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INTERACTION OF YEAST ALCOHOL DEHYDROGENASE WITH PROTOBERBERINE ALKALOIDS

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Oxidation of ethanol and reduction of aldehyde catalysed by yeast alcohol dehydrogenase is inhibited by several naturally occurring as well as semi-synthetic protoberberine alkaloids. The affinity of these compounds for the enzyme depends essentially on their hydrophobicity. Corysamine and coptisine are the most potent inhibitors among the natural alkaloids of this group. The kinetics of yeast alcohol dehydrogenase inhibition with coptisine were analysed and equilibrium measurements using optical methods were carried out. The results suggest that the binding site of the enzyme for protoberberines is not identical with those for coenzyme and substrate though it should be located near the nicotinamide ring of bound NAD. The binding of protoberberines seems to be limited to rather superficially located hydrophobic groups in the vicinity of the active site of the enzyme. The inability of these alkaloids to protrude deeply into the molecule of yeast alcohol dehydrogenase at the catalytically important region is the main difference in their behaviour towards alcohol dehydrogenases from yeast and horse liver.

KEY WORDS: Protoberberine alkaloids; berberine derivatives; yeast alcohol dehydrogenase inhibition.

INTRODUCTION

Alcohol dehydrogenases (EC 1.1.1.1) from yeast, plants and mammalian tissues are similar but not identical enzymes[1–3]. The kinetics of horse liver alchol dehydrogenase (LADH) and yeast alcohol dehydrogenase (YADH) are essentially the same and can be approximated by the ordered or the simple Theorell–Chance mechanisms (provided that suitable substrates are used and some further conditions are fulfilled[3,4]). The structure of LADH and YADH resemble each other as regards especially their content of zinc atoms in the hydrophobic catalytic centers and an analogous architecture of the coenzyme binding sites[1]. On the other hand, their substrate binding sites are not very similar (LADH possesses a large hydrophobic substrate pocket which accomodates even bulky substrates and inhibitors, whereas the substrate binding site of YADH resembles a relatively narrow cleft composed of hydrophobic amino acid residues[1]).

It has been found that protoberberine alkaloids are relatively strong inhibitors of LADH[5,6]. Their binding to the enzyme depends on the structure and hydrophobicity of the alkaloid molecule; the binding site is located in the substrate pocket of the enzyme near the catalytic zinc atom[6]. The alkaloids bind competitively (or with a complex mechanism including labilization) with respect to the coenzymes and substrates of LADH[5,6]. The interactions of some protoberberine alkaloids (esp. that of berberine) with plant (Pisum arvense) and yeast alcohol dehydrogenase have been mentioned in preliminary reports[7,8].

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The goal of this paper is to analyse the ability of these alkaloids to inhibit the YADH activity *in vitro* and to compare their action towards the enzymes from yeast and horse liver. This study is aimed at the use of these compounds as potential probes reflecting the structures of the enzymes rather than an elucidation of possible physiological consequences of the described interactions. The experiments were carried out with both natural alkaloids (compounds **1**–**8**), which occur in various species of Berberidaceae, Papaveraceae, Menispermaceae, Fumariaceae, Ranunucleceae and other families of higher plants, and several semi-synthetic derivatives containing alkyl groups at two positions in the molecule of berberine or berberrubine (compounds **9**–**15**).

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MATERIALS AND METHODS

The naturally occuring alkaloids (chlorides) (compounds **1**–**8**) were isolated and kindly donated by Prof. J. Slavík, Department of Chemistry, Medical Faculty, J. E. Purkyně University, Brno, Czechoslovakia. The derivatives of berberine and berberine (chlorides) (compounds **9**–**15**) were prepared from berberine (**6**) (E. Gurr Ltd., London) as described earlier[9], their purity was checked by the determination of melting points, by t.l.c. and by spectroscopic measurements[9].

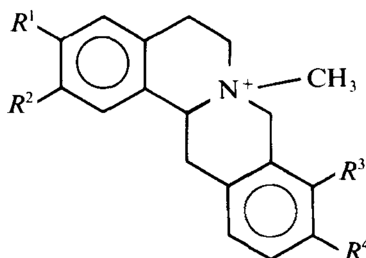
The coenzymes (NAD and NADH) were supplied from Boehringer/Mannheim (GFR). The Polybuffers 74 and 96 were the products of Pharmacia/Uppsala (Sweden). The other chemicals were of analytical purity (mostly from Lachema, Brno, Czechoslovakia).

