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The Specific Binding of Vitamin A Acid to Equine Liver Alcohol Dehydrogenase

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SUMMARY

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Addition of vitamin A acid to solutions of liver alcohol dehydrogenase (EC 1.1.1.1) results in a significant quenching of the protein fluorescence. The quenching attains a maximum value when equimolar amounts of the reactants are present. The quenching is pH-dependent, and indicates the involvement of a group on the protein with a pK value of 7.6. Vitamin A acid is a competitive inhibitor of the oxidation of ethanol, but does not significantly affect the reverse reaction. On the other hand, vitamin A amide competitively inhibits the reduction of acetaldehyde, without significantly affecting the oxidation of ethanol. The vitamin A acid protects the essential thiol groups against carboxymethylation to a lesser degree than reduced coenzymes but to a higher degree than ethanol.

INTRODUCTION

Equine liver alcohol dehydrogenase (EC 1.1.1.1) is composed of two identical subunits, each having a molecular weight of about 40,000 (1, 2) and each possessing a site for catalytic activity. Thus each subunit contains specific binding sites for Zn^{++}, DPNH, or DPN^+, and a substrate molecule (2). Ehrenberg and Dalziel (3) have shown that 2 moles of DPNH may be bound per mole of enzyme at neutral pH values; however, only 1 mole of the reduced coenzyme is bound per mole of enzyme at pH 10 or higher. In addition, van Eys *et al.* (4) showed that in the presence of hydroxylamine at pH 7.5 only 1 mole of coenzyme is bound per mole of enzyme. Van Eys *et al.* also reported that only 1 mole of a variety of thiols can be bound per mole of enzyme.

Recently Bernhard and his collaegues (5, 6) have concluded that the two catalytic sites in a mole of equine liver alcohol dehydrogenase may not operate independently, but that certain interactions between the two subunits may play an important role in ordering the course of the catalytic process. These conclusions are based on the observations that under limiting DPNH concentrations the reaction occurs in two distinct transformation steps, each leading to the same product, and each being equal to one-half the DPNH concentration. Also, when the DPNH and substrate concentrations are relatively high in proportion to the enzyme sites, a pre-steady-state "burst" reaction yields the disappearance of an amount of substrate equivalent to one-half the available enzyme sites. Sigman and Winer (7)

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concluded from studies on the formation of liver alcohol dehydrogenase–coenzyme–fatty acid amide complexes that only one binding site per mole of enzyme may be available for the substrate.

This contribution deals with the binding of vitamin A acid and vitamin A amide to equine liver alcohol dehydrogenase. This study was initiated in order to obtain more evidence about the actual number of substrate binding sites per mole of the enzyme.

MATERIALS AND METHODS

Alcohol dehydrogenases from yeast and equine liver were purchased from Boehringer and Sons, Mannheim, Germany. The liver enzyme was purified further as described below.

Crystalline preparations of vitamin A aldehyde and of the four isomers of vitamin A acid were donated by Dr. P. H. van Leeuwen, N. V. Philips-Duphar, Weesp, The Netherlands. Vitamin A alcohol was purchased from Nutritional Biochemicals Corporation. Vitamin A amide was prepared from vitamin A acid as described below. Stock solutions of 1 mM concentrations of the vitamin A derivatives were prepared in the following solvents: vitamin A acid, in 0.01 N NaOH; and vitamin A alcohol, aldehyde, and amide, in 1% *p*-dioxane in water.

All other reagents were of the highest purity and were obtained from commercial sources.

Fluorescence measurements were made with an Aminco-Bowman spectrophotofluorometer equipped with an Osram XBO 150-W xenon arc source and a constant-temperature cell holder. The measurements were made at 23°.

Optical density measurements were performed with a Zeiss spectrophotometer, type PMQ II.

