CHLOROPROTHIXENE BINDING INTO THE ACTIVE SITE POCKET OF HORSE LIVER ALCOHOL DEHYDROGENASE

J.KOVÁŘ[a], L.SKURSKÝ[a] and K.BLÁHA[b]

[a] *Department of Biochemistry, Purkyně University, 611 37 Brno and*
[b] *Institute of Organic Chemistry and Biochemistry, Czechoslovak Academy of Sciences, 166 10 Prague 6*

Received July 14th, 1975

The binding of chloroprothixene (*I*) to horse liver alcohol dehydrogenase has been studied by means of circular dichroism, kinetic and fluorescence analyses. Chloroprothixene takes on chiral conformation in binding, the conformation of the enzyme remains probably unchanged. Binding of chloroprothixene had no effect on the binding constant of NADH to the enzyme. There was, however, partial interference with the binding of 13-ethylberberinium (*II*) and of auramine O (*III*) and complete interference with the binding of ethanol and isobutyramide. It is suggested from these results that chloroprothixene probably binds to the active centre of the enzyme, but without participation of the Zn atom.

Three-dimensional X-ray structure analysis of horse liver alcohol dehydrogenase[1] has markedly improved knowledge of the topography of the active site and of the catalytic mechanism, previously deduced primarily from kinetic and equilibrium data of mixtures of apoenzyme and coenzyme, substrate and inhibitors. However, studies of interaction of the enzyme with various ligands remain of importance particularly in multi-component systems. As opposed to X-ray analysis, the latter is able to analyse dynamics and reflect phenomena which will occur in solution.

Among the heretofore known inhibitors of horse liver alcohol dehydrogenase, aromatic and heteroaromatic compounds take an important place. There are three main types of these ligands which correspond to the three different binding sites in various portions of the enzyme molecule: *1*) Inhibitors reacting with the binding site for coenzyme, interfering with the binding of the adenosine portion of the latter: 1-anilinonaphthalene-8-sulphonate and 2-toluidinonaphthalene-6-sulphonate[2], salicylate and 5-iodosalicylate[3] and Rose Bengal[4]. *2*) Inhibitors which chelate Zn, interfering with the binding of substrate and also with the binding of the nicotinamide portion of the coenzyme: *o*-phenanthroline[5], pyrazole[6] and imidazole[5]. *3*) Inhibitors interacting with lipophilic binding sites in the enzyme which bind the non-polar portion of the substrate: Auramine O (ref.[7]), Michler's ketone[8] and berberines[9].

Some drugs with a central nervous action also interact with liver alcohol dehydrogenase. This interaction with the enzyme is reflected in their ability to inhibit enzyme

activity and has been spectroscopically documented by the formation of a binary complex between the thiaxanthene derivative chloroprothixene (*I*, 2-chloro-9*E*-(3-dimethylaminopropylidene)thiaxanthene) with the enzyme[10]. The present work is an attempt to analyse the mechanism of chloroprothixene (*I*) binding to horse liver alcohol dehydrogenase and to characterize the binding sites of the enzyme for this substance, in particular its position in relation to the binding sites for coenzyme, substrate and the above-mentioned inhibitors. For this purpose we used circular dichroism, kinetic analysis and measurements of changes in fluorescence accompanying the interaction of chloroprothixene with the enzyme, in some cases in the presence of further ligands. An experimentally verified simplification of the Theorell–Chance mechanism has been used to evaluate the kinetic data.

EXPERIMENTAL

Materials

Horse liver alcohol dehydrogenase was isolated according to Theorell and coworkers[11], the concentration of the enzyme was determined kinetically[12], its purity was estimated spectrophotometrically[13] to be in the range of 70–80%. Chloroprothixene hydrochloride (*I*) was obtained from Farmakon (Olomouc), content of physiologically active *cis*-form was greater than 97% (m.p. 225°C, the literature[14] gives 224–226°C). 13-Ethylberberinium iodide (*II*) was prepared[15] from berberinium chloride (Merck); its purity was analysed spectroscopically and by thin-layer chromatography (m.p. 210°C with decomposition, the literature gives 210–215°C with decomposition[15]). Auramine O (*III*) was purified by four crystallisations from acetonitrile and its purity was measured spectroscopically[7]. Coenzyme NAD was the product of Serva and coenzyme NADH of Merck.

Methods

Enzyme activity was measured in 0·1M-NaOH–glycine buffer pH 10, the other experiments were carried out in phosphate buffer, pH 7·0, of ionic strength 0·05 at 23·5°C.

