ANALOGS OF NICOTINAMIDE ADENINE DINUCLEOTIDE AS PROBES OF THE STEREOCHEMICAL RELATIONSHIPS IN THE TERNARY COMPLEX OF ALCOHOL DEHYDROGENASES

Dissertation for the Degree of Ph. D.

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Analogs of Nicotinamide Adenine Dinucleotide As Probes of the Stereochemical Relationships in the Ternary Complex of Alcohol Dehydrogenases

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Daniel John Kroon

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ABSTRACT

ANALOGS OF NICOTINAMIDE ADENINE DINUCLEOTIDE AS PROBES OF THE STEREOCHEMICAL RELATIONSHIPS IN THE TERNARY COMPLEX OF ALCOHOL DEHYDROGENASES

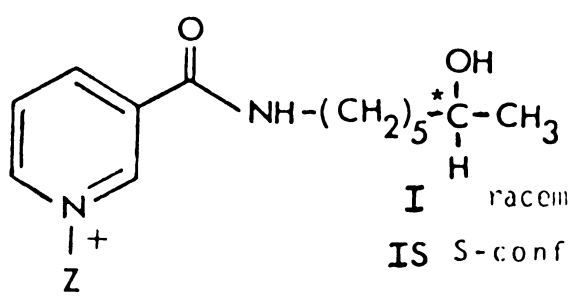
By

Daniel John Kroon

Nicotinamide adenine dinucleotide is the coenzyme for a large number of enzyme catalyzed oxidation-reduction reactions. Many of these reactions are stereospecific and much work has gone into the elucidation of the factors controlling this stereospecificity. One such reaction is the interconversion of alcohols and carbonyl compounds catalyzed by the alcohol dehydrogenases (E.C.1.1.1.1.). Prelog, Karabatsos, and others have proposed various models for the spatial arrangements of substrate and coenzyme in the enzyme active site to account for the observed stereochemistry of the reaction products. In recent studies using purified enzymes and coenzymes, Karabatsos, Nunez, and Stamoudis established that hydrophobic-hydrophilic interactions are important in determining the stereochemical outcome of the reactions catalyzed by alcohol dehydrogenases. To design a general predictive model, however, more information is required as

to the location of these points of interaction in the enzyme active site and their stereochemical relationships to the coenzyme and substrate. The available evidence for a particular orientation of the substrate with respect to the coenzyme in the enzyme ternary complex, e.g. the spectral studies of Kosower, is rather meager.

With the aim of furnishing more data relevant to this issue, the analogs of nicotinamide adenine dinucleotide (I, IS and IR) were synthesized and their activity with yeast and horse liver alcohol dehydrogenase was investigated. This analog study is a step toward eliminating the problem from which all of the previous product studies have suffered, namely, the inability to describe accurately the overall stereochemistry because of the presence of too many variables in the arrangement of enzyme, coenzyme, and substrate. The analogs of this study have the substrate covalently attached to the coenzyme, thus restricting the number of ways that the substrate can align itself with the coenzyme.



I racemic

IS S-configuration

IR R-configuration

Z = ribose-diphosphate-ribose-adenine

The investigation of the stereochemistry of the reaction of the analog with the enzyme required the synthesis and resolution of 7-amino-2-heptanol. The amino-alcohol was resolved by crystallization of its tartrate salt. Deamination to 2-heptanol and straight-forward correlations allowed the conclusion that (-)-7-amino-2-heptanol has the R configuration.

The nicotinamide adenine dinucleotide analogs functioned as coenzymes in the oxidation of ethanol with liver alcohol dehydrogenase, but the oxidation proceeded at a much slower rate than with the natural coenzyme. The analogs showed no activity with ethanol and yeast alcohol dehydrogenase.

The coenzyme-substrate analogs underwent oxidation-reduction in the presence of liver alcohol dehydrogenase as determined by UV spectroscopy. The analog prepared from the 7-amino-2-heptanol with the S configuration reacted more than the one prepared from (R)-7-amino-2-heptanol. It was determined that this reaction could be intermolecular, leaving the question of the spatial orientation of the substrate and coenzyme in the alcohol dehydrogenase ternary complex unanswered.

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Daniel John Kroon

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INTRODUCTION

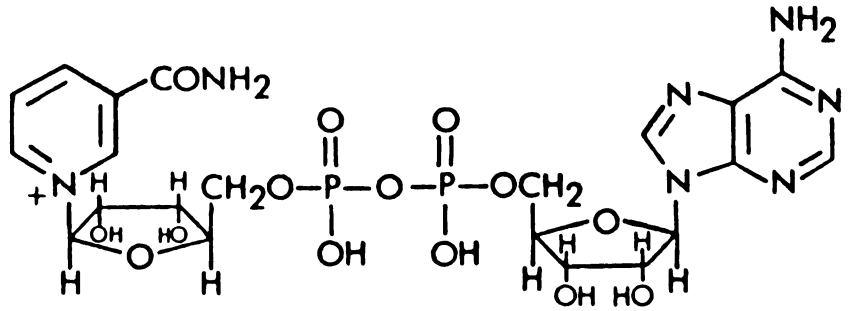
One truly amazing property of enzymes as chemical reactants is their extreme selectivity in reaction partners. Living matter is very dependent on this specificity, there being many enzymes that have been developed to sustain particular reaction sequences that are vital to the life process. Some of these enzymes operate on a single substrate and many are known that react with only one stereoisomer of a substrate. For example, in the biosynthesis of squalene from six molecules of mevalonate, Popjak and Cornforth (1) determined that there were 16,384 (2^{14}) possible stereochemical paths that the sequence of reactions could follow, yet the enzyme-catalyzed process follows one of these paths exclusively! Indeed, in living systems stereospecificity appears to be the rule rather than the exception.

During his years of work on sugar stereochemistry and fermentation reactions, Emil Fischer recognized the importance of enzyme-substrate interactions in the regulation of the specificity of enzymes. Ogston (2) extended this concept to the ability of enzymes to distinguish between two chemically identical groups bonded to the same

carbon atom, at centers now termed "prochiral." The prevalence of stereospecificity in enzyme-catalyzed reactions suggests that precise spatial orientations of substrates to the enzyme and coenzyme are an integral part of the catalysis. It is with the hope of obtaining information about these stereochemical relationships, and therefore about the catalytic mechanism, that many studies of enzyme stereospecificity are conducted.

Stereospecificity is commonly observed in the biological interconversion of alcohols and carbonyl compounds catalyzed by alcohol dehydrogenases (E.C.1.1.1.1.). The alcohol dehydrogenases isolated from yeast and horse liver (YADH and LADH) have been fully characterized and their reactions with a large number of compounds have been studied (3). This class of enzymes exhibits stereospecificity towards both substrate and the coenzyme necessary for the reaction, nicotinamide adenine dinucleotide (NAD). The structure of the oxidized form of nicotinamide adenine dinucleotide (NAD^+), confirmed by synthesis (4), is shown in Figure 1.

In the oxidation of alcohols catalyzed by YADH and LADH, a hydrogen from the α carbon of the alcohol is transferred to NAD^+ to produce the reduced form of the coenzyme (NADH). This was elegantly demonstrated by the use of deuterium labeled substrates and coenzymes (5,6). In the same studies it was shown that the transfer of the hydrogen



NAD^+

Figure 1.--Structure of oxidized nicotinamide adenine dinucleotide.

is stereospecific with respect to the coenzyme, as chemically labeled NADH lost only 50% of the label to the substrate, whereas enzymatically prepared NADH-d lost 100% of the label. The site of reduction of NAD^+ in the enzymatic reaction was shown by Colowick and coworkers (7) to be at C-4 of the nicotinamide ring. The reduction produces a methylene carbon that is a prochiral center and it was evident that the yeast and liver alcohol dehydrogenases could distinguish between the two enantiotopic hydrogens. By using tritium and deuterium to determine which enantiotopic hydrogen was transferred, various investigators classified alcohol dehydrogenases as either type A or type B. YADH and LADH are both type A enzymes. By using a degradation method on enzymatically labeled NADH-d, Cornforth and coworkers (8) determined the absolute configuration at C-4 of the coenzyme. They concluded that type A

dehydrogenases utilize the pro-R hydrogen of NADH and type B enzymes utilize the pro-S hydrogen, as pictured in Figure 2. These hydrogens are alternatively labeled as $H_A(H_R)$ and $H_B(H_S)$.

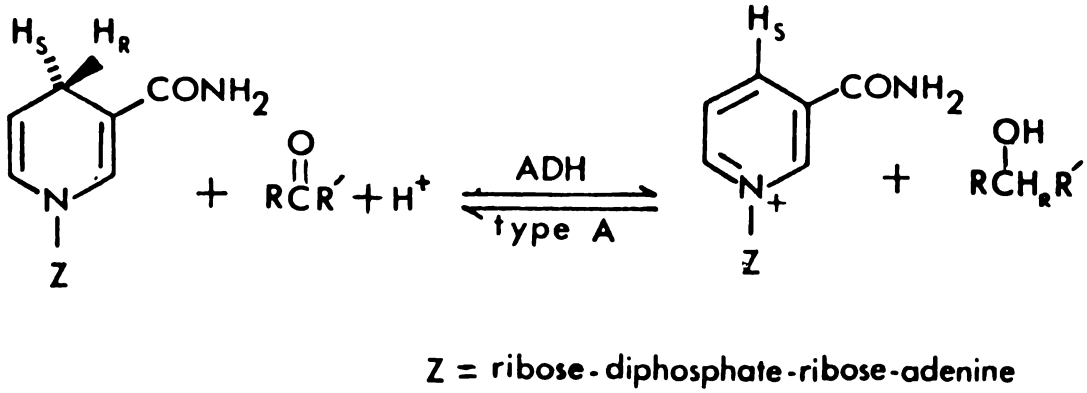


Figure 2.--Stereochemistry of hydrogen transfer from NADH.

Westheimer, Vennesland, and Loewas (9) demonstrated that YADH reduces acetaldehyde and oxidizes ethanol stereoselectively. This stereoselectivity was later shown to be absolute (10). To completely describe the stereochemical outcome of the enzymatic reduction of acetaldehyde-1-d it was necessary to know the absolute configuration of the optically active ethanol-1-d product. This was accomplished by Lemieux and Howard (11) who determined that (+)-1-deuterioethanol has the R configuration by using a correlation scheme involving the degradation of 5-deuterio-D-xylose. Since the deuterated ethanol produced by the

action of YADH and LADH on acetaldehyde-1-d was levorotatory, they concluded that the product from the enzyme reaction has the S configuration. In the Hansen nomenclature, the hydrogen is transferred to the re face of the carbonyl. Thus, it had been verified that the two alcohol dehydrogenases were stereospecific towards both their coenzyme and at least one of their natural substrates.

Studies (12,13) on the product stereospecificities of a large number of ketone reductions by fermenting yeast preparations revealed a pronounced predilection for the generation of the alcohol with the S configuration. Using purified YADH, Van Eys and Kaplan (14) examined the kinetics of oxidation of a variety of primary and secondary alcohols. From the results with (d,l)-2-butanol and (+)-2-octanol, they concluded that the enzyme utilizes only the S enantiomer of the alcohols as a substrate. From all of these studies, it is apparent that some mechanism exists whereby the enzyme orients the substrate molecule with respect to the coenzyme so that a stereochemically defined transfer of hydrogen occurs. Steric effects were proposed as the controlling factor in references 12 and 14. Hydrophobic and hydrophilic interactions between enzyme and substrate were considered to be important by other workers. As evidence of a hydrophobic binding region at the active site of alcohol dehydrogenase, the binding of long chain fatty amides was cited by Winer and Theorell (15).

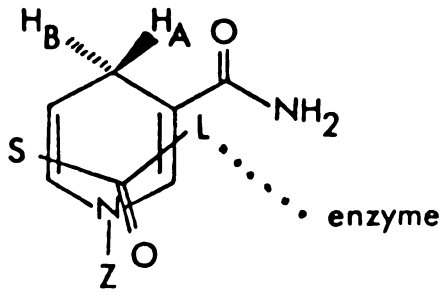


Figure 3.--Karabatsos model.

Based on the stereochemical results of the alcohol dehydrogenase reactions, Karabatsos and coworkers (16) proposed the model shown in Figure 3 for the spatial relationship of the substrate to the coenzyme in the enzyme complex. In this model the carbonyl of the substrate is lying in a plane above the plane of the NADH nicotinamide ring. To account for the substrate stereoselectivity it was suggested that a hydrophobic region of the enzyme preferentially binds the larger alkyl group of the substrate. This region is pictured as being on the same side of the nicotinamide ring as the carbamido group which would produce the alcohol with the S configuration after transfer of the pro-R hydrogen of the coenzyme. This model is similar to the one proposed by Prelog (17) as a result of his group's experiments with cyclic ketone reductions by Curvularia falcata, an organism that contains a type B dehydrogenase. Prelog's model is shown in Figure 4.

