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Alcohol as an Impurity in LADH Preparations

Determination and Removal

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A spectrofluorometric method for determining the amount of alcoholic components remaining in horse liver alcohol dehydrogenase is described. Application of the method to electrophoretically homogeneous horse liver regular alcohol dehydrogenase and purified steroid active alcohol dehydrogenase had shown that the amount of residual alcohol can be minimized by carefully controlled dialysis procedures to far less than one molecule of alcohol per coenzyme-binding site. The availability of homogeneous alcohol dehydrogenase with only trace amounts of alcohol has made experimentation with NAD^+ plus LADH mixtures possible without interference from NAD^+ reduction.

It has long been known that crystalline preparations of horse liver alcohol dehydrogenase (LADH) ** retain some alcohol despite all attempts at removal by dialysis or other means [1,2] and despite the relatively large values of the dissociation constant ($K_{E,\ alc}$), for the dissociation of ethanol from the active sites of LADH, which have been proposed (4.6 or 6 mM).[3] Our routine procedures for enzyme preparation have included a few stages of treatment with alcohol, such as a chloroform-ethanol treatment to remove hemoglobin with octanol added as an anti-foaming reagent for subsequent removal of the chloroform by evaporation. Furthermore,[4] LADH is crystallized from ethanol, and the recrystallized sample is then stored in 30 % ethanolic phosphate buffer at −20°. The enzyme solution then has to be dialysed to remove alcohol before it is used in any experiments in which the control of

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** The following abbreviations are used: LADH, liver alcohol dehydrogenase; $LADH_S$, steroid active alcohol dehydrogenase;[1] $LADH_E$, regular alcohol dehydrogenase (inactive towards steroidal alcohols); NADH and NAD^+, reduced and oxidized nicotinamide adenine dinucleotide, respectively; CM-cellulose, carboxymethyl cellulose; DEAE-cellulose, diethylaminoethyl cellulose. LADH, NADH, NAD^+ are abbreviated as E (= LADH corresponding to one coenzyme-binding site), R and O, respectively, in expressing dissociation constants and the form of the binary complex of LADH.

alcohol concentration is critical, as any side reaction involving alcohol causes complications. Under general laboratory conditions abundant opportunities exist for the enzyme solution to be contaminated with volatile alcohols. Attempts to remove reactive alcohols, *e.g.* by replacing ethanol with methanol which is known to be inactive as a substrate or by lyophilization, have not been too successful but have introduced other difficulties such as decreased stability or inactivation of the enzyme during the treatment. Precipitation of the enzyme by ammonium sulfate at neutral pH was found to be rather ineffective as a means of removing the last traces of alcohol. In fact, after the addition of ^{14}C-labeled ethanol to an enzyme solution followed by repeated precipitation and washing of the enzyme protein with 70 % saturated ammonium sulfate solution [5] nearly one ^{14}C-ethanol molecule per coenzyme-binding site was still present.

This paper describes methods which have been developed to reduce the ethanol in LADH preparations to insignificant levels. Further, it was possible to calculate the amount of ethanol in LADH solutions from the NADH formed upon addition of NAD^+. The removal of this contaminating alcohol has made possible the direct spectrophotometric determination of the dissociation constant of LADH—NAD^+, as described in a following paper.[6]

MATERIALS

Enzyme. Homogeneous $LADH_E$ was prepared by a modification of Dalziel's method.[4] The detailed description can be found in a following paper.[6]

The concentration of the dialysed enzyme preparation was determined spectrophotometrically by titrating LADH with NAD^+, in the presence of excess pyrazole, in phosphate buffer, pH 7.0, 0.1 μ,[7] and was expressed as N, the normality of the coenzyme-binding capacity. The electrophoretically homogeneous enzyme preparations used in the present studies were found to be 100 % active on the basis of a specific extinction coefficient at 280 mμ of 0.455 (mg/ml cm^{-1}) * and a molecular weight of 84 000 per two coenzyme-binding sites.

The preparation of the steroid active dehydrogenase $LADH_S$ and the determination of its purity have been described in a recent paper.[1] The purity of the steroid active dehydrogenase employed in the present studies was approximately 90 % as judged from electrophoretic criteria. The impurities (approximately 10 % of the total) were more acidic components, half of which seemed to be $LADH_E$ as analyzed by agar-gel electrophoresis and subsequent activity staining.