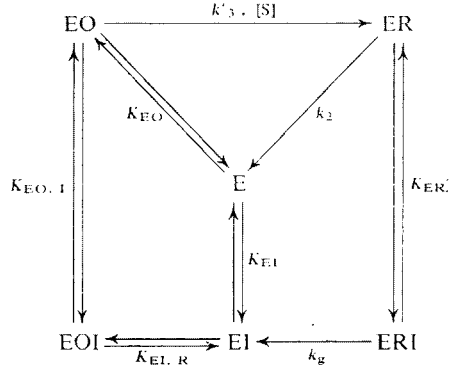
CD spectra were measured on a Roussel–Jouan Dichrographe CD 185 Model II, [Θ] values are given in deg cm^2 $dmol^{-1}$. Spectrometric measurements were carried out on a Beckman DU spectrophotometer (in some case with a Honeywell recording millivoltmeter). The same instrument was also used for fluorometric measurements with a special adapter containing a low-pressure Mercury lamp and a Schott UG 11 filter.

Kinetic measurements were carried out fluorometrically[16]; a decrease or increase in fluorescence at 460 nm (corresponding to a change in the concentration of NADH due to enzyme reaction) was the basis for calculating the initial reaction rate. Up to 40 μM concentration NADH fluorescence is a linear function of concentration; with measurements at a saturating concentration of NADH (70 μM) a correction for non-linearity was made.

Kinetics of the Inhibition

The Theorell–Chance mechanism[5], which governs the kinetics of horse liver alcohol dehydrogenase, indicates that of the enzyme species involved in the sequence of catalytic events only the

free enzyme E and binary complexes with reduced (ER) and oxidised (EO) coenzyme are kinetically significant.. When the enzymatic reaction occurs in the presence of inhibitor I, three other complexes with inhibitor can exist in the system: EI, ERI, EOI. Transitions of enzyme forms are outlined in Scheme 1, which applies to initial conditions of measurement of the rate of oxidation of ethanol, provided that the concentration of NADH and acetaldehyde are negligible. S indicates substrate (ethanol). In Scheme 1 and the subsequent equations, R and O are symbols for reduced and oxidised coenzyme. L and I indicate a general ligand or inhibitor binding to the enzyme. Combinations of basic symbols indicate the corresponding complexes. $K_{ER,I}$ and $K_{EO,I}$ are dissociation constants of ternary complexes for dissociation of inhibitor, $K_{EI,R}$ the dissociation constant of the ternary complex for dissociation of NADH and K_{EO}, K_{ER} are dissociation constants of binary complexes. Rate constants k_3, k_2 are defined according to Dalziel[17], k_g is the rate constant for dissociation of NADH from the complex ERI.



SCHEME 1

Scheme 1 shows the situation if the complex EOI is kinetically inactive (if the inhibition is not "partial"[18]). Dissociation of coenzyme from complexes ER and ERI is the step determining the rate of oxidation of ethanol; the transformation EO → ER is also slow at low concentrations of ethanol (lower than K_m). Therefore it is possible to interpret the system of interconversions in Scheme 1 as two separate equilibrium sub-systems connected by relatively slow steps ER → E, ERI → EI and EO → ER.

Using the method of "partial equilibrium"[19], we get for the oxidation of ethanol the rate equation:

$$[E_t]/v_i = \phi'_0 \frac{1 - [I]/K_{ER,I}}{1 + \gamma[I]/K_{ER,I}} + (\phi'_2/[S])\left(1 + K_{EO}/[O] + [I]/K_{EO,I} + \frac{K_{EO}[I]}{K_{EI}[O]}\right), \qquad (1)$$

where ϕ'_2 and ϕ'_0 are constants defined according to Dalziel[17], $\gamma = k_g/k_2$, $[E_t]$ is the total concentration of enzyme and v_i is the initial reaction rate in the presence of inhibitor.

An expression of the corresponding inhibitory constant of the slope K_{is}, defined by equation (1) for a variable concentration of alcohol, is a function of the concentration of NAD [O]:

$$K_{is} = \frac{[I]}{k_i/k_0 - 1} = \frac{K_{EO} + [O]}{K_{EO}/K_{EI} + [O]/K_{EO,I}}, \qquad (2)$$

where k_i and k_0 are slopes from equation (1) for inhibited and uninhibited reactions. K_{is}

takes on a limiting value K_{EI} for zero concentration of NAD which can be determined by extrapolation. With a high concentration of NAD, K_{is} approximates the value of $K_{EO,I}$ and equation (*1*) is simplified to:

$$[E_t]/v_i = \phi'_0 \frac{1 + [I]/K_{ER,I}}{1 + \gamma[I]/K_{ER,I}} + \phi'_2/[S](1 + [I]/K_{EO,I}) . \qquad (3)$$

The inhibitory constant of the intercept K_{ii} in equations (*1*) and (*3*) is defined only with the assumption that $\gamma \to 0$; it then has the significance of the dissociation constant $K_{ER,I}$.

