EXPRESSION OF ISOTOPE EFFECTS ON THE ALCOHOL DEHYDROGENASE REACTION

A thesis submitted to the Graduate School of the University of Wisconsin-Madison in partial fulfillment of the requirements for the degree of Doctor of Philosophy

by

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Steven Mark Faynor

Under the supervision of Professor Dexter B. Northrop

Primary kinetic isotope effects have been used in chemical reactions to determine if the bond to the labeled atom is broken in the rate-limiting step of the mechanism. Although it has been assumed that this principle is true for enzymatic reactions also, this has never been tested, despite recent evidence demonstrating the existance of other kinetic components not present in the simpler chemical systems. If the Vmax isotope effect is a valid indicator of the rate-limiting step, then making the rate of a non-catalytic rate-limiting step faster should increase the Vmax isotope effect by making the catalytic (non-isotopically-sensitive) step more rate-limiting.

An experimental system was designed to test this hypothesis. Hanozet et al. (Arch. Biochem. Biophys. 1979, 196, 46-53) found that deoxycholate (DOC) specifically increased the maximal velocity of rat liver alcohol dehydrogenase (RLADH). The authors attributed this effect to an increase in the rate of a rate-limiting conformational change which preceded the release of the final nucleotide product. If the Vmax isotope effect is

sensitive to changes in the rate-limiting step, then addition of DOC to RLADH should increase the Vmax effect by making catalysis more rate-limiting.

Alcohol dehydrogenase from rat liver was purified by homogenization, ammonium sulfate precipitation, anion-exchange chromatography, affinity chromatography on AMP-agarose and gel filtration. The final product had a specific activity of 1.89 units/mg and was purified 44-fold at a 34 percent yield. This product exhibited a single band on disc electrophoresis at pH 9.5 but the enzyme peak from gel filtration possessed a non-constant specific activity.

The addition of 3 mM DOC increased the Vmax of RLADH six-fold with ethanol. Contrary to the hypothesis above, the deuterium isotope effect on Vmax remained virtually constant at 1.11 $\pm$.11. Replacing ethanol with 2-propanol provided a measurable Vmax isotope effect of 1.65$\pm$.Ø9, which increased to only 2.2Ø$\pm$.Ø9. These results do not satisfy the expected increases in isotope effects that should accompany a more "rate-limiting" catalysis. The results can, however, be accomodated by using the general equation for the expression of Vmax isotope effects (Northrop, Biochemistry 1981, 2Ø, 4Ø56-4Ø61).

The effects of reductive methylation of the lysines of horse liver alcohol dehydrogenase were also studied.

Methylation activated the enzyme toward ethanol but deactivated it slightly toward 2-propanol. Despite this differential effect, the Vmax isotope effect increased for both substrates, from 1.34±.04 to 2.61±.10 for ethanol and from 2.95±.13 to 4.54±.18 for 2-propanol. The V/K isotope effect rose from 2.36±.13 to 3.75±.30 for ethanol but fell from 4.43±.39 to 3.48±.30 for 2-propanol. A theoretical basis is proposed for concluding that different sets of transistion states are utilized by the enzyme for catalyzing the oxidation of primary versus secondary alcohols.

"Luck is the residue of design."

--Branch Rickey

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To Professor Dexter B. Northrop. I started to make a list of all the things for which I wanted to thank Dr. Northrop, but it grew longer than one of these chapters. Let me instead thank him for providing the warmest and best enviornment I can imagine in which to grow as a graduate student.

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To my mother, Caroline Faynor, and to all of our neighbors and friends. Their faith and pride was a constant source of inspiration and strength, appreciated more than they will ever know.

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This dissertation is dedicated to the memory of my father, John Faynor.

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A	Substrate concentration of A or Absorbance
ADH	Alcohol dehydrogenase
AMP	Adenosine-5'-monophosphate
AMP-diol	2-Amino-2-methyl-1,3-propanediol
A.U.	Absorbance unit
BIS	N,N'-Methylene-bis-acrylamide
C	Cross-linking agent concentration (in polyacrylamide gels)
CAPS	3-(Cyclohexylamino)-propanesulfonic acid
CM	Carboxymethyl-
C_f	Forward commitment to catalysis
C_r	Reverse commitment to catalysis
DEAE	Diethylaminoethyl-
DOC	Deoxycholate
DTT	Dithiothreitol
D_k	Intrinsic deuterium isotope effect
D_V	Deuterium isotope effect on V_{max}
$D_{V/K}$	Deuterium isotope effect on V/K
E_a	Activation energy
E_f	Equilibration preceding catalysis
EtOH	Ethanol
HLADH	Horse liver alcohol dehydrogenase
HPLC	High-performance Liquid Chromatography

iPrOH	2-Propanol
I.U.	International unit of enzyme activity (1 umol of substrate consumed per min.)
K_{av}	Partition coefficient of a gel filtration column (See Fischer, 1969, for definition.)
k_{cat}	Turnover number
K_i	Inhibition constant
KIE	Kinetic isotope effect
K_m	Michaelis constant
LADH	Liver alcohol dehydrogenase
LDH	Lactate dehydrogenase
NAD	Nicotinamide adenine dinucleotide, oxidized
NADH	Nicotinamide adenine dinucleotide, reduced
PMSF	Phenylmethylsulfonylfluoride
Pz	Pyrazole
R_f	Migration distance in TLC and electrophoresis (See Stahl, 1969, p. 127, for definition.) or Ratio of catalysis
RLADH	Rat liver alcohol dehydrogenase
T	Total monomer concentration (in polyacrylamide gels)
TEMED	N,N,N',N'-Tetramethylenediamine
T_k	Intrinsic tritium isotope effect
TLC	Thin-layer chromatography
Tricine	N-Tris(hydroxymethyl)methyl glycine
$T_{V/K}$	Tritium isotope effect on V/K
v	Initial velocity

V_{max}	Maximal velocity
V/K	V_{max}/K_m
ZPE	Zero-point energy

I. INTRODUCTION

ORIGIN AND INTERPRETATION OF CHEMICAL ISOTOPE EFFECTS*. The Born-Oppenheimer Approximation allows separate consideration of the nuclear and electronic motions of atoms. For the reaction shown in Equation 1.1,

$$A{:}H + B \longrightarrow A + B{:}H \qquad (1.1)$$

substitution of deuterium for the protium atom in AH does not change the electronic potential-energy surface for the reaction when AD replaces AH as shown in Figure 1.1. The translational, rotational and vibrational energies of the nucleus are altered, however, due to the heavier deuterium atom. Of the three modes of nuclear motion, the vibrational modes have the largest effect on the magnitude of the isotope effect. The zero-point energy (ZPE) of vibration will be lower for AD than for AH, due to the heavier mass of the deuterium atom. This, too, is shown in Figure 1.1.

With the inclusion of these ZPE levels, it is evident that the activation energy (E_a) is higher for the deuterated molecule than for the protiated. Conversely, the rate constant for AD will be less than that for AH. The ratio of the rate constant for the

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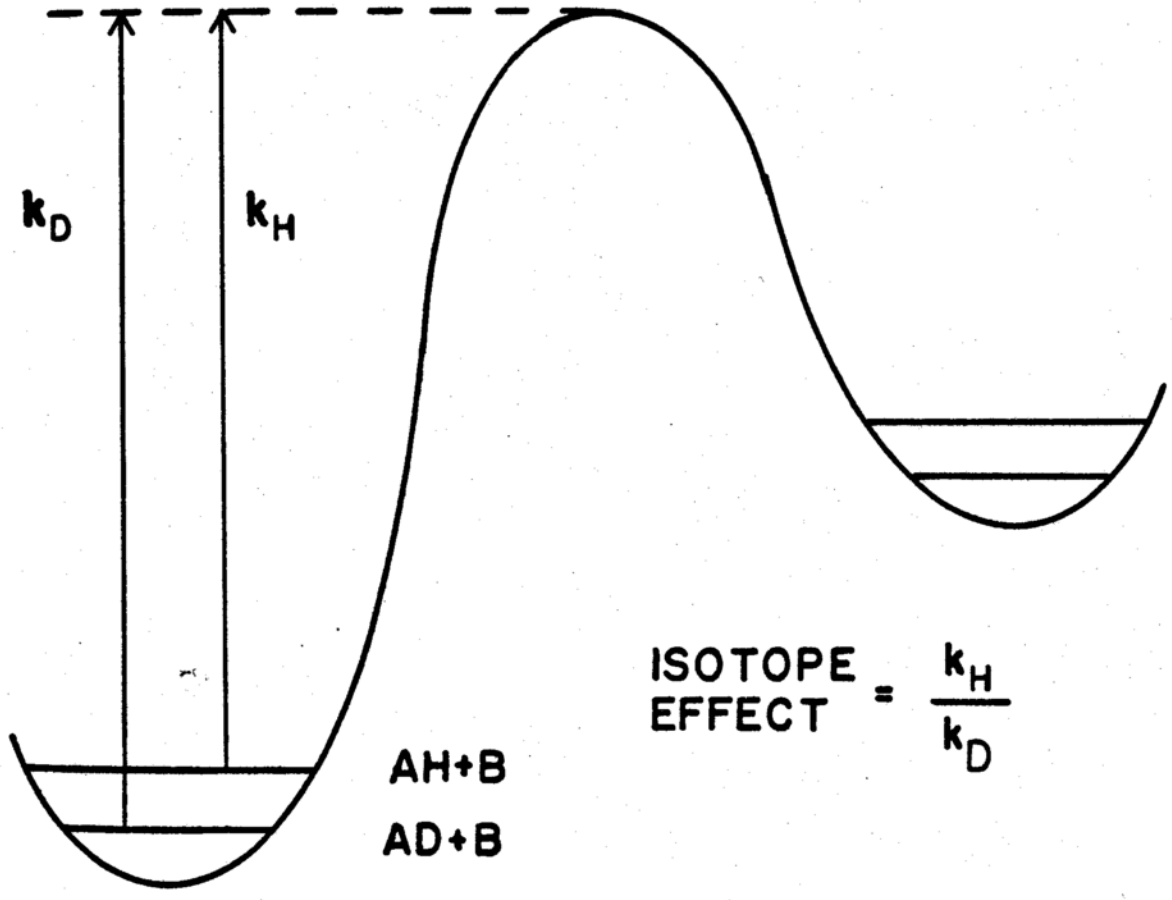
* The following discussion is taken from the list of general and classical papers on isotope effects given in Appendix A.

Figure 1.1. Electronic potential-energy surface for the reaction AH + B --> A + BH. The zero-point vibrational energy levels for the reactants are shown along with the rate constants for the hydrogen and deuterium transfers.

$$AH + B \rightarrow A + BH$$



protiated reactant to that of the deuterated is called the "kinetic isotope effect" (KIE). Since the substituted isotope is the species transferred during the reaction and this is a single-step mechanism, this is a primary intrinsic KIE.

Considering only the stretching vibrational frequencies for the isotopes of hydrogen at 25°C, one can calculate the expected maximum isotope effect using semi-classical theory. From infrared measurements, the average stretching frequencies of a C-H and a C-D bond are 2,900 and 2,100/cm, respectively. These correspond to zero-point vibrational energy levels of 4.15 and 3.00 kcal/mol. The difference of 1.15 kcal/mol gives a maximum primary kinetic isotope effect of 6.9, assuming that this ZPE ground state energy difference is uncompensated in the transition state. One can calculate a maximum tritium effect of 16 using a similar method. Bigeleisen (1949) claimed that if one considers bending vibrations, the maxima increase to 18 and 60 for deuterium and tritium, respectively.

The above discussion does not include the effect of tunneling. Some reactants with an insufficient activation energy nevertheless find their way to products, supposedly by "tunneling" through a narrow

energy barrier. Quantum mechanics predicts that tunneling will be greater for lighter atoms. The presence of tunneling will therefore increase the reaction rate for protiated molecules to a greater extent than for deuterated, raising the observed kinetic isotope effect above the expected maxima listed above.

Isotope effects are occasionally observed for single-step reactions which are lower than the expected values. Westheimer (1961) showed that an unsymmetrical transition state can diminish the isotope effect. As stated above, the isotope effect arises due to differences in the ZPE levels of the labeled and unlabeled molecules. This is shown mathematically in Equation 1.2. The linear transition

$$\frac{k_H}{k_D} = \exp\left(\frac{[ZPE_{AH}-ZPE_{AD}]-[ZPE_{[AHB]}-ZPE_{[ADB]}]}{RT}\right) \quad (1.2)$$

state for Equation 1.1, [A**H**B], will have two stretching vibrations, symmetric and asymmetric. The asymmetric vibration becomes the translational motion along the reaction coordinate. The symmetric vibration, however, is retained in the transition state. If the transition state is symmetric, the symmetrical vibration of A and B around H leaves H

motionless. Substituting D for H will not change this, so the ZPEs for the protiated and deuterated transition states are equal and cancel in Equation 1.2, maximizing the isotope effect.

In an asymmetric transition state, where either A or B is bonded more strongly to H, symmetric vibration of A and B will cause H to move also. Substituting deuterium will change the ZPE of the transition state, and the difference of the ZPEs for the two transition states will no longer be zero. This non-zero difference will diminish the value of the function for the isotope effect in Equation 1.2.

So far, only isotope effects of hydrogen transfer reactions with linear transition states have been considered. More O'Ferrall (1970) has performed model calculations of hydrogen isotope effects for reactions with non-linear transition states and found that the intrinsic isotope effects for these reactions are much smaller than for those with linear transition states. For example, a 1,2-hydride rearrangement with a bond angle of 75^{o} in the (symmetric) transition state gave a ^{D}k (intrinsic deuterium KIE) of only 1.9 at $25^{o}C$. This increased as the bond angle approached 180^{o}. More O'Ferrall attributed this supression to an out-of-plane

bending vibration in the non-linear transition state that combined with other vibrations to produce a ZPE contribution which offset the ZPE differential of the ground state molecules. Additionally, the magnitude of the isotope effect was relatively insensitive to the degree of symmetry of the transition state, unlike the linear case.

For an elementary reaction step, the deuterium and tritium isotope effects are related by the Swain-Schaad relation (Swain, et al., 1958), Equation 1.3.

$$\frac{k_T}{k_H} = \left[\frac{k_D}{k_H}\right]^{1.442} \qquad (1.3)$$

The relation considers only the zero-point vibrational energies of the species and further assumes the absence of tunneling. In spite of these restrictions, the Swain-Schaad relation appears to be obeyed in all experimental cases of elementary processses.

In multi-step chemical reactions, the observed isotope effect is sometimes less than the intrinsic, and this can reveal the kinetic mechanism of the reaction pathway. Consider the simple mechanism shown in Equation 1.4. The overall rate constant for the conversion of A to P is given in Equation 1.5.

$$A \xrightarrow{k_1} I \xrightarrow{k_3} P \qquad (1.4)$$

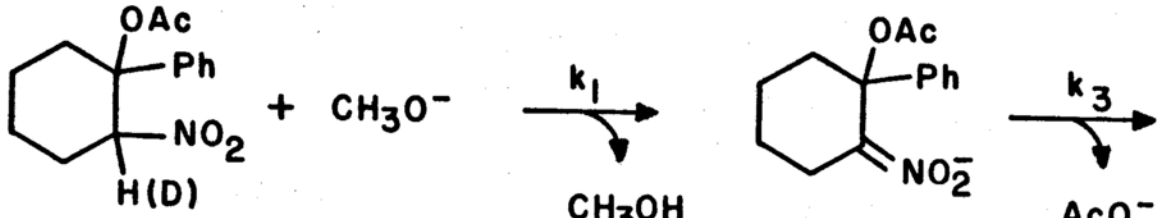
$$k_{obs} = \frac{k_1 * k_3}{k_1 + k_3} \qquad (1.5)$$

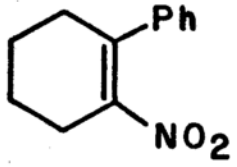
If k_1 is isotopically-sensitive and if it is the rate-limiting step (i.e., $k_3 >> k_1$), $k_{obs} = k_1$ and the isotopic substitution will cause an observable diminution of the rate. But if k_3 is rate-limiting instead ($k_1 >> k_3$), then $k_{obs} = k_3$ and the isotope effect on k_1 will not be expressed in the rate.

Isotope effects have been used extensively in this manner to study organic reaction mechanisms involving proton transfer. An example is the study by Bordwell et al. (1970), who used isotope effects to study elimination reactions involving carbanion intermediates (Elcb mechanisms).

It had been hypothesized that the elimination of acetic acid from certain nitroacetates occurs through a carbanion intermediate generated via proton transfer to a base (Fig. 1.2). If the carbanion intermediate accumulates at a steady-state concentration, then k_1, which is the isotopically-sensitive step, would be rate-limiting. The observed rate constant for the reaction would be k_1, and the observed isotope effect

Figure 1.2. Elimination reaction of acetic acid from 2-phenyl-2-acetoxy-1-nitrocyclohexane in the presence of methoxide ion.


OAc
Ph
NO2
H(D)
+ CH3O⁻
k1
CH3OH
OAc
Ph
NO2⁻
k3
AcO⁻


Ph
NO2

for this reaction ought to approach the expected intrinsic effect.

Bordwell et al. tested this hypothesis by studying the kinetics of the elimination reaction of 2-phenyl-2-acetoxy-1-nitrocyclohexane with methoxide ion. Repeating the experiment with the appropriately deuterated analog allowed calculation of a primary intrinsic isotope effect of 8.Ø, thereby confirming the proposed mechanism.

USE OF ISOTOPE EFFECTS IN ENZYMIC REACTIONS. Summarizing the preceding section, isotope effect experiments tell the researcher two basic things about chemical reaction mechanisms:

1. The absolute magnitude of the isotope effect (the intrinsic isotope effect) reflects the structure of the activated complex.
2. The degree to which the intrinsic effect is expressed in the observed value reflects the extent to which the isotopically-sensitive step limits the rate of the overall reaction.

Can these experiments reveal the same information about enzyme-catalyzed reactions?

Of course, enzymatic reactions are fundamentally more complicated than chemical reactions. Due to the saturation phenomenon, enzyme kinetics lack a simple dependence of the initial velocity on substrate concentration. This is the reason for measuring V_{max} and K_m as the fundamental kinetic parameters in steady-state enzyme kinetics, rather than the specific rate constant, k, as in chemical kinetics. Recently, the ratio of V_{max} and K_m, V/K, has come to replace K_m in terms of kinetic utility, because V/K is the apparent first-order rate constant at low substrate

concentration, and hence is more kinetically relevant and open to more precise interpretation than K_m (Cleland, 1975).

Enzymatic isotope effect experiments are run in two ways, either competitively or noncompetitively. In the noncompetitive mode, the V_{max} and V/K are first determined conventionally using unlabeled substrate, then the experiment is repeated using totally labeled substrate. The isotope effects are the ratios of the appropriate kinetic parameters. This method necessarily limits itself to isotopes available in near-100 percent concentration, e.g., deuterium. These experiments are called noncompetitive because labeled and unlabeled substrate do not "compete" for the enzyme. (Also, when the results are graphed on the traditional Lineweaver-Burk plot, the results resemble those for noncompetitive enzyme inhibition.)

In the competitive mode, both labeled and unlabeled substrates are present simultaneously in the same experiment. This is always the case in tracer experiments. Despite a long tradition of use in biochemical research, the application of tracers to the kinetics of enzymatic reactions has been mostly misunderstood. Thorn (1949) derived an expression

relating the total, observed initial velocity to the concentrations of two different substrates competing for the same site on the enzyme. Thorn demonstrated that a plot of the total velocity versus the fraction of labeled substrate in a pool of labeled and unlabeled substrate with a presumably constant total concentration would be curved if the K_m values for the two substrates were different. Although Thorn's insight into the competitive situation was useful, his method was not, because it failed to identify the kinetic function which gave rise to the difference between the two K_m values.

Abeles et al. (1960) approached the problem of enzymatic isotope effects from a similar standpoint. They derived Equation 1.6*,

$$\frac{v_H}{v_D} = \frac{V_H A_H K_D}{V_D A_D K_H} \qquad (1.6)$$

which shows that the observed isotope effect on the initial velocities in the competitive experiment is a function of the two values for V/K (although the

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* This and the following equations for competitive isotope effects are shown for deuterium; however, any isotope effect (especially tritium) can be used.

importance of V/K itself as a kinetic parameter was not recognized at this time).

A parallel development by Biegeleisen & Wolfsberg (1958) (also see Bigeleisen, 1949, and Tong & Yankwich, 1957) showed how the isotope effect in tracer experiments could be calculated for chemical reactions by measuring the specific activities of reactants and products at a low degree of conversion. Simutaneously solving the differential equations for the rates of each of the labeled and unlabeled reactions gave Equation 1.7, where $R_{a\emptyset}$ and R_{pf} are the specific activities for the substrate at time zero and for the product at some fraction of conversion, f, and ^{D}k is the intrinsic isotope effect*.

$$^{D}k = \frac{\log(1-f)}{\log[1-(f \cdot R_{pf}/R_{a\emptyset})]} \qquad (1.7)$$

When the fraction of conversion is sufficiently small, Equation 1.7 reduces to Equation 1.8.

$$^{D}k = \frac{R_{a\emptyset}}{R_{pf}} \qquad (1.8)$$

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* Bigeleisen & Wolfsberg defined the isotope effect as the inverse of the normal definitions; their equations have been rewritten to reflect the usual nomenclature of the enzymologist.

Simon and Palm (1966) recalled Bigelesien's earlier derivation and combined it with the equation of Abeles et al. to obtain Equation 1.9, which relates the V/K isotope effect to the ratio of specific activites:

$$D_{V/K} = \frac{R_{a\emptyset}}{R_{pf}} \cdot \qquad (1.9)$$

They emphasized that the competitive (tracer) experiments can only give the ratio of the V/K values, not the ratios of V_{max} or K_m alone.

In most isotope effect studies on enzymes, the observed effect on either V_{max} or V/K is less than the intrinsic effect on the cataytic step. Because it is the intrinsic isotope effect which is dependent on transition state structure, the use of this information must be preceded by a means of estimating or measuring this full value. Northrop (1975) first recognized this and derived a relationship relating the intrinsic effect to the isotope effects on V/K (Equation 1.1Ø).

$$\frac{D_{V/K} - 1}{T_{V/K} - 1} = \frac{D_k - 1}{D_k^{1.442} - 1} \qquad (1.1\emptyset)$$

This equation allows calculation of the intrinsic isotope effect from the observed V/K effects. Later, Northrop (1977) and Cleland (1977) extended this

equation to include cases where an equilibrium isotope effect was also present.

Since one estimates two fundamental kinetic parameters for enzymatic reactions, one must first decide which is the best measure of the rate-limiting step. Obviously, both cannot be equally appropriate, because V_{max} and V/K consist of different functions of the component rate constants. Northrop (1975 & 1981a) argued that the V/K effect is not the appropriate one for this question. The expression for V/K contains only rate constants of the mechanism up to and including the first irreversible step, so V/K would be insensitive to a rate-limiting step past that point. Even if the final step of the mechanism were the only irreversible step, V/K would still not be appropriate, Northrop argued, because "V/K represents an apparent rate constant at zero substrate concentration, conditions under which there is no rate (1981a, p. 111)." Therefore, only the V_{max} effect can be used to examine the relationship between the observed isotope effect and the rate-limiting step in an enzymatic mechanism.