Coenzymes and other chemicals. NAD^+ and NADH were purchased from Sigma Chemical Co. (98 and 95 % purity, respectively) and were experimentally found to be 98 % enzymatically reducible and 95 % enzymatically oxidizable, respectively, on the basis of a millimolar absorbancy index of NAD^+ at 260 mμ of 18.0 (mM^{-1} × cm^{-1}) and of NADH at 338 mμ of 6.22 (mM^{-1} × cm^{-1}).[10] NAD^+ was further purified, to essentially 100 % purity, by DEAE-cellulose column chromatography according to Dalziel's method.[12] The concentration of NAD^+ and NADH were expressed in terms of the coenzymatically reactive portion, on the basis of the 338 mμ absorption. All the chemicals used for dialysis and measurement were Merck products of analytical grade.

* In the present study ε 280 mμ = 0.455, instead of the old value of 0.420, was employed throughout. This value was found to be consistent in electrophoretically homogeneous preparations by repeated determination (personal communication from Dr. Å. Åkeson). The present value is the same as that found by Bonnichsen [8] in 1950. However, a definite determination has still to be made, since the determination is complicated by the presence of uncertain amounts of adsorbed amino acids, as reported from this laboratory.[9]

Water and buffers. The water used in the present experiments was glass distilled water, redistilled over slightly alkaline permanganate, passed through a quartz condenser, and immediately stored in tightly stoppered glass bottles. The buffer solutions used in the present studies, particularly for dialysis of the enzyme solutions, were freshly prepared by dissolving solids of the buffer components in redistilled water in a 500 ml graduated glass cylinder with stopper. It was found critical to take special care to avoid contamination by exogeneous alcohol, as already emphasized in 1961.[12] The phosphate-glycine-NaOH buffer of pH 9.0, employed throughout the present studies, was 0.1 μ phosphate buffer, pH 8.0, plus 3.6 mM glycine-NaOH buffer as used previously.[3,13] Enzymatic assay of alcohol by yeast alcohol dehydrogenase (purchased from C. F. Boehringer und Soehne, Mannheim) indicated that all chemical solutions made with the redistilled water and used in the present studies were virtually free from alcohol.

Rationale and operation of the method for determination of residual alcohol. The purpose of the present investigation was to determine the total initial alcohol concentration in an LADH solution by determination of the NADH produced after a known amount of NAD^+ had been added to the test enzyme solution. This can be done by utilizing the known dissociation constants, K_{ER} and K_{EO},[6] and the overall equilibrium constant [14]

$$K_{eq} = \frac{[R][ald][H^+]}{[O][ethanol]} = 0.90 \times 10^{-11} \text{ at } 23°C$$

The simplest way to determine the total NADH would have been spectrophotometry at 330 mμ, where free and bound NADH have an isosbestic point.[15] However, under present conditions, spectrophotometry is not sensitive enough, so spectrophotofluorometry was used. Correction was made for the widely different fluorescence intensities of free and bound NADH. The pH value of 9.0 was chosen as a compromise since an alkaline pH favours the equilibrium towards the aldehyde-NADH side but, at the same time, excessively high alkaline pH ($\gtrsim$10) allows NAD^+ to compete strongly with NADH for binding to LADH. This causes a decreased accuracy in the fluorescence measurements by losing the advantageously high fluorescence of bound NADH over that of the free form. In fact, an increase in pH decreases K_{EO} and increases K_{ER}, both dissociation constants being about the same at pH 10.0.[6] When NAD^+ (O_t μM)is added to an LADH solution (E_t μN) containing alcohol (total, X μM) at pH = 9.0, the concentration of free species, after the equilibrium is established, should be checked by the following overall equilibrium constant:[13]

$$K_{eq} = \frac{[Ald_f][R_f][H^+]}{[Alc_f][O_f]} = 9 \times 10^{-12}$$

Therefore, at pH = 9: $9 \times 10^{-3} = \dfrac{(R_f + R_b)\ R_f}{(X-a)\ (O_t-a-O_b)}$ (1)

where X = concentration of total alcohol before the addition of NAD^+ and a = aldehyde produced = $R_f + R_b$; the suffixes f and b denote the free and enzyme-bound forms of NADH, respectively. It is also assumed that most of the aldehyde produced and unreacted alcohol exist in the free form on the basis of the relatively large values proposed for the enzyme-substrate binary complex, $K_{E, ald} = 30$ μM and $K_{E, alc} = 6000$ μM or 4600 μM,[3] both determined at pH 7.0.