Approximate fulfillment of the assumption $\gamma \to 0$ can be tested either by plotting the reciprocal rate *vs* concentration of inhibitor at a saturation concentration of NAD, or by plotting intercept of the straight lines corresponding to equation (*3*) in relation to the concentration of inhibitor. If these plots are linear, then $\gamma \to 0$.

As a result of the symmetry of the Theorell–Chance mechanism, analogous schemes, equations and conclusions are also valid for the opposite reaction, *i.e.* for the reduction of acetaldehyde (constant $K_{EO,I}$ is replaced by $K_{ER,I}$ and *vice versa*).

Analogous considerations of reaction rates with saturation concentrations of substrate would formally result in similar relations, but with high substrate concentrations substrate inhibition could occur and also the conditions of validity of the Theorell–Chance mechanism could be violated.

Inhibitors I_1 and I_2 can bind to the enzyme either simultaneously (if they are not mutually competitive), or simultaneous binding of both is excluded. In the first case, the equation of the initial reaction rate shows at least one term in which the product of concentrations of both inhibitors appears. For tangents of lines in Dixon graphs ($1/v_i$ *vs* $[I_1]$) at constant concentrations of substrate in coenzyme, we would then have

$$\partial(v_i^{-1})/\partial[I_1] = f([I_2]) . \qquad (4)$$

In case of competitive inhibitors, the kinetic equation will not involve the product of concentrations $[I_1] . [I_2]$ and partial derivation of equation (*4*) is a constant. The Dixon lines for various $[I_2]$ are then parallel[20,21].

Fluorometric Evaluation of Ligand Competition

An analogous test of exclusivity (mutual competition) can be carried out for substances I_1 and I_2, which are capable of binding to enzyme E and abolish the fluorescence of a strongly fluorescent complex EL: ligands I_1 and I_2, both simultaneously present with substance L, either mutually compete and form non-fluorescent ternary complexes ELI_1 and ELI_2 or, in case of noncompetitiveness, quarternary complex ELI_1I_2. If the intensity of fluorescence is directly related to the concentration of the complex EL, changes in its reciprocal values in the presence of various amounts of ligands I_1 and I_2 can be processed in analogous fashion as the reciprocals of reaction rates in Dixon graphs. The parallelity of such lines suggests mutual competition of ligands I_1 and I_2.

The analogy between relations for initial reaction rate and fluorescence of complexes of enzyme E with fluorescent and non-fluorescent ligands can be used for fluorometric measurements of the corresponding dissociation constants. On the assumption of linearity between measured fluorescence and concentration of the complex EL, and if the concentration relations between the reactants are $[L], [I] \gg [E_t]$, then the following equation is valid for the reciprocal value of fluorescence F

$$1/F = 1/F_m(1 + [I]/K_{EL,I} + (K_{EL}/[L])(1 + [I]/K_{EI})) , \qquad (5)$$

where F_m is maximal fluorescence for saturation with ligand L and K_{ER} and K_{EI} are dissociation constants of binary complexes of enzyme with fluorescent or non-fluorescent ligand. $K_{EL,I}$ is the dissociation constant of the ternary complex enzyme–ligand–fluorescence quencher. Equation (*5*) has a similar form as the rate equation of a one-substrate inhibited enzyme reaction with Michaelis kinetics. Fluorescence F and maximal fluorescence F_m are parameters analogous to initial reaction rates and maximal rate. Ligands L and I are analogous to substrates and inhibitors.

The ratio $\alpha = K_{EL,I}/K_{EI}$ for non-competitive ligands L and I characterizes the degree of mutual interaction of bound ligands. If $\alpha < 1$, there is stabilization, if $\alpha < 1$, there is destabilization, and $\alpha = 1$ indicates that the ligands do not influence one another in binding.

RESULTS AND DISCUSSION

Enzyme binding induces chirality (CD spectrum, see Fig. 1) in the chloroprothixene (*I*) molecule. The molar ellipticities corresponding to electron transitions in chloroprothixene are fairly high and are equivalent in amplitude to the molar ellipticities of inherently chiral chromophores. It is, therefore, probable that the ligand is strongly bound to the enzyme and takes on a chiral conformation.

The weak bands corresponding to absorption of aromatic amino acids in the enzyme appear to be all present also in the CD spectrum of the complex. Since in this region of the CD spectrum there is an overlapping with two induced CD bands of the bound ligand, it is difficult to decide whether the weak bands change during ligand binding. It is therefore impossible from this data to evaluate the significance of aromatic groups in the enzyme for binding of chloroprothixene. Part of the CD spectra, corresponding to absorption of peptide bonds in the enzyme (not presented in Fig. 1) is unchanged in the presence of chloroprothixene. Binding of the latter to horse liver alcohol dehydrogenase therefore does not result in any profound changes in the conformation of the enzyme molecule.

