formally quite identical with the Lineweaver–Burk treatment of kinetic data where the measured value is the initial rate of the enzymatic reaction proportional to the concentration of the enzyme–substrate complex. The dissociation constant of the enzyme–ligand complex (similarly to K_m) is determined by the ratio of the slope to the intercept on the $1/F$ axis of the line obtained. This method does not allow the determination of the number of binding sites.

The effect of an additional ligand causing a decrease of the fluorescence intensity observed can be regarded in analogy as the effect of the inhibitor on the rate of the enzyme reaction and can be evaluated in a similar manner. The protein is titrated fluorometrically with an excess of the first ligand (analogous substrate) in the presence of the quenching ligand (analogous inhibitor). The competitive or noncompetitive character of the relation between the two ligands can be judged by the dependence of reciprocal fluorescence on reciprocal concentration of the first ligand at various concentration values of the quenching ligand. If the ligands are competitive the obtained lines intersect all at the $1/F$ axis (F_m similarly to the maximal rate, is not affected by the presence of the competitive ligand).

Fluorometric titrations of the enzyme with comparable quantities of ethylberberine were used to verify the number of binding sites of the enzyme for this ligand. The fluorescence intensity observed practically corresponds to the concentration of the bound ligand only and is a linear function of concentration at low concentration values. The dependence of fluorescence on total ligand concentration is given by equation (*5*) derived from the definition of the dissociation constant of the enzyme–ligand complex.

$$F = F_m/2 \,.\, n \,.\, c_0 \,.\, \{n \,.\, c_0 + X_0 + K - [(nc_0 + X_0 + K)^2 - 4nc_0X_0]^{1/2}\} \,. \tag{5}$$

The slope of this dependence for $X_0 = 0$ is given by

$$\partial F/\partial X_0 = F_m/2 \,.\, n \,.\, c_0 \,.\, [1 + (nc_0 - K)/(nc_0 + K)] = F_m/(nc_0 + K) \,. \tag{6}$$

At the end of the titration where $X_0 \gg K$, the slope of the dependence of fluorescence on total

ligand concentration X_0 is practically zero and fluorescence intensity approaches maximum fluorescence F_m (the fluorescence of the free ligand is negligible).

If the concentration of the protein titrated is considerably higher than the dissociation constant of the complex ($c_0 \gg K$), then the slope at the origin will approximately equal:

$$\partial F/\partial X_0 \approx F_m/n \,.\, c_0 \,. \tag{7}$$

The intersection of the tangent at the origin with the level of maximal fluorescence F_m will then determine by its coordinate on the X_0 axis the total concentration of binding sites nc_0. If condition $c_0 \gg K$ has not been fullfilled the read equivalence point gives higher values of total concentration of binding sites.

The kinetic test of mutual competitiveness of two inhibitors[12,13] was used to evaluate the relation of binding sites of the enzyme for ethylberberine and auramine. The initial rate of the enzymatic reaction was measured in the presence of a constant quantity of the enzyme, NAD, and ethanol at various concentrations of both inhibitors. If the reciprocal values of the initial reaction rates are plotted in a graph *versus* the concentration of the first inhibitor, we obtain a bundle of lines for increasing concentrations of the second inhibitor. If these inhibitors are capable of simultaneous binding to the enzyme (*i.e.* they are noncompetitive), then there is at least one term in the equation for reciprocal initial rate which contains the product of concentrations of both inhibitors (X_1, X_2). The slopes of the lines obtained then comply with

$$\partial(v_0)^{-1}/\partial X_1 = \mathrm{f}(X_2) \tag{8}$$

and the lines representing different values of X_2 are not parallel.

