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# ABSTRACT

## HYBRID COENZYME-SUBSTRATES AS CONFORMATIONALLY RESTRICTED PROBES OF DEHYDROGENASE ACTIVE SITES

By

Jelmer Andries Miedema

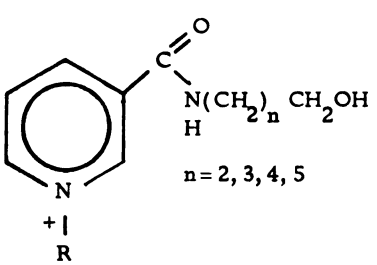
Since the early discovery of the stereospecificity of enzymatic reactions,[1] many substrate-product studies have been performed with a view towards rationalization of enzyme structure and reaction mechanism. In the case of the alcohol dehydrogenases (E. C. 1. 1. 1. 1), responsible for the nicotinamide adenine dinucleotide mediated interconversion of hydroxylic and carbonyl substrates, much ingenious experimental work by Prelog,[2] Ringold,[3] and others has gone into the construction of composite aliphatic substrates in attempts to clarify these points. Karabatsos[4], Stamoudis[5] and others have extended these results to substrates in which hydrophilic-hydrophobic interactions become significant.

Less work has been done regarding the orientation of the resulting "diamond lattice sections"[2] with respect to the enzyme-coenzyme binary complex. The solution of this problem is a necessary complement to construction of these models if any mechanistic or structural data derived from them are to be properly interpreted.

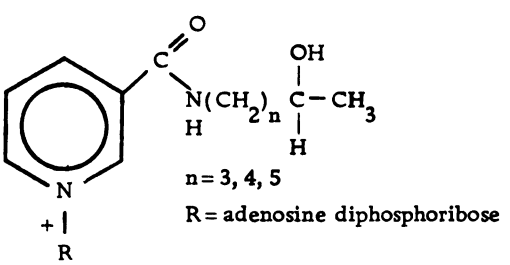
This interpretation will yield a predictive model of the binary complex-substrate interaction and considerable stereochemical information about the enzyme active site.

A model describing this interaction cannot result from further work with the conventional substrate-enzyme-coenzyme three-body systems. Such systems permit far too many degrees of conformational freedom to allow the single transition state--product correlation implicit in the confirmation of any given model. Imposition of an additional constraint would be required to make such a correlation possible.

This is simply accomplished by physically linking the substrate molecule to the coenzyme through an orientation restricting methylene bridge. To this end the following hybrid coenzyme-substrates were synthesized:




I




II

These analogs of nicotinamide adenine dinucleotide showed full activity in the horse liver alcohol dehydrogenase catalyzed oxidation of ethanol, at a rate however $10^{-3}$-$10^{-4}$ that observed in the natural

system. None showed any reactivity with yeast alcohol dehydrogenase.

Hybrid coenzyme-substrates I(n = 4, 5) and II(n = 4, 5) also had the ability to oxidize small but significant amounts of the attached hydroxyl moiety.

Further studies of I(n = 4, 5) in semicarbazide hydrochloride containing buffers indicated very significant effects on both $K_{eq}$ and $k_{initial}$ in the case of I(n = 4) but only minor effects in that of I(n = 5). The meaning of this result is not yet clear.

An inhibition experiment gave strong but not conclusive evidence for an intramolecular reaction in the case I(n = 4) (i.e. evidence that both the hydroxyl and coenzyme moieties participating in the ternary complex were provided by the same hybrid coenzyme analog). No conclusion regarding reaction mechanism could be drawn from a similar experiment with I(n = 5), but models support the likelihood of an intramolecular mechanism in this case as well as the previous. If these preliminary results are confirmed, I(n = 4, 5) and hopefully also II(n = 4, 5) will provide useful stereochemical probes of the dehydrogenase active site.

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By

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# INTRODUCTION

Since time immemorial the ability of organisms to interconvert oxo and hydroxyl compounds has been the basis of and driving force behind life. At first this process was not at all associated with cellular metabolism, even in such a relatively obvious case as fermenting yeast, but was ascribed to a mysterious and ill-defined "geist". In fact, this important association was not made until 1835-37,[1] over one hundred and fifty years following van Leewenhoek's initial microscopic observations of yeast and other cell life.

Once made, however, this association had a subtle and tenacious hold on scientific thought. It became a commonly accepted view that intracellular chemical processes were mystically linked to the "life force" of the cell and by definition could not occur outside the cell. The strength of this belief was so great that its overthrow with the 1897 demonstration of the enzymatic activity of a cell-free (sterile) yeast cell extract was sufficient to win Edward Buchner the 1907 Nobel prize. The award was granted solely for the significance of the experimental result; it was generally recognized that the work involved only application of already well-known techniques.

This discovery removed much of the awe and mystery from

the processes of life. Interest in understanding these processes grew apace with belief in their understandability. It was found that Buchner's extract contained several components, one of which was termed an alcohol dehydrogenase for its ability to oxidize alcohols and reduce carbonyls through hydrogen and electron transfer (Batelli, Wieland, and Stern, 1913).[2] The significance of this type of enzyme became clearly apparent when its presence was determined in tissues from many sources, both plant and animal.

The structure of the coenzyme forming with the substrate the reduction-oxidation couple catalyzed by dehydrogenase was postulated in 1931 by Schenk and Euler and finally proved by synthesis in 1957 by Alexander Todd.[3] This structure is shown in Figure 1.

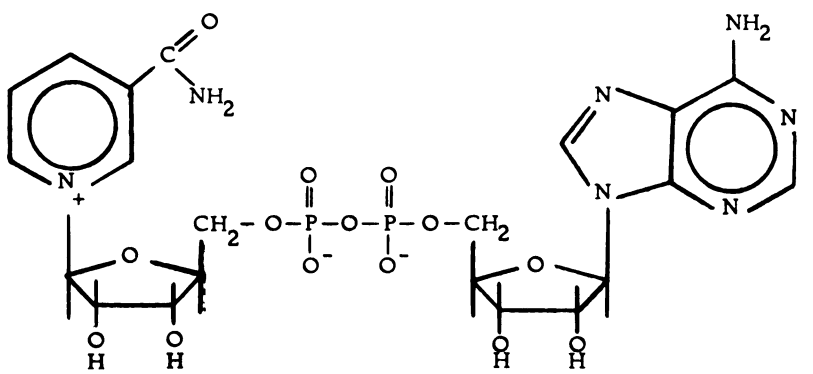


Figure 1. Structure of nicotinamide adenine dinucleotide.

The dehydrogenases and their associated coenzymes proved to be important agents both in aerobic and anaerobic respiration and in biological electron transport processes.[4] Work on these enzymes continued and quickly led to the purification by crystallization of the first alcohol dehydrogenase, that isolated from yeast (Negelein and Wulff, 1937).[5] Isolation of other dehydrogenases in pure form followed and left work in the field at a point where efforts to clarify the action of these enzymes could begin in earnest. Over the succeeding years much information has been obtained, culminating in the X-ray[6] and partial amino acid sequence analyses[7] of several dehydrogenases. Some remarkable similarities have been noted among these enzymes[8] and the growing completeness of our understanding of their static structures will eventually lead to understanding of their catalytic action.

As this research is concerned mainly with the dehydrogenase isolated from horse liver, a summary of the presently known characteristics of this enzyme is given below.

Horse liver alcohol dehydrogenase[9] (E. C. 1. 1. 1. 1.) is a metallo-enzyme of wide substrate specificity utilizing Zn as a co-factor and nicotinamide adenine dinucleotide as coenzyme. The enzyme has a molecular weight of 80,000 and is composed of two identical subunits. Although the separated subunits are inactive, they provide two active sites when combined in the enzyme molecule.

The amino acid sequence of the subunits is known,[7] as is the fact that each contains two Zn atoms, one of which seems to have structural and the other catalytic significance.[6] The enzyme exists in as many as twelve isoenzymatic forms which differ slightly in primary structure and in reactivity with acyclic versus steroidal substrates.[10] Crystal structure data to 2.9Å resolution are available for one of the isoenzymes.[6]

Shortly following the initial crystallization of horse liver alcohol dehydrogenase in more or less pure form in 1948[11] (the existence of the isoenzymatic forms was not recognized for another ten years), kinetic studies by H. Theorell and B. Chance established the enzyme as ordered bi-bi in its action; that is the enzyme binds coenzyme, then substrate, and releases product and then coenzyme in obligatory order.[12] This reaction sequence is represented by equation 1.

$$\mathrm{ROH+ENZ+NAD^+ \rightleftarrows ROH+ENZ::NAD^+ \rightleftarrows ROH::ENZ::NAD^+ \rightleftarrows} \quad (1)$$
$$\mathrm{C=O::ENZ::NADH+H^+ \rightleftarrows C=O+ENZ::NADH \rightleftarrows C=O+ENZ+NADH}$$

Almost simultaneously with this clarification of the kinetics of the catalysis some details of the mechanism became known. Through the use of deuterium labeled substrate and coenzyme, Westheimer, Vennesland and coworkers were able to show that the hydrogen transfer step of the redox equilibrium was both direct[13] and stereospecific[14] with respect to coenzyme and substrate. The

structure of the reduced coenzyme was as yet unknown, but these studies demonstrated that the hydrogen (deuterium) atom leaving the hydroxylic substrate was ultimately bonded to the reduced coenzyme and that this same atom was transferred in the reverse step to yield an alcohol stereoisomerically identical to the original. Any possibility of participation by solvent protons or exchange with other components of the system was thus eliminated.

Although the actual structure of the reduced coenzyme was uncertain, it was known that reduction took place on the nictotinamide moiety. The three positions ortho and para to the ring nitrogen which could conceivably undergo reduction were similar in that all would yield a methylene bearing diastereotopic hydrogens. That is, in all three cases if the individual members of the pair of protons bonded to the methylene were alternately replaced by any other atom, the two new molecules generated would be neither identical nor mirror images of each other. Clearly then, though bonded to the same carbon, these protons were distinct and to be consistent with the observations previously mentioned an enzyme could utilize only one of the two protons. Thus the dehydrogenases were classified in two sub-groups based on the proton utilized. Lacking any real structural information these were arbitrarily labeled as type A and type B enzymes. A fairly comprehensive listing of dehydrogenases by type is given in reference 15. Horse liver alcohol dehydrogenase is of type A.

In 1953 the methylene of the reduced coenzyme was shown to be located in the position para to the ring nitrogen.[16] It was another nine years before the absolute configuration was determined and the resulting assignment of the A and B protons made. After applying a difficult enzymatic cleavage and chemical breakdown procedure, Cornforth, Popjak, and coworkers arrived at the following structure.[17]

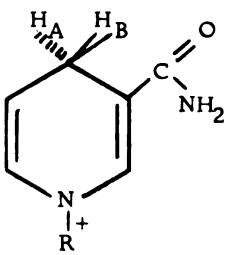



Figure 2. Absolute configuration of NADH.