That isotope effects can be used to detect the rate-limiting step in a simple, chemical mechanism is

certainly true; see Equation 1.5. That the same assumption is also true for more complicated enzymatic mechanisms had, until recently, never been questioned or tested. For example, Thomson (1963) wrote:

> Considerable use of the primary isotope effect has been made to elucidate the mechanism of biochemical reactions. By substitution of deuterium for hydrogen in a C-H bond that is broken in the course of the reaction, it is often possible to determine from the magnitude of the isotope effect whether or not the rupture of the bond is the rate-determining step. [p. 16]

Richards (1970), in a widely quoted statement of this unproven principle, said,

> As a general rule, an isotope effect of two to fifteen can be taken as evidence that the rate-determining step involves cleavage of the carbon-hydrogen bond in question. Conversely, the absence of an effect on the rate when deuterium is substituted for hydrogen indicates that the bond in question is not broken in the rate-determining step. [p. 324]

Considering the variation of intrinsic isotope effects discussed above, the lower limit of two for judging rate-determination seems unjustified. Northrop (1975) argued that, given examples of deuterium isotope effects with values of as high as fifteen in chemical systems, the minimal value to accept as evidence for a rate-limiting step cannot be less than eight in the absence of specific knowledge of the intrisic isotope

effect in the enzyme under study. In spite of these reservations, Walsh (1979), in a textbook review of the use of kinetic tools for investigating enzyme mechanisms, continues to invoke this principle:

> In general, a test for kinetic isotope effect can help determine whether or not hydrogen transfer occurs in a step that may be partially or fully rate-determining. [Italics his.] [p. 110]

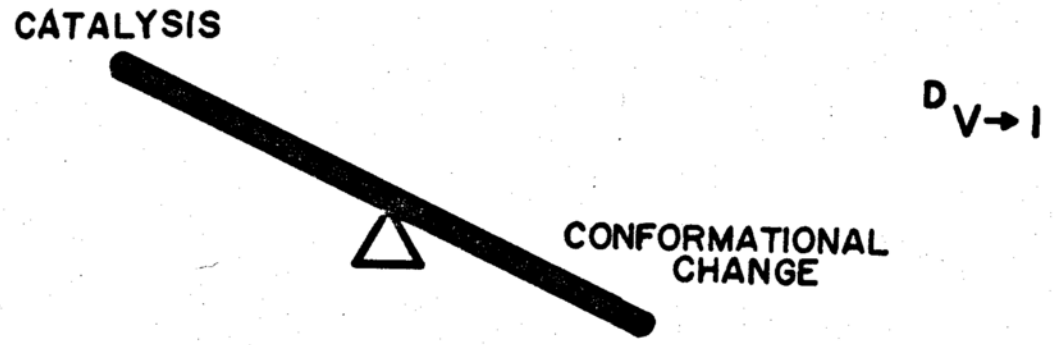
If the hypothesis that ^{D}V is a good predictor of the rate-limiting step is true, then increasing the rate for a non-catalytic rate-limiting step should increase ^{D}V, because the catalytic step will become more rate-limiting. This is shown diagramatically in Figure 1.3, where the "Rate-limitation See-saw" is tipped toward the catalytic step. This hypothesis is true for the simple mechanism shown in Equation 1.11. For this mechanism,

$$E + S \underset{k(2)}{\overset{k(1)}{<==>}} ES \xrightarrow{k(3)} EP \xrightarrow{k(5)} E + P \qquad (1.11)$$

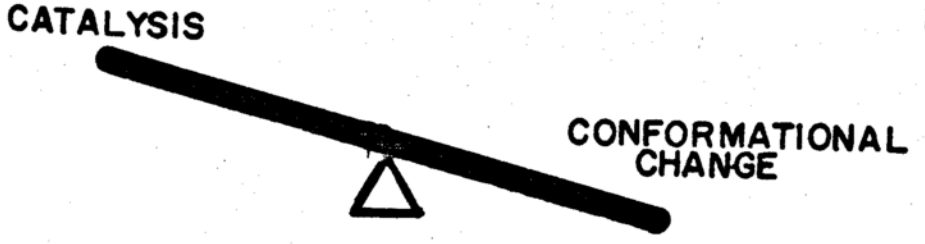
$V/E_t = k_3 * k_5 / [k_3 + k_5]$. If k_5, the product-release step, limits the rate, (i.e., $k_3 >> k_5$) then V/E_t will reduce simply to k_5 and an isotope effect on k_3 will not be expressed. Conversely, if k_3, the catalytic step limits the rate, $V/E_t = k_3$ and isotopic substitution will decrease V. (Note that the expression for V/K does not

Figure 1.3. The rate-limitation see-saw. See the text for explanation.

THE RATE-LIMITATION SEE-SAW



$D_{V \rightarrow I}$



$D_{V} \uparrow$

even include k_5, so it would be insensitive to a rate-limiting release of product.)

However, recent research has shown that enzyme action is not so simple. Enzymatic kinetic mechanisms are now understood to contain conformational changes and non-concerted, multiple catalytic steps in addition to substrate-addition, product-release and single catalytic steps. Despite this new knowledge, the belief in the applicabilty of the V_{max} isotope effect to the detection of the rate-limiting step did not change.

Finally, in 1981, Northrop (1981b) examined systematically the effects of such additional kinetic steps on the expressiion of the isotope effect. He examined mechanisms of increasing complexity and derived an expression defining the isotope effect on the maximal velocity, ^{D}V (Equation 1.12).

$$^{D}V = \frac{^{D}k+R_f/E_f+C_r*{}^{D}K_{eq}}{1+R_f/E_f+C_r} \qquad (1.12)$$

The ^{D}V was a function of ^{D}k, the intrinsic deuterium isotope effect on catalysis, R_f, the ratio of catalysis, E_f, the equilibration preceding catalysis, C_r, the reverse committment to catalysis, and $^{D}K_{eq}$, the equilibrium isotope effect. R_f, E_f and C_r are various

combinations of rate constants for the chosen mechanism. The minimal mechanism which encompasses all the requisite steps is given in Equation 1.13.

$$\begin{array}{l} \quad\quad k(1) \quad\quad k(3) \quad\quad k(5) \quad\quad k(7)^* \quad\quad k(9) \\ E1 + S \;<==>\; E2 \;<==>\; E3 \;<==>\; E4 \;<==>\; E5 \;<==>\; E6 \\ \quad\quad k(2) \quad\quad k(4) \quad\quad k(6) \quad\quad k(8)^* \quad\quad k(1\emptyset) \end{array}$$

$$\begin{array}{l} k(11) \\ \text{--}> \;\; E1 + P \end{array} \qquad (1.13)$$

When Northrop simulated ^{D}V using various values for the individual rate constants, he found, as expected, that there were some cases where ^{D}V was a good predictor of whether the rate-limiting step was the catalytic one or not. Contrary to the prevailing belief though, he also discovered some anomalous cases where this was not true.

For example, assume that k_3 in Equation 1.13 is the smallest forward rate constant and that k_7 and k_8 are the catalytic steps. If one increases k_3, that is, to make it less rate-limiting, ^{D}V should increase, as catalysis has become more rate-limiting. To test this, R_f/E_f can be written as Equation 1.14,

$$\frac{R_f}{E_f} = \frac{a*k_3 + b}{c*k_3 + d} \qquad (1.14)$$

where a, b, c and d are various combinations of the other rate constants as dictated by the model minimal mechanism. When $a/c<b/d$, increasing k_3 will decrease

R_f/E_f, and D_V will increase, as expected. But when $a/c=b/d$, R_f/E_f is constant and therefore D_V will be independent of k_3. In the least expected case when $a/c>b/d$, increasing k_3 will increase R_f/E_f and D_V will decrease, the opposite of the predicted result.

AN EXPERIMENTAL SYSTEM TO EXAMINE EXPRESSION OF THE V_{max} ISOTOPE EFFECT. The obvious requirement to test the effect of changing the rate-limiting step on the expression of the V_{max} isotope effect is a system in which one can vary the rate of a non-catalytic rate-limiting step, preferably in a continuous fashion. Originally, it was thought that this could be simply accomplished by the use of alternative substrates or extremes of pH (cf. Northrop, 1977, pp. 138ff). An alternative substrate or the usual substrate in the wrong state of ionization will usually be less sticky and will give rise to a product that will dissociate faster from the enzyme. If dissociation is rate-limiting, this change should increase the magnitude of the observed isotope effect, as discussed above. These techniques were used in preliminary studies on lactate dehydrogenase (LDH). These studies are described in detail in Appendix B; the pertinent results are summarized here. Substitution of 3-acetyl-pyridine hypoxanthine dinucleotide for NAD supressed expression of ^{D}V, as expected from the ten-fold decrease in V_{max}. However, substitution of thionico-tinamide adenine dinucleotide for NAD enhanced the expression of both ^{D}V and $^{D}V/K$, despite large decreases

in V_{max} and V/K. It was felt that the anomalous results in the thionicotinamide case could have been due to a change in catalysis for this coenzyme. This cast a shadow over this technique, because it could not be proven whether changes in the observed isotope effect were due to changes in the stickiness of the substrate or changes in the intrinsic isotope effect. Recently, Damgaard (1981) has presented evidence that ^{D}k for ADH changes when 3-acetylpyridine adenine dinucleotide and thionicotinamide adenine dinucleotide are substituted for NAD.

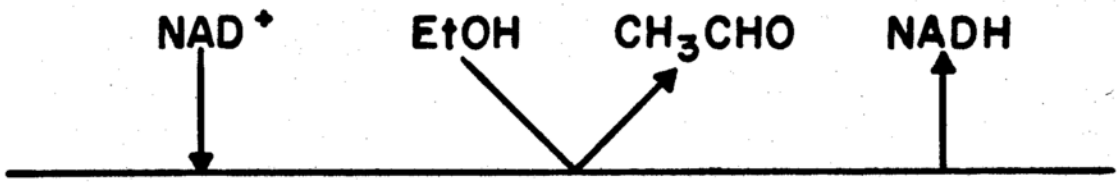
The isotope effects on LDH were also measured as a function of pH using nicotinamide hypoxanthine dinucleotide as coenzyme. Both the V_{max} and V/K isotope effects varied little with pH, despite changes in the rates. In this case, pH does not cause enhancement of the observed isotope effect. Cook and Cleland (1981a,b) have since given the theoretical basis for this originally unexpected result. In spite of useful conclusions based on their theory, the search for a system in which to study variation in the expression of the V_{max} isotope effects was continued.

Hanozet et al. (1977) reported the very interesting observation that the addition of sodium

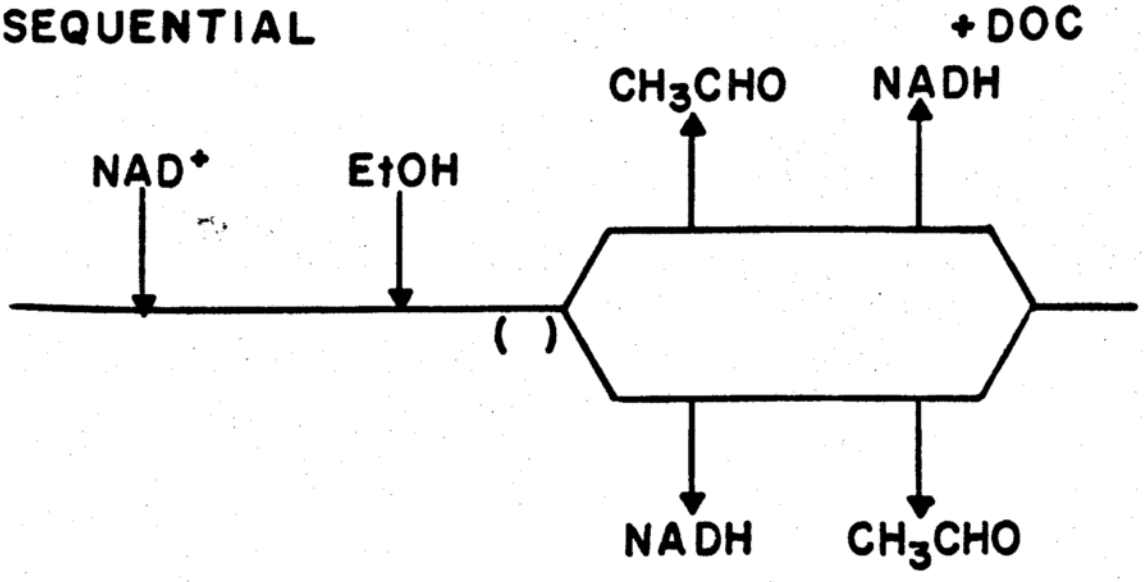
deoxycholate (DOC) to assays of rat liver alcohol dehydrogenase (RLADH) resulted in an increase in the maximal velocity for the reaction. Clearly, for the maximal velocity to increase, the rate of the rate-limiting step must have increased. Consistant with this were the results of a detailed kinetic analysis. In the absence of DOC, the reaction obeyed Theorell-Chance kinetics (see Figure 1.4), with NAD binding first and NADH leaving last. The presence of Theorell-Chance kinetics implies that catalysis is so fast that the existance of the central complex cannot be kinetically demonstrated. Catalysis is certainly not rate-limiting under these circumstances. A reasonable conclusion in this case is that the conformational change which precedes the release of the NADH limits the rate of the reaction. Upon addition of DOC, the kinetic pattern changed. The Theorell-Chance pattern was replaced with a pattern corresponding to ordered addition of substrates (NAD first) with possible random release of products. The authors hypothesized that DOC caused an increase in the rate of the conformational change which preceded the nucleotide release, thereby increasing the rate.

Figure 1.4. Mechanism of the RLADH-catalyzed oxidation of ethanol in the presence and absence of deoxycholate (DOC). The mechanism is summarized from the study of Hanozet et al (1979). This type of diagram originated with Cleland (1963).

THEORELL-CHANCE



SEQUENTIAL



Here, then, is the model system which is desired. Addition of DOC would appear to allow the experimenter to increase the rate of the rate-limiting step in a continuous fashion. As the rate of the rate-limiting conformational change increases, the catalytic step must appear more rate-limiting, based on the see-saw relationship of the two steps illustrated in Figure 1.3. The question to be considered is, will this lead to an enhanced expression of the isotope effect on V_{max}?

II. MATERIAL & METHODS

Materials for the Extraction and Purification of Alcohol Dehydrogenase. A 55 mL Potter-Elvehjem tissue homogenizer with a serrated-tipped Teflon pestle was purchased from Arthur H. Thomas Co., Philadelphia, Pa. Glass chromatographic columns were from Ace Glass, Inc., Louisville, Ky. Tricine (N-tris(hydroxymethyl)methyl glycine), dithiothreitol (DTT) and phenylmethylsulfonylfluoride (PMSF) were from Sigma Chemical Co., St. Louis, Mo. Bio-Gel P-2 and DEAE Bio-Gel A were purchased from Bio-Rad Laboratories, Richmond, Calif. Ultrogel AcA 44 was from LKB Instruments, Inc., Rockville, Md, while N^6-(6-aminohexyl)-AMP-agarose was from Sigma. Sephadex G-25 (coarse) was acquired from Pharmacia Fine Chemicals, Inc., Piscataway, N.J. "Ultra pure" ammonium sulfate was from Schwartz-Mann, Orangeburg, N.Y. Ultrafiltration was performed with in a standard stirred ultrafiltration cell using a PM 30 Diaflo membrane purchased from Amicon Corporation, Lexington, Mass.

Determination of Protein Concentrations. Relative protein concentrations were estimated in column fractions by measuring the absorbance at 280 nm. Precise protein concentrations for specific activity

measurements were determined by the method of Lowry, et al (1951), reading absorbances at 500 nm. Folin-Ciocalteau Phenol Reagent Solution, 2N, was a product of the Fisher Scientific Co., Fair Lawn, N.J., and the protein standard solution, 8 g/dL, was from Sigma (PN 540-10).

Assay of Alcohol Dehydrogenase Activity. Assays were performed in 0.5 mL total volumes in self-masking, semi-micro cuvettes containing 9 mM ethanol, 1.8 mM NAD and 0.100 M glycine buffer, pH 10.0, at 25°C. Changes in absorbance versus time were measured at 340 nm. (Dalziel, 1957.)

Polyacryamide Disc Gel Electrophoresis. Buffers 3-(cyclohexylamino)-propanesulfonic acid (CAPS) and 2-amino-2-methyl-1,3-propanediol (AMP-diol) were from Sigma and Aldrich, respectively. The reagents acrylamide, N,N'-methylene-bis-acrylamide (BIS), N,N,N',N'-tetramethylenediamine (TEMED), ammonium persulfate and Tracking Dye Solution were purchased form Bio-Rad. The protein stain Brilliant Blue R (Coomasie Brilliant Blue R-250, C.I. 42,660) was obtained from Aldrich. The electrophoresis was performed in a Bio-Rad Model 300 A disc gel

electrophoresis cell. (Bio-Rad Laboratories, 1974, and Sleeter, 1982.)

Gels of the following composition were cast in 7 x 125 mm glass tubes to heights of approximately 100 mm: 7.5 percent acryamide, 1.0 percent BIS (7.5% T, 3.3% C), 0.05 percent (v/v) TEMED and 0.05 percent (w/v) ammonium persulfate. The buffer for both the gels and electrode chambers was 0.118 M CAPS plus 0.118 M AMP-diol, pH 9.5.

Protein samples of appropriate volumes were diluted with Tracking Dye Solution (containing methylene blue and sucrose). These were applied to the top surfaces of the gels and introduced into the gel by electrophoresis at 100 volts and 1 mA per tube. After sample introduction, the voltage was increased to 150. The electrophoresis was carried out at $4^{o}C$. Electrophoresis was terminated after approximately 7-1/2 hours and the distance of migration was determined by measuring the position tracking dye in each tube. The average distance was 94 mm.

Gels were removed from the tubes and fixed in 40 percent 2-propanol, 10 percent acetic acid and 50 percent water for four hours. The gels were then stained in 0.25 percent Brilliant Blue R in 7 percent

acetic acid for two hours and destained by diffusion in 7 percent acetic acid. Relative positions of the blue dye-protein bands in the gels were determined by scanning the gels at 595 nm.

Detection of Protease Activity. To the following solution was added 1 g/dL of agarose (Sigma): Ø.Ø66 M sodium phosphate buffer, pH 7.2, Ø.45 g/dL sodium chloride, 5 mM sodium azide, 1 mM calcium chloride and 1 percent (w/v) dry skim milk (Difco Laboratories, Detroit, Mich.). The mixture was heated in a water bath until the agarose dissolved and then poured into 1ØØ mm plastic Petri dishes. Three millimeter wells were punched in the agar and filled with 3 μL samples of the protein solutions to be screened. After incubating overnight at 37°C, the plates were flodded with 5 percent (v/v) acetic acid. The presence of protease was indicated by the appearance of a clear zone of hydrolyzed protein around the well surrounded by the milky white precipitate of undigested casein. (Bjerrum, 1975.)

Purification of Deoxycholate. Sodium 7-deoxycholate (DOC) from Sigma was recrystallized thrice from 8Ø percent acetone (Makino, et al., 1973). To remove bound acetone, the product was dissolved in

water and lyophilized. To confirm homogeneity, the deoxycholate was inspected by thin-layer chromatography (TLC) on silica gel 60 plates (E. Merck) using 2,2,4-trimethylpentane:2-propyl ether:2-propanol: acetic acid, 1:1:1:1, as developing solvent (Eneroth and Sjovall, 1971). The compound appeared as a single spot at R_f=0.60 after the plate had been sprayed with 10 percent phosphomolybdic acid in 95 percent ethanol and heated. (Stahl, 1969, p. 887.)

Determinations of Isotope Effects. Nicotinamide adenine dinucleotide (NAD) (ethanol-free) was purchased from P-L Biochemicals, Milwaukee, Wisc. Ethanol was refluxed with magnesium ethoxide, then distilled. 2-Propanol was refluxed with calcium oxide instead, and distilled. Ethanol-d_6, 99 atom percent D, was purchased from Merck, Inc., Rahway, N.J. and 2-propanol-d_8, 99 atom percent D, was from either Merck or Aldrich Chemical Co., Milwaukee. Kinetic enzyme assays were performed on a Gilford Model 240 spectrophotometer equipped with a Leeds & Northrup Speedomax XL-610 recorder using a scale expansion of 0.04 to 0.2 absorbance unit full scale. Assays were done in semi-micro, self-masking quartz cuvettes with path lengths of 1 cm and volumes of 0.5 mL. All assays

were performed at a temperature of 25°C which was maintained with a circulating waterbath with flow-through thermospacers surrounding the sample chamber of the spectrophotometer. Into each cuvette were added 35 µL of 20 mg/mL NAD solution (final concentration, 1.8 mM), 0.43 mL of 58 mM sodium pyrophosphate buffer (final concentration, 50 mM), pH 8.5, and 10 µL of enzyme solution. NAD was always present at saturating concentration. Cuvette solutions were mixed by inversion thrice and incubated for several minutes at 25°C. The reaction was initiated by adding 25 µL of the appropriate aqueous dilution of substrate alcohols. Changes in absorbance at 340 nm were recorded over time on the chart recorder.

Six alcohol concentrations were chosen per determination of ^{D}V and $^{D}V/K$ with concentrations between 0.2 and 5 times K_m and assayed in duplicate, giving twelve points per determination. Pilot experiments were performed in advance to determine the appropriate concentration range for alcohol, because the K_ms for the alcohols varied with DOC concentration. Several assays were run at various concentrations of alcohol and the values for the apparent K_ms were estimated using the direct linear plot of Eisenthal and

Cornish-Bowden (1974). The pure alcohols were received and stored in sealed glass ampules. To prepare the substrate solutions for an experiment, an ampule was opened and the required amount of alcohol was delivered to a glass-stoppered volumetric flask. The solution in this flask served as the stock solution for all the other alcohol dilutions, which were prepared immediately before beginning the experiment. In this way, evaporation of the volatile alcohol was minimizied.

These procedures were repeated using the perdeuterated alcohol at appropriate concentrations to determine the isotope effects. As described above, it was necessary to do a pilot experiment to determine the correct concentration range. Deoxycholate, when added, was dissolved in the pyrophosphate buffer.

Slopes were calculated manually from the chart recordings, using ships curves when necessary. The isotope effects were estimated by fitting the data to a model (Eqn. 2.1) which included V and V/K isotope effects using the computer program ISOVKV of Cleland (1977). This program assumes equal variances for the velocities and uses Wilkinson's (1961) general approach to the solution of non-linear regression problems.

$$v = \frac{V_{max} A}{K_m [1+c(^{D}V/K-1)]+A[1+c(^{D}V-1)]} \qquad (2.1)$$

where A = substrate concentration
c = fraction of isotopic substitution

Reductive Methylation of Horse Liver Alcohol Dehydrogenase. Six and one-half milligrams of horse liver alcohol dehydrogenase (HLADH) from Sigma was added to 1.8 mL of 58 mM sodium pyrophosphate buffer at pH 8.5. 0.2 mL of 5 mg/mL sodium borohydride was added. One microliter of 37 percent aqueous formaldehyde was added and the mixture was stirred on ice for 10 minutes. This addition was repeated four times. After the reaction was completed, the enzyme was desalted on Sephadex G-25 (coarse) and stored in solution at $4^{\circ}C$. The control enzyme was treated similarily, except that water was substituted for the formaldehyde.

Other Computer Programs and Computer Facilities. Other linear regressions were performed using the BMDP Statistical Software package of the University of California, Los Angeles (Dixon, 1981). All computer programs were run at the University of Wisconsin Madison Academic Computer Center on Sperry UNIVAC equipment.

Miscellaneous Reagents. Any reagents not explicitly listed were ordinarily available and of reagent-grade quality. Water was distilled and passed through a Barnstead ion-exchange cartridge (Boston, Mass.).

III. RESULTS

ENZYME PURIFICATION. The results for a typical purification are given in Table I. Due to high interference, it was not possible to assay enzyme activity until after the Bio-Gel P-2 desalting step.

Homogenization of Tissue. Holtzman rats were killed by asphyxiation with carbon dioxide and their livers were excised and stored frozen at -20°C. To begin the purification procedure, approximately 70 g of frozen liver tissue was thawed and rinsed of extraneous blood in cold saline. Each liver was cut into sections of about 10 mm^3 with scissors and transferred to a 55 mL Potter-Elvehjem tissue homogenizer followed by 2.5 mL of homogenization buffer per gram of tissue. The homogenization buffer consisted of 50 mM Tricine, 150 mM sucrose, 0.1 mM PMSF, 1 percent (v/v) ethanol and 2.5 mM DTT at pH 8.4 and 4°C. This suspension was homogenized for ten strokes and combined with the other liver homogenates, which were kept on ice during this procedure. After homogenization was completed, the combined homogenate was centrifuged at 4°C at 39,000 x g for 45 minutes. The supernatant was decanted, the pellet was resuspended in 1.5 mL of homogenization buffer per gram of original tissue, and re-homogenized in portions for five strokes. The centrifugation was

TABLE I

Purification of Rat Liver Alcohol Dehydrogenase							
Purification Step	Volume (mL)	Total Protein (g)	ADH Activity (I.U./mL)	Total ADH (I.U.)	Specific Activity (I.U./mg)	Yield %	Purification
Liver Homogenate	231	5.27*	---	---	---	---	---
Bio-Gel P-2	215	2.89	0.562	121	0.048	100	1
DEAE-Agarose	283	1.36	0.314	88.9	0.063	73.4	1.51
AMP-Agarose	21.3	0.055	2.81	59.8	1.08	49.5	25.8
Ultrogel AcA 44	8.2	0.023	5.08	41.7	1.89	34.5	44.2

*Mass of wet tissue = 69.2 g

repeated and the supernatants from both centrifugation steps were combined. This sample is the "Liver Homogenate" identified in Table I.