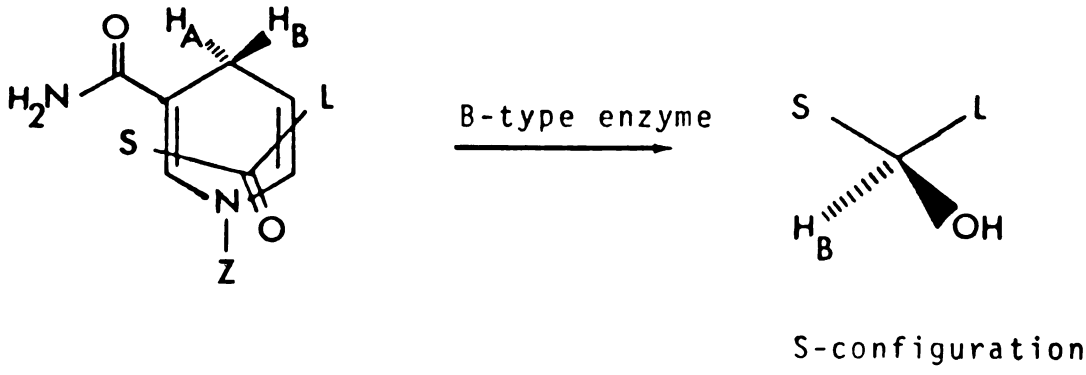


Figure 4.--Prelog model.

These two models differ in that the carbamido group of the coenzyme is placed beneath the larger carbonyl substituent of the substrate in the Karabatsos Model and below the smaller alkyl group in the Prelog Model. In devising his model, Prelog considered non-bonded interactions between the carbamido group of the coenzyme and the alkyl groups of the substrate to be important in aligning the molecules. This model, however, predicted that type A dehydrogenases would reduce ketones to alcohols with the R configuration, just the opposite of what is observed. Karabatsos felt that the binding of the large group of the substrate to a hydrophobic pocket of the enzyme is a more important factor in determining the stereochemical outcome of the reaction.

That hydrophobic and hydrophilic interactions between substrate and enzyme are important in the reactions of dehydrogenases was convincingly demonstrated by the work

of Nunez (18) and Stamoudis (19) in this laboratory. Karabatsos and Nunez determined that the product from the reduction of hydroxyacetone with purified glycerol dehydrogenase, a type A enzyme, was 100% optically pure (R)-1,2-propanediol. The stereochemistry of the product suggests a transition state for the reduction that resembles the substrate-coenzyme configurational relationship shown in Figure 5 in which the hydrophilicity of the CH_2OH group has reversed the positions of the large and small group of the Karabatsos Model.

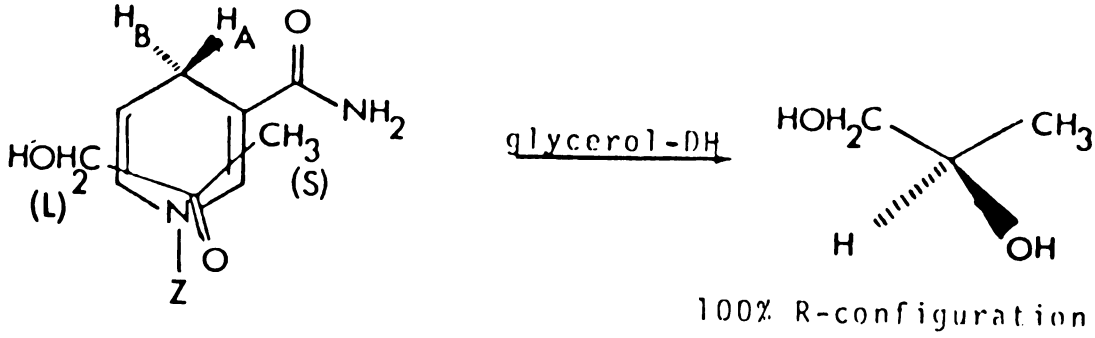


Figure 5.--Reduction of hydroxyacetone by NADH with glycerol dehydrogenase.

Using purified LADH and NAD^+, Karabatsos and Stamoudis studied the reduction of methyl ethyl ketone and chloroacetone. Methyl ethyl ketone yielded (+)-2-butanol that was 44% optically pure (72% S enantiomer) and chloroacetone gave (+)-1-chloro-2-propanol that was only 6.8% optically pure (S enantiomer slightly in excess). The larger size

and greater hydrophobicity of the ethyl group over that of the methyl group lead to a prediction based on the Karabatsos Model that is in accord with the experimental results for methyl ethyl ketone (see Figure 6). A similar product stereoselectivity of 64-67% had been observed (12) in the reduction of methyl ethyl ketone by fermenting yeast.

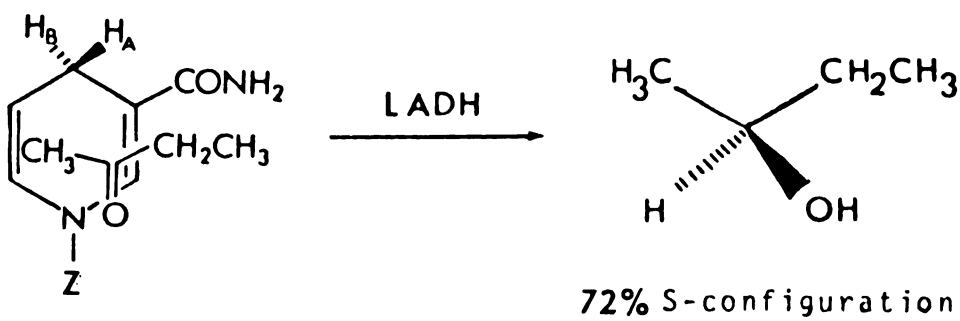


Figure 6.--Reduction of methyl ethyl ketone by NADH with LADH.

The carbonyl substituents of chloroacetone are approximately the same in size as those of methyl ethyl ketone but the hydrophilicity of the chloromethyl group is much greater than that of the ethyl group. The nearly racemic product mixture of the reduction of chloroacetone implies the configurations shown in Figure 7. Apparently the effects of steric and hydrophobic-hydrophilic interactions are nearly balanced in this system.

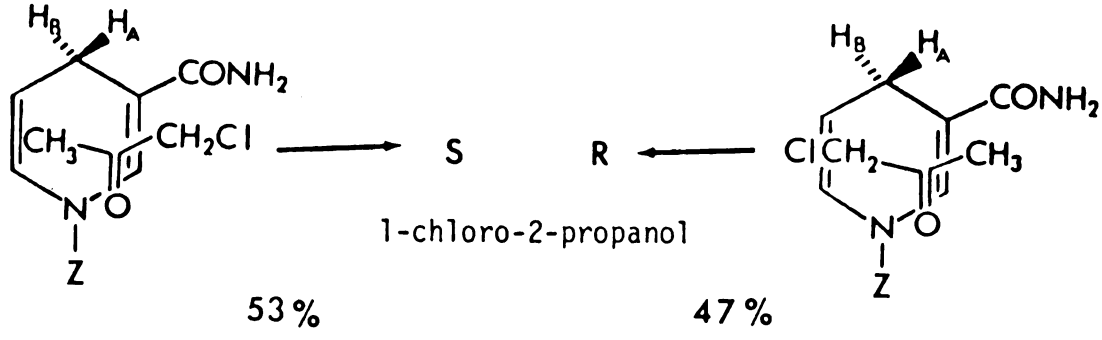


Figure 7.--Reduction of 1-chloro-2-propanone by NADH with LADH.

The product-controlling ternary complex of the alcohol dehydrogenases has been discussed up to this point in terms of representations in which the oxygen of the substrate is located over the nitrogen of the coenzyme pyridine ring. Prelog (17) had favored this orientation because he believed that it involved minimal non-bonded interactions between substrate and coenzyme. However, as the stereochemical results of the reactions with type A dehydrogenases have shown, these non-bonded interactions are probably not the dominant factor in orientating the substrate. The model proposed by Karabatsos also envisions the carbon-oxygen bond of the substrate as pointing towards the pyridine ring nitrogen. This assumption was adopted from a similar model advanced by Kosower (20) based on the observation of a shift in the absorption spectrum of NADH from 340nm to 325nm upon binding to LADH. Studies on the spectra of

model systems supported by calculations on their spectral transition energies led to his suggestion that this shift was caused by the presence of a positively charged nitrogen atom, such as a lysine residue of the enzyme, in the vicinity of the nitrogen of the dihydropyridine ring. Kosower further postulated that this quaternary nitrogen was hydrogen bonded to the ribose of the coenzyme and to the oxygen of the substrate, thus positioning this oxygen over the pyridine ring nitrogen. No direct evidence supporting this arrangement has been reported and without such confirmation, any proposal of a particular structure of the enzyme-coenzyme-substrate complex based on substrate stereospecificity must be considered as only speculation.

Examination of the literature dealing with the mechanism of alcohol dehydrogenases reveals that there is no general agreement on the structure of the ternary complex. Graves, Clark, and Ringold (21) preferred an arrangement of the coenzyme and cyclic ketone substrates such as illustrated in Figure 8. Cervinka and Hub (13), from their studies on fermenting yeast reductions of ketones, drew the pictorial representation of the substrate-coenzyme orientation shown in Figure 9. More recent studies (22) done with addition products of NAD^+ also support a model for the ternary complex in which the carbonyl of the

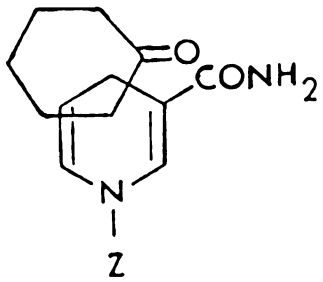


Figure 8.--Arrangement suggested by Graves.

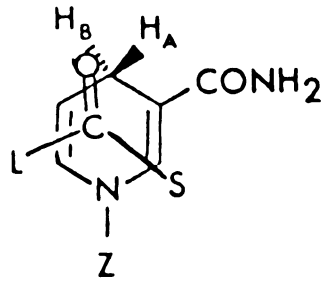


Figure 9.--Model of Cervinka and Hub.

substrate is pointing away from the pyridine ring nitrogen of the coenzyme. The author's representation for the mechanism of lactate dehydrogenase is pictured in Figure 10.

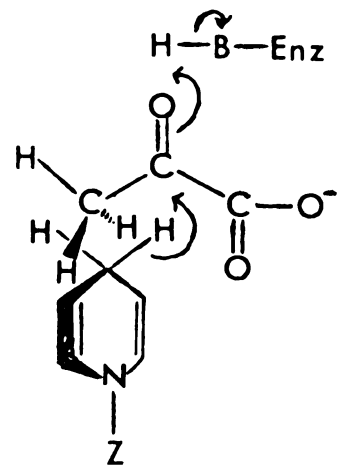


Figure 10.--Model for the mechanism of lactate dehydrogenase.

The stumbling block that this confusion has created is attested to by the following quote from this paper:

> To the best of our knowledge, there is no information available at the present time concerning the position of the substrate molecule on the enzyme, except that the substrate is located somewhere near the nicotinamide ring. However, a more detailed knowledge of the spatial orientation of the coenzyme and the substrate is important to the understanding of the reaction mechanism of the dehydrogenases.

Our approach to the solution of this difficulty was to covalently attach one end of a substrate molecule to NAD^+, thereby reducing the number of possible orientations that the substrate could assume with respect to the coenzyme. If the substrate-coenzyme analog exhibits reactivity with alcohol dehydrogenase, information concerning the spatial relationships of the species participating in the reaction could be inferred. Furthermore, studies on such analogs that are enantiomerically purified at the substrate alcohol center might yield data on the location of the hydrophobic and other binding regions of the enzyme. As illustrated in Figure 11, if only one of the stereoiosmers of the NAD^+ analog exhibited reactivity with alcohol dehydrogenase, this would be direct evidence for a particular placement of the carbon-oxygen bond of the substrate in the enzyme reaction. Initial studies of several NAD^+ analogs by J. Miedema (23) demonstrated the feasibility of using these covalently joined substrate-coenzyme molecules as probes of the alcohol dehydrogenase active sites.

