Determination of the Hydride Transfer Stereospecificity of Nicotinamide Adenine Dinucleotide Linked Oxidoreductases by Proton Magnetic Resonance†

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ABSTRACT: A facile proton magnetic resonance technique is described for the determination of the coenzyme stereospecificity during hydride transfer reactions catalyzed by pyridine nucleotide dependent oxidoreductases. The reliability of this technique was demonstrated by examining the coenzyme stereospecificity of lactate, malate, and 3-phosphoglycerate dehydrogenases, which are known to be A-stereospecific enzymes, as well as triosephosphate and octopine dehydrogenases, which are known to be B-stereospecific enzymes. Furthermore, by applying this technique, it was shown that the previously unstudied enzymes D-β-hydroxybutyrate and 4-aminobutanal dehydrogenases are B- and A-stereospecific enzymes, respectively. In addition, the nicotinamide adenine dinucleotide linked reaction of glucose-6-phosphate dehydrogenase from *Leuconostoc mesenteroides* was found to be B stereospecific, like the reaction of the nicotinamide adenine dinucleotide phosphate linked yeast enzyme.

The pyridine nucleotide linked oxidoreductases are an extensive class of enzymes which are capable of the reversible transfer of hydride from a substrate to the pyridine 4 position of NAD^+ [1] or $NADP^+$. These enzymes can distinguish the diastereotopic methylene atoms at the dihydropyridine 4 position of NADH or NADPH, transferring the hydride to the substrate stereospecifically (Fisher et al., 1953). As a result of the stereospecificity of hydride transfer, oxidoreductases have been classified into two distinct groups: the A-stereospecific enzymes, which transfer hydride to the A side of the pyridine ring, and the B-stereospecific enzymes, which transfer hydride to the B side. Not until the determination of the absolute stereospecificity by chemical means, however, was it shown that the A hydrogen of NADH had a pro-*R* configuration and that the B hydrogen had a pro-*S* configuration (Cornforth et al., 1965/1966).

Pioneering work on the stereospecificity of oxidoreductases was carried out by mass spectroscopy employing deuterium-labeled coenzymes (Levy and Vennesland, 1957). Subsequently, Jarabak and Talalay (1960) introduced a technique utilizing tritium-labeled coenzymes and this method has been used predominantly in recent years. In both of these techniques, the oxidized coenzyme, labeled with an isotope (^2H or ^3H) at the 4 position of the pyridine ring, is reduced with nonlabeled substrate and the enzyme under investigation. The resulting reduced coenzyme is then isolated, reoxidized with an enzyme of known stereospecificity, and analyzed for its isotope content. A variation of this method is possible if an isotope is stereospecifically introduced at the 4 position of the dihydropyridine ring by reducing nonlabeled coenzyme with an enzyme of known stereospecificity in the presence of its isotopically labeled substrate. The labeled reduced coenzyme thus prepared is purified, oxidized with the enzyme under investigation, and analyzed for its isotope content. However, these conventional techniques are laborious, time consuming, and require multiple purification steps.

In this report, we present a proton magnetic resonance (^1H NMR) technique which is accurate, safe, and far more facile than conventional techniques. This investigation is a continuation of the proton magnetic resonance studies conducted in this laboratory on the stereospecificity of oxidoreductases for pyridine nucleotide coenzymes (Sarma and Kaplan, 1969; Oppenheimer et al., 1971; Oppenheimer and Kaplan, 1974; Arnold and Kaplan, 1974; Oppenheimer and Kaplan, 1975).

Materials and Methods

Chemicals and enzymes were obtained from the following sources: NAD^+ from P-L Biochemicals; egg yolk lecithin, acetoacetate (lithium salt), octopine, pig heart lipoamide dehydrogenase, and chicken liver 3-phosphoglycerate dehydrogenase from Sigma; pyruvate (sodium salt), α-ketobutyrate (sodium salt), oxalacetic acid, dithiothreitol, glucose 6-phosphate (disodium salt), DL-glyceraldehyde 3-phosphate diethyl acetal (barium salt), hydroxypyruvic acid phosphate dimethyl ketal, pig heart H_4 lactate dehydrogenase, yeast alcohol dehydrogenase, glucose-6-phosphate dehydrogenase (*Leuconostoc mesenteroides*), and lecithin nonrequiring D-β-hydroxybutyrate dehydrogenase (*Rhodopseudomonas spheroides*) from Calbiochem; deuterium oxide from Wilmad; crystalline pure L-lactate dehydrogenase (turkey, halibut, and bull frog) and malate dehydrogenase (*Neurospora crassa*) from Mr. F. E. Stolzenbach in this laboratory; and 4-aminobutylaldehyde diethyl acetal as well as pure 4-aminobutanal dehydrogenase (*Pseudomonas*) from Dr. D. M. Callewaert at Oakland University.

