THE SYNTHESIS AND REACTIVITIES OF OXIDIZED NICOTINAMIDE ADENINE DINUCLEOTIDE ANALOGS WITH LIVER ALCOHOL DEHYDROGENASE

Thesis for the Degree of Ph. D.
MICHIGAN STATE UNIVERSITY
MICHAEL KENNETH MAY
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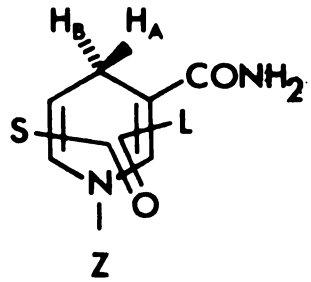
ABSTRACT

THE SYNTHESIS AND REACTIVITIES OF OXIDIZED NICOTINAMIDE ADENINE DINUCLEOTIDE ANALOGS WITH LIVER ALCOHOL DEHYDROGENASE

By

Michael Kenneth May

The stereospecificity of the nicotinamide adenine dinucleotide (NAD^+-NADH) coenzyme oxidation-reduction of carbonyl compounds with the dehydrogenase enzymes has been the topic of considerable study since these reactions were discovered in 1935. Work done initially by Prelog and extended by Karabatsos and other investigators has led to the proposal of a model which might explain the substrate-coenzyme orientation during the oxidation-reduction process (I).



L = larger group

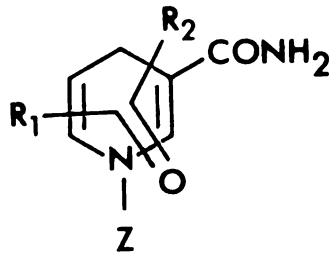
s = smaller group

Z = ribose-phosphate-phosphate-ribose-adenine

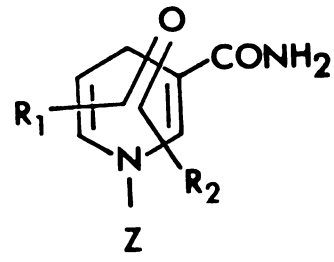
I.

The model, if proven correct, should give information which might allow predictions as to the stereochemistry of the products from the reaction.

One of the questions that must be answered is the spacial relationship of the substrate to the enzyme-coenzyme during the oxidation-reduction process (II, III).

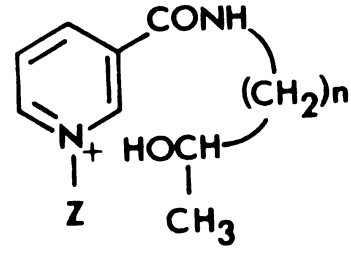


II



III

This question might be answered if the substrate were covalently bonded to the coenzyme in such a manner as to limit possible alignments by denying the substrate its freedom to rotate within its plane of the carbonyl. To accomplish this purpose, oxidized NAD^+ analogs were synthesized (IV).



n = 3-8

IV

Several of the analogs prepared react in a manner similar to the parent system but at a relatively slower rate when reacted with horse liver alcohol dehydrogenase (liver ADH, E.C. 1.1.1.1.). The most reactive ones are those with n = 5 and 7.

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Submitted to

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1976

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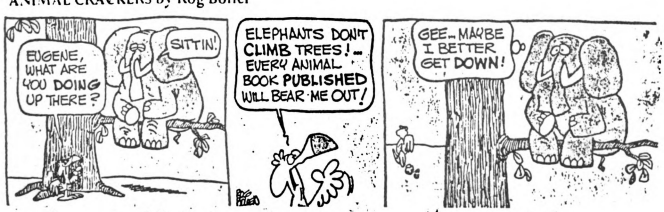

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INTRODUCTION

The naturally occurring pyridine nucleotides nicotinamide adenine dinucleotide (NAD^+) and its phosphoric acid derivative ($NADP^+$), with their reduced forms (NADH and NADPH, respectively), are the coenzymes in over a hundred enzyme catalyzed reactions. Whereas the structure of the pyridine nucleotides was postulated as early as 1931 and the chemical principles connected with the biological oxidation reactions were discovered by Warberg et al.[1] in 1935, it was not until 1957 that the structure was confirmed through synthesis by Todd.[2] The structure of the oxidized form of nicotinamide adenine dinucleotide is shown in Figure 1.

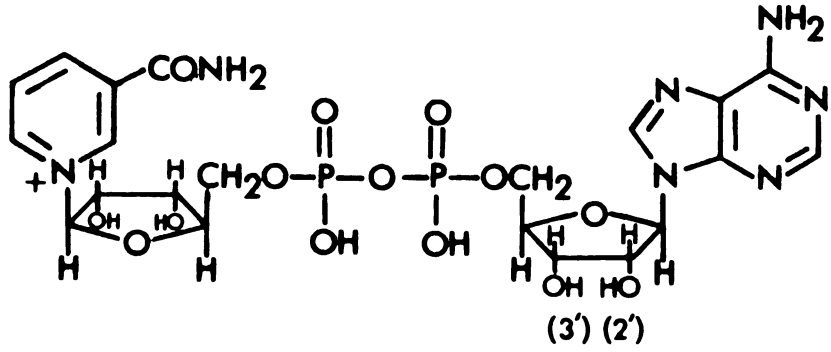
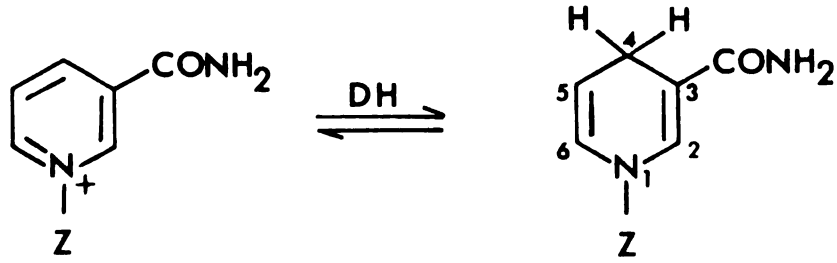


Figure 1. - Structure of Oxidized Nicotinamide Adenine Dinucleotide

The presence of a phosphoric acid group at the 2'-position of the ribose in the adenylic acid moiety is the only difference between $NADP^+$ and NAD^+. With respect to reactions and properties associated with the nicotinamide moiety the two coenzymes are identical.

The nicotinamide adenine dinucleotides are important participants in many biological oxidation-reduction reactions. Dehydrogenase enzymes (DH) catalyze the reversible electron transfer - as a proton or hydride from various substrates to the C-4 position of the nicotinamide ring of

these cofactors to produce the reduced 1,4-dihydronicotinamide structures of NADH or NADPH (Figure 2).



(z = ribose-phosphate-phosphate-ribose-adenine)

Figure 2. - Reversible oxidation and reduction of NAD^+-NADH

These enzyme catalyzed hydrogen transfers are stereospecific in that the enzymes differentiate between the pair of hydrogens at C-4 of the 1,4-dihydronicotinamide ring. Biological stereospecificity toward enantiotopic and diastereotopic hydrogens was first observed and investigated in the enzymatic reaction between yeast alcohol dehydrogenase (YADH), NAD^+, and ethanol by Leowus, Westheimer, and Vennesland.[3] These investigators found that the 1-deuterioethanol formed by the YADH catalyzed reduction of 1-deuterioacetaldehyde by NADH was distinguishable from the 1-deuterioethanol product formed by the enzymatic reduction of unlabeled acetaldehyde by NADD (the reduced form of nicotinamide adenine dinucleotide with deuterium at C-4 of the nicotinamide moiety). The two products could be clearly differentiated by running the reverse enzymatic reaction, the oxidation of ethanol by NAD^+ in the presence of YADH, the 1-deuterioethanol formed enzymatically from 1-deuterioacetaldehyde yielded non-deuterated NADH and 1-deuterioacetaldehyde; the other 1-deuterioethanol sample yielded NADD. The reaction sequence is summarized in Figure 3.

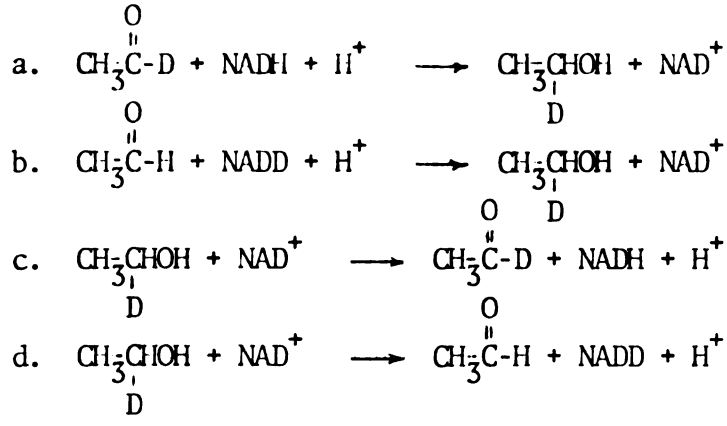


Figure 3. - Enzymatic oxidation-reduction of Acetaldehyde-1-d

Although the results of Westheimer and co-workers established that the YADH reaction displayed stereospecificity toward the pro-chiral acetaldehyde and ethanol, they did not establish the stereochemical course of the reaction.

The absolute configuration of 1-deuterioethanol obtained from the reaction of YADH, NADH and acetaldehyde-1-d was established by Levy and workers[4] who found that the 1-deuterioethanol formed was levorotatory, and by the imaginative and definitive stereospecific synthesis by Lemieux and Howard[5] of (+) R-1-deuterioethanol. These results require that an electron from NADH be transferred onto the re-face of acetaldehyde and in the reverse reaction the pro-R hydrogen of ethanol to be transferred to NAD^+ (Figure 4). Similar experiments with other enzymatic preparations

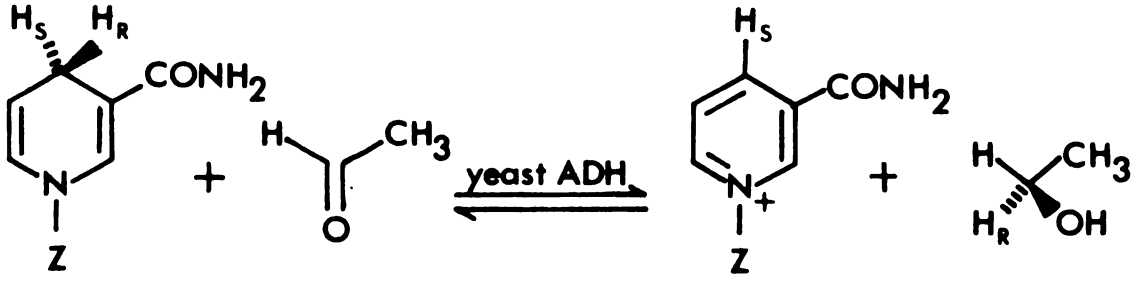
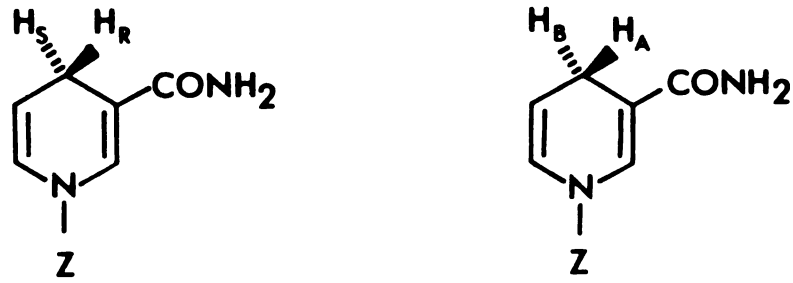