Preparation and Characterization of the Enzyme

YADH (main isoenzyme) was isolated from baker's yeast[10,11] and characterized as a single protein by FPLC[12]. The specific activity of the enzyme determined spectrophotometrically according to Ref. 10 was 4–5 μkat/mg protein. The solution of the purified enzyme in 0.2 M Na-phosphate buffer pH 7.0 containing 10 μM EDTA and 10 μM mercaptoethanol was stored in a refrigerator and used within three days. The concentration of the enzyme is given either in activity units (katals) or as subunit concentration (calculated from A_{280} of the purified sample according to Ref. 3).

Kinetic Measurements

The inhibition of YADH with the compounds tested was measured



1 $R^1 + R^2 = OCH_2O$, $R^3 = R^4 = OCH_3$ (canadine methochloride)
2 $R^1 + R^2 = R^3 + R^4 = OCH_2O$ (stylopine methochloride)

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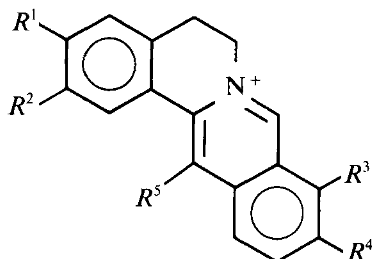
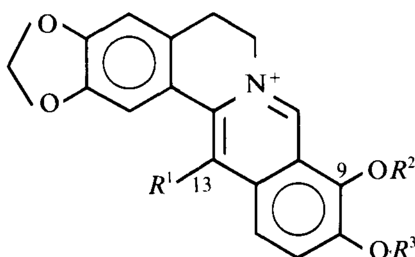


3 R^1 = OH, R^2 = OCH_3, R^3 + R^4 = OCH_2O, R^5 = H (jatrorhizine)
4 R^1 + R^2 = OCH_2O, R^3 = OH, R^4 = OCH_3, R^5 = H (berberrubine)
5 R^1 = R^2 = R^3 = OCH_3, R^5 = H (palmatine)
6 R^1 + R^2 = OCH_2O, R^3 = R^4 = OCH_3, R^5 = H (berberine)
7 R^1 + R^2 = R^3 + R^4 = OCH_2O, R^5 = H (coptisine)
8 R^1 + R^2 = R^3 + R^4 = OCH_2O, R^5 = CH_3 (corysamine)



9–12 R^1 = CH_3–C_4H_9, R^2 = OCH_3 (methylberberine–butylbeberine)
13–15 R^1 = H, R^2 = C_2H_5–C_4H_9 (ethylberberrubine–butylberberrubine)

spectrophotometrically (in cuvettes with 1 or 0.2 cm light paths) at 340 nm in 0.1 M Na–phosphate buffer pH 8 with 10 μM EDTA and 10 μM mercaptoethanol at 25°C. The reactions were started by the addition of the enzyme (the final activity of the enzyme amounted to 50 nkat/ml). The data obtained in the presence of variable concentrations of coenzymes, substrates and inhibitors were processed by a computer using a non-linear fitting program and several statistical tests as described earlier[13]. The following general equations was chosen as the mathematical model of the inhibition data:

$$1/v_0 = A\,(1 + [I]/K_1) + B(1 + [I]/K_2).1/[Alc] + C(1 + [I]/K_3).1/[\text{NAD}] + D(1 + [I]/K_4).1/[Alc].1/[\text{NAD}], \qquad (1)$$

where v_0 denotes the initial reaction rate, A, B, C and D are the kinetic coefficients for the reaction in the absence of inhibitor (which are determined separately), Alc and I refer to ethanol and inhibitor, respectively. K_1, K_2, K_3 and K_4 are the parameters to be calculated (i.e., the inhibition constants).

The inhibitory potencies of the examined alkaloids were determined by the method of Dixon[14]; the initial reaction rates were measured in the presence of increasing inhibitor concentrations in the same buffer as given above containing 0.5 mM NAD and 0.1 M ethanol at 25°C. The relative inhibitory potencies were expressed as $K_{0.5}$

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values, i.e., as the inhibitor concentrations causing the decrease in the initial reaction rate to one half under the conditions of the experiments. The analysis of mutual exclusiveness of inhibitors was carried out kinetically according to Yonetani and Theorell[15]. The experimental conditions were the same as given above. All spectrophotometric measurements were made in a Cary 118 spectrophotometer.

