I. ^{31}P and ^{13}C Nuclear Magnetic Resonance Studies of Nicotinamide Adenine Denucleotide and Related Compounds

II. ^{19}F Nuclear Magnetic Resonance Studies of Rabbit Muscle Glyceraldehyde-3-Phosphate Dehydrogenase Covalently Labeled with a Trifluoromethyl Group

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Michael Blumenstein

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ABSTRACT

The ^{31}P spectra of the reduced and oxidized forms of nicotinamide adenine dinucleotides as well as several related mono- and dinucleotides have been obtained and analyzed. From the spectral differences between the reduced and oxidized nucleotides, as well as from the determination of the pK_a values of the phosphate group in the mononucleotides, it is postulated that in the oxidized nucleotides there is an electrostatic interaction between the positively charged nitrogen of the pyridine ring and a negatively charged oxygen of the diphosphate backbone.

The natural abundance ^{13}C spectra of nicotinamide adenine dinucleotide and related compounds have been recorded and assigned. These spectra yielded further evidence for the nicotinamide-phosphate interaction in oxidized nucleotides. Small effects on the ^{13}C spectra were observed upon changes in pH or nucleotide concentration.

Rabbit muscle glyceraldehyde-3-phosphate dehydrogenase has been reacted with bromotrifluoroacetone, leading to a modified protein containing a trifluoromethyl group at each of its four active site cysteines. Addition of NAD^+ or NADH caused changes in the ^{19}F spectrum arising from the bound trifluoromethyl group. Also changes in pH affected the ^{19}F spectrum. At high pH, two resonances were observed, leading to the conclusion that the enzyme, which is composed of four subunits having identical amino acid sequences, exists as an $\alpha_2\alpha_2'$ protein. The chemical shift changes observed in this study

(up to 19 parts per million) are considerably larger than those previously observed in ^{19}F nuclear magnetic resonance studies of proteins.

Measurements of the spin-spin and spin-lattice relaxation times of the enzyme bound trifluoromethyl group have been performed. It has been shown that the bound label has very little mobility, and interacts with solvent protons, as well as protons on other residues of the protein.

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Vanity of vanities, said the preacher; vanity of vanities;
all is vanity.

Ecclesiastes 1:2

CHAPTER I: ^{31}P and ^{13}C Nuclear Magnetic Resonance Studies of Nicotinamide-Adenine Dinucleotide and Related Compounds

INTRODUCTION

Nicotinamide-adenine dinucleotide, or NAD^+* (also known as diphosphopyridine nucleotide, or DPN^+) is a coenzyme which is involved in a large number of oxidation-reduction reactions catalyzed by dehydrogenases. Its structure is shown in Figure 1.

The compound has been widely studied by pmr methods (Jardetzky et al., 1963; Jardetzky and Wade-Jardetzky, 1966; Catterall et al., 1969; Hollis, 1969; Sarma et al., 1968a, b; Sarma and Kaplan, 1969a, b, 1970a, b; Sarma et al., 1970; Griffith et al., 1970). One major conclusion from these studies is that at room temperature above pH 4, the pK_a value of N-1 of the adenine ring in aqueous medium, the dinucleotide exists partially in folded

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* Abbreviations used in Chapter I are: NAD^+, β-nicotinamide-adenine dinucleotide; NADH, reduced β-nicotinamide-adenine dinucleotide; α-NAD^+, α-nicotinamide-adenine dinucleotide; NMN^+, nicotinamide mononucleotide; $NMNH_2$, reduced nicotinamide mononucleotide; $APAD^+$, acetylpyridine-adenine dinucleotide; APADH, reduced acetylpyridine-adenine dinucleotide; ADPR, adenosine-5′-diphosphoribose; $NADP^+$, nicotinamide-adenine dinucleotide phosphate; NADPH, reduced nicotinamide-adenine dinucleotide phosphate; pmr, proton magnetic resonance spectroscopy; nmr, nuclear magnetic resonance spectroscopy; cmr, ^{13}C nuclear magnetic resonance spectroscopy.

form, with the nicotinamide and adenine rings lying in parallel planes. It has been shown, however, that raising the temperature (Sarma and Kaplan, 1970a), lowering the pH below a value of 4 (Catterall et al., 1969), or going to a methanol-water solvent mixture (Catterall et al., 1969), causes the dinucleotide to unfold.

In order to gain additional information on the structure of NAD^+ and related compounds in solution, ^{31}P and ^{13}C nmr experiments were performed. These experiments have had a threefold purpose. First, of course, they have yielded new information on the conformation of NAD^+. Secondly, since some of the experiments could possibly be repeated in the presence of enzymes, they will serve as a blank for future experiments. Finally, these experiments have given some indications of the advantages, as well as the drawbacks, of using ^{31}P and ^{13}C nmr in studying molecules of biological interest.

Using continuous wave methods, cmr studies could only be performed on material which had been specifically enriched with carbon-13. These experiments, together with the ^{31}P studies, are presented in the first part of this chapter. The acquisition of a Fourier Transform nmr system allowed the recording of natural abundance cmr spectra, as well as the performance of relaxation time experiments on the enriched material. These experiments are described in Part B.

PART A

Experimental

Materials. NAD^+, NADH, α-NAD^+, NMN^+, $NMNH_2$, NADP, NADPH, $APAD^+$, AMP, ADPR, yeast alcohol dehydrogenase, and snake venom phosphodiesterase were obtained from Sigma. Butyllithium was a product of Peninsular Chem Research and 3-bromopyridine a product of Aldrich Chemical Co. Barium carbonate-^{13}C (65% enriched) and acetic acid-1-^{13}C (60% enriched) were purchased from Merck, Sharp and Dohme. Barium carbonate-^{13}C (95% enriched) was purchased from Bio-Rad.

Preparation of NAD^+-carbonyl-^{13}C and Related Compounds. Nicotinic acid-7-^{13}C was prepared by treating 3-bromopyridine with butyllithium, followed by carbonation with CO_2-^{13}C generated by addition of acid to $BaCO_3$-^{13}C (Murray et al., 1948).

Nicotinamide-7-^{13}C was prepared from nicotinic acid-7-^{13}C via the ethyl ester according to the method of Laforge (1928).

NAD^+-carbonyl-^{13}C was prepared from nicotinamide-7-^{13}C and NAD^+ through NADase-catalyzed exchange (Zatman et al., 1953). The pig brain NADase was isolated according to the method of Zatman et al. (1954). The coenzyme was purified on a DEAE-cellulose column (Di Sabato, 1968) eluted with a 0.0015 M to 0.07 M ammonium bicarbonate gradient (pH 8.5). The enriched coenzyme was then desalted on a G-10 column. By using 65% enriched nicotinamide,

and a twofold molar excess of nicotinamide to NAD^+, a coenzyme which was about 43% enriched was prepared. (This assumes equilibration in the exchange reaction. No attempt was made to determine the exact enrichment of NAD^+.)

A solution of NADH-^{13}C was prepared from NAD^+-^{13}C using yeast alcohol dehydrogenase and ethanol.

An equimolar mixture of NMN^+-^{13}C and 5'-AMP was prepared by adding snake venom phosphodiesterase to a solution of NAD^+-^{13}C. The reduced mononucleotide was prepared from NADH-^{13}C in the same manner.

N-Methylnicotinamide-7-^{13}C was prepared by mixing nicotinamide-7-^{13}C (50 mg) with a tenfold excess of methyl iodide in acetone (1 ml). The reaction mixture was allowed to stand at room temperature for four hours, and the N-methylnicotinamide crystals which had formed during this time were collected by filtration. The purity of the product was checked by integration of the pmr spectrum, the ratio of the peak area of the methyl protons (δ 4.6) to the pyridine protons (δ 8.0-9.4) being 3:4, as expected.

Methods. ^{31}P spectra were run on a modified HR 220 nmr spectrometer operating in frequency sweep mode at 89.1 Mc. A Varian 1024 Computer of Average Transients was used to enhance signal to noise ratios. Heteronuclear proton noise decoupling was applied by use of a Varian S68625 heteronuclear decoupler, a Hewlett-Packard 6266B power supply, and a Fluke 622A frequency synthesizer. Noise decoupling was applied in all cases except where

its absence is specifically noted. In order to attain rapid temperature equilibration when using decoupling, a Varian 4540 temperature controller was used to keep the sample temperature at 24±2°.

Most ^{31}P spectra were run on 0.1 M samples in 0.1 M phosphate or pyrophosphate buffer. Some of the samples contained 0.001 M EDTA (see Results). All chemical shifts are referred to an internal standard of approximately 1% triethyl phosphate. Shifts to lower frequency (upfield) are denoted by positive numbers, and downfield shifts by negative numbers. The phosphorus resonance position of triethyl phosphate is independent of pH and comes 0.4 ppm downfield of the resonance due to 85% phosphoric acid. Shifts are accurate to ±5 cps (0.06 ppm). When greater accuracy was required (such as in comparing $APAD^+$ and NAD^+) both compounds were added to the same solution, and the spectrum was recorded.

The pH of all solutions was checked before and after the spectra were run using a Radiometer pH meter 26. NAD^+ and NADH concentrations were checked by measuring the OD_{340} (after alcohol dehydrogenase reduction in the case of NAD^+).

^{13}C spectra were obtained on a Varian XL-100-15D spectrometer operating at 25.1 Mc. A Fabritek 1062 computer was used to enhance signal strength and noise decoupling was employed in all experiments. Samples were contained in 12-mm tubes. About 2 ml of 1.5×10^{-2} M solutions was used. This corresponds to a ^{13}C concentration of about 6×10^{-3} M for NAD^+, NADH, NMN^+, and $NMNH_2$, and about 1.4×10^{-2} M for nicotinamide and N-

methylnicotinamide (where 95% enriched materials were used). By using a 50-cps sweep width and a 25-sec sweep time adequate spectra could be obtained in about 15 min. A capillary containing acetic acid-1-^{13}C (60% enriched) was used as a standard. Shifts are accurate to ±1 cps (0.04 ppm).

Potentiometric titrations were done using a Radiometer pH meter 26.

Results

^{31}P Studies. The ^{31}P spectrum (undecoupled and decoupled) of NAD is shown in Figure 2. The broadness in the undecoupled spectrum is due to incompletely resolved coupling between the phosphorus atoms and the 5′-methylene protons of each ribose ring. Even using a concentrated solution (0.5 M) and a slow sweep rate, this coupling could not be easily resolved, so all other spectra were taken using noise decoupling. From the decoupled spectrum, it can be seen that the ^{31}P spectrum of NAD^+ is composed of an AB pattern, with δ_{AB} 25.7 ± 0.5 cps and J = 20.0 ± 0.5 cps. The value for the coupling constant is consistent with the value of 19.9 ± 0.5 found for ATP (Cohn and Hughes, 1960). The ^{31}P spectrum of NADH consists of one singlet, which occurs 22 cps downfield of the center of the AB pattern seen with NAD^+.

A study of the pH dependence of the chemical shifts of NAD^+ and NADH was done, and it can be seen (Figure 3) that with the exception of a small upfield shift with NAD at pH 1, the shifts were

invariant with pH. No spectra were taken on NADH below pH 5 due to the rapid decomposition of the coenzyme in acidic solution. This decomposition leads to the appearance of new peaks in proton spectra (Griffith et al., 1970), so it was assumed that any such data from phosphorus nmr would be suspect.

It should be noted that with both NAD^+ and NADH the peaks started to broaden as the pH was lowered. The broadening was less marked in pyrophosphate than in phosphate and was almost completely eliminated by adding 10^{-3}M EDTA. The broadening was probably due to contamination by paramagnetic ions and was partially overcome in pyrophosphate due to binding of the ion to the buffer salt. Although the broadening reached 50 cps in some cases, as opposed to the normal width of about 2 cps, within experimental error, there was no change in chemical shifts when EDTA was added. Paramagnetic ion contamination is a problem when conducting ^{31}P nmr on compounds such as NAD^+, since its pyrophosphate group will strongly bind divalent cations. No large paramagnetic ion contamination effects have been reported in pmr experiments on NAD^+, nor were they observed in the ^{13}C spectra described below. It should be noted, however, that ion contamination effects did influence relaxation time measurements, as described in Part B.

The ^{31}P chemical shifts of NAD^+ and several related compounds are listed in Table I. It can be seen that in all cases, reduced nucleotides have resonances which come to lower field than the resonances in the corresponding oxidized nucleotides.

^{13}C Studies of Pyridine Nucleotides. The chemical shifts of the carbonyl ^{13}C in NAD^+, NADH, NMN^+, $NMNH_2$, nicotinamide, and N-methylnicotinamide are shown in Figure 4. It is seen that the titration curves for NAD^+ and nicotinamide show an apparent pK_a of about 4, while the shifts for NMN^+ and N-methylnicotinamide are independent of pH. No pH studies of NADH and $NMNH_2$ were done, due to the instability of these materials at low pH.

Potentiometric Titration Studies. Potentiometric titrations were done in order to determine the pK_a of the phosphate group in NMN, $NMNH_2$, and AMP (Figure 5). It can be seen that the pK_a values determined for $NMNH_2$ and AMP are about 6.4, while that of NMN is 6.0.

Discussion

^{31}P Studies of Pyridine Nucleotides. The invariance of the NAD^+ chemical shift with changing pH (Figure 3) unequivocally proves that the pK of 3.88 which NAD possesses (Moore and Underwood, 1969) is not due to a phosphate. This pK has generally been ascribed to the adenine-N-1, although it has been speculated that it could belong to a phosphate (Moore and Underwood, 1969). The titration curve for NMN^+ (Figure 3) shows that when there is a change in ionization state of a phosphate group, it is readily observed by ^{31}P nmr.

The difference in ^{31}P spectra between NAD^+ and NADH is somewhat surprising in view of the fact that the compounds are

believed to have the same overall conformation in solution (Sarma and Kaplan, 1969a), that is, both are believed to have the adenine and pyridine rings stacked in parallel planes, with the adenine in the anti conformation, and the pyridine syn. Results presented in Part B cast some doubt as to whether the pyridine ring is indeed syn to the attached ribose in NAD^+, and anti in $APAD^+$.

A possible explanation for the difference in shifts between reduced and oxidized nucleotides is that the latter contain a charged nitrogen in the pyridine ring and that there is an electrostatic interaction between this nitrogen and the negatively charged phosphate which causes a change in the ^{31}P shift. One would expect such an interaction to cause an upfield shift since an upfield shift is observed on protonation of a phosphate group, as is seen from the ^{31}P titration curve of NMN^+ (Figure 3). This upfield shift is indeed observed in going from NADH to NAD^+, APADH to $APAD^+$, NADPH to $NADP^+$, and $NMNH_2$ to NMN^+. In the dinucleotides, all of which display a singlet when in reduced form, one resonance is moved about 25 cps further upfield than the other resonance when the compound is in the oxidized form. It is believed that the resonance to higher field is due to the phosphorus which is in the nicotinamide half of the dinucleotide, since examination of space-filling models shows that this phosphorus could more easily interact with the nitrogen of the nicotinamide ring. This phosphorus undergoes a total upfield shift of about 35 cps upon oxidation, while the phosphorus which is closer to the adenine ring undergoes an upfield shift of about 10 cps. Since the upfield shift in

going from $NMNH_2$ to NMN^+ is only 29 cps one would probably expect the same upfield shift to occur for the nicotinamide phosphorus of the dinucleotide, while no shift would be expected for the adenine phosphorus. A possible explanation for the additional observed shifts of about 10 cps in the dinucleotides is that interaction of the charged nitrogen with one phosphate affects the phosphate-phosphate interaction and thereby introduces a further shift of both phosphorus atoms.

Further evidence for the electrostatic interaction was obtained by determining the pK_a for the phosphate group in NMN^+, $NMNH_2$, and AMP. Both AMP and $NMNH_2$ have an uncharged nitrogen at their glycosidic linkage, whereas the nitrogen at this position in NMN is charged. It is seen (Figure 5) that the phosphates in AMP and $NMNH_2$ possess a pK_a value of about 6.4, while that of NMN^+ has a pK_a of 6.0. The presence of a positive charge interacting with the phosphate of NMN^+ would cause this lowering of its pK. Since in addition to possessing nearly identical pK_a values $NMNH_2$ and AMP possess nearly identical ^{31}P chemical shifts (Table I) and NMN^+ has both a different pK_a and different chemical shift it would indeed appear that the chemical shift change is most likely caused by the positively charged nitrogen in NMN.