Figure 4. - Stereochemical course of YADH, NADH,

Acetaldehyde enzymatic reaction

have established that biological stereospecificity toward the diastereotopic hydrogens at C-4 of the 1,4-dihydronicotinamide ring of NADH (and NADPH) is not a unique property of YADH but a general enzymatic phenomenon.[6,7,8] These biologically stereospecific enzymatic reactions were classified as "Class-A", having the same stereospecificity as YADH; or "Class-B", having opposite stereospecificity of YADH[9] (Figure 5). The absolute configuration of the pro-R and pro-S hydrogen (Figure 5) of the dihydronicotinamide ring of NADH was established in 1964



(I) (II)

Figure 5. - Absolute configuration of the pro-R* and pro-S* hydrogens of NADH

by Cornforth and co-workers.[11]

Prelog, in studying the requirements of the enzyme active site and substrate orientation, determined the configuration of the product alcohols formed from a variety of reductions of cyclohexanones and decalones with NADH and enzymes (Class B) isolated from Curvularia falcata.[6,12] On the basis of the compounds used in these studies Prelog postulated a

---

*There is considerable disagreement in the literature as to the labelling of the paired methylene hydrogen at C-4 of the 1,4-dihydronicotinamide ring. A more descriptive - and correct - labelling would be the method developed by Hanson[10] shown in Figure 5-I. For simplification in describing those hydrogens acted upon by Class-A enzymes (H_A) or Class-B enzymes (H_B), the alternate notation in Figure 5-II will be used throughout.

model which was to define the spacial requirements of the substrate to the binary coenzyme-enzyme complex (Figure 6).

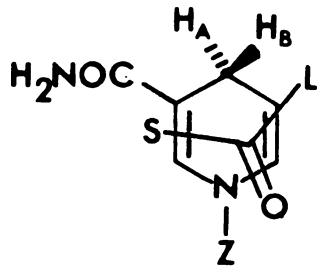
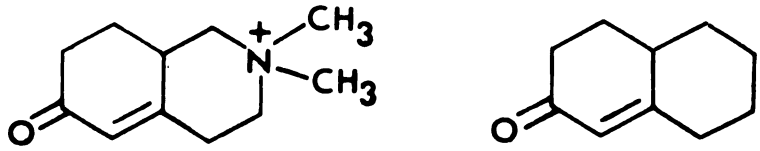


Figure 6. - Prelog Model for substrate-NADH orientation with Class B enzymes (L=larger group, s=smaller group)

Prelog justified his model by noting that steric interactions between coenzyme and substrate would be smaller when the smaller group, "s", was over the carbamido group and the carbonyl pointed down toward the pyridinium nitrogen of the dihydronicotinamide moiety, as proposed by Kosower.[13,14] He predicted that A-type enzymes would lead to products of opposite configuration, as the position of "s" and "L" would have to change.

Kosower proposed the model with the carbonyl pointed down toward the pyridinium nitrogen in order to explain the observed shift in absorption from 340 nm for the unbound NADH to 325 nm for the bound NADH-horse liver alcohol dehydrogenase (LADH) complex (this observation had also been noted previously by Kaplan[15] and Theorell[16]). He suggested that a positive charge, in close proximity (ca. 3Å) to the nitrogen of the dihydropyridine ring, could account for the hypsochromic shift, as observed with model compounds A and B below (Figure 7).[14] The calculated increase in the energy of the $\pi \longrightarrow \pi^*$ transition in the presence of an alkylammonium ion, hydrogen bonded to two groups as shown



A B

λ_{max} = 2210 Å λ_{max} = 2380 Å

Figure 7. - Kosower's model compounds used to compare hypsochromic shift to NADH

in Figure 8, corresponds closely to the observed energy change.[14]

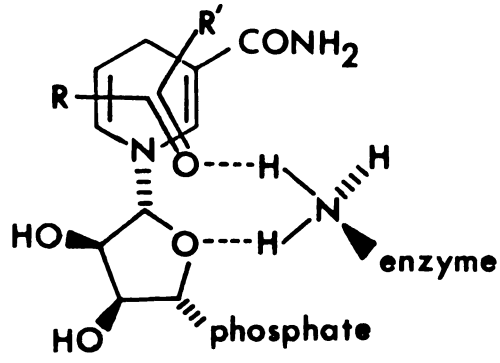


Figure 8. - Kosower's postulate for carbonyl alignment in binary complexes

Support for much of this work is based only on identification of products obtained from the previously mentioned reactions, and by analogy to the absorption spectra of reduced and oxidized dihydropyridines and pyridinium salts. It is evident therefore that the model having the carbonyl group pointed downward toward the nitrogen of the ring is not based upon the best evidence. From his model studies, Prelog further suggested that the position of the substrate groups would be controlled by

their hydrophilic-hydrophobic interactions with the enzyme. This suggestion had been made earlier by several investigators.[3,4,15]

Reflecting upon the results obtained by Levy[4] on the enzymatic reduction of acetaldehyde-1-d with NADH and YADH, Karabatsos pointed out the contradictory results obtained by applying the Prelog model in this case. He suggested[17] that the carbamido group of the coenzyme played no role in determining the stereochemistry of the product alcohols since application of the Prelog model should predict alcohols of the R-configuration. Experimental evidence clearly showed that these products had the S-configuration. The spacial arrangement of the substrate-coenzyme system was suggested to be the one shown in Figure 9, an arrangement opposite to the one Prelog had suggested.

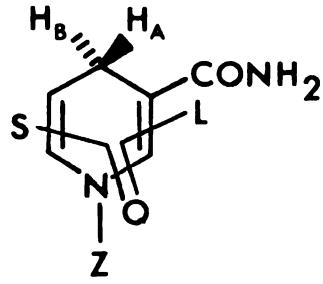


Figure 9. - Arrangement suggested by Karabatsos

As support for the model shown in Figure 9, the following evidence may be cited: Kaplan and van Eys[18] found that in the oxidation of (R,S)-2-octanol with YADH and NAD^+ only the (S)-2-octanol reacted to give the corresponding ketone (Figure 10).

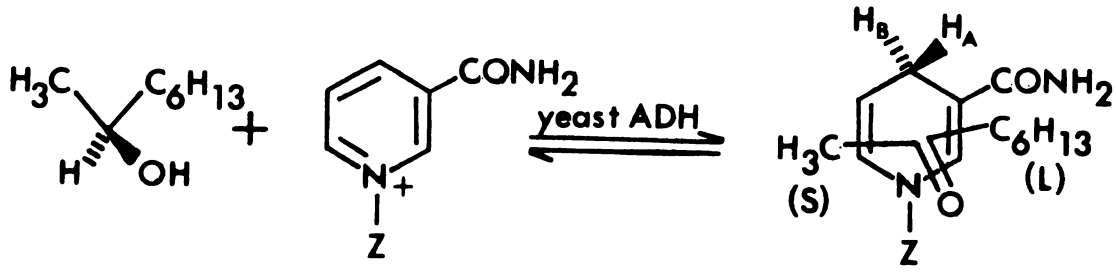


Figure 10. - Stereospecificity of NAD^+-YADH oxidation of 2-octanol

Mosher,[19,20,21] by using fermenting yeast (a Class A enzyme) to reduce trimethylacetaldehyde-1-d, benzaldehyde-α-d, and butyraldehyde-1-d, obtained products of the S-configuration. Donniger and Ryback, [22] using LADH (a Class A enzyme) to reduce geraniol-1-t, obtained alcohol of the S-configuration; and recently Gunther[23] and co-workers with 1-propanol-1-d and YADH found results which also supported the model by Karabatsos.

The evidence presented thus far in support of a model for the substrate NADH spacial requirements has generally ignored the importance of hydrophilic-hydrophobic interactions since the substrates used contained relatively nonpolar alkyl substituents bonded to the carbonyl carbon of the molecules. Karabatsos et al.[24,25] determined the absolute configuration at C-1 of 1,2-propanediol-1-d from the reduction of D- and L-lactaldehyde with NADD and LADH. These investigators found the absolute configuration at C-1 of the 1,2-propanediol-1-d produced from the reaction to be the R-configuration. This finding showed that hydrophobic interactions were less important than steric interactions as far as product stereospecificity was concerned (Figure 11).

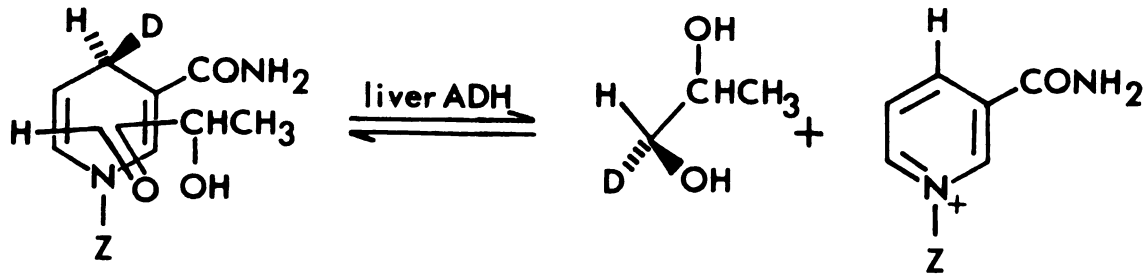


Figure 11. - Enzymatic oxidation-reduction of lactaldehyde

However, work by van Eys and Kaplan[18] with pyruvic acid and YADH yielded D-(-)-lactic acid which had the R-configuration (Figure 12). These

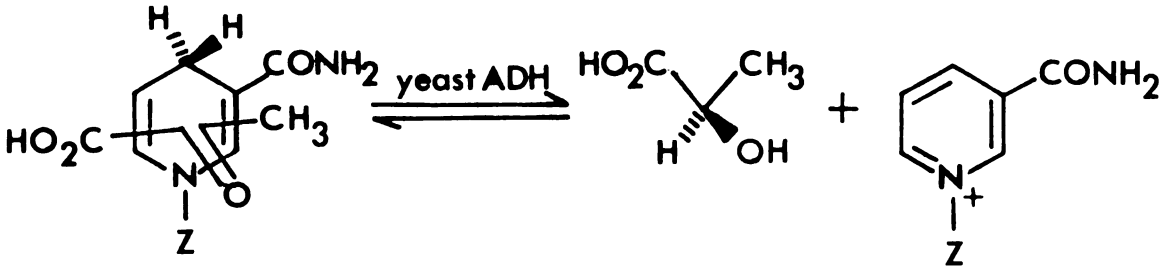
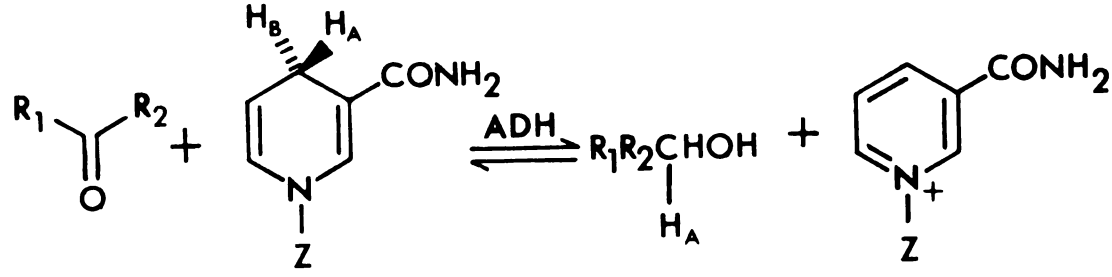


Figure 12. - Enzymatic oxidation-reduction of pyruvic acid

contradictory findings by Kaplan and others[26-31] led workers in the Karabatsos laboratories[32,33] to test the relative importance of hydrophobic interactions as well as steric interactions on substrates with other groups of comparable size but differing hydrophilicities (Table 1).