Crystalline pure triosephosphate dehydrogenases from turkey, bee, sturgeon, horse shoe crab (*Limulus polyphemus*), and *Escherichia coli* were purified as previously described (Allison and Kaplan, 1964). A sonic extract of *Bacillus subtilus* (ATCC 6633) was used as a source of the *B. subtilus*

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[1] Abbreviations used are: NAD^+, nicotinamide adenine dinucleotide; NADH, reduced NAD^+; $NADP^+$, NAD^+ phosphate; $NADPH^+$, reduced NADP; DEAE, diethylaminoethyl; EDTA, (ethylenedinitrilo)tetraacetic acid; NMR, nuclear magnetic resonance.

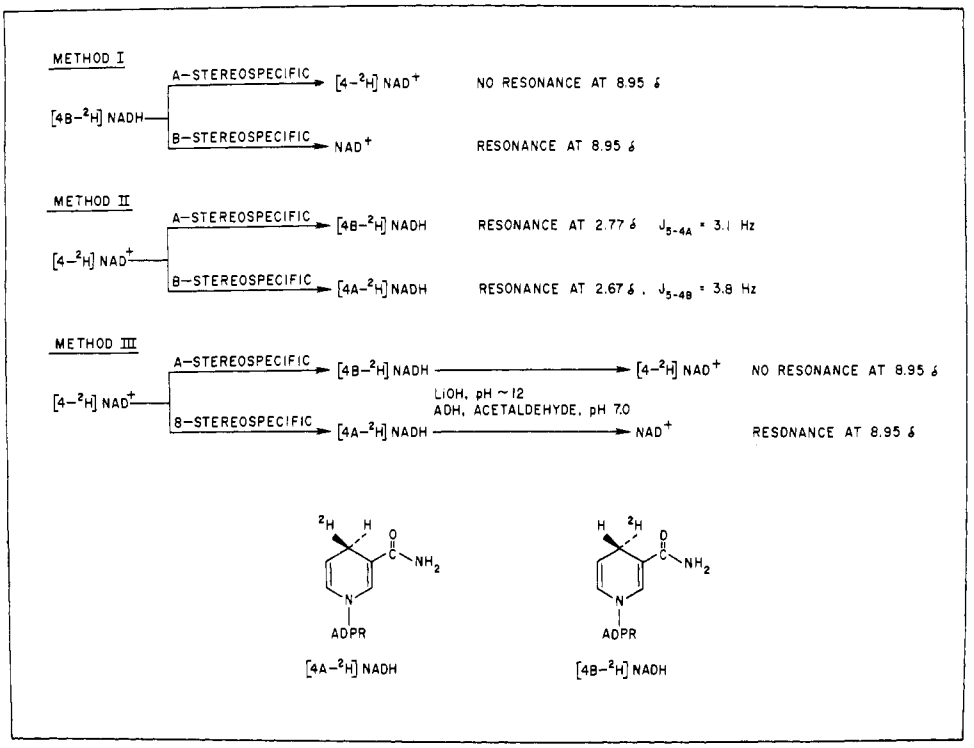

FIGURE 1: The three ^1H NMR methods employed for the determination of the stereospecificity of oxidoreductase-catalyzed reactions. Also shown are the configurations of the dihydropyridine moiety of [4A-^2H]NADH and [4B-^2H]NADH.

malate dehydrogenase. Crude extracts obtained from homogenized abalone and scallop muscle served as the sources of D-lactate dehydrogenase and octopine dehydrogenase, respectively. Lecithin requiring D-β-hydroxybutyrate dehydrogenase was prepared from bovine heart mitochondria according to the procedure of Sekuzu et al. (1963).