Rather surprisingly, the value for δ_{AB} in the AB pattern of the NAD^+ spectrum was virtually unchanged when the nucleotide was dissolved in 4 M NaCl, a medium which would be expected to greatly lessen any electrostatic interactions. It is possible, however, that the various intramolecular interactions of the NAD^+ molecule (the

base stacking as well as the nicotinamide-phosphate interaction) do not allow for the NaCl molecules to penetrate into the region where the electrostatic interaction occurs. Although δ_{AB} was unchanged in high salt, J_{AB} decreased from 20.0 ± 0.5 cps to 18 ± 0.5 cps, and the chemical shift relative to $(C_2H_5O)_3P{=}O$ decreased from 970 to 905 cps. Very similar changes in coupling constant and chemical shift were observed in the ^{31}P spectrum of ATP in 4 M NaCl, so it is likely that the effects seen with NAD^+ are of a general nature, and could be observed with most compounds which possess charged phosphate groups. It should also be mentioned that the shift of $(C_2H_5O)_3P{=}O$ relative to a capillary containing phosphoric acid changed in high salt, the shift of the $(C_2H_5O)_2P{=}O$ being 30 cps to higher field in 4 M NaCl than it was in pure H_2O. It can thus be computed that the NAD resonance actually moved 35 cps downfield in 4 M NaCl, rather than the 65 cps deduced from the fact that the shift of NAD^+ relative to $(C_2H_5O)_3P{=}O$ changed from 970 to 905 cps.

It is possible that the electrostatic interaction between the nicotinamide nitrogen and the phosphate group is not direct, but is rather mediated by a water molecule. An attempt to test this hypothesis was made by comparing spectra of NAD^+ in H_2O and in D_2O. The spectra proved to be identical within experimental error, but since even if there were an intervening water the difference between spectra run in H_2O and D_2O might be very small, the hypothesis cannot be excluded.

It should be noted that the idea of an electrostatic interaction between a phosphate and the nicotinamide ring nitrogen is not inconsistent with the model whereby the adenine and nitocinamide rings are in close proximity in parallel planes (Jardetzky and Wade-Jardetzky, 1966; Catterall et al., 1969; Hollis, 1969; Sarma et al., 1968a, b, 1970; Sarma and Kaplan, 1969a, b, 1970a, b). Examination of space-filling models reveals that both interactions may occur simultaneously. By the same token the molecule may unfold without affecting the nicotinamide-phosphate interaction. Protonation of the adenine ring below pH 4 would not be expected to produce an additional electrostatic effect because the adenine-N-1 is not sterically suitable for such an interaction, hence no change in the ^{31}P spectrum is expected due to this ionization.

The AB pattern of NAD^+ is unaffected by changing the dinucleotide concentration from 1×10^{-2} to 5×10^{-1}, demonstrating that it is unlikely that intermolecular interactions play any part in the non-equivalence of the phosphates.

It is also of interest to compare the ^{31}P spectrum of NAD^+ with that of related oxidized dinucleotides (Table I). The spectrum of NAD^+ is identical with that of $NADP^+$, but in $APAD^+$ the phosphorus closer to the pyridine moiety is 6 cps upfield of the corresponding phosphorus in NAD^+, while the phosphorus proximal to the adenine moiety retains the same position in NAD^+ and $APAD^+$. Since it has been stated (Sarma and Kaplan, 1969a) that in both NAD and NADP the nicotinamide ring is syn to the attached ribose, while in APAD

the relationship between these entities is anti, it is possible that there is a slight difference in phosphate-pyridine ring interactions in APAD from that present in NAD and NADP. Another possibility is that the difference in electron-withdrawing effect between the acetyl side chain in $APAD^+$ and the carboxamide side chain in NAD^+ alters the charge density at the pyridine nitrogen, thereby affecting the ^{31}P shift. It should be noted that the corresponding reduced dinucleotides (NADH and APADH) display identical spectra.

The ^{31}P spectrum of α-NAD^+ is an AB pattern but δ_{AB} is only 10 ± 1 cps. The peak due to the adenine phosphorus is at the same position as it is in β-NAD but the resonance of the nicotinamide phosphorus is moved downfield by 15 cps. This smaller shift relative to NADH is expected in α-NAD^+ since the nicotinamide ring is above the ribose while the phosphate is below it and the nicotinamide-phosphate interaction, while not eliminated, is lessened.

The ^{31}P spectrum of ADPR is also an AB pattern. This compound does not possess a positively charged nitrogen but the absence of the nicotinamide ring gives ADPR more rotational freedom than is possessed by NAD^+, and in addition, the molecule has a free anomeric center. It is therefore not surprising that the ^{31}P spectrum of ADPR is an AB pattern and is somewhat shifted from the positions of both NAD^+ and NADH.

<u>^{13}C Studies of Pyridine Nucleotides</u>. The resonance of the carbonyl-^{13}C in NAD^+ is about 7 ppm upfield of the corresponding resonance in NADH. This difference is not surprising in light of the

aromatic structure and positively charged nitrogen present in the pyridine ring of NAD^+ while the corresponding nitrogen in NADH is uncharged and the dihydropyridine ring is not aromatic. The effect of the charged nitrogen is seen by examining the titration curve of nicotinamide (Figure 4). At low pH, when the ring nitrogen is charged, the carbonyl chemical shift is within 1 ppm of the NAD^+ resonance. At pH 5 or above, when the nitrogen of nicotinamide is uncharged, the carbonyl chemical shift moves downfield by about 4 ppm and is then much closer to the position of the NADH resonance.

The titration curve of NAD^+ (Figure 4) shows a pK_a of about 4, with a total chemical shift difference of 10 cps to higher field in going from lower to higher pH. These data are in agreement with pmr titration data obtained by Hollis (1969) and Jardetzky and Wade-Jardetzky (1966). At low pH, the chemical shift in NAD^+ is within 5 cps of the shift in NMN^+. This would be expected if NAD^+ were unfolded at low pH, since the adenine ring would exert no influence on the chemical shift of the nicotinamide carbonyl, and the environment of the carbonyl would be very similar in NMN^+ and NAD^+. If the molecule were to fold in such a way that the carbonyl was directly over the adenine ring, an upfield shift would be observed, since the adenine ring current would exert a shielding effect on the carbonyl carbon.

At high pH, the chemical shift of NADH is about 15 cps to higher field than that of $NMNH_2$. This difference would also be expected to lessen at lower pH, for the same reasons as noted above with NAD^+.

As previously mentioned, however, the reduced nucleotides are unstable at low pH, so it was impossible to check this hypothesis.

It should be noted that the carbonyl shift in NMN^+ is not affected by the ionization of the phosphate (Figure 4). This constancy of chemical shift with changing pH is perhaps unexpected in light of the conclusion reached previously in this paper that the phosphate of NMN^+ interacts with the positively charged nitrogen of the pyridine ring. It has been found from pmr studies (Sarma and Kaplan, 1969a) however, that although the phosphate ionization in NMN^+ affects the chemical shifts of the C-2 and C-6 protons, the C-4 and C-5 protons are unaffected. The lack of an effect on the carbonyl carbon, which is attached to the C-3 carbon on the pyridine ring, is therefore not totally unexpected. It is shown in Part B, that C-4 and C-6 are the only carbons of the nicotinamide ring of NMN^+ which are substantially affected by the phosphate ionization.

PART B

Experimental

Materials. Mono- and dinucleotides used in this study were purchased from Sigma.

Methods. Spectra were taken on a XL-100-15D nmr spectrometer operating at 25.1 M Hz. Proton noise decoupling was used in all experiments, except those in which single frequence decoupling was employed to aid in peak assignments. The Fourier Transform technique was used to acquire all spectra. Conditions were such (acquisition time 0.4 sec, pulse width 25 μsec ($\pi/2$ = 105 μsec)) that all peaks were observed, though those with fairly long relaxation times were partially saturated. Most spectra were run at a 2500 Hz spectral width, with weak positions being determined from the computer generated print out, using the solvent, D_2O, as internal standard.

Most samples had a concentration of approximately 0.1 M nucleotide and also contained 0.1 M phosphate. Spectra were acquired in two hours time. Certain samples (NMN^+, $NMNH_2$, αNAD^+) contained 0.05 M nucleotide, and spectra of these samples took eight hours to accumulate. The low concentration (0.008 M) NAD^+ sample was run under different conditions (acquisition time of 0.2 sec and pulse width of 100 μsec) and also took eight hours. Under these conditions, quaternary carbons were not seen.

Most chemical shifts given are the average of two runs, performed on different days. Almost all values obtained in the two runs were within 3 Hz of each other.

Results

The positions of the ^{13}C resonances of the nucleotides used in this study are listed in Table 2. The spectra of NAD^+ and NADH are shown in Figure 6. Spectra were taken at pH values above and below the pK_a values of ionizable groups in the molecules to monitor the effects of these ionizations on the ^{13}C spectra. The differing intensities of the peaks in each spectrum arise primarily from partial saturation of quaternary carbons. It is thus observed that peaks due to the 4, 5, and 6 carbons of the adenine ring are less intense than any others. The decreased peak heights of the 4′ and 5′ peaks of the ribose rings are not due to decreased intensity of those peaks but rather to splitting caused by the phosphorus atoms of the diphosphate backbone.

Assignment of resonances: Since the ^{13}C spectrum of AMP (at pH 7) has been previously reported (Dorman and Roberts, 1970) and since it was found that the adenine ring resonance positions are very similar in all compounds containing this moiety, these resonances could be assigned in the high pH spectra of NAD^+, αNAD^+, NADH, $NADP^+$ and NADPH, in addition to AMP. Furthermore, the spectrum of ATP at pH 2.5 has been reported (Dorman and Roberts, 1970), and it was found that at low pH, resonances of the nucleotides used in this study corresponded almost exactly to the ATP resonances.

Similarly, from the AMP assignments for the ribose ring, the peaks due to the 1′, 4′, and 5′ carbons of the corresponding ribose in NAD^+, αNAD^+, and NADH could be assigned.

There has been a disagreement on the assignment of the 2′ and 3′ resonances in nucleotides, with one assignment (Dorman and Roberts, 1970) being based on a comparison of normal nucleotides with 2′-deoxynucleotides, and the other assignments (Mantsch and Smith, 1972) based on the effect of a phosphate group on the chemical shifts of the ribose ring carbons. In order to definitively assign the 2′ and 3′ carbons of AMP, single frequency decoupling was used, and it was found that the resonance due to the 2′ carbon is further downfield than that due to the 3′ carbon. This is in agreement with the newest assignment (Mantsch and Smith, 1972) made for 2′ or 3′ carbons of 5′ UMP.

The 1′, 4′ and 5′ carbons of the riboses in NMN^+ and $NMNH_2$ could be assigned as described previously (Dorman and Roberts, 1970). The 2′ and 3′ resonances of NMN^+ were assigned by single frequency decoupling, while the 2′ and 3′ resonances of $NMNH_2$ either exactly coincided or were too close to be individually assigned. Ribose assignments in the dinucleotides were made on the basis of the mononucleotide assignments, the only difficulties being encountered in the case of the 5′ carbons, which are very close to each other in the dinucleotides, and whose positions are somewhat shifted from the mononucleotides. Since the 5′ peak of AMP is downfield of the corresponding peak in NMN^+, the further downfield 5′ peak of NAD^+

was assigned to adenine ribose. Similarly, the 5′ peak of AMP is upfield of the corresponding peak in $NMNH_2$, so in NADH, the adenine ribose was assumed to give rise to the 5′ peak to highest field.

The spectrum of the ribose adjacent to the adenine ring of NADPH (and $NADP^+$) is very complex, due to the additional phosphate which is expected to split the 1′, 2′, and 3′ resonances. This ribose portion of the NADPH spectrum is shown in Figure 7 and was assigned by comparing the ribose chemical shifts with those of AMP, in a manner described for the assignment of 2′ UMP (Mantsch and Smith, 1972). The ^{13}C-^{31}P coupling constants are also similar to those found in 2′ UMP, being 8 Hz for $J_{C1'-P}$, 4 Hz for $J_{C2'-P}$, and 2 Hz for $J_{C3'-P}$ in NADPH, while the corresponding values in 2′-UMP are 9, 4.5, and 3 Hz (Mantsch and Smith, 1972). The spectrum due to the adenine ribose of $NADP^+$ was virtually identical to that of NADPH and was therefore assigned identically.

The assignment of C-2, C-4, and C-6 of the nicotinamide ring in the oxidized nucleotides was done by normal decoupling, C-3 was assigned by off resonance decoupling and the remaining peak due to this moiety was assigned to C-5. The decoupling experiments were performed on NAD^+, and due to the great similarity of the spectra, are almost certainly valid for NMN^+ and $NADP^+$. The spectrum of αNAD^+ is somewhat different (see below) but, since the proton spectrum has not been definitively assigned, peaks were assigned by assuming that each peak in αNAD^+ was due to the carbon which corresponded to the closest peak in the NAD^+ spectrum.

The peak to lowest field due to the dihydronicotinamide moiety of NADH could be decoupled by placing the decoupler at a frequency corresponding to N-2-H. Decreasing the decoupler frequency (i. e., moving upfield) by 100 cps caused decoupling of two resonances, one of which had previously been assigned to A-1′, and the other of which was now assigned to N-6. Moving the decoupler frequency 140 cps further upfield caused decoupling of two more resonances, one of which was previously assigned to N-1′ and the second of which was now assigned to N-5. The peak due to N-3 could be assigned by off resonance decoupling and C-4, being a saturated carbon, came in a totally different area of the spectrum, and was in fact outside the spectral region which was observed. The peak sometimes was "folded back" into the spectrum, but it was often not seen when using a 2500 cps sweep width. Its position was measured (in NADH and $NMNH_2$) by using a 5000 cps sweep width.

It should be noted that the ^{13}C assignments are in agreement with the newest assignments for the proton spectrum of NADH (Oppenheimer et al., 1971), and are in fact a strong confirmation of their accuracy. Using the old assignments, wherein the N-1′ and N-6 were reversed, the ^{13}C resonances of these carbons would exchange their positions, a shift of 30 ppm, and both resonances would then have phenomenally anomalous positions. The presently assigned positions of these resonances are quite reasonable.

The assignments for NAD^+ at low pH are in agreement with recently published assignments (Birdsall et al., 1972) for this compound at pH 3.

The relaxation time (T_1) of the carbonyl carbon of NAD^+-carbonyl-^{13}C was determined using the $[\ldots T \ldots 90°(S_\infty) \ldots T \ldots 180° \ldots t \ldots 90°(S_t) \ldots]_n$ pulse sequence described by Freeman and Hill (1971). The T_1 value was found to be 14 ± 2 sec, and within experimental error was pH independent.

Discussion

pH Effects. The protonation of the adenine ring in NAD^+ leads to small downfield shifts (2-6 cps) of all resonances due to the carbons on the nicotinamide ring. This is in agreement with proton results (Catterall et al., 1969), as well as the ^{13}C results of Part A and is indicative of a folding of the dinucleotide at neutral pH and an unfolding at low pH. The effect of folding of the dinucleotide is of similar magnitude (0.1-0.2 ppm) to that found in pmr spectra (Catterall et al., 1969). This is not surprising since if the effect is due to the ring current of the adenine ring, it is magnetic rather than electric in nature, and should be the same for all nuclei.

As expected, the ^{13}C spectrum of the nicotinamide portion of NAD^+ at low pH, where the dinucleotide is unfolded, is virtually identical to that of NMN^+ at pH 4, where the mononucleotide has a singly ionized phosphate group. The spectrum of NMN^+ when it is doubly ionized (pH 8) is different, however, due to an interaction

between the positively charged nicotinamide ring and the phosphate group. It is therefore not valid to determine the effect of the adenine ring on the nicotinamide spectrum by comparing NAD^+ and NMN^+ when both are at pH 7, as was done with proton data by Sarma and Kaplan (1969a). On the basis of this comparison they reached the conclusion that the adenine ring shielded certain protons of the nicotinamide ring more than others. If, however, they had compared the shifts of NAD^+ at pH 7 with NMN^+ at pH 4.5 they would have found (using data presented later in that paper) that all of the protons were shielded virtually the same amount. Again, the differential shift which they claimed existed between acetylpyridine adenine dinucleotide and its corresponding mononucleotide vanishes when one uses the mononucleotide data at pH 4.5 rather than pH 8. The fact that the nicotinamide ring protons are shielded virtually equally by the adenine ring is expected, since this is what one would expect from a maximum interaction between the rings. Other workers (Catterall et al., 1969) have determined the adenine shielding in NAD^+ by comparing high and low pH pmr spectra of the dinucleotide and have indeed found similar shieldings on all protons.

As already mentioned, the chemical shift of certain nicotinamide carbons are affected by a change in ionization state of the phosphate group in NMN^+. These changes are not present in the $NMNH_2$ spectrum and confirm results of the ^{31}P experiments presented in Part A, that the presence of a positive charge in the nicotinamide ring causes an interaction with the phosphate group. Somewhat

surprisingly, while in the PMR work (Sarma and Kaplan, 1969a) H-2 is most affected by a change in phosphate ionization state (0.1 ppm) and H-6 less affected (0.05 ppm) and other protons are unchanged, in the carbon spectrum C-6 is most shifted (0.7 ppm), C-4 affected somewhat (0.3 ppm), and other carbons very slightly changed. In both the carbon and proton spectra, shifts are to higher field at low pH. The proton results have been interpreted as indicating that the nucleotide exists in the syn conformation, and since recent theoretical studies (Berthod and Pullman, 1971) support this view one must wonder as to the origin of the carbon shifts, which at first glance appear to support an anti conformation. It can, however, be deduced from the direction and magnitude of the carbon shifts that they must arise from causes somewhat different than the proton shifts since neither of the reasons given (Schweizer et al., 1968) for the specific shielding of aromatic ring protons by the phosphate group of mononucleotides explain the shifts. Thus if the shift were caused by magnetic anisotropy due to the negative charge, it would have to be of the same magnitude (in ppm) in carbon and proton spectra, while in fact the observed carbon shifts are much larger. Alternatively, it is suggested (Schweizer et al., 1968) that the negative charge pulls the proton away from the carbon, decreasing the charge density at the proton, and thereby shifting it downfield with increasing phosphate ionization. Such an effect, however, would increase the charge density at the carbon and cause the shift to be opposite to that of the proton, while in fact shifts in the same direction are observed.