Kinetic Analysis of Enzyme Inhibition by Chloroprothixene (I)

Chloroprothixene inhibits enzyme activity both in terms of oxidation of ethanol[10] and reduction of acetaldehyde. The kinetics of inhibited oxidation of ethanol were measured with changing and low concentration of substrates (lower than the corresponding Michaelis constant) and with changes in the concentration of NAD. Processing of the data according to Webb[18] showed that the lines of dependence of $1/i$ *vs* $1/[I]$ cross the $1/i$ axis at unity. The condition for validity of Scheme 1 is therefore fulfilled. The experimental data were further processed according to Lineweaver and Burk in terms of the concentration of ethanol. The inhibition constants of K_{is} read off from the graphs were processed according to equation (*2*), *cf.* Fig. 2. The resulting dissociation constants K_{EI} and $K_{EO,I}$ are presented in Table I.

The kinetics of inhibited reduction of acetaldehyde were measured at saturating concentration of NADH and varied concentration of the aldehyde. Processing

of the results according to Dixon showed a straight-line relationship of $1/v_i$ *vs* [I], which is evidence for approximate validity of equation (*3*). (The same condition is fulfilled for kinetic reaction in the opposite direction). From the Lineweaver–Burk relationship we calculated, using equation (*3*), dissociation constants $K_{ER,I}$ and $K_{EO,I}$, *cf.* Table I.

The Behaviour of Chloroprothixene (I) *with Fluorescent Complexes Containing* NADH

The fluorescence of free NADH (λ_{max} = 460 nm) increases with NADH binding to enzyme and the emission maximum is shifted[5] to 450 nm. Addition of chloro-

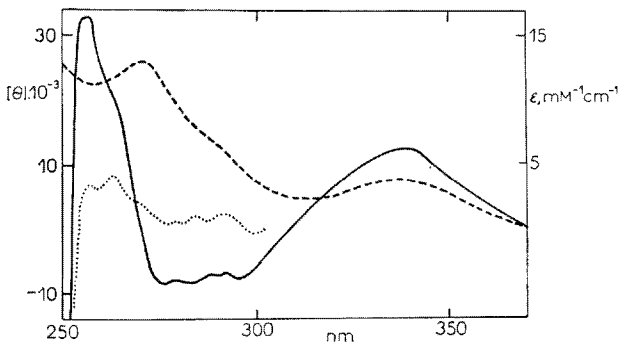


FIG. 1

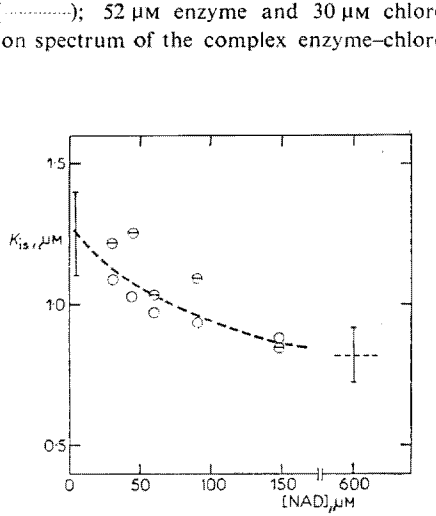
CD Spectrum of Chloroprothixene (*I*) Bound to Liver Alcohol Dehydrogenase

Conditions of measurement: 52 μM enzyme (·····); 52 μM enzyme and 30 μM chloroprothixene (———). For comparison the absorption spectrum of the complex enzyme–chloroprothixene is given (ref.[10]) (– – – –).

FIG. 2

Graphic Determination of the Dissociation Constant of the Complex Enzyme–Chloroprothixene (K_{EI}) from Kinetics of Inhibition

K_{is} the inhibition constant of the slope calculated according to equation (*2*). Experimental conditions: 0·1 μM enzyme, ethanol 0·1—0·5 mM, chloroprothixene 1·2 μM (○) and 2·4 μM (⊖). The interval shown on the horizontal axis indicates the estimated scatter of extrapolated value of the constant K_{EI}. The constant $K_{EO,I}$ for saturating concentrations of NAD at 600 μM (*cf.* ref.[10]) was determined from equation (*3*).



prothixene to a mixture of enzyme and NADH has no influence on the intensity of fluorescence or on the position of the emission maximum (Fig. 3), the binding of chloroprothixene does not therefore affect the binding of NADH to the enzyme. The binary complex enzyme–NADH is able to bind isobutyramide, an inhibitor competing with substrate: this causes a λ_{max} shift to 440 nm. The resulting ternary complex is very stable[22]. Gradual addition of chloroprothixene to this complex decreases the intensity of fluorescence to the level of that of the binary complex enzyme–NADH (Fig. 3). Isobutyramide is therefore forced to dissociate by chloroprothixene.