If the inhibitors are competitive then the kinetic equation is lacking the term containing product $X_1 X_2$ and the lines obtained are parallel:

$$\partial(v_0)^{-1}/\partial X_1 = \text{const} \,. \tag{9}$$

The thermodynamic analysis of the binding of berberines to the enzyme was carried out on the basis of the knowledge of dissociation constants of the corresponding complexes at two different temperatures. The thermodynamic parameters of association were calculated from the following equations:

$$\Delta H^0 = \frac{\boldsymbol{R} \,.\, T_1 \,.\, T_2 \,.\, \ln K_2/K_1}{T_1 - T_2}, \quad \Delta G^0 = \boldsymbol{R} \,.\, T \,.\, \ln K, \quad \Delta S^0 = \frac{\Delta H^0 - \Delta G^0}{T}, \tag{10}$$

where ΔH^0 is the change in standard enthalpy, ΔG^0 the change in standard free enthalpy, and ΔS^0 the change in standard entropy (these parameters characterize the association), K the dissociation constant, $\boldsymbol{R}$ the universal gas constant, and T the absolute temperature.

The type of inhibition (the differentiation between partial and total inhibition) was determined by spectrophotometric measurement[5] of enzymatic activity at pH 10 in the presence of increasing concentrations of the corresponding inhibitors. In Webb's[14] concept the inhibition is partial if the complex of the enzyme with the inhibitor shows any fractional activity. This type of inhibition can be determined best from the dependence of reciprocal fractional inhibition ($1/i$) on reciprocal inhibitor concentration. If the inhibition is partial the interpolated dependence intersects the $1/i$ axis at a point lying above unity (*i.e.* when the enzyme is saturated with the inhibitor inhibition is not complete, *i.e.* equal unity).

The estimate of the distance between auramine bound to the enzyme and the tryptophan residue in the molecule of the enzyme was carried out on the basis of the Perrin–Förster theory of the resonance energy transfer between the excited donor and the acceptor. According to Förster[15] the distance between the molecules of the donor and of the acceptor involved in half energy transfer from the excited donor to the acceptor (R_0) is given by

$$R_0^6 = \frac{9 \cdot 10^6 \cdot (\ln 10)^2 \cdot k^2 \cdot c \cdot \tau_s}{16 \cdot \pi^4 \cdot n^2 \cdot N^2 \cdot \tilde{\nu}_0^2} J(\tilde{\nu}) , \qquad (11)$$

where N is the Avogadro number, c the velocity of light, τ_s the lifetime of the excited state of the donor, $\tilde{\nu}_0$ the wave number of the 0–0 transition of the donor, n the refractive index of the medium, k the orientation factor of the chromophore molecules, and $J(\tilde{\nu})$ the overlap integral defined by

$$J(\tilde{\nu}) = \int \varepsilon_a^A(\tilde{\nu}) \cdot \varepsilon_e^D(\tilde{\nu}) \, d\tilde{\nu} , \qquad (12)$$

where A stands for the acceptor, D for the donor, a for the absorption, and e for the emission.

This overlap integral can be determined by graphical integration of the overlap curve $\varepsilon_e^A(\tilde{\nu}) \cdot \varepsilon_a^D(\tilde{\nu})$ of the absorption spectrum of the acceptor (auramine) and of the emission spectrum of the donor (tryptophan), normalized for the maximum of the longest wavelength absorption band. Since the actual refractive index of the medium is unknown the value of 1·6 usually used for proteins[16] was considered in the calculations. The value of the orientation factor, which is also unknown, was enumerated as $\sqrt{2}/3$ (ref.[15]), *i.e.* the value for random orientation between the chromophores.

RESULTS AND DISCUSSION

Absorption Spectrum of Bound Ethylberberine

The binding of ethylberberine to liver alcohol dehydrogenase is paralleled by similar changes in the absorption spectrum of this substance as the binding of berberine[1] and auramine[9] to this enzyme (Fig. 1). A weak bathochromic shift of the two long-wavelength bands and a very weak hyperchromic effect take place. Similarly to the binding of auramine to the enzyme, a new, very weak absorption band with a maximum around 480–490 nm appears in the spectrum of the bound ethylberberine. This absorption band corresponds in its position and intensity to the electron transfer from some donor in the enzyme molecule to ethylberberine which because of its structure (heteroaromatic cation) has good prerequisites to act as an electron acceptor. The binding site of the enzyme for berberines therefore contains some group capable of charge transfer to these ligands. The changes in absorption properties of ethylberberine which accompany its binding to the enzyme can be utilized for the determination of the affinity of the enzyme for ethylberberine and for the determination of the number of binding sites of the enzyme for this ligand. This analysis can be effected best by the Scatchard approach (equation (*1*)).