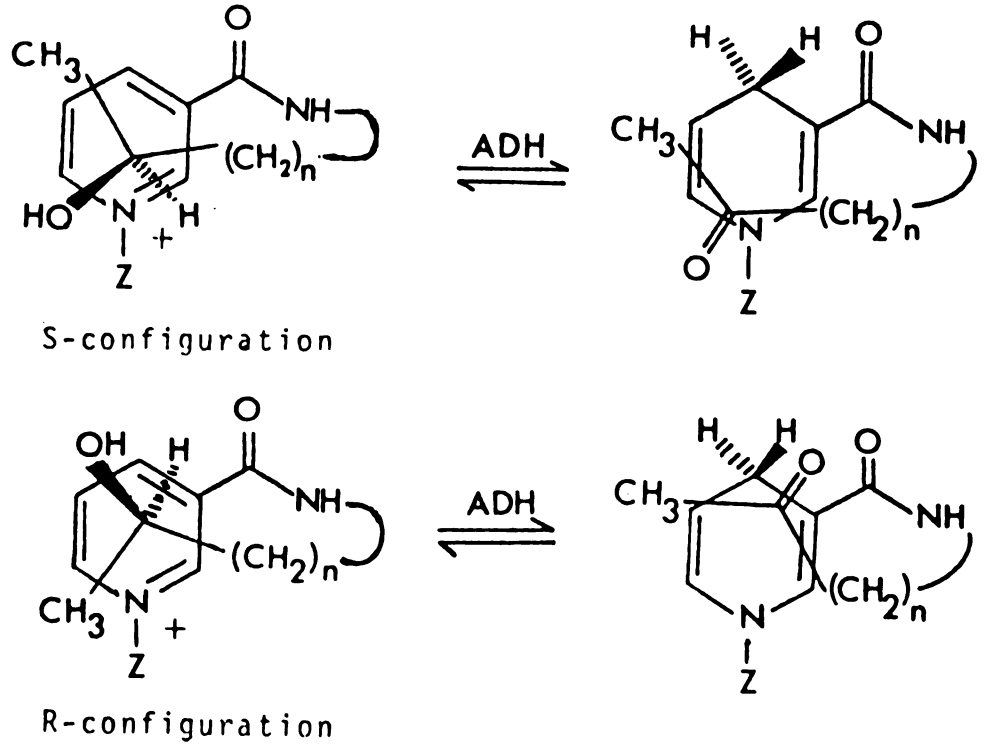
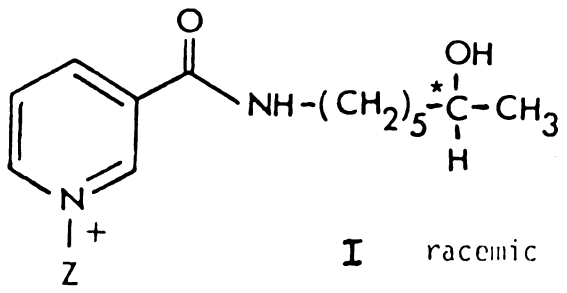


Figure 11.--Stereochemistry of the reaction of NAD^+ analogs.

This thesis describes the synthesis and reactivity studies of the NAD^+ analogs (I), (IR), and (IS) shown in Figure 12. These studies required the synthesis and resolution of 7-amino-2-heptanol and the determination of the resolved aminoalcohols' enantiomeric purity and absolute configurations. In the course of this investigation, 6-amino-2-hexanol and its corresponding nicotinamide were



I racemic

IS S-configuration

IR R-configuration

Z = ribose-diphosphate-ribose-adenine

Figure 12.--NAD^+ analogs (I).

also synthesized and these two compounds along with several other aminoalcohols and nicotinamides were tested as substrates for liver alcohol dehydrogenase.

EXPERIMENTAL

Instrumentation

Proton NMR spectra were recorded on a Varian T-60 NMR spectrometer. Fluorine NMR spectra were obtained with a Varian A-56/60D spectrometer. All infrared spectra were recorded on a Perkin Elmer 237B IR grating spectrophotometer.

Melting points were determined with a Hoover capillary melting point apparatus.

A Bodenseewerk model 141 polarimeter with automatic readout was used to measure optical rotations. The instrument was equipped with sodium and mercury lamps to allow readings at wavelengths of 589nm, 578nm, 546nm, 436nm, and 365nm.

The pH measurements of buffer solutions and enzyme reaction solutions were done with an Instrumentation Laboratory model 245 pH meter.

A Sorvall RC2-B refrigerated centrifuge was used to isolate the products of the NAD analog synthesis.

Column separations were monitored by passing the eluate through a 0.1 cm flow cell in a Beckman DB-G grating spectrophotometer connected to a Sargent SR recorder.

Fractions were collected with a Buchler Fractomette 200 collector that had an output to the recorder to record tube changes.

Ultraviolet spectra for the reactivity studies were recorded on a Unicam SP800 spectrophotometer.

Synthesis of 7-amino-2-heptanol

Synthesis of ethyl (3-cyanopropyl)-acetoacetate

Ethanol 800ml was distilled from magnesium methoxide directly into a 2 liter three-necked round-bottomed flask that had been oven-dried. To this flask was added 23g of sodium metal under a flow of dry nitrogen. When all of the sodium had reacted, 50g of powdered potassium iodide and 220g of freshly distilled ethyl acetoacetate was added to the flask. The flask was fitted with a reflux condenser and mechanical stirrer and the reaction mixture heated to reflux for ten minutes. The contents of the flask were allowed to cool somewhat, then 100g of 4-chlorobutyronitrile (Aldrich) was added slowly with stirring. The nitrogen flow was discontinued at this point and the reaction mixture heated at reflux for seven hours. During this time a white precipitate of sodium chloride formed and the pH of the solution decreased to about 9. The condenser was then replaced with a distillation head and the ethanol was distilled. After addition

of 600ml of a 50/50 mixture of benzene and water to the cooled residue, the organic layer was separated, and the aqueous layer was extracted three times with 75ml of ethyl ether. The combined organic layers were washed with saturated sodium chloride solution and dried over anhydrous magnesium sulfate.

Following filtration the solvent was removed from the reaction products by rotary evaporation. Fractional distillation of the residue under vacuum yielded several fractions. The first fraction consisted mainly of ethyl acetoacetate; the second fraction, b.p. 72-75° at 0.16mm, was a pale yellow liquid; the third fraction, b.p. 96-122° at 0.14mm, was a mixture; and the fourth fraction, b.p. 122-125° at 0.14mm, was the desired product. The NMR spectrum of this fraction was consistent with the structure of ethyl (3-cyanopropyl)-acetoacetate. The yield was 103.9g, 55% of theoretical.

A second reaction using the same procedure gave another 110g of product.

Synthesis of 6-cyano-2-hexanone

To a solution of 200g of sodium carbonate in 1600ml of water in a 2000ml round-bottomed flask equipped with a reflux condenser was added 213.7g (1.08 mol) of ethyl-(3-cyanopropyl)-acetoacetate. The reaction flask was connected to a gas bubbler filled with barium hydroxide

solution to detect the evolution of carbon dioxide. The reaction mixture was refluxed for six hours, by which time carbon dioxide formation had ceased. After cooling, the reaction mixture was saturated with potassium carbonate. The two layers that formed were separated. The aqueous layer was extracted four times with ether and the combined organic layers were dried over anhydrous magnesium sulfate. Solvent removal and distillation yielded only one fraction, b.p. 78-79° at 0.5mm. The NMR spectrum of this material had only a methyl singlet (δ2.05ppm) and peaks for the eight methylene protons. IR (CCl_4): 1720cm^{-1}, 2245cm^{-1}. Yield: 114.7g (0.92mol), 85% theoretical.

Synthesis of 6-cyano-2-hexanol

The 114.7g of 6-cyano-2-hexanone was dissolved in 400ml of methanol in a 1000ml three-necked round-bottomed flask equipped with a thermometer, mechanical stirrer, and addition funnel. The contents of the flask were chilled to 0° with a salt-ice bath. To it was added a solution of 11.5g (0.30mol) of sodium borohydride in cold water, slowly with stirring so as to maintain the temperature of the reaction mixture below 10°. After completion of the addition, stirring was continued for one hour. This was followed by the addition of 25% sulfuric acid until the reaction mixture was slightly acidic. The

reaction mixture was allowed to stand overnight, then the precipitate was filtered. Most of the methanol was removed from the filtrate by rotary evaporation. Addition to the residue of 300ml of ether resulted in the formation of two layers. The organic layer was washed with saturated sodium chloride solution and dried over anhydrous potassium carbonate. Distillation of the solvent, followed by vacuum distillation of the residue, produced 101.6g (0.80mol) of the alcohol, b.p. 92-94° at 0.25mm. Yield: 87% of theoretical.

Synthesis of 7-amino-2-heptanol

Raney nickel W-2 was prepared by the standard method (24). Approximately 9g of the catalyst was added to a 500ml Parr bomb containing a solution of 34g of 6-cyano-2-hexanol in 300ml of 10N ammonia in methanol. The bomb was fitted to a Parr hydrogenation apparatus and hydrogen introduced to a pressure of 50psi. After twelve hours of shaking, the pressure in the bomb had fallen by the theoretical amount (43psi). The nickel was removed by filtration through celite. The filtrate was distilled yielding a single product, b.p. 96-97° at 0.9mm, which solidified in the receiver.

Two more portions of the cyanoalcohol were hydrogenated to give a total yield of 95.9g (0.732mol) 7-amino-2-heptanol, 92% of theoretical. NMR ($CDCl_3$):

δ1.1 doublet (3), δ1.4 broad multiple (8), δ1.95 singlet (3-OH and NH_2), δ2.6 triplet (2), and δ3.65 multiplet (1).

Synthesis of 6-amino-2-hexanol

Synthesis of 5-cyano-2-pentanone

Ethyl (2-cyanoethyl)-acetoacetate was prepared by a Michael addition of ethyl acetoacetate and acrylonitrile using the method of Albertson (25). Of this ester, 154g (0.842mol) was hydrolyzed and decarboxylated by refluxing with a solution of 160g of sodium carbonate in 1400ml of water. After four hours of refluxing the solution had become homogeneous. Saturating with potassium carbonate caused an organic layer to separate. The aqueous layer was extracted five times with ether and the organic layers were combined. After drying over anhydrous magnesium sulfate, the ether was removed from the product by rotary evaporation. The residue was vacuum distilled through a short Vigreaux column to give 68.9g of product. The NMR and IR spectra of the product were consistent with the structure of 5-cyano-2-pentanone. The yield was 74% of theoretical.

Synthesis of 5-cyano-2-pentanol

Into a 200ml three-necked round-bottomed flask equipped with a thermometer and stir bar was added a

solution of 63.5g (0.572mol) of 5-cyano-2-pentanone in 200 ml of methanol. After chilling the contents of the flask to 0° with an ice bath, a solution of 7.2g (0.19mol) of sodium borohydride in a small amount of cold water was added at a rate such that the temperature of the reaction mixture did not rise above 10°. When all of the borohydride had been added, the reaction mixture was allowed to warm solwly to room temperature. The reaction was completed by acidifying with 25% sulfuric acid. The precipitated salts were removed by filtration and the bulk of the methanol was removed from the filtrate by rotary evaporation. The residue was dissolved in ether; this solution was washed with water and saturated sodium chloride solution, then dried over anhydrous magnesium sulfate. Solvent removal and vacuum distillation yielded 60.8g of product, 94% of theoretical. IR (CCl_4): 2240cm^{-1}, 3480cm^{-1}, 3630cm^{-1}, no carbonyl absorption.

Synthesis of 6-amino-2-hexanol

Into a 500ml glass Parr bomb were added 19.36 (0.170mol) of 5-cyano-2-pentanol, 4g of Raney nickel W-2, and 300ml of approximately 10N ammonia in methanol. Hydrogen was introduced into the bomb to a pressure of 45psi. After shaking for twenty hours the pressure had decreased the theoretical amount (27psi). The beautiful blue-green reaction mixture was filtered through celite.

Distillation of the filtrate gave a single product, b.p. 63-65° at 0.08mm. The NMR and IR spectra confirmed that this was 6-amino-2-hexanol. The yield was 18.3g (0.156mol), 92% of theoretical.

Resolution of 7-amino-2-heptanol

Resolution with d-tartaric acid

Resolution of 7-amino-2-heptanol was accomplished by the method of repeated recrystallizations of its tartrate salt. A mixture of methanol and ethyl acetate was found to be a suitable solvent for crystallization.

102.9g of 7-amino-2-heptanol was dissolved in 100ml of methanol and this solution added to a solution of 118.1g of d-tartaric acid (Eastman Organic) in 200ml warm methanol in an Erlenmeyer flask. The solution was stirred for one half hour, then 150 ml of hot ethyl acetate was added and the flask set aside for crystallization. After standing in the refrigerator overnight, the crystals that had formed were collected by suction filtration and washed with a little cold methanol. This tartaric acid salt was repeatedly recrystallized from 80/20 (v/v) methanol/ethyl acetate. Melting points and optical rotations of the salt were used to follow the progress of the resolution. Table 1 summarizes the data from the successive recrystallizations.

TABLE 1.--Crystallizations of the d-tartrate of 7-amino-2-heptanol.

	Volume of Solvent	Product Weight	Melting Point (°)	$[\alpha]_D$(°)
I	450ml	200g	122-125	
II	400ml	167g	123-125	
III	310ml	134g	124.5-126	+16.7
IV	380ml	120.5g	125-126.5	
V	200ml	112.5g	126-127	+15.2
VI	200ml	104g	127-128.5	
VII	250ml	87.5g	127.5-128.5	+14.3
VIII	175ml	74g	128-129.5	
IX	150ml	60g	129-130	+13.2
X*	150ml	68.7g	128.5-130	
XI	150ml	59.8g	129.5-130.5	+12.9
XII	150ml	51.6g	130-131.5	+12.4
XIII	125ml	49.1g	130.5-131.5	+12.2
XIV	125ml	42.3g	131.5-132	
XV	100ml	38.3g	132-132.5	+12.1

*For this recrystallization the product from the ninth crystallization was combined with another 14.7g of tartrate obtained by taking a second crop of crystals from the mother liquors of the fourth cyrstallization. It was then carried through successive crystallizations using as a solvent the mother liquors of the original crystallizations, then from fresh solvent for the last two crystallizations.