The competition of binding of chloroprothixene and isobutyramide is shown by a series of fluorometric titrations of the complex enzyme–NADH with isobutyramide in the presence of various concentrations of chloroprothixene. Graphic processing of the data according to equation (5) results in a series of lines with intercepts on the axis of the reciprocal of fluorescence (Fig. 4). The parameter analogous to the inhibition constant K_{is} corresponds here to the dissociation constant of the ternary complex enzyme–NADH–chloroprothixene $K_{ER,I}$ and is in good agreement with the value of $K_{ER,I}$ determined from kinetic measurements (Table I).

Competition of chloroprothixene with isobutyramide can be the result either of an overlapping of binding sites of the enzyme for these ligands or of a change

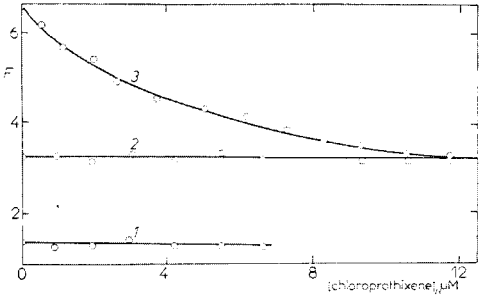


FIG. 3

The Effect of Chloroprothixene (*I*) on the Fluorescence of Free and Bound NADH

1 7·2 μM-NADH; *2* 7·2 μM-NADH + 0·8 μM enzyme; *3* 7·2 μM-NADH + 0·8 μM enzyme — 0·75 mM isobutyramide; excitation 365 nm, emission 440 nm.

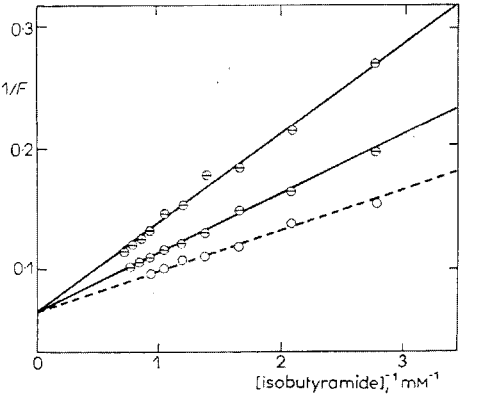


FIG. 4

Competition of Chloroprothixene (*I*) and Isobutyramide for the Complex Enzyme–NADH, Tested according to Eq. (5)

Conditions of measurement: 0·3 μM enzyme, 9 μM-NADH, concentrations of chloroprothixene (in the direction of increasing slope): 0·0, 1·2, 3·6 μM. $1/F$ is the reciprocal fluorescence at 440 nm; excitation 365 nm.

Collection Czechoslov. Chem. Communs. [Vol. 41] [1976]

TABLE I

Values of Dissociation Constants of Binary and Ternary Complexes Containing Enzyme and Chloroprothixene at pH 7

Complex	Constant, μM	Definition and methods	Experimental conditions
Enzyme–chloroprothixene	$K_{EI} = 1{\cdot}3 \pm 0{\cdot}2$	$K_{EI} = \lim K_{is}$, Eq. (*1*) $[O] \to 0$	Fig. 2
	$K_{EI} \sim 1{\cdot}3$	$K_{EI} \sim I_{0,5}$ (concentration producing 50% inhibition)	ref.[10]
	$K_{EI} = 1{\cdot}3 \pm 0{\cdot}3$	Eq. (*5*); titration of enzyme with 13-ethylberberinium in the presence of chloroprothixene	Fig. 6
Enzyme–NAD–chloroprothixene	$K_{EO,I} = 0{\cdot}8 \pm 0{\cdot}1$	$K_{EO,I} = K_{is}$, Eq. (*3*) for oxidation of ethanol	ref.[10]
	$K_{EO,I} \sim 0{\cdot}8 \pm 0{\cdot}2$	$K_{EI,I} \sim K_{ii}$, Eq. (*3*) for reduction of acetaldehyde	60 μM-NADH, 0·2 – 1·3 mM acetaldehyde
Enzyme–NADH–chloroprothixene	$K_{ER,I} = 1{\cdot}4 \pm 0{\cdot}2$	$K_{ER,I} = K_{is}$, Eq. (*3*) for reduction of acetaldehyde	60 μM-NADH, 0·2 – 1·3 mM acetaldehyde
	$K_{ER,I} \sim 1{\cdot}5 \pm 0{\cdot}4$	$K_{ER,I} \sim K_{ii}$, Eq. (*3*) for oxidation of ethanol	ref.[10]
	$K_{ER,I} = 1{\cdot}4 \pm 0{\cdot}1$	Eq. (*5*) titration of complex enzyme–NADH–isobutyramide in the presence of chloroprothixene	Fig. 4
Enzyme–13-ethylberberinium–chloroprothixene	$K_{EL,I} = 7 \pm 2$	Eq. (*5*) titration of enzyme with 13-ethylberberinium in the presence of chloroprothixene	Fig. 6
Enzyme–auramine O–chloroprothixene	$K_{EL,I} = 10 \pm 2$	Eq. (*5*) titration of enzyme with auramine O in the presence of chloroprothixene	Fig. 6, but auramine in concentration range 0 – 50 μM