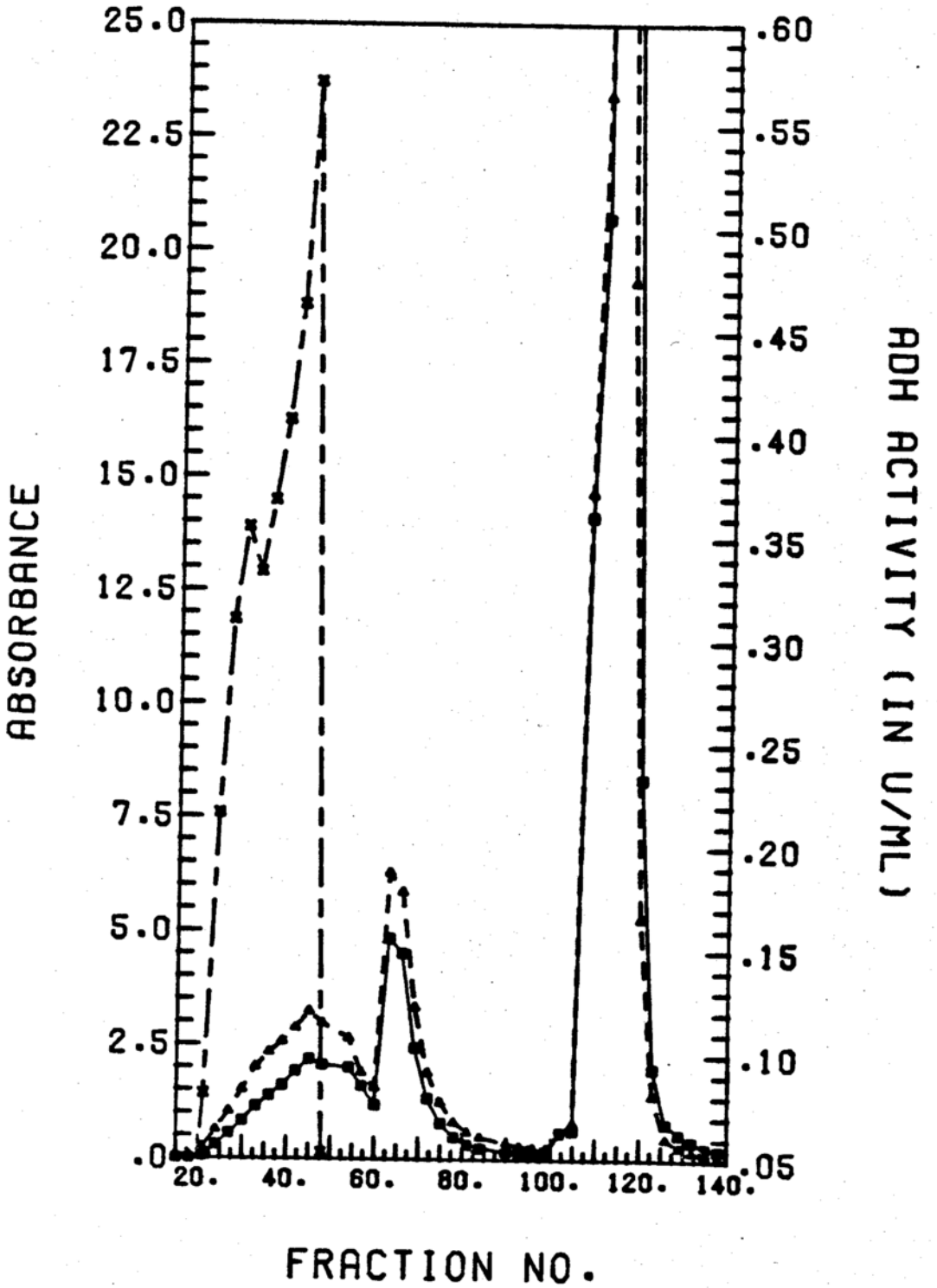
Ammonium Sulfate Precipitation of Proteins. The liver homogenate was brought to 35 percent saturation with solid ammonium sulfate and stirred for approximately one hour. The suspension was centrifuged for 15 minutes at 18,000 x g and the precipitates discarded. Solid ammonium sulfate was added to the supernatant until 75 percent saturation was reached. The suspension was stirred and recentrifuged as described above. The supernatant fluid was decanted and the pellets were stored at -20°C.

The pellets were redissolved by stirring in DEAE buffer (ca. 200 mL). Addition of small amounts of 1-butanol suppressed frothing. Insoluble protein was removed by centrifugation. The redissolved protein was desalted on a 1-L Bio-Gel P-2 column equilibrated with DEAE buffer.

DEAE-Agarose Chromatography. The desalted protein solution was passed through a 2.5 x 60 cm DEAE Bio-Gel A column (100-200 mesh) which had been equilibrated with 20 mM Tricine, 2.5 mM DTT, pH 8.4 (DEAE buffer). The results are shown in Figure 3.1. The alcohol

Figure 3.1. Anion-exchange chromatography of the desalted supernatant from homogenized rat livers on DEAE-agarose. The plots represent: Absorbances at 280 (-▲-) and 260 nm (-■-), and ADH activity (I.U./mL) (-✱-). The column dimensions were 2.5 x 60 cm, the flow rate was 2 mL/min and the fraction size was 10 mL. The sample size was 295 mL and after application, the column was developed with 20 mM Tricine buffer, pH 8.4, plus 2.5 mM DTT. After all of the unretained protein had been washed from the column, the eluent was changed to 2 M NaCl at fraction 85 and the strongly bound protein and nucleic acids were eluted. This peak is off-scale on the figure. Fractions 21 through 48 were combined and used as the sample for the AMP-agarose column.

DEAE-AGAROSE ELUTION PROFILE



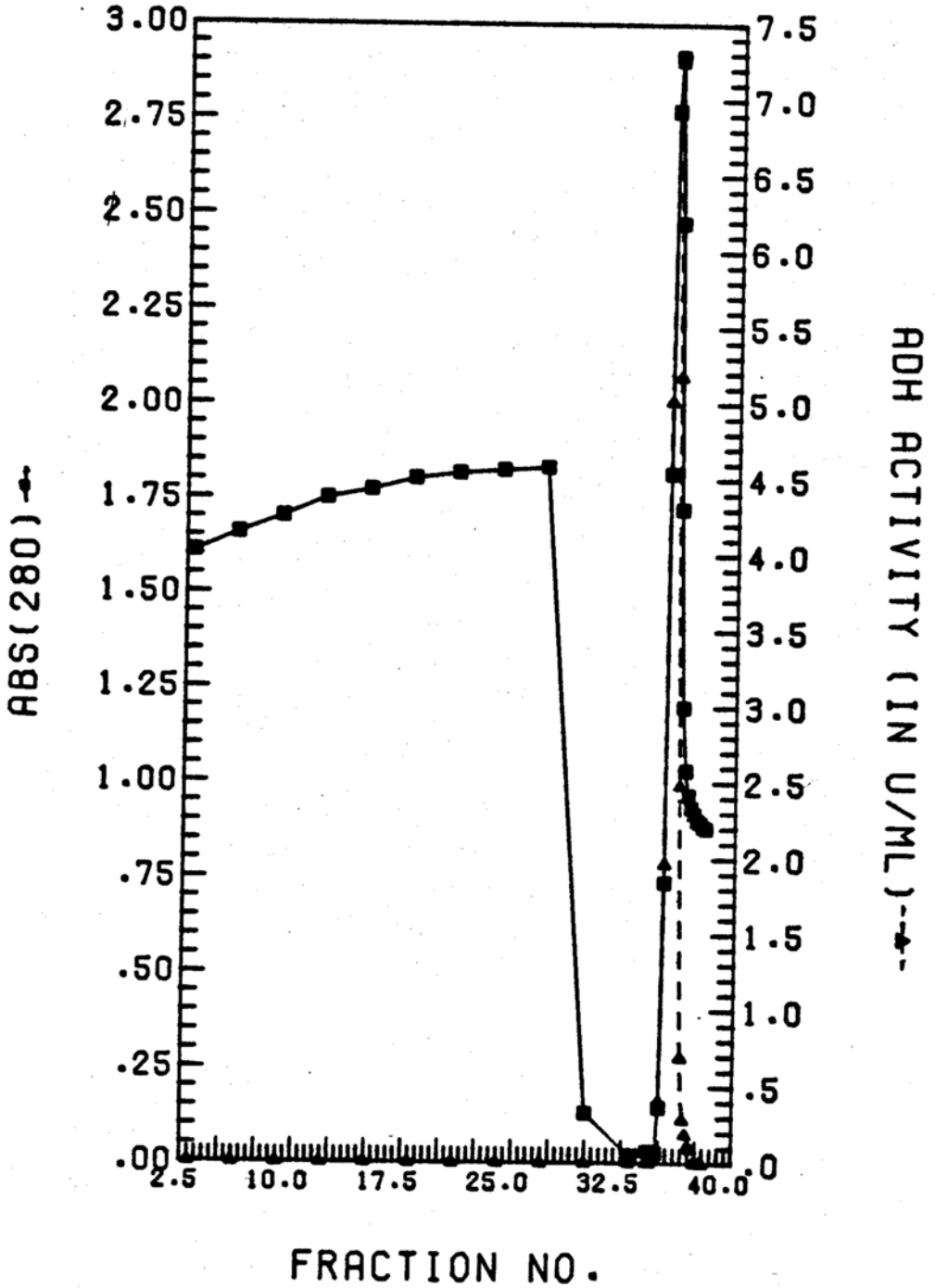
dehydrogenase, which was only slightly retarded, was eluted on the leading edge of the first protein peak (fractions 21-48). A second protein peak was also eluted under the condition of starting buffer. A large amount of contaminating protein and nucleic acid adsorbed to the column and were eluted with 2 M sodium chloride. This fraction had a peak absorbance of about 60 A.U. and is off-scale on the figure.

AMP-Agarose Chromatography. The ADH fraction from the DEAE-agarose column was applied to an N^6-(6-aminohexyl)-AMP-agarose column. After the entire sample had been applied, the unbound protein was washed off of the column with DEAE buffer. The profile from this experiment is shown in Figure 3.2. The large, broad, first peak represents the unretained protein in the sample. After the A_{280} reapproached zero, the bound ADH was eluted from the column by the addition of 0.25 mM NAD plus 10 mM 1-butanol to the buffer (fractions 34.9-37.1). (However, the A_{280} did not return to zero after the second peak had been eluted because the NAD in the eluent has some absorbance at 280 nm.)

Ultrogel Chromatography. The ADH peak from the AMP-agarose column was concentrated using a stirred

Figure 3.2. Affinity chromatography of RLADH on N^6-(6-aminohexyl)-AMP-agarose. The plots represent: Absorbance at 280 nm (—■—) and ADH activity (I.U./mL) (- ★ -). The bed volume was 5 mL and the flow rate was 1 mL/min. The sample size was 283 mL and after application, unbound protein was eluted with 20 mM Tricine buffer, pH 8.4, plus 2.5 mM DTT (DEAE buffer). Fraction volumes were 10 mL. At fraction 32, the eluent was changed to 0.25 mM NAD + 10 mM 1-butanol in DEAE buffer and the fraction volume was reduced to 1 mL. (These fractions are represented as tenths of a fraction number.) Fractions 34.9 throught 37.1 were combined and used as the sample for the Ultrogel AcA 44 column after concentration on an ultrafiltration cell.

AMP-AGAROSE ELUTION PROFILE



ultrafiltration cell and a PM 30 membrane having a molecular weight cut-off of 30,000 daltons. The concentrated sample was then fractionated on a 1.1 x 45 cm Ultrogel AcA 44 gel filtration column equilibrated and run in 100 mM Tricine, 2.5 mM DTT, pH 8.4. The elution profile for this column is shown in Figure 3.3. The ADH was eluted in the main peak with K_{av}=0.31. The specific activity for this peak is also shown on the figure. The specific activity was not constant here, although it did reach a peak value of 3.2 I.U./A_{280}. An apparent impurity was eluted on the leading shoulder of this peak, but it was not included in the final collected product from this column. A minor peak was eluted late with K_{av}=0.90.

Fractions 24 through 32 were combined, portioned into small polypropylene vials and stored frozen at -20°C. A sample was subjected to polyacrylamide disc gel electrophoresis at pH 9.5 and consisted of a single, although broad, band at R_f=0.12, as shown in Figure 3.4. A screen for contaminating protease activity was negative.

Figure 3.3. Molecular sieve chromatography of RLADH on Ultrogel AcA 44. The plots represent: Absorbance at 280 nm (—■—), ADH activity (I.U./mL) (—◆—) and Specific Activity (I.U./A_{280}) (—✕—). Column dimensions were 1.1 x 45 cm, the flow rate was 0.1 mL/min and the fraction size was 0.87 mL. The sample size was 1.3 mL and the column was developed with 100 mM Tricine buffer, pH 8.4, plus 2.5 mM DTT. Fractions 24 through 32 were combined and used as the enzyme in the isotope effect determinations.

ULTROGEL ACA 44 ELUTION PROFILE

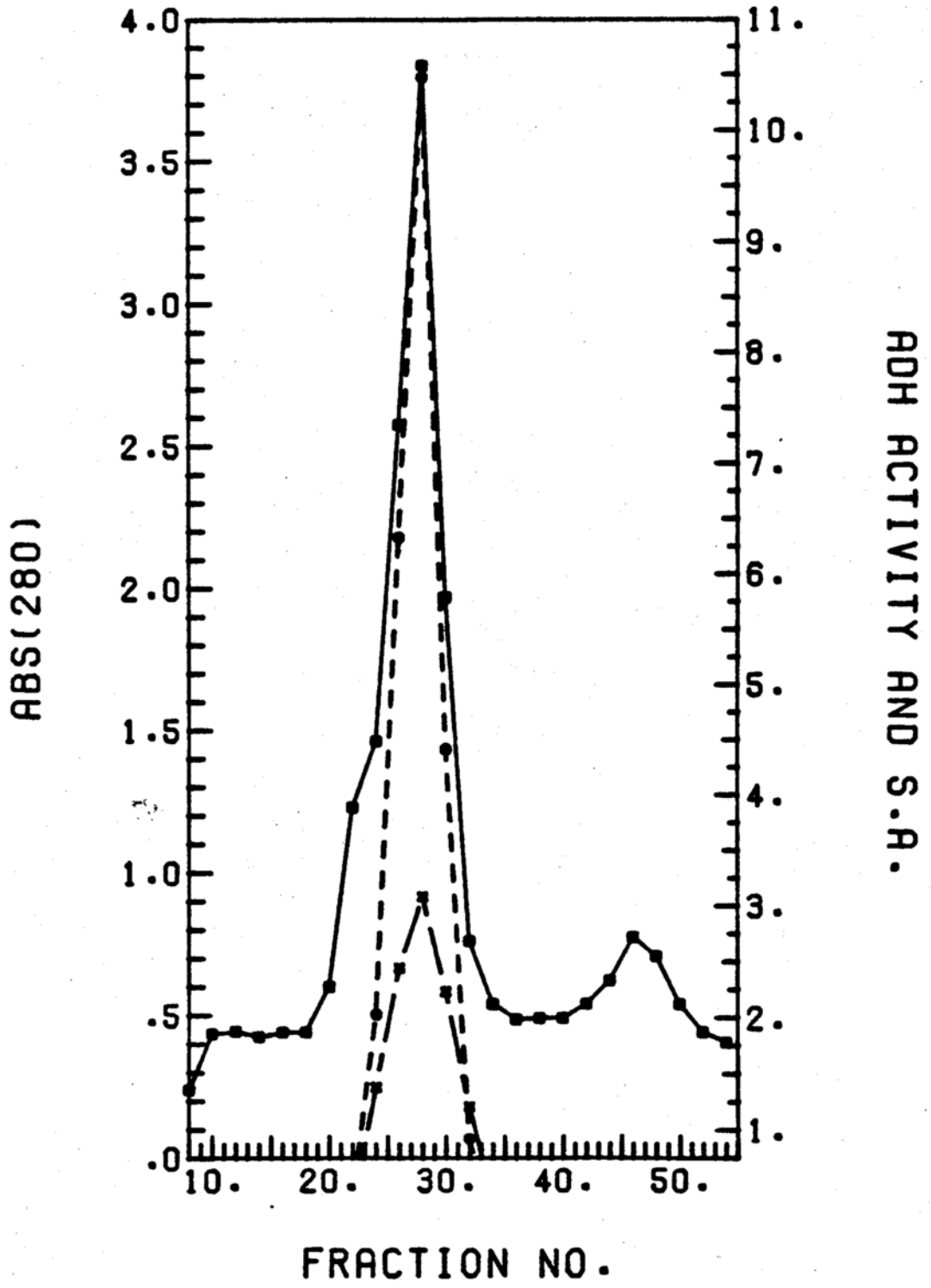
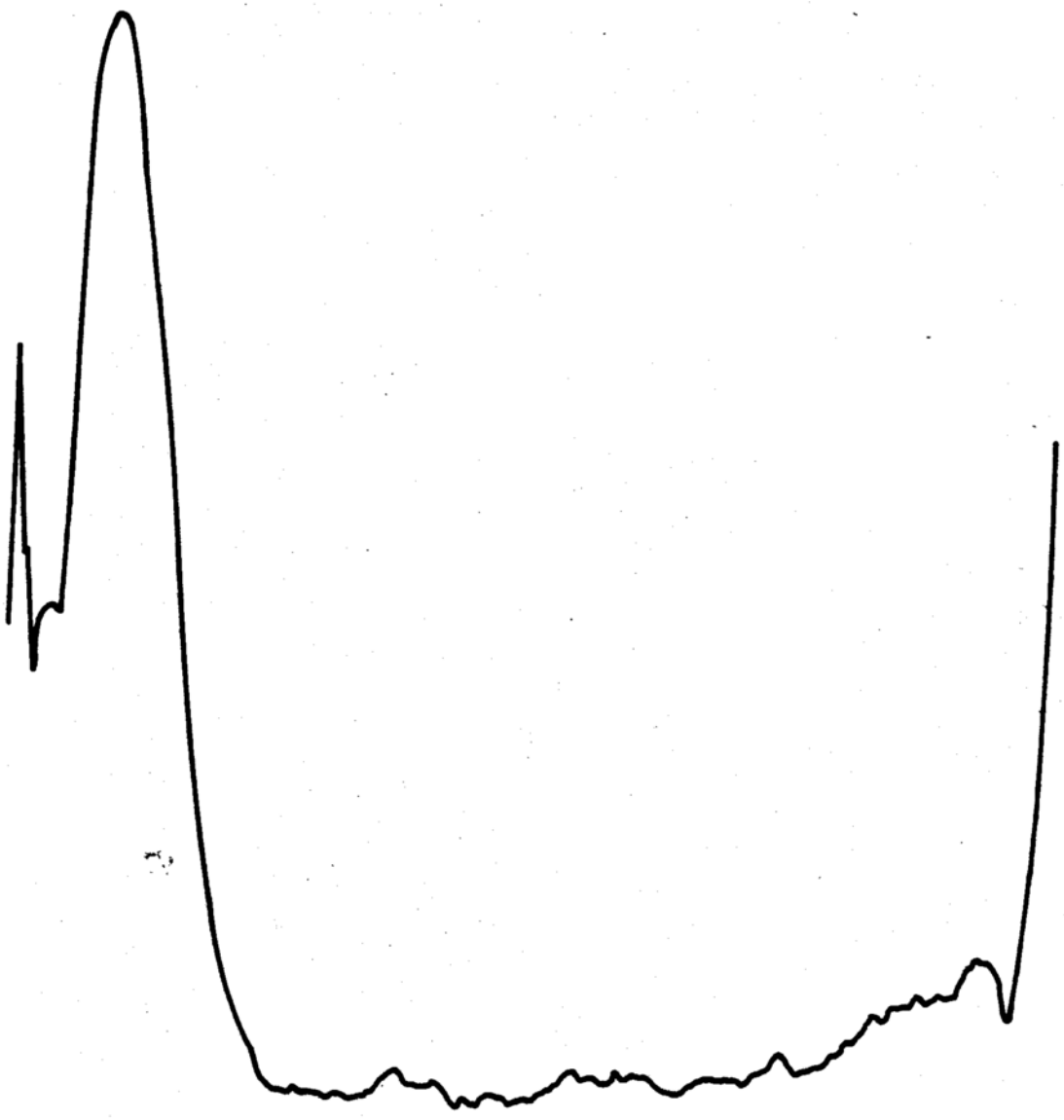


Figure 3.4. Electropherogram of the Ultrogel sample of the RLADH purification. The electrophoresis was performed as described in the Materials & Methods chapter. The gel was scanned for the blue dye-protein complex with Gilford 240 spectrophotometer at 595 nm using a slit width of 0.05 mm and a linear scan rate of 1 cm/min. The cathode (-) is at the left and the anode (+) is at the right. The origin is at the left. The length of the gel was 97 mm and is represented by the length of the imaginary horizontal axis. The imaginary vertical axis is in arbitrary absorbance units.



VARIATION OF THE STEADY-STATE KINETIC CONSTANTS OF RLADH WITH DEOXYCHOLATE. Consistant with the original experiments of Hanozet et al. (1979), adding DOC to assays of RLADH increased the maximal velocity*. This was true with ethanol as substrate, as reported by Hanozet et al., but also true with 2-propanol, which is a poorer substrate. The addition of 3 mM DOC resulted in a six-fold increase in the V_{max} for ethanol oxidation and a three-fold increase for 2-propanol oxidation. In addition, this new work shows that the DOC increased the K_m and decreased the V/K for RLADH. The V/K decreased by 75-80 percent in the presence of 3 mM DOC with either ethanol or 2-propanol (Figures 3.5 through 3.10).

The effect of the DOC concentration on V_{max} and V/K appears hyperbolic (Fig. 3.5, 3.6, 3.8 & 3.9). However, since the critical micellar concentration of DOC at this pH is about 3.3 mM (Makino et al., 1973),

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* Because these experiments were done over a period of days, some inactivation of the enzyme occurred. To correct for this, a standard activity assay was done at the start of each day and the steady-state parameters obtained were normalized accordingly. To insure that the trends so obtained were not an artifact of this procedure, V_{max} values were measured at saturating ethanol concentrations at pH 8.5 with varying DOC concentrations on a single day, and the trends were found to be real (data not shown).

Figure 3.5. Variation of V_{max} for RLADH vs. DOC concentration using ethanol as substrate. V_{max} is in units of A.U. at 340 nm per min. The line is freely drawn.

VMAX VS. [DOC] FOR ETOH AS SUBSTRATE

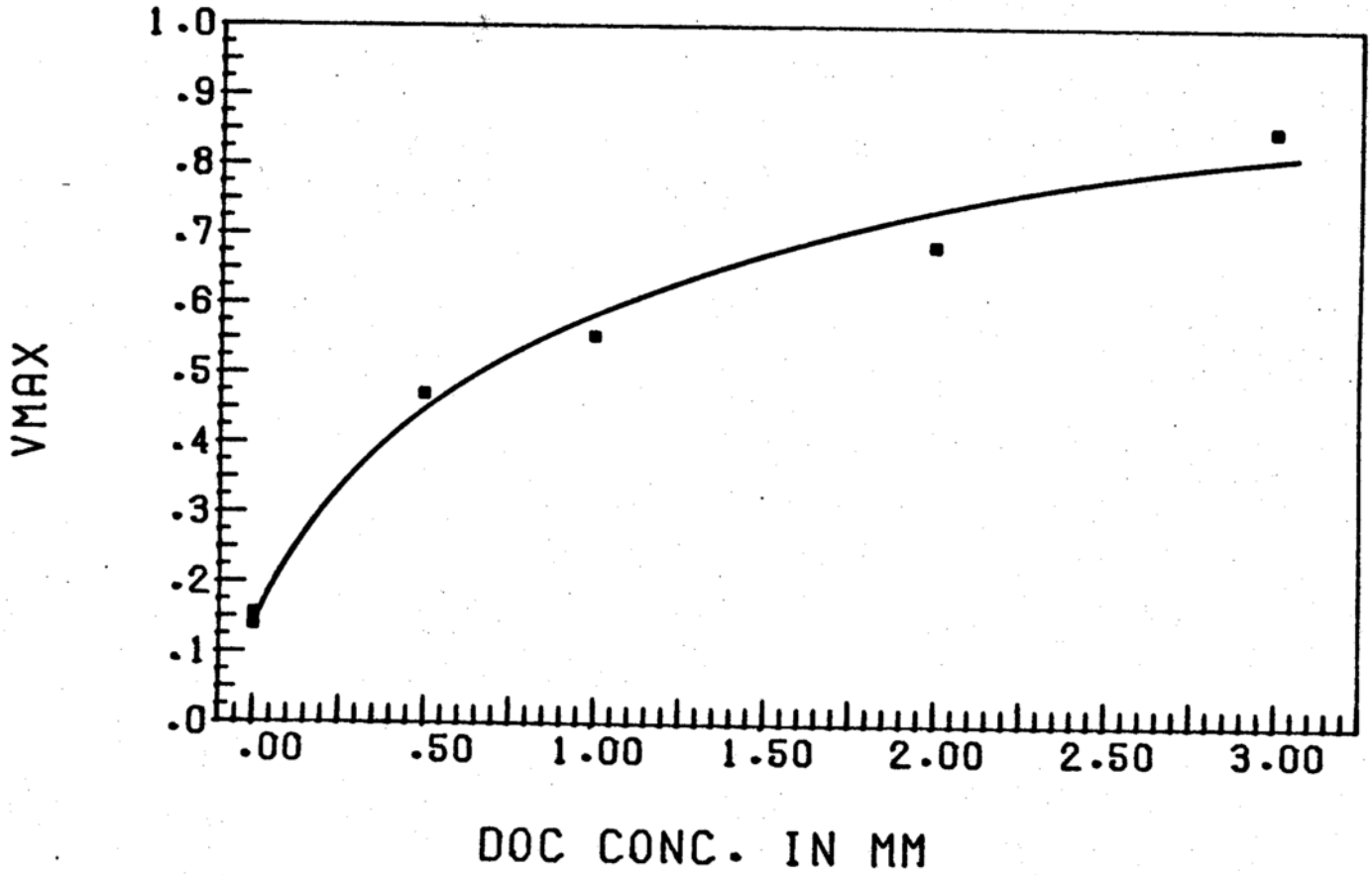


Figure 3.6. Variation of V/K for RLADH vs. DOC concentration using ethanol as substrate. V/K is in units of l/min. The line is freely drawn.