With the elucidation of these basic details of the enzyme's mechanism, attempts to probe the active site of this and other dehydrogenases began to intensify. Initially this work focused on attempts to determine the steric dimensions of the active site by the construction of "diamond lattice sections".[18, 19, 20]

In the case of the dehydrogenase isolated from Curvularia falcata, V. Prelog approached this problem by determining the stereoisomeric distribution of products arising from the reduction of the isomeric decalin-1, 4-diones.[18] By aligning those products forming at measurable rates in such a way that the $CR_2HOH$ tetrahedra

were coincident Prelog was able, by virtue of conformational restrictions on the decalin skeleton, to sketch out the apparent volume which could be occupied by substrate at the active site. By also taking into consideration the differing rates of reaction of the various substrates he was able to identify "forbidden" positions which if occupied would greatly reduce substrate reactivity. Similar work reported in the same paper with horse liver alcohol dehydrogenase as catalyst and methyl substituted cyclohexanols and decalin derivatives as substrates resulted in the diamond lattice shown in Figure 3.

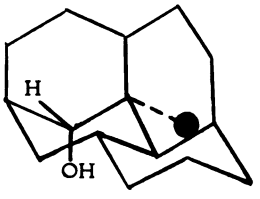



Figure 3. Characteristic diamond lattice for HLAD, after Prelog.

This work was later greatly elaborated by Ringold and collaborators. These workers arrived at the modified diamond lattice section for horse liver alcohol dehydrogenase shown in Figure 4[19] and performed detailed studies on the 3-hydroxysteroid dehydrogenase isolated from P. testosteroni.[20]

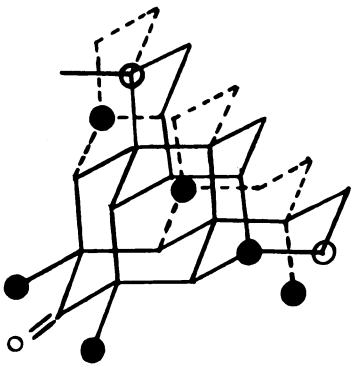


Figure 4. Modified diamond lattice for HLAD, after Ringold et al.

Still, the precise significance of these lattice sections remained somewhat obscure. If the enzyme active site were a rigid structure these models could be considered simply as defining the volume at the catalytic site not occupied by either solvent, bonded ions, or protruding members of the enzyme protein. This explanation clearly did not account for the possibility of the enzyme undergoing consecutive changes in conformation with the binding of coenzyme and substrate molecules, changes which perhaps were not independent of the substrate bound. Although this hypothesis has yet to be completely confirmed or denied, it is implicit in the Theorell-Chance mechanism and the enzyme is known to lose a symmetry element on binding of nicotinamide adenine dinucleotide.[6] The likelihood of similar conformational changes upon substrate binding cannot be ignored.

The existence of these diamond lattice models did, however, suffice as a starting point for speculation regarding the orientation of the carbonyl bond axis with respect to the coenzyme, or to be more precise, with the coenzyme-enzyme binary complex. On the basis of his work with the type B dehydrogenase associated with Curvularia falcata, Prelog postulated the following steric relationship between substrate and coenzyme.[18]

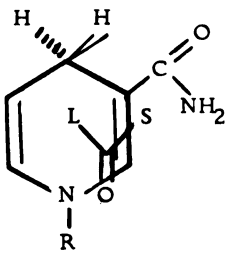



Figure 5. Prelog's model of carbonyl orientation with respect to NADH.

He supported this model by stating that this orientation, with the larger substituent further from the bulky carboxamide and both substituents neatly straddling the methylene bridge, minimized non-bonded interactions in the transition state. Prelog carefully noted that it was the structure of the enzyme protein which would play the decisive role in the stereochemistry of this complex, thus acknowledging both the probability of hydrophilic-hydrophobic interactions

with the enzyme protein and the primacy of enzyme-substrate (not coenzyme-substrate) steric interactions.

This model generally supported another, developed on independent evidence, by E. M. Kosower.[21] It had previously been noted that the 340nm absorption peak of reduced nicotinamide adenine dinucleotide shifted to a lower wavelength, 325nm, upon binding of the coenzyme to horse liver alcohol dehydrogenase. Kosower rationalized this observation by postulating the presence of a quaternary nitrogen about 3Å from the nitrogen of the dihydropyridine ring. Model compound studies indicated that such a structure would indeed shift the energy of the excited state dipole relative to the ground state species sufficiently to account for the experimental result. Incorporating this ammonium ion, he hypothesized the following ternary complex with the ion covalently linked to the enzyme protein and hydrogen bonded to both substrate and coenzyme ribose.

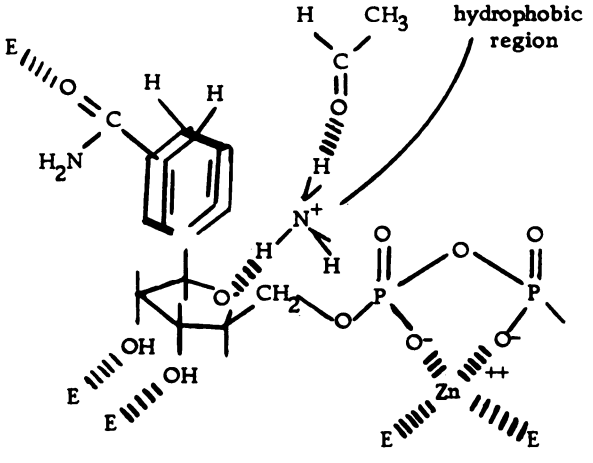



Figure 6. Kosower's model of the ternary complex.

Clearly this model supported Prelog's conclusions regarding the orientation of the carbonyl, but again no inference regarding the relative positions of the carbonyl substituents could be drawn unless further assumptions regarding non-bonded interactions and hydrophilic and/or hydrophobic enzyme regions were made.

A third model, offered by Graves and Ringold,[19] completely contradicts the previous two. On the basis of their work with horse liver alcohol dehydrogenase they arrived at the following structure.

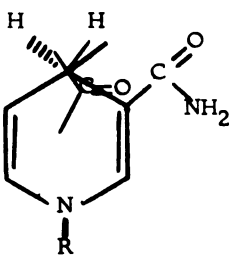



Figure 7. Graves' and Ringold's model of carbonyl orientation with respect to NADH.

It is interesting to note that all of these models could predict the same product structure in a given reduction. Thus it becomes a doubly worthwhile problem to test the correctness of one or another of these models. This question is complicated by the fact that it has become clear that enzyme-substrate interactions do take precedence over interactions with coenzyme and so the model may be different for every enzyme. This is most succinctly shown by noting the

existence of two A type lactic acid dehydrogenases, one specific for d-lactic acid and the other for l-lactic acid.[22] It is also reflected in the work of Karabatsos,[23] Stamoudis, and Nunez[24] who demonstrated the fallacy of applying Prelog's model for C. falcata to alcohol dehydrogenase. They arrived at the model shown in Figure 8; the opposite of that of Prelog.

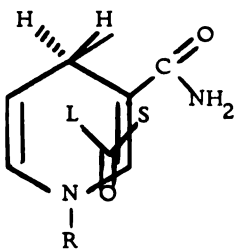
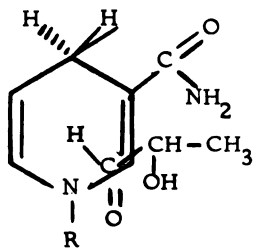
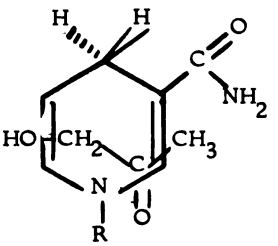



Figure 8. Karabatsos' model of carbonyl orientation with respect to NADH.

In addition, through studies utilizing polar hydrogen bonding substrates, these workers showed the significance of hydrophilic-hydrophobic interactions and the ability of such forces to counterbalance and even overwhelm those resulting from steric interactions. If, as in Figure 9, one of the carbonyl substituents is sufficiently hydrophilic, the relative orientation predicted on steric grounds may be completely reversed.

a. b.





100% (ref. 22) 100% (ref. 25)

Figure 9. Competition between hydrophilic-hydrophobic and steric interactions for control of substrate orientation.

All these models seem reasonable on the basis of the evidence offered in their defense. In no sense however, does this evidence constitute a proof of any given model. This is clearly apparent when considering Figure 10.

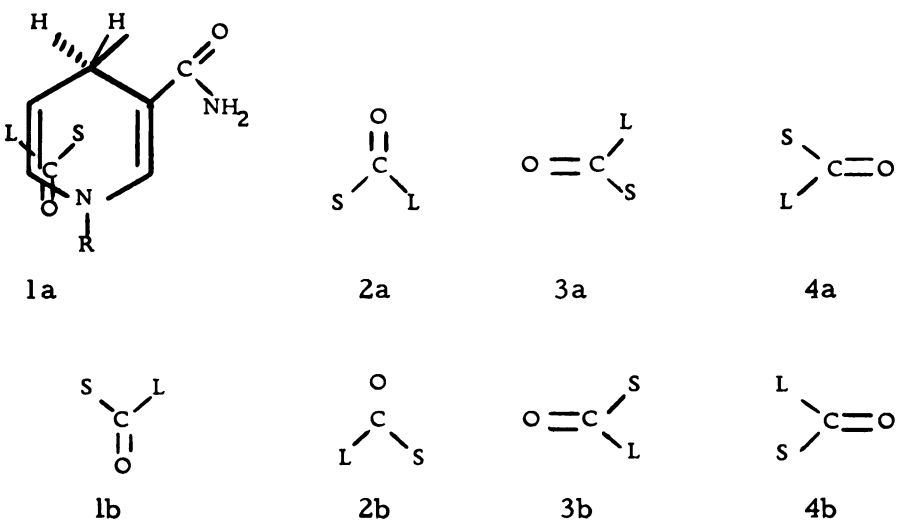


Figure 10. Tabulation of possible models of carbonyl orientation with respect to NADH.

L and S represent any two different substituents. 1, 2, 3, and 4a will yield the same product and 1, 2, 3, and 4b its enantiomer. The sigma $sp^2$ bonds are shown as projections on the dihydropyridine ring and the C = O bond axis is shown aligned with the coordinate on which its projection is longest. Coplanarity is not implied.

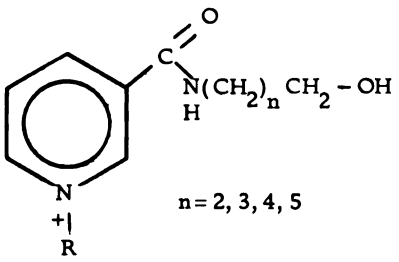
These eight models represent the possible orientations of the carbonyl with respect to the enzyme-coenzyme binary complex. Each represents an approximation to reality, for of course an infinite number of actual alignments are conceivable. Even ignoring this detail, at most two possible products (enantiomeric alcohols) may be formed from each carbonyl undergoing reduction. Thus a one to one correspondence between product and model does not exist and it follows that the choice of model cannot be made on the basis of product analysis alone. This conclusion extends to the diamond lattice studies of Prelog and Graves, based merely on the composites of individual product studies, and to all other known work in this field. Quite simply, not only is the previously available evidence insufficient to come to any conclusion regarding the structure of a ternary complex but the methods used to obtain the evidence are not suitable to this end.