The enzyme was titrated with ethylberberine and the concentration of the bound ligand was determined by absorbance measurement at 350 nm where the difference in the absorption coefficients of bound and free ethylberberine is maximal (Eq. (3)). The total ethylberberine concentration was known from the size of the additions of this ligand to the enzyme solution and was also checked by absorbance measurement at 343 nm (isosbestic point in Fig. 1). The Scatchard plot is shown in Fig. 2. The dissociation constant read from the line obtained is 1·1 μM (negative reciprocal slope of the curve obtained, *cf.* equation (1)) and the number of binding sites in 1·85, *i.e.* two moles of ethylberberine bind to one mole of the enzyme. Since horse liver alcohol dehydrogenase consists of two identical subunits[17], it is practically proved that each enzyme subunit bears an identical binding site for ethylberberine.

Fluorescence Emission Spectrum of Bound Ethylberberine

The binding of ethylberberine to the enzyme is paralleled by considerable changes in its fluorescence emission spectrum, similarly to berberine and some of its derivatives[1,3]

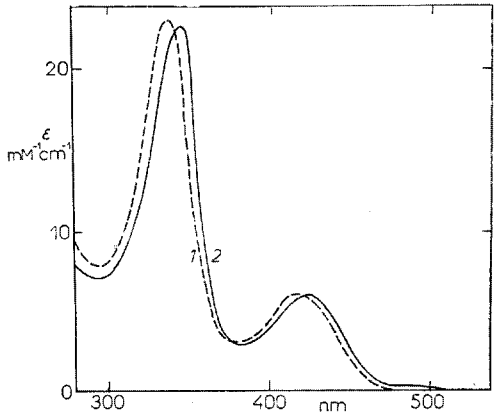


FIG. 1

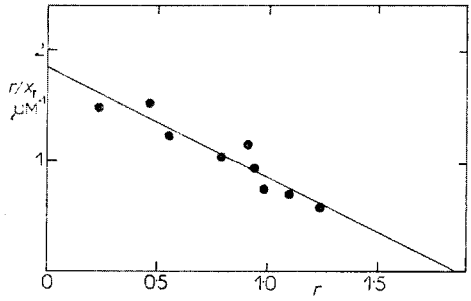
Absorption Spectrum of Ethylberberine Free *1* and Bound to Enzyme *2*

Concentration of enzyme 25 μM, concentration of ethylberberine 10 μM. The conditions of the measurement are specified in the experimental part.

FIG. 2

Scatchard Graphical Treatment of Titration of Enzyme (2 μM) with Ethylberberine (0·4—3·6 μM)

X_f concentration of free ligand, r binding ratio. The r and X_f values were determined from absorbance at 350 nm. The conditions of the measurement are specified in the experimental part.



or auramine[9] (Fig. 3). The aqueous ethylberberine solution is weakly fluorescent, the fluorescence intensity dramatically increases (more than a hundred times) after its binding to the enzyme and simultaneously a slight hypsochromic shift of the emission maximum takes place. Analogous changes in fluorescence accompany the shift of berberine alkaloids from a polar to a nonpolar solvent[2,3]. It may therefore be concluded that berberines bind to the strongly hydrophobic domain of the enzyme molecule.

The intensity of ethylberberine fluorescence is increased approximately twenty times after its transition from water to dioxane and approximately hundred times after its binding to the enzyme. Since it is improbable that the ethylberberine molecule comes to a domain more hydrophobic than dioxane in the process of its binding to the enzyme, other factors influence most likely the increase of fluorescence intensity. The chief of these influences is obviously an increase in the rigidity of the ethylberberine molecule during its binding to the enzyme. This hypothesis is supported by the fact that the fluorescence of free ethylberberine is approximately ten times higher in ice at 0°C than in water at the same temperature. These factors also play an important role in the increase of fluorescence intensity of bound auramine[18].