Fluorimetric Experiments

The fluorescence data were measured in a standard Aminco-Bowman spectrofluorimeter using the same conditions as given above. The quenching of protein fluorescence (λ_{exc} = 280 nm, λ_{em} = 340 nm) with the addition of the compounds tested was analysed as described earlier[15]. The corrected quenching curve (obtained by the comparison of protein and *N*-acetyltryptophan quenching) was fitted by a computer to the following equation[15]:

$$F = (K + F_m[I])/(K + [I]), \tag{2}$$

where F stands for the corrected fluorescence intensity, K is the dissociation constant of the protein-quencher complex and F_m denotes the fluorescence at a saturating concentration of the quencher (I). The fluorescence polarization was measured by means of the same instrument equipped with Glan-prism polarizers, the values of polarization (p) being calculated as described earlier[15]. The same experimental conditions as given above were used.

Stopped-flow Measurements

An Aminco–Morrow apparatus in the fluorescence mode served for these experiments. A high pressure Xe lamp was the excitation source (λ_{exc} = 275 nm, 2 mm slit) and the emitted light was passed through a UG-2 filter with the transmission maximum at 355 nm. 90 μl of both enzyme (10 μM) and alkaloid (20–120 μM) solutions in the above buffer were mixed. The experiments were carried out either at 25°C or 5°C.

RESULTS AND DISCUSSION

Comparison of Various Derivatives

The inhibition of YADH with several naturally occurring protoberberine and tetrahydroprotoberberine alkaloids is summarized in Table I. YADH did not interact with tetrahydroprotoberberines (canadine and stylopine, **1** and **2**), whereas some of the protoberberines were fairly good inhibitors, their affinity to YADH depending on their substituents. Two factors seem to be important for the inhibitory potency of these compounds. Firstly, methylenedioxy groups are preferred to methoxy or hydroxy groups (cf. coptisine *vs*. berberine, palmatine and jatrorhizine, the anomalous behaviour of berberrubine is explained below). Secondly, the inhibitory potency of alkylderivatives seems to be greater than that of lower homologues (cf. corysamine *vs*. coptisine in Table I). These structural requirements of YADH are similar to those of LADH[6,16,17]. The influence of the presence of hydrophobic substituents on inhibitory behaviour of berberines was analysed more thoroughly using several semisynthetic derivatives of these compounds (**9**–**15**). This analysis revealed some differences in the binding of these compounds to YADH and LADH. The essential

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TABLE I
Inhibition of yeast alcohol dehydrogenase with several naturally occurring protoberberine alkaloids

Compound	$K_{0.5}$ (mM)	Compound	$K_{0.5}$ (mM)
1 Canadine	No inhibition[a]	**5** Palmatine	Approx. 0.5
2 Stylopine	No inhibition[a]	**6** Berberine	0.45
3 Jatrorhizine	Approx. 0.5	**7** Coptisine	0.095
4 Berberrubine	0.13	**8** Corysamine	0.035

[a]No decrease in the enzyme activity in the presence of 0.5 mM alkaloid was observed.

feature of the effects of alkyl groups in position **9** and **13** was the enhancement in the inhibitory potency towards YADH with increasing hydrophobicity of the substituents. When the inhibition constants were plotted on the logarithmical scale against the hydrophobicity of substituents (expressed in terms of π parameter[18]) straight-lines with slopes of 0.48 and 0.44 respectively, were obtained (Figure 1). The observed values are very close to the theoretical values of 0.5 which should be attained if the compounds tested bound superficially with both varied substituents[19] (i.e., both substituents should be exposed both to the relatively hydrophobic surface of the enzyme molecule and to aqueous surroundings). On the other hand, LADH exhibited a different effect towards C-13 derivatives[6]; the affinity increased with the slope close to one from –H to $-C_2H_5$ and then it decreased steeply (at least the part of the molecule of alkaloid around C-13 seemed to be buried into the structure of the horse liver enzyme). That being so, one of the differences between the binding of protoberberine alkaloids to YADH and LADH concerns rather a superficial binding mode of the whole inhibitor molecule in the case of YADH. The binding of berberrubine (**4**) to YADH seems to be anomalous from this point of view (cf. Figure 1). This compound is bound more tightly than it should be if only hydrophobic effects were influencing the interactions of the substituents with the enzyme. The structural changes of this compound at different pH might explain its behaviour towards YADH. It has been found[20,21] that the pK value for the ionization of the phenolic group of this compound is extremely low (between 5 and 6) and that the formed phenolate ("zwitterion") is able to transform into a neutral quinoid structure. This quinoid form of berberrubine prevails at pH 8 at which the experiments were carried out. Consequently the binding of berberrubine to YADH could be regarded as the interaction of a molecule of another type which deviates from the structure of compounds 5–15 (e.g., an involvement of the formed carbonyl group might enhance the affinity of this compound to YADH). This finding also suggests that the positive charge on the alkaloids under study is not essential for their binding to YADH (whereas the opposite seems to be valid for LADH[17]). Supporting evidence for the presented explanation is afforded by jatrorhizine (3) which also contains a hydroxy group but it is a poor inhibitor of YADH (Table I). This compound is not able to transform easily into the corresponding quinoid since the pK value[21] of its phenolic group is above 8.