Our incomplete knowledge of the enzyme active site can be expanded only by the application of different techniques. In recent years the X-ray studies of Branden,[6] Rossman,[8] and other workers, the work of Weiner and Mildvan with spin labeled analogs of

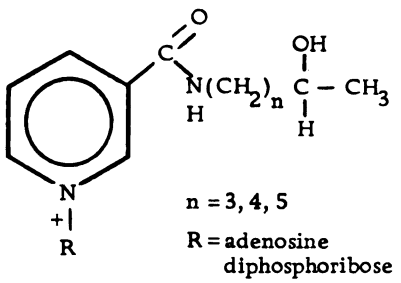
nicotinamide adenine dinucleotide,[26] and Takahashi's[27] analysis of the fluorescence properties of Co-modified horse liver alcohol dehydrogenase provide examples.

This thesis describes the development of another potentially useful probe of the active site. Consideration of the weaknesses of the product analysis based probes described previously quickly leads to apprehension of the common flaw. In all cases the experiment is carried out such that the ternary complex has an excessive number of degrees of freedom. In consequence a single rationale of the experimental data, and thus a single model, cannot be given. The solution of the problem self-evidently lies in the removal of the excess degrees of freedom. In principle this can be accomplished simply by binding the reacting species together and this has been the approach taken in this instance.

Specifically, this research project has involved the synthesis of and initial reactivity studies on analogs of nicotinamide adenine dinucleotide in which the hydroxylic substrate is incorporated into the coenzyme molecule. To the best of our knowledge this is the first research undertaken involving such hybrid coenzyme-substrate systems. The structures synthesized are shown on the following page.


O
C
N(CH2)n CH2 – OH
H
N
+
R
n = 2, 3, 4, 5

I


O
C
OH
N(CH2)n C – CH3
H
H
N
+
R
n = 3, 4, 5
R = adenosine diphosphoribose

II

# EXPERIMENTAL

## I. Synthesis

### Preparation of potassium 3-pyridinecarboxylate

To a 2000ml three-necked round-bottomed flask equipped with a mechanical stirring apparatus, powder funnel, and reflux condenser was added 1200ml of 95% ethanol. With stirring and some cooling, 60g (1.07mol) potassium hydroxide was dissolved in the alcohol. On complete dissolution of the base, stirring was continued and 123.1g (1.00mol) nicotinic acid (Sigma Chemical Co.) was added. The flask was then stoppered and brought to reflux. Reflux was continued for one half hour, the product was cooled to room temperature, and the clear solution was transferred to a 2000ml beaker. The solution was cooled further to 0°, allowed to stand at this temperature overnight, and the white crystalline product was filtered. After washing with cold ethanol and oven drying at 120° a first crop of 92g (0.571mol, 57% yield) of potassium 3-pyridinecarboxylate was obtained. Upon reducing the total volume to 250ml a second crop of 23g was obtained. The overall yield was 115g (0.714mol, 71% of the theoretical amount).

Preparation of 3-chloroformylpyridine[28]

To a 1000ml three-necked round-bottomed flask equipped with a mechanical stirring apparatus, reflux condenser terminated with a gas bubbler, and a 50ml addition funnel was added 400ml of carbon tetrachloride. Suspended with stirring in the carbon tetrachloride was 64.8g (0.402mol) of potassium 3-pyridinecarboxylate which had been dried under vacuum for 24 hours at 115°. The flask and addition funnel were thoroughly flushed with dry nitrogen, 36.0ml (59.7g, 0.501mol) thionyl chloride (Fisher Scientific, purified) was pipetted into the closed addition funnel, and the system was closed. The thionyl chloride was added dropwise with stirring to the suspension and the exothermic reaction was cooled as necessary. On completion of the addition the mixture was heated to reflux. With the cessation of $SO_2$ evolution the bubbler was replaced with a drying tube and the reflux was continued overnight. The solution was then cooled, filtered, and the carbon tetrachloride was removed in a rotary evaporator. The crude material was refiltered. Distillation under reduced pressure yielded 41.26g (0.291mol, 72% theoretical) of 3-chloroformylpyridine, bp. 70° at 4-5mm. The reported bp. was 98-100° at 21mm.

Preparation of potassium phthalimide[29]

The phthalimide used in this preparation was a very crude technical grade product (Eastman Organic, practical). As a

preliminary step, 100g (0.68mol) of the crude phthalimide was recrystallized from 2000ml 95% ethanol.

The recrystallized material was redissolved in 2000ml fresh 95% ethanol boiling in a 3000ml Erlenmeyer flask on a magnetic stirrer-hot plate. To the hot solution was added 46g (0.82mol) potassium hydroxide in 300ml 95% ethanol. The combined solutions were quickly mixed and immediately plunged into an ice bath. By replenishing the ice as necessary, the temperature of the solution was brought below 10° as quickly as possible. The product precipated immediately and was removed from solution by filtration, then washed twice with 100ml portions of 95% ethanol and a 100ml portion of acetone. After drying at 115°, 95g (0.51mol, 75% of theoretical) of potassium phthalimide was obtained.

Preparation of 5-chloro-2-pentanone[30]

5-chloro-2-pentanone was prepared from 2-acetylbutyrolactone (Aldrich) by a procedure submitted to Organic Syntheses by G. W. Cannon, R. C. Ellis, and J. R. Leal. The reaction was carried out on a four molar scale. Vacuum distillation yielded 390g (3.23mol, 81% of theoretical) of 5-chloro-2-pentanone, bp. 60-61° at 10mm. The reported bp. was 70-72° at 20mm.

Preparation of 5-chloro-2-pentanol

To a 500ml three-necked round-bottomed flask equipped with a magnetic football stirrer, thermometer, and 100ml addition funnel were added 175ml 95% ethanol and 28.50ml (30.14g, 0.250mol) 5-chloro-2-pentanone. The solution was cooled to 0° with stirring in an ice slush.

During the cooling period a solution of sodium borohydride (4.72g, 0.125mol, Metal Hydrides, Inc.) in 60ml of distilled water was prepared. This was added dropwise to the stirring ketone solution at a rate to keep the reaction temperature below 20°. On completion of the addition the solution was stirred vigorously for 12-14 hours at 0°.

The reaction mixture was titrated to neutrality with 20% sulfuric acid, again with cooling, and the precipated salts were removed by filtration. The solids were washed with 50ml of 95% ethanol which was then combined with the filtrate. Distillation under reduced pressure removed the bulk of the ethanol and some water (28-30° at 85mm). The residue was washed into a 250ml separatory funnel with 25ml ether to which was added sufficient extra water to provide an aqueous phase of about 100ml volume. The organic layer was separated and the remaining aqueous phase extracted twice with 50ml portions of ether. The combined ether fractions were washed with 50ml saturated sodium chloride solution and dried over potassium

carbonate. After removal of the ether by rotary evaporation the NMR spectrum of the product showed no starting material and only minor detectable impurities. Although distillation proved unnecessary and is not recommended due to the ready cyclization of the product, a bp. of 30° at 0.4-0.5mm was obtained. The reported bp. is 66-68° at 3mm.[31] The yield of crude 5-chloro-2-pentanol was 27.92g (0.228mol, 91% of theoretical).

Preparation of 2-(trimethylsiloxy)-5-chloropentane

An apparatus consisting of a dry 1000ml three-necked round-bottomed flask equipped with a mechanical stirrer, thermometer, and 100ml addition funnel terminated with a drying tube was prepared and flushed with dry nitrogen. To the flask were added 700ml anhydrous ether, 27.92g (0.228mol) 5-chloro-2-pentanol, and 35.5ml (30.4g, 0.280mol) chlorotrimethylsilane (Aldrich). The solution was cooled to 0° with stirring in an ice slush bath.

The addition funnel was closed, flushed with nitrogen, and filled with 50.0ml (36.3gm, 0.359mol) of triethylamine (Mallinckrodt) which had been dried over potassium hydroxide pellets. With the flask remaining in the ice bath, the triethylamine was added dropwise with stirring such that the reduction temperature did not exceed 20°. Since the reaction was accompanied by formation of a copious precipitate, the stirrer speed was adjusted to maintain proper mixing. On completion of the addition the reaction mixture was stirred for

an additional hour at room temperature.

The precipitate was removed by filtration and washed with 100ml anhydrous ether which was then combined with the filtrate. The ether and most of the residual triethyl amine and chlorotrimethylsilane were removed by rotary evaporation. This procedure was accompanied by more precipitation and the solution was again filtered. The solid was washed with 25ml of anhydrous ether which was then added to the filtrate. Further rotary evaporation yielded a crude product which showed no remaining hydroxyl and a strong absorption in the tetramethylsilane region of the NMR spectrum. The 2-(trimethylsiloxy)-5-chloropentane was not purified but used immediately to minimize hydrolysis losses.

Synthesis of 5-amino-2-pentanol — The Gabriel Synthesis[32]

Preparation of N-(4-trimethylsiloxypentyl)-phthalimide--To a 500ml three-necked round-bottomed flask equipped with a stirbar, thermometer, serum cap, and condenser terminated by a gas bubbler was added 45g (0.243mol) of potassium phthalimide. This was followed by addition of a solution of the crude 2-(trimethylsiloxy)-5-chloropentane (0.228mol assumed) in 200ml of dimethyl formamide that had been dried by storage over molecular sieve type 4A (J. T. Baker). The system was flushed with dry nitrogen, and the serum cap and bubbler were replaced with a glass stopper and drying tube respectively. The reaction mixture was stirred and heated gradually

to reflux at about 125°. After three hours of reflux the flask was cooled to room temperature.

The contents of the flask were poured into a 2000ml separatory funnel and diluted with 1000ml distilled water and 300ml chloroform. Shaking brought about the dissolution of all solids. The organic layer was removed and the aqueous phase was re-extracted twice with 100ml portions of chloroform. The chloroform layers were combined and washed with 200ml of 0.1N sodium hydroxide in order to remove excess phthalimide. Washes with 150ml distilled water and 150ml saturated sodium chloride followed. The solution was dried over anhydrous potassium carbonate and the chloroform removed by rotary evaporation. The crude N-(4-trimethylsiloxypentyl)-phthalimide was recovered as a yellowish oil. Some hydrolysis to N-(4-hydroxypentyl)-phthalimide was inevitable and of no consequence. Purification was not attempted.

Preparation of 4-trimethylsiloxypentylammonium phthalhydrazide -- To a 1000ml single-necked round-bottomed flask equipped with a stirbar and condenser were added the crude N-(4-trimethylsiloxypentyl)-phthalimide (0.228mol assumed), 11.5ml (11.85g, 0.237mol) hydrazine hydrate (Matheson, Coleman, and Bell), and 500ml methanol. The reaction mixture was refluxed for one and a half hours. Upon cooling to room temperature a white asbestos-like solid was deposited.