Determination of enzyme activity. The enzymatic activities of the alcohol dehydrogenases were determined under the following conditions. For the forward reaction the assay solution contained 67 mM ethanol, 170 μM DPN^+ or $(AcPy)DPN^+$,[1] a suitable amount of enzyme, and 0.1 M glycine-NaOH buffer, pH 9.5, in a total volume of 3 ml. For the reverse reaction the solution contained 6 mM acetaldehyde, 140 μM DPNH, a suitable amount of enzyme, and 0.05 M glycylglycine buffer, pH 7.5, in a total volume of 3 ml. The reactions were initiated by the addition of the enzyme, and readings were taken every 15 sec at 340 nm. Assays were carried out at room temperature.

Determination of protein concentration. The concentration of the alcohol dehydrogenase was determined by titrating the enzyme solution with DPNH in the presence of isobutyramide, as described by Winer and Theorell (8).

Purification of liver alcohol dehydrogenase. Crystalline, commercial preparations of liver alcohol dehydrogenase may contain a certain amount of a substrate that cannot be completely removed by dialysis. The presence of this substrate in dialyzed enzyme preparations was demonstrated by adjusting the pH of the solution to 10, followed by the addition of a small amount of $(AcPy)DPN^+$. An enzyme-dependent increase in absorption at 363 nm indicated the formation of (AcPy)DPNH.

Several attempts were made to remove this substrate from the enzyme preparations. The following procedure proved to be successful: 20 mg of the commercial enzyme were dialyzed overnight against 0.1 M Tris, pH 10, at 4°. Then 10 mg of solid DPN^+ were added to the enzyme solution, and the mixture was kept at room temperature for 1 hr to stimulate the oxidation of the substrate. The enzyme was then separated from the pyridine nucleotides using a Sephadex G-100 column. The column (a 50-ml burette) was equilibrated with 0.05 M KCl in 0.05 M Tris buffer, pH 10, prior to use. The protein was eluted at room tem-

[1] The abbreviations used are: $(AcPy)DPN^+$ and (AcPy)DPNH, acetylpyridine analogues of DPN^+ and DPNH.

perature using the same buffer, and fractions of 1 ml were collected. The enzyme was recovered as a sharp band with a maximum at approximately fraction 30. The nucleotides were completely separated from the enzyme, and were recovered around fraction 55. The alcohol dehydrogenase was subsequently pooled and dialyzed against 0.1 M phosphate buffer, pH 7.5. In all preparations the recovery in enzyme units was better than 85%.

Preparation of vitamin A amide. All-*trans* vitamin A amide was prepared by converting the all-*trans* vitamin A acid into the acid chloride with the method described by Huisman *et al.* (9). This conversion was followed by treatment of the acid chloride with ammonia gas to yield the desired product.

Crystalline all-*trans* vitamin A acid (300 mg) was dissolved in 20 ml of pure dry benzene. Then 0.06 ml of redistilled PCl_3 was added, and the mixture was kept for 3 hr at room temperature in a desiccator that contained some solid NaOH to bind the formed hydrochloric acid. The solution rapidly became dark yellow and crystallized when cooled to 10°. The crystals were identified as vitamin A acid chloride by their absorption spectrum in cyclohexane (9).

To redissolve the crystals, 20 ml of dry benzene were added, and dry ammonia gas was slowly bubbled through the solution at 5°. The solution soon became pale yellow. The addition of ammonia was continued for 30 min. The reaction mixture was then transferred into a separatory funnel and extracted twice with a 1% sodium carbonate solution to remove unreacted vitamin A acid. The organic phase was collected, washed twice with water, and dried over anhydrous Na_2SO_4. The benzene was removed under vacuum, and the remaining solid was dissolved in 15 ml of boiling acetone. Upon cooling, the material crystallized, forming small, pale yellow needles. The crystals were collected and recrystallized from acetone. Yield, 163 mg; m.p. 165–166° (uncorrected). The absorption spectrum of the amide in cyclohexane is presented in Fig. 1. The molar extinction coefficient at 351 nm was calculated to be 2.2×10^4.