Kinetics of YADH Inhibition with Coptisine

This compound was chosen as the representative of protoberberines since its inhibitory effect on YADH activity was fairly high (Table I). It was found (by plotting the relative inhibition against the reciprocal alkaloid concentration – not shown) that

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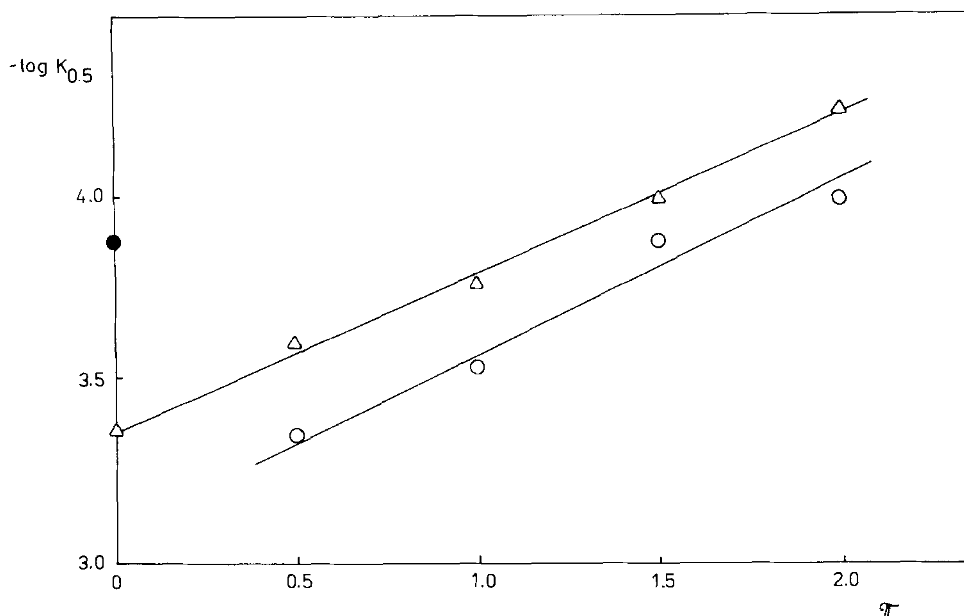

FIGURE 1 Dependence of the affinities of berberine derivatives on the hydrophobicity (π) of the substituents. Conditions of measurements are given in Methods, the values of π were taken from Ref. 18. (△) C-13 alkyl-derivatives (compounds **6** and **9**–**12**), (○) C-9 alkoxy-derivatives (compounds **6** and **13**–**15**), (●) the "anomalous" C-9 derivative (compound **4** – see text).

this compound acts as a total inhibitor of YADH for the obtained straight-line crossed the ordinate at about unity (cf. Ref. 14). The total effect (i.e., the inability of the enzyme complexes, in which the inhibitor is bound, to yield reaction products) is a common feature characterizing the behaviour of both enzymes compared towards alkaloids (cf. Ref. 17).

The mechanism of YADH inhibition with coptisine was investigated by kinetic experiments performed in both reaction directions. As it is known that the reaction catalyzed by YADH is a preferred pathway mechanism (coenzyme binding is preferred to substrate binding[1–4]) the concentrations of substrates (ethanol and acetaldehyde) were kept relatively low (in comparison with the published K_m values[3]). Under such conditions, the mechanism can be approximated as an ordered sequence of coenzyme association followed by the binding of substrate. The dissociation of aldehyde (or alcohol) is preferred to the dissociation of coenzyme, the break-down of the enzyme–coenzyme complexes being the rate limiting step in both reaction directions (the binary enzyme–aldehyde complex may occur but it is not kinetically significant[4]). If the kinetic mechanism of YADH were approximated to the Theorell–Chance type[2], in which the enzyme–NAD and enzyme–NADH complexes and the free enzyme (E) should be the only kinetically significant enzyme forms (as claimed in Ref. 4), the interpretation of the kinetic data would be further simplified (see Table II).