Preparation of [4B-^2H]NADH, [4A-^2H]NADH, and [4-^2H]NAD$^+$. [4B-^2H]NADH was prepared by reducing 300 μmol of NAD$^+$ with 10 μmol of lipoamide and 10 units of lipoamide dehydrogenase (a B-stereospecific enzyme) in the presence of 600 μmol of dithiothreitol and 0.05 M $(NH_4)_2CO_3$ in 50 ml of ^2H_2O; the pD was maintained at 8.5 by additions of [U-^2H]ammonium hydroxide throughout the reaction. When the A_{260}/A_{340} was less than 3.0, the reaction mixture was diluted to 200 ml and applied to a DEAE-11 column (carbonate form, 2.5 × 25 cm). The [4B-^2H]NADH was then purified by eluting the column with a linear 1-l. gradient of 0–0.5 M $(NH_4)_2CO_3$. The [4B-^2H]NADH was found to elute at approximately 0.15 M $(NH_4)_2CO_3$ and those fractions having A_{260}/A_{340} of less than 2.4 were pooled and lyophilized.

[4-^2H]NAD$^+$ was obtained by oxidizing the [4B-^2H]-NADH thus prepared with yeast alcohol dehydrogenase (an A-stereospecific enzyme) in the presence of excess acetaldehyde and 0.05 M $(NH_4)_2CO_3$. The reaction mixture was then applied to a Dowex-1 column (formate form, 2.5 × 25 cm) and the [4-^2H]NAD$^+$ was purified by eluting the column with a 1-l. linear gradient of 0–1.5 N formic acid. The [4-^2H]NAD$^+$, which eluted at approximately 0.4 N formic acid, was assayed by reducing it with yeast alcohol dehydrogenase in the presence of ethanol. Those fractions giving A_{260}/A_{340} of less than 2.6 upon assay were pooled and lyophilized. The [4-^2H]NAD$^+$ thus prepared had a deuterium content of 97 ± 1% at the pyridine 4 position, as determined by ^1H NMR spectroscopy (see Figure 2).

[4A-^2H]NADH was prepared by reducing [4-^2H]NAD$^+$ with lipoamide dehydrogenase as described above for the preparation of [4B-^2H]NADH, except that the reaction was carried out in H_2O.

Stereospecificity Determinations. Enzyme stereospecificity was determined by employing one of the three methods illustrated in Figure 1. This figure also shows the structure of stereospecifically deuterated NADH. The particular method employed for each enzyme under investigation was dependent upon the availability of necessary substrates and also upon the equilibrium of the reaction being examined.

Method I was employed for the determination of the stereospecificities of lactate, malate, 3-phosphoglycerate, and D-β-hydroxybutyrate dehydrogenases. The reaction mixtures contained 5–20 units of enzyme, 10–15 μmol of [4B-^2H]-NADH, 50–100 μmol of substrate, and 150 μmol of potassium phosphate in a final volume of 30 ml at pH 7.5. The reaction mixture for the mitochondrial D-β-hydroxybutyrate dehydrogenase also contained 100 μg of lecithin and 30 μmol of dithiothreitol. When there was no further decrease in A_{340}, the reaction was quenched by lowering the pH of the medium to 2 with concentrated HNO_3 and the mixture was lyophilized. Usually, it was not necessary to purify the product NAD$^+$ from the reaction mixture, but when crude extracts were used as enzyme sources, NAD$^+$ was purified after the initial lyophilization by acid–acetone precipitation (Kornberg, 1957). The ^1H NMR spectrum of the resulting NAD$^+$ was then analyzed for the retention of deuterium at the pyridine 4 position. A B-stereospecific enzyme removes deuteride from [4B-^2H]-NADH and the formed NAD$^+$ shows a doublet near 8.95 δ (pD 3, 23 °C) for the pyridine 4 proton. An A-stereospecific enzyme, on the other hand, transfers hydride from [4B-^2H]NADH and the ^1H NMR spectrum of the resultant NAD$^+$ shows no resonance for the pyridine 4 proton (see Figures 1 and 2).

Method II can be applied to enzymes that catalyze reactions predominantly in the direction of coenzyme reduction and in cases where only the reduced form of the substrate is available. The stereospecificity of the *L. mesenteroides* glucose-6-phosphate dehydrogenase for NAD^+ was established by method II. The reaction mixture contained 10 units of enzyme, 15 μmol of [4-^{2}H]NAD^+, 30 μmol of glucose 6-phosphate, and 150 μmol of Tris-Cl in a final volume of 30 ml at pH 8.8. When there was no further increase in A_{340}, 1 N LiOH was added to raise the pH to 12 and the solution was lyophilized. After lyophilization, the sample was dissolved in 0.5 ml of H_2O and the NADH was purified by barium thiocyanate–acetone precipitation and converted to its sodium salt (Oppenheimer et al., 1971). The ^{1}H NMR spectrum of the deuterated NADH (20 mM at pD 8, 23 °C) permits easy identification of the enzyme stereospecificity, since an A-stereospecific enzyme produces [4B-^{2}H]NADH, the 4A proton of which has a chemical shift of 2.77 δ and an *unresolved* coupling constant ($J_{5\text{-}4A}$) of 3.1 Hz, whereas a B-stereospecific enzyme produces [4A-^{2}H]NADH, the 4B proton of which has a chemical shift of 2.67 δ and a *resolved* coupling constant ($J_{5\text{-}4B}$) of 3.8 Hz (Arnold and Oppenheimer, unpublished results[2]) (see Figure 4).