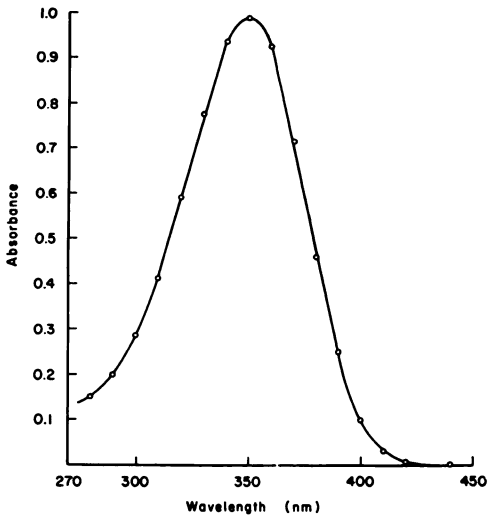

FIG. 1. *Absorption spectrum of vitamin A amide* Concentration: 13.5 mg/liter in cyclohexane.

RESULTS

Effect of vitamin A acid on fluorescence of liver alcohol dehydrogenase. Ten additions of all-*trans* vitamin A acid, each containing 1.2 nmoles, were made to a solution of 6 nmoles of liver alcohol dehydrogenase in 2 ml of 0.1 M sodium phosphate buffer, pH 7.5. The decrease in the tryptophan fluorescence was determined after each addition, and the results are presented in Fig. 2. A 30% decrease in the protein fluorescence is observed when 1 mole of vitamin A acid is present per mole of enzyme. Further additions of the acid do not result in any further decrease of the fluorescence. When the reaction is performed in glycine buffer at pH 9.5, the decrease in protein fluorescence is about 45%; this is also obtained when equivalent amounts of enzyme and vitamin A acid are present in the solution.

When the reaction is performed in glycylglycine buffer, pH 7.5, maximum quenching of the protein fluorescence is also reached at

the point where equimolar amounts of the reactants are present; however, 69% quenching of the protein fluorescence is obtained under these conditions. When the Δ^3-*trans*-Δ^7-*cis* isomer of vitamin A acid is used under comparable conditions, the maximal quenching is 53%. Similarly, the Δ^3-*cis*-Δ^7-*trans* and the Δ^3-*cis*-Δ^7-*cis* isomers yield 54% and 63% quenching, respectively. No quenching of the protein fluorescence was observed when all-*trans* vitamin A aldehyde or all-*trans* vitamin A alcohol was used under comparable conditions.

Effect of carboxymethylation on binding of vitamin A acid to liver alcohol dehydrogenase. Ten milligrams of liver alcohol dehydrogenase, dissolved in 1 ml of 0.1 M phosphate buffer, pH 7.5, were treated with 7.4 mg of iodoacetate (320 moles/mole) containing some [^{14}C]iodoacetate for 55 min at room temperature. The reaction was terminated by the addition of 0.1 ml of β-mercaptoethanol. The solution was then dialyzed against 0.1 M ammonium carbonate. An enzymatic assay of the dialyzed material indicated that 12% of the original activity still remained in the protein. Another aliquot of the dialyzed material was analyzed for its ^{14}C content. The results indicated that 4.15 carboxymethyl groups were bound per mole of enzyme. In a parallel experiment the incubation with iodoacetate was performed in the presence of 0.05 ml of 95% ethanol. Under these conditions 2.55 carboxymethyl groups were bound per mole of enzyme, and 63% of the original activity still remained in the protein.

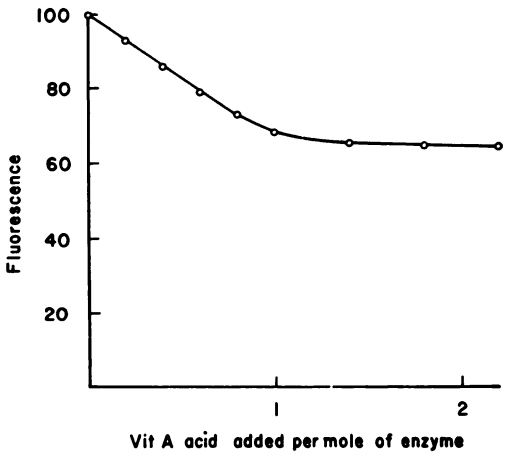

FIG. 2. *Quenching of fluorescence of liver alcohol dehydrogenase upon addition of vitamin A acid*
Concentration of enzyme; 3.0 μM; excitation, 288 nm; emission, 340 nm.