Also, the general effect of a negative charge would be to increase the charge density at both the proton and the carbon, thereby causing downfield shifts while as just noted, the observed shifts are upfield. A likely explanation for the ^{13}C shifts is that the phosphate-nitrogen interaction causes a redistribution of charge around the nicotinamide ring leading to shifts of certain carbons. Examination of the chemical shift changes which occur in the adenine ring carbons of NAD^+ when the adenine-N-1 is protonated illustrates the large charge effects seen in the ^{13}C spectra of aromatic systems. Thus as expected, both C-2 and C-6 of the adenine ring of NAD^+ exhibit large chemical shifts when the ring is protonated, but while C-4 and C-5 display very slight shifts, C-8 exhibits a very large shift. It is clear, therefore, that the shifts induced in the nicotinamide ring of NMN^+ by the phosphate group are not necessarily an indication of which carbons are spacially closest to this group. It should be pointed out that a redistribution of charge around the nicotinamide ring will also affect the proton spectrum, so the observation of a shift in H-2 upon ionization of the phosphate does not necessarily imply the syn conformation for NMN^+.

Dihydronicotinamide Conformation. As was mentioned previously, the ^{13}C resonances due to the nicotinamide ring in NMN^+ are virtually identical (at low pH) to those in NAD^+. This equivalence is not the case for the dihydropyridine moieties in $NMNH_2$ and NADH, since examination of Table II shows a 15 Hz shift difference for C-3 and a 22 Hz shift difference for C-6, in both cases the higher field shift being observed in NADH. One explanation for these results is

the recent suggestion (Oppenheimer et al., 1971) that the dihydropyridine ring is puckered at the C-4 in NADH, while it is flat in $NMNH_2$. Such a change, however, would be expected to have a large effect on the C-4 chemical shift, while no chemical shift difference between NADH and $NMNH_2$ is seen at this position. It is possible that the chemical shift difference observed at C-6 is due to a different orientation of the dihydropyridine ring relative to the attached ribose in $NMNH_2$ and NADH. Thus if the C-6 experiences steric hindrance in NADH (Sarma and Kaplan, 1969a), it is possible that in $NMNH_2$, the dihydropyridine ring, no longer constrained by the parallel adenine ring, can twist around the glycoside bond to avoid this strain. The origin of the shifts of C-3 is unclear, though perhaps it involves the attached carboxamide side chain rather than a direct interaction involving C-3.

α-NAD^+. The position of the nicotinamide resonances in NAD^+ differs by 1-1.5 ppm from the corresponding resonances in αNAD^+. The shifts go in both directions but the largest shifts, and the "total" shift (Perlin, 1971) is upfield in going from βNAD^+ to αNAD^+. This corresponds to greater steric interaction between the nicotinamide ring and the adjacent ribose when the latter is in the α configuration. It is of interest that the nicotinamide resonances of αNAD^+ are more pH dependent than are the corresponding peaks in βNAD^+. A possible explanation is that there is greater nicotinamide-adenine interaction in αNAD^+ than in βNAD^+. Another explanation is similar to that for the difference in the C-6 resonances of NADH and

$NMNH_2$; i.e., in αNAD^+, when the dinucleotide unfolds at low pH, the nicotinamide ring twists to relieve some steric strain.

Ribose Conformation. The ^{13}C spectrum of ribose rings of the mono- and dinucleotides used in this study is much clearer than the 220 M Hz proton spectrum, and it is observed that the shifts in the dinucleotides are virtually identical to those in the mononucleotide (except in the case of the 5′ carbons, where the phosphate present in the mononucleotides causes a somewhat different shift than does the dinucleotides' pyrophosphate). It would thus appear that the ribose conformations are identical in the mono- and dinucleotides and are solely a function of the base to which the ribose is attached.

It can be seen (Table II) that the ribose chemical shifts are very dependent on the base which is attached. It is observed that the largest chemical shift differences are seen in the C-1′ and C-2′ carbons, so these shifts can be partially explained in terms of different electron withdrawing abilities of the adenine, nicotinamide and dihydronicotinamide rings. The C-3′ and C-5′ carbons are affected very slightly by a change in the moiety attached to the ribose but, surprisingly, the 4′ carbons show rather large shift differences, with the resonance in AMP occurring 90 cps to higher field of that in NMN^+, and the $NMNH_2$ resonance occurring 40 cps to higher field of the AMP resonance. It is not clear whether these shifts reflect different ribose conformations or are due to long-range electric effects caused by the bases attached to the riboses.

One effect common to the three mononucleotides, NMN^+, $NMNH_2$, and AMP, is that in going from a singly ionized phosphate to a doubly ionized phosphate the 5′ carbon shifts upfield by about 1 ppm, while the 4′ carbon resonance shifts downfield by about 0.8 ppm. Comparing the work of Grant (Jones et al., 1970) on nucleosides with that of Roberts (Dorman and Roberts, 1970) on nucleotides, one sees that addition of a phosphate to nucleosides causes a downfield shift on the 5′ resonance of 2-2.5 ppm and an upfield shift of ~1 ppm on the 4′ resonance. The anomalous shifts often observed with ^{13}C have been discussed elsewhere (Horsley and Sternlicht, 1968) where it is noted that redistribution of charge between carbons and different nuclei or other carbons often leads to shifts which are opposite to those seen in pmr work and which are often surprisingly small.

It must be noted that far more work must be done on correlating cmr data with sugar conformation, but the large shift differences which are observed among various sugars indicate that cmr spectroscopy should be a very valuable tool in this area.

Many small shifts are seen in both ribose rings of NAD^+ when its adenine ring is titrated, the largest shift of course being observed in C-1 of the ribose bound to adenine. The shifts in the nicotinamide ribose are again indicative of an effect on the entire molecule which occurs when the adenine ring is protonated.

Base Stacking. Changing the concentration of NAD^+ from 0.1 M to 0.008 M has a very small effect on the spectrum, with changes of less than 5 Hz being observed. A comparison of the

spectral positions of the AMP resonances in this study, using 0.1 M nucleotide, with those of a previous study (Dorman and Roberts, 1970), using 1-2 M nucleotide again reveal that very small changes (less than 5 cps at 25.1 M Hz) occur even with fairly large changes in concentration.

It is clear from the pH studies of NAD^+, as well as the concentration studies of NAD^+ and AMP, that the study of intermolecular and intramolecular base stacking, as well as aromatic ring current effects in general by cmr techniques requires exceedingly great precision of measurement and would often better be studied by pmr, where the shifts in Hz will be large due to the higher frequency of the proton absorptions. There are, however, many cases where the cmr study of such effects will no doubt prove profitable, such as in complex molecules where by dint of greater spectral resolution the ^{13}C peaks can be more easily resolved and identified than the corresponding proton peaks, or in cases where the carbon atom is not bonded to any protons, and is therefore the only probe one has at a given position.

Relaxation Times. The T_1 value of 14 ± 2 secs determined for the carbonyl carbon of NAD^+ indicates that the amide nitrogen does not provide an efficient mechanism for the relaxation of this carbon. Furthermore, the fact that the T_1 is not greatly altered in going from low to high pH indicates that folding of the molecule also has no large effect on the relaxation. It should be noted that the purity of the NAD^+ has a very large effect on the T_1, with values of from 1-5 sec being

obtained when the dinucleotide was used without first being purified by ion exchange chromatography. It was observed in Part A that paramagnetic ion concentration has a very great effect on the ^{31}P spectra of NAD^+ and NADH, and also causes a substantial shortening of the T_1's for the ^{31}P atoms of ATP (M. P. Klein, personal communication).

Conclusions

In solution, NAD^+ and NMN^+ exist in a conformation so as to allow an interaction between the positively charged nitrogen of the nicotinamide ring and a negatively charged oxygen of the pyrophosphate group. This interaction is absent in the corresponding reduced nucleotide, in which the pyridine moiety is uncharged, and is diminished in αNAD^+, in which stereochemistry dictates an increased distance between the nicotinamide ring and the pyrophosphate group.

The ribose rings of corresponding mono- and dinucleotides have the same conformations. Base stacking of NAD^+ occurs in solution and is affected by changes in pH as well as nucleotide concentration, but cmr is fairly insensitive to these effects.

TABLE I: ^{31}P Chemical Shifts of NAD and Related Nucleotides

Nucleotide	Δ_P	Δ_A	δ_{PA}
NAD	983	957	26
NADH	948	948	0
NADP	983	957	26
NADPH	948	948	0
APAD	988	957	31
APADH	948	948	0
αNAD	967	957	10
NMN	-342	–	–
NMNH	-371	–	–
AMP	–	-371	–
ADPR	950	926	24

All spectra taken in 0.1 M pyrophosphate +0.001 M EDTA buffer, pH 8.3. Nucleotide concentrations are 0.1 M in all cases. Shifts are in cps at 89.1 mc and are referred to a 1% $(C_2H_5O)_3$ P = 0 internal standard. Positive numbers signify upfield shifts. Δ_P is the shift of the phosphorus atom in the pyridine half of the nucleotide, Δ_A the shift of the phosphorus in the adenine half, and δ_{PA} the chemical shift difference between the two phosphorus atoms.

Table 2: ^{13}C Chemical Shifts of NAD^+ and Related Nucleotides[a]

		Adenine Ring					Adenine Ribose				
	pH	A-2	A-4	A-5	A-6	A-8	A-1′	A-2′	A-3′	A-4′	A-5′
AMP	2.5	801	718	1468	674	868	2233	2569	2681	2331	2829
AMP	5.0	627	713	1476	558	931	2256	2576	2680	2338	2830
AMP	8.5	611	707	1473	544	926	2262	2576	2673	2320	2853
NAD^+	2.5	799	720	1471	677	865	2236	2275	2683	2333	2811
NAD^+	7.5	607	706	1471	545	933	2265	2587	2678	2340	2806
NAD^+ (0.008M)	8.0	601				931	2264	2591	2678	2342	2810
NADH	8.0	608	708	1469	541	937	2255	2682	2575	2350	2810
αNAD^+	2.5	795	719	1470	671	869	2237	2681	2563	2332	2805
αNAD^+	7.5	609	710	1475	543	932	2267	2680	2574	2335	2802
$NADP^+$	2.5	799	718	1474	676	853	2264	2685	2510	2337	2806
$NADP^+$	5.1	631	710	1480	565	920	2287	2683	2527	2349	2806
$NADP^+$	8.0	610	706	1475	546	929	2281	2685	2537	2361	2803
NADPH	8.1	610	700	1464	539	931	2264	2684	2529	2360	2804

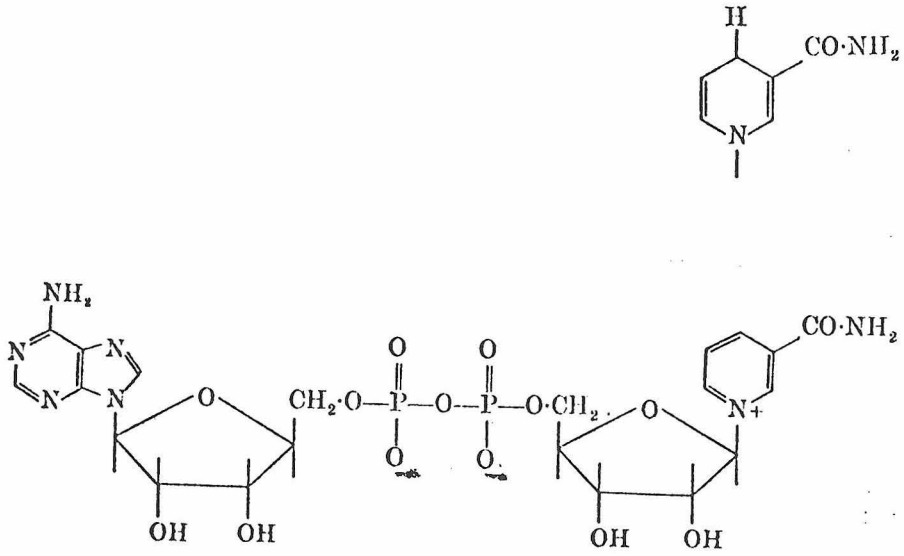
Table 2 continued

		Nicotinamide Ring					Nicotinamide Ribose				
	pH	N-2	N-3	N-4	N-5	N-6	N-1′	N-2′	N-3′	N-4′	N-5′
NMN^+	4.0	929	1078	774	1116	863	1933	2493	2663	2253	2837
NMN^+	8.0	932	1078	768	1116	846	1922	2492	2652	2228	2863
NAD^+	2.5	930	1081	776	1111	862	1934	2497	2668	2257	2811
NAD^+	7.5	931	1087	782	1113	867	1933	2497	2674	2249	2815
NAD^+ (0.008M)	8.0	929		779	1111	865	1931	2497	2674	2242	2816
αNAD^+	2.5	904	1127	795	1151	836	1914	2647	2669	2256	2804
αNAD^+	7.5	914	1139	808	1156	844	1914	2647	2668	2254	2802
$NADP^+$	2.4	933	1083	776	1111	863	1936	2499	2671	2264	2815
$NADP^+$	5.1	933	1089	783	1114	870	1936	2500	2676	2265	2820
$NADP^+$	8.0	935	1094	788	1116	875	1936	2500	2679	2273	2817

		Dihydronicotinamide Ring					Dihydronicotinamide Ribose				
	pH	N-2	N-3	N-4	N-5	N-6	N-1′	N-2′	N-3′	N-4′	N-5′
$NMNH_2$	5.2	974	1919		1798	1307	2061	2673 2668		2372	2819
$NMNH_2$	8.5	971	1918	3897	1797	1309	2059	2671	2671	2357	2841
NADH	8.0	971	1933	3899	1801	1330	2056	2676 2668		2380	2791
NADPH	8.2	974	1934		1804	1334	2058	2674 2668		2378	2794

[a] Shifts are in cps at 25.1 Mc and are referred to a $CH_3{}^{13}CO_2H$ standard. All shifts are upfield relative to the standard.

FIGURE 1. The structure of NAD^+. In upper right is shown the dihydropyridine ring of NADH.



NAD+ (DPN+)

FIGURE 2. (A) The ^{31}P spectrum of 0.5 M NAD^+, pH 8.2, proton decoupled; (B) the undecoupled spectrum of the same solution; (C) the spectrum of 0.1 M NADH, pH 8.2, proton decoupled.