V/K VS. [DOC] USING ETOH AS SUBSTRATE

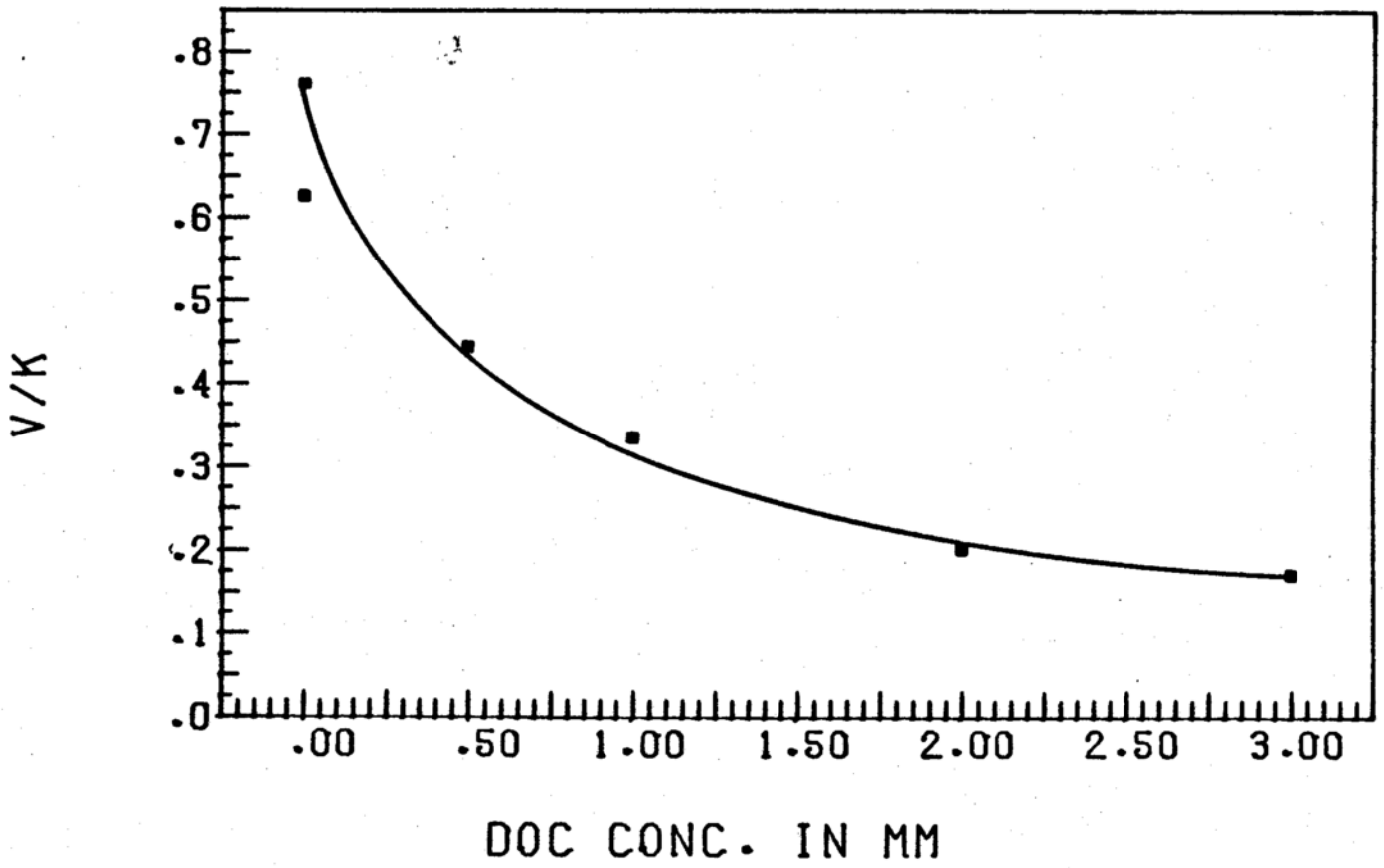


Figure 3.7. Variation of K_m for ethanol for RLADH. K_m is in units of mM. The line represents the best linear fit and gives a correlation coefficient of Ø.999 (p>Ø.99).

KM VS. [DOC] FOR ETOH AS SUBSTRATE

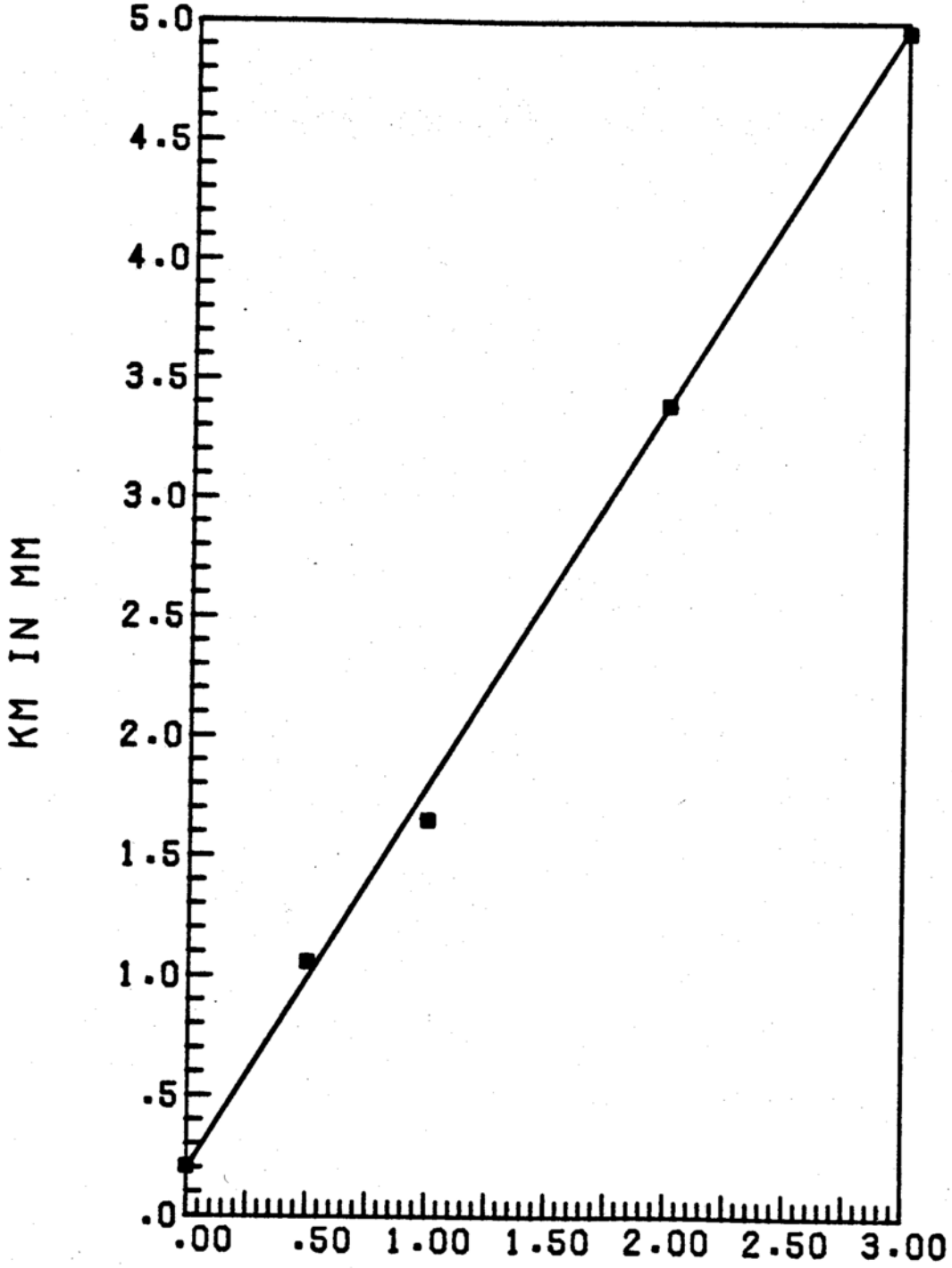


Figure 3.8. Variation of V_{max} for RLADH vs. DOC concentration using 2-propanol as substrate. V_{max} is in units of A.U. at 340 nm per min. The line is freely drawn.

VMAX VS. [DOC] USING I-PROH AS SUBSTRATE

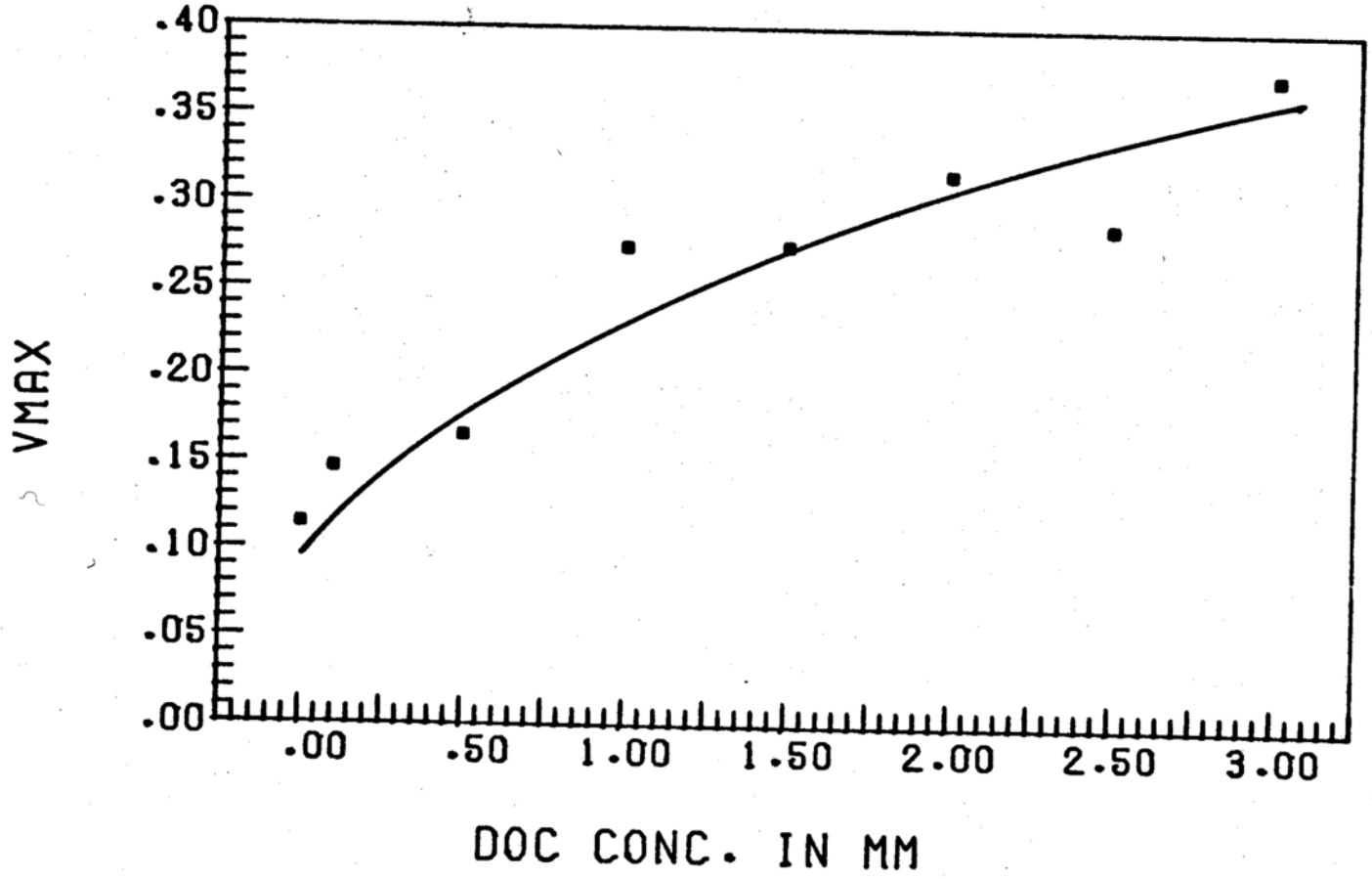


Figure 3.9. Variation of V/K for RLADH vs. DOC concentration using 2-propanol as substrate. V/K is in units of 1/min. The line is freely drawn.

V/K VS. [DOC] USING I-PROH AS SUBSTRATE

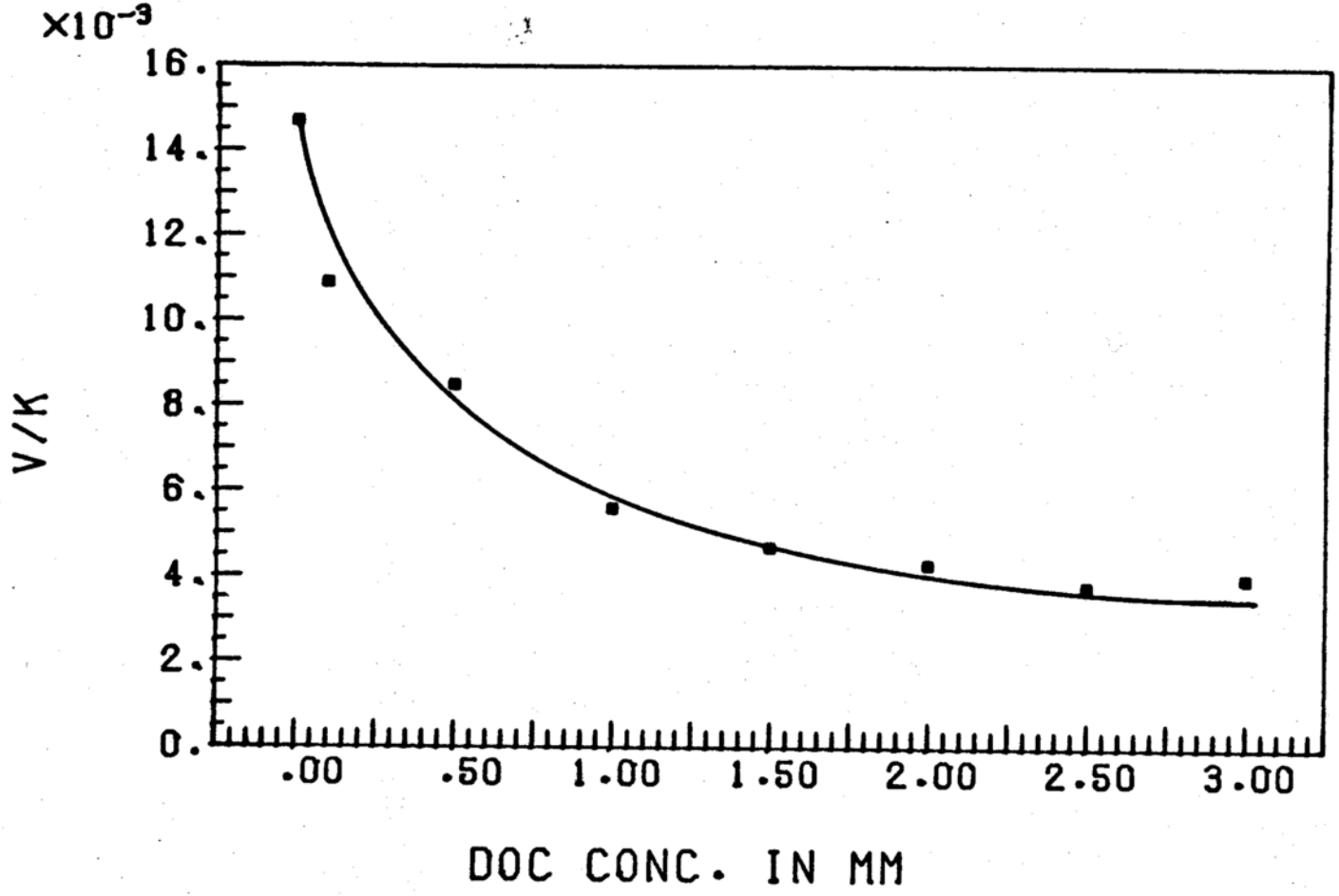
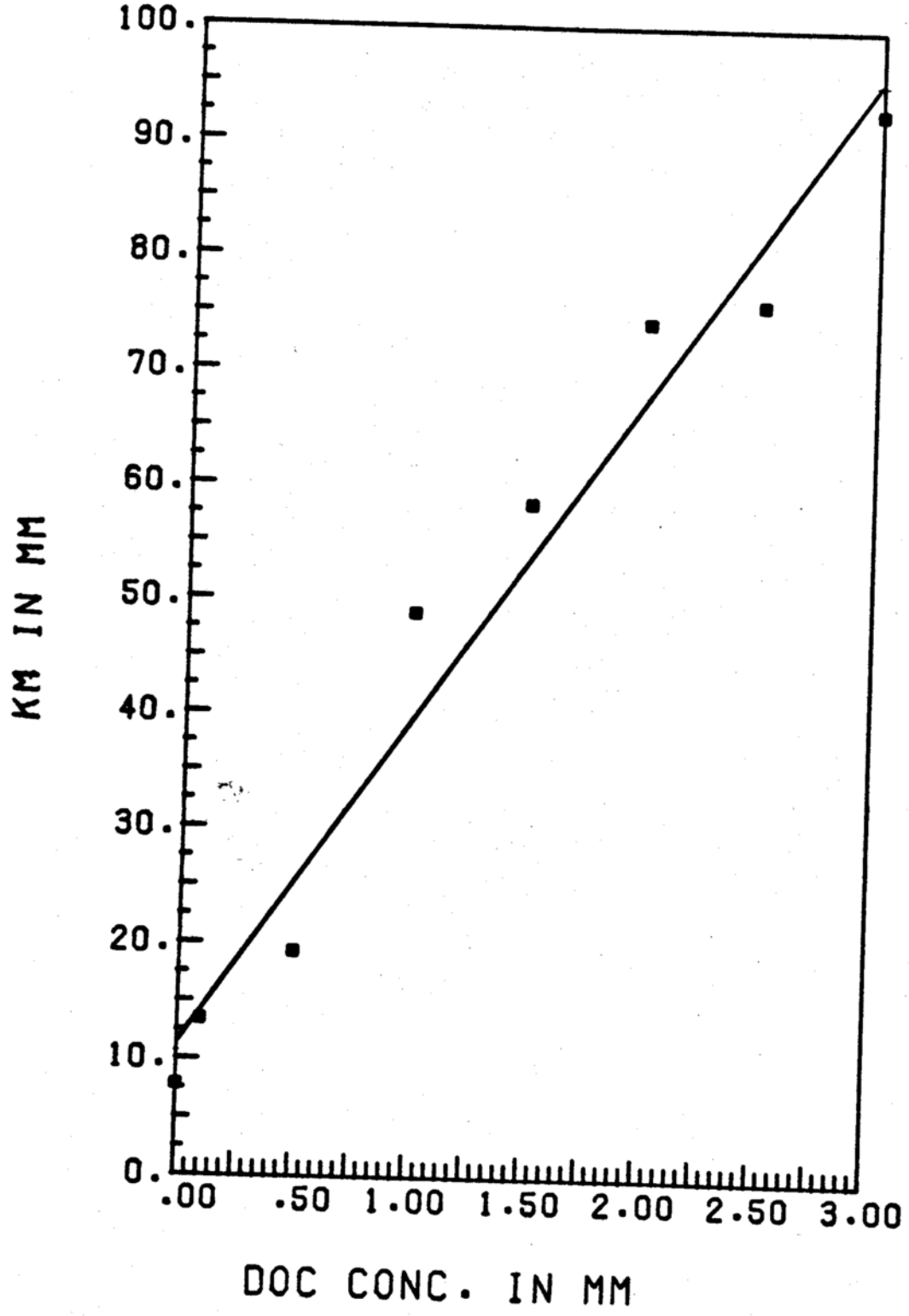


Figure 3.1Ø. Variation of K_m for 2-propanol for RLADH. K_m is in units of mM. The line represents the best linear fit and gives a correlation coefficient of Ø.983 (p>Ø.99).

KM VS. [DOC] USING I-PROH AS SUBSTRATE



it was pointless to attempt to demonstrate saturation at higher concentrations. Hanozet et al. (1979) reported no significant change in the V/K value for ethanol, but they determined the V/K at only two DOC concentrations (0 and 1 mM). Even with a large standard deviation of 20 percent on the value at 0 mM DOC, these two values demonstrated a decrease of 30 percent in V/K (0.0021 at 0 mM vs. 0.0015 at 1 mM DOC).

Curiously, the relationship between DOC concentration and K_m appears linear, suggesting a direct correlation between DOC and the kinetic constants held in common by V_{max} and V/K (Fig. 3.7 & 3.10). Regression of K_m versus DOC concentration gave a correlation coefficeient of 0.999 for ethanol and 0.983 for 2-propanol.

Variation of the Observed Isotope Effects with Deoxycholate. Figures 3.11 and 3.12 give the observed isotope effects for the RLADH reaction using ethanol and 2-propanol as substrates. The data reveal no convincing enhancement of the observed isotope effect upon addition of DOC. According to the hypothesis outlined in the Introduction, an increase in the V_{max} isotope effect is expected. To test for the existence

Figure 3.11. Variation of the observed kinetic isotope effects for RLADH vs. DOC concentration using ethanol as substrate. The broken line is the V_{max} isotope effect and the solid line is the V/K isotope effect. Error bars denote $\pm$ 1 S.D.