Each preparation was then tested for its ability to bind vitamin A acid by measuring the quenching of the protein fluorescence. The results, presented in Fig. 3, indicate that no quenching of the fluorescence was obtained with the enzyme that contained 4.15 carboxymethyl groups per mole, whereas the protein with 2.55 carboxymethyl groups showed a maximum quenching of 20% when equimolar amounts of the

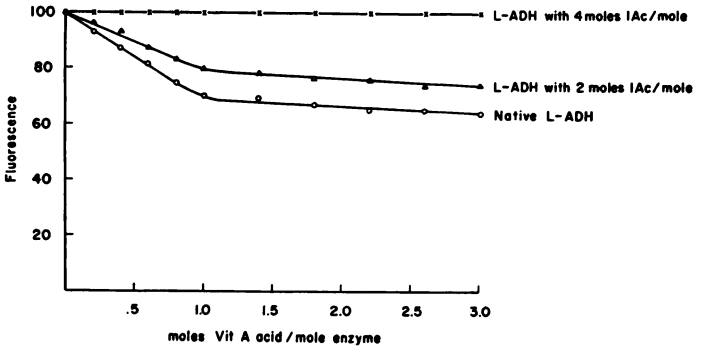

FIG. 3. *Quenching of fluorescence of carboxymethylated liver alcohol dehydrogenase (L-ADH) by vitamin A acid*
Concentration of protein, 3.0 μM; excitation, 288 nm; emission, 340 nm. IAc, iodoacetate.

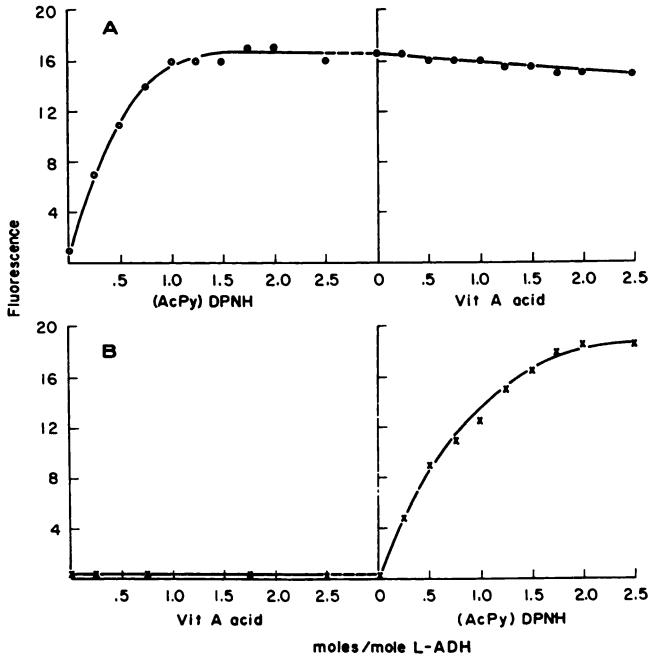

FIG. 4. *Enhancement of coenzyme fluorescence*

A. Titration of 3.0 μM liver alcohol dehydrogenase (L-ADH) with vitamin A acid, followed by titration with (AcPy)DPNH. B. Titration of 3.0 μM enzyme with (AcPy)DPNH, followed by titration with vitamin A acid. Excitation, 366 nm; emission, 450 nm.

enzyme and of the vitamin A acid were present. This decrease in quenching effect may indicate that the vitamin A acid is bound somewhat differently in the partially carboxymethylated enzyme than it is in the native protein.

Effect of coenzymes on binding of vitamin A acid. The addition of DPNH to liver alcohol dehydrogenase containing 2 moles of vitamin A acid per mole at pH 7.5 did not result in any further quenching of the tryptophan fluorescence. Nor were further changes observed in an experiment in which the reduced coenzyme was added prior to the vitamin A acid. Similarly, the enhancement of the coenzyme fluorescence of (AcPy)-DPNH upon binding to liver alcohol dehydrogenase is not affected by the presence of vitamin A acid, as shown in Fig. 4A and B. These results may indicate that vitamin A acid does not disturb the binding of the coenzyme, and vice versa.