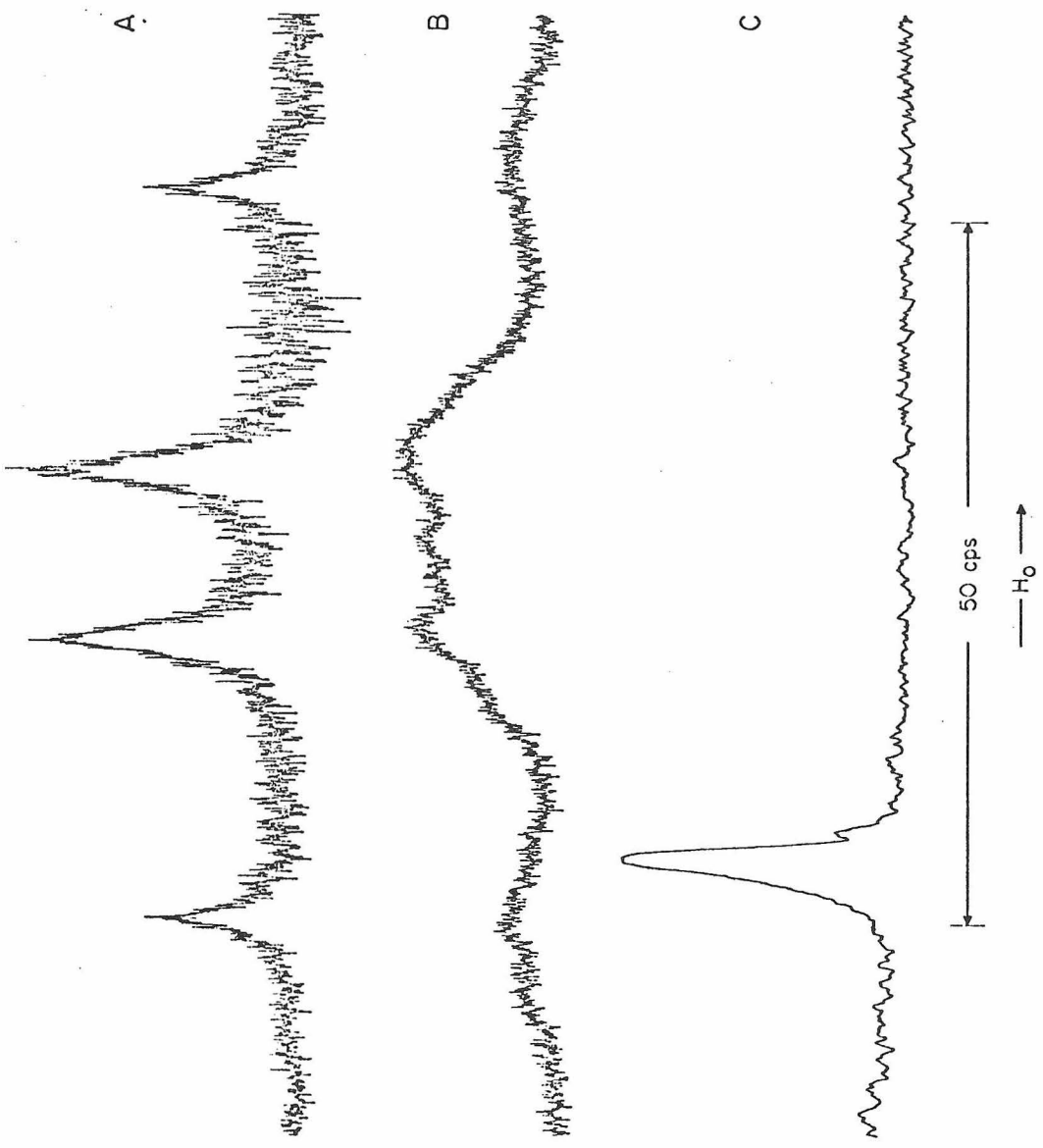

A.
B
C
50 cps
H_o

FIGURE 3. Upper, ^{31}P nmr titration data for NAD^+ and NADH. Shifts are in cps relative to $(C_2H_5O)_3P{=}O$. Lower; ^{31}P titration data for NMN.

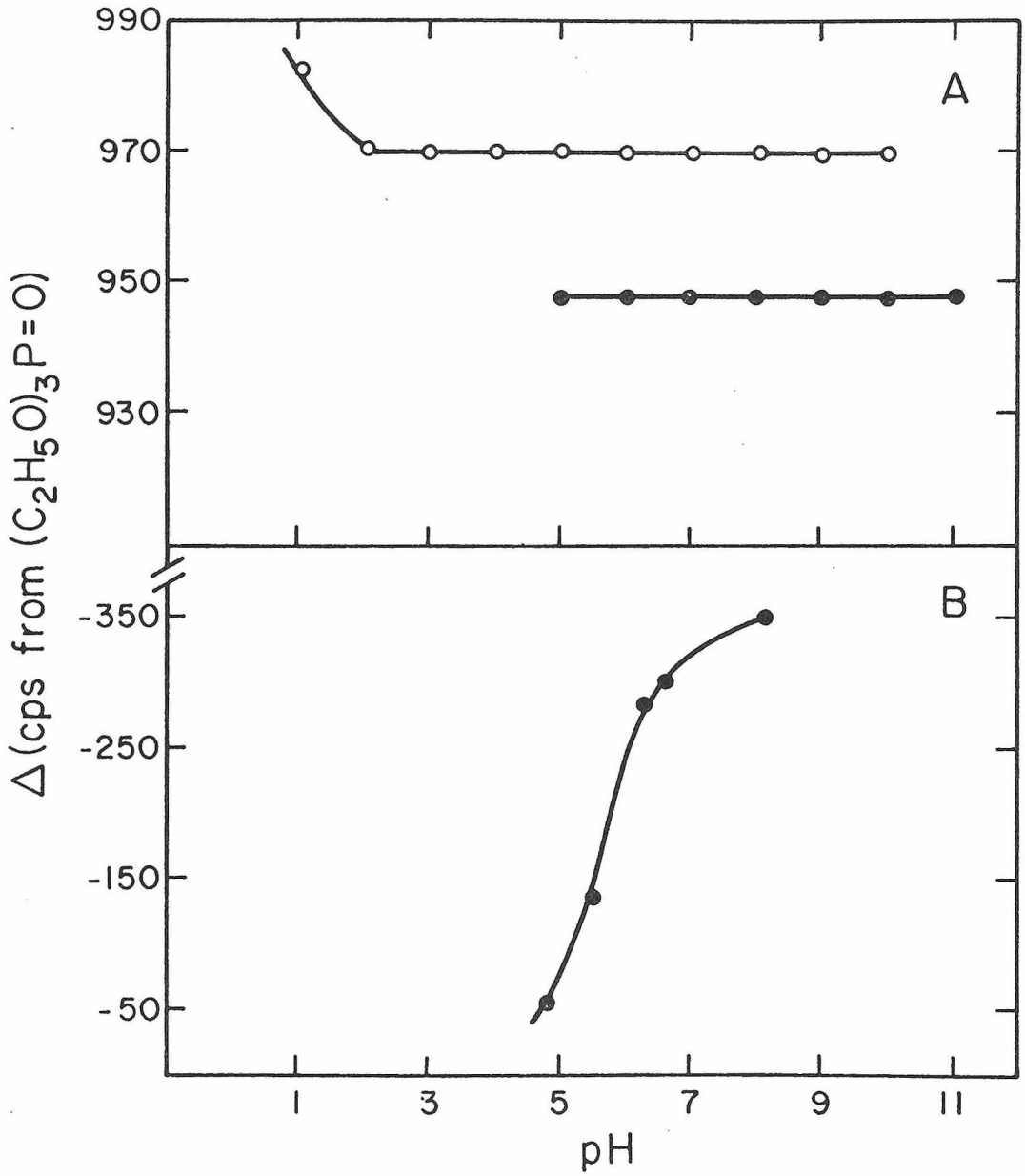

990
970
950
930
A
-350
-250
-150
-50
B
Δ (cps from $(C_2H_5O)_3P=O$)
1
3
5
7
9
11
pH

FIGURE 4. ^{13}C nmr titration data for the carbonyl carbon of NAD^+, NADH, NMN, $NMNH_2$, nicotinamide (NA), and N-methylnicotinamide (N-Me-NA). Shifts are in cps relative to a capillary of enriched $CH_3CO_2H-^{13}C$.

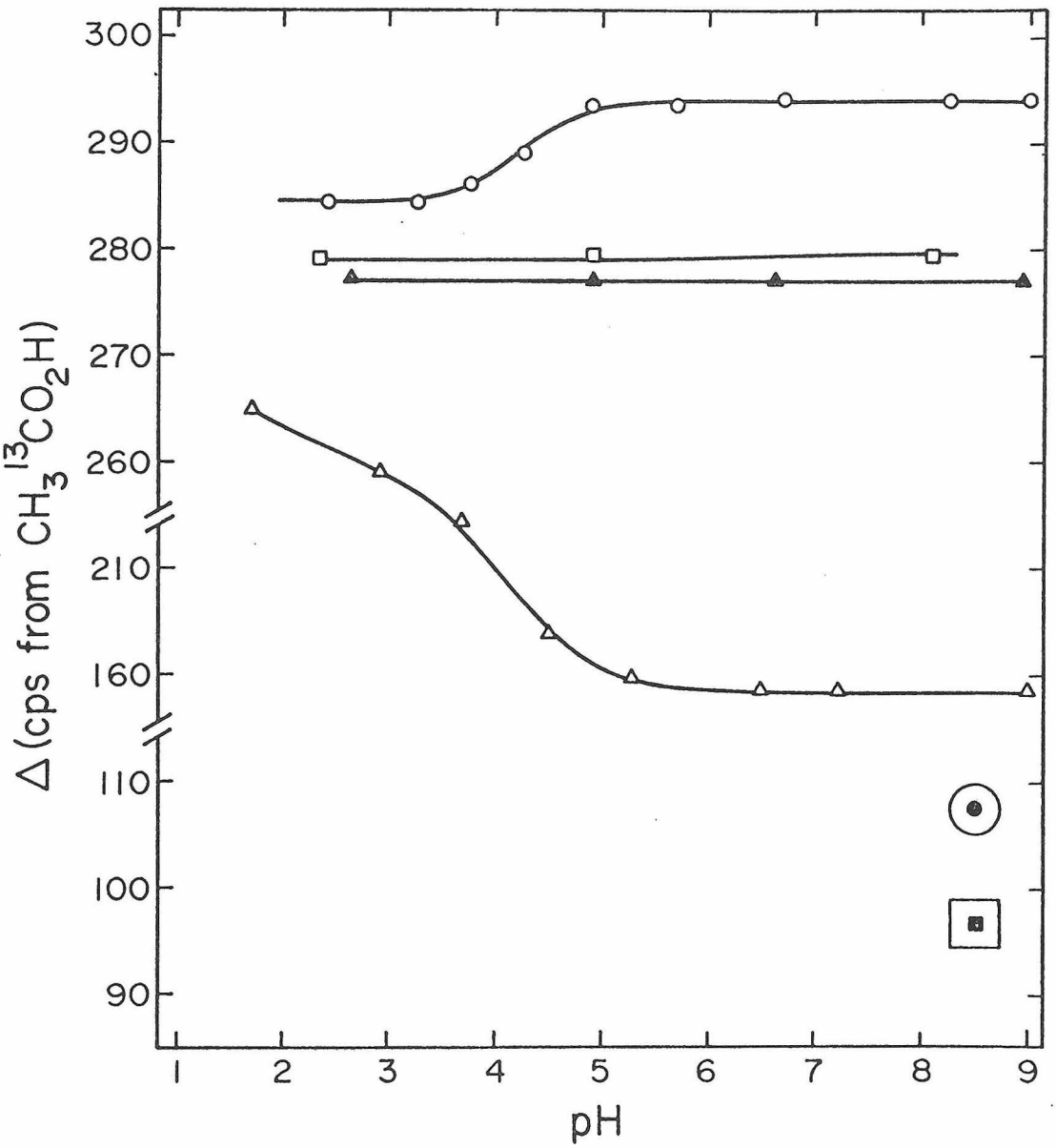

300
290
280
270
260
210
160
110
100
90
1
2
3
4
5
6
7
8
9
pH
Δ (cps from $CH_3{}^{13}CO_2H$)

FIGURE 5. Potentiometric titration of NMN, $NMNH_2$, and AMP. Titrations performed on 0.01 M solutions at 25°.

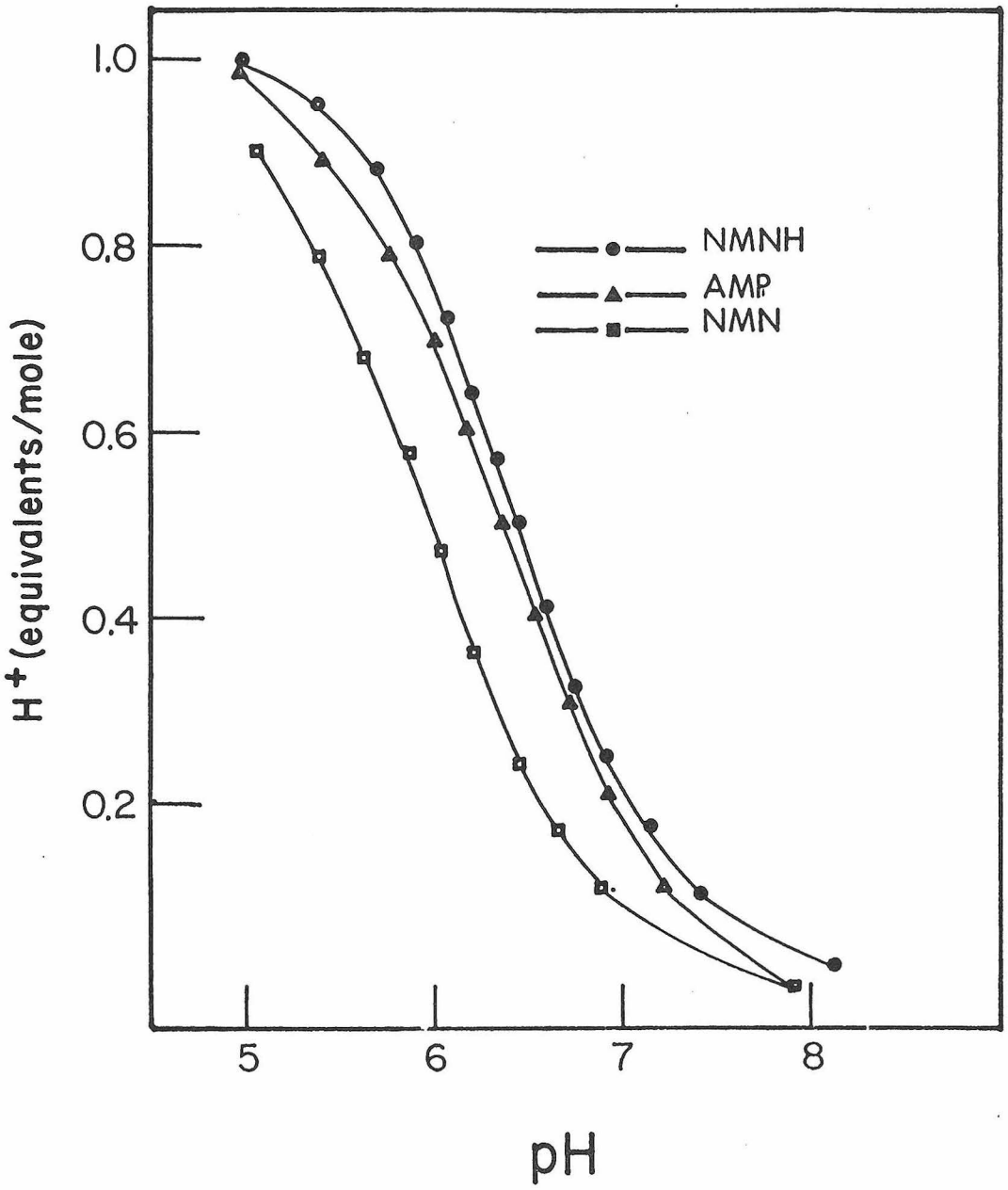

1.0
0.8
0.6
0.4
0.2
H+ (equivalents/mole)
NMNH
AMP
NMN
5
6
7
8
pH

FIGURE 6. Natural abundance, proton noise decoupled ^{13}C spectra of 0.1 M NAD^+ (lower) and NADH (upper). Accumulation time for each spectrum was two hours. A and N refer to the adenine and nicotinamide (or dihydronicotinamide) rings, respectively. In each spectrum, the carbonyl resonance is folded in from the left side, while in the NADH spectrum the N-4 peak is folded in from the right side. In each spectrum, the peak marked with a cross at 1800 cps is probably due to the methyl group of acetone, which is an impurity in the nucleotide samples. This peak is folded in from the right side.