The flask contents were cooled to 0° and the solid was filtered and saved. Rotary evaporation of the methanol from the filtrate resulted in the progressive precipitation of more product which was periodically collected by filtration. No attempt was made to purify the 4-trimethylsiloxypentylammonium phthalhydrazide from the combined solids and the small amount of oily residue remaining after the removal of the methanol.

Preparation of 5-amino-2-pentanol -- To a 1000ml single-necked round-bottomed flask equipped with a stirbar and condenser was added the 4-trimethylsiloxypentylammonium phthalhydrazide (0.228mol assumed) dissolved in 500ml distilled water. After stirring was begun, 21ml of concentrated hydrochloric acid (8.31g HCl, 0.251mol) was added to the clear yellow-green solution. A heavy precipitate of free phthalhydrazide was immediately evident.

In order to complete hydrolysis of the silyl ether the mixture was heated and then refluxed gently for two hours. After the mixture was cooled to room temperature the phthalhydrazide was removed by filtration, resuspended in 200ml distilled water, and refiltered. The combined filtrates were subjected by rotary evaporation to reduce the total volume to about 200ml. Any additional precipitate was again removed by filtration.

The hydrochloric acid and salts in solution were neutralized by the addition of 10g (0.250mol) of sodium hydroxide. Additional

acid or base was added as required to bring the pH of the reaction mixture to 10. The mixture was poured into a 500ml separatory funnel and twice extracted with 50ml portions of chloroform to remove discoloring side-products. These chloroform layers were discarded and the aqueous phase was made 30% in base by the addition, with cooling, of 60g of sodium hydroxide.

This solution was extracted with 100ml portions of chloroform. A few drops of each chloroform extract were evaporated on filter paper and checked for amine concentration with a ninhydrin spray reagent (Nin-Sol®, Pierce Chemical Company). After eight to ten extractions, the intensity of the developed color fell and the combined extracts were dried over anhydrous potassium carbonate. Following filtration the chloroform was removed by distillation through a fifteen inch asbestos-wrapped Vigreaux column. Especially in the latter stages care was taken not to overheat the product nor entrain it with distilling solvent. Gradual application of a vacuum was helpful. The product distilled at 56-59° at 0.2-0.4mm. The reported bp. is 80-81° at 1mm.[31] The yield was 14.5g (0.141mol, 62% of the theoretical based on 5-chloro-2-pentanol).

Anal. Calcd. for $C_5H_{13}NO$: C, 58.21; H, 12.70; N, 13.58.

Found: C, 58.06; H, 12.81; N, 13.40.

Preparation of 1-methylcyclohexyl hydroperoxide[33]

To a 2000ml three-necked round-bottomed flask equipped with a long condenser, thermometer, and slit serum cap was added 1200ml (923g, 9.40mol) of methylcyclohexane (Fisher Certified). A fritted glass gas diffusion tube was inserted through the serum cap, which should fit snugly, and connected to an air supply which was largely dried and freed from carbon dioxide by passage through a drying tower containing layers of potassium hydroxide and indicating Drierite® (W. A. Hammond). The liquid was heated to 98-100° and its level was marked on the flask. Air was then bubbled through the material at a rate such that the aerosol droplets visible in the condenser air column seemed, as nearly as possible, neither to rise nor fall. The rheostat controlling the heating mantle was adjusted to maintain the required temperature, and then both rheostat and flow rate were periodically readjusted until equilibrium was attained. The reaction was allowed to continue for seven days, with additional methylcyclohexane being added as required to maintain approximately the marked volume. No more than 100ml should be lost in the initial twenty-four hours and the rate of loss should drop as the reaction proceeds.

During the latter half of the reaction period the hydroperoxide concentration was monitored by iodometric titration.[34] (See method of Wagner, Smith, and Peters.) The final hydroperoxide content

reached 1.06meq/ml. When no further increase in hydroperoxide titer was noted, air flow was halted, a stirbar was added, the condenser was replaced with a distillation head, and the excess methylcyclohexane was immediately removed. Distillation at atmospheric pressure was discontinued when the pot temperature approached 105° and the collected distillate, mainly starting material and water, was dried over molecular sieve and reused.

The product mixture was then transferred to an appropriately sized single-necked round-bottomed flask equipped with a stirbar and vacuum distillation head. During vacuum distillation it was essential to cool the distillation flask to room temperature if for any reason the vacuum had to be broken. Failure to take this precaution resulted in product decomposition accompanied by violent deflagration.

Following a forerun of residual methylcyclohexane, a second fraction was removed, bp. 27-28° at 2mm. The product distilled at 54-60°, 0.15mm. (literature bp. 38° at 0.03mm) Iodometric titration showed the hydroperoxide content to be 100% within experimental error.

The yield of hydroperoxide was 104g (0.799mol, 8.5% of theoretical). The product is a mixture of the desired 1-methylcyclohexyl hydroperoxide (60%) and the isomeric 2, 3, and 4-methylcyclohexyl hydroperoxides (40%).[33] Thus the yield of the desired isomer is 62.4g (0.479mol, 5% of theoretical). No attempt was made to

separate the 1-methylcyclohexyl hydroperoxide from its isomers.

Preparation of 7-chloro-2-heptanone[35]

To a 2000ml three-necked round-bottomed flask equipped with a thermometer, football stirrer, and 1000ml addition funnel was added a solution of the mixed hydroperoxide product (0.720mol total, 0.432mol 1-methylcyclohexyl hydroperoxide) in 210ml petroleum ether (Matheson, Coleman, and Bell; bp. 30-60°). The solution was placed in an ice bath and cooled to 0° with stirring.

In a 1000ml beaker equipped with a stirbar was prepared a solution containing 207g (0.745mol) ferrous sulphate heptahydrate (Matheson, Coleman, and Bell), 485ml distilled water, and 170ml (2.04mol) concentrated hydrochloric acid. After cooling to room temperature this solution was added dropwise to the cooled and vigorously stirred hydroperoxide mixture. The rate of addition was controlled such that the temperature of the reaction medium rose to and was maintained at 14-16°. The initially water-white organic phase quickly turned a dark red-brown and then increasingly yellow-green as the addition proceeded. Upon completion of the addition, stirring was continued and the ice bath was removed. The reaction mixture was brought to room temperature and stirred an additional half hour.

After the aqueous and organic layers were separated by using

a 2000ml separatory funnel, the aqueous phase was reextracted with 75 and 50ml portions of petroleum ether. The combined ether layers were washed with 25ml $H_2O$, 25ml saturated sodium chloride and finally dried over anhydrous potassium carbonate. Following filtration the petroleum ether was removed by rotary evaporation. Vacuum distillation of the residue yielded 51.19g (0.344mol, 80% of theoretical) of 7-chloro-2-heptanone, bp. 78-80° at 3mm. The reported bp. is 101-102° at 16mm.

Preparation of 7-chloro-2-heptanol

To a 500ml three-necked round-bottomed flask equipped with a thermometer, stirbar, and 100ml addition funnel was added 51.19g (0.344 mol) of 7-chloro-2-heptanone in 120 ml methanol. The mixture was cooled to 0° by stirring in an ice bath.

A solution of 4.88g (0.129mol) of sodium borohydride (Metal Hydrides, Inc.) in 70ml distilled water was prepared and added dropwise to the stirring methanolic ketone solution. Cooling was continued to maintain the reaction temperature below 20°. On completion of the addition the reaction mixture was stirred for 12-14 hours at 0°.

The homogenous product mixture was titrated to neutrality with 30% sulfuric acid and the precipitated salts were removed by filtration. The solide were washed with some methanol which was then combined with the filtrate. The bulk of the methanol was

removed by using a rotary evaporator. Approximately 100ml of distilled water was added to the residue and the mixture poured into a 500ml separatory funnel. The evaporation flask was rinsed with ether which was then poured into the funnel. The product was extracted with three 75ml portions of ether. The combined ether layers were washed with 50ml of saturated sodium chloride, dried over potassium carbonate, and the ether removed by rotary evaporation. The crude 7-chloro-2-heptanol weighed 46.55g (0.309mol, 90% of theoretical) and boiled at 54-55°, 0.15mm.

Synthesis of 7-amino-2-heptanol — The Gabriel Synthesis[32,36]

Preparation of N-(6-hydroxyheptyl)-phthalimide -- To a 250ml three-necked round-bottomed flask equipped with a stirbar, condenser, and thermometer was added 33.7g (0.182mol) of potassium phthalimide. A solution of 20.18g (0.134mol) of 7-chloro-2-heptanol in 100ml dry dimethylformamide was prepared and added with stirring.

While stirring was continued, the mixture was heated to 120-150° for three hours. The flask contents were then cooled to 100° and 10ml of distilled water was added. The mixture was reheated to reflux at 117° and left for 12-14 hours.

The flask contents were cooled to room temperature and poured into a 1000ml separatory funnel. The reaction flask was rinsed with 150ml of chloroform which was added to the separatory

funnel along with 500ml of distilled water. The mixture was shaken, the chloroform layers were washed with 150ml 0.1N sodium hydroxide, 150ml distilled water, and 150 ml saturated sodium chloride. The solution was then dried over anhydrous potassium carbonate and the chloroform and some dimethylformamide were removed by rotary evaporation. The residual oil was used without purification.

Preparation of 6-hydroxyheptylammonium phthalhydrazide -- To a 500ml single-necked round-bottomed flask equipped with a stirbar and condenser were added a solution of the crude N-(6-hydroxyheptyl)-phthalimide (0.134mol assumed) in 300ml methanol, and 6.6ml (6.8g, 0.136mol) hydrazine hydrate. After refluxing the mixture for one and a half hours, most of the methanol and excess hydrazine hydrate were removed by rotary evaporation. The residue was used without purification.

Preparation of 7-amino-2-heptanol -- The solid 6-hydroxy-heptylammonium phthalhydrazide (0.134mol assumed) was dissolved in 250ml distilled water and the pH of the solution was adjusted to 10 by the addition of concentrated potassium hydroxide solution. The mixture was poured into a 1000ml separatory funnel and extracted twice with 50ml portions of chloroform to remove any colored reaction side products. When the chloroform extracts were colorless the solution was made 30% in potassium hydroxide and the product was extracted with 100ml portions of chloroform. The aminoalcohol

content of the successive extracts was monitored as described under the preparation of 5-amino-2-pentanol. The tendency for fine needles of potassium phthalhydrazide to form during extraction was counteracted by the addition as necessary of extra 30% potassium hydroxide.

The combined extracts were dried over anhydrous potassium carbonate and the chloroform was removed with efficient stirring and careful distillation through a Vigreaux column to minimize entrainment. The yield of 7-amino-2-heptanol was 11.79g (0.0898mol, 67% of theoretical based on 7-chloro-2-heptanol), bp. 68-69° at 0.10-0.15mm, mp. 37-39°.

Anal. Calcd. for $C_7H_{17}NO$: C, 64.07; H, 13.06; N, 10.68.
Found: C, 64.01; H, 13.06; N, 10.53.