Incubation of 6 nmoles of liver alcohol dehydrogenase in 2 ml of phosphate buffer, pH 7.5, with 60 nmoles of DPN^+ and 12 nmoles of vitamin A acid for 1 hr at room temperature did not result in quenching of the tryptophan fluorescence to a greater extent than is observed with vitamin A acid alone. Evidence for the formation of a ternary complex among enzyme, DPN^+, and vitamin A acid could also not be gained from the absorption spectrum, owing to the very high extinction coefficient of vitamin A acid around 340 nm.

Effect of pH on quenching of liver alcohol dehydrogenase fluorescence. Aliquots of 6 nmoles of liver alcohol dehydrogenase, each contained in 2 ml, were prepared in 0.1 M phosphate buffers ranging in pH from 5.5 to 9.5. To each solution was added 0.02 ml of vitamin A acid solution, containing 6 nmoles, and the effect on the fluorescence of the enzyme was recorded. The effect of pH on the quenching of the protein fluorescence is illustrated in Fig. 5. Below pH 7.0 the

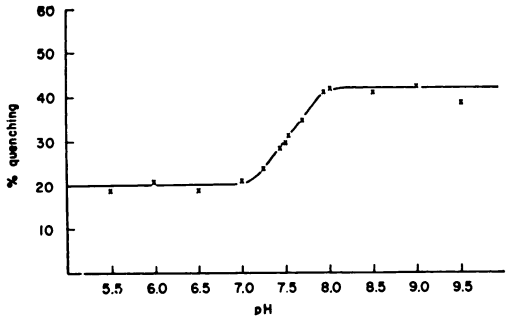

FIG. 5. *Quenching of fluorescence of liver alcohol dehydrogenase by vitamin A acid in phosphate buffers, pH 5.5–9.5*

Concentrations: enzyme, 3.75 μM; vitamin A acid, 6.0 μM.

quenching is about 20%, whereas an increase to about 47% is observed at pH values above 8.0. The pK is 7.6. These data indicate that a group in the protein with a pK of 7.6 may be involved in the binding of the vitamin A acid.

Inhibitory effects of vitamin A acid. The oxidation of ethanol by liver alcohol dehydrogenase, with DPN^+ as the oxidizing agent, was inhibited by 5% when 1 mole of vitamin A acid per mole of enzyme was added to the reaction mixture. However, the compound shows much stronger inhibitory properties when the DPN^+ is replaced by its acetylpyridine analogue. Table 1 summarizes data showing the effects of increasing concentrations of the acid on the ethanol reaction with (AcPy)DPN^+ as the coenzyme. It should be pointed out that the inhibition levels off at concentrations of about 10 μM vitamin A acid, because of its limited solubility at neutral pH. In another experiment the inhibitory effect of a constant amount of vitamin A acid was measured at various concentrations of ethanol. The results are presented in Fig. 6 in the form of a double-reciprocal plot. The data show that the inhibition is competitive with the substrate. The K_i value was calculated to be 1.94 μM. The inhibition is noncompetitive with the coenzyme.

Vitamin A acid in a concentration of 3 μM does not inhibit the reduction of acetaldehyde by liver alcohol dehydrogenase with DPNH as the reducing agent. Yeast alcohol dehydrogenase is not inhibited in either direction by the vitamin acid. The compound also does not affect the activity of several lactate dehydrogenases.

Effect of vitamin A amide on activity of liver alcohol dehydrogenase. Vitamin A amide at a concentration of 3.4 μM does not affect the oxidation of ethanol by liver alcohol dehydrogenase. However, the compound was found to be a competitive inhibitor of the reduction of acetaldehyde as catalyzed by this enzyme. Data showing the competitive inhibition are presented in Fig. 7. The K_i was calculated to be 8.6 μM.

No quenching of the fluorescence was observed when vitamin A amide was added to liver alcohol dehydrogenase at pH 7.5 or 9.5.