Table 1. - Effects of hydrophilic groups on product stereospecificity (Ref. 33)



R_1	R_2	Enzyme(s)	Class	%R-config.	%S-config.
CH_3-	$HOCH_2$-	Gly-DH	A	∿100	0
CH_3-	CH_3CH_2-	LADH,Gly DH	A	33-36	64-67
CH_3-	$ClCH_2$-	LADH	A	48	52

The findings summarized in Table 1 above might imply that the orientation predicted from either model may be completely reversed if the substituents on the carbonyl bearing substrate are sufficiently hydrophilic. Indeed,

a model may be needed for every enzyme tested.

There is, however, one obvious fact which has not been taken into account in the studies of the various model systems yet proposed. To date there has been no model presented which may account for the possibility of having the carbonyl of the substrate aligned in a manner other than that suggested by Kosower.[13,14] Any model now proposed must account for the possibility of having the carbonyl group either "up" of "down", or similar orientations, on face A or face B of the nicotinamide ring of the coenzyme. Available data from product stereospecificities agree with either orientation of the carbonyl group (Figure 13).

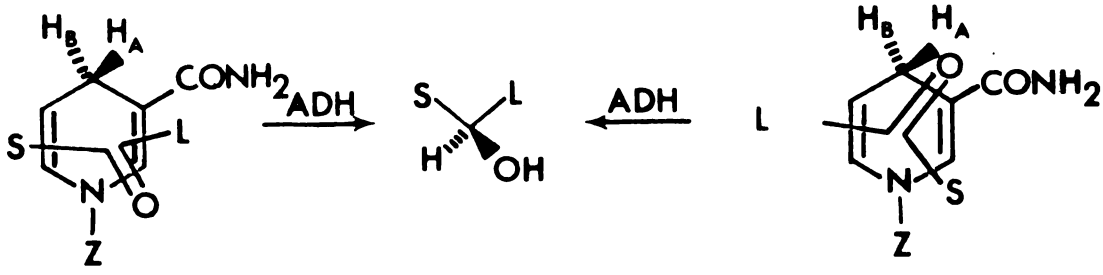


Figure 13. - Possible carbonyl orientations of non-bonded substrates

This thesis describes an attempt to answer the question of the substrate alignment by covalently bonding the substrate to the coenzyme and thus removing its freedom to rotate within its plane (Figure 14).

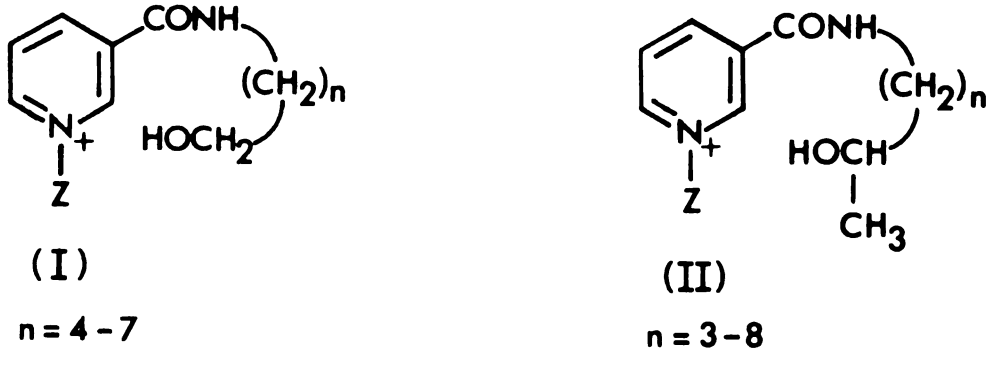


Figure 14. - NAD^+-Analogs with covalently bonded substrates

By restricting the "face" which the bonded substrate must use, with appropriate choice of enzyme, only two orientations of the carbonyl should be allowed (Figure 15). Isolation of products with concomitant analysis

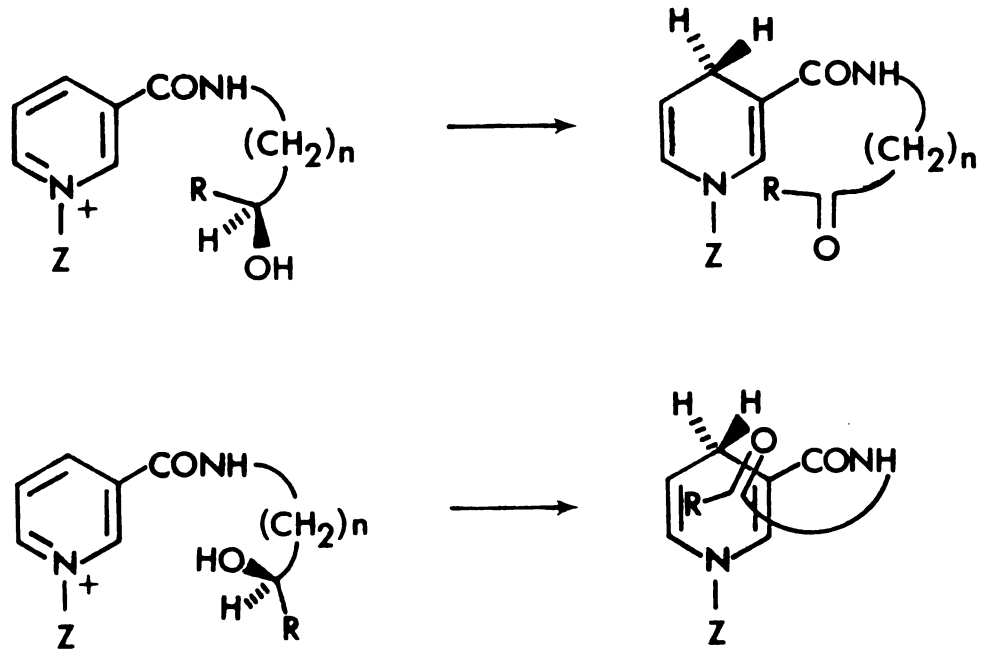


Figure 15. - Possible orientations of the carbonyl in covalently bonded NAD^+-Analogs

of the configuration of the unreactive alcohols should give a clearer indication of substrate and coenzyme arrangement. The NAD^+ analogs shown in Figure 14 have been synthesized and their relative reaction rates determined.[34] The results of these studies should provide further insight into the elucidation of a model to fit this system.

EXPERIMENTAL

PART I. Synthesis and purification of NAD^+ analogs

I. Synthesis of β,ω-Hydroxyamines

A. Alkylations of Ethyl Acetoacetate

To a three-necked round-bottomed flask fitted with an addition funnel, mechanical stirrer, and Friedricks Condenser was added 400 ml of absolute ethanol which had been refluxed six hours over magnesium methoxide and distilled prior to use.[35] The reaction vessel was maintained under a slow stream of dry nitrogen while 11.5 g (0.500 mol) of cleaned sodium metal was added and allowed to dissolve with gentle stirring. After the solution had cooled to room temperature, 122.3 g (0.940 mol, 119 ml) of freshly distilled ethyl acetoacetate (Aldrich) was added and the solution was stirred for 5-10 minutes at reflux on a steam bath. To the heated solution was added 25 mol percent of potassium iodide (Mallinkrodt, reagent grade) which had been ground and oven dried at 150° for two hours.

The stirring was increased as 0.500 mol of the appropriate pure halonitrile was added to the heated solution of the sodio-salt of the ester over a period of 10-15 minutes. The nitrogen flow was discontinued and reflux was maintained until the mixture was neutral to moist litmus. The reflux period varied from 1-4 hours depending upon which halonitrile was used. In all cases a white precipitate of the insoluble sodium iodide began to form within 30 minutes.

Upon completion of the reaction the majority of the ethanol was distilled from the mixture and the residue was allowed to cool to room temperature. After cooling, the residue - and precipitated salts - were

transferred to a separatory funnel. A 1:1 mixture of 320 ml benzene-water was added to the separatory funnel whereupon two layers formed. The organic layer was separated and the aqueous layer extracted with three 100-ml portions of ether. The ethereal extractions and organic layer were combined and dried over anhydrous magnesium sulfate.

The solvents were removed by rotary evaporator and the yellow, oily residue was distilled under high vacuum. Three fractions were obtained with the last fraction containing the desired products. (Table 2)

Table 2. - Alkylations of Ethyl Acetoacetate

$$X\text{-}(CH_2)_n\text{-}CN + CH_3COCH_2CO_2CH_2CH_3 \longrightarrow CH_3COCHCO_2CH_2CH_3$$
$$\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\qquad\quad (CH_2)_n\text{-}CN$$

n	X	Product	b.p.,°C/mm	%Yield
1	Cl	ethyl (1-cyanomethyl)acetoacetate	95.5°-100.5°/0.18	38.5
2	--	ethyl (2-cyanoethyl)acetoacetate[36a]	132.0°-143.0°/0.20	42.7
3	Br	ethyl (3-cyanopropyl)acetoacetate	80.0°-82.5° /0.02	60.4
	(Cl)	ethyl (3-cyanopropyl)acetoacetate	------- *	(36.2)
4	Br	ethyl (4-cyanobutyl)acetoacetate	------- *	----
5	Br	ethyl (5-cyanopentyl)acetoacetate	136.5°-139.0°/0.02	69.0
6	Br	ethyl (6-cyanohexyl)acetoacetate	------- *	----

* Product could not be distilled, crude residue used without further purification

B. Decarbethoxylation of alkylated β-ketoesters

Procedure I:[38]

To a solution of 200 g of sodium carbonate in 1800 ml of water was added 1.09 mols of the ester. The two layer mixture was refluxed for

four hours during which time it became homogeneous. The product was salted out with potassium carbonate and the aqueous layer extracted with three 100-ml portions of ether. The organic layer and ether extracts were combined and dried over anhydrous potassium carbonate. The ether was removed by rotary evaporator and the product distilled under vacuum. In all cases, only one fraction was obtained with a small amount of residue remaining (Table 3).