in conformation of the enzyme upon binding chloroprothixene which renders the isobutyramide binding site of the enzyme inaccessible for the latter ligand. Since interference of chloroprothixene binding and that of isobutyramide is complete, the second possibility is not very probable.

Localisation of the Binding Site for Chloroprothixene (I)

In all probability, chloroprothixene binds in the "active site pocket". This is an extensive and markedly hydrophobic region of the enzyme in close vicinity with the Zn ion of the active centre[5] and functions in binding the non-polar portion of substrates, and inhibitors which compete with substrates. Isobutyramide belongs among the latter.

For a closer characterization of the binding site for chloroprothixene we can apply its relation to *o*-phenanthroline which binds to the same region and chelates Zn[5] and to 13-ethylberberinium (*II*) which primarily interacts with the non-polar portion of the binding site for substrates[9].

For evaluation of the relation of the binding of chloroprothixene and *o*-phenanthroline we used a kinetic test[20,21]. The resulting relation for concentrations of enzyme 0·1 μM, NAD 0·3 μM, ethanol 3 mM, chloroprothixene 0 – 3 μM, and *o*-phenanthroline 0 – 30 μM equation (*4*) was found to apply. This means that the binding sites of the enzyme for chloroprothixene and *o*-phenanthroline are not identical; chloroprothixene, therefore, does not bind directly to the catalytic Zn atom.

For evaluation of differences in interaction of enzyme with chloroprothixene and with berberinium, we compared first of all the corresponding CD spectra, Fig. 5. On binding to the enzyme chirality is induced also in ethylberberinium[23]. Fig. 5 demonstrates the difference in binding sites for the two types of ligand. There is here also a three-component mixture: enzyme, chloroprothixene, and 13-ethylberberinium. In the long wave-length region this mixture has a CD band approximating the mean between the bands of both ligands bound separately to the

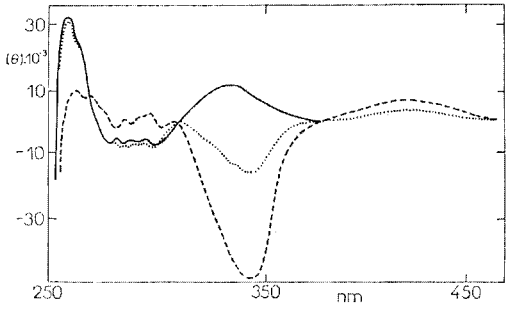


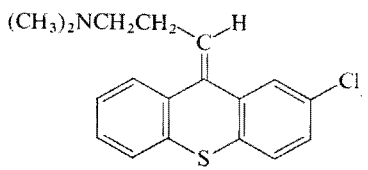
FIG. 5

CD Spectra of Complexes of Enzyme with Chloroprothixene (*I*) and with 13-Ethylberberinium (*II*)

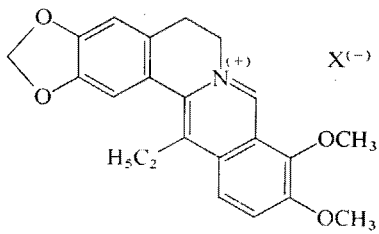
Conditions of measurement: 52 μM enzyme and 30 μM chloroprothixene (———); 52 μM enzyme and 30 μM 13-ethylberberinium (– – – –); 52 μM enzyme, 30 μM chloroprothixene and 13-ethylberberinium of the same amount (·····).

enzyme, which would be in agreement with their competition. However, the short wave-length band of bound chloroprothixene in the region at about 258 nm practically did not change in the presence of 13-ethylberberinium (*II*). Changes in molar ellipticity in a mixture which are not parallel at the various wave-lengths clearly indicate the formation of a ternary complex enzyme–13-ethylberberinium–chloroprothixene.