Method III, a combination of methods I and II, was devised to avoid the handling of NADH, which is relatively unstable and requires a careful determination of ^{1}H NMR chemical shifts and coupling constants. The stereospecificities of triosephosphate, octopine, and 4-aminobutanal dehydrogenases were determined by this method. The reaction mixture for triosephosphate dehydrogenase contained 20 units of enzyme, 4 μmol of [4-^{2}H]NAD^+, 35 μmol of glyceraldehyde 3-phosphate, 100 μmol of sodium arsenate, and 150 μmol of Tris-Cl, pH 8.8, in a final volume of 30 ml. The reaction mixture for octopine dehydrogenase contained 20 units of enzyme, 15 μmol of [4-^{2}H]NAD^+, and 150 μmol of octopine in 30 ml of the above buffer. The reaction mixture of 4-aminobutanal dehydrogenase contained 10 units of enzyme, 4 μmol of [4-^{2}H]NAD^+, 90 μmol of 4-aminobutanal in 30 ml of the above buffer. When the reactions were completed, 1 N LiOH was added to raise the pH to 12 and the mixtures were placed in a boiling water bath for 90 s. This procedure denatures the protein and rapidly destroys any residual NAD^+, but leaves NADH intact. By rapidly destroying residual NAD^+, the possibility of the nonstereospecific exchange known to occur between NAD^+ and NADH is eliminated (Ludowieg and Levy, 1964). The mixtures were then neutralized by adding 0.1 N HCl with stirring and the NADH formed was oxidized with excess acetaldehyde by yeast alcohol dehydrogenase. When there was no further decrease in A_{340}, the pH was lowered to 2 with concentrated HNO_3 and the samples were lyophilized. The ^{1}H NMR interpretation is the same as that of method I.

Proton Magnetic Resonance Measurements. To remove water, the samples were lyophilized twice from 99.8% ^2H_2O and dissolved in 100% ^2H_2O containing 1 mM tetramethylammonium chloride (TMAC) and 1 mM EDTA. The sample volume was 0.3 ml and Teflon vortex plugs (Wilmad) were used. Spectra were taken at 23 °C (the ambient temperature of the probe) with a Varian HR-220 nuclear magnetic resonance spectrometer interfaced to a Transform Technology 220 Fourier transform system. In order to improve the signal-to-noise ratio, as few as 16 transients were accumulated, but when sample concentrations were low, as many as 200 transients were acquired.

Spectra were generally taken at a sweep width of 2500 Hz with a recycle time of 1.6 s and processed with an exponential multiplier of 0.2–0.4 Hz. Chemical shifts were obtained by determining chemical shifts relative to internal TMAC, and adding 3.2 ppm, the chemical shift difference between TMAC and sodium trimethylsilyl[U-^{2}H]propionate (TSP).

The retention of a proton at the pyridine 4 position in methods I and III could be quantitatively determined by comparing the integration of its resonance to that of the resonance for the proton at the pyridine 6 position (see Figure 2a for the designation of the pyridine proton resonances). In this regard, one should not use the resonance of the proton at the pyridine 2 position as an integration standard, since this proton