Procedure II:[39]

A solution containing 50 ml of dimethylsulfoxide (Fischer certified), 0.20 mol of water, 0.07 mol of sodium chloride, and 0.065 mol of the ester was added to a 3-necked round-bottomed flask fitted with a magnetic stir bar, thermometer, and condenser attached to a trap filled with a saturated solution of barium hydroxide. This mixture was heated slowly at 150°-170° on an oil bath with stirring and kept at that temperature until the evolution of carbon dioxide ceased. Heating was continued for 1-6 hours depending upon the ester used. The solution remained colorless or slightly yellow throughout the reaction period.

On completion of the reaction, the solution was cooled to room temperature and transferred to a separatory funnel. Addition of a volume of cold water equal to the volume of dimethylsulfoxide used resulted in the formation of two layers. The organic layer was separated and the aqueous layer was extracted with three 50-ml portions of ether. The extracts were combined and washed with several small portions of saturated sodium chloride solution and dried over anhydrous potassium carbonate. The ether was removed under rotary evaporation and the product distilled. Only one fraction was recovered (Table 3).

It is of interest that for the substrates where n = 1-3 (Table 2), Procedure II caused decomposition of the β-ketoester; and for substrates

where n = 4-6 (Table 2), Procedure I failed to accomplish the desired decarbethoxylation reaction.

Table 3. - Decarbethoxylation of Alkylated β-ketoesters

$$CH_3COCHCO_2CH_2CH_3 \;(\text{with } (CH_2)_n\text{-CN on CH}) \longrightarrow CH_3CO(CH_2)_{n+1}\text{-CN}$$

n	Procedure	b.p. °C/mm	% Yield
1*	I	-----	----
	II	-----	----
2	I	66.0°-68.5°/1.0	78.0(36)
3	I	65.5°-67.0°/0.10	85.3
4	II	88.0°-89.5°/0.10	86.0
5	II	91.2°-94.5°/0.20	75.9
6	II	90.5°-94.0°/0.05	62.0

* Product hydrolyzed when either method was used.

C. Reduction of ketonitriles

Into a 3-necked round-bottomed flask fitted with an addition funnel, thermometer, and magnetic stir bar was added 200 ml of absolute methanol (Matheson, Coleman, and Bell) and 0.57 mol of the ketonitrile. The solution was cooled to 0° in an ice-salt water bath with rapid stirring. To the cooled mixture was added in small amounts a solution containing 0.19 mol of sodium borohydride (Metal Hydrides, Inc.) in cold water maintained at 0°. Only the minimum amount of water required to dissolve the sodium borohydride was used. Hydride was added at such a rate as to maintain the reaction mixture at a temperature less than 10°. Addition of the hydride solution was achieved over a period of

30-50 minutes in all cases.

Upon completion of the addition, the stirred solution was allowed to gradually warm to room temperature. A solution of cold 10% sulfuric acid was added until the solution reached a pH of 5-6. This solution was then stirred for approximately 3-4 hours - or until the flocculent precipitate became granular. The solid was filtered from the mixture with a vacuum aspirator.

After most of the solvent was removed by rotary evaporator, the residue was taken up in a small amount of ether and dried over anhydrous magnesium sulfate. The ether was removed and the products were distilled under vacuum (Table 4).

Attempts to reduce both the carbonyl and nitrile groups in one reaction, using a variety of reducing agents, failed in all cases. Only cyclised or polymerized products were obtained.

Table 4. - Reduction of Ketonitriles

$$CH_3CO(CH_2)_n\text{-}CN \longrightarrow CH_3CH(OH)(CH_2)_n\text{-}CN$$

n	b.p. °C/mm	% Yield
3	55.0°-56.6°/0.10	95.0[36]
4	81.5°-83.0°/0.20	91.0
5	106.0°-106.5°/0.20	84.7
6	118.0°-119.5°/0.20	86.7
7	116.5°-118.0°/0.10	85.8

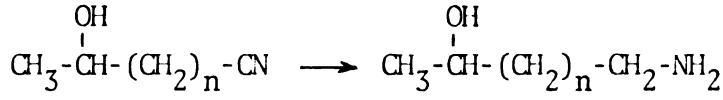
D. Reduction of β-hydroxynitriles with Raney Nickel (W-2) catalyst

A solution of 0.1 mol of the nitrile in approximately 400 ml of 10N

methanolic ammonia[40] and 2-3 g of freshly prepared W-2 Raney nickel catalyst (PCR Chemicals)[41] was placed under 50 psi of hydrogen pressure in a Parr apparatus at room temperature. Reductions were generally completed within 18 hours with a pressure drop to within 2 psi of the calculated value. Yields were quantitative for the liquid aminoalcohols (Table 5).

After evaporation of the solvent, aminoalcohols were recovered initially as pale green oils. These oils were dissolved in acetonitrile (Fisher) to which a small amount of decolorizing charcoal had been added, boiled for a short period, and precipitated as solids. Recrystallization of the solids led to constant melting points (Table 5).

Table 5. - Reduction of β-hydroxynitriles



n	m.p.,°C[b.p.,°C]	% Yield
2	[56°-59°/0.2-0.4][(a)]	62.0[a]
3	[62.0°-62.5°/0.10]	∿100
4	[68.0°-69.0°/0.10]	92.0
	37°-39°[(b)]	
5	43.3°-44.1°	75.4
6	47.9°-48.1°	52.2
7	55.3°-54.0°	68.6

a. Prepared by J. Miedema, Ph.D. Thesis, Michigan State University, 1974, p. 26.

b. Distillable liquid which solidifies on standing at room temperature

II. Preparation of 8-Hydroxy-1-octylamine

A. Alkylation of Ethyl Malonate

Into a 3-necked round-bottomed flask fitted with an addition funnel, mechanical stirrer, and Friedricks condenser was added 400 ml of freshly distilled dry ethanol[35] and 1.05 moles of cleaned sodium metal. The system was protected from moisture with dry nitrogen. When the metal had completely dissolved, 27.66 g of pre-dried potassium iodide and 1.05 moles of freshly distilled ethyl malonate (Matheson, Coleman, and Bell) were added and the solution was stirred for 15 minutes.

A solution of 1.00 mol of 6-bromocapronitrile (Fairfield) in 120 ml dry ethanol was added slowly to the rapidly stirring solution. On completion of the addition the reaction mixture was refluxed on a steam bath until neutral to moist litmus. A precipitate of the insoluble sodium iodide began to be formed immediately upon heating.

Most of the ethanol was distilled from the mixture and the residue was allowed to cool to room temperature. A volume of water equal to the volume of ethanol removed was added to the cooled residue without removal of the precipitated salts. Two layers formed on addition of water and the organic layer was separated from the mixture. This layer was washed with three 50-ml portions of a saturated sodium chloride solution and the washings combined with the aqueous layer previously separated. The combined aqueous solution was extracted with three 50-ml portions of ether which were combined with the organic layer and dried over anhydrous potassium carbonate.

The solvent was removed by rotary evaporation and the product was obtained by vacuum distillation; b.p. 131.2°-132.0°/0.16 mm, in 71.7% yield.

B. Decarbethoxylation of Ethyl (5-Cyanopentyl) malonate[39]

In a 3-necked round-bottomed flask outfitted with magnetic stir-bar, condenser, and thermometer were mixed 40 ml of dimethylsulfoxide (Fischer), 0.15 mol of water, 0.05 mol of sodium chloride, and 0.04 mol of the diester. The mixture was stirred vigorously as the temperature was raised to 150°-170° in an oil bath. Heating was continued until the evolution of carbon dioxide, monitored by means of a trap containing a saturated solution of barium hydroxide, ceased.

The colorless solution was cooled in an ice-water bath to 0° and transferred to a separatory funnel. A volume of cold water equal to the dimethylsulfoxide used was added, resulting in the formation of two layers. The organic layer was separated and the aqueous layer was extracted with three 50-ml portions of ether and combined with the organic layer. The combined extracts were dried over anhydrous potassium carbonate.

The ether was removed by rotary evaporation and the residue was distilled under vacuum. The one recovered fraction contained the product, b.p. 93.0-94.0°/0.2 mm, in 73.9% yield.

C. Preparation of 8-Hydroxy-1-octylamine

1. Preparation of Raney Nickel (W-4) Catalyst[42]

To a well-stirred solution containing 128 g of sodium hydroxide in 500 ml of water was added 100 g of Raney Nickel catalyst powder (PCR Chemicals) at such a rate as to maintain the temperature at 50° ± 2°. A few drops of ethanol were added to quench subsequent foaming when necessary. After the addition had been completed, the mixture was digested for 50 minutes at 50° with moderate stirring.

The catalyst was washed by decantation several times with water and transferred to a 500-ml graduated cylinder. Approximately 15 liters of

water was passed through the suspended catalyst while the solution was stirred in an arrangement described by Adkins.[42] The solid was kept below the surface of the liquid at all times. When the washings became neutral to litmus, the catalyst was transferred to a 250-ml centrifuge bottle and stirred (not shaken) with three 150-ml portions of 95% ethanol. The catalyst was centrifuged after each washing at 10,000 rpm for 10 minutes.

The washing and centrifugation was repeated with three 150-ml portions of absolute ethanol and the activated powder stored at 5° in a tightly closed bottle filled to the top with absolute ethanol. This highly active form of Raney catalyst was used immediately upon preparation since it tended to lose its activity upon storage.

2. Reduction of Ethyl 7-Cyanoheptanoate

A solution containing 0.10 mol of the ester in 400 ml of 10N methanoic ammonia with 5-6 g of the Raney Nickel (W-4) catalyst was placed in a Parr apparatus under 50 psi of hydrogen pressure. The reduction of both the ester function and the nitrile was completed in 36 hours as determined by the recorded hydrogen uptake.

The solvent was removed and the dark green oil boiled for a few minutes in acetonitrile to which a small amount of decolorizing charcoal had been added. The white crystals were obtained after several recrystallizations from acetonitrile in 60.0% yield; m.p. 48.0°-48.3°.

III. Synthesis of N-(hydroxyalkyl)nicotinamides

A. Preparation of Nicotinyl Chloride

To a suspension of 400 ml of carbon tetrachloride and 64.8 g (0.400 mol) of potassium nicotinate was added slowly, with stirring, 36.0 ml (0.500 mol) of thionyl chloride. The system was protected from atmospheric moisture with dry nitrogen. After the addition was completed,

the solution was allowed to reflux for 12-24 hours during which time the evolution of sulfur dioxide ceased. The precipitated salts were filtered from the mixture in an inert atmosphere and the solvent was removed under vacuum. The residue was again filtered and distilled to yield a colorless liquid, b.p. 30.0°-31.5°/0.08 mm. The yield was not determined since the product hydrolyzes readily.

B. Preparation of the Trimethylsilyl Ethers of the Aminoalcohols

Equimolar amounts (ca. 0.10 mol) of the aminoalcohol and 1,1,1,3,3,3,-hexamethyldisilizane (Aldrich) with approximately 0.1 g of dried ammonium chloride were placed in a three-necked round-bottomed flask equipped with mechanical stirrer, condenser, and rubber septum. All glass-to-glass connections were fitted with teflon sleeves (Chem. Tec). The heterogeneous mixture was allowed to heat slowly to 125° with stirring, during which time a vigorous evolution of ammonia was observed. When the evolution of ammonia had ceased and the solution had become homogeneous, the mixture was allowed to cool to room temperature. In order to avoid losses due to hydrolysis, the product was not isolated (previous attempts at preparation and purification[36b] showed the product to be formed in nearly quantitative yield but to hydrolyze during purification).