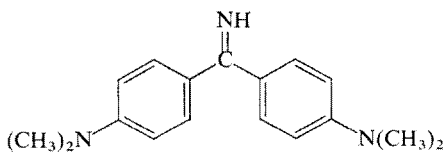
For detailed characterization of this ternary complex we can use an approach analogous to the analysis of the binding of chloroprothixene and isobutyramide to the complex enzyme–NADH. Just as berberinium, also auramine O weakly fluoresces in aqueous solutions, the intensity of the fluorescence in the region 500–550 nm markedly increases on binding to the enzyme[7,9]. Addition of chloroprothixene to a mixture of enzyme and 13-ethylberberinium (*II*) or auramine O (*III*) results in a decrease in the intensity of fluorescence to the level of that of the free ligands. Again, one can consider competition between chloroprothixene and the fluorescent ligand, or formation of a non-fluorescent ternary complex. The situation in general is simpler than binding of isobutyramide to the complex enzyme–NADH, since both ligands, chloroprothixene and 13-ethylberberinium, bind to the free apoenzyme.



I



II



III

The fluorometric titration of the enzyme by 13-ethylberberinium in the presence of increasing concentration of chloroprothixene, processed according to equation (5), is shown in Fig. 6. Since the point of intersection of the obtained lines lies above the axis of the reciprocal concentration, chloroprothixene forms a labilized ternary complex with the complex enzyme–ethylberberinium. The relationship of 13-ethylberberinium to chloroprothixene can be labelled as "mixed-competitive".

Auramine O (*III*) behaves with chloroprothixene (*I*) in an entirely analogous fashion as 13-ethylberberinium (*II*). The dissociation constants of ternary complexes

are given in Table I. Comparison with the dissociation constant of the binary complex enzyme–chloroprothixene shows labilization of the enzyme–chloroprothixene bond by the third partner. 13-Ethylberberinium increases the constant by a factor of 5 and auramine O by a factor of about 7. The fact that the binding of chloroprothixene is labilized in the presence of 13-ethylberberinium or auramine O suggests that the binding site for these ligands in the region of the "active site pocket" are not identical, but probably lie in close vicinity to one another. The greater degree of interference of chloroprothixene with auramine O might be the result of the fact that both substances have a dimethylamine group which can orient to the same site during binding to the enzyme.

The Effect of Ethanol on the Complex Enzyme–Chloroprothixene

Chloroprothixene competes with isobutyramide, an inhibitor analogous to substrate, and it is therefore probable that it competes with the substrate itself. To a mixture of enzyme (0·1 μM) and 13-ethylberberinium (1·3 μM), and increasing concentrations of ethanol (0 – 5 mM), we gradually added increasing amounts of chloroprothixene (0 – 4 μM). Measured values of fluorescence were plotted as reciprocals against concentration of chloroprothixene. The parallelity of the obtained lines indicates that chloroprothixene actually competes with ethanol. This confirms the localization of the binding site for chloroprothixene to the region of the enzyme responsible for substrate binding.

It is interesting that 13-ethylberberinium (*II*) and chloroprothixene (*I*) compete with ethanol, but not with each other. Their interference in binding to the enzyme is manifested only in a labilization of the resulting ternary complex. One reason for this can be the different size of molecules of the two ligands on the one hand, and the molecule of ethanol on the other. Chloroprothixene and 13-ethylberberinium are large molecules which are clearly bound to several functional groups in the enzyme. Part of these groups in the enzyme are probably common for binding

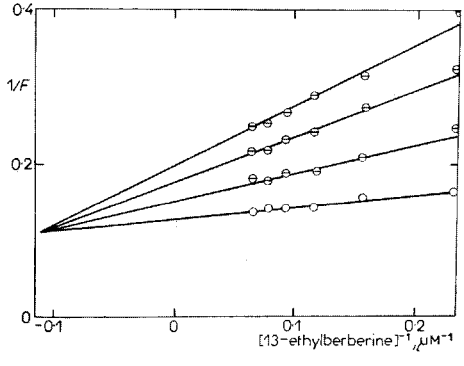


FIG. 6

Non-Competitive Relation between Chloroprothixene (*I*) and 13-Ethylberberinium (*II*) for Enzyme Binding

Conditions of measurement: 0·1 μM enzyme; concentration of chloroprothixene (in the direction of increasing slope) 0·0, 2·5, 4·0, and 6·0 μM. $1/F$ indicates the reciprocal fluorescence at 510 nm, excitation 365 nm.

of one and the other ligand. In a ternary complex enzyme–chloroprothixene–ethylberberinium both ligands bind only partially in comparison with the corresponding binary complexes, and probably with a somewhat altered geometry. This results in labilization of the binding of chloroprothixene and 13-ethylberberinium in the ternary complex. On the other hand, a small molecule such as ethanol probably binds to only a few functional groups of the enzyme. If these latter groups are parts of the binding sites for chloroprothixene or 13-ethylberberinium, then ethanol cannot bind at all to complexes of enzyme and chloroprothixene and 13-ethylberberinium, (Preventing ethanol from binding in the presence of these ligands can also be the result of a conformational change in the binding site for ethanol produced by the ligands.)