We also have the following relationship between R_b and R_f for a known K_{ER} value:

$$K_{ER} \text{ at pH } 9.0 = 0.46\ \mu M^* = \frac{E_f \times R_f}{ER} = \frac{R_f}{R_b}(E_t - R_b - O_b) \quad (2)$$

Equations (1) and (2) give

$$X = \underbrace{\frac{a \times R_f \times 110}{O_t - R_f - E_t + (0.46\ R_b/R_f)}}_{\text{(unreacted alcohol)}} + \underbrace{a}_{\text{(oxidized alcohol)}} \quad (3)$$

* This new value of the dissociation constant was determined spectrofluorometrically in equilibrium experiments with homogeneous $LADH_E$ samples.[6]

Thus, X can be determined if the concentration of both R_b and R_f in the system at equilibrium is experimentally determined. Furthermore, since there is an additional relationship with O_b;

$$K_{EO} \text{ at pH } 9.0 = 11.0\ \mu M\ ^{6} = \frac{E_f \times O_f}{EO} = \frac{1}{O_b}\,(E_t - R_b - O_b)\,(O_t - a - O_b) \qquad (4)$$

this can be used to check the overall consistency of the experimentally determined values of R_b and R_f by calculating the O_b value in the following two different ways:

$$O_b = A - (0.46\ R_b/R_f) \text{ (derived from (2), where } A = E_t - R_b) \qquad (5)$$

or, by solving the following equation derived from (4):

$$O_b^2 - O_b\,(A + B + 11.0) + AB = 0, \text{ where } B = O_t - a \qquad (6)$$

Experimental determination of the R_b and R_f values was carried out by means of a recording spectrofluorimeter [16] (phosphate-glycine-NaOH buffer, pH 9.0, was used throughout the present studies). Instrumental conditions were a fixed slit-opening (2 mm) for excitation light (330 mμ) and 1 mm for fluorescence emission light and a fixed sensitivity scale on the recorder. Fluorescence emission spectra of a 1.0 μM NADH solution and of a 1.0 μM NADH plus 10 μN LADH solution were obtained over the range 260 to 650 mμ, curve A and B, respectively, after correction as described below in Fig. 1. The references were phosphate-glycine-NaOH buffer, pH 9.0, and the same buffer containing 10 μN enzyme, respectively. These two emission spectra are standards for the following calculation, the latter representing the spectrum of 0.95 μN ER complex (calculated on the basis of K_{ER} = 0.46 μM at pH = 9.0) plus 0.05 μM free NADH. To obtain the theoretical spectrum of ER alone, the emission of 0.05 μM NADH was subtracted to yield curve B, Fig. 1, which represents the emission of 0.95 μM ER. The ratio between the emission intensity at 410 mμ 1.0 μM R_b, which could be calculated from curve B, and that for

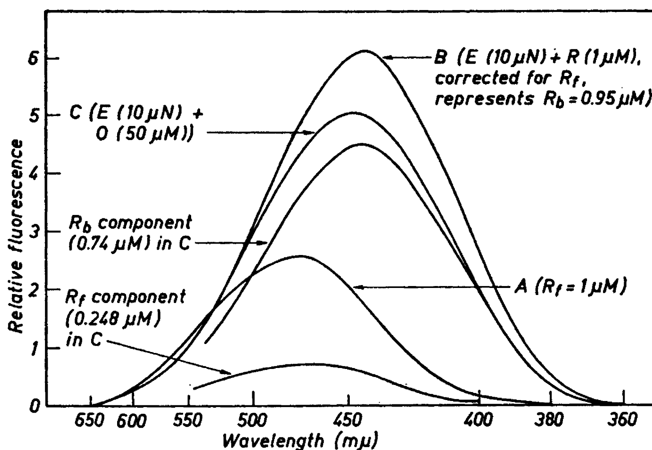

Fig. 1. Records of fluorescence emission spectra of bound NADH, free NADH, and a mixture of both in an $LADH_E$ sample in which alcohol is to be determined. Exciting wavelength 330 mμ; 33.5° and phosphate-glycine-NaOH buffer, pH 9.0. These fluorescence emission spectra were not corrected for variations in the intensity with wavelength of the exciting light and sensitivity of the photocell. This correction would shift the maxima about 15 mμ towards shorter wavelengths on this apparatus. For details, see the text. Curve A: 1 μM NADH, curve B: 0.95 μM bound NADH, and curve C: 50 μM NAD^+ added to a 10 μN test sample of $LADH_E$.