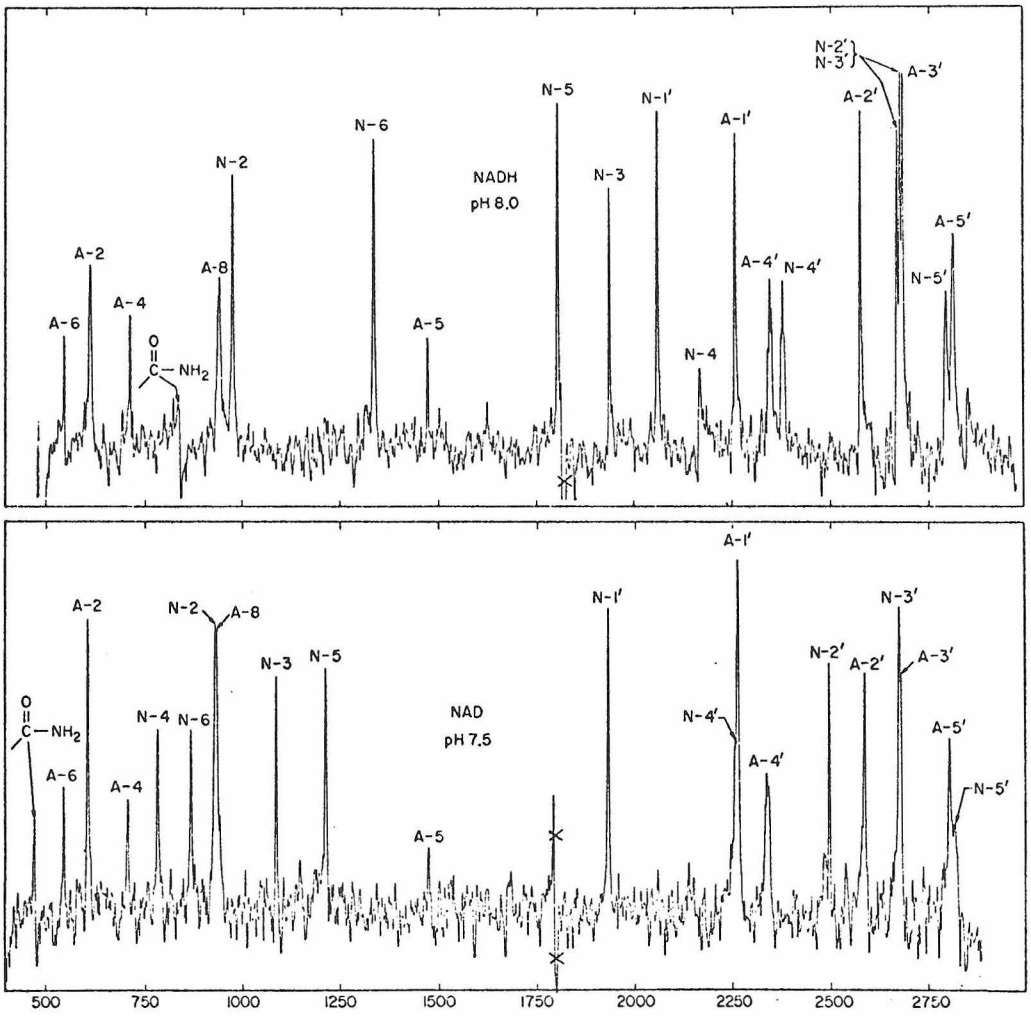
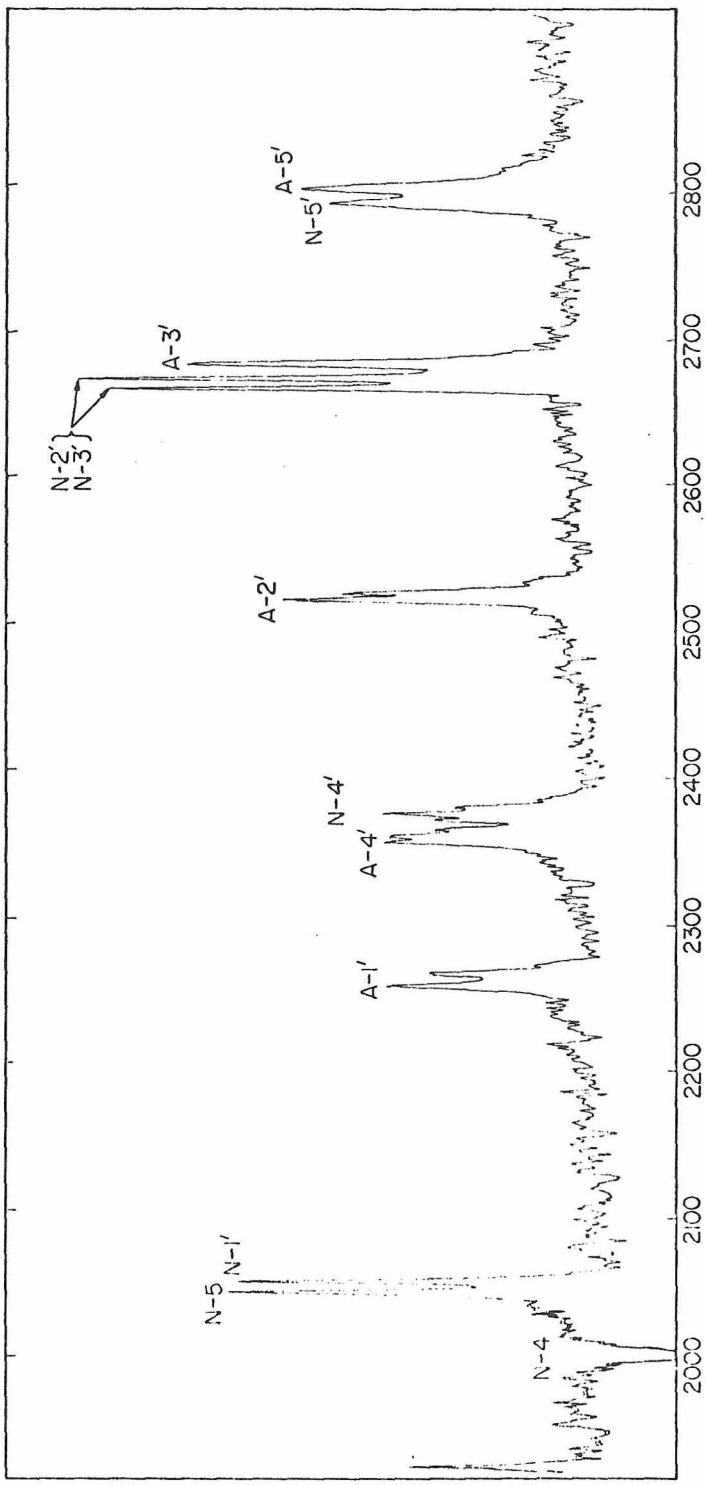

NADH
pH 8.0
A-6
A-2
A-4
A-8
N-2
N-6
A-5
N-5
N-3
N-1′
A-1′
N-4
A-4′
N-4′
N-2′
N-3′
A-2′
A-3′
N-5′
A-5′
NAD
pH 7.5
500
750
1000
1250
1500
1750
2000
2250
2500
2750
cps from $CH_3\ ^{13}CO_2H$

FIGURE 7. Ribose region of the ^{13}C spectrum of NADPH. The N-4 peak is folded in from the right side of the spectrum, while the N-5 peak (which is completely in phase) is folded in from the left side.


N-5
N-1'
N-4
A-1'
N-4'
A-4'
A-2'
N-2'
N-3'
A-3'
N-5'
A-5'
2000
2100
2200
2300
2400
2500
2600
2700
2800
cps from $CH_3\,{}^{13}CO_2H$

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CHAPTER II: ^{19}F Nuclear Magnetic Resonance Studies of Rabbit Muscle Glyceraldehyde-3-Phosphate Dehydrogenase Covalently Labeled with a Trifluoromethyl Group

INTRODUCTION

Glyceraldehyde-3-phosphate dehydrogenase (D-dlyceraldehyde-3-phosphate: nicotinamide adenine dinucleotide oxidoreductase (phosphorylating), EC 1.2.1.12) is an important enzyme in the glycolysis cycle, catalyzing the conversion of glyceraldehyde-3-phosphate to 1,3-diphosphoglyceric acid. In vitro, the enzyme has been found to catalyze several other reactions (Colowick et al., 1966), including the hydrolyses of acetyl phosphate and p-nitrophenyl acetate. These reactions have been useful in studying the properties and mechanism of the enzyme.

The enzyme was first isolated from yeast (Warburg and Christian, 1939). It has since been isolated from other sources, with the most work being done on the enzyme from yeast, rabbit muscle, and lobster. Though the amino acid composition varies slightly in GPD* isolated from different sources (Allison and Kaplan, 1964), most properties of the enzyme are the same, regardless of source.

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*Abbreviation for Chapter II: GPD, glyceraldehyde-3-phosphate dehydrogenase; F_3-GPD, the 3,3,3-trifluoroacetonyl-cysteine-148 derivative of GPD; DTNB, 5,5′-dithiobis(2-nitro-benzoic acid). All other abbreviations are as described in Chapter I.

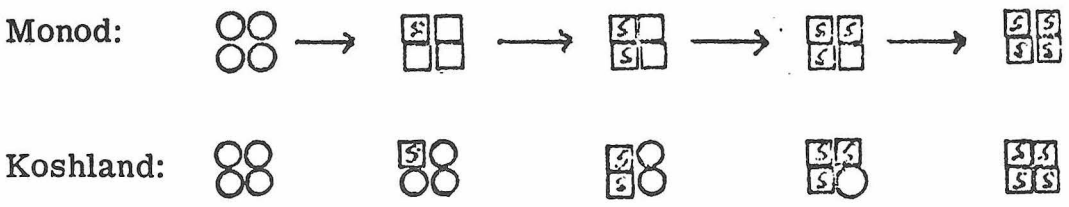
The enzyme has a molecular weight of 144, 000 and is tetrameric. The complete primary sequence of the lobster enzyme has been determined (Harris and Kaplan, 1965), and it was found that the four chains had an identical primary sequences. Low-resolution x-ray analysis has shown (Watson and Banaszak, 1964) the enzyme to possess at least twofold symmetry, and the possibility of the enzyme's possessing fourfold symmetry was not ruled out. The active site has been located by performing the hydrolysis of labeled p-nitrophenyl acetate, precipitating the resulting acetyl-enzyme complexes, and then performing suitable proteolytic digestion to find where the label was attached to the enzyme (Park et al., 1961). It would found that a thioester bond was formed between a cysteine and the substrate. The same cysteine was found to react rapidly with iodoacetate (Harris et al., 1964), leading to the formation of a thioether, and causing inactivation of the enzyme.

GPD can bind one molecule of its coenzyme, NAD, per subunit (Conway and Koshland, 1968). The binding is noncovalent, as indicated by the fact that the coenzyme can be removed by treatment with charcoal (Fox and Dandliker, 1956). An interesting feature of the binding of NAD^+ to GPD is the presence of negative cooperativity (Conway and Koshland, 1968), i. e., as each molecule of NAD^+ binds to the enzyme, the binding of the next molecule becomes less favorable. Thus, while the dissociation constant of the $E\text{-}(NAD)_1$ complex is approximately 10^{-11} M, the dissociation constant for the reaction

$$E\text{-}(NAD)_4 \rightleftharpoons E\text{-}(NAD)_3 + NAD$$

is approximately 10^{-5} M. While positive cooperativity has been an often observed phenomenon, this was the first reported occurrence of negative cooperativity. The latter type of cooperativity has since been observed in phosphoenolpyruvate carboxylase (Corwin and Kaplan, 1964) and cytidine triphosphate synthetase (Levitski and Koshland, 1969), and it has been suggested (Levitski and Koshland, 1969) that it is a general phenomenon.

The finding of negative cooperativity casts much light on the mechanism of allostery, the phenomenon whereby the catalytic function of enzymes is affected by small molecules which interact with the protein at a location other than the active site. There are two main theories as to the mechanism of allosteric transitions, the first due to Monod (Monod et al., 1965), and the second to Koshland (Koshland et al., 1966). The two mechanisms may be summarized pictorially as follows:



□ : conformation favoring binding of ligand(S)

○ : conformation not favoring binding of S

In both models, each subunit of the enzyme can exist in two states, one of which favors substrate binding, and one of which does not. In the Monod model all subunits are in the same state at all times (conservation of symmetry), while in the Koshland model, sequential changes in subunit conformation occur. When only one kind of ligand is involved in the allosteric transitions (homotropic case), the Monod model predicts only positive cooperativity, while the Koshland model predicts that either positive or negative effects are possible. Since the interaction of GPD with NAD belongs to the homotropic case, the finding of negative cooperativity is evidence for the Koshland model.

In order to study the various conformational transitions which GPD undergoes, the enzyme was reacted with 3-bromo-1, 1, 1-trifluoroacetase, a reagent which has previously been employed in this laboratory for use in ^{19}F NMR studies on hemoglobin (Heustis, and Raftery, 1972). The reagent was found to react specifically with cysteine 93 of the β chain of hemoglobin, and the ^{19}F chemical shift of the trifluoromethyl group was very sensitive to changes in pH and/or ligation, as well as further chemical modification of the protein.

Since, as mentioned previously, GPD possesses one cysteine per chain which is especially reactive to iodoacetate, it was hoped that this residue could be specifically labeled with trifluorobromo-acetone, and this proved to be true. Also, the chemical shift of the trifluoromethyl group proved sensitive to change in coenzyme binding

and pH, so that information concerning the structure of the protein in solution could be obtained.

PART A

Experimental

All nucleotides used in this study were purchased from Sigma, with the exception of APADH, which was purchased from P-L Biochemical.

Preparation of GPD. Rabbit muscle GPD was prepared and assayed according to the method of Bloch, MacQuarrie and Bernhard (1971). The enzyme which eluted from the carboxymethyl cellulose column had an A_{280}/A_{260} ratio of 1.3-1.5. The enzyme solution was saturated with NAD^+, the pH adjusted to 6.5-7.0, and the solution ultrafiltered through either a PM-10 or PM-30 membrane until the enzyme concentration was 50-70 mg/ml. The solution was then stored at 4°C until it was to be used.

Modification of GPD. To the enzyme solution (50-70 mg/ml) was added trifluorobromoacetone in a ratio of six moles of reagent per mole of tetramer (i.e., 1.5 moles per mole of monomer). The solution was stirred in the cold for 15 minutes, and then dialyzed at 4° for several hours against 0.1 M KCl, 5 mM EDTA pH 7. Immediately before use, the bound coenzyme was removed from the modified protein by charcoal treatment (Fox and Dandliker, 1956). Enzyme concentrations were determined by using the extinction coefficient for the unmodified enzyme (Bloch et al., 1971).

Nucleotides were added as either weighed solids, or from solutions whose concentrations were determined from known extinction coefficients.

Changes in pH were effected by adding either HCl or NaOH to the enzyme solution, with rapid stirring. The pH of the solutions were determined with a Radiometer 26 pH meter, and were checked both before and after recording of the spectra.

The sulfhydryl content of the enzyme was done according to the procedure described for lobster GPD (Wassarman and Major, 1969).

NMR spectra were recorded at 94.1 mc using an XL-100-15D NMR spectrometer operating in the Fourier transform mode. The spectrometer was locked to the H_2O signal, which also served as internal standard. A spectral width of 5000 cps was employed, and the position of the pulse was occasionally changed, to make sure that the observed positions of the peaks were correct, i.e., they were not "folded back" into the spectrum from outside the observed range.

In spectra in which peak intensities were compared, the acquisition time was at least three times the spin-lattice relaxation time of all peaks in the spectrum (see Part B for determination of relaxation times).

Results

The reaction of GPD with trifluorobromoacetone was found to cause greater than 98% inactivation of the enzyme. Sulfhydryl analyses with DTNB showed that the modified enzyme contained 4 ± 0.5 less SH groups than did the native enzyme, which possessed the expected 16 ± 0.5 SH groups per tetramer.

Some representative spectra of F_3-GPD both in the presence and absence of added coenzymes, are shown in Figure 1. It is seen that the spectrum of apo-F_3-GPD consists of one peak, about 50 cps wide, which falls 485 cps upfield of the position of the trifluoroacetic acid resonance.

The addition of NADH to the solution leads to a new peak, about 600 cps downfield of the peak originally present (Figure 1). As more NADH is added, the downfield peak increases in intensity and the apo-enzyme peak gets smaller, and finally disappears. No peaks having intermediate positions are observed, and neither peak undergoes significant broadening. While the peak which grows with added NADH always has the same position, independent of its intensity, the same is not true for the original peak. This peak keeps its original position as its relative intensity decreases from 100% to 50%, but then starts to move upfield with added NADH. At the smallest intensity at which the shift can be accurately measured ($\sim 10\%$), it has undergone an upfield shift of about 25 cps.

In contrast to the large shift induced by the binding of NADH, the binding of NAD at pH 7 causes a small shift of 30 cps in the upfield direction (Figure 1). Due to the small shift relative to the linewidth it is very difficult to tell whether or not the original peak gradually moves (fast exchange) or a new peak slowly grows in intensity (slow exchange). A binding study of NAD^+ to F_3-GPD shows somewhat greater linewidth at intermediate saturation values, indicative of either slow, or at least intermediate, exchange. At high pH, where the shift difference between F_3-GPD and F_3-GPD with added NAD^+ is about 120 cps (see below), the binding of NAD^+ to F_3-GPD clearly displays slow exchange.

A complete binding study of NADH to F_3-GPD in the presence and absence of NAD^+ is shown in Figure 2. As can be seen there is a slight lag in the growth of the downfield peak, but after about 20% saturation, the relative intensity of this peak is equal to the ratio of NADH added/subunit concentration until nearly total saturation. In the presence of a twofold excess of NAD^+, the NADH effect shows the same characteristics as with the apo-enzyme though the growth of the downfield peak is slightly inhibited (~10%). As is expected, if one first saturates the enzyme with NADH it takes a very large excess of NAD^+ to significantly reduce the intensity of the downfield peak. From these data, one can estimate the dissociation constant of the F_3-GPD-NADH complex to be 20 μm, while that of the F_3-GPD-NAD^+ complex is 20-50 times larger. These values may be compared with the results obtained from kinetic data (Smith and Velick, 1972) for

the acyl-enzyme complex, which were a dissociation constant of 10 μm for acyl-GPD-NADH, and about 1000 μm for acyl GPD-NAD^+.

In order to compare NADH binding to modified enzyme with binding to native enzyme, equal amounts of native and modified enzyme were mixed, and an ^{19}F NMR NADH saturation experiment performed. The results were virtually identical with those shown in Figure 2, indicating that modification of the enzyme has a small effect on NADH binding. This is again in agreement with kinetic results (Smith and Velick, 1972).