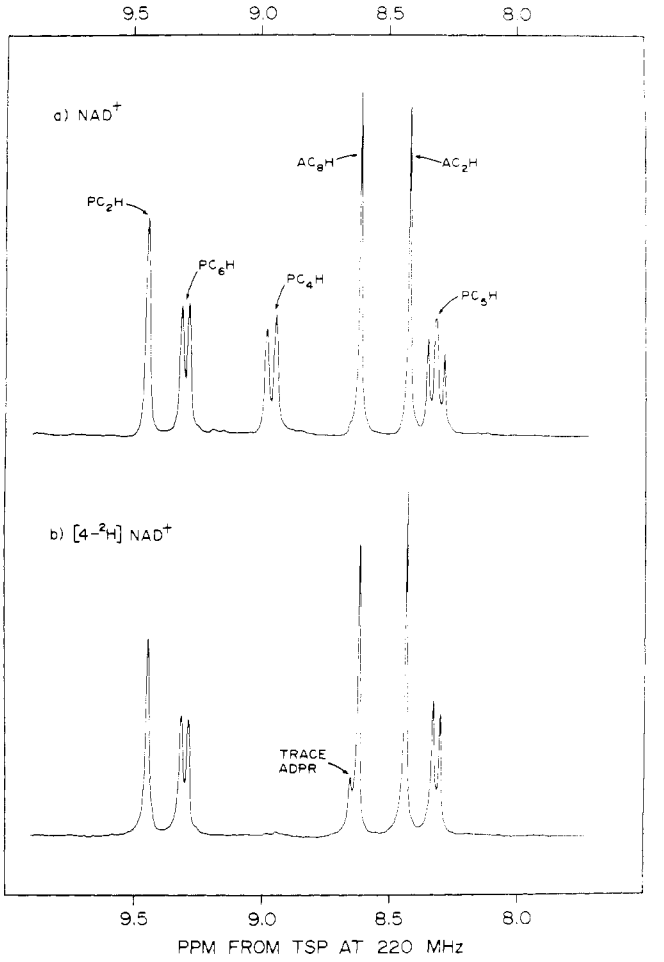

FIGURE 2: The ^{1}H NMR aromatic region of (a) NAD^+ and (b) [4-^{2}H]NAD^+ (20 mM, pD 3, 23 °C, 100 scans). The deuterium content at the pyridine 4 position was found to be 97 ± 1% by integration. The resonances denoted by PC_2H, PC_4H, PC_5H, and PC_6H are from the protons at carbon 2, 4, 5, and 6 of the pyridine ring, and AC_2H and AC_8H are those at carbon 2 and 8 of the adenine ring. The small resonance on the left side of the AC_8H resonance in [4-^{2}H]NAD^+ is caused by contaminating ADP ribose.

[2] It should be pointed out that these values differ slightly from those previously reported (Oppenheimer et al., 1971). In the earlier studies, the coupling constants were measured directly from the PC_4H (see the legend to Figure 2 for the designation of the pyridine protons) resonances while spin decoupling the PC_6H resonance. Under these conditions, the PC_4H resonances sharpened sufficiently to measure both the $J_{5\text{-}4A}$ and the $J_{5\text{-}4B}$ coupling constants. However, due to coupling to the PC_2H resonance, the PC_4H resonances were still broad and this led to distortion of the "true" coupling constants. The coupling constants of the PC_4H presented in this report were obtained by determining them directly from the PC_5H resonance.

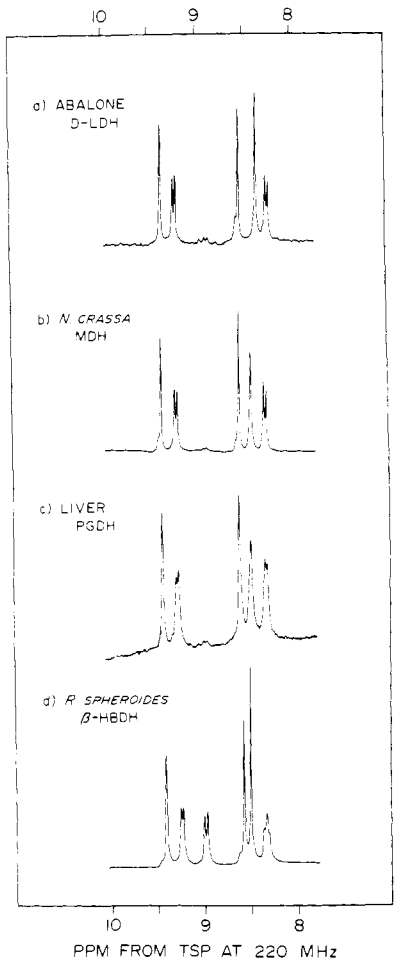

FIGURE 3: The ^{1}H NMR aromatic region of NAD$^+$ formed from [4B-^{2}H]NADH by various enzymes according to method I (pD 3, 23 °C). The enzymes employed are: (a) abalone D-lactate dehydrogenase (LDH), 32 scans; (b) *N. crassa* malate dehydrogenase (MDH), 64 scans; (c) chicken liver 3-phosphoglycerate dehydrogenase (PGDH), 64 scans; and (d) *R. spheroides* D-β-hydroxybutyrate dehydrogenase (β-HBDH), 64 scans.