1.0 μM R from curve A, was found to be 13.2, in agreement with the "Q" value for the ER complex already reported.[16]

Under constant instrumental conditions, 50 μM of purified NAD^+ * was added to 10 μN LADH in a fluorimeter cuvette and the time course of the increase in fluorescence intensity at 440 mμ due to the formation of R_f and (mainly) R_b was traced by the recorder. The fluorescence intensity reached a constant value some minutes after the addition of NAD^+.** The fluorescence emission spectra of the final equilibrium mixture were obtained with, as reference, the spectrum of 10 μN LADH alone in the same buffer, as recorded in Fig. 1, curve C. Curve C is regarded as a superimposed spectrum of R_b and R_f in the final equilibrium mixture. The fraction of the contributing R_b component (n) is calculated on the figure at several wavelengths between the measured intensity for curve A, B, and C: $B \times n + A\ (1-n) = C$. From Fig. 1, the average value of n thus graphically obtained was 0.752; R_b and R_f are therefore determined as $0.95 \times 0.752 = 0.714$ μM and $1.0 \times 0.248 = 0.248$ μM, respectively.

A similar experiment was carried out with varied LADH concentration (5 and 15 μN) using the same enzyme preparation.

RESULTS

The results obtained with an enzyme thoroughly dialysed against the phosphate-glycine-NaOH buffer, pH 9.0, are summarized in Table 1. The total amount of alcohol in the test LADH solution (X) was calculated according to eqn. (3). In order to check the consistency of these values, two O_b values were calculated in each case according to eqn. (5) or (6). The two values obtained for O_b, as given in Table 1, are in good agreement, indicating the consistency of the values of R_b, R_f, and consequently X, obtained by this method.

Table 1. Results of determination of alcohol in a dialysed homogeneous $LADH_E$ sample, with varied concentration of $LADH_E$.

$LADH_E$ tested (E_t μN)	5	10	15
NAD^+ added (O_t μM)	50	50	50
Graphically determined			
R_b	0.39	0.71	1.30
R_f	0.24	0.25	0.20
Residual alcohol calc. by eqn. (3):			
alcohol reacted (a)	0.63	0.96	1.50
alcohol unreacted	0.37	0.53	0.87
Total (X)	1.00	1.49	2.37
O_b calculated by eqn. (5)	3.9	7.95	11.7
by eqn. (6)	3.8	7.3	10.6

* No measurable quenching effect was observed with 50 μM of purified NAD^+ under these experimental conditions.

** We recently found that NADH was modified to so-called NADHX relatively slowly at this pH.[17] The effect of this side reaction on the present determination of the spectrum of curve C would be negligible since the small concentration of NADH produced, and the presence of excess NAD^+, effectively inhibited the modification reaction at this pH.

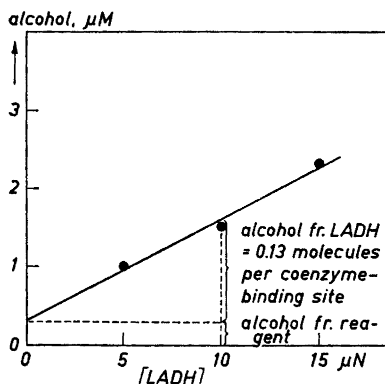

Fig. 2. Plot of residual alcohol concentration with varied concentration of dialysed $LADH_E$ sample (summary of the data presented in Table 1).

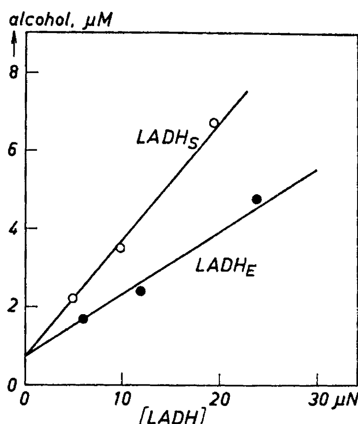

Fig. 3. Plot of alcohol concentration with varied concentration of $LADH_E$ and $LADH_S$ dialysed under identical conditions.