Some spectra of the enzyme at different pH's both in the presence and absence of NAD^+ and NADH are shown in Figure 3. It is observed that with both the apo-enzyme and the enzyme with NAD^+ added, there is a new peak at pH, ~1000 cps downfield of the original peak, while with NADH, there is a new peak about 75 cps downfield of the original peak. With apo-F_3-GPD, as well as when NAD^+ is added, the downfield field is sharper than the original peak, its width being 25-30 cps. The relative intensities of these peaks vs. pH is shown in Figure 4. Rather surprisingly, in all cases, the new peaks only reach a total relative intensity of about 50%. Also the pK values of the transition differ among F_3-GPD, F_3-GPD-NAD^+ and F_3-GPD-NADH.

The pH dependence of F_3-GPD and F_3-GPD-NAD^+ not only displays a different pK value but also somewhat differing chemical shift characteristics. Thus at high pH, with the apo-enzyme, the original (upfield) peak moves downfield by 70 cps, while with NAD,

this peak moves upfield by about 30 cps (Figure 3). Furthermore, the downfield peak is 100 cps further downfield with the apo-enzyme than with F_3-GPD-NAD^+. Due to the 120 cps shift differences which exist between F_3-GPD and F_3-GPD-NAD^+ at high pH, it is possible to determine whether the NAD^+ induced shifts exhibit slow or fast exchange. At partial saturation four peaks are observed, thus showing that slow exchange is present.

The binding of a number of coenzyme analogues was observed and the pH dependence studied. The analogues generally showed either no effect (the spectral characteristics were very similar to those of the apo-enzyme) or showed binding and pH effects similar to NAD^+ or NADH. The binding and pH behavior of pyridine-3-aldehyde adenine dinucleotide was different from that of any other compounds used. The results are summarized in Table I.

It is noticed that the active oxidized analogues exhibit the same behavior as NAD^+ (pK about 7.5 and high pH peaks 100 cps upfield of corresponding peaks in apo-enzyme), while the active reduced analogues mimic NADH (600 cps shift on binding). Actually, due to fairly weak binding between the analogues and F_3-GPD, the spectra generally contained peaks due to apo-enzyme as well as enzyme which contained bound nucleotide. In all cases, slow exchange among all species present in the solution was observed.

The components of NAD^+ and NADH do not exhibit any effects on the ^{19}F spectra, nor do the related coenzymes $NADP^+$ and NADPH. Some spectra obtained in the presence of pyridine-3-aldehyde adenine

dinucleotide are shown in Figure 5. A new peak, 500 cps downfield of the peak due to apo-F_3-GPD is seen, but even with a large excess of nucleotide, this peak only reaches a total intensity of 50%. Also, the remaining upfield peak moves further upfield by 50 cps. At high pH, the situation becomes much more complex. Two new peaks are seen, one 300 cps downfield of the apo-enzyme peak and the other 1700 cps downfield. The peak which was seen 500 cps downfield has disappeared. Also peaks are seen in the positions where high pH apo-enzyme peaks appear.

It has been shown (Wassarman and Major, 1969) that after the active site cysteine of GPD has been carboxymethylated, reaction of the native protein with DTNB causes modification of one additional cysteine residue per polypeptide chain. This DTNB reaction was carried out on F_3-GPD and indeed 1.0-1.5 cysteines per chain were modified. Several ^{19}F spectra of this doubly modified enzyme are shown in Figures 6 and 7. At around pH 7 the spectra of the apo-enzyme and the enzyme with NAD^+ or NADH are similar to spectra obtained without DTNB modification. However, with apo-F_3-GPD or F_3-GPD-NAD^+ as the pH is raised, a new peak, about 1700 cps downfield, is observed. In apo-F_3-GPD, this peak is 50 cps further downfield than in F_3-GPD-NAD^+. Also, the upfield peak totally disappears as the pH is raised further. The pK_a for the transition in both apo-F_3-GPD and F_3-GPD-NAD^+ is about 7.5.

As the pH is raised with doubly modified GPD-NADH, the center of the observed peak moves downfield. Due to poor spectral quality, caused by decomposition following the DTNB reaction, it is difficult to tell whether the observed spectra show the same characteristics as before DTNB modification. It is clear that reaction of one addition SH group per chain does not have as large an effect on the spectra observed in the presence of NADH as on spectra of F_3-GPD or F_3-GPD-NAD^+.

Discussion

In order to serve as a useful probe of enzyme structure a modifying reagent should react specifically at a known site on the enzyme, and should not affect the structure of the enzyme.

There have been many studies done involving the reaction of α-halo-carbonyl compounds with GPD (Harris et al., 1963; Moore and Fenselau, 1972; Kirtley and Koshland, 1970) and in all cases, it has been found that when the reagent is added to the enzyme in a small excess, reaction occurs specifically at cysteine 148, the active site residue. When GPD was reacted with trifluorobromoacetone in 50% excess, a total of four SH groups per tetramer were found to react, and all enzymatic activity was lost. These two facts, together with the large body of data for similar compounds,lead to the conclusion that trifluorobromoacetone reacts specifically with cys 148 of GPD.

A good indication of whether the enzyme's structure has been perturbed is a comparison of coenzyme binding in native and modified enzyme. The dissociation constant for NADH from the modified enzyme, 10^{-5} M^{-1}, is very similar to that bound for the native enzyme. The dissociation constant of the GPD-TFA-NAD^+ complex was found to be about 10^{-3} M^{-1}, which is several orders of magnitude lower than that observed with the native enzyme (Conway and Koshland, 1968). However, kinetic studies (Smith and Velick, 1972) have indicated that the dissociation constant of NAD^+ from acyl-GPD (the covalent enzyme-substrate complexes formed from GPD and glyceraldehyde-3-phosphate) is also about 10^{-3} M^{-1}. Thus, it would appear that F_3-GPD- possesses affinities for coenzymes almost exactly identical to those of the active enzyme substrate complex, and can thus yield structural information concerning this complex. Other workers (Kirtley and Koshland, 1970) have modified GPD with a spectrophotometric probe, 2-bromoacetamide-4-nitrophenol, and have also concluded that the modified enzyme resembled the enzyme-substrate complex. A recent report (Bloch et al., 1971) has indicated that, in vivo, the enzyme exists in the acylated state, so studies on its structure take on even greater significance.

NADH Binding. The binding of NADH leads to a new peak in the ^{19}F spectrum of F_3-GPD. This peak is in a slow exchange condition relative to the peak due to apo-enzyme, i.e., the exchange rate between the species giving rise to the two peaks is much smaller than the chemical shift difference between them. For the slow

exchange condition, the following equation holds (Carrington and McLachlan, 1967):

$$\frac{1}{\pi T_{2E}} - \frac{1}{\pi T_2} = \frac{P}{\pi}$$

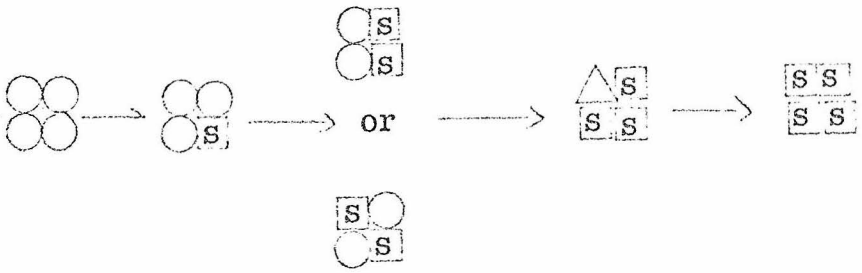
where $\frac{1}{\pi T_{2E}}$ is the observed linewidth in the presence of exchange, $\frac{1}{\pi T_2}$ is the observed linewidth in the absence of exchange, and P is the jumping rate between species. Since examination of spectra due to F_3-GPD partially saturated with NADH reveals no line broadening in either peak relative to spectra obtained at either 0% or 100% saturation, one can say that the exchange rate between NADH free and NADH bound forms is less than π times the experimental error in linewidth determination, or about 15 sec^{-1}. With other reduced coenzymes (see below) which bind to GPD much more weakly than do NADH and would therefore be expected to have greater off rates, the same lack of exchange broadening is observed. This exceedingly slow rate of exchange indicates that the peak which is observed on NADH binding is probably due to a conformation change of the enzyme, since if it were a direct effect of NADH on the trifluoromethyl group the exchange rate between states would be expected to be 10^2 sec^{-1} or greater. (The off rate of NADH from yeast GPD has been determined to be 1900 sec^{-1} (von Ellenrieder et al., 1972).) The existence of such a conformation change has previously been postulated on the basis of kinetic data (Smith and Velick, 1972).

An interesting feature of the binding of NADH to F_3-GPD is the observed lag, which is present both in the absence and presence of NAD^+. A possible artifactual explanation for this lag is that a small portion of GPD was unmodified, and that this species binds NADH more strongly than the modified enzyme. This possibility was eliminated by the observation that when the NADH binding experiment was performed on a 50-50 mixture of GPD and F_3-GPD-NADH binding was unaffected. Stronger binding to GPD would of course have lead to a greater observed lag in binding to F_3-GPD. This experiment also showed that NADH binding to unmodified GPD displays an initial lag.

The most likely explanation for the observed lag is the presence of positive cooperativity in the binding of NADH to both native and modified GPD. Positive cooperativity has been reported by one group (Amiguet and Luisi, 1972) to occur in the binding of NADH to yeast GPD, though other workers (von Ellenrieder et al., 1972) indicate that the binding is noncooperative. Detailed studies of the binding of NADH to rabbit muscle GPD have not been performed.

The fact that the amount of conformation change induced by addition of NADH between 20% and 80% saturation is proportional to the amount of NADH added is consistent with the KNF (Koshland et al., 1966) model of sequential changes in quaternary structure occurring upon addition of ligand. If the MWC model (Monod et al., 1965) were to be applicable in this case, one would expect the observed conformation change to either lag or lead the binding throughout most of the saturation curve.

The small shift of the upfield peak with added NADH is indicative of inter-subunit interaction; i. e., a change in the quaternary structure of the enzyme with added NADH. This change appears to take place when a third molecule of NADH is added to the tetramer. It should be pointed out that any quaternary change which occurs upon binding of the fourth molecule of NADH obviously cannot be detected in this manner, since the upfield peak is not present in the species containing four NADH per tetramer. The simple KNF model, wherein conformation changes only occur in subunits which have bound ligand, cannot account for this shift in the apo-enzyme peak so one must postulate a more complex form of the KNF model which can account for such changes. The binding of NADH to F_3-GPD may be summarized pictorially as follows:



The above scheme is still somewhat oversimplified, for it treats all four subunits as being inherently equivalent, while as shown below, they are not.

pH Effects. The pH studies give strong evidence for the non-equivalence of the subunits of F_3-GPD. At high pH, in apo-F_3-GPD,

as well as F_3-GPD-NAD^+ and F_3-GPD-NADH, there are two peaks of roughly equal intensity. Thus, in spite of the identical sequence of the GPD subunits, it would appear that the tetrameric association induces a nonequivalence and that the enzyme therefore has an $\alpha_2 \alpha_2'$ structure. This nonequivalence has previously been postulated (Malhotra et al., 1968; MacQuarrie and Bernhard, 1971) from experiments which showed that two subunits were much more reactive toward acylation than the other two subunits. The ^{19}F experiments show that the non-equivalence is present in the apo-enzyme, and is not induced by addition of coenzyme.

It is again of interest to look for any possible line broadening so as to determine the rate at which the pH dependent transitions occur. As was the case with the shift induced by NADH, virtually no line broadening is observed. Since protonation rates for residues such as cysteine, lysine, and histidine, which are most likely to be titrated in the pH range 7-9 are all 10^3 sec^{-1} or greater, it again appears that the observed shifts are due to conformational changes occurring in the enzyme.

A study of the binding or pH induced spectral changes caused by various coenzyme analogues has yielded very interesting results. It is thus observed that the changes observed in the presence of NAD^+ are also observed in the presence of active analogues of NAD^+, and the changes observed with NADH are seen with the active analogues of NAD^+, and the changes observed with NADH are seen with the active analogues of NADH. No changes, however, are observed with

the components of these coenzymes such as AMP, NMN^+, NMNH, and ADPR. These components have been shown to be inhibitors of GPD (Eby and Kirtley, 1971), and furthermore it has been shown (Kirtley and Koshland, 1970) that AMP and ADPR cause difference spectra when added to GPD which has had cys 148 modified with 2-bromoacetamido-4-nitrophenol. Thus, they almost surely bind to F_3-GPD, but apparently do not cause conformation changes to occur. It seems likely that the difference spectra obtained with AMP and ADPR in the above mentioned study (Kirtley and Koshland, 1970) were due to a direct interaction of the nucleotides with the spectrophotometric probe, rather than significant enzyme-nucleotide interactions.

Modification of the nicotinamide or adenine rings of NAD^+ or NADH leads to decreased catalytic activity, as well as decreased binding to GPD (Eby and Kirtley, 1971). Also, the modified coenzymes bind much less strongly to F_3-GPD, as evidenced by the fact that fourfold excesses of coenzyme were in some cases not sufficient to saturate the enzyme. In spite of the above, the conformation changes induced in GPD by thionicotinamide adenine dinucleotide, 3-acetylpyridine adenine dinucleotide, and nicotinamide deaminoadenine dinucleotide were identical to those caused by NAD^+, and the changes caused by the corresponding reduced coenzymes were identical to those caused by NADH. It can thus be concluded that the conformational changes observed with NAD^+ and NADH are very specific and essential to the catalytic mechanism of the enzyme.

The binding of pyridine-3-aldehyde adenine dinucleotide to F_3-GPD causes effects differing from those of any other compound tested. This compound, while it is an active coenzyme for most dehydrogenases, is totally inactive with GPD, and is in fact a very powerful inhibitor. The fact that its binding causes a new peak which reaches only 50% intensity is again indicative of the nonequivalence of the GPD subunits. It would appear that pyridine-3-aldehyde adenine dinucleotide does induce conformational changes in F_3-GPD which are different from those caused by NAD and its active analogues.

In an effort to determine the residue(s) responsible for the pH behavior of F_3-GPD, the enzyme was reacted with DTNB. The binding of NADH seemed largely unaffected by this treatment but the pH behavior of apo-F_3-GPD, F_3-GPD-NAD^+ was greatly affected. Of prime importance was the fact that the downfield peak which arose at high pH was shifted 700 cps further downfield, and that it reached an intensity of 100% on titration, rather than 50% as was observed with the unmodified F_3-GPD. It would thus appear that the nonequivalence of the subunits was destroyed by DTNB treatment. It is of course not clear whether this is due to the importance of the cysteines for this nonequivalence, or residues near the cysteine which are sterically affected by DTNB treatment. It seems likely, however, that since a pH dependent shift remains after DTNB treatment, there is another kind of residue, perhaps lysine or histidine, which is responsible for the observed pH dependent shifts.

It is interesting to note that both before and after DTNB treatment the behavior of F_3-GPD-NAD^+ is similar, though not identical, to that of apo-F_3-GPD, while the ^{19}F spectra of F_3-GPD in the presence of NADH display startling different characteristics. It would thus appear that the addition of NADH to F_3-GPD causes more extensive conformation changes than does the additio of NAD^+.

The chemical shift changes observed in this study have been considerably larger than those observed in any previous ^{19}F study of a macromolecule, and are over an order of magnitude larger than shift changes observed when the same reagent, trifluorobromo-acetone, was used to monitor conformation changes in hemoglobin (Huestis and Raftery, 1972). The various factors which can cause chemical shifts in studies such as this have been delineated (Millett and Raftery, 1972) and are four in number: a) ring current effects due to aromatic groups; b) van der Waals interactions with neighboring groups; c) effects due to static electric fields arising from charged groups; and d) specific bonding interactions with neighboring residues. It has been calculated (Giessner-Prettre and Pullman) that the first of these interactions can only lead to shifts of 100-200 cps. Also, one would not expect charge interactions to have a large effect on the trifluoromethyl group of the $S-CH_2-\overset{O}{\overset{\|}{C}}-CF_3$ moiety, since it is uncharged. Thus, while both of these effects may influence the observed shifts, and could indeed dominate the small shifts, it is unlikely that they would contribute substantially to the observed shifts of 600, 1000, and 1700 cps. It is thus likely the

effects giving rise to the large shifts are due to hydrophobic interactions and/or bonding interactions. Among the latter, hydrogen bonding to the carbonyl adjacent to the trifluoromethyl group is a possible explanation of the observed shifts.

PART B

Experimental

Values for the spin-lattice relaxation time (T_1) were determined by the progressive saturation method (Freeman and Hill, 1971). Spin-spin relaxation times (T_2) were determined from the observed linewidth (corrected for field inhomogeneity and artificial peak broadening which is introduced to improve sensitivity) according to the equation:

$$\nu_{1/2} = \frac{1}{\pi T_2},$$

in which $\nu_{1/2}$ is the full width at half height.

Enzyme solutions in D_2O were prepared by dialysis.

All other procedures were as described in Part A.