The double reciprocal plots of some of the inhibition data are presented in Figures 2 and 3. The qualitative analysis of these plots shows that the inhibition pattern for the reaction approaches the un-noncompetitive type, whereas the mixed competitive

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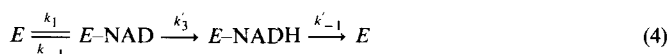
TABLE II

Analysis of the influence of coptisine (I, comp. 7) on the kinetics of the ethanol oxidation catalyzed with YADH The interpretation assumes the validity of the ordered (Eq. 3) or the Theorell-Chance (Eq. 4) mechanisms, the inhibitor is considered to bind with all enzyme forms appearing in Eqs. 3 and 4 (the free coenzymes, substrate and product are not included in the equations for the sake of simplicity). A, B, C, D, K_1, K_2, K_3, and K_4 are defined in Eq. (1).

$$E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{–NAD} \underset{k_{-2}}{\overset{k_2}{\rightleftharpoons}} E\text{–NAD–}Alc \underset{k'}{\overset{k}{\rightleftharpoons}} E\text{–NADH–}Ald \xrightarrow{k'_{-2}} E\text{–NADH} \xrightarrow{k'_{-1}} E \quad (3)$$



$$E \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} E\text{–NAD} \xrightarrow{k'_3} E\text{–NADH} \xrightarrow{k'_{-1}} E \quad (4)$$

Kinetic coefficients:	A	B	C	D
Interpretation of the kinetic coefficients[1]:				
Ordered mechanism	$1/k'_{-1} + 1/k'_{-2} + Q/K$	$\frac{Qk_{-2} + k}{kk_2}$	$1/k_1$	$k_{-1}/k_1.B$
Theorell–Chance mechanism	$1/k'_{-1}$	$1/k'_3$	$1/k_1$	$k_{-1}/k_1.B$
Inhibition constants:	K_1	K_2	K_3	K_4
Inhibition constants reflect the binding of I with[1,14,22]:				
Ordered mechanism	E–NADH and ternary complexes	E–NAD	E	E
Theorell–Chance mechanism	E–NADH	E–NAD	E	E
Calculated values of inhibition constants (μM) ($\pm$SD)	35 ± 6	236 ± 106	46 ± 8	59 ± 19

$Q = 1 + k/k'_{-2}$.

pattern can be observed for the reverse reaction. If the Theorell–Chance approximation were assumed these results suggested that the binding of coptisine to the enzyme–NAD complex (reflected by the effects on the intercepts in the plots for the backward reaction) should be weaker than that to the enzyme–NADH complex (influence on the intercepts in the plots for the forward reaction–see Table II). If the ordered mechanism were assumed the interpretation of the data would be less straightforward since the binding of the inhibitor to the ternary complexes would influence the values of intercepts (cf. Table II).

The kinetics of inhibition were analysed in greater depth for the forward reaction. More than one hundred initial reaction rates at changing concentrations of NAD (0.02–0.06 mM), ethanol (5–90 mM) and coptisine (0–80 μM) were measured. These data were fitted by a computer to the general equation describing the influence of an inhibitor on the kinetics of a two-substrate enzyme (which is valid regardless of the kinetic mechanism) (Eq. (1) in Methods). The values of A–D constants were computed from the data obtained in the absence of coptisine. The results of the computer analysis are presented in Table II along with the interpretation of the data. The calculated values for the inhibition constants are compatible with the supposed kinetic mechanisms and with the results of the qualitative analysis presented above. The value of K_2 (which should reflect the binding of the inhibitor to the E–NAD complex) is