KIE VS. [DOC] USING ETOH AS SUBSTRATE

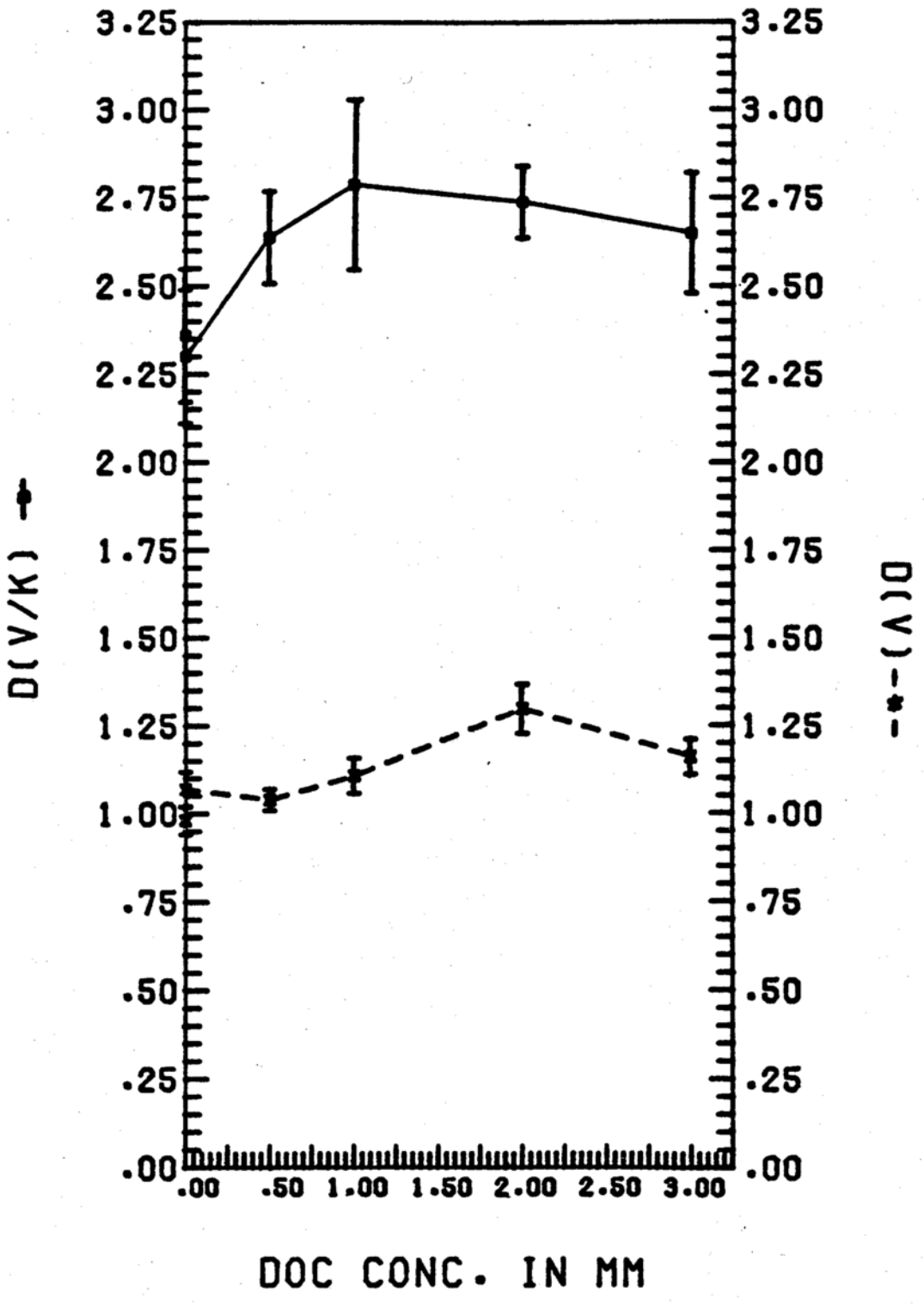


Figure 3.12. Variation of the observed kinetic isotope effects for RLADH vs. DOC concentration using 2-propanol as substrate. The broken line is the V_{max} isotope effect and the solid line is the V/K isotope effect. Error bars denote $\pm$ 1 S.D.

KIE VS. [DOC] USING I-PROH AS SUBSTRATE

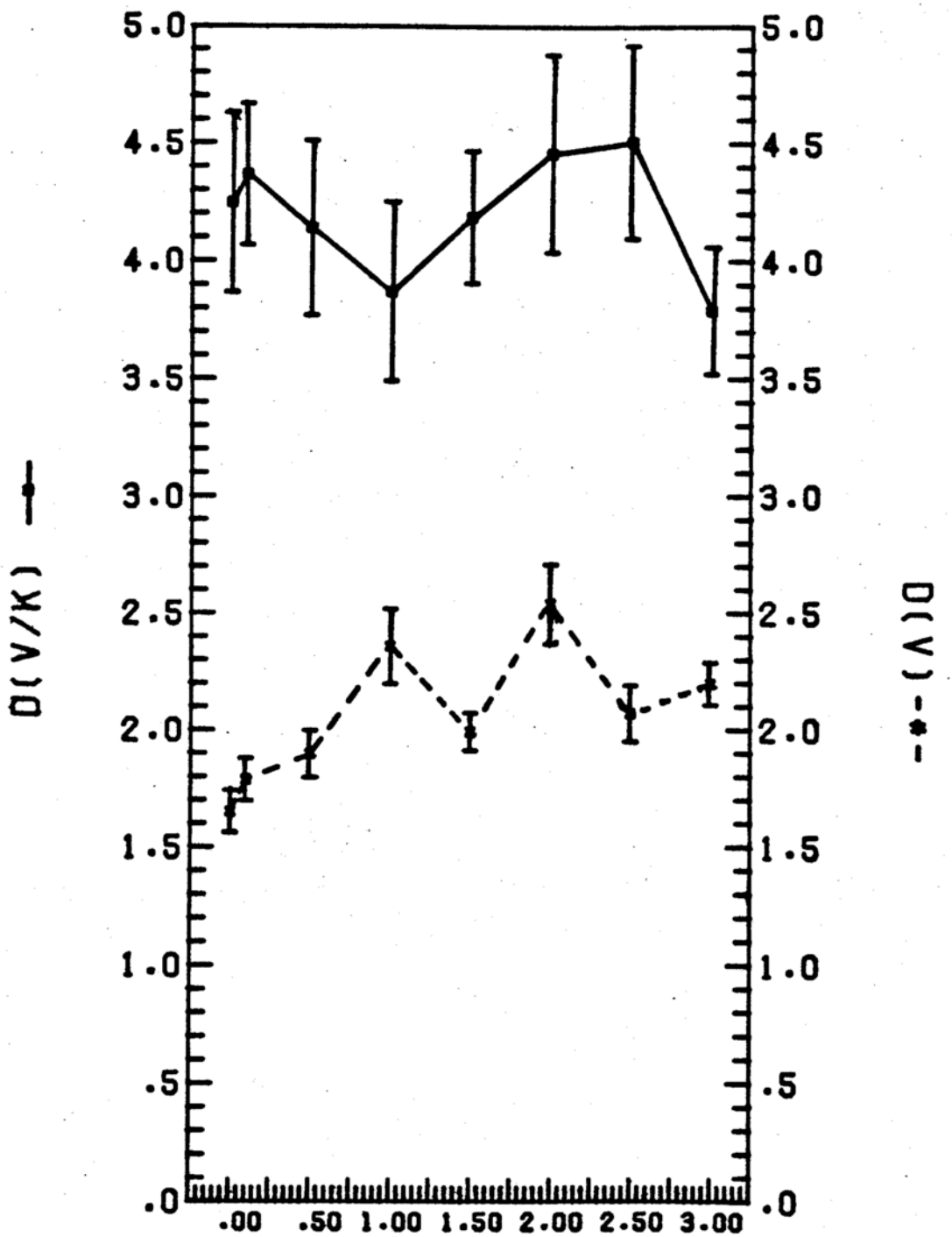


TABLE II

Results of Weighted Linear Regression of Isotope Effects vs. DOC Concentration for RLADH

Substrate	Isotope Effect	df	Correlation Coefficient	Slope (± S.D.)
Ethanol	D_V	4	Ø.771	Ø.Ø653±.Ø27Ø
	$D_{V/K}$		Ø.669	Ø.1Ø3 ±.Ø57
2-Propanol	D_V	6	Ø.763*	Ø.161 ±.Ø56 *
	$D_{V/K}$		-Ø.41Ø	-Ø.Ø922±.Ø838

*Significant at p>Ø.95.
df = Degrees of freedom.

of trends in the presence of experimental uncertainty, weighted linear least-squares regressions were performed using the BMDP routine P1R (Dixon, 1981). The values for the isotope effects were weighted according to the recipricols of their variances. Two hypotheses were tested according to this procedure: (a) that the isotope effects and DOC concentrations were significantly correlated; and (b) that the slopes of the regression lines were significantly different than zero. The results of these regressions are shown in Table II. In three of the sets of data, the statistical analyses rejected both hypotheses at a confidence level of 95 percent. In the third set, ^{D}V vs. DOC using 2-propanol, significance is assigned to both the correlation and positive slope. However, the regression line predicts that the $^{D}V/K$ will increase only from 1.78 to only 2.26 with 3 mM DOC.

VARIATION OF THE KINETIC CONSTANTS OF HLADH WITH REDUCTIVE METHYLATION. Tsai et al. (1978) reported that reductive methylation of the lysine residues of horse liver alcohol dehydrogenase (HLADH) (Equation 3.1) using formaldehyde and sodium borohydride resulted in an increase in activity toward primary alcohols and a decrease in the activity toward secondary alcohols. Attempts here to repeat their activation procedure (Tsai et al., 1974, and Tsai, 1977) resulted in negligible activation.

$$Lys\text{-}NH_2 + HCHO + NaBH_4 \rightarrow Lys\text{-}NHCH_3 \qquad (3.1)$$

The chemical modification was repeated, using higher reactant concentrations according to the procedure of Means and Feeny (1971). The time course of the activation, as measured by the standard ADH activity assay, is shown in Figure 3.13. Maximal activation (1.8-fold) was achieved within 40 minutes, although the reaction was allowed to continue for slightly longer than one hour.

Specific effects of methylation of the enzyme on the steady-state kinetic parameters are shown in Table III. In accordance with the results of Tsai (1978), methylation greatly increased the V_{max} when ethanol was used as substrate and slightly decreased the V_{max} with

Figure 3.13. Time course of the activation of HLADH by reductive methylation by formaldehyde and sodium borohydride. The vertical axis represents the ADH activity of samples of the activation reaction mixture. The horizontal axis is the time of the activation reaction, in minutes.

ACTIVATION OF HLADH BY REDUCTIVE METHYLATION

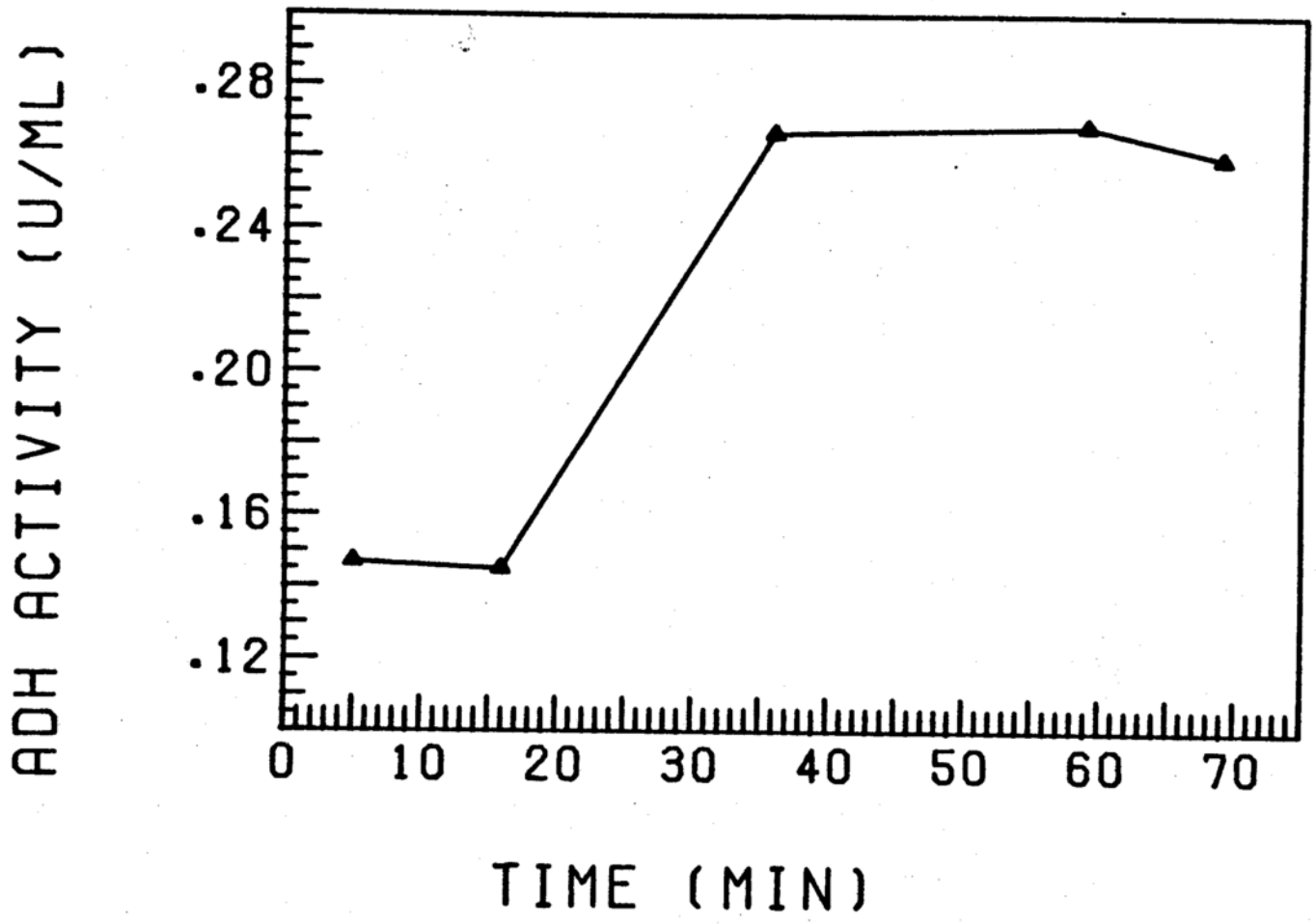


TABLE III

Effect of Methylation on the Expression of the Isotope Effects of HLADH

Treatment	Substrate	V_{max} (A.U./min)	V/K (min^{-1})	K_m (mM)	$D_{V/K}$	D_V
Control	Ethanol	Ø.448	1.Ø1	Ø.465	2.36±.13	1.34±.Ø4
Methylated	Ethanol	2.54	Ø.35Ø	7.26	3.75±.3Ø	2.61±.1Ø
Control	2-Propanol	Ø.Ø485	Ø.ØØ376	12.9	4.43±.39	2.95±.13
Methylated	2-Propanol	Ø.Ø442	Ø.ØØ129	34.2	3.48±.3Ø	4.54±.18

2-propanol. In both cases the V/K values decreased*.

Methylation caused large, significant (t-test, $p>0.99$) increases in the V_{max} isotope effects. The ^{D}V value increased from 1.34 to 2.61 when ethanol was used as substrate, and from 2.95 to 4.54 using 2-propanol. However, the V/K isotope effects behaved differently. With ethanol, $^{D}V/K$ rose from 2.36 to 3.75, but with 2-propanol, the isotope effect fell from 4.43 to 3.48. Note that this large change in the $^{D}V/K$ with 2-propanol occured despite the presence of only a slight change in its V_{max} values upon methylation.

* As was the case for RLADH, corrections were necessary for decay in enzyme activity over time. Because these experiments were performed on two different enzyme solutions, it was also necessary to correct for differences in enzyme concentration. This was adjusted using the A_{280} values.

IV. DISCUSSION

ENZYME PURIFICATION. Previous purification procedures for rat liver alcohol dehydrogenase revealed several undesirable characteristics peculiar to the enzyme from this animal, namely instability at pH values other than 8.5 and in the absence of substrate and dithiothreitol (DTT). Instability in the absence of substrates is inconvenient to the kineticist because the substrate concentration is usually the parameter that he wishes to vary. Restriction to such a narrow pH range limits the use of ion-exchange resins since most proteins will adsorb to any given ion-exchange column only within certain limits of pH. In the case of RLADH, the enzyme is retarded slightly or not at all on every conventional ion-exchange resin at pH 8.5.

There are five published procedures for the purification of RLADH (Reynier, 1969; Okuda and Takigawa, 1970; Markovic et al., 1971; Arslanian et al., 1971; Duncan et al., 1976). Steps common to all five are homogenization of the rat liver tissue followed by centrifugation and ammonium sulfate fractionation. Additionally, in four of the procedures the enzyme is passed over a DEAE anion-exchange column. Although the enzyme is only slightly retarded, at the crude stage there are many other contaminating proteins

and nucleic acids which will adhere, thus giving this step some utility. These four steps were included at the beginning of the present purification procedure.

In four of these procedures a cation-exchange column is used. The enzyme is not retained at the higher pH values necessary to protect the enzyme against inactivation (Reynier, 1969; Okuda and Takigawa, 1970; Arslanian et al., 1971; Duncan et al., 1976), and dropping the pH to lower values gives variable results. For example, Duncan et al. found that the enzyme was not retained at pH 7 on CM-cellulose, while Reynier found that part of the ADH activity was retained at pH 7.2. The utility of this technique is lowered by the lack of consistent retention by the column and the pH-dependent inactivation of the enzyme. A cation-exchange step was omitted in the present procedure.

One powerful technique that has been utilized in only one of the above procedures (Duncan et al.) is affinity chromatography. Since ADH is an NAD-dependent dehydrogenase, it ought to bind to an NAD-analog resin with greater specificity than in the costly cation-exchange step. The first NAD-analog affinity resin utilized in the present work was reactive blue

2-agarose (blue agarose). NAD-dependent dehydrogenases are believed to bind to this resin because the reactive blue 2 dye molecule mimics the conformation of the pyridine nucleotide coenzyme as it sits in the "dinucleotide fold" of the enzyme (Stellwagen, 1977). The resin is convenient to use because the industrial dye ligand Cibachrome Blue F3GA (the blue functionality of Blue Dextran) is much less expensive than the biological ligands NAD or AMP. The second resin examined, AMP-agarose, is also extensively used as an affinity medium for NAD-dependent dehydrogenases (Mosbach et al., 1972). The AMP portion of NAD is believed to be involved in the binding of the coenzyme to the protein, and therefore NAD-dependent dehydrogenases will bind to AMP-agarose. Although more expensive than blue agarose, it is widely available commercially.

RLADH was found to bind to both resins, but attempts to displace the it from these immobilized ligands with NAD alone resulted in poor yields or very slow elution. Apparently the binding constant favors sorption of the enzyme on the column matrix. A means was therefore sought to make the displacement of the enzyme more efficient. One approach to this problem

would have been to raise the NAD concentration in the eluent. However, there are several NAD-dependent dehydrogenases present in rat liver, and they all presumably adsorb to the coenzyme-analog affinity resins. Some specificity would be lost by merely increasing the NAD concentration to the point where they all might be released. A better way to proceed was to take advantage of the kinetics of the enzyme in question (see Figure 1.4). Since in this case the reaction sequence for RLADH is probably ordered (Crabb et al., 1982), the following equilibria are present on the column matrix:

$$\text{Ag-L*ADH} \;\overset{}{\Longleftrightarrow}\; \text{Ag-L} + \text{ADH} \;\overset{\text{NAD}}{\Longleftrightarrow}\; \text{NAD*ADH} \;\overset{\text{S}}{\Longleftrightarrow}\; \text{ADH*NAD*S} \qquad (4.1)$$

where Ag-L is the agarose matrix with the covalently-attached affinity ligand and S is the substrate alcohol or a competitive analog. The equation shows that addition of NAD plus S in the eluent will effect desorption by shifting the equilibria towards the right. For S, an inhibitor is generally used because it cannot be consumed during elution.

This procedure has been used previously by Andersson et al. (1974, 1975) to elute HLADH from

AMP-agarose using NAD and cholic acid or pyrazole (Pz). Both cholic acid and pyrazole are competitive inhibitors versus alcohol. Duncan et al. (1976), using NAD plus Pz, extended the method to RLADH. Despite the success of this procedure in this laboratory (Fig. 4.1), a problem was suspected in using a tight-binding inhibitor during the purification. If the inhibitor is not removed during the subsequent steps, its presence can give erroneous results in the kinetic experiments. Dr. Bryce Plapp (personal communication) pointed out the uncertainty of the removal of pyrazole by a gel filtration column due to its small dissociation constant (K_i=4.2 uM, Reynier, 1969)*.

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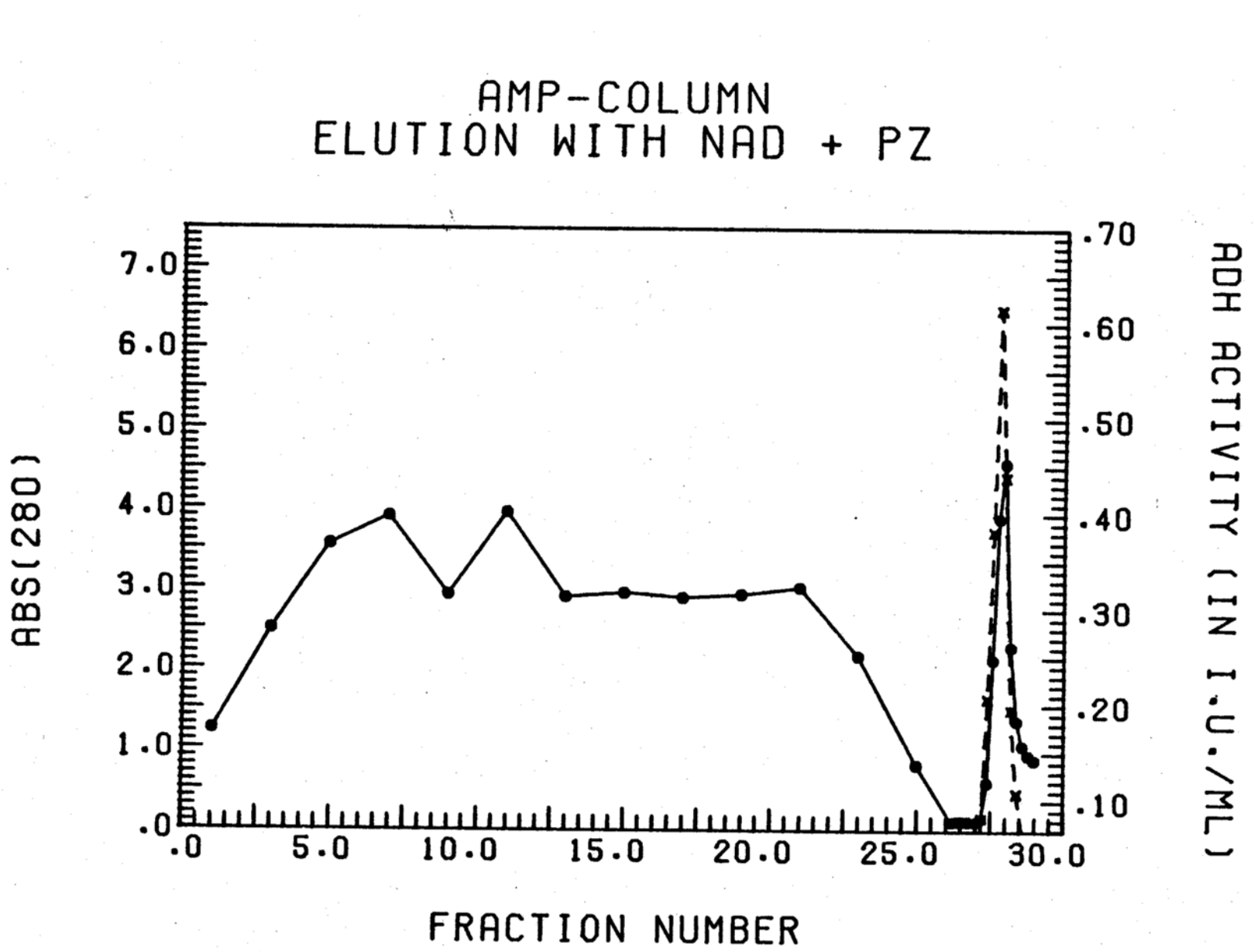
* Lange and Vallee (1976) circumvented this problem by instead immobilizing the inhibitor, a 4-substituted pyrazole. ADH was retained by the resin in the presence of NAD, and eluted upon the addition of NAD and ethanol. Unfortunately, the synthesis of this resin ("CapGapp-Sepharose") required eleven steps. It was thought that by using a different coupling procedure, the synthesis could be shortened by seven steps. 4-(3-Hydroxypropyl)pyrazole was synthesized by the method of Lange and Vallee and added to epoxy-activated agarose (Sundberg & Porath, 1974, and Vretblad, 1976). Sadly, HLADH was not retained by this column. Apparently the hydroxyl of the substituted pyrazole did not couple to the oxirane group of the agarose, because no bound nitrogen was detected on the column (method of Kohn & Wilchek, 1981).

Figure 4.1. Affinity chromatography of RLADH on AMP-agarose, elution with NAD and pyrazole (Pz). The plots represent: Absorbance at 280 nm (—●—) and ADH activity (I.U./mL) (—✱—). Conditions were similar to the column in Fig. 3.2, but elution was accomplished with 0.25 mM NAD plus 1.1 mM Pz in DEAE buffer beginning at fraction 26.


AMP-COLUMN
ELUTION WITH NAD + PZ
ABS(280)
7.0
6.0
5.0
4.0
3.0
2.0
1.0
.0
.70
.60
.50
.40
.30
.20
.10
ADH ACTIVITY (IN I.U./ML)
.0
5.0
10.0
15.0
20.0
25.0
30.0
FRACTION NUMBER

According to Equation 4.1, any compound capable of forming a ternary complex with ADH and NAD in solution ought to be useful as a desorbing agent. Instead of a dead-end inhibitor, a substrate can also form a ternary complex, albeit a transient one. Because of their much higher dissociation constants (in the millimolar range), the substrates ought to be removable on a subsequent gel filtration column. Although the kinetic parameters for alcohols other than ethanol were not known for RLADH, 1-butanol was chosen by analogy with HLADH (Sund & Theorell, 1963) to replace pyrazole, because it ought to bind well yet turn over slowly.

As shown in Figure 3.2, the combination of Ø.25 mM NAD plus 1Ø mM 1-butanol eluted the RLADH specifically and in a very sharp peak--a convienient result, because a small volume was desirable for the next step, gel filtration.

