sembled that of the abnormal whole serum.

Although the viscosity of the purified material varies considerably from preparation to preparation, it is usually very high and tends to increase with time. Intrinsic viscosity as high as 1.0 dl g^{-1} (25°C) has been observed under conditions of both high and low shear, which indicates the formation of highly asymmetrical aggregates. The relative viscosity of fresh whole serum from affected patients is above the normal range (up to 2.2 at a concentration of 1.9 g of the abnormal lipoprotein per deciliter). Since the expected intrinsic viscosity of disks with an axial ratio of five as calculated by the Simha equation (*21*) is 0.045 dl g^{-1}, the increased viscosity observed in whole serum can be accounted for either by a monomeric dispersion of the disks or by linear aggregates of axial ratio not exceeding four. The marked increase observed in the viscosity of the material in the purified state suggests that other elements of serum interfere with the formation of highly asymmetrical aggregates. That serum albumin and perhaps other proteins exert such an effect is suggested by the finding that incubation of a viscous purified preparation for 1 hour at 25°C with an amount of serum albumin equal to the protein content of the abnormal lipoprotein resulted in a marked decrease in viscosity. The hypothesis that protein molecules in the serum coat the surfaces of the abnormal lipoprotein will account for both the x-ray pattern of the whole serum and the effect of albumin upon viscosity of the purified material.

The viscometric behavior indicates that only moderately asymmetrical particles exist in the native serum of patients with cholestasis. The electron microscopic images and x-ray diffraction patterns are consistent with particles in the form of partially flattened vesicles, the walls of which are a continuous lipid bilayer of the width expected for an equimolar mixture of choline phosphatides and cholesterol. The unique disklike appearance of the abnormal lipoprotein can be expected to facilitate investigation of its origin and metabolic fate.

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Deuterium Effects on Binding of Reduced Coenzyme Alcohol Dehydrogenase Isoenzyme EE

Abstract. *Determination of dissociation constants by two different methods yield the following mean values in 20 millimolar phosphate, pH 7.0, 25°C: 0.27 micromolar for reduced nicotinamide adenine dinucleotide (NADH); 0.29 micromolar for NADH with deuterium in the nicotinamide 4-B position (B-NADD); and 0.46 micromolar for NADH with deuterium in the nicotinamide 4-A position (A-NADD). These results indicate that dehydrogenases are capable of recognizing and distinguishing the appropriate hydrogen in the coenzyme already in the initial binding reaction.*

Various class A dehydrogenases—enzymes responsible for the stereospecific proton transfer to and from the A side of reduced nicotinamide adenine dinucleotide (NADH)—exhibit significant kinetic isotope effects in the hydrogen transfer step. These include yeast alcohol dehydrogenase (*1, 2*), malate dehydrogenase (*3*), lactate dehydrogenase (*4, 5*), and liver alcohol dehydrogenase (*1, 6–9*). Some of the results obtained suggested direct interaction between the enzyme and the coenzyme involving the proton to be transferred.

However, these interpretations are not without ambiguity; it therefore seemed desirable to make a direct comparison of the dissociation constants of the enzyme-NADH complexes with those of stereospecifically labeled enzyme-NADD complexes (*10*). Any significant interaction between the enzyme and the transferable proton should produce an observable isotope effect on this dissociation constant. Although such effects have been reported for dissociation constants calculated from rate constants (*1, 3, 4, 7*), no direct equilibrium measurements have been reported. To remedy this situation we have studied the LADH-NADD complexes; this enzyme is perhaps the most widely investigated example among the class A dehydrogenases.

The necessity for a purified LADH sample is quite evident in equilibrium studies which require relatively large amounts of enzyme. We observed that the commercial preparations of LADH, used in many laboratories for kinetic studies, slowly destroyed NADH in binary complexes under equilibrium conditions, a fact also reported by Theorell *et al.* (*11*). In order to remove this contaminant we adopted a purification procedure that resulted in the isolation of LADH isoenzyme EE (*12*). Thus, in contrast to all but the most recent results (*13*), the equilibrium constants reported here refer to homogeneous reactions involving only one isoenzyme.

We purified LADH (Boehringer) in lots of 8 to 10 mg by chromatography

on a carboxymethyl cellulose (*14*) column (Whatman CM52; 0.7 by 10 cm) at 8°C with 20 m*M* phosphate, *p*H 6.4. Peak fractions were homogeneous upon rechromatography on carboxymethyl cellulose as well as on Sephadex G-100 and starch-gel electrophoresis. Concentrations of LADH were determined from the absorbance at 280 nm and with an extinction coefficient of 0.455 per milligram of protein per centimeter (*15*).

Both NAD and NADH as purchased (Sigma, grade III; ~ 98 percent pure) were used without further purification. The deuterated coenzymes (A-NADD and B-NADD) were prepared as described by Colowick and Kaplan (*16*); the enzymatic method was used for reduction; [4-d]NAD was prepared by cyanide exchange in deuterium oxide. The reduced coenzymes were purified by a modification of Winer's chromatographic method (*17*). Deuterium content [by nuclear magnetic resonance (NMR), peak height integration] of the various deuterated coenzymes used was > 0.95 g atom/mole for the A form, and ≥ 0.90 g atom/mole for the B form.