Effects of DPNH, vitamin A acid, and ethanol on carboxymethylation of liver alcohol dehydrogenase. Eight 1-ml aliquots, each containing 2 mg of the enzyme in 0.1 M phosphate buffer, pH 7.5, were incubated at room temperature with 12.5 moles of iodoacetate per mole of enzyme. Prior to the addition of iodoacetate, DPNH was added to samples 1 and 2 at concentrations of 58 and 13 moles/mole of enzyme, respectively. Similarly, samples 3 and 4 contained, respectively, 50 and 10 moles of vitamin A

TABLE 1

Inhibition by vitamin A acid of oxidation of ethanol by equine liver alcohol dehydrogenase

The reaction mixture contained 670 nM enzyme, 29 mM ethanol, 170 μM (AcPy)DPN^+, and 0.1 M glycine-NaOH buffer, pH 9.5.

Concentration of vitamin A acid	Inhibition
M	%
1.7×10^{-7}	8
3.4×10^{-7}	13.5
3.4×10^{-6}	26
3.4×10^{-5}	35.5[a]

[a] Saturated.

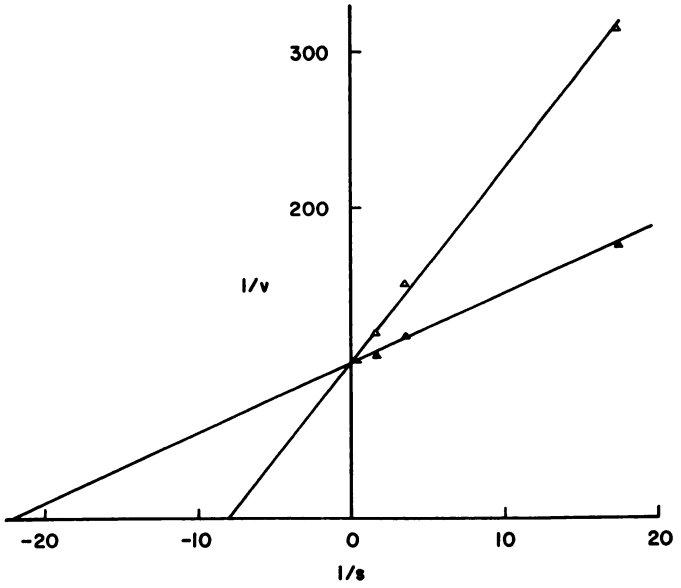

FIG. 6. *Lineweaver-Burk plot of inhibitory effect of vitamin A acid on oxidation of ethanol*
Concentration of vitamin A acid, 3.4 μM. (AcPy)DPN$^+$ was used as the coenzyme.

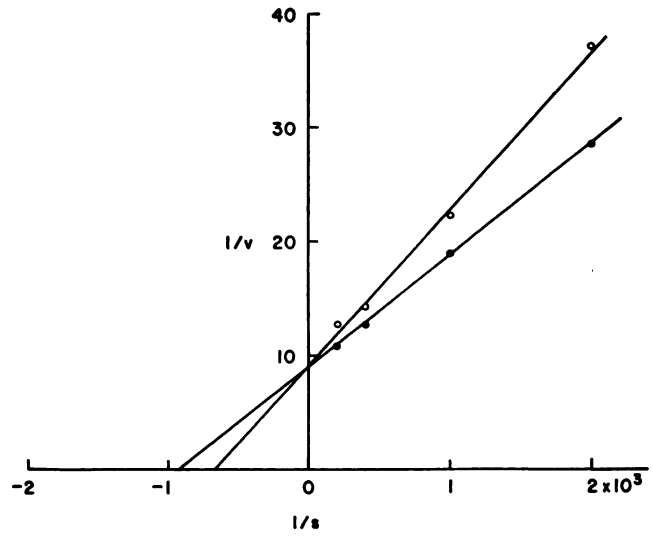

FIG. 7. *Lineweaver-Burk plot of inhibitory effect of vitamin A amide on reduction of acetaldehyde*
Concentration of amide, 3.4 μM. The coenzyme used was DPNH.