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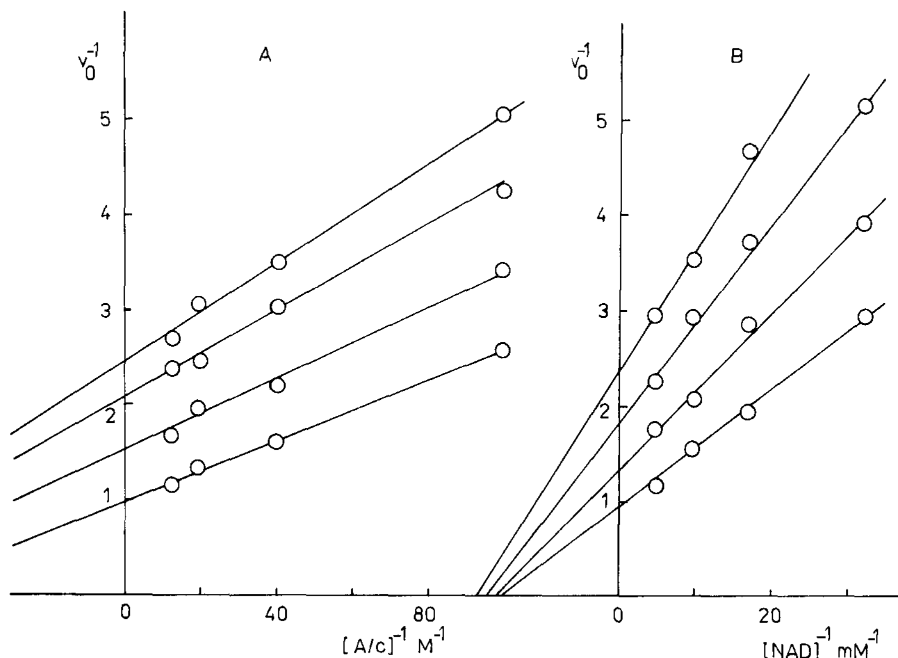

FIGURE 2 Inhibition of YADH with coptisine (forward reaction). The conditions of experiments are described in Methods; v_0 is the initial rate. Coptisine concentrations: 0, 20, 40 and 60 μM. (A) Variable ethanol (*Alc*) concentration ([NAD] = 0.6 mM), (B) Variable NAD concentration ([ethanol] = 90 mM).

essentially higher than those of the other inhibition constants; this can be interpreted as a pronounced labilization of the inhibitor binding to YADH in the presence of bound NAD. The values of K_3 and K_4 can be regarded as identical (these constants should reflect the binding of the inhibitor to the free enzyme as imposed by both assumed mechanisms). The K_1 constant cannot be interpreted unequivocally if the ordered mechanism is assumed, only if the Theorell–Chance approximation is considered then this constant should reflect the binding of coptisine to the *E*–NADH complex. However, the closeness of its value to the values of K_3 and K_4 suggests that the affinity of coptisine to *E*–NADH (and to the ternary complexes if they were kinetically significant) does not diverge substantially from that to the free enzyme.

Equilibrium Measurements

In order to confirm the results of the kinetic analysis (which could be biassed to some extent due to the approximations made regarding the kinetic mechanism of YADH) we carried out the measurements of the changes in optical properties of coptisine and corysamine in the presence of YADH and coenzymes or substrates. Additions of YADH (30 μM) to the solutions of these compounds (20 μM) brought about slight hypochromic effects in the UV-VIS absorption spectra (at about 350 and 420 nm). These effects were partially reversed by NAD additions (0.5 mM). As a comparable hypochromism was observed in ethanol or dioxane the supposed relative hydrophobicity of the YADH binding site for protoberberines seems to be confirmed.

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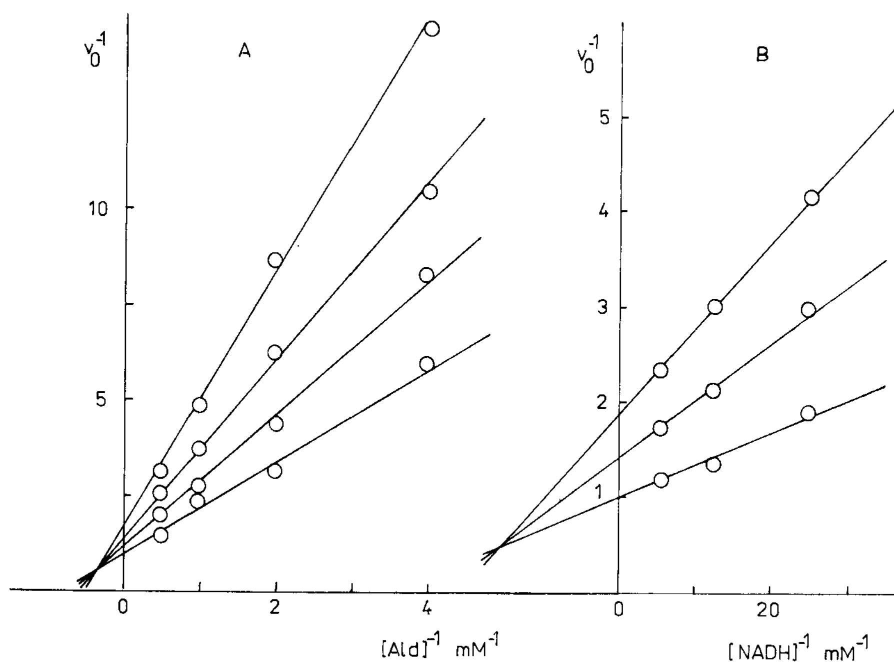