C. Preparation of N-(trimethylsiloxyalkyl) nicotinamides

To the reaction vessel described above was added 70 ml of pyridine (distilled from barium oxide) and 30 ml of triethylamine (distilled from sodium metal). The flask was heated to 105° and an equivalent amount of nicotinyl chloride (0.10 mol) was added slowly to the solution by injection through the septum using a disposable syringe with a neoprene plunger. When the addition of nicotinyl chloride had been completed,

the septum was replaced with a glass stopper and the mixture was refluxed for one half-hour. The solution was cooled to 0° in an ice-water bath and the precipitated triethylammonium chloride filtered from the mixture. The product was not isolated.

D. Preparation of N-(hydroxylalkyl) nicotinamides

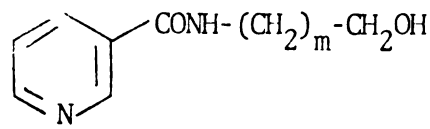
The residue from the previous filtration was treated with 10 ml of water and the solution was refluxed for one hour. The pyridine-water azeotrope was distilled from the solution and the remaining solvent was removed under vacuum with heating to yield a dark brown oil. The oily residue was dissolved in chloroform to a concentration of approximately 0.5 g/ml based upon the theoretical yield.

Aliquots containing approximately 10 g of the crude product were applied to a 2.5 x 80 cm column packed with alumina (Matheson, Coleman, and Bell, Alcoa type F-20, 80-200 mesh) prepared with 100:2 chloroform-methanol. Collection of the product by gravity elution using 100:2 chloroform-methanol as solvent was monitored by absorption at 254 nm using a 1.0 mm flow cell with a Beckmann DB spectrophotometer.

Removal of the solvent by rotary evaporator left a pale yellow oil. The oil was taken up in acetone and recrystallized twice in the same solvent. The shorter chain N-(hydroxalkyl) nicotinamides crystallized spontaneously upon treatment with acetone. The longer chain substrates had to be chilled to below 0° or allowed to stand for 10-14 days at room temperature in a minimum of solvent. The yields, after successive recrystallizations, were typically 55-60% except for N-(8-hydroxynonyl) nicotinamide. This compound could not be recovered as a white crystalline

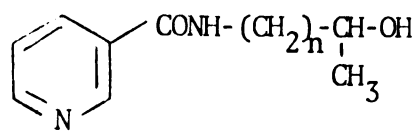
solid in any solvent system tried. The brown oily residue solidified on standing at room temperature after one month, but ^{1}H-nmr of the tan solid showed it to be reasonably free of impurities. The products and their melting points are listed in Table 6.

Table 6. - Melting points of N-(hydroxyalkyl)nicotinamides



m	Product	m.p., °C
4	N-(5-hydroxypentyl)nicotinamide*	91.0°-92.0°
5	N-(6-hydroxyhexyl)nicotinamide*	79.2°-80.0°
6	N-(7-hydroxyheptyl)nicotinamide	60.1°-61.0°
7	N-(8-hydroxyoctyl)nicotinamide	90.1°-91.0°

* Amino alcohols purchased from Aldrich



n	Product	m.p., °C
3	N-(4-hydroxypentyl)nicotinamide	64.8°-66.0°[43]
4	N-(5-hydroxyhexyl)nicotinamide	48.9°-50.5°[36b]
5	N-(6-hydroxyheptyl)nicotinamide	41.1°-42.0°
6	N-(7-hydroxyoctyl)nicotinamide	51.5°-52.1°
7	N-(8-hydroxynonyl)nicotinamide	-----
8	N-(9-hydroxydecyl)nicotinamide	69.0°-70.3°

IV. Preparation of NAD^{+} analogs[42]

To a 25 ml Reacti-flask (Pierce) with a screw cap having a teflon-rubber laminated insert and magnetic stir-bar was added 1.144 mmols of

the nicotinamide dissolved in 6.0 ml of 0.1M sodium phosphate buffer, pH 8.0. A second solution was prepared containing 0.286 mmols of oxidized β-nicotinamide adenine dinucleotide (Sigma, grade III) in 6.5 ml of the same buffer. On mixing the solution had a pH of 7.6. To this solution was added 0.5000 g (0.015 unit/mg) pig brain NADase (Sigma, E.C. 3.2.2.5., NAD glycohydrolase) and the heterogeneous mixture was incubated at 37.00° ± 0.10° for 3.0 hours.

At the end of the incubation period the solution was made up to 5% in trichloroacetic acid (ca. 0.5 g) and chilled to 5°. The denatured protein was removed from the mixture by refrigerated centrifugation at 10,000 rpm for 10 minutes. The supernatant liquid was transferred to a 250-ml polyethylene centrifuge bottle with screw cap and 5 volumes (62.5 ml) of cold acetone was added to precipitate the solid products. After standing at 5° for a few minutes the suspension was centrifuged at 5,000 rpm for 15 minutes and the supernatant liquid discarded. The residual acetone was evaporated with a stream of nitrogen and the solid was dissolved in 10.0 ml of cold, de-ionized distilled water. The solutions were stable for several months when stored at 5°.

V. Isolation and purification of NAD^+ analogs

The coenzyme analogs were separated on a 0.9 x 55 cm Polyethyleneimine cellulose (PEI cellulose, Sigma, 1.20 meq/g) column prepared in the following manner. All operations were carried out at 5° unless otherwise noted.

One and one-half column volume of dry resin was allowed to stand in 220 ml of 0.003M ammonium bicarbonate solution for one-half hour. The solvent was decanted from the settled suspension and the process repeated.

After the second decantation, the resin was suspended in 100 ml of the bicarbonate solution and the entire mixture was poured into the column as a slurry. The resin was allowed to settle for approximately two hours without solvent flow. A small layer of sand was placed above the settled resin and the column was fitted with a pressure head connected to a Polystaltic pump (Büchler instruments). The solvent pressure was slowly increased to allow even settling of the column until a flow rate of 1.8 ml/min was obtained. Column equilibration was continued until 1 liter of 0.003M ammonium bicarbonate had passed through the resin.

A 1.00 ml aliquot of the crude reaction mixture of a coenzyme analog was loaded onto the column and those fractions containing analog and unreacted NAD^+ were collected separately. Previous identification of the fractions had been determined.[42] The eluate was monitored at 260 nm in a 0.1 cm flow cell using a Beckmann DB-G spectrophotometer. Fractions from three column loadings were combined for each analog isolated. The column was washed for five hours with the eluting solvent between successive separations. Of the substrates prepared, those analogs with seven- and eight- carbon alkyl chains and a primary hydroxyl group, and the ten-carbon chain with a secondary hydroxyl group could not be resolved. Variation of concentration of the eluting buffer as well as several gradient elution methods failed to resolve the analog and NAD^+ fractions.

A 50-ml aliquot of each of the eluted fractions was further purified by molecular filtration. The solution was placed in a 47 mm stirred cell containing a PSAC membrane (Millipore, nominal molecular weight limit 1000). The solution was concentrated to approximately 10 ml under 50 psi nitrogen pressure with stirring and then diluted to 60 ml with 0.10M

sodium phosphate buffer, pH 7.5, which was 0.01M with respect to semicarbazide. Dilution and concentration steps were repeated 5 times to insure a sample purity of greater than 95% in terms of buffer exchange and contaminating impurities. The final volumes of all samples purified in this manner were 10 ml.

Part II. Initial reactivities of coenzyme analogs

A. Methods

Initial reaction velocities were principally determined by monitoring the increase in fluorescence due to the formation of the reduced 1,4-dihydropyridinium moeity reported to be at 462 mm. The excitation wavelength used was 350 nm while recording fluorescence at 450 nm. A computer centered spectrophotometer-spectrofluorimeter with PDP-8/I unit[43] was used to record any changes in fluorescence in samples whose average concentration was approximately 8.4×10^{-5}M (based on $\varepsilon_{260\ NAD^+} = 18 \times 10^6\ cm^2\ mol^{-1}$). Absolute concentrations could not be determined since the reactions were run on a scale which precluded isolation of adequate quantities for accurate determinations of extinction coefficients for each of the analogs. Extreme care was taken to exclude particulate matter which would have distorted the trace at the high sensitivities employed.

Those analogs tested were run by using the following methods: (a) Freshly prepared enzyme solution was mixed with the analog and the scan begun immediately upon mixing. Approximately 2-3 seconds lapsed between mixing and starting the scan, but, since the reactions were relatively slow, this proved to be no handicap. Samples were scanned for 5 minutes with data points taken every 3.00 seconds. (b) A second sample of the analog was incubated at 31.0° for two hours prior to the run. Fluorescence readings were taken for 10.0 seconds to check for the presence of reduced 1,4-dihydropyridinium in the analog preparation. Baseline checks were run on buffer solution alone, buffer and analog alone, buffer with enzyme added, and buffer, purified NAD^+, and enzyme to correct for impurities that might affect the results.

As a secondary check on the reactivity of the analogs an ultraviolet spectrum was run on the samples by using Unicam SP800 spectrophotometer with a 1.0 cm cell. The spectra were recorded directly as absorbance versus a constant buffer reference. Recordings were made of sample absorbance alone, sample with added enzyme, and sample with enzyme and ethanol as a check to determine whether the analog would function as a natural substrate in the liver ADH system. Readings were recorded of analog alone in buffer solution, and analog with enzyme added at time intervals t = 0, 1, 3, 5, 7, and 9 minutes of mixing. Solutions were transferred to small vials and incubated at 31.0° with readings made at 1.0 hour and 24.0 hour intervals. Baselines were run against buffer solutions with care taken to maintain that baseline for incubated samples.

Separate samples containing analog and ethanol were run at t = 0, 1, 3, and 5 minutes after addition of enzyme. No incubation of these samples was attempted.

A diagnostic check was made by observing the formation of the fluorescent cyanide addition complex reported to show an absorption increase at 325 nm similar to the enzymatic reduction of NAD^+ to NADH.[44]

B. Materials and sample preparation

Samples used for both absorption and fluorimetry were prepared in a similar manner. A 1.50 ml aliquot of the purified coenzyme analog was diluted prior to use with 0.10M sodium phosphate buffer which was 0.01M in semicarbazide-HCl to a total volume of 3.00 ml. The buffer had been previously titrated to pH 7.50 $\pm$ 0.05 using a 0.10M sodium hydroxide. Solutions were allowed to equilibrate at ambient temperature for 15 minutes

before study without added enzyme or incubated with enzyme added for the appropriate time. Ethanol, when present, was added as 50µl of a 95% solution.

Horse liver alcohol dehydrogenase (Sigma, liver ADH, E.C. 1.1.1.1) was obtained as the lyophilized powder containing 20 units activity and reconstituted using 1.00 ml buffer stock solution. The enzyme solutions were used without further purification since a fluorimetric scan of a solution containing purified β-NAD^+ (Sigma) and the enzyme showed no fluorescence increase due to the presence of oxidizable impurities. One unit, 50µl, of enzyme was added to all samples checked for reactivity.