The final product from the resolution was dissolved in 100ml of warm methanol. To this solution was added a solution of 15.3g of potassium hydroxide in 75ml of methanol. After stirring for twenty minutes, the precipitate was filtered and washed with 50ml of methanol. The filtrate was distilled yielding 13.5g of 7-amino-2-heptanol, b.p. 86-87° at 0.6mm.

The optical rotation of a 10.0ml solution of 0.3462g of this 7-amino-2-heptanol in purified ethanol was measured at five wavelengths using ethanol as a blank. The measured rotations at 20° and the specific rotations are reported in Table 2.

TABLE 2.--Optical rotations of (-)-7-amino-2-heptanol.

Wavelength	α(°)	$[\alpha]^{20°}$(°)
589nm	-0.271	- 7.83
578nm	-0.282	- 8.16
546nm	-0.321	- 9.28
436nm	-0.528	-15.25
365nm	-0.797	-23.0

Another crop of the d-tartrate crystals was obtained by evaporating the mother liquors of the last three recrystallizations to a smaller volume. These

crystals were recrystallized twice from fresh solvent to give a product, m.p. 129-130°, which was saved for work on the determination of the absolute configuration of the resolved aminoalcohol.

Resolution with l-tartaric acid

The mother liquors of the recrystallizations of the d-tartrate were evaporated to obtain the salt. An aqueous solution of the salt was treated with sodium hydroxide, then saturated with sodium chloride and extracted six times with methylene chloride. After drying over anhydrous potassium carbonate, the organic layers were distilled to yield 63.4g of 7-amino-2-heptanol.

The 7-amino-2-heptanol was dissolved in 100ml of methanol and to this solution was added a solution of 72.6g of l-tartaric acid (Aldrich) in 150ml of warm methanol. After stirring for one half hour, 100ml of warm ethyl acetate was added and the solution was allowed to cool slowly, then refrigerated overnight. The crystallized product was collected by suction filtration. The tartrate was dissolved in fresh methanol/ethyl acetate, the solution was filtered while hot, and then cooled for crystallization. Table 3 reports the weights and melting points of the products of the successive crystallizations.

TABLE 3.--Crystallizations of the l-tartrate of 7-amino-2-heptanol.

	Volume of Solvent	Product Weight	Melting Point
I	350ml	115g	126.5-128
II	300ml	89g	127-128
III	210ml	62g	128-128.5
IV	175ml	53.5g	128.5-129.5
V	150ml	47.5g	129-130
VI	100ml	43.4g	129.8-130.5
VII	90ml	39.2g	130-131
VIII*	100ml	40.1g	131-131.8
IX	90ml	34.9g	131-132
X	80ml	32.2g	132-132.5

*A second crop of crystals was obtained from the mother liquors of the fourth crystallization and recrystallized from the mother liquors of the succeeding crystallizations. Finally, it was recrystallized three times from fresh solvent. This afforded another 5.6g of tartrate which was combined with the product of the original seventh cyrstallization.

The product from the tenth crystallization was dissolved in 50ml of water. A solution of 9.20g of sodium hydroxide in 25ml of water was added with stirring to the tartrate solution. The solution was then saturated with sodium chloride and extracted with chloroform until the extract gave only a faint color with ninhydrin spray. The

chloroform extracts were dried over anhydrous sodium sulfate, then distilled to give 13.285g of 7-amino-2-heptanol.

The optical rotation of this product was determined using a 10.0ml solution of 0.1826g of the aminoalcohol in pure ethanol. The observed and specific rotations are listed in Table 4.

TABLE 4.--Optical rotations of (+)-7-amino-2-heptanol.

Wavelength	α(°)	$[\alpha]^{20°}$(°)
589nm	+0.142	+7.76
578nm	+0.148	+8.12
546nm	+0.168	+9.22
436nm	+0.281	+15.4
365nm	+0.428	+23.4

Determination of the Absolute Configuration of (-)-7-amino-2-heptanol

The (-)-7-amino-2-heptanol was reductively deaminated by a procedure modified from Nickon and Hill's method (26). The aminoalcohol for this reaction was obtained from the second crop of salt from the resolution with d-tartaric acid.

The (-)-7-amino-2-heptanol was first converted to its sulfonamide by dissolving 1.257g (9.61mmol) of it in

10ml of 10% sodium hydroxide solution and adding 1.24ml (9.63mmol) of benzenesulfonyl chloride. The mixture was stirred vigorously for one hour, then acidified and extracted three times with chloroform. The extracts were dried over anhydrous magnesium sulfate and the solvent removed to yield 2.25g (87% of theoretical yield) of an almost colorless oil.

The sulfonamide was dissolved in 100ml of 10% sodium hydroxide solution and this solution added to a 200ml round-bottomed flask equipped with a reflux condenser and stir bar. The contents of the flask were heated to about 60°. Over the period of one hour, 12g of hydroxylamine-O-sulfonic acid (Alfa) was added in portions to the flask with vigorous stirring. The reflux condenser was then replaced with a distillation head and distillation was begun. When 50ml of steam distillate had been collected, 25ml more water was added to the reaction flask and the distillation was resumed until another 50ml of distillate had been collected. The distillates were neutralized with 10% hydrochloric acid, saturated with sodium chloride, and extracted four times with ether. The combined extracts were dried over anhydrous sodium sulfate. The solvent was removed by distillation through a Vigreaux column leaving a residue with the characteristic odor of 2-heptanol. GLC analysis of the residue showed that it

contained ether, 2-heptanol (identified by mixed injection with a known sample), and very little else. The product was short-path vacuum distilled to give 0.1018g of 2-heptanol, GLC pure.

The 2-heptanol was dissolved in 6.0ml of pure ethanol for the determination of its optical activity. The observed optical rotation at 589nm was -0.109° and at 365nm was -0.289°. The calculated specific rotations at ambient temperature are -6.43° and -17.0° at 589nm and 365nm, respectively.

NMR Method for the Determination of Enantiomeric Purities

The aminoalcohols were converted to their sulfonamides by reaction with an equivalent amount of benzenesulfonyl chloride in 10% sodium hydroxide solution. The sulfonamides were obtained pure by acidification of the reaction mixture and extraction with chloroform. Yields were generally 75-80% of theoretical.

(+)- and (-)-α-methoxy-α-trifluoromethylphenylacetic acid were obtained from Aldrich Chemical Co. The acid chlorides were prepared by refluxing the acid with purified thionyl chloride and a small amount of sodium chloride for fifty hours followed by vacuum distillation.

About 0.35mmol of the sulfonamide was dissolved in 10 drops of dry chloroform. To this solution was added

a slight molar excess of the acid chloride, ten drops of dry carbon tetrachloride, and ten drops of pyridine. The solution was allowed to stand in the stoppered flask for twelve hours. Two ml of water was added and the reaction mixture transferred to a separatory funnel with 25ml of ether. The ether solution was washed twice with 10% hydrochloric acid solution, then once with sodium carbonate solution, water, and saturated sodium chloride solution. After drying over anhydrous magnesium sulfate, the ether was removed from the product. The residue was taken up in deuteriochloroform for NMR analysis, using approximately 15% trifluoroacetic acid as an internal standard for the fluorine NMR spectra.

Resolution of 6-amino-2-hexanol

The 6-amino-2-hexanol was partially resolved by using dibenzoyl-d-tartaric acid. A quantity of 23.95g (0.205mol) of the aminoalcohol was added to a warm solution of 79.1g (0.210mol) of dibenzoyl-d-tartaric acid monohydrate (Aldrich) in 150ml of methanol. After stirring for one half hour the solution was filtered hot, 400ml of ether was added to the filtrate, and the flask was set in a refrigerator overnight. The crystals that had formed were collected and recrystallized from 75/25 ethyl acetate/methanol. A summary of the crystallizations is given in Table 5.

TABLE 5.--Crystallizations of the dibenzoyl-d-tartrate of 6-amino-2-hexanol.

	Volume of Solvent	Product Weight	Melting Point (°)
I		112g	147-150 dec.
II	300ml	98g	151-153.5
III	200ml	66g	155-156.5
IV	150ml	38g	156-157
V	100ml	25.9g	157-158
VI	75ml	11.5g	159-160.5
VII	55ml	7.6g	160.5-161.6

The product from the seventh cyrstallization was added to 2.4g of sodium hydroxide in water. The solution was saturated with potassium fluoride and extracted ten times with chloroform. The chloroform extracts were dried over anhydrous sodium sulfate. Solvent removal and vacuum distillation yielded 1.13g of 6-amino-2-hexanol. The specific rotation of the product in ethanol was $[\alpha]_D^{20} - 2.6°$.

Synthesis of the N-(hydroxyalkyl)-nicotinamides

Nicotinyl chloride was prepared by the slow addition of 36ml of purified thionyl chloride to a suspension of 64.5g of potassium nicotinate in 400ml of dry carbon tetrachloride. The reaction mixture was refluxed for

twelve hours, then the solids were filtered and the solvent removed from the filtrate under vacuum. Vacuum distillation gave one fraction, b.p. 38-39° at 0.3mm.

The synthesis of (-)-N-(6-hydroxy-1-heptyl)-nicotinamide is described herein as representative of the procedure used to prepare all of the N-(hydroxyalkyl)-nicotinamides.

To an oven-dried 500ml four-necked round-bottomed flask was added 12.62g of the (-)-7-amino-2-heptanol obtained from the resolution with d-tartaric acid. The flask was fitted with a mechanical stirrer, reflux condenser, rubber septum, and nitrogen inlet. All glass connections were sealed with Teflon© sleeves. After flushing the flask with dry nitrogen, the aminoalcohol was heated until it melted. A catalytic amount of ammonium chloride was added, followed by 10.1ml of hexamethyldisilazane (Aldrich). Stirring was begun, the flask was heated, and ammonia began to evolve. The contents of the flask were heated at 100° for one hour, by which time the evolution of ammonia had just about ceased. To the reaction flask was added 70ml of pyridine and 20 ml of triethylamine, both having been dried and distilled under nitrogen. An amount of 10.6ml of nicotinyl chloride was then added slowly from a disposable syringe. The reaction being exothermic, the

heat source was removed from the flask. The colorless solution became yellow, then orange. When the nicotinyl chloride had been completely added the septum was replaced with a glass stopper and the reaction mixture was refluxed for one hour.

The reaction mixture was cooled and the precipitated hydrochloride salt was filtered and washed with a little pyridine. The filtrate was transferred to a 250ml round-bottomed flask and the remaining triethylamine was distilled off. A reflux condenser was attached to the flask, 15ml of water was added, and the solution was refluxed for two hours. All of the solvent was then removed under vacuum, leaving a viscous brown oil. The NMR of the product indicated it to be mainly the desired N-(6-hydroxy-1-heptyl)-nicotinamide with a few minor impurities. TLC of the product on silica gel with 10/1 $CHCl_3$/MeOH produced five spots: the major component with an Rf of 0.11, the others with Rf's of 0.92, 0.56, 0.36, and 0.0.

The N-(hydroxyalkyl)-nicotinamides were purified by chromatography on a 2.5 X 80cm column of alumina (type F-1, 80-100 mesh) with 100/2 chloroform/methanol as the eluant. A 10ml aliquot of a solution of the nicotinamide product in 30ml of chloroform was loaded onto the column and eluted at a flow rate of about two ml per minute, while the column eluate was continuously

monitored at 254nm with a Beckman DB-G UV spectrophotometer connected to a recorder. Several fractions were observed before the main component began coming off the column. This fraction was collected and the solvent distilled off, leaving a slightly yellow oil.

The N-(hydroxyalkyl)-nicotinamides were further purified by dissolving the oil in 40ml of acetone, boiling with a little Norit-A, then gravity filtering. The filtrate was evaporated to a volume of about 15ml and set in a freezer at -20°. Scratching led to the slow formation of crystals. The products were recrystallized one or two more times until a constant melting point was obtained. Crystallization was greatly facilitated by seeding with a crystal from the previous recrystallization.