Two methods were used for the determination of equilibrium constants, gel exclusion (*18*), and fluorometry. Freshly packed columns of extensively washed Sephadex G-50 (fine bead form; Pharmacia) were used to give a gel volume of about 20 ml in glass columns of 1.0 or 1.2 cm diameter. Equilibrium solutions were prepared by dissolving small amounts of coenzyme in 20 m*M* phosphate, *p*H 7.0, with the coenzyme molarity equal to or slightly larger than the enzyme normality in the binding mixture. Coenzyme concentrations used were 2 μM and 1 μM, respectively; enzyme normality was determined (*13*) on the basis of the coenzyme binding sites per molecule (*19*).

The Sephadex column was equilibrated with the coenzyme solution, and a 1.0-ml portion of the solution was added to a portion (0.15 to 0.20 ml) of LADH. From this LADH-NADH binding mixture 1.0 ml was removed and placed on the gel surface for 3 minutes to allow for temperature equilibration at 25°C. The mixture was then washed into the gel with several small amounts of the NADH solution, which was also used to elute the sample at constant flow, usually 30 to 40 ml per hour, and monitored at 340 nm. Fractions were collected automatically at fixed time intervals and measured by drawing the samples into pipettes calibrated to contain 2.0 ml in 0.01-ml divisions.

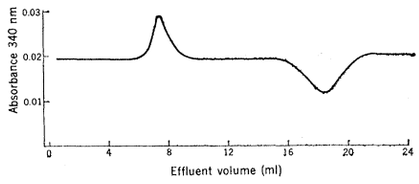

Fig. 1. Elution of LADH-NADH complex ($3.1 \times 10^{-6} N$) from a Sephadex G-50 column (1.0×26 cm) equilibrated with NADH in 20 m*M* phosphate, *p*H 7.0 at 25°C.

The amount of coenzyme bound to the LADH was determined from the absorbance trough appearing at the end of the elution profile, as described by Fairclough and Fruton (*20*). Because the original binding mixture was diluted 15 to 20 percent with LADH, the actual value for "micromoles bound to LADH" was determined by subtracting this 15 to 20 percent deficit of NADH from "apparent micromoles in trough."

Fluorimetric determinations of LADH-coenzyme dissociation constants were performed at 25°C by means of an Aminco Bowman spectrophotofluorometer with a Sargent model SRL Recorder. An exciting wavelength of 340 nm was used with an emission wavelength of 430 nm. The different preparations of purified LADH and stock buffer (20 m*M* phosphate, *p*H 7.0) were used after filtration through Millipore filters to remove dust and lint which interfered with the fluorescence measurements. Coenzyme solutions were prepared in the filtered buffer to give concentrations of 6.3 ± 1 μM as determined by their absorbance at 340 nm.

Determinations of dissociation constants were carried out by titrating 0.3 μM LADH (14 to 17 μl in 2.0 ml of buffer) with 2-μl portions of NADH or NADD, which were added to the cuvette on a plastic mixing spoon. After the recorder pen reached equilibrium the fluorescence intensity was recorded for about 30 seconds. At least 20 μl of NADH or NADD was added to each enzyme solution (ten additions) so that the final amount of NADH added represented a total coenzyme concentration of 0.6 μM. Similar determinations were also performed with the free coenzyme.

Dissociation constants were calculated according to the method of Theorell and Winer (*21*) with the aid of a CDC 6500 computer for each NADH addition with the use of the average enhancement value, with the K_d reported being equal to the average of all K_d values within two standard deviations.

Dissociation constants determined by these methods were extremely sensitive to a number of external influences since small amounts of aldehyde or alcohol contamination in any of the solutions, or in the laboratory air, significantly altered the results with both methods as the LADH-coenzyme complex was destroyed or modified. Because the Sephadex method also required extreme instrumental sensitivity, those experiments exhibiting more than 10 percent experimental uncertainty were rejected from further consideration.

Dissociation constants for binary complexes of LADH with NADH, A-NADD, or B-NADD are shown in Table 1. Other values for the K_d with NADH are those of Anderson and Weber (*22*), who report 0.29 μM at *p*H 7.4; of Dalziel (*23*), 0.27 μM at *p*H 7.4; and of Theorell and McKinley-McKee (*24*), 0.31 μM at *p*H 7.0; all these values were obtained fluorimetrically with isoenzymically heterogeneous samples of LADH. Taniguchi *et al.* (*13*) using double difference spectrophotometry have reported a K_d value for NADH and homogeneous LADH of 0.20 μM at *p*H 7.0.

The values for the constants with NADH and B-NADD may be considered identical within experimental error, and exclude any secondary interactions between the proton in the B position and the enzyme. Although this result appears different from the calculated

Table 1. Dissociation constants for binary complexes of homogeneous LADH with NADH, A-NADD, or B-NADD in 20 m*M* phosphate, *p*H 7.0 at 25°C.