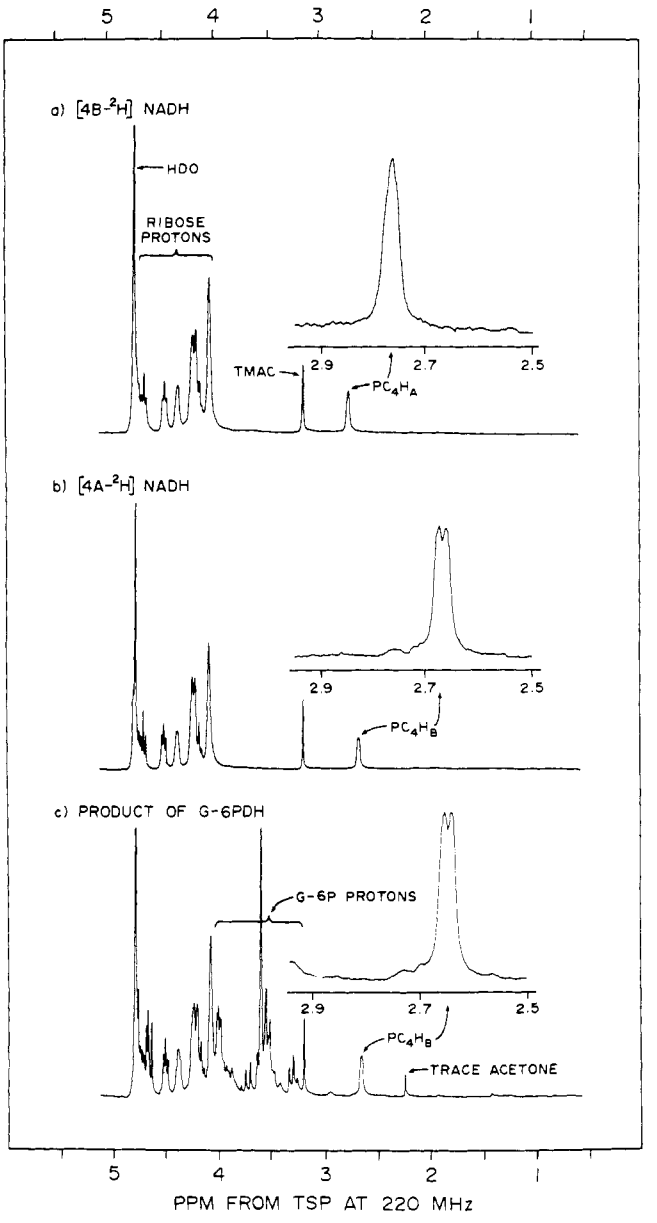

FIGURE 4: The upfield portion of the ^{1}H NMR spectra of (a) [4B-^{2}H]NADH, (b) [4A-^{2}H]NADH, and (c) NADH formed from [4-^{2}H]-NAD$^+$ by *L. mesenteroides* glucose-6-phosphate dehydrogenase (G-6PDH) according to method II. The resonances seen between 3 and 4 ppm in spectrum (c) are from glucose 6-phosphate (G-6P) protons. The inserts show the expansions of the dihydropyridine 4 proton region in order to clearly reveal the difference in chemical shift and coupling constants of the stereospecific labels (pD 8.0, 23 °C, 100 scans).

undergoes partial deuterium exchange with ^2H_2O during the formation of [4B-^{2}H]NADH (San Pietro, 1955; Dubb et al., 1958).

Results

Figure 2 compares the ^{1}H NMR aromatic region of NAD$^+$ and [4-^{2}H]NAD$^+$ which was obtained by oxidizing [4B-^{2}H]NADH with acetaldehyde and yeast alcohol dehydrogenase. It is clear from these spectra that the pyridine 4 proton in [4-^{2}H]NAD$^+$ is largely absent, showing a residual proton absorption of only 3 ± 1%. This [4-^{2}H]NAD$^+$ was used subsequently for methods II and III.