Fig. 2 gives data for the total alcohol concentration, as determined with various LADH concentrations, as recorded in Table 1. The intercept (equivalent to 0.30 μM alcohol) is believed to represent the fraction of alcohol emanating from the reagents used. The concentration of alcohol in the test LADH solution is, therefore, 0.13 molecules per coenzyme-binding site, or only 1/8 of the previously proposed value of 1 molecule alcohol per coenzyme-binding site.

Three different LADH samples, homogeneous $LADH_E$, $LADH_S$ of 90 % purity and a crude LADH preparation (60 % purity, obtained before the stage of the first CM-cellulose column chromatography), all approximately 100 μN, were dialysed in the same vessel against the same redistilled water; the pH was adjusted to 9.5 by concentrated ammonia. The remaining alcohol was then determined by addition of 50 μM NAD^+ at pH 9.0.

The fluorescene increase induced in a 10 μN solution of the crude sample was very high and was still increasing slightly after 15 min.

Fig. 3 shows the results of the alcohol determination with only homogeneous $LADH_E$ and $LADH_S$. The calculation of alcohol in $LADH_S$ was carried out by assuming its K_{ER} at pH 9.0 to be = 0.82 μM * and the same overall equilibrium constant = 9×10^{-12} M.

As Fig. 3 shows, extrapolation of both lines to zero enzyme concentration gave nearly identical intercepts, representing 0.70 μM alcohol brought into the test solution by the reagents used. The calculated amount of the remainder of the alcohol per coenzyme-binding site was 0.165 molecules for $LADH_E$ and 0.305 molecules for $LADH_S$.

* The dissociation constant tentatively determined for $LADH_S$ (0.82 μM) was applied. As seen in eqn. (3), uncertainty in the value of K_{RE} is not critical in calculating the concentration of alcohol (X), if the concentration of NAD^+ added is high enough.

DISCUSSION

The method described for determining residual alcohol appears to provide consistent results. The results given in Fig. 3 show that this alcohol concentration was considerably higher in $LADH_S$ than in $LADH_E$ but not high enough to explain the higher value previously obtained with heterogeneous samples.[5] Both LADH samples gave a lower value than previously proposed, far less than unity per coenzyme-binding site. Furthermore, judging from the kinetics of the fluorescence increase observed at 410 mμ after the addition of NAD^+, which is shown to be far slower than the "on" reaction between LADH and NAD^+, the alcohol detected here cannot be bound at the catalytic site of the LADH molecule. From the above observations it seems reasonable to assume that the lowered value for alcohol found in homogeneous $LADH_E$ preparations results from:

1. The elimination of some alcohol-adsorbing impurities (besides $LADH_S$) since the crude preparation at this stage is known to contain at least three active components, distinguishable by electrophoresis, as well as inactive impurities.

2. Elimination of some alcohol (such as the octanol used as an antifoaming reagent) during the course of CM-cellulose column chromatography which was used twice in the present purification procedure.[6] The latter possibility rather accords with our experience that the difficulty of removing residual alcohol is encountered especially when the samples are prepared omitting the CM-cellulose chromatography.

3. A carefully controlled dialysis, among all the possibilities, appears to be the main factor which determines the residual alcohol concentration. In fact, we have found that even with homogeneous $LADH_E$, determination of K_{EO} was totally impossible if the dialysis was not carefully carried out, for instance in an open vessel. Carefully controlled dialysis never failed to provide an enzyme solution from which an accurate K_{EO} determination could be made. The enzyme solution appears, from our experience, to absorb trace amounts of alcohol from the air. The same is very probably true for any water solution in a laboratory, where the air will always contain some ethanol. However, these minute amounts of ethanol mostly do not cause any observable effects at all except under the very special condition that alcohol dehydrogenase and NAD^+ are both present.

In so far as the "bound" alcohol value is now found to be lower than unity per coenzyme-binding site, it seems too low for any significance whatever to be attached to it. In fact, more thorough dialysis, if carried out carefully, was found to remove still more of the alcohol than shown in Figs. 2 and 3, and it is routinely possible to prepare samples with less alcohol than 0.1 molecules per coenzyme-binding site. Such preparations were used in the studies of the EO complex.[6]

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