FIGURE 3 Inhibition of YADH with coptisine (backward reaction). The conditions of experiments are given in Methods. Variable acetaldehyde (*Ald*) concentration ([NADH] = 0.1 mM, [coptisine] = 0, 15, 30 and 60 μM). Variable NADH concentration ([acetaldehyde] = 10 mM, [coptisine] = 0, 10 and 20 μM).

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The measurements of fluorescence were carried out since this optical property of protoberberine alkaloids is more sensitive to environmental changes than the absorption spectra[16]. Coptisine fluoresces in water or buffers at about 530 nm (λ_{exc} = 420 nm); its fluorescence increases in non-polar media[16]. Addition of 10 μM YADH to 10 μM coptisine in the buffer pH 8 brought about a slight (but reproducible) increase in fluorescence intensity and a nearly negligible hypsochromic effect. This change was not sensitive enough to allow a precise evaluation of the data (at variance with LADH for which the fluorescence changes were very distinct[5,6]). On the other hand, polarization of the coptisine fluorescence increased essentially upon addition of YADH (Figure 4a). The presence of NAD in the cuvette resulted in a decreased effect on enzyme addition (Figure 4a), whereas NADH (50 μM), ethanol (0.1 M) and acetaldehyde (0.05 M) exhibited only negligible changes in the obtained titration curve. These results confirm unequivocally the labilization in coptisine binding in the presence of the oxidized coenzyme and indicate that the effects of NADH and substrates are small.

Coptisine, which has a high absorption coefficient at the wavelengths at which proteins fluoresce (i.e., around 350 nm), could act as a quencher of the intrinsic fluorescence of the enzyme via the energy-transfer mechanism. As this compound was also found to quench the fluorescence of tryptophan or acetyltrytophan (by a simple absorption mechanism) the quenching curve measured in the presence of the enzyme was corrected for this effect (see Methods). The corrected quenching curve (Figure 4b) could be described by Eq. (2) (see Methods) with $K = 52 \pm 6\,\mu M$ and $F_m = 0.72 \pm 0.06$. The value of the dissociation constant (K) is identical with the

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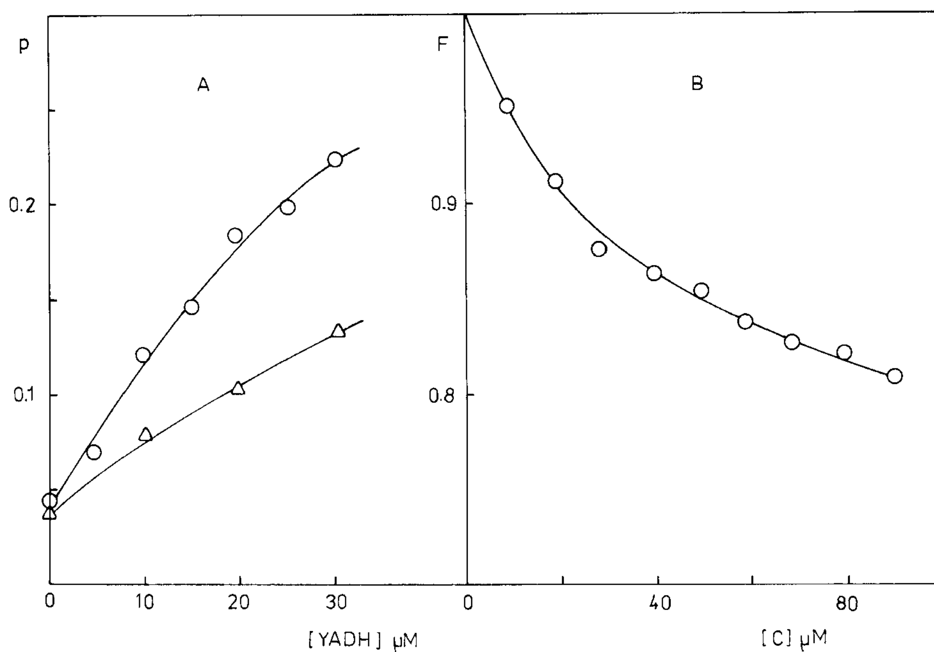