acid per mole of enzyme, and samples 5 and 6 contained 50 and 10 moles of ethanol per mole of protein. No additions were made to samples 7 and 8. The effects of these compounds on the rate of carboxymethylation, as measured by the decrease in enzyme activity, are indicated in Fig. 8. The reduced coenzyme, in concentrations as low as 13 moles/mole of enzyme, protects the enzyme completely from inactivation by iodoacetate. Partial protection is found with vitamin A acid and with ethanol. Vitamin A acid seems to be the more potent protector of the two compounds, giving better protection at a concentration of 10 moles/mole than ethanol does at a concentration of 50 moles/mole.

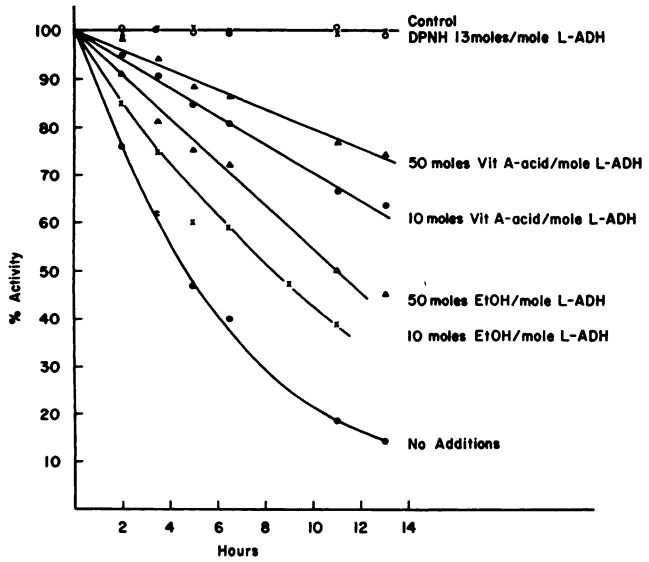

FIG. 8. *Effects of DPNH, vitamin A acid, and ethanol on reaction of iodoacetate with liver alcohol dehydrogenase (L-ADH)*

Concentrations: enzyme, 24 μM; iodoacetate, 300 μM. The reaction was performed in 0.1 M Tris buffer, pH 7.5, at room temperature.

In a separate experiment it was determined that ethanol at a concentration of 7500 moles/mole of enzyme gives protection comparable to that given by 10 moles of vitamin A acid per mole of protein.

DISCUSSION

Vitamin A acid was found to be a competitive inhibitor of the oxidation of ethanol by DPN^+, whereas vitamin A amide affects the reduction of acetaldehyde by DPNH, as catalyzed by liver alcohol dehydrogenase. These observations indicate that vitamin A acid and its amide affect the liver enzyme-catalyzed reactions in a way similar to that observed with a variety of other fatty acids and fatty acid amides (8, 10). It is thus reasonable to assume that the vitamin A derivatives bind to the enzyme in a manner similar to other fatty acids and their amides.

Since the inhibitors are competing with the substrate for the substrate-binding site, it follows that the maximum number of moles of inhibitor that can bind to a mole of enzyme represents the number of available substrate binding sites per mole of alcohol dehydrogenase. The data in this paper suggest that only 1 mole of vitamin A acid binds per mole of enzyme, whereas presumably two substrate-binding sites are available. Analogous observations were made by van Eys *et. al.* (4). These authors observed that various thiols are able to bind to liver alcohol dehydrogenase; the maximum amount of thiol that may be bound to the enzyme, however, is only a single mole per mole. Sigman and Winer (7) studied the kinetic properties of alcohol dehydrogenase–coenzyme–fatty acid amide complexes. They concluded that their data indicate the presence of a single substrate-binding site per molecule. The evidence obtained with binding studies thus strongly suggests the presence of a single substrate-binding site on the liver alcohol dehydrogenase molecule.