Ultrogel was chosen as the medium for gel filtration. Ultrogel is an acrylamide cross-linked agarose which posesses certain advantages over dextran and pure agarose gels. Agarose has the desired biological compatibility but the noncross-linked variety has poor mechanical integrity. The cross-linked Ultrogel has a rigid bead structure which

allows higher flow rates and gives more reproducible results. Because most mammalian alcohol dehydrogenases have molecular weights around 80,000 (Branden et al, 1975, and Jornvall & Markovic, 1972), Ultrogel AcA 44 was chosen for its fractionation range of 10,000 to 130,000.

The RLADH was eluted from the Ultrogel column as a single peak with a probable contaminant eluting on the leading shoulder (see Fig. 3.3). This contaminant was not included in the fractions chosen for study. A minor peak without ADH activity eluted later and probably contained other protein and peptide contaminants plus the NAD and butanol (and NADH and butanal) which were used in the elution of the enzyme from the previous step. The low number of peaks on this column demonstrates the specificity of the AMP-agarose and the eluent chosen*.

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* Recent results released from the laboratory of T.-K. Li (Bosron, 1981, and Crabb et al., 1982) are in substantial agreement with those presented above. Apparently they, too, were unsatisfied with previous purificiation procedures for RLADH and sought to develop their own. Their procedure included: (1) anion-exchange chromatography on DEAE-cellulose at pH 7.9; (2) affintiy chromatography on CapGapp-Sepharose; and (3) cation-exchange chromatography on CM-cellulose at pH 7.5. The ADH activity, however, was eluted from this last column in two peaks. The enzyme demonstrated ordered, Theorell-Chance kinetics, which agrees with

The specific activity of 1.89 I.U./mg of the collected product from the gel filtration column compares favorably to the value of 1.73 obtained by Markovic et al., and is only a little lower than the value of 2-2.5 of Duncan et al. Nevertheless, homogeniety is not assured because a constant specific activity across the peak was not attained, despite a single electrophoretic band. Control assays of the enzyme minus alcohol gave only a minimal background reaction, and this rate decreased nearly to zero after incubating the assay cuvettes for the several minutes necessary for them to come to the required constant temperature. A screen for contaminating proteases was negative.

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the earlier experiments of Hanozet et al. (1979). Li et al. determined the K_m for ethanol to be Ø.47 mM at pH 7.3 and 37° C, and this value is acceptably close to the value of Ø.21 mM at pH 8.5 and 25° C for this work.

EFFECTS OF DOC ON THE ISOTOPE EFFECTS OF RLADH. The conventional theory for the interpretation of isotope effects predicts that observed effects will be smaller than the intrinsic effect if a step other than the isotopically-sensitive one limits the rate of a reaction. If an enzymatic mechanism consisted only of substate-binding, catalytic and product-release steps such as in Equation 4.2,

$$E + S \leftrightarrows ES \rightarrow EP \rightarrow E + P \qquad (4.2)$$

then making the rate of the rate-limiting product-release step faster will shift the burden of rate-limitation to the catalytic step and the observed isotope effect should increase. This distribution of rate-limitation between the catalytic and product-release steps is shown in Figure 1.3, the "Rate-limitation See-saw." The see-saw can be tipped either toward the catalytic or product-release step, depending on which is more rate-limiting.

Enzyme mechanisms are now known to be more complicated than portrayed in Equation 4.2. Conformational changes, known to be kinetically important for many mechanisms, are usually not included in the models used to interpret isotope effect results. Also, the irreversibility of certain steps is often

assumed, despite evidence to the contrary. For example, Rao et al. (1979) have shown that the equilibrium constant for the interconversion of ternary complexes on pyruvate kinase is one, despite an equilibrium constant for the overall reaction of 3 x 10^{-4}. This indicates that this catalytic step is readily reversible, even though the overall equilibrium constant favors the back-reaction. With these considerations in mind, an obvious need arose for experimental data to test how changes in the rate-limiting step would affect expression of the isotope effect.

The reactions of rat liver alcohol dehydrogenase provided an attractive experimental system in which to test this hypothesis. First of all, the rate-limiting step for this reaction was believed known and highly documented. Theorell and Chance (1951) were the first to propose a mechanism for (horse) liver alcohol dehydrogenase that involves compulsory ordered binding of nucleotide first, then alcohol binding second. The existence of a central complex, while chemically necessary, was not kinetically demonstrable. The addition of the second substrate appeared to be followed immediately by the release of the first

product, the aldehyde. According to this mechanism (top half of Fig. 1.4), the release of the reduced nucleotide was the rate-limiting step of this reaction. Small observed steady-state hydrogen isotope effects (less than 2) were interpreted as being consistent with this mechanism (Gershman & Abeles, 1973). Bush et al.(1973) later showed that small kinetic isotope effects observed with deuterated NADH were abolished by saturating with aldehyde, also in accordance with this mechanism. Furthermore, the observed isotope effect was seen to increase (to greater than 4) in transient-state kinetic studies of Shore & Gutfreund (1970) and Brooks & Shore (1971). These studies indicated that the intrinsic isotope effect, being at least as large as four, was somehow suppressed in the steady-state, and that the suppression was related to events (viz., product release) that occured after the "burst" of NAD reduction that appeared on the enzyme during the time-course of the transient-state experiment.

For the enzyme from rat liver, conditions were known which caused a change in the rate-limiting step. Hanozet et al. (1979) and Crabb et al. (1982) found that rat liver ADH obeyed the same Theorell-Chance

mechanism with ethanol. Hanozet et al. also found that the addition of deoxycholate (DOC) increased the maximal velocity, with the results of their steady-state kinetic analysis indicating that DOC increased the rate of a conformational change which preceded nucleotide release. This was reflected in a change of mechanism from Theorell-Chance to ordered sequential (Fig. 1.4). The ability to demonstrate kinetically the existence of the central complex with DOC implies that the catalytic step now bears some of the burden of rate-limitation--in other words, the "See-saw" had been tipped from a non-catalytic step to a catalytic one. The theory predicts that the observed isotope effect on V_{max} should increase in the presence of DOC.

The rat liver ADH purified by the procedure described in this dissertation shared many of the properties described above. The Michaelis constant of Ø.21 mM for ethanol is acceptably close to the value of Ø.34 obtained by Hanozet et al. In addition, the six-fold increase in the maximal velocity with 3 mM DOC confirms the stimulatory effect of DOC. A V_{max} isotope effect of Ø.98 was obtained with this enzyme using ethanol, and this is consistent with the proposed

Theorell-Chance mechanism in the absence of DOC. The continuity of this new work and the earlier proposals is therefore assured.

In spite of the change in the rate-limiting step with the addition of DOC, the observed isotope effects on V_{max} did not show the expected trend of enhancement with increasing DOC concentration (Table II). The low correlation coefficient obtained when ^{D}V for ethanol was regressed against the DOC concentration indicates the lack of a linear relation between these two variables, and the negligible slope indicates that no enhancement is expected from the best-fit line. These results illustrate the existence of a case where the prevailing theory failed to predict the observed result.

A reasonable rebuttal to this argument is that the baseline V_{max} isotope effect with ethanol was too small to enable measurement of any change. The V_{max} effect using 2-propanol, on the other hand, was reasonably large (1.65) without DOC. Although the effect of 3 mM DOC on the maximal velocity for this reaction was still significant (three-fold, see Fig. 3.8), the regression line of ^{D}V on the DOC concentration had a very small slope, indicating an expected enhancement from 1.78 to

only 2.26 (Table II). The magnitude of this change is smaller than what one would ordinarily expect in the face of a three-fold increase in the rate and reflects a low sensitivity of the isotope effect to these changes.

(It should be noted that the use of perdeuterated alcohols as substrates imposed secondary isotope effects on the primary ones. Because primary and secondary isotope effects obey the same functions governing their expression, polydeuterated substrates should give increased baseline isotope effects, enabling more precise detection of changes.)

Returning to the analogy of the "Rate-limitation See-saw" of Figure 1.3, there is another possible case not yet considered which is superficially consistent with these results. Instead of tipping the see-saw toward the catalytic step, the addition of DOC might have increased the rates of <u>both</u> the catalytic and product-release steps in constant ratio--thus raising the entire see-saw. But if this were true, the effects of DOC on the V and V/K isotope effects should have been the same when 2-propanol was used in place of ethanol. This was not observed, as shown in Table II. The V_{max} isotope effect for 2-propanol was

significantly, although only slightly enhanced, showing that the catalytic step was not affected in the same way as the other step(s) governing expression of the isotope effect. Also, raising the see-saw would not result in a change in mechanism from Theorell-Chance to ordered sequential.

The lack of significant enhancement in the isotope effects in the face of a change in the "rate-limiting step" implies that there is not a direct one-to-one relationship between these two factors, but that a more complex relationship is required to explain the data. Northrop's (1981b) equation (Eqn. 4.3) proposes

$$^{D}V = \frac{^{D}k + R_f/E_f + C_r * {}^{D}K_{eq}}{1 + R_f/E_f + C_r} \qquad (4.3)$$

that the ^{D}V is a function of several parameters, C_r, E_f, R_f, ^{D}k and $^{D}K_{eq}$, the latter two being insensitive to changes in the rate-limiting step. While an individual parameter, R_f for instance, might obey the rule of the see-saw (decreasing as catalysis becomes more rate-limiting, in this case), the combination of the parameters as they occur in the equation appears to have remained constant here, despite obvious perturbations of individual rate constants. Therefore, although changes in the rate-limiting step of this

reaction must have occured to cause changes in the V_{max}, they are not reflected in ^{D}V for this case.

EFFECTS OF METHYLATION ON THE OBSERVED ISOTOPE EFFECTS OF HLADH. In preliminary studies on LDH (Appendix B), substituting NAD for 3-acetylpyridine adenine dinucleotide as coenzyme in the oxidation of lactate caused an increase in V_{max}. The ^{D}V increased for this reaction, in accordance with the conventional prediction. In the case discussed above, addition of DOC to assays of RLADH caused an increase in V_{max}, but without the expected increase in ^{D}V. In the case to be discussed below, methylation of HLADH caused a minor decrease in the V_{max} for 2-propanol, yet the ^{D}V increased significantly.

Methylation of the lysine residues of HLADH with formaldehyde and sodium borohydride gave a five-fold increase in activity toward ethanol and a slight decrease in activity toward 2-propanol (Table III), consistent with the results of Tsai (1978). In accordance with conventional theory, methylation enhanced ^{D}V with ethanol. Despite the decreased V_{max} with 2-propanol, this ^{D}V was also enhanced. This variation of behavior of the V_{max} isotope effect can now be accomodated by the general function for ^{D}V, Equation 4.3.

The V/K isotope effects also exhibited interesting behavior. With ethanol, the isotope effect rose after methylation; with 2-propanol, it actually fell. This is unlike the effect of DOC on RLADH, where the effects were similar for both substrates. These results illustrate a case where the V/K isotope effects can be used to gain insight into the kinetic mechanism.

Equation 4.4 shows that $^{D}V/K$ is a function of ^{D}k, C_f, C_r and $^{D}K_{eq}$, where the C-functions are the commitments to catalysis. The commitment factors, in turn, are functions of ratios of pairs of rate constants leading from a common ground state, e.g., k_3 and k_2 in Equation 4.5.

$$^{D}V/K = \frac{^{D}k + C_f + C_r * ^{D}K_{eq}}{1 + C_f + C_r} \qquad (4.4)$$

$$E + S \underset{k(2)}{\overset{k(1)}{<==>}} ES \underset{k(4)}{\overset{k(3)}{<==>}} EX \underset{k(6)*}{\overset{k(5)*}{<==>}} EP \overset{k(7)}{-->} E + P \qquad (4.5)$$

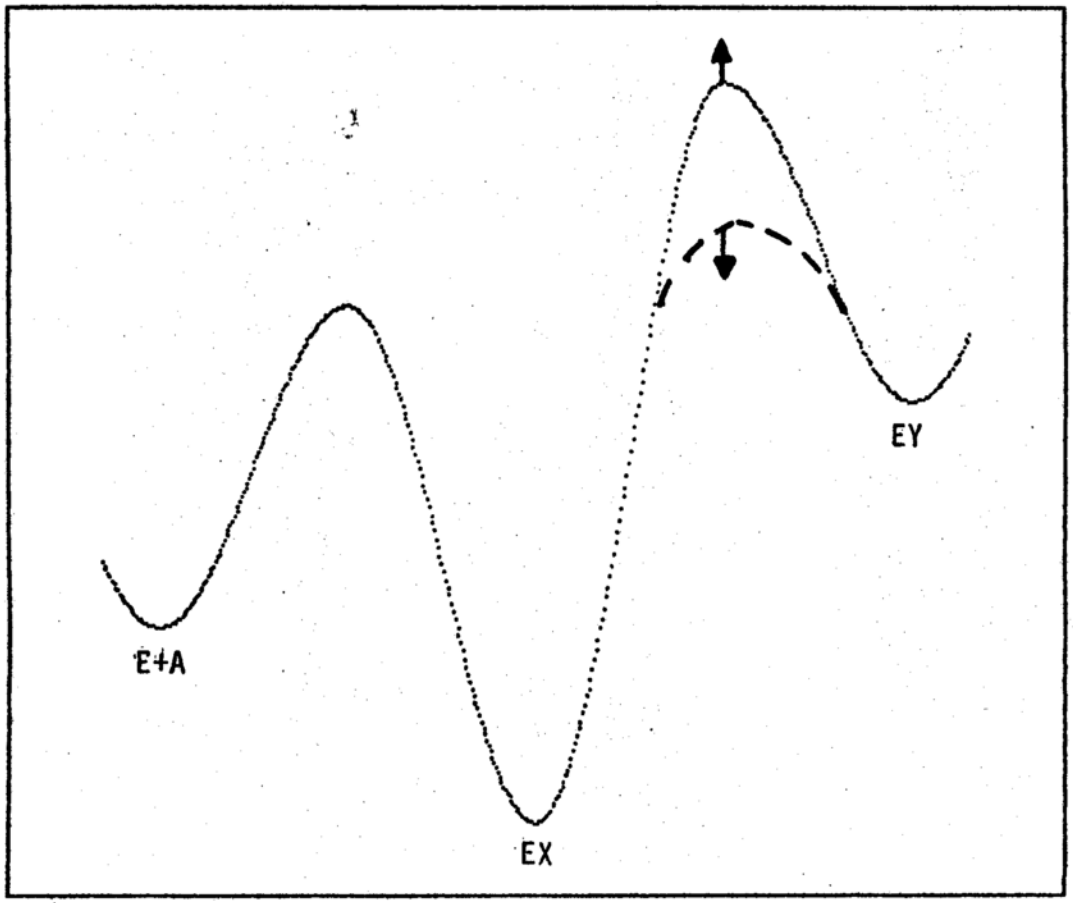
$$C_f = \frac{k_5}{k_4}\left(1 + \frac{k_3}{k_2}\right) \qquad (4.6)$$

Changing the level of this common ground state must necessarily change both of these k values. However, they in turn must change so that their ratio remains constant, because k_3 appears in the numerator and k_2 in the denominator of the equilibrium constant for the

overall reaction, and changing this intermediate ground state will not change the overall equilibrium constant. Hence, $^{D}V/K$ is independent of the levels of the ground states. On the other hand, changing the level of a common transition state will also change the values of two rate constants, e.g., k_3 and k_4 But because the commitments are dependent on more than direct ratios of these two rate constants (cf. Eqn. 4.6), alterations of a transition-state energy level will change the value of $^{D}V/K$.

The changes in $^{D}V/K$ resulting from methylation therefore indicate that alterations occured in the transition states for both ethanol and 2-propanol oxidation. However, had the transition states for both reaction mechanisms started out the same, methylation would have affected the $^{D}V/K$ values for each substrate in a similar manner, instead of one rising and one falling. Because the changes in $^{D}V/K$ for each substrate after methylation were in different directions, the transition states involved in the oxidation of primary and secondary alcohols by HLADH must have been different also. This is shown diagramatically in Figure 4.2, where methylation is depicted as raising the transition state for one

Figure 4.2. Partial reaction-coordinate diagram for the case where methylation of HLADH affects a single transition state. The broken line is for primary alcohols and the dotted line is for secondary alcohols. The arrows show the directions of change of the transition-state heights upon methylation.

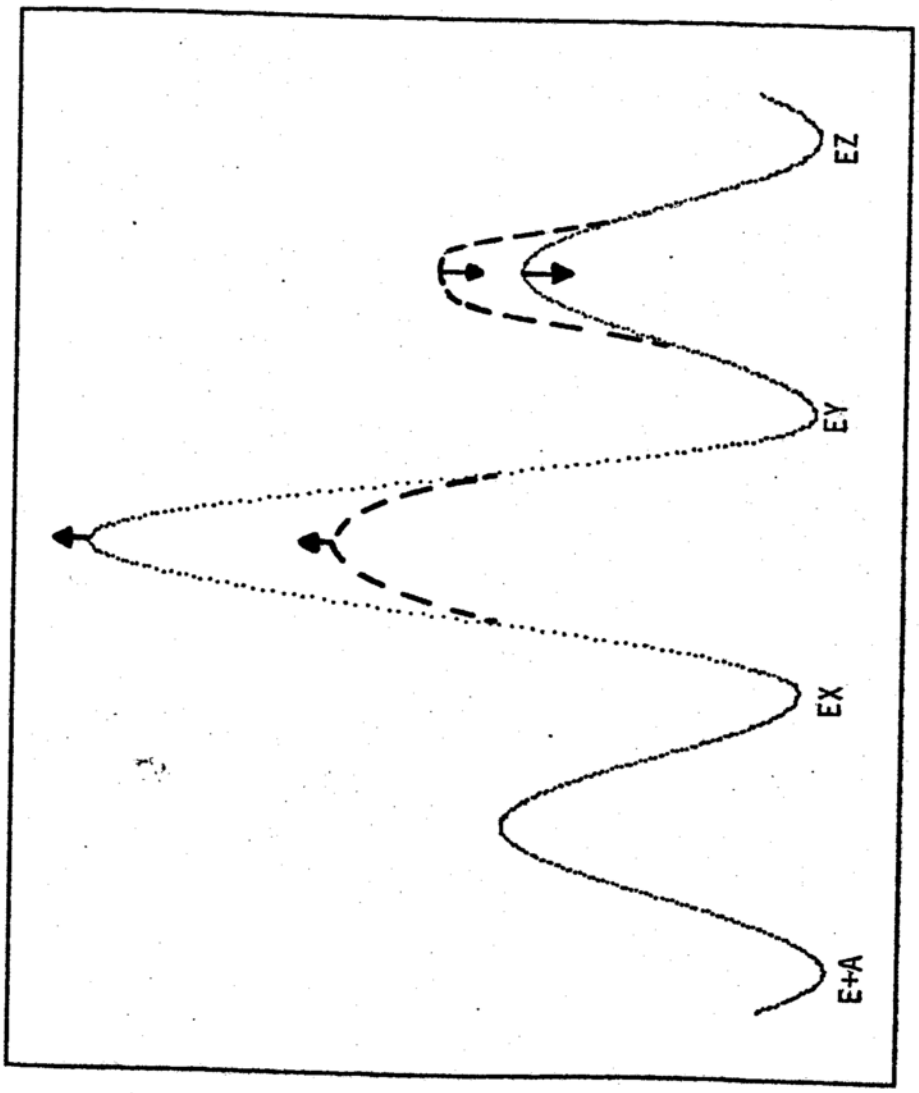

ENERGY
E+A
EX
EY
REACTION COORDINATE

substrate while lowering it for the other. This scheme is likely if the methylation affects the alcohol binding site directly. Methylation might in this case assist in oxidation of a primary alcohol by partially masking the polar amino nitrogen, making entrance of the substrate to the active site easier. But this extra methyl group might have the opposite effect if it overlaps with the C-2' methyl of 2-propanol.

An alternate explanation for the effects of methylation is given by Figure 4.3. Unlike the model above, which proposes opposite changes in the transition states for primary and secondary alcohol oxidation upon methylation, the changes in the transition states in this scheme are in the same direction. However, this restriction requires there to be two transition states for each reaction which are perturbed by methylation, one being raised and one being lowered. The contrasting effects on $^{D}V/K$ arise because each of these steps contributes in differing degrees to the functions defining V_{max} and $^{D}V/K$ for each substrate.

A possible mechanism consistent with this model would have methylation affecting the transition states of catalysis and release of the aldehyde product.

Figure 4.3. Partial reaction-coordinate diagram for the case where methylation of HLADH affects two transition states. The broken line is for primary alcohols and the dotted line is for secondary alcohols. The arrows show the directions of change of the transition-state heights upon methylation.


E+A
EX
EY
EZ
ENERGY
REACTION COORDINATE

Methylation might change the orientation of the reactants slightly, making catalysis more difficult. However, this same change might make the exit of the aldehyde easier.

Based on additional data for HLADH, it appears that the latter scheme is the more likely. Although Jornvall (1973) found that many of the thirty lysines per subunit (Jornvall, 1970) were modified by methylation, Dworschack et al. (1975) found that it was lysine-228 that was modified at the active site. X-ray data (Branden et al., 1975 and references therein) showed that this lysine is hydrogen-bonded to aspartate-223, which is in turn hydrogen-bonded to a hydroxyl of the adenosine ribose of NAD(H). Because the coenzyme was the same regardless of which alcohol was tested in the studies reported in this dissertation, it seems unlikely that chemical modification would cause opposite changes in a single transition state by acting at a common site distal to the alcohol. It would seem more feasible that methylation would cause changes in the two transition states and in the same direction, but expressed differently due to the differences in the energy levels

of these transition states during the two different oxidation reactions.

A recent analysis by Dutler and Branden (1981) of substrate orientation by alkyl-substituted cyclohexanones in the active site of HLADH provides a precedence for the existence of different transition states for the two oxidation reactions. Their analysis suggests that an unfavorable interaction of the C-2' carbon of 2-propanol with amino acid residues in the active site would change the orientation of the C-1 carbon of the alcohol to the nicotinamide ring and to the catalytic zinc atom. This spatial rearrangement could obviously lead to an alteration of transition-state structure.

The new information presented here helps to explain the results of previous studies on this enzyme. Dalziel and Dickinson (1966) observed different kinetic mechanisms for the oxidation of primary and secondary alcohols with HLADH, and Tsai (1978) observed the different effects of methylation on V_{max} as discussed above. That these differences are at least in part due to differences in transition states between primary and secondary alcohols is provided by the $^{D}V/K$ data. Furthermore, since V/K includes only rate constants up

to and including the first irreversible step of the mechanism, these transition states which are sensitive to methylation must be crossed before the release of the first product. Hence, an examination of the changes in the expression of the V/K isotope effects upon methylation enables the researcher to draw several conclusions about differences in the mechanisms of oxidation of primary and secondary alcohols that would not be possible in the absence of the isotope effect data.

This information about the V/K isotope effect can be applied retrospectively to provide some new conclusions about some old data. Plapp and his co-workers picolinimidylated (Plapp et al., 1973), pyridoxylated (Sogin & Plapp, 1975) and hydroxybutyrimidylated (Dworschack & Plapp, 1977) acetimidyl-HLADH and found that the activity increased 10-, 15- and 20-fold, respectively. In each case, the increase in velocity was attributed to an increased rate of nucleotide release that was considered rate-limiting for the unmodified enzyme. The V_{max} isotope effects for these same modified enzymes were 4.8, 2.8 and 3.1 versus control values of 1.2-1.35. Considering the multiplicity of effects caused by chemical modification

at the amino group of the active site (Fig. 4.3) and the complex expression for ^{D}V (Eqn. 4.3), the fact that ^{D}V values do not rise smoothly with the increase in V_{max} is not suprising. Although the V/K isotope effects were not calculated in these papers, a value of 2.28 with ethanol can be calculated from Figure 4 of Plapp et al. (1973). Unfortunately, no control value was given. However, the ^{D}V for their control experiment is 1.35 at pH 8 and 25°C. This value is identical to the control value of 1.34 at pH 8.4 and 25°C obtained for the experiments on HLADH described in this dissertation. Therefore, it is permissible to use the $^{D}V/K$ of 2.36 from Table III as a control to compare the $^{D}V/K$ results. The value of 2.28 for picolimidyl-HLADH is very close to the control value of 2.36, suggesting that picolinimidylation does not affect the transition states preceding the first irreversible step of the mechanism for the modified enzyme, but rather causes changes in ground states or changes in transition states after release of the first product.