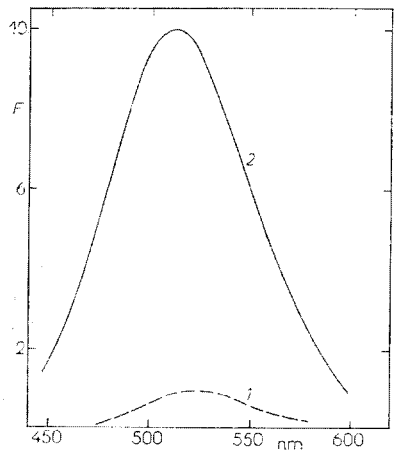


FIG. 3

Fluorescence Emission Spectra (uncorrected)

Free ethylberberine 3·8 μM *1*, equal concentration of ethylberberine in the presence of 0·2 μM enzyme, *2*. *F* relative fluorescence intensity, excitation 365 nm.

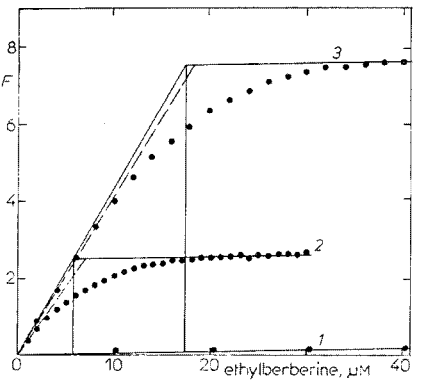


FIG. 4

Fluorometric Titration of Enzyme with Ethylberberine

Buffer *1*, 3 μM enzyme solution *2*, 8·9 μM enzyme solution *3*. The theoretical equivalence points are marked by the intersection of full lines, dotted lines are actual tangents to the titration curve at the origin. *F* fluorescence intensity at 510 nm and with excitation at 365 nm.

The fluorescence characteristics of ethylberberine can again be used for the determination of the affinity of this ligand for the enzyme and for the determination of the number of binding sites for this ligand on the enzyme. To determine the dissociation constant the enzyme was titrated with an excess of the ligand and the titration was evaluated according to equation (*4*). As shown in Fig. 5 (bottom line) the value of the dissociation constant is 1·3 μM, *i.e.* in good agreement with the value of 1·1 μM obtained by the Scatchard method from absorption data.

The number of binding sites can be determined from the titration curve obtained under conditions where the order of enzyme concentration approaches the ligand concentration and markedly exceeds the value of the dissociation constant (Fig. 4). The titration curves do not show a sharp bent and since $K \approx 1{\cdot}2$ μM the error of determination of the total number of binding sites (nc_0) will be lower than 5% at an enzyme concentration higher than 20 μM (*cf.* equations (*6*) and (*7*)). The titration of such large enzyme quantities is inconvenient from the practical viewpoint and the interpretation of the results troublesome (the relation between fluorescence and concentration, is no longer linear at ethylberberine concentrations exceeding 20 μM under the given experimental conditions). Other methods[5,19,20] are more suitable for the practical determination of the enzyme concentration. Nevertheless the equivalence points read from Fig. 4 show that the molarity of ethyberberine at equivalence is double the enzyme molarity. This finding supports the hypothesis that two ethylberberine molecules bind to one enzyme molecule.

Fluorometric Analysis of Binding of Ethylberberine and Ethanol

The relation between the binding site of the enzyme for the inhibitor and for ethanol and acetaldehyde can be determined from kinetic data[21] with difficulties; it is more convenient to examine the effect of ethanol on the fluorescence of the enzyme–ethylberberine complex. Ethanol added to the mixture of enzyme and ethylberberine gradually decreases the fluorescence intensity down to the level of free ethylberberine (in analogy to berberine[2]). This finding shows that ethanol either competes with ethylberberine or forms with the enzyme–ethylberberine complex a new, ternary enzyme–ethylberberine–ethanol complex and quenches the fluorescence of bound ethylberberine. We investigated the effect of increasing ethanol concentrations on the titration curve of the enzyme with an excess of ethylberberine. The titration curves were treated graphically according to equation (*4*) (Fig. 5).