Boiled, deionized-distilled water was used in preparation of all solutions used throughout the study. Samples used in the cyanide test were prepared by dissolving 0.60-0.80 ml in enough 1.0M potassium cyanide solution (pH 12.0) to bring the total volume to 3.00 ml.[44] Spectra were run against a potassium cyanide blank.

Results and Discussion

The work presented here represents a continuing effort to understand the mechanism of enzymatic oxidation and reduction in one of the earliest discovered and most studied systems in biological chemistry. The initial approach to the problem, first suggested by Karabatsos, was to restrict the many degrees of freedom allowed the substrate. This move toward eliminating some of the variables was first attempted with amino alcohols possessing a primary amino and primary hydroxy function.[32,42] Many of these primary amino alcohols were commercially available and it was believed that through isotopic labelling experiments, stereochemical assignments could be obtained. Once the initial reactivity of these coenzyme analogs had been established, we realized that amino alcohols bearing a secondary hydroxyl group would simplify the problem of stereochemistry by eliminating, for a time, the need for labelling experiments. The absolute configurations of a large number of asymmetric alcohols have been in the literature for years. Methods for the resolution of racemic compounds also appear throughout much of the current literature; and, if a general method for the preparation of these so-called secondary amino alcohols could be obtained, it remained then a simple matter of relating the stereochemistry to findings already established.

Since no commercial preparation of secondary amino alcohols was available, work was started by May[36] to outline a good general method for the preparation of these compounds in reasonable yields. Initial attempts to synthesize secondary amino alcohols resulted in low yields for several reasons: (a) the lack of selectivity in group transformation (b) the tendency towards cyclization due to the nucleophilic character

of these groups, and (c) the extreme water solubility and hygroscopicity of the compounds which complicated isolation procedures.

The synthetic route in equations 1-4 below, appears to have solved the problems mentioned. All compounds used in this study have been prepared by these methods and obtained in yields greater than 50%.

1. $CH_3COCH_2CO_2C_2H_5 + X\text{-}(CH_2)_n\text{-}CN \longrightarrow CH_3COCHCO_2C_2H_5$ with $(CH_2)_n\text{-}CN$ on the CH

2. $CH_3COCHCO_2C_2H_5$ (with $(CH_2)_n\text{-}CN$ on the CH) $\longrightarrow CH_3COCH_2\text{-}(CH_2)_n\text{-}CN$

3. $CH_3COCH_2\text{-}(CH_2)_n\text{-}CN \longrightarrow CH_3CH(OH)\text{-}CH_2\text{-}(CH_2)_n\text{-}CN$

4. $CH_3CH(OH)\text{-}CH_2\text{-}(CH_2)_n\text{-}CN \longrightarrow CH_3CH(OH)CH_2\text{-}(CH_2)_n\text{-}CH_2NH_2$

Variations in the alkyl chain length, n, and the halide, X, have relatively little effect in the preparation of these compounds.

The oxidized nicotinamide adenine dinucleotide analogs were prepared by a method outlined by Kaplan[45] and isolated by column chromatography on a PEI cellulose column. Eluates were monitored by untraviolet spectrophotometer and the fractions identified as (1) unresolved nicotinamide and N-(alkylhydroxy) nicotinamide, (2) NAD^+ analog, and (3) unreacted NAD^+ (Figure 16). The retention times and peak elution profiles for all analogs isolated were identical. Additionally, thin layer chromatography of crude reaction mixtures on preformed PEI cellulose 300 (UV_{254}) plates developed with 0.15M ammonium chloride were shown to have R_f values for each of the fractions which were essentially identical. Yields were typically 20-30% by this method.

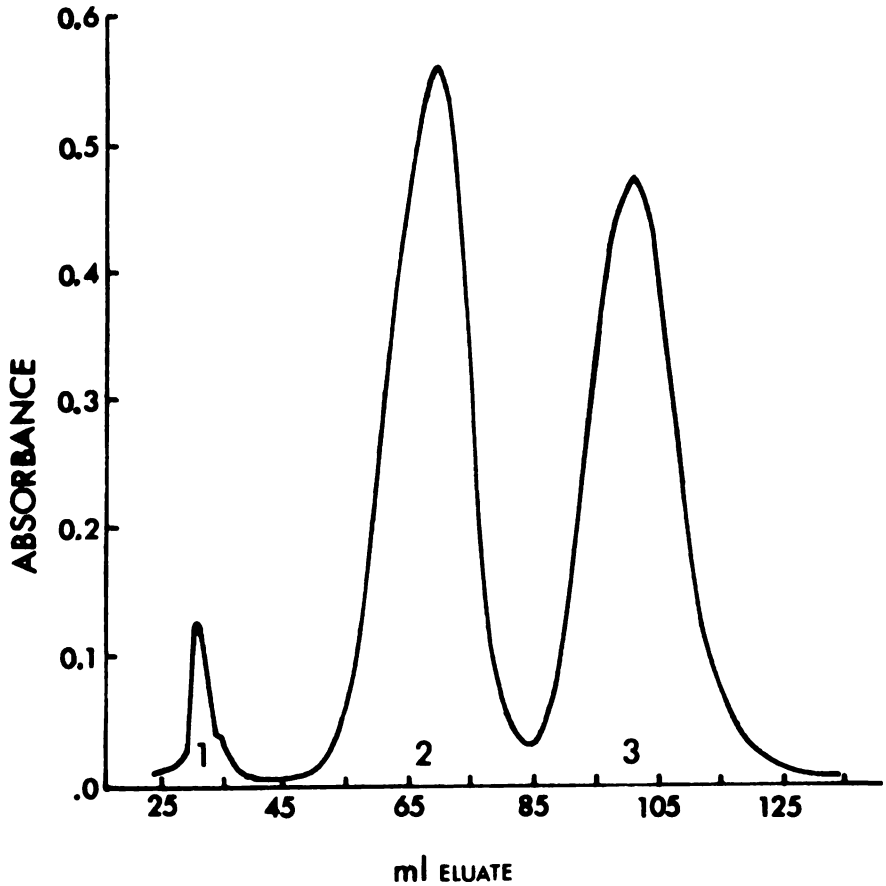


Figure 16. - Elution curves for analog purification

Preliminary characterization of the analogs was made by analogy to the parent system. All compounds obtained showed a λ_{max} at 260 nm and formed fluorescent addition complexes with cyanide leading to an increase in absorbance at 325 nm with a corresponding increase in extinction at 260 nm (Figure 17). This ability to form a complex with cyanide is a general property of N-substituted nicotinamide analogs as well as NAD^+ and has been used as the basis for the analytical determination of NAD^+ in unknown samples.[44]

An earlier study in this laboratory[42] showed that all the analogs

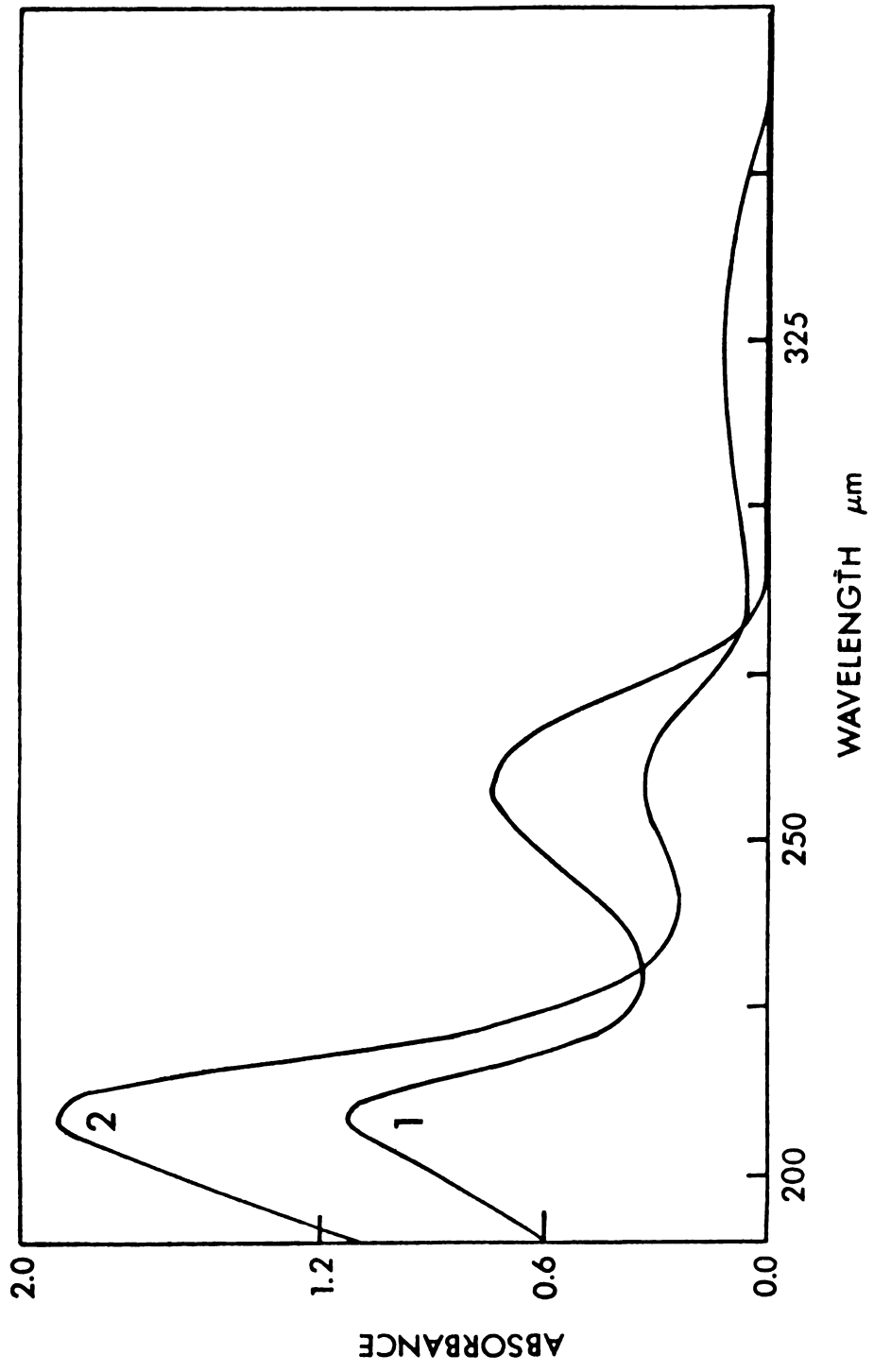


Figure 17. - Typical cyanide addition complex of NAD^+ analogs

1. NAD^+ analog alone
2. NAD^+ analog with cyanide

from C_5 - through C_7 (i.e.: possessing an alkyl chain with 5-7 total carbon atoms) underwent complete reduction in pyrophosphate buffer with liver ADH and ethanol on incubation. That study was extended to include the highly purified substrates through C_9 in phosphate buffer and those original findings have been confirmed. This indeed shows that the analogs can function alone as active cofactors in the natural system; although at a much slower rate in most cases. The longer chained N-alkylhydroxy derivatives appear to react at nearly identical rates as NAD^+ itself when ethanol is added (Figure 18), and without the need for incubation.