The utility of $^{D}V/K$ for detecting changes in transition-state heights is not unexpected, based on recent theories of the kinetic meaning of V/K. Two

groups have been advocating the use of V/K as a fundamental parameter in enzyme kinetics: those recognizing its kinetic value in interpreting observed isotope effects (Northrop, 1975, and Cleland, 1975); and those recognizing its mechanistic value as a measure of enzyme-transition state complimentarity (Fersht, 1974 & 1977, and also Wolfenden, 1974, Paige, 1979 and Jencks, 1980). With regards to this second group, Jencks (1975 & 1980) has advanced the proposal that an enzyme converts binding energy into strain energy, allowing it to force the substrate into the transition state and through to products. Fersht (1974 & 1977, p. 246f) has demonstrated that V/K is at a maximum when the enzyme and transition state are complimentary to one another--in other words, when conversion of binding energy into strain energy is maximized. Northrop's (1981b) proposal that $^{D}V/K$ is sensitive to transition-state energies provides a further refinement by identifying a parameter in which ground-state effects have been eliminated.

THE CONCEPT OF THE RATE-LIMITING STEP. The results of the experiments with DOC and RLADH indicate that the simple mechanism of Equation 4.2 is not an adequate model for the interpretation of isotope effect data, and that recent discoveries regarding additional components of enzymatic mechanisms cannot be ignored in the context of isotope effect experiments. Equation 4.3 identifies certain cases for more complex mechanisms where the isotope effect will no longer be sensitive to changes in the rate-limiting step or where it will behave in an anomalous fashion.

The use of a simple chemical reaction illustrates that the mechanism does not have to be very complex for the isotope effect to fail to identify the rate-limiting step. Let k_2 be the reverse rate constant for the first step of Equation 4.7 and assume that k_3 is isotopically-sensitive.

$$A \underset{k(2)}{\overset{k(1)}{<==>}} I \xrightarrow{k(3)} P \qquad (4.7)$$

Application of the steady-state approximation gives an observed rate constant for the overall reaction of $k_1*k_3/(k_2+k_3)$. Designating the rate constant for the protiated reactant as k_{3H} and for the deuterated

reactant as k_{3D}, the isotope effect on the observed rate constant is

$$^{D}k_{obs} = \frac{k_{3H}}{k_{3D}}\left(\frac{k_2+k_{3D}}{k_2+k_{3H}}\right) = \frac{\left(\frac{k_{3H}}{k_{3D}}\right)+\left(\frac{k_3}{k_2}\right)}{1+\left(\frac{k_3}{k_2}\right)}. \quad (4.8)$$

The value for k_1 does not even appear in the expression, so the isotope effect would be insensitive to a slow k_1. (Note that $^{D}V/K$ is insensitive to k_1 for the same reason.) This result is not widely appreciated; March (1977, p. 455), for example, argued that isotope effects can be used to distinguish between (1) a slow attack of an electrophile on an aromatic hydrocarbon to form an arenium ion intermediate (k_1 slow), versus (2) slow decomposition of the intermediate to form the substituted product (k_3 slow) (Equation 4.9).

$$ArH + Y^{+} \underset{k(2)}{\overset{k(1)}{<==>}} AHY^{+} \overset{k(3)}{-->} AY + H^{+} \quad (4.9)$$

Equation 4.8 shows that isotope effects will not distinguish between these two cases. In this mechanism, however, the isotope effect is useful for determining what March calls the "partitioning factor," k_3/k_2 (cf. the commitment factor, Eqn. 4.6). When

more intermediate partitions backward to reactants than goes on to form products ($k_2 >> k_3$), Equation 4.9 simplifies to ${}^{D}k_{obs} = {}^{D}k_3$, and the intrinsic isotope effect is fully expressed.

Biochemists appear to have borrowed the term "rate-limiting step" from chemical kineticists without a clear understanding of its meaning and limitations. Under conditions of saturating substrate, only two candidates exist for the rate-limiting step in Equation 4.2: catalysis and product release. The rate-limitation see-saw can be either tipped left or right. However, when additional steps, such as conformational changes, or reversible steps are added, it is not clear that the concept of a single rate-limiting step can be applied to a more complicated mechanism.

One commonly-accepted definition of the rate-limiting step is the one "which has a much slower rate than the others." (Gardiner, 1969, p. 30). Consider the mechanism given in Equation 4.10.

$$E + S \underset{k(2)}{\overset{k(1)}{<==>}} ES \underset{k(4)}{\overset{k(3)}{<==>}} EP \overset{k(5)}{-->} E + P \qquad (4.10)$$

Application of the steady-state assumption to the intermediate EP yields the following expression for the change in EP concentration:

$$\frac{d[EP]}{dt} = \emptyset = k_3[ES]-k_4[EP]-k_5[EP]. \qquad (4.11)$$

Rearranging this equation demonstrates that the net flux through the second step of the mechanism is equal to the flux through the third.

$$k_3[ES]-k_4[EP] = k_5[EP] \qquad (4.12)$$

By extension, one can show that in a more complex mechanism, the net fluxes through all the step are equal. Clearly, Gardiner's definition of the rate-limiting step cannot apply to an enzymatic mechanism.

Moore (1972) offers an alternative definition of the rate-limiting step:

> If one of the stages has a specific rate much lower than any one of the others, the overall rate will be controlled by the specific rate (rate constant) of this particular reaction. [p. 346]

This criterion is quite adequate for a mechanism consisting of only unimolecular, irreversible steps, as shown in Equation 1.4. Of course, enzymatic mechanisms are not so simple. This criterion implies that k_{cat} ought to be equal to the smallest rate constant of the

mechanism, which is seldom true. V_{max} is a complex function of the rate constants which occur after substrate addition. "In more complicated schemes," writes Boyd (1978),

> ...involving for example reversible reactions or steps of different kinetic orders, no conveniently simple definition of a "rate-determining step" seems possible, and the analysis must revert to a detailed examination of the algebraic structure of the kinetic equations.

This line of reasoning suggests that the concept of a rate-limitation see-saw that can be tipped alternately between two steps is inadequate to describe a real enzymatic reaction mechanism. A more accurate representation might be a multi-armed see-saw, where the movement of one arm causes a response in all the others, but the degrees of coupling between the various arms are unequal and in different directions. Certainly some rate constants of the mechanism are greater than others, just as some arms are tipped higher than others. However, this is not necessarily the same as saying that a unique one is "rate-limiting." Because of this complicated state of affairs, any reseacher who uses the term "rate-limiting step" has the burden of supplying a definition for it which is consistent with the conditions and mechanistic

complexities of his experimental system (if one exists). When viewed from this more complicated perspective, it is not suprising that the observed isotope effect on V_{max} is not related in a simple fashion to the various factors which limit the rate of the reaction.

VI. BIBLIOGRAPHY

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VI. APPENDIX A
REFERENCES ON THE THEORY OF CHEMICAL ISOTOPE EFFECTS

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VII. APPENDIX B
PRELIMINARY EXPERIMENTS ON THE ISOTOPE EFFECTS OF LACTATE DEHYDROGENASE WITH ALTERNATIVE SUBSTRATES AND EXTREMES OF pH

INTRODUCTION

Calculating intrinsic isotope effects by Equation B.1 for enzyme-catalyzed reactions requires precise determinations of the observed V/K effects.

$$\frac{D_{V/K} - 1}{T_{V/K} - 1} = \frac{D_k - 1}{D_k^{1.442} - 1} \qquad (B.1)$$

Because it is easier to measure larger signals accurately and with more precision, and because many enzymes give small observed isotope effects, methods were sought which would enhance expression of the observed effects. The rationale for enhancement is illustrated for the simple mechanism shown in Equation B.2.

$$E + S \underset{k(2)}{\overset{k(1)}{<==>}} ES \underset{k(4)}{\overset{k(3)}{<==>}} EP \overset{k(5)}{\longrightarrow} E + P \qquad (B.2)$$

The V/K isotope effect (assuming $^{D}K_{eq}$ is equal to one) is described by the function

$$^{D}V/K = \frac{^{D}k + C_f + C_r}{1 + C_f + C_r} \qquad (B.3)$$

where the C-terms represent the commitments to catalysis, C_f being equal to k_3/k_2 and C_r equal to k_4/k_5 for this mechanism. When product-release is rate-determining ($k_3 >> k_5$), C_r is large and $^{D}V/K$

approaches unity. If, however, the experiment is done under conditions where the substrates and products are not very sticky ($k_5 > k_4$ and $k_2 > k_3$), the commitment factors will be very small and the observed isotope effect will approach the intrinsic, ^{D}k. Two such conditions investigated for lactate dehydrogenase (LDH) that ought to have resulted in less sticky subsrates and products are the use of poorer, alternative substrates and extremes of pH. The results presented below show that the use of poorer substrates did not always lead to enhancement. Reasons for this behavior are suggested in the main body of this dissertation. Isotope effect determinations at extreme pH values also failed to produce enhancement of the observed effects. However, a theoretical basis for such pH-behavior has recently been published by Cook and Cleland (1981), and the results are now interpretable in terms of new information about the kinetics of the LDH reaction.

MATERIALS AND METHODS

The following compounds were purchased from Sigma Chemical Co., St. Louis, Mo.: tris(hydroxymethyl)-aminomethane (Tris), dithiothreitol (DTT), activated charcoal (acid-washed with HCl), L(+)lactic acid, sodium pyruvate, yeast alcohol dehydrogenase (YADH), yeast aldehyde dehydrogenase (YAlDH), lactate dehydrogenase-5 from rabbit muscle (LDH-M_4) and lactate dehydrogenase (LDH, a mixture of isozymes) from rabbit muscle. NAD and NAD-analogs were from P-L Biochemical, Inc., Milwaukee, Wisc. Perdeuteroethanol (EtOH-d_6) was manufactured by Merck & Co., Inc., Rahway, N.J. The ion-exchange resin AG 1-X8 (100-200 mesh) was a product of Bio-Rad Laboratories, Richmond, Calif. Glycine was purchased from Aldrich Chemical Co., Milwaukee, and hydrazine from Eastman Kodak Co., Rochester, N.Y. The ether extraction apparatus was similar to the Kontes (Vineland, N.J.) model K-581500, 500 mL size. Other chemicals not listed were of ordinary reagent-grade quality.

[2-^{2}H]-Lactate (lactate-d) was synthesized in a system containing NAD, pyruvate, perdeuteroethanol and

the enzymes YADH, YAlDH and LDH, according to Equation B.1:

$$CD_3CD_2OD + NAD \xrightarrow{ADH} CD_3CDO + NADD + H^+$$

$$CD_3CDO + NAD \xrightarrow{AlDH} CD_3COOH + NADD + H^+$$

$$NADD + H^+ + \text{pyruvate} \xrightarrow{LDH} \text{lactate-d} + NAD \qquad \text{(B.1)}$$

The initial reaction mixture contained 50 mM Tris buffer, pH 7.4, 10 mM potassium chloride, 0.15 mM DTT, 13.6 mM sodium pyruvate, 0.10 g/L NAD, 1100 I.U./L LDH, 1100 I.U./L YADH, 6.25 I.U./L YAlDH and 1 mL/L ethanol-d_6. The mixture was stirred overnight at room temperature, after which the volume was decreased by rotary evaporation to about 500 mL. The denatured protein was removed by centrifugation, and the nucleotides were removed by stirring with activated charcoal. The charcoal was removed by vacuum filtration. The lactate was separated from other ions by acidifying the remaining aqueous solution and extracting with ether on a 500 mL liquid-liquid continuous extraction apparatus. After 36-48 hours of extraction, the ether layer was collected, water added to it and the ether removed by distillation. The resulting aqueous layer contained the product.

The sample was applied to an AG 1-X8 (100-200 mesh) anion-exchange column (Busch et al., 1952), which had previously been converted to the formate form and then washed with water until the conductivity had fallen to less than 25 meq/L. The lactic acid was eluted using a linear gradient of 0-1.5 N formic acid (250 mL water plus 250 mL 1.5 N formic acid). Fractions of 150 drops were collected. The presence of lactic acid in the fractions was detected by either thin-layer chromatography (Passera et al., 1964) or enzymatically (Gutmann & Wahlefeld, 1974, see below). The apex of the lactic acid peak appeared at Fraction 30 (with Fraction 1 being the first after the gradient was begun). The fractions containing lactic acid were combined and the formic acid removed by steam distillation. The product was assayed by titration against a standard sodium hydroxide solution and the yield was ca. 25 percent. A proton magnetic resonance spectrum at 90 MHz in deuterium oxide showed complete deuteration at C-2 with the methyl protons appearing as a singlet 1.42 ppm downfield from 3-(trimethylsilyl-1-propanesulfonate. No evidence of a proton signal at C-2 was observed.

Kinetic assays were performed on a Gilford Model 240 spectrophotometer equipped with a Leeds & Northrup Speedomax XL-610 recorder. Assays were done in semi-micro, self-masking quartz cuvettes with path lengths of 1 cm and volumes of 0.5 mL. All assays were performed at a temperature of 25°C which was maintained with a circulating waterbath with flow-through thermospacers surrounding the sample chamber of the spectrophotometer.

Isotope effects were determined on LDH-M_4 using an assay consisting of the following: 0.01 mL of a 15-25 mg/mL solution of pyridine nucleotide, 0.1 mL of lactate solution, 0.38 mL of 0.2 M buffer and 0.01 mL of an appropriate dilution of LDH-M_4 in 0.1 M phosphate buffer, pH 7.4. The first three solutions were added first, the contents mixed by inversion thrice, and the reaction started by the addition of enzyme with a plastic adder-mixer. The nucleotide was always present at saturating concentration (ca. 500 μM). Six lactate concentrations were chosen and assayed in duplicate, giving twelve points per determiniation. These procedures were repeated using deuterated lactate to determine the isotope effects. The volume of the

lactate-d solution was increased to Ø.15 mL and the buffer volume decreased to Ø.33 mL in these assays.

Experiments with alternative pyridine nucleotides were performed in glycine-NaOH buffer at pH 9.5. Changes in absorbance over time were recorded on the chart recorder. The reduced forms of the alternative pyridine nucleotides absorbed light at different wavelengths and their respective absorption maxima and extinction coefficients can be found in the publication by P-L Biochemicals (1961). Experiments at various pH values were determined with nicotinamide hypoxanthine dinucleotide (NHD) as coenzyme. The absorbance at 338 nm was recorded over time for these experiments. Buffers used for the various pH values were: pH 6.5 and 7.5, phosphate; pH 8.5, Tris; pH 9.5 and 1Ø.5, glycine-NaOH.

Slopes were calculated manually from the chart recordings using ships curves. The isotope effects were estimated by using a computer program supplied by Dr. W.W. Cleland of the Department of Biochemistry (see p. 4Ø).

The lactate concentrations of the stock solutions were determined enzymatically using the procedure of Gutmann and Wahlenfeld (1974). Into a cuvette were

added: Ø.Ø1 mL of a fifty-fold dilution of the sample, Ø.Ø2 mL of 3Ø mg/mL NAD, Ø.45 mL of Ø.5 M glycine-Ø.4 M hydrazine buffer, pH 9.Ø and Ø.Ø2 mL of undiluted LDH suspension in 2.1 M ammonium sulfate. The absorbance was monitored at 34Ø nm until equilibrium was reached and the total absorbance change calculated by subtracting the absorbance from a blank assay in which 2.1 M ammonium sulfate was substituted for the LDH. The lactate concentration was then calculated by assuming that each lactate molecule produces one NADH, and that the extinction coefficient for NADH is 6.22 $(mM\text{-}cm)^{-1}$.

RESULTS AND DISCUSSION

The results of the isotope effect determinations with alternative pyridine nucleotides are shown in Table B.I. Substituting NHD for NAD decreased both the ^{D}V and $^{D}V/K$ values, as would be expected from the decreases in V and V/K with NHD. However, substituting thionicotinamide adenine dinucleotide (ThNAD) resulted in still slower steady-state parameters, yet both the ^{D}V and $^{D}V/K$ increased from values close to two with NAD to values approaching three with ThNAD. On the other hand, 3-pyridine aldehyde adenine dinucleotide gave the lowest V/K value, and also gave the lowest V/K isotope effect, Ø.93.

These results indicated that V and V/K values could not be used to predict the direction of change in the observed isotope effects. Clearly, the functions governing the expression of the isotope effects are dependent on parameters not fully expressed on V and V/K alone. One of the additional parameters could be the intrinsic isotope effect itself. Damgaard (1981) has presented evidence that ThNAD and 3-acetylpyridine adenine dinucleotide give different intrinsic effects than NAD for ADH. In any case, the functional

TABLE B.I

Isotope Effects on Lactate Dehydrogenase with Alternative Nucleotide Substrates				
Dinucleotide	V_{rel}	V/K_{lac}	D_V	$D_{V/K}$
Nicotinamide Adenine	171.	8.61	1.93±.14	1.87±.34
Nicotinamide Hypoxanthine	150.	4.92	1.52±.13	1.71±.15
Thionicotinamide Adenine	11.0	1.15	2.81±.72	2.98±.31
3-Pyridine Aldehyde Adenine	53.2	0.0305	1.90±.16	0.93±.02
3-Acetylpyridine Hypoxanthine	17.0	6.23	1.38±.04	1.09±.08
3-Acetylpyridine Adenine	19.3	25.7	0.96±.04	1.83±.14

combination of the various parameters precludes a simple prediction of the behavior of the observed isotope effects resulting from nucleotide substitution, and this route as a method of investigating enhancement was abandoned.

Figures B.1 and B.2 give the isotope effects when the pH of the reaction is varied. The results show no noticeable enhancement, with $^{D}V/K_b$ remaining at approxomately 1.65 and ^{D}V at approximately 1.6. (The ^{D}V value at pH 10.5 was not considered due to is poor precision. The enzyme is fairly unstable at this point and it is difficult to obtain precise assays.) This result was not expected originally, because extreme pH condidtions should have made an ionic substrate less sticky, thereby decreasing the commitment to catalysis and increasing the isotope effect. However, Cook and Cleland (1981) have recently shown that no variation of ^{D}V and $^{D}V/K_b$ with pH would be expected if the pH-sensitive step of the mechanism serves only to divert the E*NAD binary complex away from the catalytic pathway but does not affect the catalytic step itself.

This finding is consistent with the mechanism proposed for LDH by Holbrook and Gutfrenund (1973) from transient-state kinetics. In this case, both enzyme

Figure B.1. Variation of the observed V_{max} isotope effect vs. pH for LDH. Nicotinamide hypoxanthine dinucleotide was used as coenzyme and was present at saturating concentration. Error bars denote $\pm$ 1 S.D.

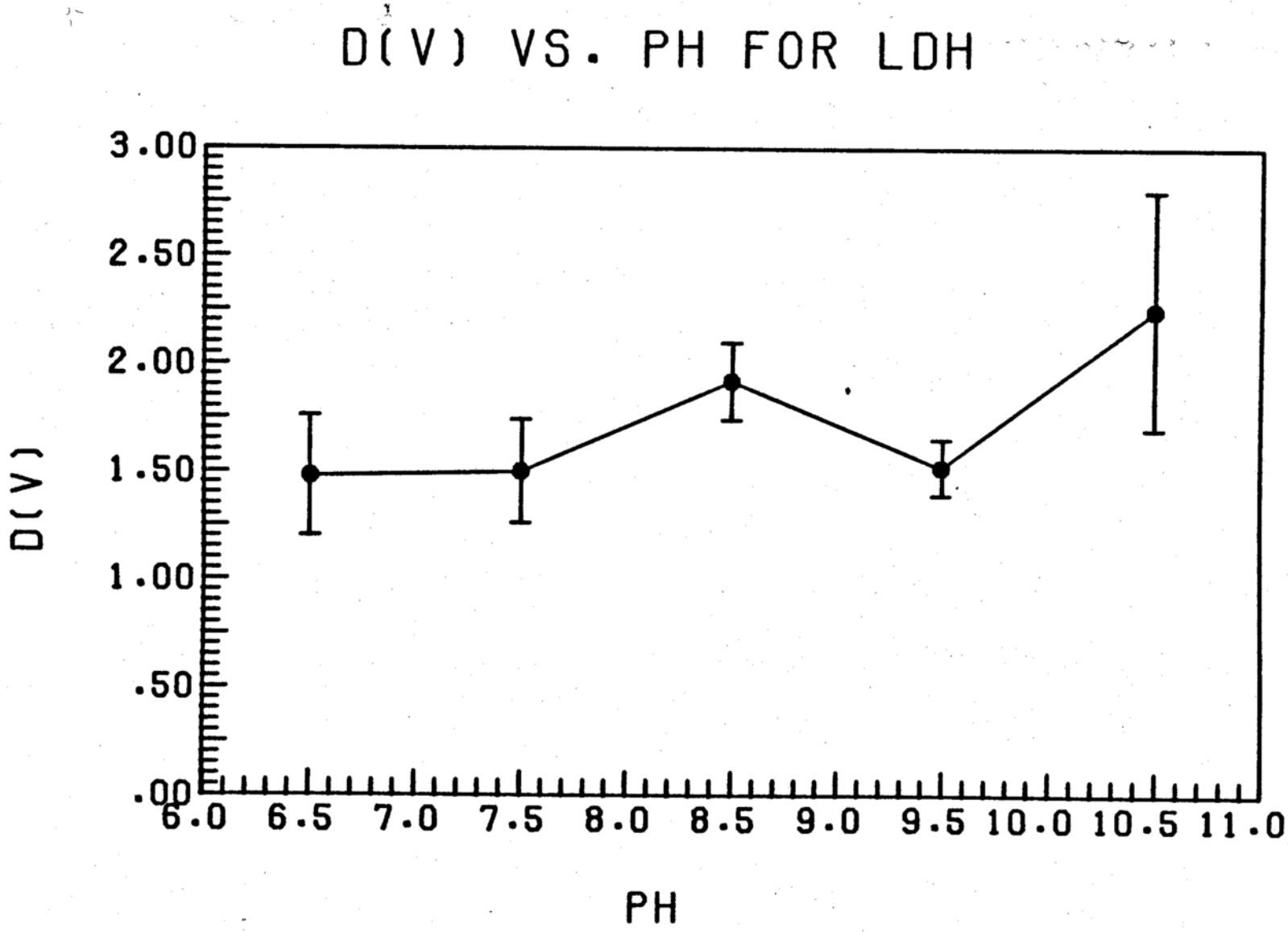
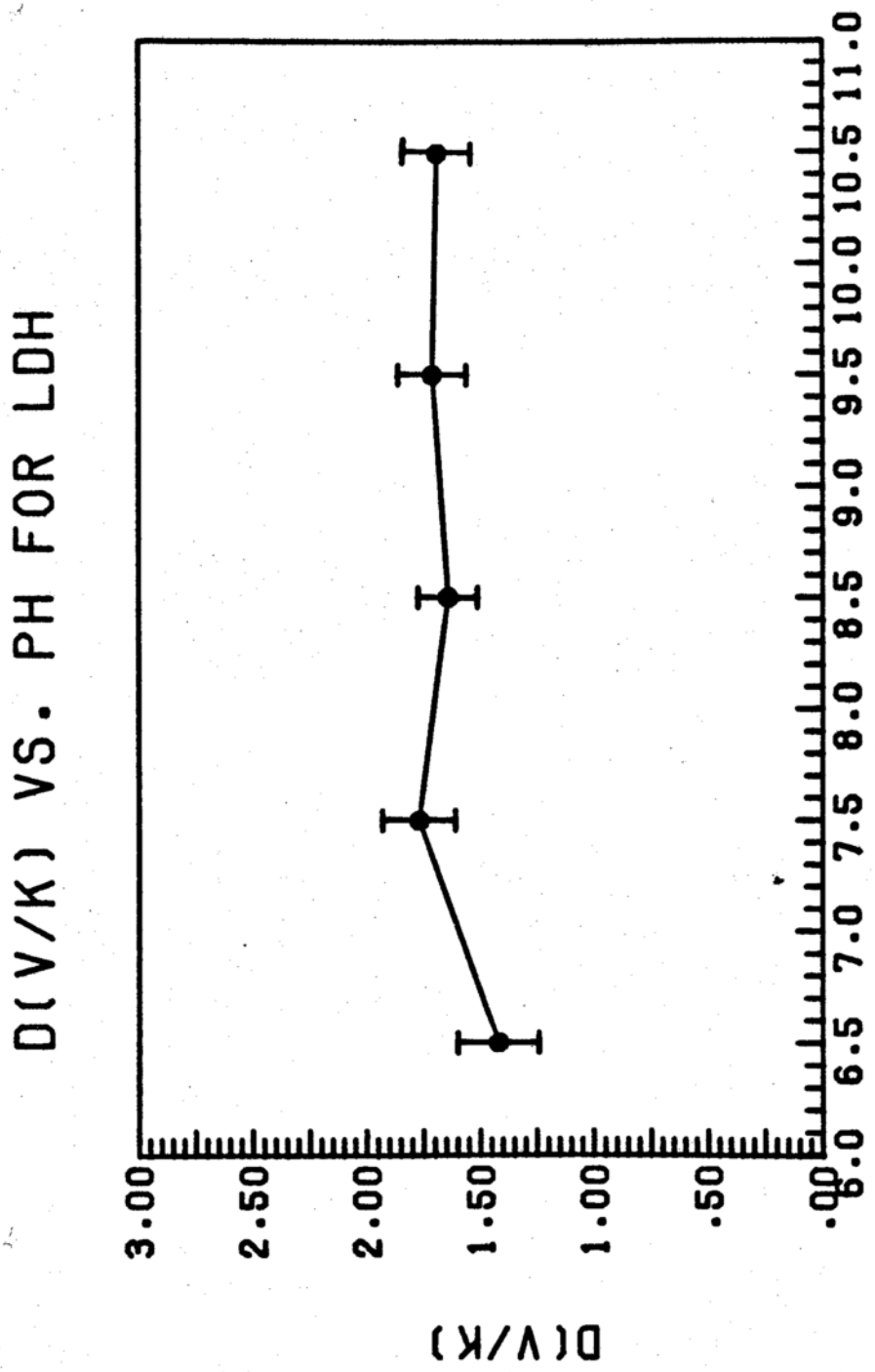
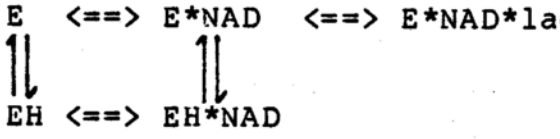

D(V) VS. PH FOR LDH
3.00
2.50
2.00
1.50
1.00
.50
.00
D(V)
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5
11.0
PH

Figure B.2. Variation of the observed V/K isotope effect vs. pH for LDH. Nicotinamide hypoxanthine dinucleotide was used as coenzyme and was present at saturating concentration. Error bars denote $\pm$ 1 S.D.