Fig. 5 shows that the intersection of the bundle of lines for various ethanol concentrations lies on the $1/F$ axis. Hence, ethanol and ethylberberine do compete, similarly to ethanol and berberine, as shown in a slightly different manner earlier[2]. Berberines completely interfere with ethanol during their binding to the enzyme, the binding sites for these compounds overlap or the binding of one ligand induces such conformational changes of the enzyme molecule which make the binding site

for the other ligand completely inaccessible. In any case, the immediate vicinity of the binding site for berberines and for the substrate is practically sure.

Kinetic Analysis of Binding of Ethylberberine and Auramine

The behavior of berberine alkaloids toward liver alcohol dehydrogenase is strikingly similar to the behavior of auramine toward this enzyme. Likewise, there are also certain analogies in the structure of berberines and auramine. A striking feature of both molecules is their nonplanarity (caused in berberines by the hydrogenated B ring and in auramine by steric hindrance of the free rotation of phenyls). Auramine inhibits enzyme activity and forms with the enzyme a complex which manifests itself by completely analogous changes in absorption and fluorescence emission spectrum[9]. Similarly, the behavior of auramine toward the substrates of the enzyme is similar and there is competition especially with higher alcohols[22].

The question arises whether berberines bind at the same binding site on the enzyme as auramine. One of the reliable tests determining the relation between the binding sites for two inhibiting ligands is the kinetic test (equation (*8*) and (*9*)). The initial reaction rate was measured of oxidation of ethanol at various ethylberberine and auramine concentrations and the results were treated as shown in Fig. 6. The fact that the

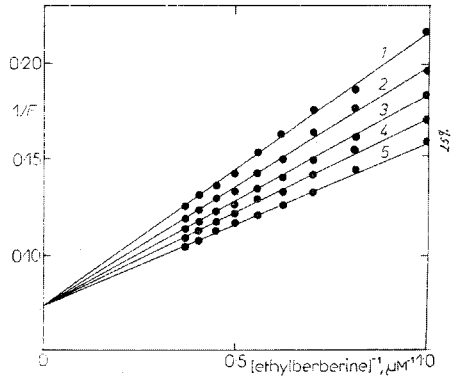


FIG. 5

Treatment according to Equation (*4*) of Fluorometric Titration of Enzyme (2 μM) with Excess of Ethylberberine in Presence of Ethanol

Ethanol concentration for individual curves (from bottom): 0, 0·6, 1·2, 2·0, and 3·0 mM. *F* fluorescence intensity at 510 nm and with excitation at 365 nm.

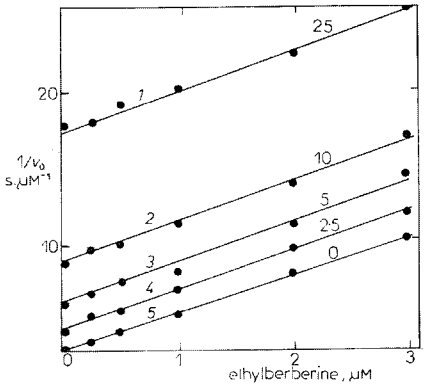


FIG. 6

Kinetic Test of Competitiveness of Ethylberberine and Auramine O

Concentration of enzyme 0·05 μM, concentration of ethanol 3·5 mM, concentration of NAD 0·2 mM. The concentration of auramine in μM is given at each individual line. v_0 initial rate of the enzymatic reaction.

curves obtained are parallel provides evidence showing that auramine and berberine bind most likely to an identical site on the enzyme molecule.