Information regarding the intra (or inter) molecular reaction of the substrates without external ethanol added is much less conclusive. Because of the small scale on which the reactions have been run (ca. 3×10^{-7} to 8×10^{-5}M) and an unfavorable equilibrium constant,

$$K_{eq} = \frac{[NADH][CH_3CHO][H^+]}{[NAD^+][CH_3CH_2OH]} \sim 8 \times 10^{-12}M,$$

for the ethanol system,[46] the accurate determination of any reaction by ultraviolet methods has proven inconclusive. The general phenomena of increasing extinction at 340 nm could not be accurately measured since the sample concentrations were well below - or at the extreme lower limits of - the sensitivity of ultraviolet spectroscopy. In the case of N-(6-hydroxyalkyl)- and N-(8-hydroxyalkyl)nicotinamide adenine dinucleotides a decrease at 260 nm was observed on incubation of the substrates with enzyme for 24 hours (Figure 19). No usch decrease was seen for any of the other tested substrates.

Due to the greater sensitivity of fluorescence analysis (10^{-9} to 10^{-11}M) it was decided that information could be obtained as to initial

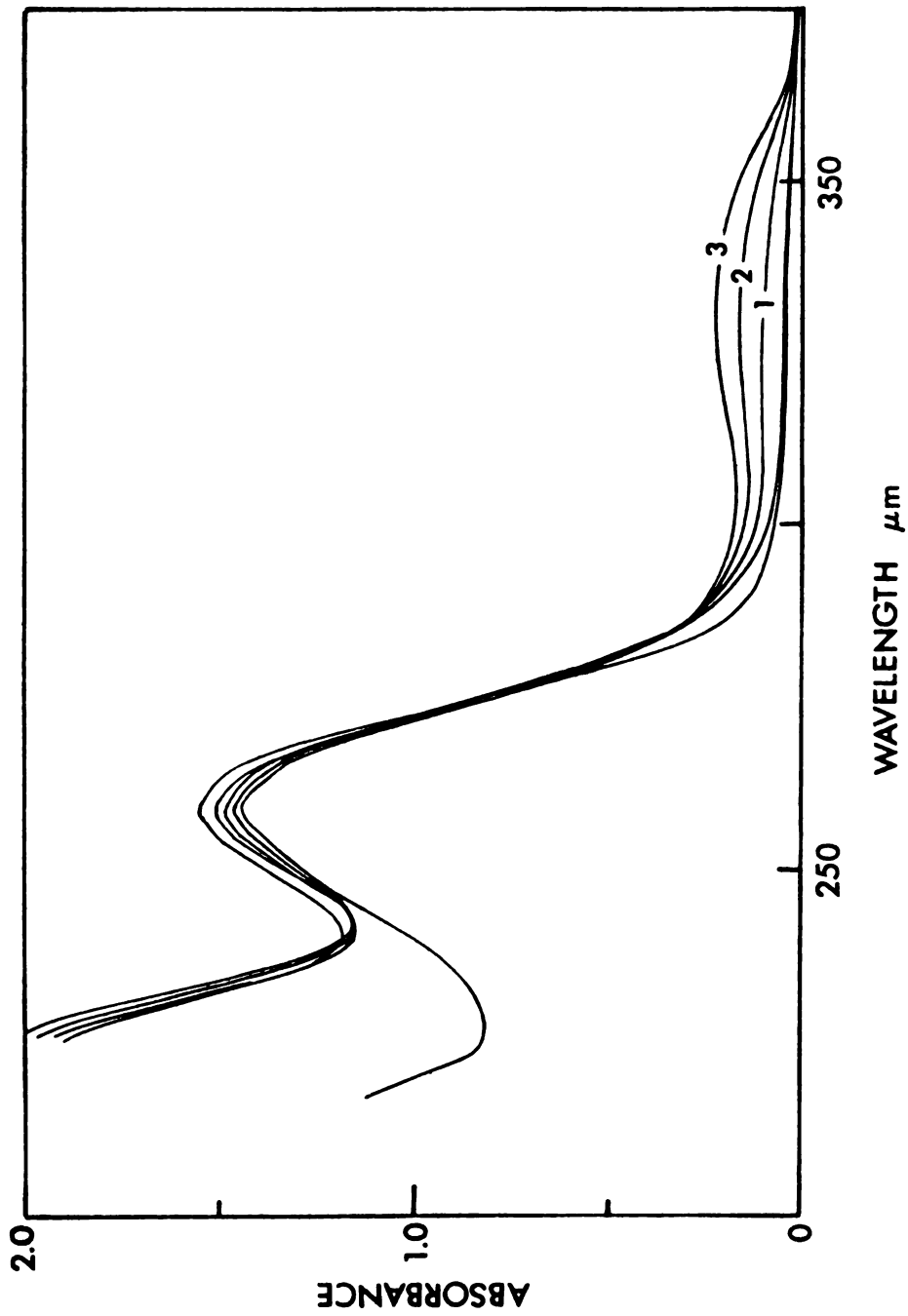


Figure 18. - Spectrum of N-(6-hydroxyheptyl)NAD^+ with added ethanol

1. Increase in absorbance after 1 min.
2. Increase in absorbance after 3 mins.
3. Increase in absorbance after 5 mins.

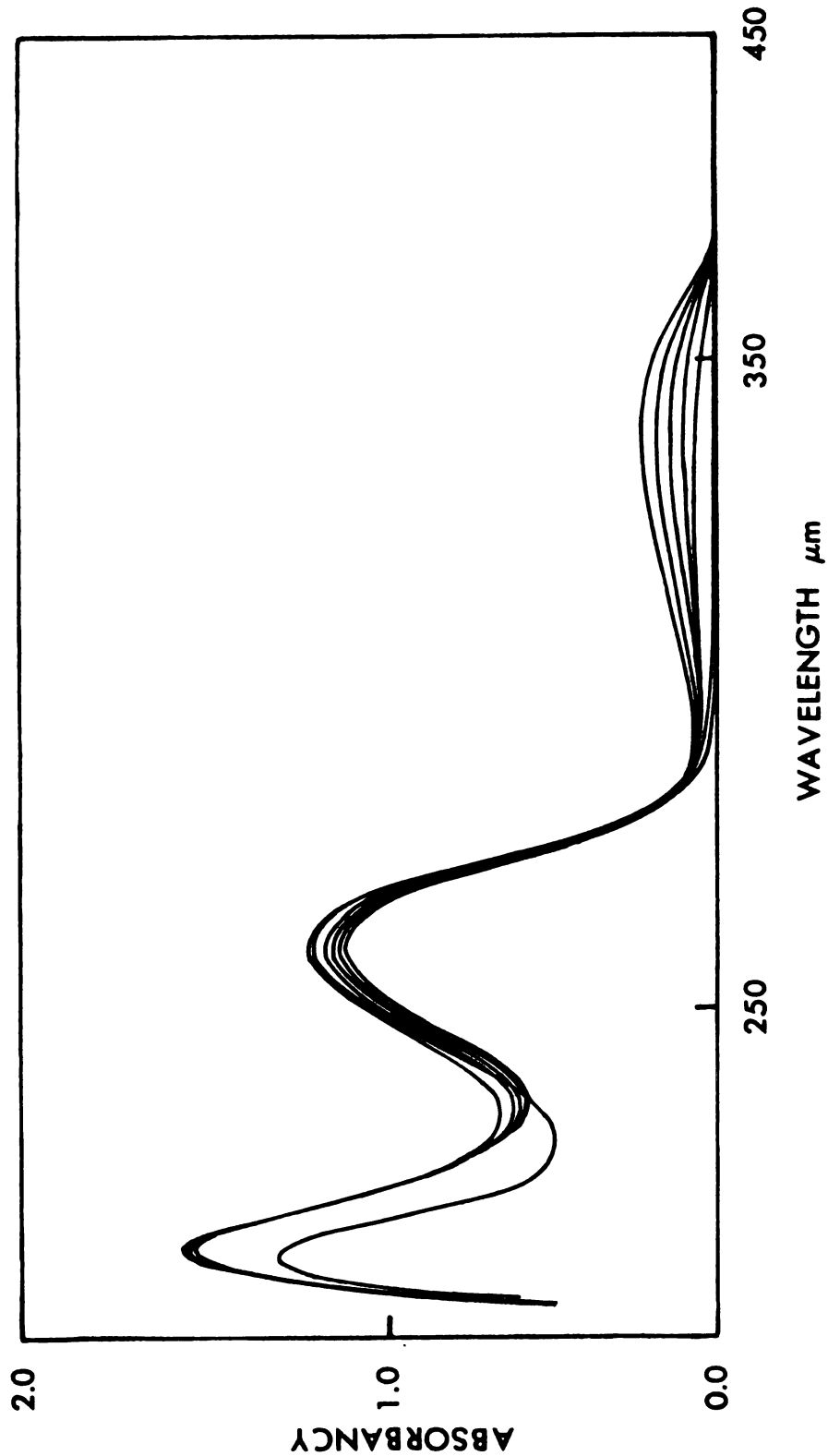


Figure 18. (cont.) - Spectrum of β-NAD^+ with added ethanol

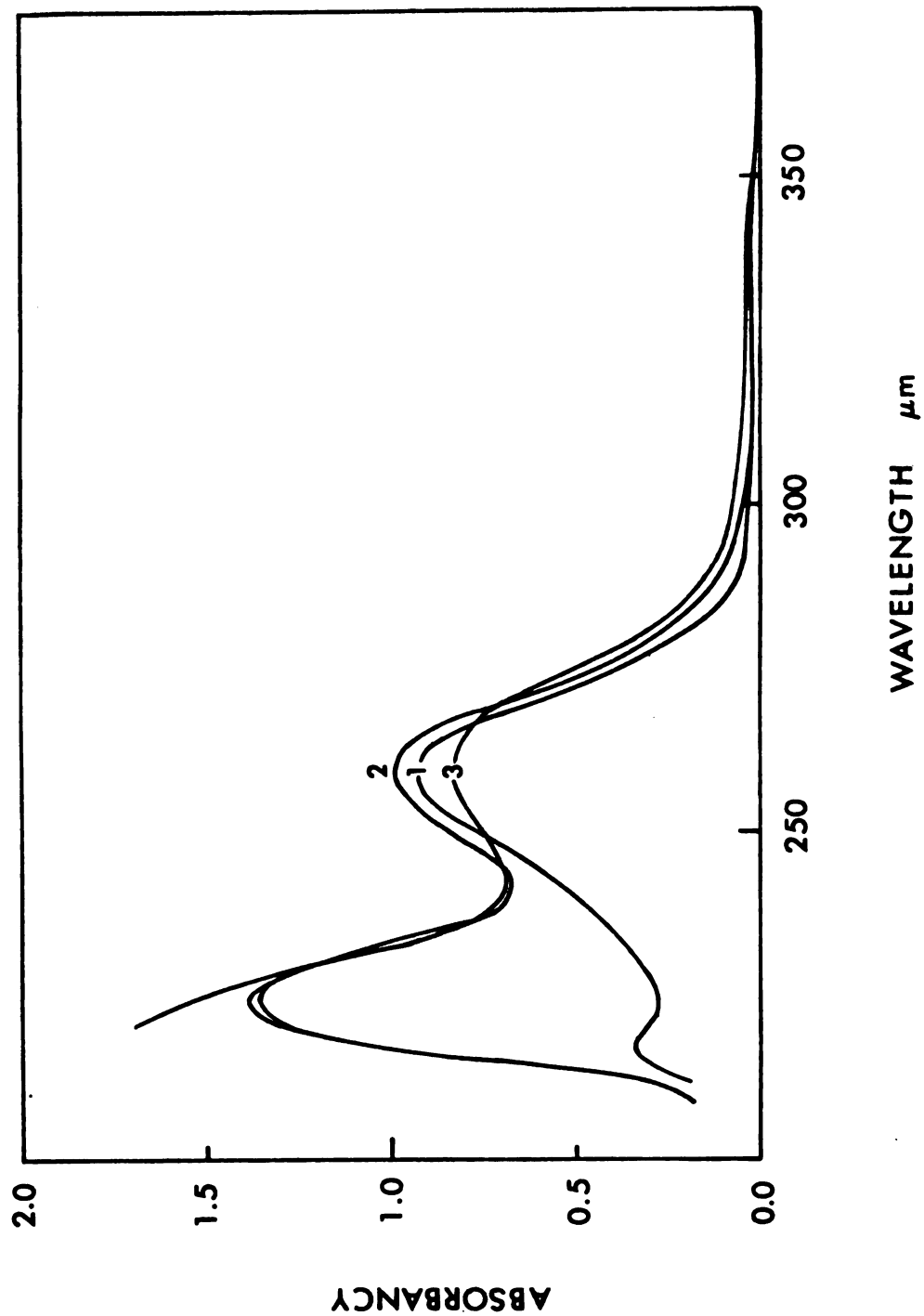


Figure 19. - Spectrum of N-(6-hydroxyheptyl)NAD^+ without ethanol

1. Analog alone
2. Analog with HLAD after 1, 3, 5, 7, and 9 mins.
3. Analog with HLAD after incubation for 24 hrs.

velocities under extremely dilute conditions. Spectra were run for five minutes of continuous scan on a computer centered instrument which gave simultaneous, corrected fluorescence and absorbance data. Scanning time was restricted to five minutes due to instrumental limitations. This process attenuates the excitation beam such that the detector observation geometry changes as the concentration of the absorbing species changes which eliminates wavelength dependent errors.[43] As a check on the reaction beyond the five minute limitation, samples were incubated two hours prior to investigation with enzyme in buffer that was 0.01M in semicarbazide. It was hoped that by trapping the carbonyl as it was formed, the equilibrium could be shifted enough to determine whether a reaction had occurred. The results of these experiments are shown in Table 7. No attempts have been made to quantitate the data with repetitive experimentation. Of the compounds tested only 6-HHNAD$^+$ and 8-HNNAD$^+$ showed any significant reactivity, thus confirming preliminary

Table 7. - Fluorescence study of analog reactivity

Compound*	Fluorescence increase	
	t = 5 mins	t = 2 hrs
4-HPNAD$^+$	-	-
5-HHNAD$^+$	-	-
6-HHNAD$^+$	+	+
7-HONAD$^+$	-	-
8-HNNAD$^+$	+	+

* Abbreviations used: 4-HPNAD$^+$ = N-(4-hydroxypentyl)NAD$^+$
5-HHNAD$^+$ = N-(5-hydroxyhexyl)NAD$^+$; 6-HHNAD$^+$ = N-(hydroxyheptyl)NAD$^+$
7-HONAD$^+$ = N-(7-hydroxyoctyl)NAD$^+$; 8-HNNAD$^+$ = N-(8-hydroxynonyl)NAD$^+$

work done by ultraviolet spectroscopy. Studies made with NAD^+ and yeast ADH on alcohols of varying chain lengths lend some support to these findings. Kaplan observed a similar alternating trend for the free alcohols when the chain length was varied.[18]

Space filling models of the various compounds would seem to indicate that 4-HPNAD$^+$ would have an alkyl chain too short to place the hydroxyl group near the presumed reaction site. The fact that this compound does not show a reaction under the conditions outlined is encouraging in terms of tentatively eliminating a possible intermolecular reaction. This same argument may be used for 7-HONAD$^+$ since the alkyl chain length would undoubted enable the hydroxy bearing carbon to fit into the enzyme active site. Admittedly these are naive assumptions which require more extensive investigation.

Several additional anomolies still exist and much more information will be required. Essentially no information is known about the pH dependence, buffer effects, or inhibition due to enzyme or cofactor concentrations in the systems studies. It is fortunate that work done here supports that done previously in this laboratory under significnatly different conditions.[32,33,42]