Results

Spectra obtained in a ^{19}F T_1 experiment in F_3-GPD saturated with NAD^+ in H_2O at pH 8.5 are shown in Figure 8. These data (together with data from a similar experiment run in D_2O) are graphically displayed in Figure 9. It is observed that in both H_2O and D_2O, the downfield peak has a T_1 value which is about 60% longer than that of the upfield peak. Furthermore, the T_1 values for both peaks obtained in D_2O are about 60% longer than those in H_2O. A complete summary of T_1 values at different pH's for apo-F_3-GPD, as well as

enzyme saturated with NAD^+ or NADH is shown in Table II. No value for apo-enzyme at high pH could be obtained due to rapid decomposition of the enzyme.

Linewidths of 45 ± 3 cps, indicating T_2 values of 6.5-7.5 msec were observed for all peaks, except the downfield peaks present at high pH with apo-F_3-GPD or enzyme with NAD^+ added. These peaks displayed linewidths of 25 ± 3 cps, or T_2 values of 11-14 msec. T_2 values in D_2O were determined at high pH for resonances arising from enzyme saturated with NAD^+, and were identical to those observed in H_2O.

Discussion

The fact that T_1 is much longer than T_2 for all species observed in this study indicates that τ_c, the correlation time for the trifluoromethyl group, is considerably longer than $\frac{1}{\omega_0}$ (Carrington and McLachlan, 1967), where ω_0 is the observing frequency (in radians) for the NMR experiment. In the experiments reported here, at 94 MHz, the value for $\frac{1}{\omega_0}$ is 1.7×10^{-9} sec. The inequality of T_1 and T_2 is expected, since the correlation time of GPD which can be calculated using the Debye equation

$$\tau_c = \frac{4}{3} \frac{\pi \eta a^3}{kT}$$

where η is the macroscopic viscosity and a is the radius of the protein (which is assumed to be spherical) is 8×10^{-8} sec, which is obviously much larger than $\frac{1}{\omega_0}$.

There are three major types of motion which will contribute to the correlation time of the CF_3 group attached to GPD. First, is the overall tumbling rate of the macromolecule. Second is any local mobility which the trifluoroacetonylcysteine residue may possess and finally there is the rotation of the CF_3 group about its own axis. It is important to note that the first two of these motions are isotropic, while the third is distinctly anisotropic.

In the present case, one may assume that the relaxation of the ^{19}F nucleus is dominated by dipolar contributions, since the values of T_1 and T_2 are too small to be greatly affected by spin rotation, which is indeed often the major relaxation mechanism of trifluoromethyl groups of small molecules in solution (Burke and Chan, 1970), and chemical shift anisotropy has been shown to have a negligible effect on the relaxation of CF_3 groups (Burke and Chan, 1970).

For dipolar relaxation between like nuclei, the relation between T_2 and the correlation time is as follows (Solomon, 1955):

$$\frac{1}{T_2} = \frac{3}{40}\frac{\gamma^4\hbar^2}{r^6}\left(6\tau_c + \frac{10\tau_c}{1+\omega_0{}^2\tau_c^2} + \frac{4\tau_c}{1+4\omega_0{}^2\tau_c^2}\right).$$

In the case of a CF_3 group, in which each fluorine nucleus is relaxed by two others, the right side of the equation is doubled. Also, when $\omega_0{}^2\tau_c^2 \gg 1$, the last two terms drop out, leaving

$$\frac{1}{T_2} = \frac{9}{10}\frac{\gamma^4\hbar^2}{r^6}\tau_c.$$

Using the value for τ_c obtained from the Debye equation, one can now calculate the expected linewidth of the CF_3 group, assuming that the only contribution to the dipolar relaxation of each fluorine atom is that caused by the other two fluorine atoms of the CF_3 group. The value which one obtains is 100 cps. Since the observed linewidths are up to four times narrower than the calculated linewidths, it is clear that the CF_3 groups possess greater mobility than does the protein as a whole.

One additional motion which the CF_3 group probably possesses is rotation about its own axis. The effect of this rotation is to decrease the efficiency (i. e., decrease the effective correlation time) of dipolar relaxation (Woessner, 1965). In the limiting case, where rotation of the methyl group is much faster than the overall tumbling time of the molecule to which it is attached, the dipolar contribution to T_2 will be reduced by a factor of four (Burke and Chan, 1970; McMurray et al., 1972; Navon and Lanir, 1972). It should be pointed out that since methyl rotation can effect T_2 by at most a factor of four while the correlation time for methyl rotation may differ by several orders of magnitude from the overall correlation time of the macromolecule, one cannot deduce a correlation time for methyl rotation itself from studying T_2 values, as has recently been claimed (Nowak and Mildvan, 1972).

If one assumes rapid CF_3 rotation to be present, the observed linewidths are in good agreement with the calculated linewidths (it will be shown later that the linewidth of the 45 cps wide lines are

probably effected by other groups on the enzyme). The fact that the observed linewidths can be rationalized through considerations of only the overall tumbling of GPD, plus the rotation of the CF_3 group, indicates that the $-S-CH_2\overset{O}{\overset{\|}{C}}CF_3$ group possesses very little segmental motion and is thus probably located at the interior of the GPD molecule, in a fairly narrow cleft.

The internal rotation of the CF_3 group also affects the value of T_1, but very differently than it effects T_2. In the limiting case, where rotation is much faster than the overall tumbling rate, the value of T_1 will again be increased by a factor of four. However, due to the shape of the $\frac{1}{T_1}$ vs. τ_c curve, to reach this limiting case for T_1, when $\omega_0^2\tau_c^2 \gg 1$, the effective correlation time must be reduced by several orders of magnitude. Thus, in the present case, the effect of rotation of the trifluoromethyl group is to cause a large decrease in the value of T_1, as the value calculated from the expression

$$\frac{1}{T_1} = \frac{3}{10}\frac{\gamma^4\hbar^2}{r^6}\left(\frac{2\tau_c}{1+\omega_0^2\tau_c^2} + \frac{8\tau_c}{1+4\omega_0^2\tau_c^2}\right)$$

(obtained from doubling the right side of the usual (Solomon, 1955) expression for T_1) is about 5 sec, while the observed values (in H_2O) are 120-200 msec. As will be shown later, part of the lower value for T_1 is due to intermolecular effects rather than methyl rotation.

Since the T_1 and T_2 values for the CF_3 group remain unchanged (at low pH) upon addition of NAD^+ or NADH to the apo-enzyme (Table II), it would appear that the conformation changes induced by these

substances (Part A) do not affect the group's mobility. A previous study (Balthasar, 1971), employing nitroxide spin labels covalently attached to cys 148,has reported a mobility change of the spin label upon addition of NAD^+, but the spin labels employed were considerably larger than the ^{19}F probe used in this experiment and this fact could account for the discrepancies between the two experiments.

At high pH, there are two peaks in the ^{19}F spectrum of F_3-GPD (Part A). In the presence of NADH, both peaks display the same relaxation characteristics as are observed in the low pH position (Table II). Also, the high field peaks in the spectra of apo-F_3-GPD and F_3-GPD-NAD^+ are unchanged. However, the low field peaks display increased values of T_2, and in the case of F_3-GPD-NAD^+, an increased T_1 (this quantity could not be accurately measured in apo-F_3-GPD).

The most likely explanation for the increased values of T_1 and T_2 is a change in "intermolecular" effects (here "intermolecular" is used in the sense of an effect caused by atoms in a different part of the same enzyme molecules, as well as atoms on different molecules). Thus, in the high field peaks, there could be a contribution due to the juxtaposition of hydrogen atoms in one of the protein residues and the trifluoromethyl group. If this interaction were absent in the conformation which gives rise to the downfield peaks, both the T_1 and T_2 would decrease, as observed. The decreasing T_1 and T_2 can be explained neither by increasing rotation of the CF_3 group nor by increased segmental motion of the $S\text{-}CH_2\overset{O}{\overset{\|}{C}}CF_3$ moiety,

since the former would leave T_2 unchanged, while the latter, since $\tau_c \gg \frac{1}{\omega_0}$, would lead to a decrease in T_1.

A good indication that intermolecular interactions play an important role in the relaxation of the CF_3 group is obtained from the results in D_2O. The longer T_1 values in this solvent relative to H_2O can almost certainly be attributed to intermolecular interactions between the solvent protons and the CF_3 group. It is not likely that a conformational change is responsible for the different T_1 values because the chemical shifts in H_2O and D_2O are identical. This relaxation mechanism becomes unimportant in D_2O, due to the smaller magnetogyric ratio of the deuteron relative to the proton. As expected, the T_2 values are unaffected by going from H_2O to D_2O. This is because the correlation time for the solvent-protein interaction is equal to the correlation time for the water molecule. Since this correlation time is much less than $\frac{1}{\omega_0}$, $\frac{1}{T_{1\text{inter}}} = \frac{1}{T_{2\text{inter}}}$ (Carrington and McLachlan, 1967). However, since $\frac{1}{T_{2\text{intra}}} \gg \frac{1}{T_{1\text{intra}}}$, the total relaxation, $\frac{1}{T_{\text{inter}}} + \frac{1}{T_{\text{intra}}}$, is virtually unaffected in the case of T_2, though T_1 does change. The fact that solvent molecules can thus cause relaxation of the CF_3 group makes it not unlikely that other protons on the enzyme can also cause relaxation. Studies of T_1 values of protons in proteins (Navon and Lanir, 1972; Benz et al., 1972) have also shown interresidue effects to be of great importance.

The observation of a relaxation mechanism which affects T_1, but not T_2, points up the dangers of determining correlation times from the $\frac{T_1}{T_2}$ ratio, as has recently been done (Nowak and Mildvan, 1972), since this method is based on the assumption that both T_1 and T_2 are affected by the same relaxation effects. Also, internal rotation may cause a large error in such calculations, since as has been discussed above, this motion affects T_1 and T_2 differently and causes the effective correlation time for these relaxation to differ from one another.

Conclusions

Rabbit muscle GPD can be alkylated with bromotrifluoroacetone and the modified enzyme processes the same affinity for coenzymes as does the enzyme-substrate (acyl-enzyme) complex. Addition of NAD^+ or NADH to the modified enzyme induces slow ($<15\ sec^{-1}$) conformation changes. Reduced and oxidized coenzymes cause different conformation changes, while active coenzyme analogues cause the same changes as NAD^+ or NADH. Changes in pH also cause slow conformational transitions, and at high pH, there is evidence for the enzyme's existing as an $\alpha_2\alpha_2'$ protein.

The trifluoromethyl group attached to the protein is highly immobilized, and interacts with solvent molecules as well as with protons on other residues of the protein.

Table 1: Effects of Coenzyme Analogues on ^{19}F-GPD.

Similar to Apo-enzyme	Similar to addition of NAD^+	Similar to addition of NADH	Other effects
adenosine-5′-monophosphate	3-acetylpyridine adenine dinucleotide	reduced 3-acetylpyridine adenine dinucleotide	pyridine-3-aldehyde adenine dinucleotide
nicotinamide mononucleotide	nicotinamide deamino-adenine dinucleotide	reduced nicotinamide deaminoadenine dinucleotide	
reduced nicotinamide mono-nucleotide	3-thiolpyridine adenine dinucleotide		
adenosine monophosphate + nicotinamide mononucleotide			
adenosine monophosphate + reduced nicotinamide mono-nucleotide			
adenosine-5′-triphosphate			
adenosine-5′-diphosphoribose			
nicotinamide adenine dinucleo-tide phosphate			
reduced nicotinamide adenine dinucleotide phosphate			

Table 2: T_1 Values of the CF_3 Group Attached to GPD in the Presence and Absence of Coenzymes.

Coenzyme Present	pH	T_1 Values of High Field Peak (in sec.)	T_1 Values of Low Field Peak (in sec.)
None	6.8	0.13	
NAD^+	6.8	0.13	
NAD^+	8.5	0.13	0.22
NAD^+	8.5 (D_2O)	0.22	0.32
NADH	6.8	0.13	
NADH	8.5	0.12	0.12

The T_1 values listed above are averages of at least two runs and are reproducible to ± 0.02 sec. Protein concentrations were about 60 mg/ml. All solutions contained 0.1 M KCl and 0.005 M EDTA. The solvent was H_2O, except where the use of D_2O is indicated.

Figure 1: ^{19}F nmr spectra of F_3-GPD in the absence and presence of NADH and NAD^+. The numbers next to the peaks refer to chemical shifts, in cps, at 94.1 MHz, and are referenced to the average position of the peak from apo-F_3-GPD, which is arbitrarily set to 0. Positive numbers indicate downfield shifts, and negative numbers, upfield shifts. Shifts were reproducible to ± 5 cps. The accumulation time for each spectrum was about 20 minutes.

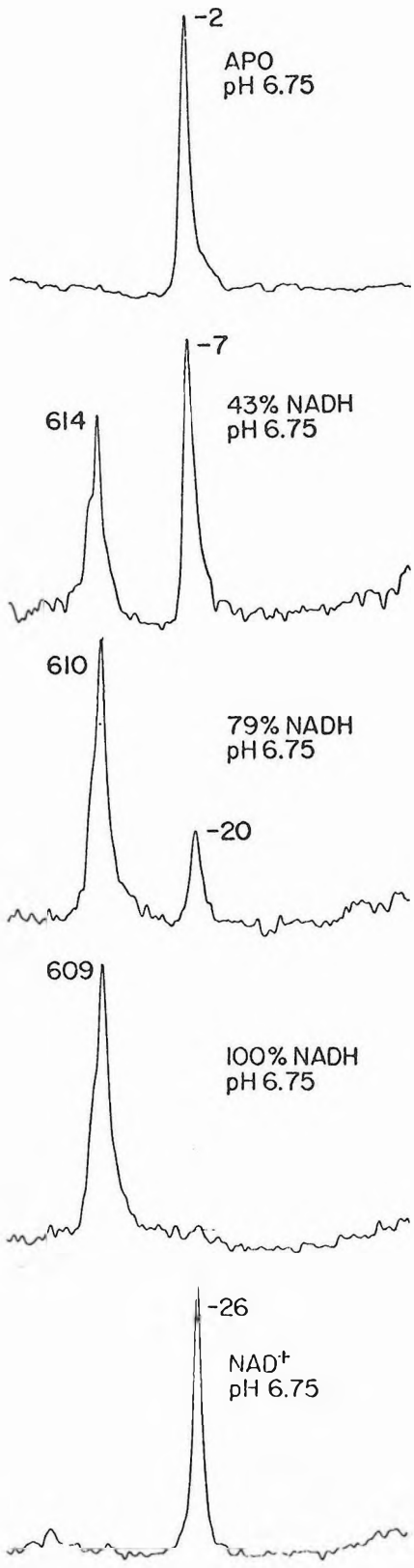

-2
APO
pH 6.75
-7
614
43% NADH
pH 6.75
610
79% NADH
pH 6.75
-20
609
100% NADH
pH 6.75
-26
NAD+
pH 6.75

Figure 2: Saturation curve of F_3-GPD with NADH in the absence and presence of NAD^+ obtained from ^{19}F nmr data. The downfield peak is observed upon binding of NADH (see Fig. 1 and text). The dashed line is a 45° angle line and represents what would be observed if all added NADH were bound to the enzyme. Note the initial lag, which was highly reproducible.