Figure 3 shows the spectra of NAD$^+$ produced from reaction mixtures in which [4B-^{2}H]NADH was oxidized with abalone D-lactate, *N. crassa* malate, chicken liver 3-phosphoglycerate, and *R. spheroides* D-β-hydroxybutyrate dehydrogenases (method I). The spectra indicate that the *R. spheroides* D-β-hydroxybutyrate dehydrogenase is B stereospecific, while the abalone D-lactate, *N. crassa* malate, and chicken liver 3-phosphoglycerate dehydrogenases are A stereospecific. The following enzymes also were found to be A stereospecific by this method, although the results are not shown: malate dehydrogenase from *B. subtilus;* L-lactate dehydrogenase from turkey, bull frog, and halibut; and porcine H_4 L-lactate dehydrogenase using α-ketobutyrate, instead of pyruvate, as the substrate.

The results obtained with the lactate, malate, and 3-phosphoglycerate dehydrogenases verify previous determinations of the stereospecificity of these enzymes by conventional techniques (Slaughter and Davies, 1968; Popják, 1970; Davies et al., 1972; Long and Kaplan, 1973; Winicov, 1975; You and Kaplan, 1975). Like the *lecithin-nonrequiring R. spheroides* enzyme, the *lecithin-requiring* D-β-hydroxybutyrate dehydrogenase from bovine heart mitochondria was also found to be B stereospecific (result not shown).

Glucose-6-phosphate dehydrogenase from *L. mesenteroides* utilizes both NAD$^+$ and NADP$^+$ as a coenzyme (Olive and Levy, 1967). The NADP$^+$ linked reaction was previously found

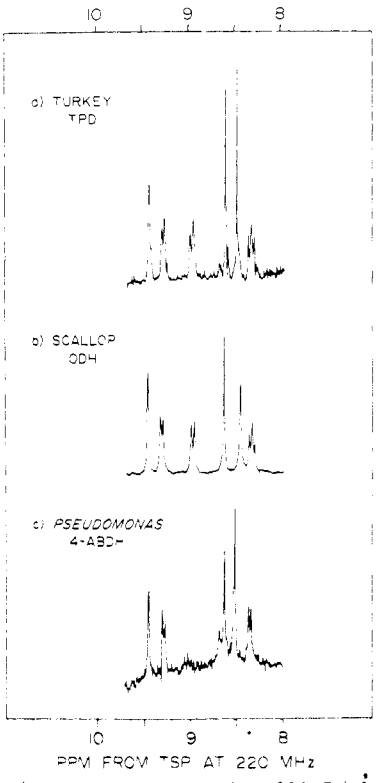

FIGURE 5: The ^{1}H NMR aromatic region of NAD$^+$ formed from [4-^{2}H]NAD$^+$ by various enzymes according to method III (pD 3, 23 °C). The enzymes employed are: (a) turkey triosephosphate dehydrogenase (TPD), 200 scans; (b) scallop octopine dehydrogenase (ODH), 16 scans; and (c) *Pseudomonoas* 4-aminobutanal dehydrogenase (4-ABDH), 200 scans.

to be B stereospecific with the yeast enzyme (Stern and Vennesland, 1960). However, the stereospecificity of the NAD$^+$ linked reaction of the enzyme has not been determined to date. Figure 4 presents the spectrum of the NADH generated from [4-^{2}H]NAD$^+$ by the action of the *L. mesenteroides* enzyme (method II) along with the spectra of authentic [4B-^{2}H]-NADH and [4A-^{2}H]NADH. These spectra clearly show that the NADH generated by *L. mesenteroides* is identical to authentic [4A-^{2}H]NADH. It can therefore be concluded that hydride was transferred to the 4B position of NADH.

Figure 5 shows the results obtained by employing method III with turkey triosephosphate, scallop octopine, and *Pseudomonas* 4-aminobutanal dehydrogenases. The spectra indicate that triosephosphate and octopine dehydrogenases are B-stereospecific enzymes and that 4-aminobutanal dehydrogenase is A stereospecific. The results obtained with the triosephosphate and octopine dehydrogenases are in accord with previously published data obtained by conventional techniques (Popják, 1970; Biellman et al., 1973).

In the cases of triosephosphate and 4-aminobutanal dehydrogenases, unequivocal results were obtained even though less than 4 μmol of the coenzyme were present in the reaction mixture before purification.

The stereospecificity of triosephosphate dehydrogenase purified from sturgeon, *E. coli*, bee, and *Limulus* all invariably showed B stereospecificity (results not shown).

Discussion

The data presented show that the ^{1}H NMR technique described in this report can be efficiently applied for the determination of the stereospecificity of pyridine nucleotide linked oxidoreductases.