Synthesis of the N-(hydroxyalkyl)-3-pyridinecarboxamides

A procedure suitable for the preparation of this series of compounds from the corresponding aminoalcohols is described. A list of the aminoalcohols and their sources follows in Table 1.

Table 1

Reactant aminoalcohols

| Aminoalcohols | Source |
|---|---|
| 3-aminopropanol | Aldrich |
| 4- aminobutanol | Chemicals Procurement Lab. |
| 5-aminopentanol | Aldrich; Pfaltz and Bauer |
| 6-aminohexanol | Pfaltz and Bauer |
| 5-amino-2-pentanol | this work |
| 6-amino-2-hexanol | Michael K. May, M. S. thesis, M. S. U., 1973. |
| 7-amino-2-heptanol | this work |

Preparation of the trimethylsiloxyalkylamine -- A 100ml bantam three-necked round-bottomed flask fitted with a stirbar, serum cap, and condenser terminated by a gas bubbler was flushed with dry nitrogen. About 0.050mol of dry aminoalcohol was added and the exact amount was determined by difference weighing. While keeping the system under a blanket of dry nitrogen an equivalent (0.025mol, 5.32ml, 4.11g) of hexamethyldisilazane, (Aldrich) followed by a catalytic amount of oven-dried ammonium chloride, was added. Stirring was begun.

Nitrogen flow was discontinued and the heterogeneous reaction mixture was slowly heated. Upon warming, the evolution of gaseous ammonia began. Heating was continued until the oil bath temperature

reached 130°. At this point no reflux was visible, ammonia evolution had ceased, and the reaction mixture had become a homogeneous water-white solution. The reaction flask was maintained at this temperature for a brief period, the gas bubbler was replaced by a drying tube, and the solution was allowed to cool. Purification of the silyl ether amine was not necessary.

Preparation of N-(trimethylsiloxyalkyl)-3-pyridinecarboxamide -- Keeping the silyl ether amine under a blanket of dry nitrogen, 35ml of pyridine (stored over molecular sieve type 4A) and 15ml of triethylamine (stored over potassium hydroxide) were added to the previously described reaction flask. The flask was heated to 105° and an equivalent (0.050mol, 7.20g, 5.57ml) of 3-chloroformylpyridine was slowly added by using a disposable syringe equipped with a neoprene plunger. On completion of the addition the serum cap was replaced with an ungreased glass stopper and the mixture was refluxed for one half hour.

After cooling to 0° the precipated triethylammonium hydrochloride was removed by filtration. The N-(trimethylsiloxyalkyl)3-pyridinecarboxamide was not removed from the solvent.

Preparation and isolation of N-(hydroxyalkyl)-3-pyridinecarboxamide -- A 100ml bantam round-bottomed flask was fitted with a stirbar and distillation head. The N-(trimethylsiloxyalkyl)-3-pyridinecarboxamide solution was added and the remaining triethylamine was removed. The distillation head was replaced by a reflux

condenser, 5ml of distilled water was added, and the solution was refluxed for about an hour or until hydrolysis of the silyl ether was complete. This reaction was easily monitored by taking successive NMR spectra of neat samples of the mixture. Hydrolysis was accompanied by a distinctive 7hz downfield shift of the protons in the silane region.

On complete hydrolysis the pyridine-water azeotrope and remaining pyridine were completely removed by vacuum distillation followed by several air flushes and depressurizations at about 120°. The crude N-(hydroxyalkyl)-3-pyridinecarboxamide was consistently obtained in 98-100% of the theoretical yield. Analysis by infra-red spectroscopy and thin-layer chromatography (silica gel, 8:2/$CHCl_3$:$CH_3OH$) showed the desired major product and minor amounts of 3-pyridinecarboxylic acid and other unidentified compounds. The product was taken up in dry chloroform and purified by chromatography on alumina.

A 2.5x80cm alumina column (Matheson, Coleman, and Bell, Alcoa type F-20, 80-200 mesh) was prepared in 100:2/$CHCl_3$:$CH_3OH$ solvent, an aliquot of chloroform solution containing about 5g crude product was applied, and the column was eluted with the same solvent at a flow rate of 1.5-2.0ml/min. Due to the straightforward nature of the synthesis and lack of intermediate work-up, isolated yields of 85-90% were typical. The products were viscous

yellowish oils. Some crystallized spontaneously and most could be crystallized from various solvents. A table of melting points follows.

Table 2

Melting points of solid N-(hydroxyalkyl)-3-pyridinecarboxamides

| N-(hydroxyalkyl)-3-pyridinecarboxamide | Solvent | M. P. |
|---|---|---|
| N-(3-hydroxypropyl)* | Ethyl acetate | Between 5-20° |
| N-(4-hydroxybutyl) | Ethyl acetate | 71-73° |
| N-(5-hydroxypentyl) | Ethyl acetate | 86-88° |
| N-(6-hydroxyhexyl)* | Ethyl acetate | 79-80° |
| N-(4-hydroxypentyl) | Not yet obtained | -- |
| N-(5-hydroxyhexyl)** | Acetone | 45-51° |
| N-(6-hydroxyheptyl) | Not yet obtained | -- |

*I would like to express my appreciation to Vassilios C. Stamoudis for his preparation of these compounds.

**Michael K. May, M. S. thesis, Michigan State University, East Lansing, 1973.

## II. Preparation and isolation of $NAD^+$ analogs[37]

A five dram glass vial equipped with a polyethylene cap and miniature stirbar served as an incubation vessel for this enzymatic synthesis. The incubation was performed in a circulating water bath thermostatically controlled at 37 ± 0.25°.

To the vial were added 0.185g (0.251mmol based on a hydrated $M_w$ of 735) nicotinamide adenine dinucleotide (Sigma Chemical Company, grade III) and 1.04mmol of the precursor nicotinamide derivative. This was followed with 0.53ml 1.0M potassium phosphate buffer, pH 7.5, and 12ml of boiled distilled water. All solids were dissolved with gentle stirring and the pH of the solution was adjusted to 7.7 ± 0.1 by dropwise addition of concentrated potassium hydroxide. After the vial was brought to temperature, 0.50g (3.5 units; 1 unit = 1.0 mol per min at 37°, pH 7.3) of pig brain NADase (Sigma, E. C. 3. 2. 2. 5., NAD glycohydrolase) suspended in solution was added. The mixture was incubated with vigorous stirring for three hours and the reaction was ended by making the solution 5% in trichloroacetic acid. A procedure for monitoring the conversion of $NAD^+$ to analog will be described subsequently.

Denatured protein was removed by centrifugation at 10,000 rpm for ten minutes. The supernatant liquid was added to cold acetone (1:5 volume ratio), let stand one minute and centrifuged at

5,000 rpm for ten minutes. The acetone solution was discarded, the centrifuge bottle was dried with a gentle stream of air, and the crude product coating its bottom and sides in a barely perceptible film was taken up in 10ml of boiled distilled water. This working solution was stable for up to a year if maintained at 4°. The analog was isolated by chromatography on polyethyleneimine cellulose. A 1ml aliquot of the working solution containing about 20mg of solid was loaded on a preconditioned 0.9 x 55cm PEI cellulose (Sigma, 1.17meq/g) column and eluted with 0.0030M $NH_4HCO_3$ at a flow rate of 1.6ml/min. The eluate was monitored at 254nm in a 0.1cm flow cell and collected in 5ml aliquots. A sample separation is shown in Figure 12. The identification of the fractions will be discussed later.

### III. High pressure liquid chromatographic monitoring of analog synthesis

In order to establish reaction conditions for analog synthesis, a method, preferably quantitative, was needed for following the decrease in $NAD^+$ and the corresponding increase in analog concentration as the incubation of precursor with NADase progressed. The usual procedures, analyses based on evaluation of the ultraviolet spectrum of the reduced pyridine nucleotides or the coenzyme $CN^-$ complexes,[37,38] were not felt to be suitable as

1. it was not certain that the analogs would undergo reduction at a rate sufficient to enable real-time analysis, and

2. it seemed likely that the spectral properties of the analogs and their complexes would differ little from those of the parent nicotinamide adenine dinucleotide.

As a point of interest both of these objections proved to be valid.

Other attempts to directly follow the increase in analog concentration were futile. An indirect approach relying on high pressure liquid chromatography to quantitate the uptake of N-(hydroxyalkyl) nicotinamide was then developed. As the nicotinamide derivatives were present in four-fold excess, the errors involved were large, but the method nevertheless proved useful.

The liquid chromatograph was equipped with a 37-50μ 2'x0.093" Corasil II column (Waters Associates) eluted with 97:3/$CHCl_3$:MeOH at 60% pump stroke. The chloroform had previously been purified by passage through an alumina column.

Samples were prepared for analysis as follows: The enzymatic synthesis was carried out as described but with the abstraction at intervals of aliquots somewhat over 0.5ml in volume. The samples were immediately centrifuged to remove particulate matter and 0.500ml of the clear supernatant liquid was added to a tapered, stoppered test tube. An internal standard solution containing 0.675g 3-acetylpyridine (Sigma) per 10ml solution had been prepared, and after adding a 5-10μl aliquot to the test tube the mixture was extracted with 5.00ml of an 8:2/$CHCL_3$:MeOH solution. The organic phase was removed, dried for five minutes over a reproducible volume of

anhydrous potassium carbonate, and filtered into a vial. Chromatography of the mixture and comparison of the areas under the nicotinamide and N-(hydroxyalkyl) nicotinamide peaks with the height of the 3-acetylpyridine peak yielded information of progress of the reaction. A discussion of results will follow.

IV. Determination of analog activity in enzyme systems

On enzyme-catalyzed reaction with a hydroxylic substrate, nicotinamide adenine dinucleotide and the analogs herein described undergo a 1, 4 reduction of the pyridine moiety. This transformation is easily observed through the appearance of an absorption at 340nm. The presence and intensity of this absorption were taken to indicate the degree of reaction.

The procedure was as follows: The coenzyme analogs were isolated in aqueous ammonium bicarbonate solution following elution from the cellulose ion exchange column. Depending on concentration, 0.50 to 1.00ml of this solution was used per sample and in each case sufficient blank column eluate added to make the total volume 1.00ml. To this was added 2.00ml of a stock solution of the buffer of interest, whose molarity and pH were adjusted so as to yield the desired values on dilution. Alcohol, if present, was added as 10μl 95% ethanol. These mixtures were made up and later incubated in one dram screw-top glass vials with Teflon®-lined caps. An initial (t = 0)

spectrum of each sample was taken within two minutes of the addition by syringe of the enzyme. In all cases reaction was sufficiently slow that the delay was insignificant. The spectrum was then rescanned at the desired internals and the results were recorded either directly as absorbance versus a constant reference, or indirectly as the difference spectrum of two samples. Care was taken to establish the same base-line for spectra taken some time apart.

The buffer stock solutions used were: (pH adjusted to the indicated value with KOH).