The N-(6-hydroxy-1-heptyl)-nicotinamides synthesized from the racemic and optically active 7-amino-2-heptanols were characterized as follows: The melting points were (R,S) 39-41°, (R) 48-49.5°, and (S) 48.5-50°. The NMR spectrum of (S)-N-(6-hydroxy-1-heptyl)-nicotinamide is shown in Figure 13. By deuterium exchange, the singlet at δ3.1ppm was identified as the OH peak and the triplet at δ7.4ppm as the NH peak. Irradiation of the peak at δ7.4ppm caused decoupling of the peak at δ3.3ppm. IR spectrum (CH_2Cl_2): 3610, 3450, 3325, and 1660cm^{-1}. The optical rotations of

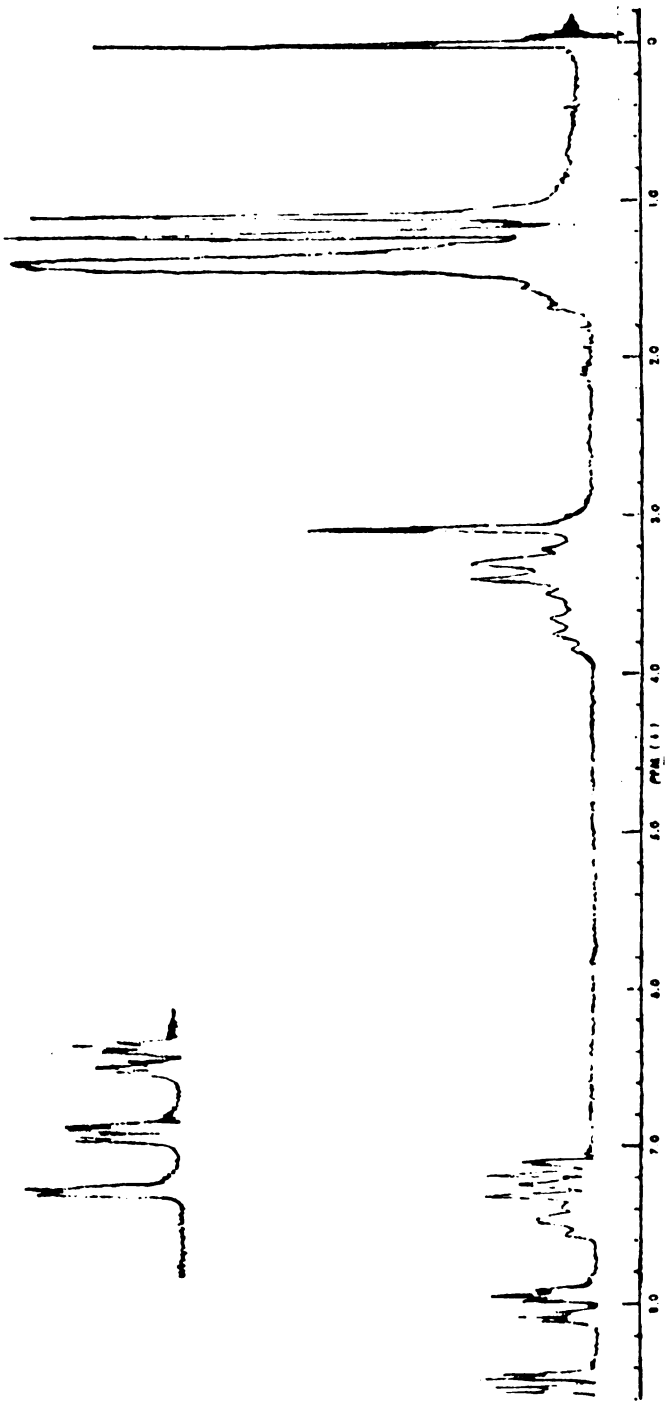


Figure 13.--NMR spectrum of (S)-N-(6-hydroxy-1-heptyl)-nicotinamide.

(R)-N-(6-hydroxy-1-heptyl)-nicotinamide, as measured on a 10ml solution of 0.2785g of it in ethanol, are given in Table 6.

TABLE 6.--Optical rotations of (-)-N-(6-hydroxy-1-heptyl)-nicotinamide.

Wavelength	α(°)	$[\alpha]^{23}$(°)
589nm	-0.189	- 6.78
578nm	-0.196	- 7.04
546nm	-0.223	- 8.01
436nm	-0.368	-13.2
365nm	-0.563	-20.2

Preparation of NAD^+ Analogs

To a reaction flask with Teflon© seal cap was added 0.180g (0.25mmol) of β-NAD^+ (Sigma, grade III) and 0.235g (1.00mmol) of N-hydroxyalkyl-nicotinamide. Twelve milliliters of 0.1M phosphate buffer, pH 7.5, was added and the solids were dissolved with gentle stirring. The pH of the solution was adjusted to 7.5 with 0.2M sodium hydroxide solution. The reaction flask was equilibrated in a constant temperature bath at 37.0°. Then, 0.30g (4.5 units) of pig brain NADase (Sigma) was added and the reaction vial was put back in the bath. The reaction

mixture was stirred vigorously for three hours at 37°. The reaction was stopped by chilling in an ice bath and adding 0.5g of trichloroacetic acid. The contents of the flask were transferred to a centrifuge tube and centrifuged at 10,000rpm for ten minutes in a refrigerated centrifuge. The supernatant liquid was decanted and diluted with 60ml of cold acetone. After standing a few minutes, the mixture was centrifuged at 5000rpm for fifteen minutes. The supernatant liquid was discarded. The small amount of solid lining the bottom of the centrifuge bottle was dried under a flow of nitrogen, then dissolved in 5ml of deionized water and the solution transferred to a sealed vial which was stored at 3°.

A 0.9X 5-cm column was constructed of PEI cellulose anion exchange resin (Sigma, 1.17meq/g) and 0.003OM ammonium bicarbonate in deionized water. The column, which was kept in a refrigerator, was fully equilibrated before use. A 1ml aliquot of the analog product solution was loaded onto the column and eluted with 0.003M ammonium bicarbonate solution using a polystatic pump to obtain a flow rate of 1.4ml per minute. The eluate was monitored by passing it through a 0.1cm flow cell in a UV spectrophotometer. Four fractions were separated.

Activity Determinations in Enzyme Systems

Horse liver alcohol dehydrogenase (LADH) and yeast alcohol dehydrogenase (YADH) were obtained in vials of lyophilized protein from Sigma Chemical Co. The enzyme was reconstituted prior to use by adding 1.0ml of phosphate buffer, pH 7.5. These solutions were stored at -15°. β-NAD (Grade III) was also obtained from Sigma.

The buffer stock solutions used in the enzyme studies were prepared as described in Methods in Enzymology (27). The following buffers were used: pH 7.5 phosphate, pH 10 glycine/NaOH, and pH 8.8 glycine/NaOH.

All water used in these studies was house-supplied distilled water that was passed through an ion exchange column and boiled.

The extent of the enzyme reactions were measured as the increase in the UV spectrum at 340nm due to the reduction of the pyridine ring of the nicotinamide adenine dinucleotide. Blank solutions were used as references and care was taken to establish the same baseline for spectra taken over long time intervals.

The activity of the NAD^+ analogs with the alcohol dehydrogenases was determined in solutions made with 1.0ml of the column eluate containing the analog and 2.0ml of the buffer stock solution. One unit of LADH or

25 units of YADH was added and the UV spectrum taken immediately and again at various time intervals. The solutions were incubated in the UV cuvettes either at ambient temperature or at 30° in a constant temperature bath. Measured amounts of a 3.00×10^{-2}M solution of ethanol in water were added to the enzyme reaction solution for testing the coenzyme function of the analogs.

More concentrated solutions of the NAD^+ analogs were obtained by two processes: lyophilization and molecular filtration. The molecular filtration method involved the use of a Millipore© molecular filtration apparatus with a Pellicon© PSAC membrane to concentrate the analog fractions from several column runs. The remaining ammonium bicarbonate was removed by twice diluting with a buffer solution and reconcentrating. These concentrated solutions of analog and buffered solutions of the lyophilized analogs were tested for reactivity with LADH. Column fractions containing NAD^+ were also concentrated by these methods as a check of their capability to produce intact coenzymes.

The general procedure for determining a substance's activity as a substrate for LADH was to add 1.00ml of an aqueous solution of the compound to 2.00ml of a stock solution of NAD^+, about 5.2×10^{-4}M, in the buffer of interest. One or one-half unit of LADH was added and the UV spectrum determined at various time intervals. Relative

initial rates of NADH production were measured as the increase in absorbance at 340nm. Equilibrium concentrations of NADH were determined after incubating for 24 hours or more. When no further increases in the peak at 340nm were noted, fresh enzyme was added to insure that a true equilibrium had been reached before enzyme denaturation. Alternatively, ethanol was added to check the activity of the enzyme system.

Equilibrium constants for the general reaction:

$$NAD^+ + RCHOHR' \rightleftharpoons NADH + RCOR' + H^+$$

were calculated by using the expression

$$K_{eq} = \frac{x^2 \cdot [H^+]}{([NAD]_0 - x)([RCHOHR']_0 - x)}$$

where X = [NADH] = [RCOR'] at equilibrium and $[NAD^+]_0$ = initial concentration of NAD^+ and $[RCHOHR']_0$ = the initial concentration of the alcohol. The concentration of NADH was determined from the absorbance at 340nm by using the reported (28) extinction coefficient of 6.22×10^6 cm^2/mol. The $[H^+]$ was fixed by the pH of the buffer solution. For equilibrium constants determined on the analog systems, the extinction coefficients at 260nm and 340nm were assumed to be equivalent to those of NAD^+ for calculating the necessary concentrations.

RESULTS AND DISCUSSION

Resolution of 7-amino-2-heptanol

Enantiomeric Purity of the Resolved 7-amino-2-heptanols

Since interpretation of the type of results which were the goal of this research project depended on having NAD^+ analogs of known stereoisomeric composition, it was necessary to have some means of determining the extent of resolution of the aminoalcohols from which the analogs were synthesized. Two methods were investigated.

The first attempt at solving this problem was to study the effect of an optically active shift reagent on the NMR spectrum of the aminoalcohol. Europium (III)-tris-(3-heptafluoropropyl-hydroxymethylene)-d-camphor (Optishift II, Willowbrook Laboratories) was added in portions to a chloroform-d solution of 7-amino-2-heptanol and the NMR spectrum was recorded after each addition. Since the europium reagent complexes with both alcohols and amines, it was necessary to add large amounts of this shift reagent to the solution (1/1 molar ratios of shift reagent to alcohol were found to be needed to produce significant chemical shift differences for enantiomers of simple alcohols)(29). The peak broadening that resulted

from the use of two equivalents of shift reagent made the integration of any slightly separated peaks impractical. Because of this and the expense of the optically active shift reagents, this approach was abandoned.

The next approach proved more successful. Use was made of fluorine NMR to measure the relative amounts of the two diastereomeric esters formed from the reaction of optically active α-methoxy-α-trifluoromethylphenylacetyl chloride with the sulfonamide of the resolved amino-alcohols. In 1969 Mosher, Dale, and Dull (30) reported the successful use of this reagent in the determination of the enantiomeric composition of a number of secondary alcohols. Since the acid chloride also reacts with amines, this complication in the present study was avoided by protecting the amino group by conversion to its sulfonamide before reaction with the acid chloride. Using the procedure described in the experimental section, the sulfonamides were obtained cleanly in good yields. The NMR spectrum (triplet at 5.1ppm, doublet of triplets centered at 2.9ppm, singlet at 1.8ppm in $CDCl_3$) and IR spectrum (sulfonamide absorptions at 1325cm^{-1} and 1160cm^{-1} and hydroxy absorptions at 3530cm^{-1} and 3620cm^{-1}) confirmed the identity of the products.

The reaction of the MTPA chloride with the sulfonamides was clean and yielded nearly theoretical yields of

the esters. The product of the reaction of (R)-α-methoxy-α-trifluoromethylphenylacetyl chloride with the sulfonamide of unresolved 7-amino-2-heptanol exhibited two single peaks of equal intensity, separation 10Hz, in its fluorine NMR spectrum. The chemical shifts and peak separation varied somewhat with the concentration of trifluoroacetic acid used as an internal standard.

The relative amount of the two diastereomers resulting from the reaction of an optically active acid chloride with an alcohol depends on the enantiomeric purity of the acid chloride as well as the alcohol. Since the MTPA purchased from Aldrich is presumably produced by a resolution, it is not safe to assume that it is optically pure. The optical purity of the (+)-MTPA was checked by fluorine NMR analysis of the esters of l-menthol (EKC Chemicals) and 2-octanol obtained from the reduction of 2-octanone with fermenting yeast. The l-menthol is extracted from a natural source and thus is likely to be 100% optically pure; and the reduction of 2-octanone by yeast has been shown (14) to be at least 98% stereospecific. Both of these esters had two peaks in the fluorine NMR spectrum with an area ratio of 94/6, indicating that the (R)-MTPA chloride was only 88% optically pure. This was taken into account in determining the enantiomeric purity of the resolved aminoalcohols.

The fluorine NMR spectrum of the (R)-MTPA esters of (-)-7-amino-2-heptanol and (+)-7-amino-2-heptanol showed two peaks with the area ratios of 80/20 and 81/19 respectively. The downfield peak was the major one in the ester of (-)-7-amino-2-heptanol; the upfield one was the major peak in the spectrum of the (+)-7-amino-2-heptanol ester. Correcting for the enantiomeric purity of the acid chloride, we calculated that the (-)-7-amino-2-heptanol was 68% optically pure (84/16 ratio of enantiomers) and the (+)-7-amino-2-heptanol was 70% optically pure (85/15 ratio of enantiomers). In the following discussions (-) and (+)-7-amino-2-heptanol refer to the compounds of these enantiomeric purities.