Although both oxidized and reduced forms of the coenzyme can be analyzed easily, analysis of oxidized form is preferable because: (1) NAD$^+$ is more stable than NADH, since NADH is susceptible to nonstereospecific air oxidation; (2) the determination of the presence or absence of the pyridine 4 proton (8.95 δ) is much simpler than determining the chemical shifts and coupling constants of the closely situated pyridine 4A- and 4B-proton resonances of NADH; (3) purification of NAD$^+$ from the reaction mixture is not usually required (especially if the reaction components are known), since impurities having absorptions in the aromatic proton region are limited. Thus, methods I and III are more conveniently employed than method II.

It should also be pointed out that the results obtained with triosephosphate dehydrogenase and 4-aminobutanal dehydrogenase (method III) indicate that satisfactory ^{1}H NMR spectra can be obtained with the use of less than 4 μmol of [4-^{2}H]NAD$^+$, even when acid–acetone purification is included. If purification of the coenzyme is unnecessary, as little as 0.6 μmol[3] of the coenzyme can be used. It should be mentioned, however, that operation at these lower concentrations requires instrumentation with signal averaging capabilities.

In order to avoid possible nonstereospecific exchange of the pyridine 4 hydrogen between NAD$^+$ and NADH at high concentrations, we carried out the reactions at low concentrations of coenzyme. Ludowieg and Levy (1964), for example, found that a nonstereospecific hydrogen exchange occurred between 30 mM [4-^{3}H]NAD$^+$ and 30 mM NADH to the extent of 39% in 8 h at pH 8.0 and 30 °C. When the coenzyme concentrations were lowered to 0.3 mM, however, the chemical exchange was only 7% in the same period of time. Under our experimental conditions, the combined concentrations of oxidized and reduced coenzyme were never greater than 0.5 mM and reactions were completed within 30 min.

When compared with conventional mass spectroscopic and radioisotopic techniques, the described ^{1}H NMR technique has numerous advantages. These include: (1) the accurate and rapid monitoring of the *exact* oxidoreduction site, rather than the entire coenzyme molecule (the accuracy of this ^{1}H NMR technique is limited by the accuracy of integration, which is approximately ±2% in spectra with good signal-to-noise ratios); (2) the elimination of extensive and tedious purification steps; (3) the use of crude extracts (especially if a control experiment, i.e., an experiment without substrate, is carried out, as shown in the cases of abalone D-lactate and octopine dehydrogenases); (4) the alleviation of the hazards associated with the use of radioisotopes; (5) the use of μmol quantities of coenzyme when instrumentation with Fourier transform analysis is available.

[3] The minimum quantity of material required for analysis by any of the methods depends upon the strength of the magnetic field and upon the availability of signal averaging capabilities. From our experience when using a 5-mm sample tube, the following formula expresses the minimum concentration of sample needed for the reliable determination of signal intensities

$$\text{Minimum concentration} = \frac{2M}{\frac{S}{N}(\text{transient})^{1/2}}$$

where S/N represents the signal-to-noise ratio of the instrument. Thus, if the S/N is 50 and the number of transients is 400, the sample concentration required is 2 mM. If one employs vortex plugs, in order to reduce the sample volume required to 0.3 ml, only 0.6 μmol of the coenzyme is needed for a concentration of 2 mM. Where possible, however, higher concentrations should be employed in order to eliminate time-consuming signal accumulations.

It should be emphasized that these studies can be carried out with most commonly available nuclear magnetic resonance instruments. However, the instrument employed may restrict the methods used, as well as the quantity of material required. For methods I and III, any instruments having a magnetic field of 40 MHz or more can be used, but for method III a 100 MHz or higher field instrument is recommended in order to achieve accurate resolution of chemical shifts.

Employing the technique presented here, it is possible to determine the stereospecificity of $NADP^+$ linked enzymes as well. It was previously shown that at pD 7.0 the resonance for the pyridine 4 proton of $NADP^+$ appears at 8.78 δ (Sarma and Kaplan, 1969) and that at pD 8.5 the pyridine 4A and 4B protons of NADPH appear at 2.82 δ ($J_{5\text{-}4A}$ = 3.1 Hz) and 2.72 δ ($J_{5\text{-}4B}$ = 3.8 Hz), respectively (Arnold and Oppenheimer, unpublished results[2]). [4B-^{2}H]NADPH may be readily prepared with the use of $NADP^+$ linked glutathione reductase in the presence of reduced glutathione and dithiothreitol in ^2H_2O.

With the aid of this facile ^{1}H NMR technique, the stereospecificity of a large number of previously unstudied enzymes is being established in this laboratory. These results will be presented elsewhere.

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