Table 3

Buffer systems used in coenzyme analog incubation with dehydrogenase

| |
|---|
| 0.05M sodium pyrophosphate - pH 10 |
| 0.05M sodium pyrophosphate; 0.10% gelatine; 0.001M semicarbazide hydrochloride - pH 10 |
| 0.05M sodium pyrophosphate; 0.01M semicarbazide hydrochloride - pH 10 |
| 0.05M sodium pyrophosphate; 0.10% gelatine; 0.01M semicarbazide hydrochloride - pH 10 |
| 0.075M glycine; 0.01M semicarbazide hydrochloride - pH 8 & 10 |
| 0.075M glycine; 0.10% gelatine; 0.01M semicarbazide hydrochloride - pH 10 |
| 0.075M Tris; 0.01M semicarbazide hydrochloride - pH 10 |

The horse liver alcohol dehydrogenase (Sigma, E. C. 1.1.1.1.) was obtained in vials containing lyophilized protein equivalent to 20 units activity (one unit converts 1μ mol ethanol per minute to acetaldehyde at pH 8.8, 25°) and reconstituted with 0.01M potassium phosphate buffer pH 7.50. Once reconstituted, the material was stored in dry ice. Incubation was at ambient temperature (25°) with one unit (0.05ml) of enzyme.

Yeast alcohol dehydrogenase (Sigma, E. C. 1.1.1.1.) was similarly reconstituted and the incubation with 40 units enzyme in 0.2ml buffer per sample was otherwise carried out in the same way.

Boiled, house-supplied distilled water was used in all buffers. An attempt to improve procedures by using water distilled from alkaline permanganate resulted in no noticeable benefits and considerable inconvenience.

Blanks containing co-eluted $NAD^+$ in place of analog were run with all samples in order to monitor contamination by oxidizable impurities. Generally only small and quickly apparent background absorptions were found. Interestingly, those analogs not reactive in the absence of external ethanol also showed no blank, reflecting the expected lesser activity of the analogs in the dehydrogenase system.

# V. Instrumentation

## Spectrometers and Spectrophotometers

NMR: Varian T-60

Mass: Hitachi Perkin Elmer RMU-6

Infra-red: Perkin Elmer 237B

Visible ultra-violet: Unicam SP. 800
Beckman DB-G

## pH meter

Instrumentation Laboratory model 245 fitted with a Beckman 39183 probe.

## Melting point apparatus

Hoover capillary

## Centrifuges

Waco Separator (Wilkens-Anderson Co.)

Sorvall RC2-B refrigerated

## Temperature bath

Regulated temperature incubations were performed in a stirred water bath set at 37 ± 0.25°. A vermiculite insulated battery jar was equipped with a mechanical stirrer (Talboys Instrument Co., model no. 104) and a Beckman differential thermometer. The bath

temperature was maintained within ±0.01° of the set-point by a mercury microset thermoregulator (Precision Scientific Co., model no. 62541). The thermoregulator switched an electronic relay (Precision Scientific Co., model no. 62690), energizing a 125 watt blade heater (Cenco) through a Powerstat rheostat (The Superior Electric Co.) set to equalize on and off times. A submersible magnetic stirrer (Henry Troemner, Inc.) was used to agitate the incubation mixture.

Liquid chromatography

The low pressure liquid chromatography apparatus consisted of a Buchler Polystaltic pump, Glenco column, and Buchler Fracto-Mette 200 fraction collector, all maintained at 4°. Column eluate was monitored with a Beckman DB-G spectrophotometer equipped with a 0.1cm flow cell and operating at 254 or 260nm. The output was converted to the absorbance mode by a logarithmic amplifier (see Appendix) and recorded on a Sargent model SR recorder. In order to minimize dead-space and mixing effects, 0.027" i.d. Teflon® tubing was used at all key points.

High pressure liquid chromatography utilized a Waters ALC 202* equipped with a 2'x0.093" Corasil II column (Waters). The output of an ultraviolet absorption detector operating at 254nm was

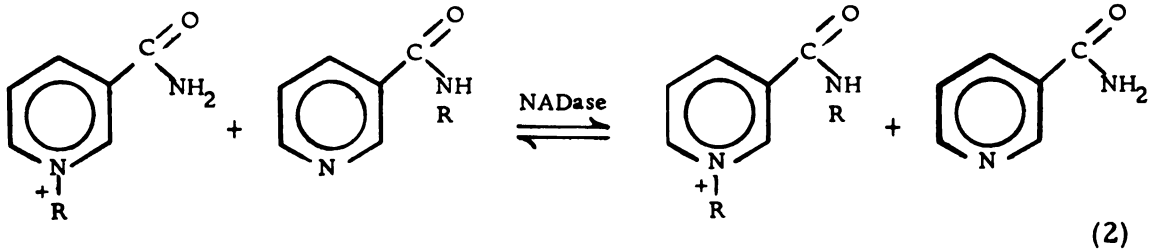
*We are indebted to Professor Harold Hart.

recorded on a Heath model EU-205-11 strip chart recorder driven through a Heath EU-200-01 potentiometric amplifier.

## RESULTS AND DISCUSSION

### I. Monitoring analog synthesis by high pressure liquid chromatography

A major problem with analytical methods which monitor a reaction through measurement of the uptake of a reactant present in excess is that of minimizing error. For example, if a reactant present in five-fold excess could be determined to ±10%, the error in a percent completion calculation would be 50% and the analyst had better seek elsewhere. The leverage present in the present situation is similar; the reactant monitored is present in four-fold excess. As a result it might seem more appealing to follow nicotinamide generation rather than N-(hydroxyalkyl) nicotinamide uptake. Unfortunately the equilibrium shown in equation (2) is very much idealized and the NADase may cleave $NAD^+$, freeing nicotinamide, with no concommitant assembly of analog.




(2)

R = adenosine diphosphoribose

In order to establish procedures for this analysis preliminary work was done with aqueous dummy solutions containing N-(hydroxyalkyl) nicotinamide, nicotinamide, and 3-acetylpyridine. Two major sources of error were found. The first originated in the sample preparation procedure, specifically in volume reproducibility and primarily in the approximately 2% limitation on the precision with which the internal standard could be dispensed from a microliter syringe. The second error lay in the peak integration process.

The 3-acetylpyridine, nicotinamide, and N(hydroxyalkyl) nicotinamide eluted from the column in that order and as relatively symmetrical peaks with little tailing. However, while the nicotinamide and N-(hydroxyalkyl)-nicotinamide gave broad and substantial peaks having a high area/edge ratio and thus well-suited to cut-and-weigh integration, the 3-acetylpyridine standard eluted as a sharp spike. It seemed that application of the cut-and-weigh technique here might lead to excessive error, and comparison of results using cut-and-weigh with those obtained using the height of the 3-acetylpyridine peak confirmed this possibility.

Data calculated for a series of seven injections of a standard mixture indicated the following: For peak area calculations based on reference peak weight the average deviation from the mean was 3.9%. For those based on reference peak height this value was 2.3%. Expression of the standard deviation as a percentage of the mean

provided corresponding values of 4.6 and 3.2%.

Clearly, the minimum error of about 3.2% was obtained if the integrated peak areas were referenced to the height and not weight of the reference peak. This experiment had been undertaken with no specific precautions to assure optimal equilibration of the chromatograph. In a later study, careful conditioning of the column by operation under experimental conditions for an hour or two prior to sample injection resulted in a maximum deviation from the mean of 1.5% for calculations by this method. This marked increase in precision was duly noted and every effort subsequently made to precondition the column routinely. Reasonable equilibration throughout a run was assumed if identical first and last injections agreed within 2%. An error of 2% for this stage of the analysis was then assumed.

By considering both error sources, the total error in a percent completion calculation would be (4(2+2))% or 16%. This is large, but certainly allows for sufficient precision to crudely optimize the incubation time. If necessary, greater accuracy could be achieved through use of an electronic or ball and disc integrator.

A plot of results for two analog syntheses, Figure 11, incicated that 180 min incubation resulted in a 40-60% conversion of $NAD^+$ to analog. This degree of conversion conveniently left behind sufficient $NAD^+$ to serve as a contamination monitor during isolation procedures, a use to be described later.

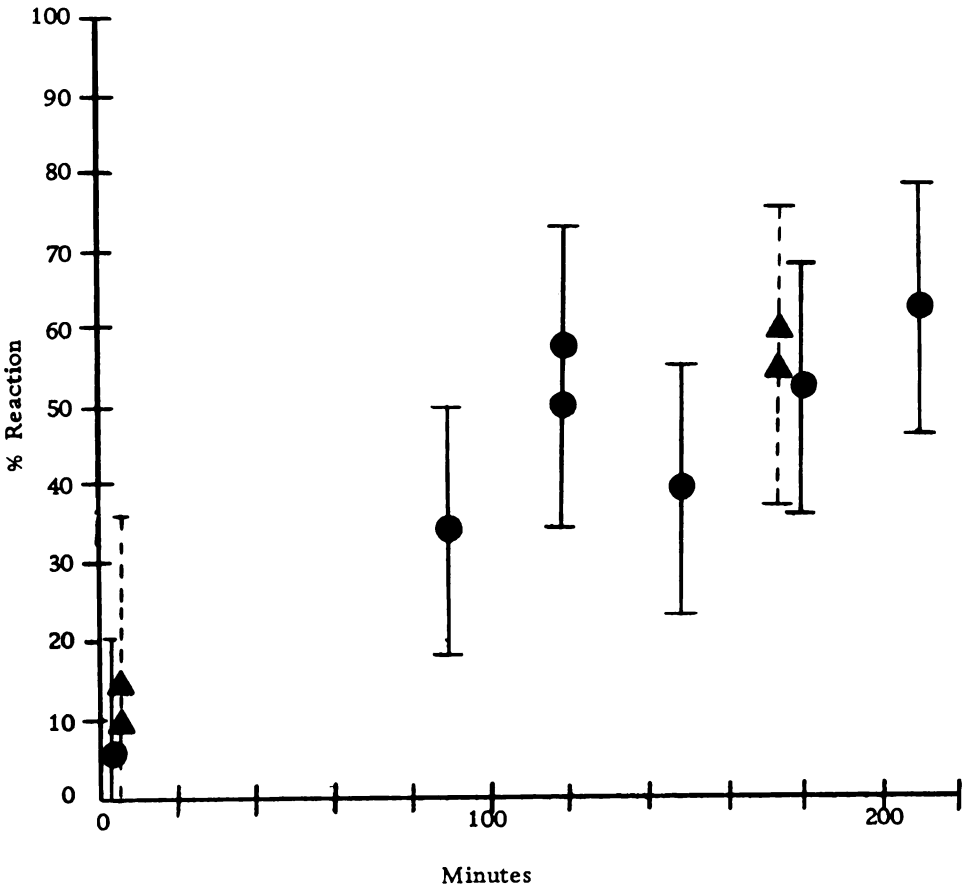
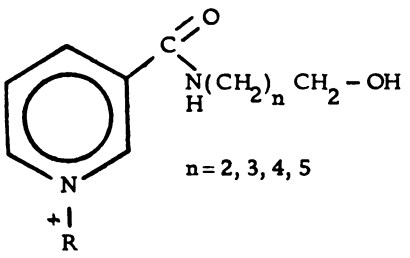
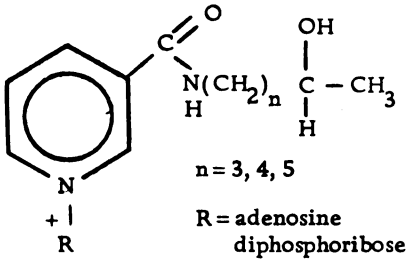



Figure 11. Plot of percent analog synthesis vs. time.