D(V/K) VS. PH FOR LDH
D(V/K)
3.00
2.50
2.00
1.50
1.00
.50
.00
6.0
6.5
7.0
7.5
8.0
8.5
9.0
9.5
10.0
10.5
11.0
PH

and protonated enzyme are capable of forming a binary complex with NAD or NADH. In addition, the protonated and unprotonated binary complexes are capable of interconverting. However, only the unprotonated form in the case of E*NAD, or the protonated form in the case of E*NADH, is capable of binding the second substrate and continuing forward through catalysis.



(B.5)

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Holbrook, J.J. & Gutfreund, H. (1973) FEBS Lett. 31, 157-169.

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VIII. APPENDIX C
PURIFICATION OF NADH

INTRODUCTION

The original plan for this dissertation involved the determination of isotope effects on dehydrogenases in the direction of reduction of substrates using NADH as coenzyme. The presence of dehydrogenase inhibitors in NADH preparations was identified quite some time ago by Dalziel (1961) and Fawcett et al. (1961). These inhibitors are known to affect the results of enzymatic kinetic experiments (Dalziel, 1962, Strandjord & Clayson, 1966, Howell et al., 1976, and Howell, 198Ø). Additionally, Grimshaw (1979) showed that the presence of an inhibitor in substrate preparations would give erroneous isotope effect results, even if the inhibitor was present in equal amounts in labeled and unlabeled preparations. For this reason, it was necessary to develop methods to synthesize and purify NADH with deuterium incorporated into the transferable position. Although isotope effects were never determined using NADH as coenzyme, the methodology is still valuable and is described here.

Chromatography on DEAE-cellulose (Pastore & Friedkin, 1961) was originally employed for purification. Other anion-exchange resins have also

been used, including DEAE-Sephadex (Wenz et al., 1976, and Loesche et al., 1980), QAE-Sephadex (Haid et al., 1975) and benzyolyated DEAE-cellulose (Kurz & Frieden, 1977). However, Loshon et al. (1977) have shown that one of these dehydrogenase inhibitors cannot be separated from NADH on DEAE-cellulose or DEAE-Sephadex. This was demonstrated using the reversed-phase high-performance liquid chromatographic (HPLC) procedure of Margolis et al. (1976)*. Hence, HPLC is now the method of choice for analyzing samples of NADH.

Because the reversed-phase partition chromatographic system provided the only mean of detecting all of the impurities, it followed that it might also be useful in their removal on a preparative scale. This method did separate mononucleotides very effectively, but was not capable of resolving NADH from large amounts of NAD, as might be present in an enzymic reaction mixture. Anion-exchange chromatography separated NAD and NADH well, but does not resolve all

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* Alternatively, the purity of NADH might have been assessed by measuring its absorbance in solution at 260 and 340 nm and calculating the ratio of the absorbances (Lehninger, 1952), with a value of less than 2.3 being accepted as evidence of reasonable purity. However, impuritites have been isolated with spectral properties similar to NADH (Margolis et al., 1977, and Bielmann et al., 1979).

the inhibiting impurities. Hence, both methods are required in combination. But rather than using a cellulose or Sephadex ion-exchange resin, a new, macroporous, strongly basic anion-exchanger, AG MP-1, was investigated after it had been reported by Hsu and Chen (1980) that adenine nucleotides could be separated on this resin. The macroporous styrene-divinylbenzene copolymer matrix offers higher flow rates and a higher surface area than cellulose- and Sephadex-based resins. In addition, the polystyrene matrix imparts a hydrophobic quality which appears to assist separation. Dr. Colin Newton of this laboratory found that this resin was capable of separating pyridine nucleotides in addition to adenine nucleotides.

MATERIALS AND METHODS

NAD was purchased from P-L Biochemicals, Milwaukee, Wisc. The enzymes yeast alcohol dehydrogenase (YADH) and aldehyde dehydrogenase (YAlDH) were from Sigma Chemical Co., St. Louis, Mo. A microburet was purchased from Gilmont Instruments, Inc., Great Neck, N.Y. Ultrogel AcA 202 cross-linked agarose gel filtration medium was a product of LKB Instruments, Inc., Rockville, Md. AG MP-1 macropourous anion-exchange resin (100-200 mesh, chloride-form) was acquired from Bio-Rad Laboratories, Richmond, Calif. Ace Glass, Inc. (Louisville, Ky.), chromatographic columns were used for the conventional chromatography.

For HPLC, analytical μBondapak C_{18} (3.9 x 300 mm, 10 μm particle diameter) and preparative Bondapak C_{18}/Porasil B (0.78 x 61 cm x 2 columns in tandem, 37-50 μm particle diameter) reveresed-phase columns were purchased from Waters Associates, Inc., Milford, Mass. An Altex Model 110A HPLC pump and a Rheodyne Model 7010 sample injection valve were acquired from Altex Scientific, Inc., Berkeley, Calif. LKB Instruments manufactured the Model 2138 Uvicord S absorbance monitor.

1,2-Propanediol was dried over sodium sulfate and distilled at reduced pressure. Davison molecular sieves, Type 4A (Grade 514, 8-12 mesh beads) were packaged by Fisher Scientific Co., Fair Lawn, N.J. These were activated by heating in a vacuum oven. Other chemicals used were of ordinary reagent-grade.

The NADH was synthesiszed by the method of Viola et al. (1979), which uses ADH and AlDH to catalyze the transfer of two hydrogen atoms from C-1 of ethanol to each of two NAD molecules. To 10 mL of 10 mM ammonium bicarbonate and 1 mM potassium chloride, pH 9.0, were added 40 mg NAD, 50 I.U. YADH (0.16 mg), 100 I.U. YAlDH (22.2 mg) and 2 μL of ethanol. The mixture was stirred at room temperature and the pH held constant by addition of small amounts of 1 N sodium hydroxide using the microburet. The reaction was virtually complete after one hour. The enzymes were separated from the nucleotides on a 1.5 x 30 cm Ultrogel AcA 202 gel filtration column using 10 mM ammonium bicarbonate, pH 9, as the eluent and collecting 5 mL fractions. Those fractions containing protein and those containing the nucleotides were combined and lyophilized separately.

The purified NADH was analyzed by reversed-phase HPLC according to the method of Margolis et al.

(1976). The separation was run on the μBondapak C_{18} column with 20 mM sodium potassium phosphate, pH 7.05, as the mobile phase. The flow rate was 2.0 mL/min and the effluent was monitored at 254 nm with the absorbance monitor.

RESULTS

The lyophilized nucleotide fraction was redissolved in 0.5 M ammonium bicarbonate, pH 9.0, and applied to a 1.1 x 30 cm AG MP-1 anion-exchange column which had previously been equilibrated with this same buffer at 4°C in the dark. The column was eluted with starting buffer until the absorbance at 254 nm returned to baseline levels. At this point, the eluent was changed to 1 M ammonium bicarbonate, pH 9.0. Fractions of effluent were analyzed for absorbance at 260 and 340 nm and results are given in Figure C.1.

The profile shows that a small peak was eluted at Fraction 6 which was probably unreacted NAD. After the ammonium bicarbonate concentration had been increased to 1 molar, a large peak with 340 nm-absorbing components was eluted. This peak contained the desired NADH, but the A_{260}/A_{340} ratio showed that an impurity also eluted on the leading edge of this peak. This behavior is similar to that exhibited on the DEAE resins, although on the AG MP-1 resin, fewer NADH fractions seemed to be contaminated, permitting higher yields. Fractions 50-100, which had ratios of

Figure C.1. Anion-exchange chromatography on AG MP-1 of the synthesized NADH. Column dimensions were 1.1 x 30 cm. The column was run at 4°C in the dark. The starting buffer was 0.5 M ammonium bicarbonate, pH 9.0, and the flow rate was 3.0 mL/min. The eluent was changed to 1 M ammonium bicarbonate at Fraction 24. Fractions of 5 mL were collected and analyzed for absorbance at 260 and 340 nm. In addition, the ratios of A_{260}/A_{340} are indicated on the figure.

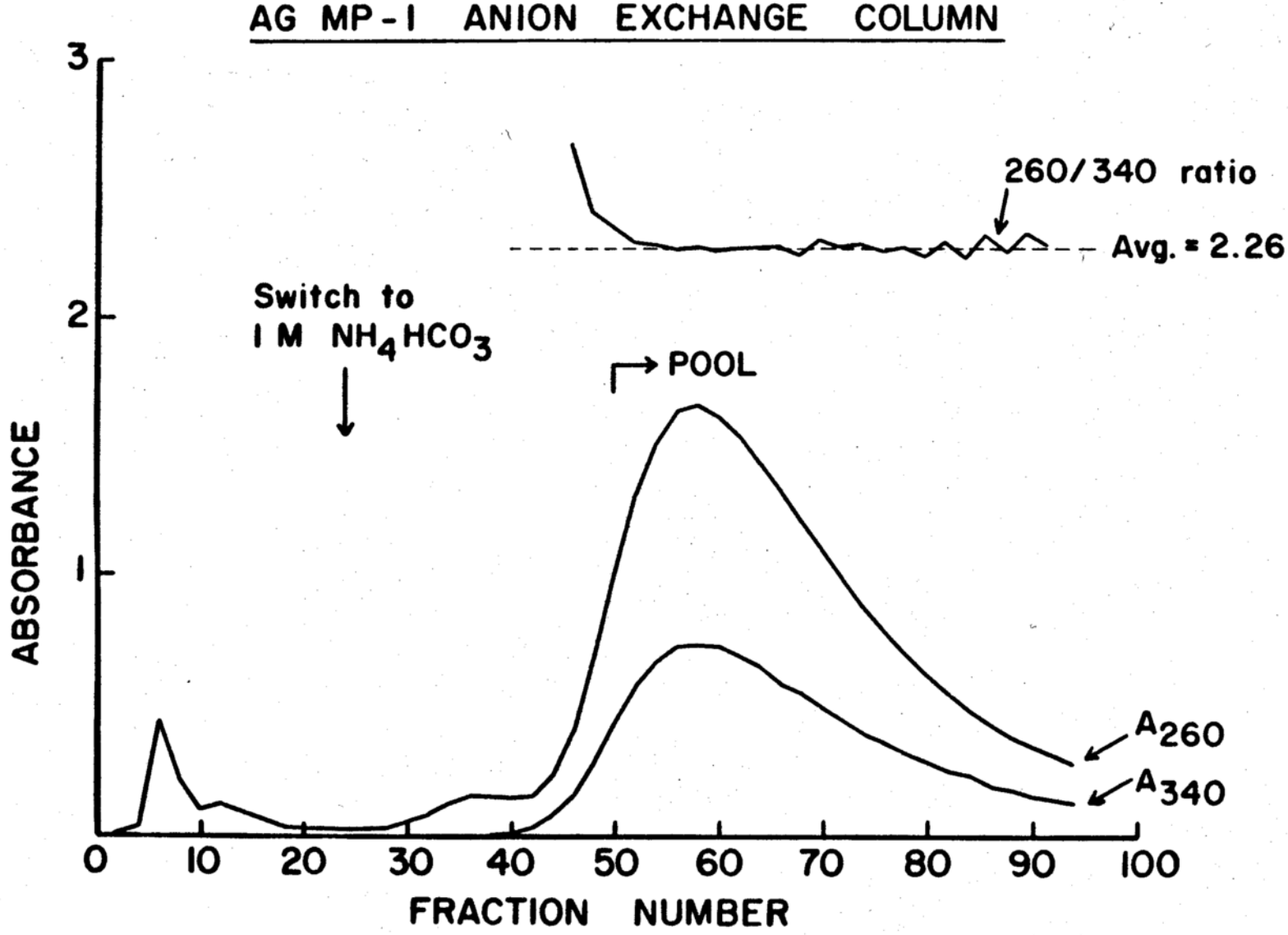
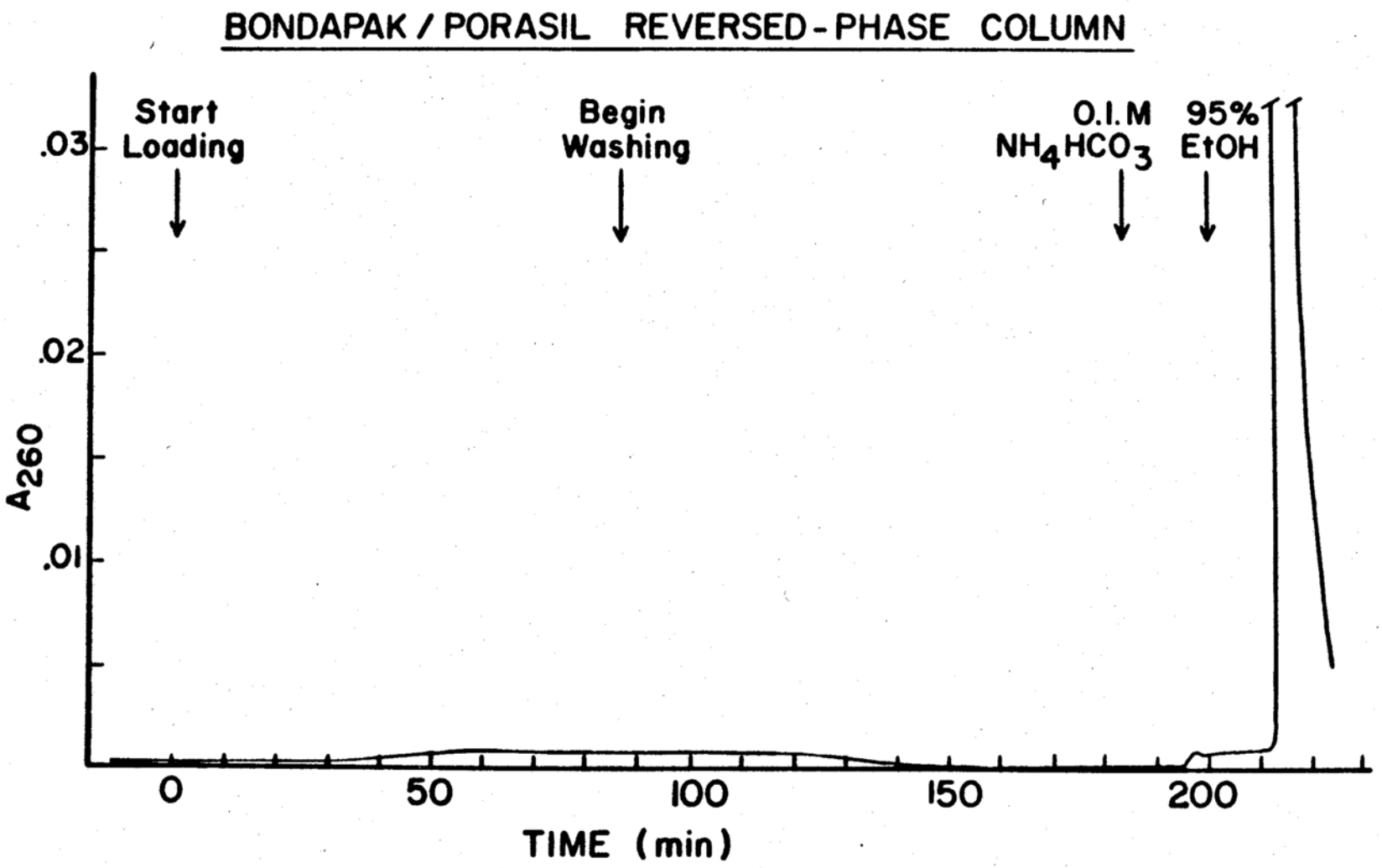

AG MP-1 ANION EXCHANGE COLUMN
260/340 ratio
Avg. = 2.26
Switch to
1 M NH_4HCO_3
POOL
A_{260}
A_{340}
ABSORBANCE
FRACTION NUMBER
0
10
20
30
40
50
60
70
80
90
100
1
2
3

Figure C.2. Preparative reversed-phase high-performance liquid chromatography on Bondapak C_{18}/Porasil B. Two Ø.78 x 61 cm columns were used in tandem. The columns were equilibrated with 1 M ammonium bicarbonate, pH 9.Ø, at 25°C. The flow rate was 3.Ø mL/min and was controlled with an Altex Model 11ØA HPLC pump. After the 268 mL-sample from the AG MP-1 column had been applied, elution was continued with 1 M ammonium bicarbonate. The eluent was changed again at the times indicated on the profile. The absorbance of the effluent was monitored at 254 nm.


BONDAPAK / PORASIL REVERSED-PHASE COLUMN
Start Loading
Begin Washing
0.1 M NH_4HCO_3
95% EtOH
.03
.02
.01
A_{260}
0
50
100
150
200
TIME (min)

2.26±.Ø5, were combined. The total volume of this product was 268 mL.

The product from the AG MP-1 column was pumped onto the preparative reversed-phase Bondapak C_{18}/Porasil B HPLC columns, which had previously been equilibrated with 1 M ammonium bicarbonate at pH 9.Ø and 25°C. The NADH adsorbed to the columns under these conditions. After sample loading was completed, the columns were washed with 1 M ammonium bicarbonate and a broad, low peak was eluted. This is shown in Figure C.2. When the elution of this impurity was complete, the eluent was changed to Ø.1 M ammonium bicarbonate, pH 9.Ø, to decrease the ionic strength. Fifty milliliters of this eluent were used. The NADH was eluted with 95 percent ethanol which had been made basic with a small amount of concentrated ammonium hydroxide. The NADH appeared as a sharp peak.

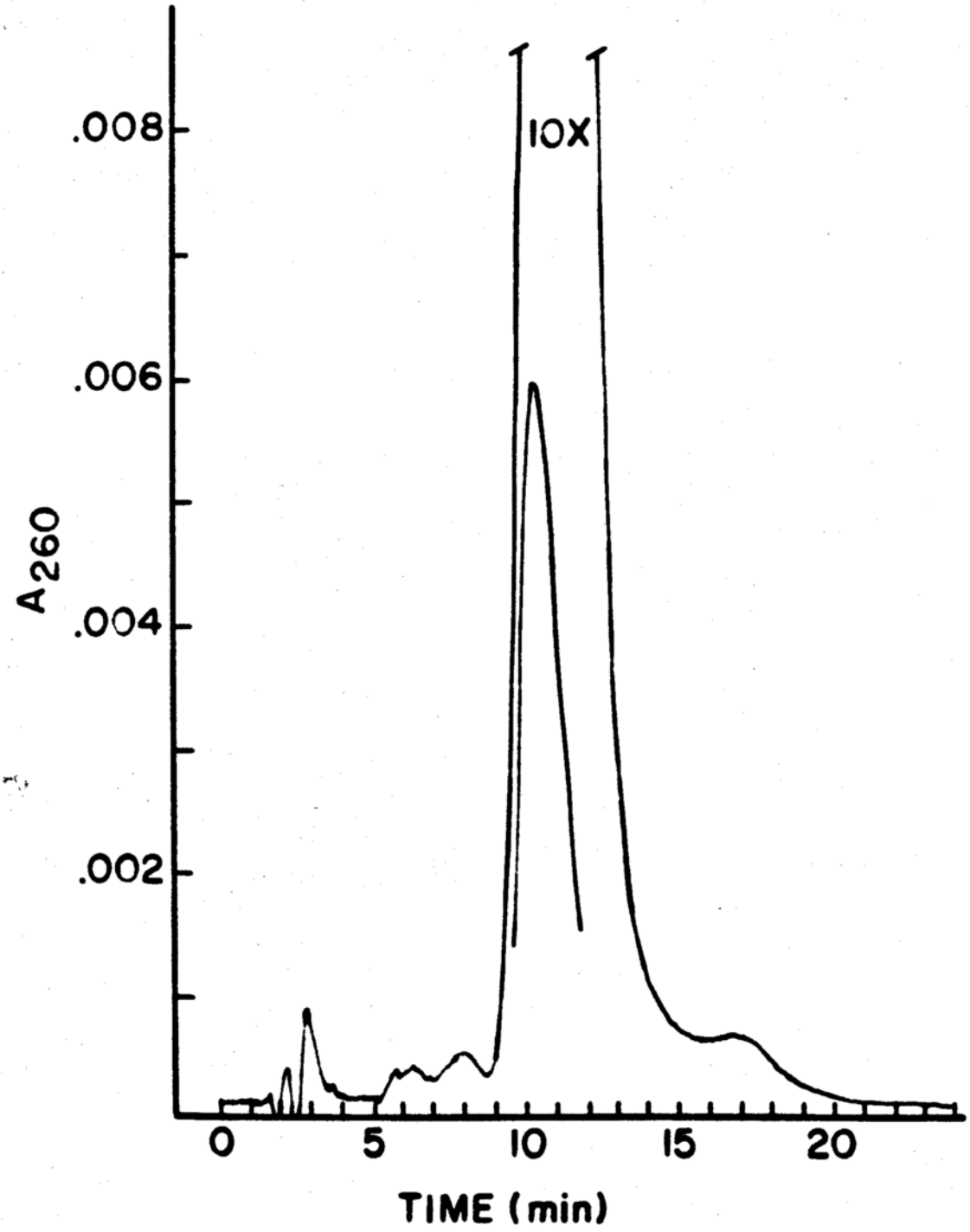
Because moisture accelerates the decomposition of NADH (Strandjord & Clayson, 1966), Gallati (1976) investigated storage of NADH in organic solvents and found that this method enhanced stability. Modrovich (1979) patented a process of storing NADH in 1,2-propanediol over molecular sieves to maintain dryness. Based on this evidence, the product from the

preparative HPLC column was stored in this vehicle. The pale yellow peak from the preparative HPLC columns was dried over anhydrous sodium sulfate and the volume reduced by rotary evaporation. The resultant slurry was suspended in dry 1,2-propanediol. Sonication assisted dissolution. A small amount of material failed to dissolve in the propanediol and was removed by decantation. Several activated molecular sieves were added to each milliliter of NADH solution and it was stored at $-20^{\circ}C$.

The results of the analytical reversed-phase HPLC analysis of the final, soluble product on μBondapak C_{18} using the method of Margolis et al. (1976) are shown in Figure C.3. The NADH appeared as the only major peak on the chromatogram with a retention time of 10.4 min. Several small peaks which eluted between 2 and 4 min also appeared when the propanediol vehicle was analyzed alone.

Figure C.3. Analytical reversed-phase high-performance liquid chromatography on μBondapak C_{18} of the purified NADH in 1,2-propanediol. Column dimensions were 3.9 x 300 mm. The mobile phase was 20 mM sodium potassium phosphate buffer, pH 7.05. Flow rate was 2.0 mL/min and was controlled with an Altex Model 110A HPLC pump. Effluent was monitored at 254 nm. The sample size was 25 μL and the temperature was 25°C.

μBONDAPAK C_{18} ANALYTICAL
REVERSED-PHASE HPLC COLUMN



CONCLUSIONS

These results show that combining ion-exchange with reversed-phase partition chromatography accomplished purification of NADH that neither technique alone could provide. When the product NADH was stored in a dry vehicle, it was found to give a single, major peak when analyzed by reversed-phase analytical HPLC.

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