No conclusions should be drawn, however, from work done to this point other than that the substrates prepared do mimic the natural system without the necessity of added substrate. Considering also that the N-alkylhydroxy analogs of NAD^+ were reacted as d,l mixtures, the influence of one or the other of these forms could drastically alter the reactions shown here. Work is currently being done to optimize the preparation and isolation of all the analogs as well as the resolution of the racemic aminoalcohols. It is hoped that future work will soon be able to answer the many unsolved problems presented here.

BIBLIOGRAPHY

Bibliography

1. O. Warburg, W. Christian, and A. Griese, Biochem Z, 282, 157 (1935).

2. A. Todd, J. Roy. Inst. Chem., 82, 309 (1958).

3. F. A. Loewus, F. H. Westheimer, and B. Vannesland, J. Amer. Chem. Soc., 75, 5018 (1953).

4. H. R. Levy, F. A. Loewus, and B. Vannesland, J. Amer. Chem. Soc., 79, 2947 (1957).

5. R. V. Lemieux and J. Howard, Can. J. Chem., 41, 308 (1963).

6. V. Prelog, Ind. Chim. Belge., 27, 1309 (1962).

7. J. W. Cornforth, Bioch. Bioph. Res. Comm., 9, 371 (1962).

8. G. J. Karabatsos and H. Nunez, unpub. results (1971).

9. For a listing of Class A and Class B enzymes see R. Bentley, "Molecular Assymetry in Biology," Vol II, Academic Press, New York, 1970, Table I, pp. 6-9; Table II, p. 11 and N. O. Kaplan in P. D. Boyer, H. Lardy, K. Myrback, "The Enzymes," Vol 3, Academic Press, New York, 1960, Table II, p. 115.

10. K. R. Hanson, J. Amer. Chem. Soc., 88, 2731 (1966).

11. J. W. Cornforth, R. H. Cornforth, C. Donniger, G. Popjak, G. Ryback, and G. J. Schroepter, Jr., Proc. Roy. Soc. Ser. B., 163, 436 (1966).

12. V. Prelog, Pure Appl. Chem., 9 (1), 119 (1964).

13. E. M. Kosower and D. C. Remy, Tetrahedron, 5, 281 (1959).

14. E. M. Kosower, Bioch. Biophys. Acta, 56, 474 (1962).

15. J. van Eys, F. E. Stolzenbach, L. Sherwood, and N. O. Kaplan, Biochem Biophys. Acta, 27, 63 (1958).

16. H. Theorell and R. Bonnichsen, Acta. Chem. Scand., 5, 1105 (1951).

17. G. J. Karabatsos, J. S. Fleming, N. Hsi, R. H. Abeles, J. Amer. Chem. Soc., 88, 849 (1966).

18. J. van Eys and N.O. Kaplan, J. Amer. Chem. Soc., 79, 2782 (1957).

19. V. E. Althouse, K. Veda, and H. S. Mosher, J. Amer. Chem. Soc., 82, 5938 (1960).

20. R. MacLeod, H. Prosser, L. Fikentscher, J. La-yi, and H. S. Mosher, Biochem., 3, 838 (1964).

21. V. E. Althouse, D. M. Fiegel, and W. A. Sanderson, and H. S. Mosher, J. Amer. Chem. Soc., 88, 3595 (1966).

22. C. Donniger and G Ryback, Biochem. J., 91, 115 (1964).

23. A. Gunther, F. Biller, M. Kellmer, and H. Simon, Angew Chem. Inter. Ed., 12, 146 (1973).

24. P. A. Frey, G. J. Karabatsos, and R. H. Abeles, Biochem Biophys Res. Comm., 18, 551 (1965).

25. B. Zagalak, P. A. Frey, G. J. Karabatsos and R. H. Abeles, J. Biol. Chem., 241, 3028 (1966).

26. C. Neuberg, Adv. Carbohyd. Chem., 4, 75 (1949).

27. C. Neuberg, and F. F. Nord, Ber., 52B, 2248 (1919).

28. P. A. Levene and A. Walti, J. Biol. Chem., 94, 361 (1933).

29. S. Grzycki, Biochem. Z., 265, 195 (1933).

30. S. Fumise, Biochem. Z., 236, 231 (1931).

31. R. V. Lemieux and J. Giguerre, Can. J. Chem., 29, 678 (1951).

32. H. Nunez, unpub. results 1972.

33. V. C. Stamoudis, Ph.D. Thesis, Michigan State University, 1973, Table 14, p. 57.

34. See B. M. Anderson and N. O. Kaplan, J. Biol. Chem., 234, 1226 (1958) and refs. therein for prep., rel. rates of redn., and λ_{max} for other NAD^+ Analogs; also H. Sund, "Pyridine Nucleotide Dependent Dehydrogenases," Springer-Verlag, Heidelberg, 1970, Table I, pp. 606-607.

35. A. J. Gordon and R. A. Ford, "The Chemists Companion," J. Wiley and Sons, New York, 1972, p. 433.

36. a) Prepared via Michael Condensation with acrylonitrile, see M. K. May, M.S. Thesis, Michigan State University, 1973, p. 12.

    b) Ibid., Idem., pp. 17-18.

37. N. F. Albertson, J. Amer. Chem. Soc., 72, 2594 (1950).

38. A. P. Krapcho and A. J. Levy, Tet. Lett., 957 (1973).

39. L. F. Fieser and M. Fieser, "Reagents for Organic Synthesis," Vol I, J. Wiley and Sons, New York, 1967, p. 725.

40. R. Monzingo, "Organic Synthesis Coll. Vol II," 181 (1955).

41. A. A. Pavlic and H. Adkins, J. Amer. Chem. Soc., 68, 1471 (1946).

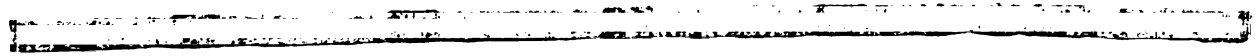
42. J. Miedema, Ph.D. Thesis, Michigan State University, 1974.

43. J. F. Holland, R. E. Teets, and A. Timnick, Anal. Chem., 45 (1), 145 (1973).

44. S. P. Colowick, N. O. Kaplan, and M. M. Ciotti, J. Biol. Chem., 197, 447 (1951).

45. L. J. Zatman, N. O. Kaplan, S. P. Colowick, and M. M. Ciotti, J. Biol. Chem., 209, 467 (1954).

46. H. Theorell, A. P. Nygaard, and R. Bonnischen, Acta. Chem. Scand., 9, 1148 (1955).

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