II. Identification of the products of the analog synthesis




I




II

*This compound was not studied.

The nicotinamide adenine dinucleotide analogs shown in structures I and II were synthesized and then isolated by chromatography on polyethyleneimine cellulose, as described in the experimental section. A typical separation with experimental conditions and fraction identifications is shown in Figure 12. All isolations proceeded within the same range of yields (20-25% based on reactant $NAD^+$, assuming identical $\epsilon_{260nm}$ for analogs as for $NAD^+$) and, with the proviso that resolution deteriorated with decreasing analog alkyl chain length, all analogs behaved similarly on chromatography.

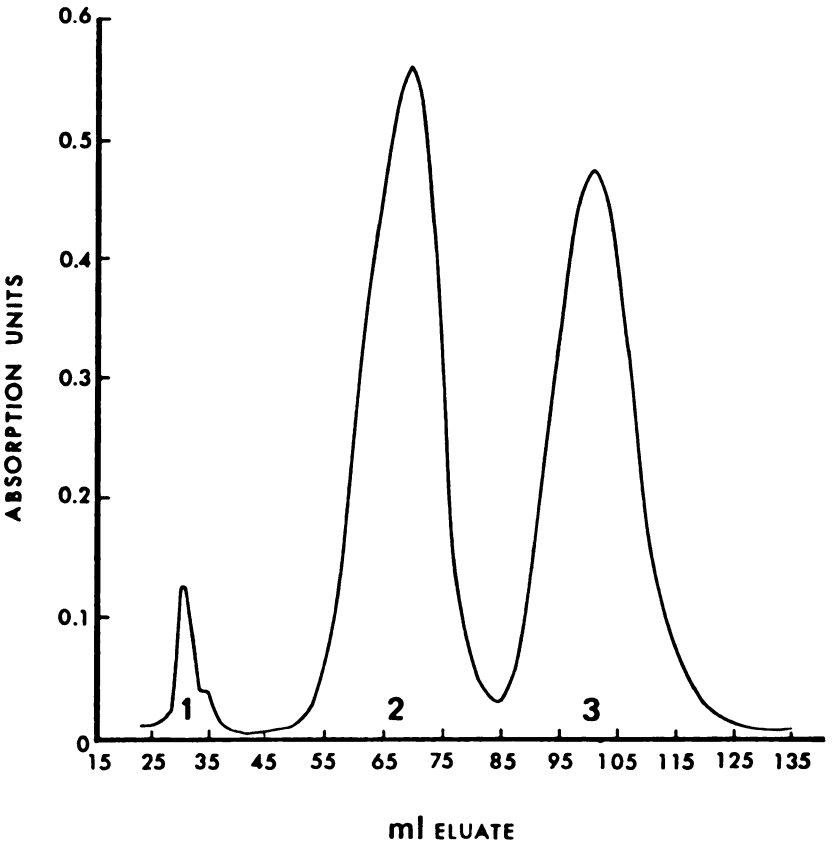



Figure 12. Chromatographic isolation of pyridine nucleotide II(n = 5).

Isolation of pyridine nucleotide II(n = 5). About 20mg of the crude acetone precipitate in 1ml $H_2O$ was loaded on a preconditioned 0.9 x 55cm PEI cellulose column (1.17meq/g) and eluted with 0.0030M $NH_4HCO_3$ at a flow rate of 1.6ml/min. The eluate was monitored at 254nm and collected in 5ml aliquots. The fractions were identified as described -- (1) unresolved nicotinamide and N-(alkylhydroxy) nicotinamide, (2) analog II(n = 5), and (3) $NAD^+$.

The fraction containing nicotinamide and N-(hydroxyalkyl) nicotinamide was identified on the basis of UV spectra and by thin layer chromatography against known samples (unactivated silica gel G developed with 5:4:1/chloroform:acetone:acetic acid or 73:27:1/ isobutyric acid:$H_2O$:conc. $NH_4OH$). The identity of the $NAD^+$ fraction was established by thin layer chromatography (PEI cellulose developed with 0.15M $NH_4Cl$) and further substantiated by its immediate reduction in buffers containing ethanol and either horse liver or yeast alcohol dehydrogenase.

The analogs were characterized as follows: Within experimental error both the analogs and $NAD^+$ were characterized by a $\lambda_{max}$ at 260nm in an otherwise featureless UV spectrum. Both formed fluorescent addition complexes with $CN^-$, a reaction diagnostic of the pyridinium salts possessing a meta $sp^2$ carbon substituent. Associated with this reaction in all instances was a decrease in the extinction coefficient at 260nm and the appearance of a new absorption at about 325nm. The absence of any frequency shift in the newly generated absorption in the case of the analogs confirms that these are in fact nicotinamide nucleotides.

All the analogs underwent apparently complete reduction when incubated in pyrophosphate buffer with horse liver alcohol dehydrogenase and ethanol, but at a rate $10^{-3}$–$10^{-4}$ that of the natural system. As was the case with the natural coenzyme, this reaction was

indicated by the appearance of a second $\lambda_{max}$ at 340nm. Unlike $NAD^+$, however, none of the analogs has yet been observed to function as a coenzyme with yeast alcohol dehydrogenase.

## III. The intramolecular* reactivity of substrate-coenzyme hybrids

The reactivity of these coenzymes in the ethanol-horse liver alcohol dehydrogenase system, albeit kinetically unspectacular, provided a convincing demonstration of the ability of the hybrids to bind to the enzyme protein and be manipulated by it throughout the conformational changes accompanying formation of the binary and ternary complexes essential to enzymatic action. This sequence, first postulated by Theorell and Chance[12] and substantiated many times since, is shown in equation 3.

$$\begin{aligned} &ROH+ENZ+NAD^+ \rightleftarrows ROH+ENZ::NAD^+ \rightleftarrows ROH::ENZ::NAD^+ \rightleftarrows \\ &C=O::ENZ::NADH+H^+ \rightleftarrows C=O+ENZ::NADH \rightleftarrows C=O+ENZ+NADH \end{aligned} \quad (3)$$

---

*In the context of this discussion the following definitions apply:

intramolecular reaction-reaction in which the "ternary" complex is a two-body system with a single hybrid coenzyme-substrate molecule providing both reacting moieties.

intermolecular reaction-reaction in which the ternary complex is a three-body system containing two hybrid coenzyme-substrate molecules, one providing the reacting coenzyme moiety and the other the substrate.

Discovery of this reactivity was important, but viewed in light of the main goal of this project it meant only that a precondition had been met without which this goal would have been unattainable. Not only was it necessary for the coenzyme moiety of the analog to bind the enzyme active site, but also essential was the intramolecular binding and oxidation of the substrate moiety. This was a much larger order.

Determination of intramolecular reduction-oxidation could most logically be broken into two experimental steps, answering two ordered questions. The first was: Knowing that the hybrid coenzyme-substrates form binary complexes with horse liver alcohol dehydrogenase capable of binding and oxidizing ethanol, is it also possible to find evidence for the reactivity of one or more of these binary complexes with the hydroxyl moiety of the hybrid coenzyme? The second, naturally enough, was: Can any such reaction be shown to be intramolecular? As is often the case, the simplicity of the answers was inversely related to that of the questions.

In principle, the first question could be answered quite readily by the observation of reaction, or lack of it, in an enzyme-hybrid coenzyme system free from added alcohol. Due to experimental difficulties and our lack of sophistication in dealing with biochemical reactions it was some time before reliable results were obtained.

Long incubations of 16 to over 24 hours proved to be necessary, that resulted in occasional but unpredictable enzyme instability. Another problem was that of contamination arising from many potential sources. The presence of atmospheric ethanol or acetone, incomplete removal of ethanol during processing of the enzyme, and contamination of buffer were important considerations and led us to doubt some early positive results because of an uncomfortable realization of just how little such contamination would be required to destroy an experiment. Each trial was carried out with $3 \times 10^{-7}$ mol of coenzyme, requiring only $1.5 \times 10^{-5}$ g ethanol to completely react. The real danger of course lay in the possibility that even smaller amounts of some contaminant, or enzyme decomposition, might raise false hopes by mimicking the small amount of reaction we realistically might see. Clearly, we would never be able to trust our results until we had developed a method of detecting such spurious "reductions". Fortunately this was easily done.

Because the enzymatic synthesis of the hybrid analogs was an equibilibrium reaction, some $NAD^+$ always remained in the product mixture even after optimization of analog yield. It was only necessary to recover this $NAD^+$ along with the coenzyme and use it in an incubation blank. The $NAD^+$, isolated on the same column and almost simultaneously with the hybrid, incubated in the same buffer, with the same enzyme and under the same conditions, served as an ideal

monitor. These blank runs always indicated some contamination, but with proper technique and unless either the enzyme or coenzyme had been stored improperly or too long, the amount was usually small. For the purpose of these experiments the useful life of the enzyme was a week or so with storage of the solution on dry ice. The isolated $NAD^+$, if stored under refrigeration in an air-tight container, often gave low blanks even after two weeks.

With this means of verifying the validity of our results, we were able to conclude that four of the hybrid coenzymes did show some reactivity over and above that caused by the oxidation of contaminants. These were I(n = 4, 5) and II(n = 4, 5), that is, those hybrid coenzyme-substrated in which the hydroxyl was separated from the coenzyme by chains of five and six methylenes. Interestingly, those hybrids which did not show this reactivity showed no reaction at all, i.e. not even the small contamination reaction of the $NAD^+$ blank. This reflects the expected lower activity of the dehydrogenases with these analogs than with $NAD^+$ and implies that the small reaction of the blanks was not due to ethanol. The identity of the oxidizable impurity has so far not been established.

The first question had been answered in the affirmative, but the degree of coenzyme reduction found is worthy of further discussion. It corresponded to only 5-15% of the complete reduction found on incubation with excess ethanol, and lesser amounts being

obtained with coenzymes of type II and the greater with those of type I. We were naturally quite disappointed and at first tended to ascribe these results to kinetic control of the reaction, i.e. deactivation of the enzyme prior to reaction completion. Consistently, however, we found the dehydrogenase to be still viable upon addition of ethanol, even though further reduction did not occur in its absence. Of course it is always possible that only one of the isozymes processes our hybrid system and that for some reason this isozyme inactivates at a greater rate than the others. Although some precedent for this possibility does exist this explanation admittedly seems far-fetched.[10] Much more likely is the presence of the true equilibrium point in the 5-15% reduction range. Attempts to test the fast-decaying isozyme theory by periodic additions of fresh dehydrogenase unfortunately failed. Contamination from inactivated enzyme made the results impossible to interpret.