Conversely, it has been shown that liver alcohol dehydrogenase accommodates 2 coenzyme molecules per enzyme molecule (11–14). This led van Eys *et al.* (4) to suggest that 2 moles of DPN^+ may be required for the oxidation of 1 mole of substrate, 1 mole of DPN^+ serving as the coenzyme and being

reduced to DPNH, and the other DPN^+ serving in some form of regulator capacity. Recently Bernhard and his colleagues (5, 6) investigated the course of the transient kinetics of this enzyme, using a chromophoric aromatic aldehyde as the substrate. Their results show the occurrence of a rapid initial "burst" of activity preceding the attainment of the steady state. During this rapid initial process an amount of substrate equivalent to one-half the available coenzyme-binding sites is converted to product, and 1 mole of DPNH per mole of aldehyde is simultaneously oxidized to DPN^+. The occurrence of such a "burst" is expected if the rate-limiting step in the dehydrogenase reaction involves the dissociation of products; however, the amount of product formed during the burst should be equivalent to the concentration of enzymatic active sites. Heck *et al.* (15) demonstrated the occurrence of such a "burst" in activity in the lactate dehydrogenase-catalyzed oxidation of lactate; in this case the amount of DPNH formed during the burst was equivalent to the number of active sites of lactate dehydrogenase, i.e., 4 moles of DPNH formed per mole of enzyme, as expected. Unfortunately, no data were given concerning the transient kinetics of the reverse reactions.

Available information thus points more and more toward the idea that only one active site per mole of liver alcohol dehydrogenase is operative during catalysis (4–7), and the data presented here provide additional support for this theory. X-ray crystallographic data have shown that the apoenzyme is a symmetrical molecule, containing two subunits. However, if the enzyme is saturated with DPN^+, the symmetry in the molecule no longer exists (16). It is thus possible that the binding of the 2 coenzyme molecules causes the two subunits to become different in conformation, with only one having the capacity to bind a substrate molecule.

Unlike vitamin A acid, vitamin A amide does not quench the fluorescence of liver alcohol dehydrogenase, although it is a competitive inhibitor of the reduction of acetaldehyde. The different effects on the enzyme fluorescence that are exerted by the inhibitors may support the conception that alcohols and fatty acids on the one site and aldehydes and amides on the other site bind to different sites on the enzyme (7, 17); another explanation may be that both inhibitors bind to the same site and that the quenching of the protein fluorescence by vitamin A acid is simply the result of an interaction of the negatively charged carboxyl group with a tryptophan residue.

It is of interest that only small differences are found in the fluorescence quenching upon binding of the four isomeric forms of vitamin A acid. Since the carbon-carbon chains in these isomers vary from being linear (in the all-*trans* vitamin A acid) to possessing two angles (in the all-*cis* acid), this seems to indicate that the area on the enzyme molecule that is occupied by the nonfunctional groups of the substrates or the inhibitors is a large open space, or that these nonfunctional groups mostly reside outside the protein region.

Carboxymethylation of the sulfhydryl groups prevents the quenching of the enzyme fluorescence by vitamin A acid. However, bound vitamin A acid does not influence the binding of DPNH. Whether bound coenzyme influences the binding of the inhibitor cannot be concluded from the fluorescence studies, since the quenched fluorescence of the enzyme-DPNH complex is not changed upon addition of vitamin A acid. The inhibition of the carboxymethylation of the enzyme by vitamin A acid may indicate the proximity of the inhibitor to the coenzyme-binding site. The protection against carboxymethylation by coenzymes was also shown by Li and Vallee (18). These authors were unable, however, to show any influence by ethanol on the reaction.

The pH effect on the quenching of the protein fluorescence by vitamin A acid shows a pK value of 7.6. This indicates that

a group on the protein with such a pK is affected by the binding of the inhibitor. It seems somewhat premature to speculate on the nature of this residue, which may be a histidine or an α-amino group, as well as possibly a sulfhydryl or an ϵ-amino group. It is also possible that it is the result of the binding of the inhibitor to a zinc atom. Harris (19) has indicated that the active site of the liver enzyme contains a histidine residue that is in a relatively favorable position for binding to the substrate. On the other hand, evidence has been obtained in Theorell's (20) and Vallee's (21, 22) laboratories that a zinc atom may be involved in the active site, possibly by binding to the substrate.

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