Optical Rotations of the Resolved 7-amino-2-heptanols

The optical rotatory dispersion curve of the (-)-7-amino-2-heptanol obtained from the resolution with d-tartaric acid is shown in Figure 14. The (+)-7-amino-2-heptanol gave a similar curve. By using the optical purities of these aminoalcohols, the specific rotation for 100% optically pure 7-amino-2-heptanol was calculated to be 11.3° and the molecular rotation to be 14.8° at 589nm and 20°. This can be compared with the molecular rotation of (+)-2-heptanol which has been reported (31) to be +12.0° at 20°.

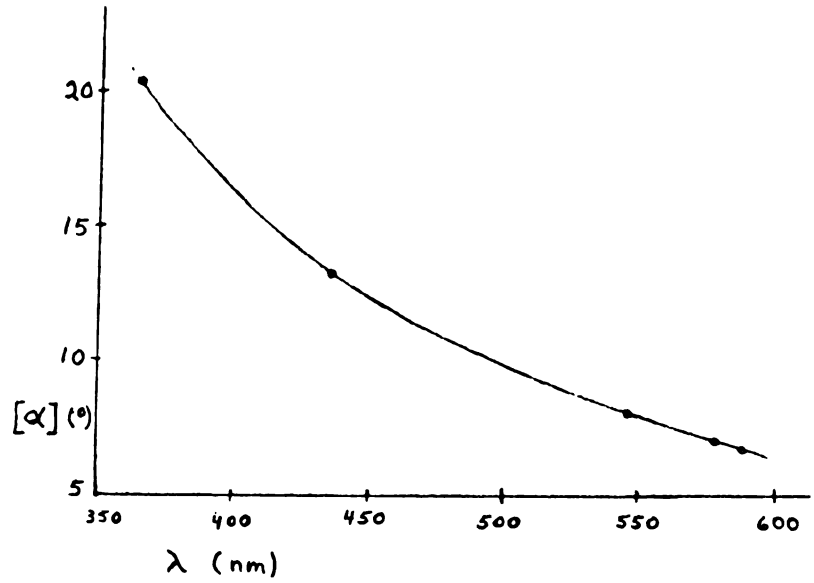


Figure 14.--ORD curve of (-)-7-amino-2-heptanol.

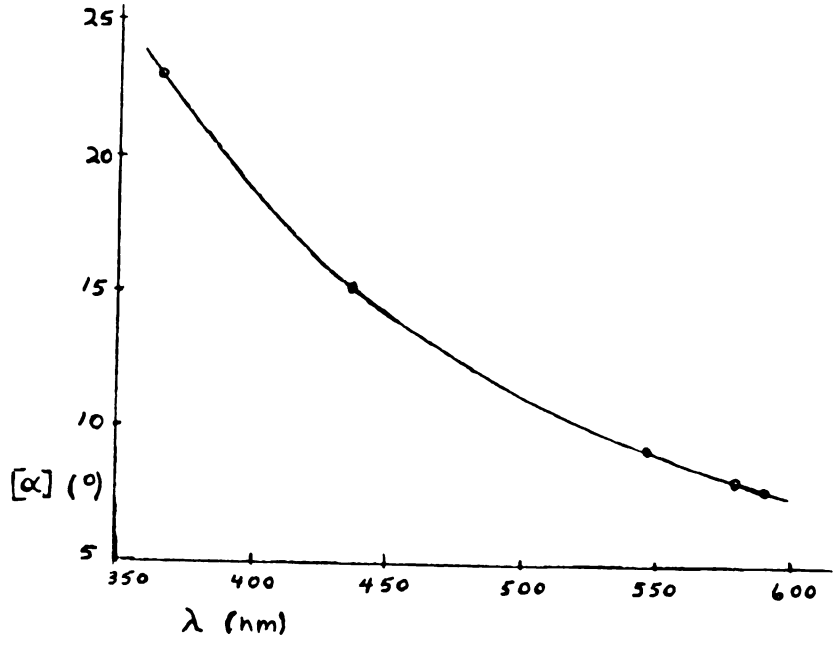


Figure 15.--ORD curve of (-)-N-(6-hydroxy-1-heptyl)-nicotinamide.

Absolute Configurations of (-)- and (+)-7-amino-2-heptanol

The absolute configurations of the methyl n-alkyl carbinols have been established as being (S):(+) and (R):(-) (32,33). The absolute configuration of the resolved 7-amino-2-heptanols could be assigned by reductively deaminating them to 2-heptanol and correlating the optical rotations. Very few methods have been reported for carrying out this type of reaction, but use was made of the procedure described by Nickon and Hill (26) wherein the amine was first converted to its sulfonamide and then reductively deaminated with hydroxylamine-O-sulfonic acid in basic solution. For this reaction the (-)-7-amino-2-heptanol obtained from a second crop of the d-tartrate was used since it was only necessary to have an excess of one enantiomer for the determination of the sign of rotation of the 2-heptanol product. Since the 2-heptanol isolated from the reaction mixture had a specific rotation at 589nm of -6.43°, the (-)-7-amino-2-heptanol has the R configuration and the (+)-7-amino-2-heptanol has the S configuration.

As a further indication that these configurational assignments are correct, it was noted that with all of the methyl alkyl carbinols studied in the experiments of Mosher (30) and the present work, the ester diastereomer

made with the (S)-alcohol and (R)-MTPA chloride produced the trifluoromethyl peak with a lesser downfield shift than the one made with the (R)-alcohol. This NMR method might prove useful in assigning absolute configurations to optically active alcohols and amines.

Resolution of 6-amino-2-hexanol

The reaction of 6-amino-2-hexanol with d-tartaric acid and (+)-mandelic acid produced oils that could not be crystallized. The 6-amino-2-hexanol did form a crystalline salt with d-10-camphorsulfonic acid, but after six recrystallizations the recovered aminoalcohol was still nearly racemic. Considerable difficulty was encountered in recovering the aminoalcohol from the salt in good yield. Apparently 6-amino-2-hexanol is more soluable in water than in most organic solvents; salting out and numerous extractions were necessary to recover a reasonable amount of the aminoalcohol.

Better success was obtained by using dibenzoyl-d-tartaric acid as the resolving agent. Several grams of partially resolved 6-amino-2-hexanol were obtained which had a specific rotation of -2.6° at 598nm. Analysis of the enantiomeric purity of this product by the fluorine NMR method indicated it to be 34% optically pure (67/33 ratio of enantiomers).

Synthesis of Optically Active N-(6-hydroxy-1-heptyl)-nicotinamides

(+)-, (-), and (d,l)-N-(6-hydroxy-1-heptyl)-nicotinamides were prepared from the resolved aminoalcohols with the same sign of optical rotation. The products were identified by their NMR and IR spectra. Synthesis of these compounds involved protection of the alcohol group of the aminoalcohol by formation of its trimethylsilyl ether and subsequent hydrolysis of the ether. The possibility that racemization occurs in this reaction can be eliminated on the basis of experiments done with O^{18} exchange (34) and optically active dimethyldi-d-2-butoxysilane (35) which demonstrated that hydrolysis of silyl ethers proceed with breakage of the Si-O bond giving alcohols with retention of configuration. It can be assumed then that the enantiomeric purities and absolute configurations of the N-(6-hydroxy-1-heptyl)-nicotinamides are the same as the 7-amino-2-heptanols from which they were made.

An optical rotatory dispersion curve of the (-)-N-(6-hydroxy-1-heptyl)-nicotinamide (predominately R configuration) is presented in Figure 15. Assuming an optical purity of 68%, the $[\alpha]_D^{20°}$ for 100% (R)-N-(6-hydroxy-1-heptyl)-nicotinamide is -9.98° and the molecular rotation is -23.5°.

Preparation of the NAD^+ Analogs

The NAD^+ analogs (I), (IR), and (IS) were prepared by using the procedures developed by Kaplan and Ciotti (36), wherein pig brain NADase catalyzes the exchange of the nicotinamide portion of the coenzyme as shown in Figure 16. Excess amounts of NAD^+ were used to drive the equilibrium to greater production of the analog. After separating the protein from the products, analysis of the product mixture by TLC on polyethyleneimine cellulose plates with fluorescence indicator (Brinkman) using 0.15M aqueous ammonium chloride solution as the developing solvent showed two spots of about equal size, Rf 0.50 and 0.37, and two smaller spots with Rf 0.045 and Rf 0.023.

The NAD analogs were isolated by anion exchange chromatography on polyethyleneimine cellulose as described in the Experimental section. A UV absorption-time trace of one column separation is shown in Figure 17. The first two fractions off the column were nicotinamide and N-alkylnicotinamide. The fourth fraction was identified as NAD^+ by thin layer chromatography against a known sample of NAD^+ and by its rate of reduction by ethanol with LADH. Fraction number three was identified as the NAD^+ analog. This fraction had a peak in the UV spectrum at 260nm, as does NAD^+, and formed an addition compound with cyanide ion as evidenced by a decrease in the absorbance at 260nm

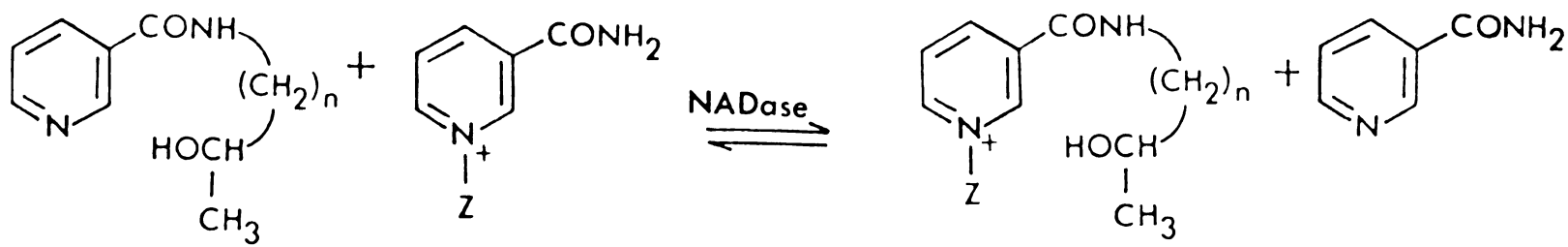


Figure 16.--Preparation of NAD^+ analogs.

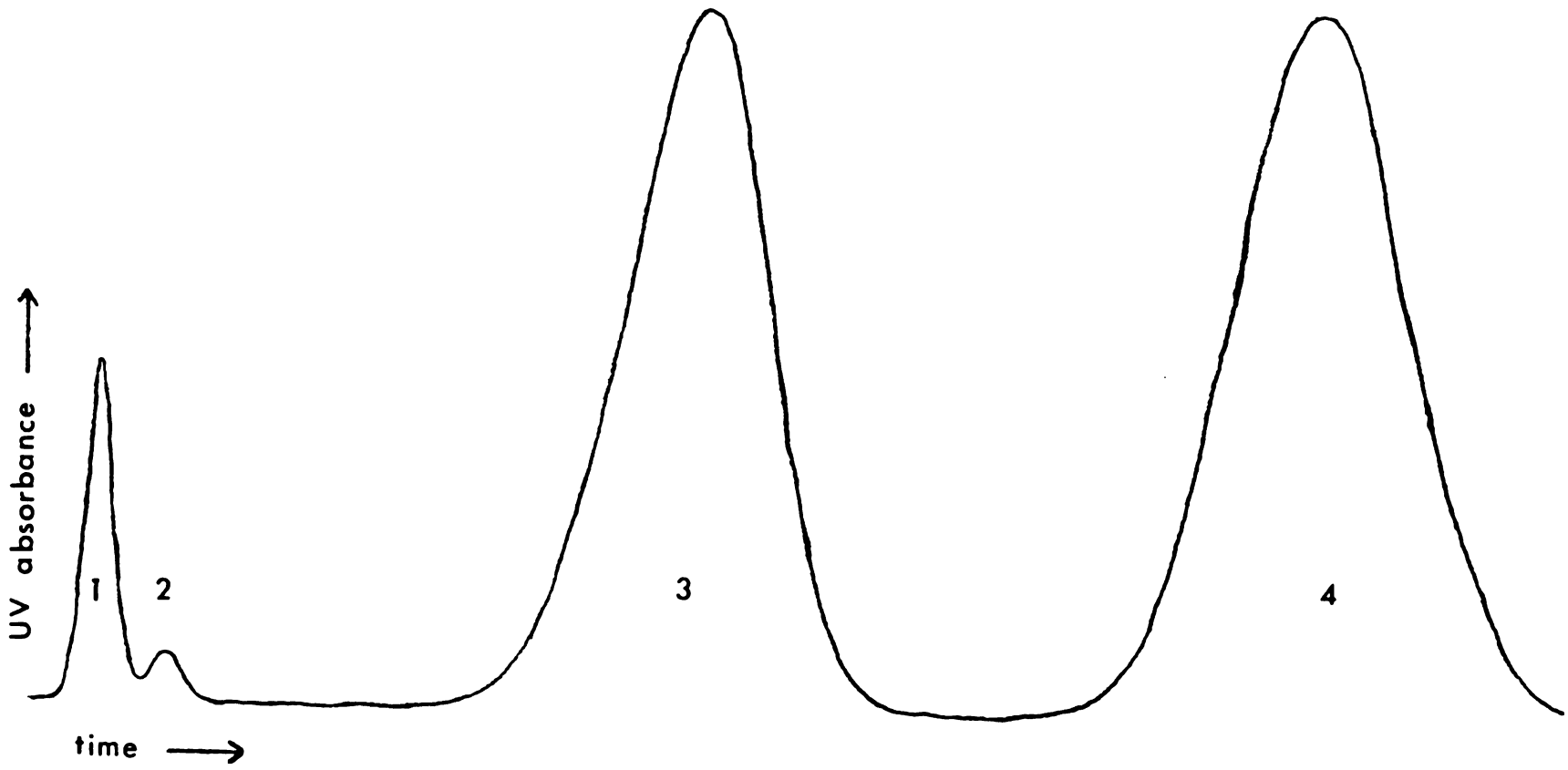


Figure 17.--Column separation of NAD^+ analog.

and the appearance of a new peak at 325nm. The formation of cyanide complexes is characteristic of pyridine nucleotides (37). Like the natural coenzyme, the analog also reacted with ethanol in the presence of LADH as indicated by the appearance of a new maximum in the UV spectrum at 340nm; this reaction, however, was much slower than reaction with NAD^+.