Effect of Ionic Strength on Affinity of Enzyme for Ethylberberine

It is obvious from the changes in the fluorescence emission spectrum of berberines and auramine during their binding to the enzyme that these compounds bind to some hydrophobic domain of the enzyme molecule. Hence, the hydrophobic interaction between the enzyme and these ligands plays an important role. Berberines and auramine, however, are cations and the question arises whether such ionic interactions will also contribute to the total affinity of these compounds for the enzyme. The contribution of the ionic interaction can be determined from the dependence of the dissociation constant of the enzyme–ethylberberine complex on the ionic strength of the medium.

The dissociation constant was determined by fluorometric titration of the enzyme with an excess of the ligand (equation (*4*). The experiments were carried out at pH 5·9 and 7·0 (phosphate buffer) and at pH 10·8 (borate buffer), the ionic strength was adjusted by additions of sodium chloride to values of 0·05–2. The dissociation constant practically does not vary with either ionic strength or pH. (By contrast, the fluorescence intensity of ethylberberine bound to the enzyme increases with the increasing ionic strength; this suggests possible conformational changes in the enzyme molecule in media of different ionic strength). The share of ionic interaction on the binding of ethylberberine to alcohol dehydrogenase appears therefore negligible. The fact that ionic interaction does not play an important role in the binding of auramine to the enzyme is evidenced by the finding that a structural analog of auramine – Michler's ketone (4,4'-dimethylaminobenzophenone) – is also capable of binding to liver alcohol dehydrogenase[23].

If we compare the information obtained on the binding of berberines and auramine to liver alcohol dehydrogenase with the characteristics of the indivudual parts of the enzyme subunits as provided by Eklund and coworkers[17] we can allocate with a high probability the binding site for these ligands to the region known as the "active site pocket". This is a relatively extensive region of the enzyme subunit which contains a zinc ion important for the mechanism of the enzymatic reaction at its periphery. The main role of this region is to bind the nonpolar part of the substrate (alcohol or aldehyde). This region is built up of exclusively nonpolar amino acid residues and does not contain any amino acid residue with an integral charge at physiological pH-values.

Thermodynamics of Binding of Berberines to Alcohol Dehydrogenase

The determination of the fundamental thermodynamic characteristics of the binding of berberines to the enzyme can contribute to a more detailed elucidation of the

mechanism of interaction of the enzyme with these ligands. The thermodynamic parameters were calculated according to equation (*10*) from the values of dissociation constants of the corresponding complexes (determined by titration with an excess of ligands) at two different temperatures. Table I presents the parameters determined of the association of berberine, ethylberberine, and other berberine derivatives with the enzyme. As can be seen the enthalpy changes which accompany the association lie in the range of weak bonds (*e.g.* hydrogen bonds or van der Waals forces) and attain almost the same values for all the four derivatives examined.

The changes in the association entropy are more substantial; mainly these changes contribute to the differences in affinity of the berberine derivatives for the enzyme. According to Westley[24] the change in entropy accompanying the interaction of a macromolecule with a ligand is given by the changes in entropy of the electrostatic influences, in entropy of hydrophobic influences, and in entropy of the conformational state of the enzyme and ligand molecules. (In addition to these factors the contribution of approximately − 8 e.u. ascribed to the decrease of the number of particles in the solution because of association must be taken into account).

Electrostatic forces play a negligible role in the binding of berberines to alcohol dehydrogenase. On the assumption that identical conformational changes take place in the macromolecule during its association with the compounds assayed (the structural differences between individual ligands are negligible), we can regard the differences in entropy changes which parallel the binding of these compounds to the

TABLE I

Thermodynamic Parameters of Association of Berberines with Liver Alcohol Dehydrogenase

The values of dissociation constants were obtained from three fluorometric titrations of the enzyme with excess of ligands after evaluation according to equation (*4*). The thermodynamic parameters were calculated from equations (*10*). The error of the results is approximately 5%.