Method	Analyses (No.)	LADH complex with:	K_d (μM)
Sephadex	12	NADH	0.30 ± 0.08*
Sephadex	16	A-NADD	0.46 ± 0.07*
Sephadex	14	B-NADD	0.29 ± 0.08*
Fluorimetric	4	NADH	0.24 ± 0.04†
Fluorimetric	4	A-NADD	0.46 ± 0.02†

* Uncertainty expressed for 90 percent confidence level. † Standard deviation.

isotope effect, reported by Baker (*6, 7*), for B-NADD of 0.50 ± 0.10 based on kinetic measurements, it is in complete agreement with our most recent kinetic results (*9*). Our observations also indicate that the B-NADD preparation is more sensitive to deterioration on storage than the usual NADH sample. This is probably the cause of the discrepancy between Baker's results and those reported here.

The isotope effect for equilibrium dissociation constants with A-NADD was determined by both the Sephadex and the fluorimetric methods, and the two results are identical within experimental error:

$$\overline{K}_d \equiv K_d^{E\text{-}NADH}/K_d^{E\text{-}(A\text{-}NADD)}$$
$$= 0.65 \pm 0.14 \text{ (Sephadex method)}$$
$$= 0.52 \pm 0.07 \text{ (fluorimetric method)}$$

This kind of ("inverse") isotope effect indicates that A-NADD is bound less tightly to the enzyme than is NADH:

$$\text{LADH} + \text{NADH} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{LADH} - \text{NADH}$$

and that the zero point energies for the isotopically sensitive vibrations are lower in the complex than in the free NADD.

Since $\overline{K}_d$ (observed) $\equiv K^H/K^D = \overline{k}_2/\overline{k}_1$; $\overline{k}_1 \equiv k_1^H/k_1^D$, $\overline{k}_2 \equiv k_2^H/k_2^D$, then if $\overline{K}_d < 1$ and $\overline{k}_2 > 1$ it follows that $\overline{k}_1 > \overline{k}_2 > 1$ (conversely for $\overline{k}_2 < 1$, $\overline{k}_1 < \overline{k}_2$). Therefore if the isotope effect on the dissociation reaction (rate constant k_2) is normal ($\overline{k}_2 > 1$), a possibility suggested from kinetic data (*6, 7, 9*), then the isotope effect for the binding step (rate constant k_1) must also be normal and even larger, thus indicating an ability of the enzyme to discriminate kinetically against the coenzyme deuterated at the transferable hydrogen.

Previously Mahler *et al.* (*7*) reported $\overline{K}_d$ for the LADH-(A-NADD) dissociation constant to be 1.31 ± 0.64; however, this value was based on an extrapolation from kinetic constants derived from initial rate measurement and as indicated, exhibited a large experimental uncertainty. Isotope effects for the corresponding equilibrium constants for yeast alcohol dehydrogenase (*1*) and malate dehydrogenase (*3*) were reported to be of the same magnitude as that for LADH. However, "inverse" deuterium isotope effects similar to the one reported here, but based on inverse rate effects for k_1, were reported from Thomson's laboratory (*4*) for the binding of A-NADD to heart lactate dehydrogenase ($\overline{K}_d = 0.69$) and muscle lactate dehydrogenase ($\overline{K}_d = 0.43$).

These various results in conjunction with the absence of any real difference in the binding constant between NADH and B-NADD, which eliminates the possibility of secondary interaction between the B proton and the enzyme, indicate that dehydrogenases are capable of recognizing and discriminating against coenzymes isotopically substituted in the appropriate position ("A" for A enzymes) even in the initial binding reaction. The properly deuterated coenzyme is bound less tightly than is its protonated counterpart, so that during the binding reaction the equilibrium (and perhaps also the rate) constant already reflects the specificity of the subsequent hydrogen transfer step.

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Bacteriophage and the Toxigenicity of Clostridium botulinum Type C

Abstract. *Nontoxigenic and bacteriophage-sensitive bacterial cultures have been isolated from toxigenic* Clostridium botulinum *type C, strain 468C, after treatment with either ultraviolet light or acridine orange. Two bacteriophages, designated CEβ and CEγ, were isolated from toxigenic strain 468C. Both of these bacteriophages were capable of infecting the nontoxigenic type C cultures, but only bacteriophage CEβ was involved in the change from nontoxigenicity to toxigenicity.*

Nontoxigenic clostridia resembling toxigenic *Clostridium botulinum* have frequently been isolated from marine and terrestrial environments (*1*); occasionally, pure cultures of toxigenic *C. botulinum* have also become nontoxigenic (*2*). It has been suggested that the production of toxin by *C. botulinum* might be governed by the presence of a specific converting bacteriophage in a system analogous to the production of diphtheria toxin by *Corynebacterium diphtheriae* (*3, 4*). In a recent report, Inoue and Iida (*5*) presented data which strongly suggested that bacteriophage may be involved in the toxigenicity of *C. botulinum* type C. They were able to recover toxigenic isolates from nontoxigenic cultures incubated in broth with filtrates of a toxigenic type C (strain Stockholm). Marked lysis occurred in the cultures; however, plaques were not demonstrated on solid medium.

In our studies, two bacteriophages, designated CEβ and CEγ, were isolated from toxigenic *C. botulinum* type C,