Activity of the NAD^+ Analogs in Enzyme Systems

The utility of any model system to provide insights into the functioning of a natural system depends on its ability to mimic that system in most aspects of its mechanism of action. In the case of the NAD^+ analogs that this study is concerned with, maximum usefulness as a model system requires that the analogs become bound to the enzyme at the active site and that they function as coenzymes, that is, a hydrogen from an alcohol substrate is transferred to the pyridine ring of the analog. Also, to allow any definite conclusions to be drawn about the stereochemical relationships in the alcohol dehydrogenase active site, the alcohol portion of the analog must be accepted by the enzyme as a substrate and finally, the oxidation-reduction reaction should be intramolecular--one molecule of the analog should serve as both coenzyme and substrate.

To determine whether the first two of these criteria are met, the ability of the N-(6-hydroxy-1-heptyl)-NAD^+ analog to serve as a coenzyme in the oxidation of ethanol with LADH and YADH was tested. It was found that the analog is reduced by ethanol in the presence of LADH but at a much slower rate than the natural coenzyme under comparable reaction conditions (the reaction with the analog took two hours to reach equilibrium, the reaction with NAD^+ achieved equilibrium within a few minutes). Whether this difference in rates is due to a lesser ability of the enzyme to bind the analog than the NAD^+, or to a diminished rate at some other step in the reaction sequence could not be concluded from these experiments. A factor to be borne in mind when considering this question is that it has been determined (38,39) that the rate-determining step in alcohol dehydrogenase catalyzed oxidation of primary alcohols is the dissociation of the reduced coenzyme from the enzyme. In contrast to the results with LADH, the NAD analog (I) showed no activity with the ethanol-YADH system. Perhaps this inability of YADH to use the analog as a coenzyme is a manifestation of a more "hindered" enzyme active site as proposed (40) to account for the greater substrate specificity and stereospecificity of yeast alcohol dehydrogenase over that of the dehydrogenase obtained from horse liver.

The equilibrium constant for the overall reaction:

$$\text{ethanol} + \text{OXD} \rightleftharpoons \text{acetaldehyde} + \text{RED} + H^+$$

where OXD and RED are the oxidized and reduced forms of the coenzyme, was smaller for the analog than for NAD^+ as the coenzyme. At pH 10, ionic strength 0.04, and 23°, the equilibrium constant with NAD^+ was found to be 8.86×10^{-12}. This is in good agreement with the reported (41) value of 9.2×10^{-12} at 23°, ionic strength 0.1, considering that the equilibrium constant decreases with ionic strength (42). Under the same conditions, the calculated equilibrium constant for the NAD^+ analog (I) system was 9.05×10^{-13}, a factor of about ten less than the equilibrium constant with NAD^+. Thus, there appears to be a thermodynamic effect as well as a kinetic effect on the enzyme-catalyzed reaction when the analog is substituted for the natural coenzyme.

Having determined that the NAD^+ analog (I) could function as a coenzyme with LADH, the next important question to be investigated was whether or not the alcohol group on the alkyl side chain of the analog could serve as a substrate for LADH with the analog as the coenzyme. Buffered solutions of the analogs (I), (IR), and (IS) were incubated with LADH and the reaction was followed by UV spectrophotometry as described in the Experimental section.

At pH 7.5 there were no discernable changes in the UV spectrum with time for any of the systems. At pH 10, however, small increases (about 0.01 absorbance units) were observed in the spectrum at 340nm and decreases at 260nm, the greatest increases being observed in the (IS) system and the least in the (IR) system. NAD^+ isolated on the column from the analog synthesis reaction, when subjected to the same reaction conditions, showed no or very slight changes in the UV spectrum, implying that the reaction observed with the analogs was not due to some impurity in the system. The increase in the UV absorption at 340nm of the reaction mixture containing the analog (IS), after thirty hours, amounted to approximately 10% of the amount observed in the reaction of the analog with excess ethanol. Although these reactions were slow and small in magnitude, the results do allow the conclusion that the substrate-coenzyme analog does undergo oxidation-reduction in the presence of liver alcohol dehydrogenase. It is significant that the analog with the majority of the molecules having the S configuration at the alcohol center exhibited an amount of activity with the enzyme at least four times as great as the amount observed with the analog consisting of mainly the R configuration. That this reaction was not detected at pH 7.5 is not surprising, since the equilibrium

amount of reduced coenzyme depends on the pH (one of the products of the reaction is H^+).

In an attempt to enhance the observed amount of reaction, thereby lessening the uncertainty of the results, the column fractions containing the analog and NAD^+ were concentrated by two different methods. One method was lyophilization. By this process more concentrated solutions of the analogs could be prepared, but a possible disadvantage of this method is that it also concentrates the small amount of ammonium bicarbonate that was used in the column eluant. The pH's of the enzyme reaction solutions prepared from the lyophilized analogs were only slightly reduced by the ammonium bicarbonate; the ionic strengths, however, were certainly greater. Using the lyophilized analogs, the magnitude of the spectral changes of the enzyme incubation mixtures were considerably enhanced. At pH 10 the reduction of the pyridine ring of the analog (IS) was apparent within three hours after the addition of the enzyme; after twenty-four hours there was an increase in the UV absorbance at 340nm of 0.05 absorbance units, corresponding to a concentration of reduced coenzyme of about 8×10^{-6}M. Even at pH 7.5 a small but definite reaction was evident using the lyophilized analog (IS). The analog (IR) on the other hand, exhibited only a very small amount of reaction with LADH after twenty-four hours at pH 10,

measured on a solution of comparable concentration. The lyophilized NAD^+ also showed a small reaction, but considering that NAD^+ is a much more effective coenzyme for LADH than the analog as indicated by the results with the oxidation of ethanol, this reaction is probably amplified relative to the amount of reaction that this apparent impurity would cause in the analog system. Addition of ethanol to the analog reaction mixture after 24 hours of incubation brought about further increases in the UV absorbance at 340nm, evident within a few minutes. This indicates, for one thing, that the enzyme was still active at this time, but more significantly, that at least a good portion of the analog molecules were still intact to function as a coenzyme--no apparent decomposition of the analogs had occurred in the lyophilization or under the reaction conditions.

Another method of concentration was employed in an attempt to circumvent any artifacts that could have produced spurious results in the analog systems prepared by the lyophilization process. The analog solutions were concentrated by molecular filtration using a membrane which retained most of the materials with a molecular weight greater than 800. After the initial concentration, the solutions were twice diluted with pH 8.8 buffer solution and reconcentrated, a process by which the residual ammonium bicarbonate was removed. The analog (I)

solutions prepared in this manner also exhibited activity with LADH over that of NAD^+ solutions prepared in the same way and used as a reference. The results with the analogs (IS) and (IR) again demonstrated the greater activity of the analog (IS). The NAD^+ concentrated by molecular filtration appeared to retain full activity, oxidizing ethanol at a rate comparable to NAD^+ not subjected to this treatment.

The results of the experiments to this point have demonstrated that the substrate portion of the substrate-coenzyme analogs is oxidized by the coenzyme portion with the aid of liver alcohol dehydrogenase. This implies that both the coenzyme and substrate binding sites of the enzyme are able to accommodate the appropriate portions of the analog molecule and conduct them through the steps in the catalytic mechanism. However, one more condition must be met for the differences in the reactivity of analog (IS) and analog (IR) to be used as evidence for a particular model of coenzyme-substrate spatial relationship and that is the oxidation-reduction reaction of the analog must be intramolecular. There remains the possibility that the observed reduction of the pyridine ring of the coenzyme analog was accompanied by the oxidation of the hydroxyl substrate portion of another molecule of the analog. If this were the case, the greater activity of the analog (IS) over that of analog (IR) would only be

another confirmation of the propensity of alcohol dehydrogenase to react with the S enantiomer of methyl alkyl carbinols; it would not answer the question of how the substrate is aligned with respect to the coenzyme at the active site.

In previous work done in this laboratory by Jelmer Miedema (23) with the NAD^+ analogs (IIa,b,c,d) (see Figure 18), it was found that the analogs with alkyl side chains of five and six carbon atoms (IIc and IId) displayed some activity with LADH, while those with shorter alkyl side chains (IIa and IIb) showed very little if any reactivity. At the time it was felt that these results supported an intramolecular mode of reaction; a carbon chain of sufficient length would be necessary for it to be able to "fold back" to bring the hydroxyl-bearing carbon in position for the direct transfer of hydrogen to the nicotinamide ring. Although this argument is plausible, it is possible that some alternative explanation exists involving some other factor causing the same reactivity differences in an intermolecular reaction.

We decided to test the possibility that the NAD^+ analogs were reacting in an intermolecular fashion by observing the result of adding NAD^+ to the reaction mixture. If the oxidation-reduction reaction were intermolecular, we anticipated that the rate and/or extent of

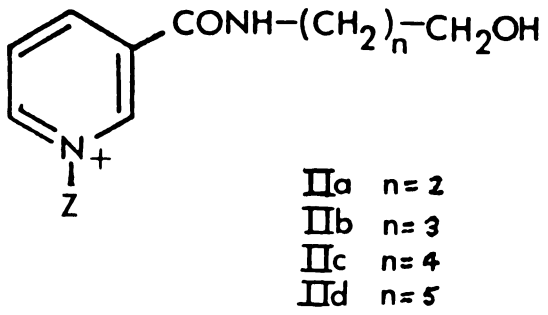


Figure 18.--NAD^+ analogs (II).

reaction would be increased as a result of the presence of a greater number of oxidizing species which are more active as coenzymes than the NAD^+ analog. If on the other hand the reaction of the analog were intramolecular, we expected that the addition of NAD^+ would not increase the amount of reaction, and would possibly decrease the reaction rate by competing with the analog for the coenzyme binding sites of the enzyme. The effect of adding NAD^+ to the analog incubation mixtures was determined in three different systems. In one experiment 0.50ml of analog (I) solution from the column separation and 0.50ml of column solution of NAD^+ were added to 2.0ml of pH 10 buffer to make a solution with the total coenzyme concentration nearly equal to a solution made with 1.0ml of analog fraction. A third solution made with 1.0ml NAD^+ solution was used as a blank. The three solutions were simultaneously incubated with LADH. Another experiment made use of the concentrated solutions of the analog (I)

obtained by molecular filtration to make mixtures of analog and NAD^+ that would produce concentration changes less subject to the limitations of the UV detection system. In this experiment pH 8.8 buffer solutions were used and the concentration of NAD^+ in the reaction solutions was 3.5×10^{-4}M. A third experiment was done using the solutions of lyophilized analog (IS) and analog (IR) in pH 10 buffer. The effects of NAD^+ addition on these two stereoisomers were compared.

The results were the same in all three methods: the addition of NAD^+ to the reaction mixture caused large increases in the amount of observed reaction. The mixtures containing both analog (I) and NAD^+ column solutions produced more reduced coenzyme in a given time span than either one alone. The solution containing the analog concentrated by molecular filtration produced in thirty hours time an amount of reduced coenzyme equal to 8.5% of the amount of oxidized coenzyme at the start of the reaction; the analog alone reacted to the extent of 2-3% in this time. The results with the lyophilized analogs demonstrated a pronounced stereoselectivity in the reaction. After 24 hours the analog (IS) solution had an increase in the UV absorbance at 340nm of 0.55 absorbance units; the analog (IR) solution had an increase of only 0.08 absorbance units. Clearly, the NAD^+analogs are supplying the substrate for enzymatic reduction by the NAD^+ coenzyme.