FIGURE 4 Fluorescence changes accompanying the binding of coptisine to YADH. (A) (○) Polarization (p) changes of 10 μM coptisine upon additions of YADH (λ_{exc} = 420 nm, λ_{em} = 430 nm), (△) the same in the presence of 0.3 mM NAD. (B) The corrected quenching curve of YADH (3 μM) fluorescence (F) upon additions of coptisine (C) (λ_{exc} = 280 nm, λ_{em} = 340 nm). The other conditions are described in Methods; the data are averages of three measurements.

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corresponding values of K_3 and K_4 obtained from the inhibition data (see above). The value of maximal quenching (i.e., about 28% of the initial protein fluorescence at saturation with coptisine) cannot be easily interpreted due to a high content of tryptophan residues in the molecule of YADH[1]. No reliable conclusion about the presence or absence of tryptophan residues in the binding site of YADH for alkaloids can be drawn from these data.

The equilibrium between bound and unbound coptisine in the presence of YADH is achieved very quickly. This was proved by the stopped-flow experiments in which the time depedence of the quenching of YADH fluorescence upon mixing the enzyme with coptisine was measured. The half-time of this reaction (measured at both 25 and 5°C) was found to be shorter than the dead-time of the instrument (about 4 ms). This result is compatible with the supposed superficial binding of protoberberine alkaloids to YADH and suggests that no essential conformational changes of the enzyme molecule are induced by the bound alkaloid. The corresponding process in the case of LADH is essentially slower[23] since the molecule of alkaloid protrudes deeply into the substrate pocket of this enzyme and brings about some conformational changes of the enzyme molecule at least in the vicinity of the alkaloid binding site[6].

Location of the Binding Site

YADH binds several heteroaromatic compounds[24] (such as *o*-phenanthroline and

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benzoquinoline) which resemble the examined alkaloids to some extent. However, the binding site of the enzyme for protoberberines is not identical with that for these compounds since the Yonetani–Theorell plots in the presence of 0–16 μM coptisine and 0–500 μM *o*-phenanthroline yielded nonparallel straight-lines (see Methods for the other experimental conditions). The observed behaviour (not shown) indicated clearly the non-exclusive binding of both inhibitors (though some labilization effects seemed to be involved). This difference in binding sites is quite conceivable since the binding site of YADH for phenanthrolines and benzoquinolines is claimed to be identical with that for the nicotinamide moiety of NAD[24], whereas protoberberine alkaloids do not compete with the coenzyme.

Further tentative conclusions on the location of the alkaloid binding site of YADH might be drawn from the observed difference in the effects of NAD and NADH on the binding of coptisine. In principle, two possibilities might be involved. First, the bound NAD could elicit some conformational changes of the enzyme molecule which would decrease the affinity of coptisine to YADH (the binding sites for NAD and coptisine could be relatively far from each other in this case). Considering the absence of the labilization effect in the presence of NADH, this explanation does not seem very probable (it would imply an essential difference in the binding modes of NAD and NADH and their influences on the enzyme conformation; this is unlikely in the case of YADH[1–3]). Second, the binding site of YADH for protoberberine alkaloids could be located close to the nicotinamide binding site of the enzyme. In this case, the positive charge of the NAD nicotinamide ring would decrease the affinity of alkaloids to YADH by an electrostatic repulsion between their positively charged molecules (the uncharged nicotineamide ring of NADH being without any appreciable effect). The structural, kinetic and binding characteristics of YADH[1–3] as well as the fact that no detectable labilization by NAD was observed for berberrubine at pH 8 (this substance is uncharged at pH above 6 – see above) seem to be compatible with the second possibility, i.e., with the assumption that the binding site of YADH for protoberberines is located at the surface of a relatively hydrophobic region near the nicotinamide binding site of the enzyme.

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