The Character of the Binding Site in the Enzyme for Chloroprothixene

Chloroprothixene binds to the active centre of the enzyme and prevents simultaneously binding of substrate and its analogues. In all probability, the hydrophobic part of the side chain of chloroprothixene binds in a similar manner as the hydrophobic part of the substrate, and thus gets into closer proximity of H_2O or OH^-, which occupy one coordination site of Zn in the active centre[1]. Interference of chloroprothixene and 13-ethylberberinium and Auramine O could result from non-binding interaction of the side-chain of chloroprothixene and the oxygen-containing substituents on the protoberberinium skeleton, which are probably oriented towards this region of the enzyme. In binding of the aromatic portion of chloroprothixene, such residues as His 31 and Phe 93 (found in this region[1]) could play a role. For this binding dispersion forces and dipole–dipole interactions might be responsible.

The affinity of chloroprothixene to the enzyme–NADH (ER) complex is approximately the same as that to the free enzyme. Dissociation constants K_{EI} and $K_{ER,I}$ (for I = chloroprothixene) have the same value of 1·4 μM. On the other hand, the ternary complex formed from the complex enzyme–NAD (EO) and chloroprothixene has a lower dissociation constant (about 0·8 μM). In the presence of NAD the binding of chloroprothixene to the enzyme is therefore stabilized. This appears to be understandable if we take into account the positive charge of the nicotinamide ring of the coenzyme acting on the splitting of proton from a water molecule which binds to the active centre Zn. It is possible to assume that the resulting hydroxyl then diminishes electrostatic repulsion between the positive charges of chloroprothixene and Zn in the active centre of the enzyme.

REFERENCES

1. Eklund U., Nordström B., Zeppezauer E., Söderlund G., Ohlsson I., Boiwe T., Brändén C. I.: FEBS (Fed. Eur. Biochem. Soc.) Lett. *44*, 200 (1974).
2. Brand L., Gohlke J. R.: Fed. Proc. Fed. Amer. Soc. Exp. Biol. *25*, 460 (1966).

3. Einarsson R., Eklund H., Zeppezauer E., Boiwe T., Brändén C. I.: Eur. J. Biochem. *49*, 41 (1974).
4. Brand L., Gohlke J. R., Rao D. S.: Biochem. *6*, 3510 (1967).
5. Sund H., Theorell H. in the book: *The Enzymes* (P. D. Boyer, Ed.), Vol. 7. p. 25. Academic Press, New York and London 1963.
6. Theorell H., Yonetani T.: Biochem. Z. *338*, 537 (1963).
7. Conrad R. H., Heitz J. R., Brand L.: Biochem. *9*, 1540 (1970).
8. Wicken J. S., Woody R. W.: Unpublished results.
9. Kovář J., Skurský L.: Eur. J. Biochem. *40*, 233 (1973).
10. Skurský L., Kovář J., Michalský J.: FEBS (Fed. Eur. Biochem. Soc.) Lett. *51*, 197 (1975).
11. Theorell H., Taniguchi T., Åkeson Å., Skurský L.: Biochem. Biophys. Res. Commun. *24*, 603 (1966).
12. Dalziel K.: Acta Chem. Scand. *11*, 397 (1957).
13. Bonnichsen R.: Acta Chem. Scand. *4*, 715 (1950).
14. Jílek J. O., Rajšner M., Pomykáček J., Protiva M.: Českoslov. Farm. *14*, 294 (1965).
15. Naruto S., Kaneko H.: Yakugaku Zasshi *92*, 1017 (1972).
16. Theorell H., McKinley - McKee J. S.: Acta Chem. Scand. *15*, 1797 (1961).
17. Dalziel K.: Acta Chem. Scand. *11*, 1706 (1957).
18. Webb J. L.: *Enzyme and Metabolic Inhibitors*, Vol. 1, p. 57. Academic Press, New York and London 1963.
19. Cha S.: Biol. Chem. *243*, 820 (1968).
20. Yonetani T., Theorell H.: Arch. Biochem. Biophys. *106*, 243 (1964).
21. Cleland W. W. in the book: *The Enzymes* (P. D. Boyer, Ed.) Vol. 2, p. 1. Academic Press, New York and London 1970.
22. Yonetani T., Theorell H.: Arch. Biochem. Biophys. *99*, 433 (1962).
23. Kovář J., Pavelka S.: This Journal, in press.

Translated by J. H. Cort.