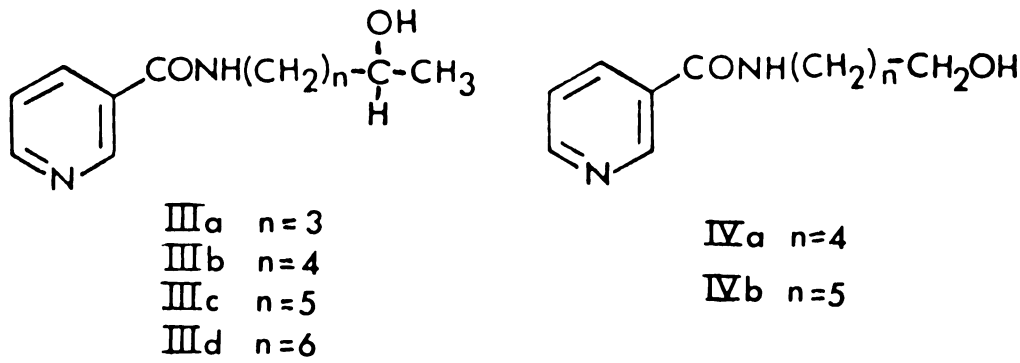
These results suggest that the small amount of reaction observed with the analogs and enzyme alone may be due at least in part to an intermolecular oxidation-reduction.

Substrate Specificities and Stereospecificities of Liver Alcohol Dehydrogenase

A few comments on the likelihood of LADH being able to use the large NAD^+ analog molecule as a substrate seem in order. Horse liver alcohol dehydrogenase is known to bind large molecules such as pentadecanoate anion (15) and reduce bulky ketones such as 3β-keto-5β-cholanic acid(43). In fact, for many series of related compounds, the tightness of binding to LADH increases with increasing carbon chain length for chains with up to fifteen carbon atoms. Hansch and coworkers (44) present a good review and discussion of this "hydrophobic effect." Recent x-ray crystallographic determinations of the structure of LADH (45) suggest that the active site of the enzyme is a deep cleft or groove in the molecule. A substrate binding site of this type could accommodate a rather large molecule. As a test case more relevant to the present research, it was thought that if the NAD analogs were being oxidized and reduced intermolecularly with LADH, the enzyme ought also to act on N-(6-hydroxy-1-heptyl)-nicotinamide. This was tested by adding LADH to solutions

that were 1.0×10^{-3}M in the nicotinamide and 5.2×10^{-4}M NAD^+ in pH 10 and pH 7.5 buffers. At pH 10, after eighteen hours of incubation, 43% of the NAD^+ had been reduced to NADH. At pH 7.5 reduction of the NAD^+ was also evident. It is apparent that N-(6-hydroxy-1-heptyl)-nicotinamide is a possible substrate for liver alcohol dehydrogenase. The substrate ability of the (hydroxyalkyl)-nicotinamide lends support to the idea that two molecules of the NAD^+ analog could be involved as substrate and coenzyme in the enzymatic reaction, but also raises the possibility that the reaction observed in the analog systems could be due to decomposition of the analog molecule by cleavage at the nicotinamide-ribose bond to give a (hydroxyalkyl)-nicotinamide which could then act as a substrate for the enzyme-analog complex.

If the whole coenzyme-substrate analog or a portion of it operates as the substrate in an intermolecular reaction with another molecule of the analog serving as the coenzyme, as the results discussed so far seem to indicate, one might return to the question of why no activity was observed in the NAD^+ analog (IIa,b) systems and in the analogous secondary-hydroxyl systems. In an attempt to provide an answer to this question, the reactivity of the series of N-alkylnicotinamides IIIa,b,c,d and IVa,b (see Figure 19) as substrates for LADH with NAD^+ as the coenzyme was determined.



IIIa n=3
IIIb n=4
IIIc n=5
IIId n=6

IVa n=4
IVb n=5

Figure 19.--N-(hydroxyalkyl)-nicotinamides.

The reaction solutions were 3.50×10^{-4}M NAD, approximately 1.0×10^{-3}M nicotinamide, and buffered at pH 10. All of the nicotinamides displayed activity as substrates. Under the same experimental conditions, the relative amount of NADH produced in ten minutes from the oxidation of the nicotinamides IIIa, IIIb, IIIc, and IIId was 1/3/27/110 which gives an idea of the relative rates of reaction. Equilibrium was established at 30° within 36 hours, less for the faster reacting systems. The equilibrium constants for the overall reaction were calculated and are presented in Table 7. The results were significantly different in the primary alcohol systems IVa and IVb. They reacted so rapidly that substantial reaction had occurred between addition of the enzyme and recording of the initial UV spectrum. These systems reached equilibrium in about

TABLE 7.--Equilibrium constants for the oxidation-reduction of N-(hydroxyalkyl)-nicotinamides.

Substrates	[RCHOHR']	[NADH]	K
IIIa	1.06×10^{-3}M	3.46×10^{-5}M	3.8×10^{-3}
IIIb	1.08×10^{-3}M	1.82×10^{-4}M	2.2×10^{-1}
IIIc	1.03×10^{-3}M	2.62×10^{-4}M	1.0
IIId	1.01×10^{-3}M	2.75×10^{-4}M	1.4
IVa	9.95×10^{-3}M	8.37×10^{-5}M	2.9×10^{-2}
IVb	1.00×10^{-3}M	9.49×10^{-5}M	3.9×10^{-2}

twenty minutes; the equilibrium constants, however, were smaller than those of all but one of the slower-reacting secondary alcohol systems (see Table 7).

The results of the experiments with the N-(hydroxyalkyl)-nicotinamides suggest that a relationship exists between reactivity with LADH and the length of the alkyl side chain of the nicotinamide. This trend in reactivities may account for the greater activity of the analogs (I), (IIc), and (IId) over that of the analogs (IIa) and (IIb) if the same factors are assumed to be operating. The equilibrium constants in Table 7 are a measure of the free energy difference between the hydroxy form and the keto form of the N-substituted nicotinamides. Although the elements operating on the free energies of substances are numerous, it is not obvious why there should be such a

large variance in the free energy differences among the alkylnicotinamides that differ only in the number of methylene groups in the alkyl chain. One explanation for these differences is offered here. The N-(hydroxyalkyl)-nicotinamide can exist in an intramolecularly hydrogen-bonded form such as pictured in Figure 20. This stabilized structure is lost when the alcohol group is oxidized to the carbonyl in the enzyme reaction. The stability of the hydrogen bonded structure could vary considerably with the size of the ring, consequently shifting the equilibrium between the oxidized and reduced states of the molecule.

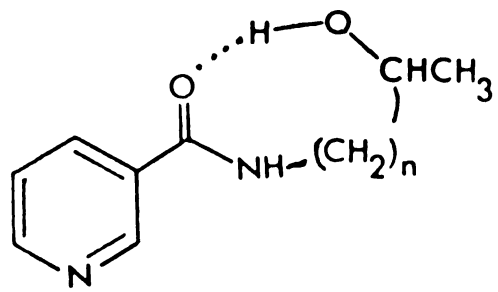


Figure 20.--Possible intramolecular hydrogen bonding.

The aminoalcohols: $CH_3CHOH(CH_2)_nNH_2$ where n= 3,4,5,6 were also tested as substrates for LADH, and a similar correlation of reactivity to alkyl chain length was found. Over the first twenty minutes of reaction, the relative reaction rates were about 1.0/1.5/2.0/34 for

n=3,4,5, and 6 respectively. The equilibrium constants at 30° are reported in Table 8. Except for the eight carbon aminoalcohol, the equilibrium constants were smaller for the aminoalcohols than the corresponding nicotinamides. Perhaps this is a reflection of greater hydrogen bonding stabilization in the aminoalcohols, but certainly there are other factors operating in the equilibrium.

TABLE 8.--Equilibrium constants for the oxidation-reduction of aminoalcohols.

aminoalcohol	[RCHOHR']	[NADH]	K
5-amino-2-pentanol	1.00×10^{-3}M	1.37×10^{-5}M	5.6×10^{-4}
6-amino-2-hexanol	1.05×10^{-3}M	2.09×10^{-5}M	1.3×10^{-3}
7-amino-2-heptanol	1.02×10^{-3}M	5.22×10^{-5}M	9.0×10^{-3}
8-amino-2-octanol	1.02×10^{-3}M	3.06×10^{-4}M	3.0

In the reactions of liver alcohol dehydrogenase with analog (IS) and analog (IR), a marked stereoselectivity was observed. It was relevant to the interpretation of these results to determine if this stereoselectivity also existed in the reaction of LADH and NAD^+ with N-(6-hydroxy-1-heptyl)-nicotinamide (IIIc) and with 7-amino-2-heptanol. Solutions of known concentration of

(+), (-), and (d,l) 7-amino-2-heptanol and N-(6-hydroxy-1-heptyl)-nicotinamide were incubated with 0.5 units of LADH and 3.47×10^{-4}M NAD^+ in pH 10 buffer at 23°. The reaction with the aminoalcohols was also run at 30°. In all three experiments the (+)-substrate produced NADH at an initial rate about five times greater than the (-)-substrate. The equilibrium amounts of NADH in the (+)-substrate solutions were 2-2.5 times as much as in the (-)-substrate solutions. The rate and equilibrium measurements suggest that the enzyme reacts only with the S enantiomer of the substrate. The slower reactions with the (-)-substrates were probably due to the minor amount of the S enantiomer in the mixture. As discussed previously, the actual concentrations of the S enantiomer of the resolved aminoalcohols and nicotinamides was 85% of the total concentration of the (+) compounds and 16% of the (-) compounds. Assuming that the R enantiomer of the compounds is unreactive with the enzyme, the equilibrium constants for the reactions were calculated using the concentrations of the S components as the substrate concentrations. Tabulation of these results appears in Table 9. The good agreement of the values from the (+) and (-) systems suggests that the assumption that the enzyme is stereospecific for the S enantiomer of both N-(6-hydroxy-1-heptyl)-nicotinamide and 7-amino-2-heptanol is valid.

TABLE 9.--Oxidation-reduction equilibrium in optically active systems.

System	[(S)-RCHOHR']	K
(+)-IIIc	8.95×10^{-4}M	1.73
(-)-IIIc	1.76×10^{-4}M	1.77
(d,l)-IIIc	5.15×10^{-4}M	1.89
(+)-7-amino-2-heptanol	1.25×10^{-2}M	1.44×10^{-2}(23°)
(-)-7-amino-2-heptanol	2.39×10^{-3}M	1.30×10^{-2}(23°)
(+)-7-amino-2-heptanol	1.25×10^{-2}M	1.88×10^{-2}(30°)
(-)-7-amino-2-heptanol	2.39×10^{-3}M	1.79×10^{-2}(30°)

Conclusion

This thesis has described the synthesis of two aminoalcohols and their corresponding nicotinamides. Resolution of these aminoalcohols gave (+)- and (-)-7-amino-2-heptanol and (-)-6-amino-2-hexanol. In the course of this work a general method for the determination of the extent of resolution and the absolute configuration of optically active aminoalcohols was developed. Analogs of nicotinamide adenine dinucleotide were prepared from the optically active 7-amino-2-heptanols and these were used to explore the stereochemical relationships in the active site of alcohol dehydrogenases. It was found that the analog (I) does function as a coenzyme for liver alcohol

dehydrogenase but not for yeast alcohol dehydrogenase. This was interpreted as evidence of a more sterically hindered coenzyme binding site in the yeast enzyme. The ability of the NAD^+ analog to oxidize ethanol when in the presence of LADH indicates that the amino group of the amide portion of the coenzyme is not essential for the coenzyme function. The NAD^+ analog (I), which consists of both a coenzyme and a substrate portion, displayed a small amount of activity with LADH. The analog (IS) exhibited a much greater amount of reaction with LADH than did the analog (IR). If this stereoselectivity represents an intramolecular oxidation-reduction reaction of the substrate and coenzyme portions of the analog, it would provide support for the Karabatsos model for the ternary complex of the enzyme. However, intramolecularity could not be established. In fact, evidence was obtained that an intermolecular mode of reaction is available to the system. It is possible that the observed reaction of the analogs is the oxidation of the N-(hydroxyalkyl)-nicotinamides produced under the reaction conditions by cleavage of the nicotinamide-ribose bond of the analog, since these substituted nicotinamides were also found to serve as substrates for LADH. Stereospecificity for the enantiomers with the S configuration was observed in the reaction of N-(6-hydroxy-1-heptyl)-nicotinamide and

7-amino-2-heptanol with NAD^+ and LADH, confirming the hypothesis that, in substrates with alkyl groups of greatly differing size, the enzyme has some mechanism of discriminating between the two stereoisomers, probably by binding of the larger group to a hydrophobic region. The question of the actual spatial orientation of the substrate and coenzyme in the ternary complex remains unanswered. Determination of the location of the hydrophobic region and other binding sites of the enzyme, perhaps with the aid of other analog systems, would help to clarify this matter. In conclusion, it is hoped that the results of this study will stimulate further research into the elucidation of the structure of the active site of alcohol dehydrogenases and the mechanism by which these structural features control the stereospecificities of enzyme reactions.

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