Ligand	Temperature °C	Dissociation constant μM	ΔG^0 kcal/mol	ΔH^0 kcal/mol	ΔS^0 e.u.
Ethylberberine	10	1·05	−7·77	−3·8	12
	30	1·65	−8·05		
Methylberberine	10	7·0	−6·73	−3·5	9·5
	30	10·2	−6·94		
Ethoxyberberine	10	12·0	−6·40	−3·6	8·0
	30	18·5	−6·58		
Berberine	10	36·5	−5·83	−3·7	6·0
	30	55·0	−5·92		

enzyme as a manifestation of individual changes in ligand conformation during the association and especially as a manifestation reflecting differences in hydrophobic features of the ligands. Groups $—OC_2H_5$ and —H are almost identically hydrophobic[25], thus the difference in the change of association entropy of ethoxyberberine and berberine is determined practically only by differences in changes of conformational entropy of these ligands during their binding to the enzyme. Berberines are nonplanar structures permitting the existence of two conformers. The transition of one conformer to the other is easier with unsubstituted berberine than with the derivatives substituted at position 13 because of the higher nonbinding interactions of groups at positions 13 and 1 during the tilting of the A-ring. Likewise, the angle between ring A and rings C and D is most likely larger with substituted berberines[7]. The ligand molecule is practically immobilized during the binding to the enzyme; most likely only one of the conformers binds, as postulated by Wicken and Woody for auramine[8]. The entropy drop which parallels the transition from free to bound state is therefore bigger with berberine than with its derivatives substituted at position 13 and is reflected by the difference in entropy changes during the association of ethoxyberberine and berberine with the enzyme (approximately 2 e.u. – *cf.* Table I).

Simultaneously with the increase of the hydrophobic character of the ligand (substitution by methyl and ethyl) increases also the entropy change of hydrophobic forces and contributes to an affinity increase even more than a change in ligand conformation. The hydrophobic parts of the ligand and of the macromolecule in free state are surrounded by organized water molecules which are liberated during the association and pass to a less organized state. Since the quantity of structural water in free ethylberberine is larger than in methylberberine, the latter again being larger than in berberine, the corresponding increases in entropy of hydrophobic effects are equally related (Table I).

Orientation of Binding Site for Berberines toward Catalytic Center Zinc Atom

The mere fact that the affinity of ethylberberine for liver alcohol dehydrogenase does not depend on the ionic strength of the medium excludes the possibility of direct binding of berberinium cations to the active center zinc cation of the enzyme.

Berberines which bear in A and D rings of the protoberberine backbone relatively hydrophilic substituents bind to the enzyme more firmly than unsubstituted protoberberine[3]. Similarly, the character of inhibition of enzymatic activity by protoberberine and berberine is slightly different (Fig. 7). Protoberberine causes a partial inhibition only since – unlike with berberine – the inhibition of the enzyme is not complete even after it has been saturated with protoberberine. Protoberberine obviously is not capable of complete displacement of the substrate; its presence

leads most likely merely to changes in the orientation of the substrate and of its neighborhood, changes which bring about a decrease of the activity only.

It follows from these facts that the molecule of berberine is oriented during its binding to the enzyme most probably by relatively hydrophilic groups (in A or D ring) directly to the region of the binding site for ethanol. These hydrophilic substituents can perhaps come to a distance suitable for formation of hydrogen bonds with, *e.g.* water molecules bound to one of the coordination sites of the active center zinc atom and/or to serine residue No 48 which is placed in its immediate vicinity[17].

Mechanism of Interaction of Enzyme with Berberines and Auramine

The binding of berberines and auramine to the enzyme has a complex character. In addition to the considerable contribution of hydrophobic forces to the affinity, a certain role play probably also the hydrogen bonds of the hydrophilic parts of the substituents as well as the rigidity and orientation of aromatic and heteroaromatic parts of ligand molecules with respect to the specifically formed hydrophobic binding site of the enzyme.