In an effort to compare our results with those to be obtained in a more commonplace situation, we located literature values for the equilibrium constants on some alcohol-$NAD^+$ systems.[39] Using equimolar ethanol-$NAD^+$ and 2-propanol-$NAD^+$ as models, these values implied that at pH 10 and 25°, our incubation conditions, 24% and 89% respectively of the $NAD^+$ would be reduced at equilibrium. Clearly we were nowhere in sight of these values.

The discrepancy is difficult to explain, for even though the

model systems superficially seem very different from the synthesized hybrids, this is not altogether true. Equilibrium constants are controlled by simple thermodynamic parameters. The component parts of the redox pairs in both cases are essentially identical; both systems oxidize aliphatic alcohols and reduce pyridinium carboxamides. Linking the pieces together wouldn't be expected to have much effect on the separately considered oxidation-red-uction potentials, or through them on the equilibrium constant. Apparently then, any such effect would have to be the result of some interaction between the linked coenzyme and substrate moieties. Perhaps the most reasonable explanation for the observed reduction in the equilibrium constant would involve possible intramolecular hydrogen bonding between the hydroxyl of the internal substrate and the ribose or pyrophosphate linkages of the analog coenzyme. Such bonding would reduce the free energy of the oxidized coenzyme-alcohol relative to the reduced coenzyme-carbonyl forms and lead to the observed smaller amount of reaction to the analogs.

All speculation as to its cause aside, this small amount of reaction was a potentially serious problem. Its significance was redoubled because the final conclusions of this project would have to be based on the relative reactivity of two diastereomers of structure II; here we had a degree of reactivity mediocre even in an absolute sense. Even though the experiments would be run with proper

controls, it would be difficult for us to present with complete confidence conclusions based on such potentially small changes, and even more so for outsiders to accept them. A means of displacing the equilibrium and/or increasing the reaction rate was a necessity.

After trying and failing with a number of other approaches, the expedient of including semicarbazide hydrochloride in the incubation buffer proved effective. Incubation of I(n = 4) with dehydrogenase in 0.05M glycine/KOH-0.01M semicarbazide hydrochloride, pH 10, resulted in the establishment of an equilibrium in the range of 30-50% reduction. An initial attempt with a buffer 0.001M in semicarbazide hydrochloride had yielded no detectable results, thus the implicit dependence of coenzyme reduction on semicarbazide concentration could almost certainly be used to force the equilibrium even further. As things were, this was a substantial and probably sufficient increase in the extent of reaction. Even more remarkable was the effect of semicarbazide on the rate of the reaction. After approximately four hours incubation, an extent of reaction equivalent to a full twenty-four hours incubation with a semicarbazide-free buffer had been attained.

Interestingly enough, these results were not repeated with I(n = 5). Any effects induced by semicarbazide on either the equilibrium on the reaction rate were in this instance relatively small. We will return to this observation shortly.

With the advent of success in obtaining substantial amounts

of reaction with our hybrid coenzyme-substrates, perhaps the most important problem of all began to loom larger. This was really not so much a problem as a question having only one acceptable answer. Earlier in this discussion the second question posed was: Can any such reaction be shown to be intramolecular? A stereochemical determination could not be made unless the observed reduction were shown to occur intramolecularly. Our hope was that it would and the problem was to find a means of determining if this were indeed the case.

Our first thought was to use a labeling experiment. If coenzyme analogs of structure I were to be synthesized with the carbon alpha to the hydroxyl nonstereospecifically monodeuterated, any intermolecular reaction catalyzed by dehydrogenase would result in deuterium scrambling and the consequent presence in the equilibrium mixture of dideuterated and nondeuterated species. This would not be the case in an intramolecular reaction, and subsequent cleavage and mass spectral analysis of the N-(hydroxyalkyl)nicotinamide moiety would readily discriminate between the possibilities. Although this experiment should eventually be performed and probably stands alone in terms of realiability and freedom from possible misinterpretation, we were unable to do so due to difficulties in obtaining the analogs in sufficiently large amounts. An alternate experiment applying the principle of competitive inhibition was developed in its

place. It should be noted that all results from this point on must be considered preliminary.

Although the Theorell-Chance mechanism (equation (1)) for horse liver alcohol dehydrogenase catalyzed hydrogen transfer reactions has been accepted for many years, some of its kinetic implications have recently been called into question.[40] It now appears that the kinetics of the reaction are not invariant, but may change with the concentration of substrate relative to enzyme. Considering this present uncertainty regarding the behavior of the dehydrogenase and our lack of any quantitative kinetic data regarding these coenzyme-substrates, it seems premature to enter into any prolonged discussion of their reaction kinetics. Even from a simplistic viewpoint, however, it is clear that comparison of the reaction rates of analogs incubated with and without addition of supplemental nicotinamide adenine dinucleotide might constitute a useful probe into the mode of reaction. If reaction occurred by an intermolecular mode the additional $NAD^+$ might generate an increase in $v_{initial}$ by functioning as a source of easily utilized coenzyme. On the other hand, an intramolecular reaction might reveal itself by inhibition brought about by competition for enzyme active site between analog and $NAD^+$.

The experiment was easily carried out. Three cuvettes, one containing the analog in question, the second $NAD^+$, and the third amounts of both $NAD^+$ and analog identical to those in the first

two, were prepared and incubated with dehydrogenase in glycine/KOH-semicarbazide hydrochloride buffer, pH 10. Difference ultra-violet spectroscopy was used to determine relative reaction rates. The results were clear cut. Addition of $NAD^+$ substantially in increased the initial reaction rate of I(n = 5) and this rate increase fell with time. With I(n = 4), the $NAD^+$ caused no change in the initial reaction rate; after about four hours a very slight inhibition became apparent.

The effects of both semicarbazide hydrochloride and $NAD^+$ on reactivity are tabulated below.

Table 4

Qualitative effects of the addition of $NAD^+$ and/or semicarbazide HCl on substrate oxidation

| coenzyme | semicarbazide | | $NAD^+$ + semicarbazide |
|---|---|---|---|
| | $K_{eq}$ | $k_{initial}$ | $k_{initial}$ |
| I(n = 4) | +++ | +++ | 0 |
| I(n = 5) | (a) | 0 | ++ |

(a) final equilibrium may not be attained before dehydrogenase denaturation

The interpretation of these results is straight-forward. The semicarbazide experiment indicates that the rate determining steps of the two analog-dehydrogenase systems are quite different. This

difference in kinetics might also lead to speculation that the analogs react by different mechanisms, one intermolecular and the other intramolecular, but at this stage any such conclusion must be pure conjecture.

The data from the $NAD^+$ inhibition experiment may be rationalized on the following energy diagram.

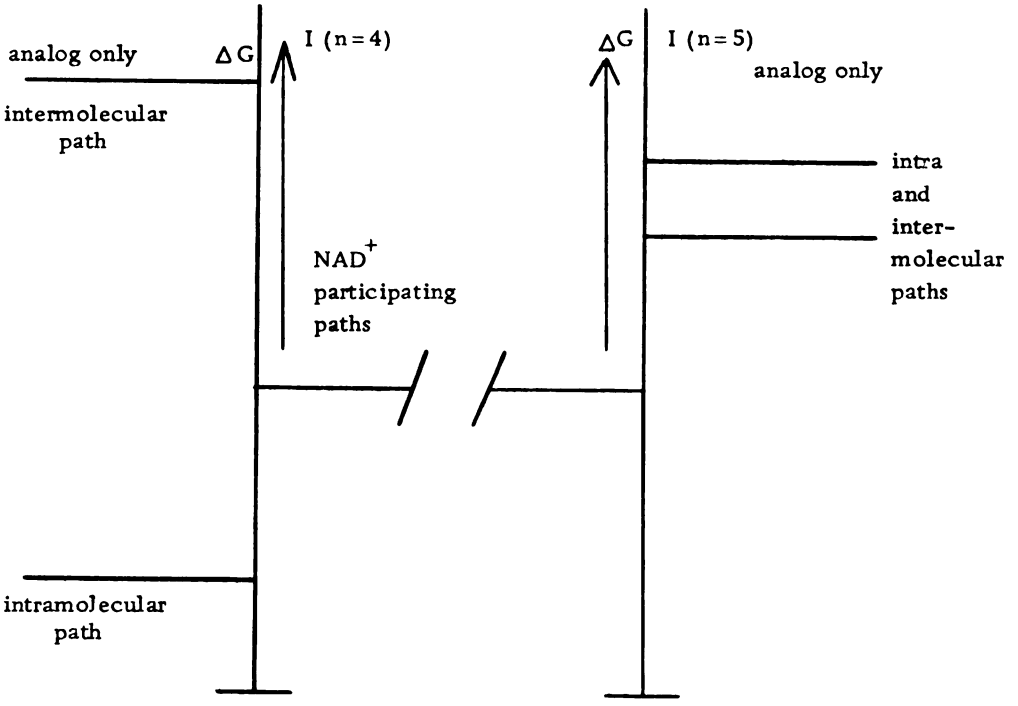



Figure 13. Relative energy levels of the possible reaction modes.

The energies shown are those of the rate limiting steps of the systems' three possible modes of reaction: (1) intramolecular, (2) intermolecular (analog only), and (3) intermolecular ($NAD^+$ participation). All energy levels are relative to those of the intermolecular ($NAD^+$ participation) modes, which are assigned the same level as a matter of convenience only.

Addition of $NAD^+$ brought about a definite increase in the reaction rate of analog I(n = 5). Apparently, the energy level of the newly available intermolecular ($NAD^+$ participation) mode lay somewhat below that of both analog-only modes, intra- and intermolecular. On the basis of this evidence we can again reasonably place the intermolecular (analog only) mode of coenzyme I(n = 4) at an energy above that of the corresponding intermolecular ($NAD^+$ participation) mode. Experimental results with this analog indicated that addition of $NAD^+$ had little or no effect on the initial rate of reaction. Clearly then, some lower energy mechanism is followed both in the presence and absence of $NAD^+$.[7] This can only be the intramolecular mechanism. This conclusion is strengthened by a study of space-filling models of the hybrid substrate-coenzymes. The hydroxyl bearing carbon of I(n = 4) is readily aligned in close proximity with the nicotinamide ring and in such a way that the carbon-oxygen bond orientation fulfills the requirements of the Prelog and Karabatsos models, (see Figures 7 and 10).

Having come to this fortunate result for analog I(n = 4) we are left with the problem of analog I(n = 5). At this stage we do not have sufficient data to make a positive determination on whether the reaction path followed here in the absence of $NAD^+$ is intra or intermolecular. Space filling models however show no reason why an intramolecular path would not be followed in this case also. The six methylene chain allows what seems to be almost as good as orientation with respect to the nicotinamide ring as the five methylene chain. Analogs I(n = 2, 3) on the other hand, have alkyl chains too short to allow such an orientation and their non-reactivity with horse liver alcohol dehydrogenase may be taken as additional evidence of intramolecular reaction in those analogs which are reactive. Work to confirm these initial results and extend them to analogs of structure II is continuing.

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