There exists probably a charge transfer to the molecules of these ligands with inherent charge acceptor character from some charge donor in the enzyme molecule. This is indirectly evidenced by some conclusions drawn from studies on the relations between structure and affinity of different protoberberine derivatives[3] and directly evidenced by the occurrence of a new, weak absorption band in the long wavelength range observed during the binding of ethylberberine (Fig. 1) and auramine[9] to the enzyme.

Charge donors on a protein molecule are represented above all by residues of aromatic amino acids (tryptophan, tyrosine, phenylalanine, and histidine). Tyrosine and histidine can hardly participate on the binding of these ligands since the affinity

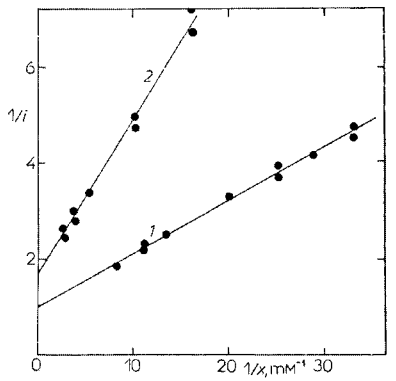


FIG. 7

Determination of Type of Inhibition by Berberine *1* and Ethylberberine *2*

The fractional inhibition (*i*) was calculated from the enzyme activity measured spectrophotometrically[5] at pH 10 in the presence of the corresponding quantity of the inhibitor (*x*).

of ethylberberine for the enzyme is practically independent of both ionic strength and pH (the pK values of histidine and tyrosine residues lie between pH 5·9 and 10·8).

The role of tryptophan in the binding of auramine was checked because of the fact that tryptophan is responsible for the fluorescence of the enzyme quenched as a result because of energy transfer in the presence of bound auramine. Equation (*10*) was used to estimate the distance necessary for half energy transfer from excited tryptophan to auramine. The lifetime of the excited state of tryptophan is approximately $2{\cdot}6 \cdot 10^{-9}$ s (ref.[26]). The wave number 0–0 of the tryptophan transition is approximately 32000 cm^{-1} (ref.[27]). The value of the overlap integral $J(\tilde{\nu})$ was determined by graphical integration of the overlap curve of the absorption spectrum of auramine[9] and of the normalized emision spectrum of tryptophan[27] according to equation (*11*) ($J(\tilde{\nu}) \approx$ $\approx 2{\cdot}8 \cdot 10^{-11}$ cm^4 mM^{-2}). After substitution of these values into equation (*10*) the value obtained for the distance between tryptophan and auramine required for half energy transfer is approximately 22 Å. Since the actual energy transfer from the tryptophan residue of alcohol dehydrogenase is lower than one half[9], the distance between bound auramine and the nearest tryptophan residue on the enzyme molecule is greater than 22 Å. This excludes the possibility of a direct participation of the tryptophan residue on the binding of auramine and therefore also of the berberines.

Since histidine, tyrosine, and tryptophan residues do not participate on the binding of berberines and auramine, it is quite likely that a phenylalanine residue is responsible for charge transfer from the enzyme molecule to these acceptors.

Berberines and auramine have been shown to bind at the part of the enzyme molecule named the "active site pocket"[17]. This domain of the enzyme subunits contains two phenylalanine residues (Phe 93 and Phe 110) whose α-carbons lie close to each other and at the same time to a considerable degree also rigidly oriented toward each other by the presence of bulky hydrophobic amino acid residues occurring in this domain (Leu 57, Leu 116, Leu 141, and Ile 138). The periphery of this considerably hydrophobic domain bears on the one side the catalytic zinc atom and a hydrophilic serine residue (Ser 48) and other relatively hydrophilic amino acid residues (especially Ser 117) on the other.

It is probable that the molecules of berberine and auramine bind through their aromatic and heteroaromatic parts, respectively to the oriented phenylalanine residues (Phe 93 and Phe 110); it is also possible that their relatively hydrophilic side-chain substituents are involved in hydrogen bonds to some of the serine residues mentioned above (Ser 48 and Ser 117), or alternatively to a water molecule which occupies one coordination site of the catalytic zinc atom.

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