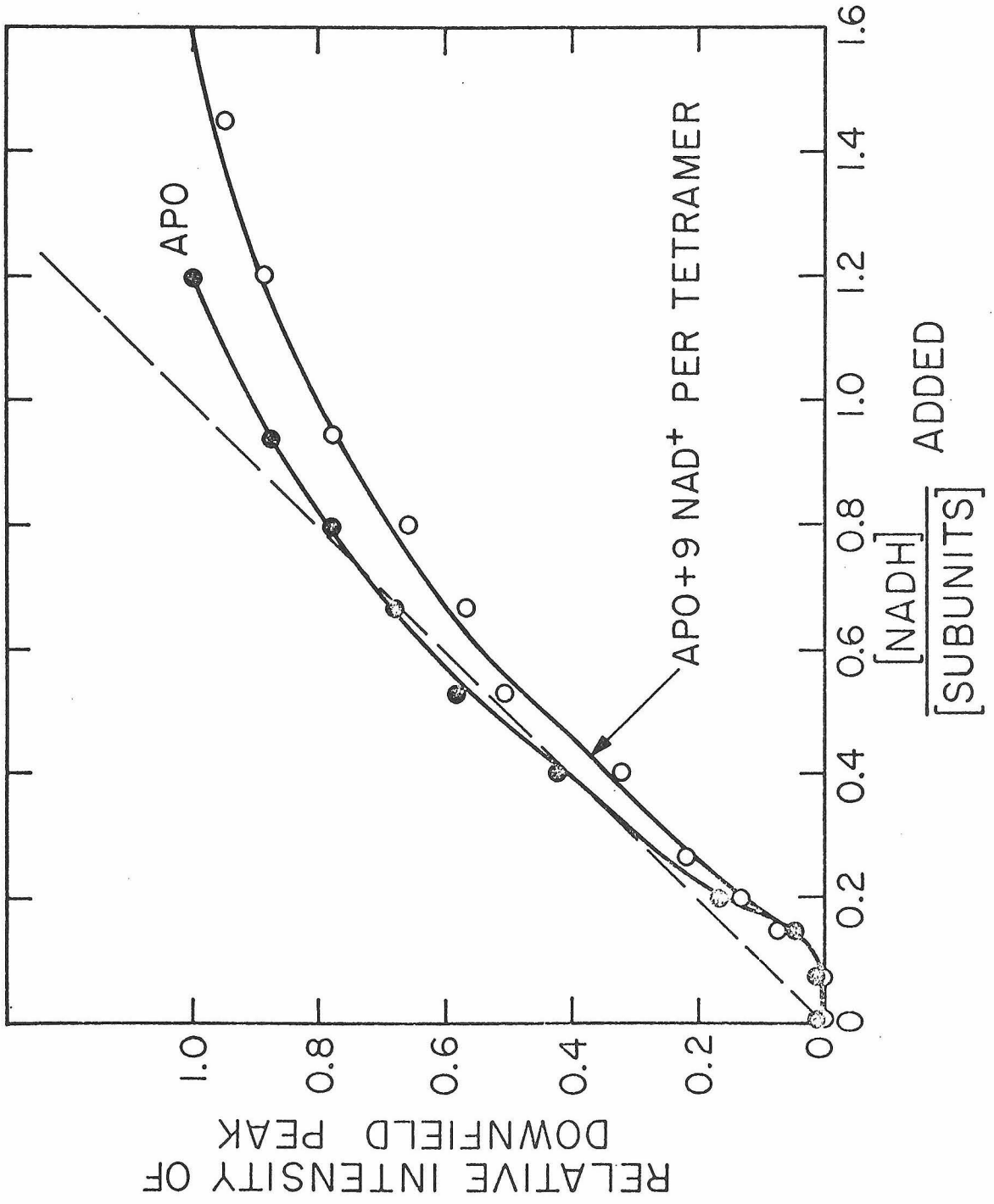

RELATIVE INTENSITY OF DOWNFIELD PEAK
1.0
0.8
0.6
0.4
0.2
0
0
0.2
0.4
0.6
0.8
1.0
1.2
1.4
1.6
[NADH] / [SUBUNITS] ADDED
APO
APO+9 NAD+ PER TETRAMER

Figure 3: ^{19}F nmr spectra of F_3-GPD, with and without added coenzyme, at various pH values. See Fig. 1 for meaning of numbers next to peaks.

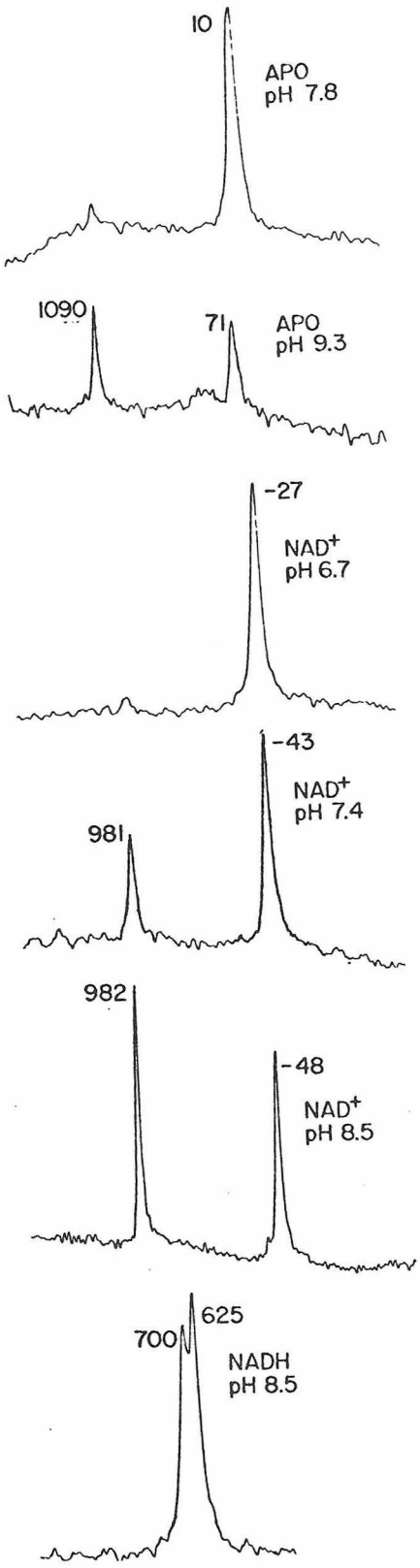

10
APO
pH 7.8
1090
71
APO
pH 9.3
-27
NAD+
pH 6.7
-43
NAD+
pH 7.4
981
982
-48
NAD+
pH 8.5
625
700
NADH
pH 8.5

Figure 4: ^{19}F nmr pH titration curves of F_3-GPD in presence and absence of coenzymes. The downfield peak is observed at high pH (see Fig. 3 and text).

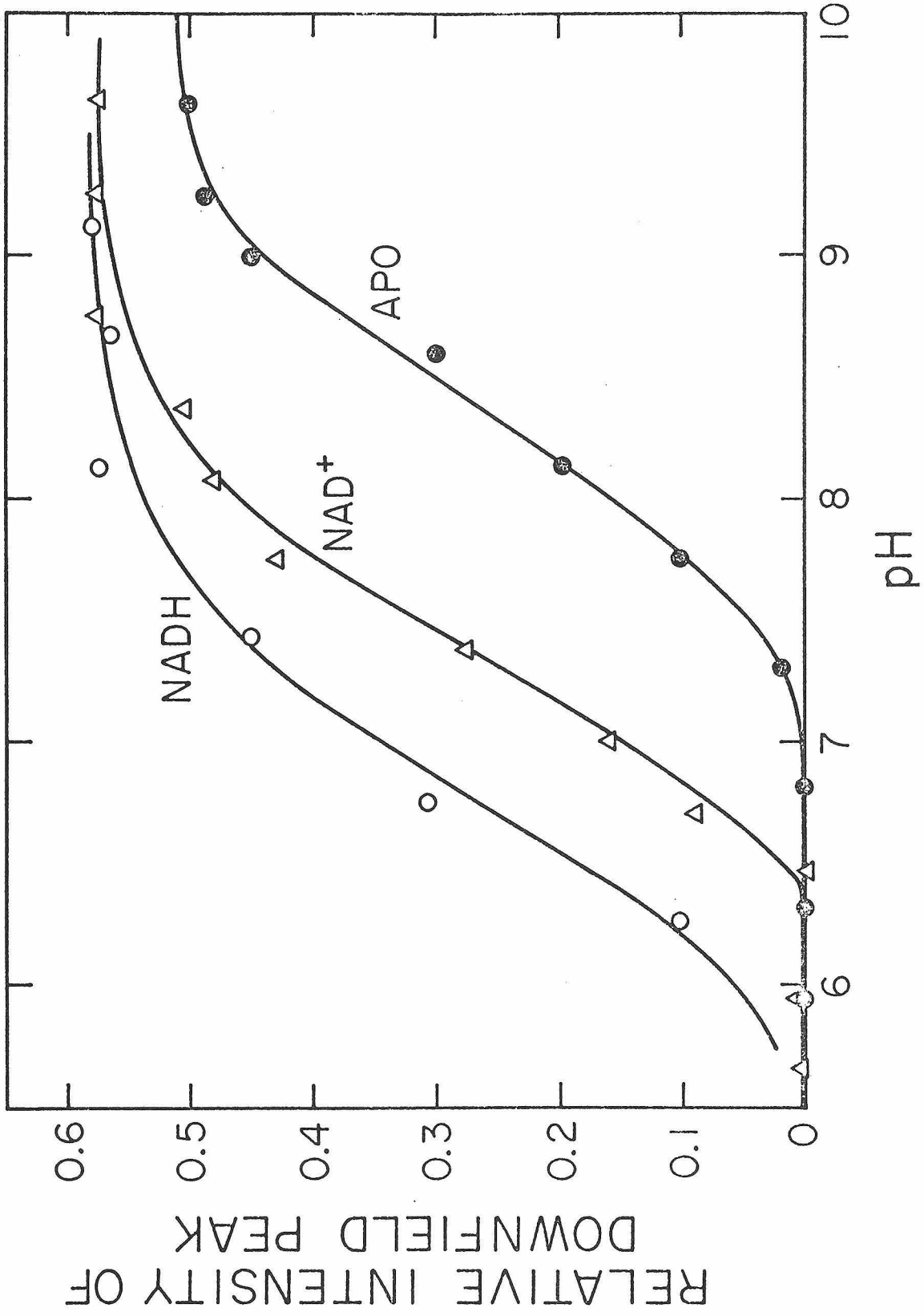

RELATIVE INTENSITY OF
DOWNFIELD PEAK
0
0.1
0.2
0.3
0.4
0.5
0.6
6
7
8
9
10
pH
APO
NAD+
NADH

Figure 5: ^{19}F nmr spectra of F_3-GPD in presence of saturating amounts of pyridine-3-aldehyde adenine dinucleotide.

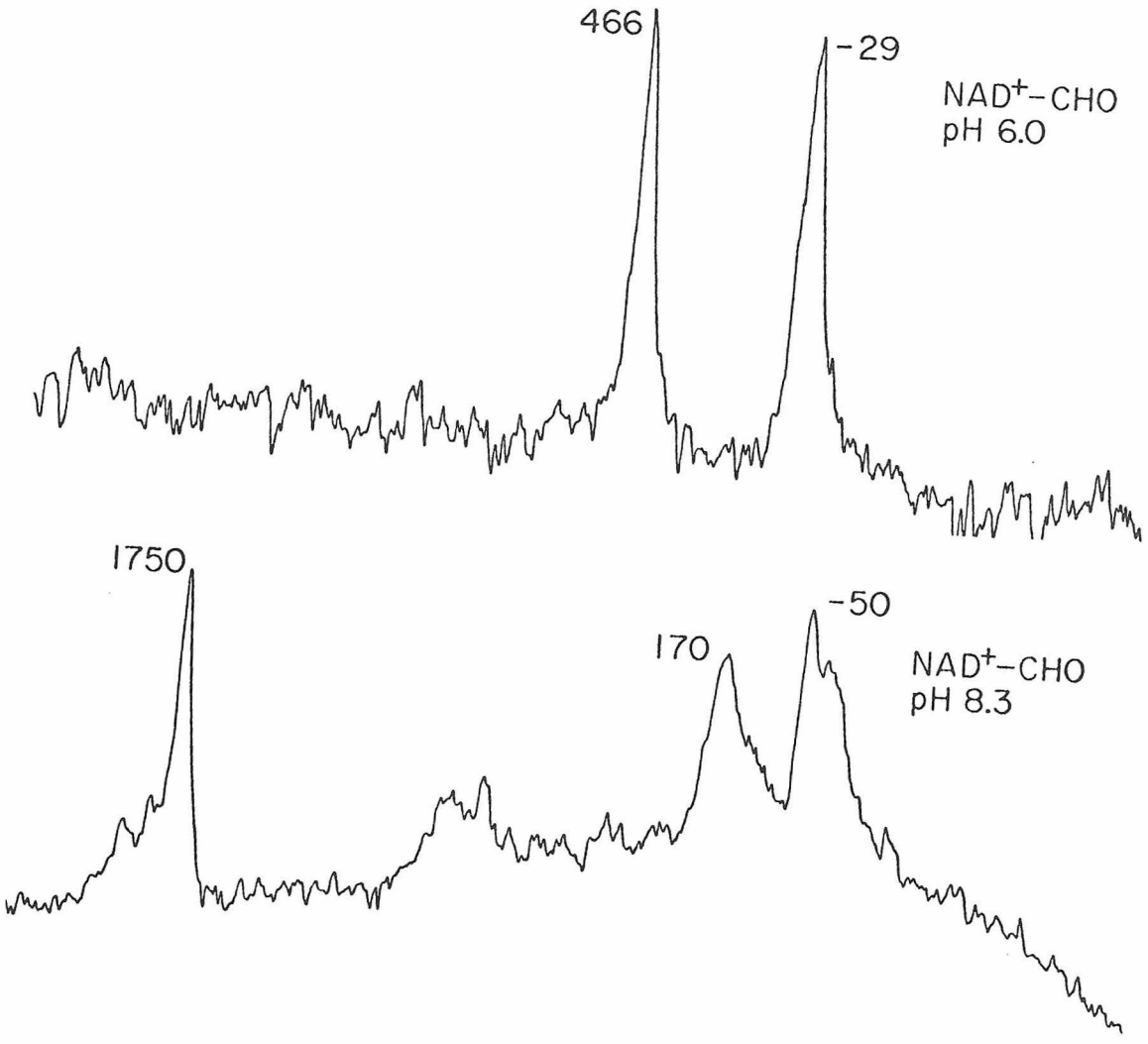

466
-29
NAD+-CHO
pH 6.0
1750
170
-50
NAD+-CHO
pH 8.3

Figure 6: ^{19}F spectra of F_3-GPD which was further modified with DTNB. Above spectra for enzyme with and without added NAD^+, at various pH values.

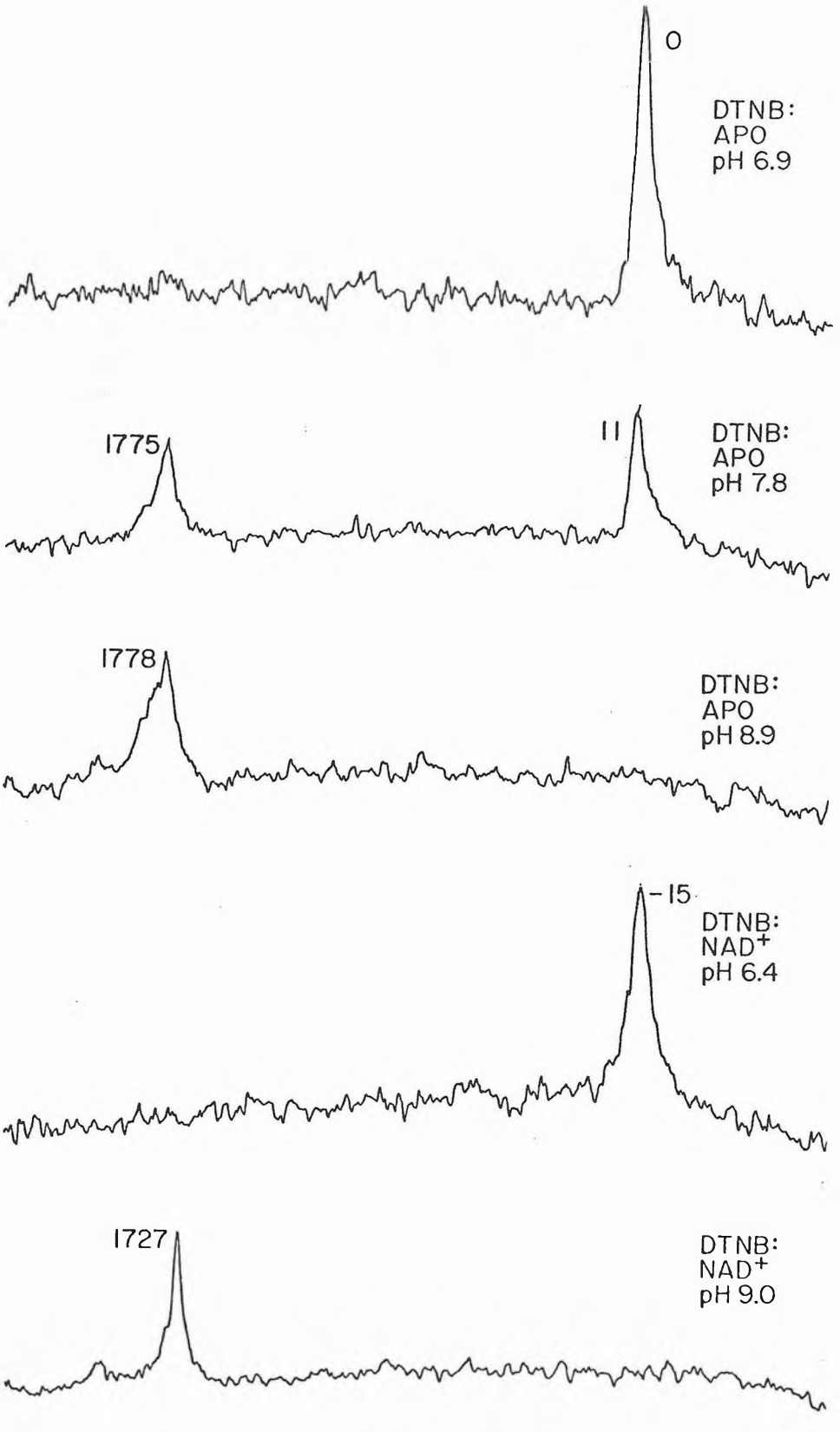

0
DTNB:
APO
pH 6.9
1775
11
DTNB:
APO
pH 7.8
1778
DTNB:
APO
pH 8.9
-15
DTNB:
NAD+
pH 6.4
1727
DTNB:
NAD+
pH 9.0

Figure 7: Same as Fig. 6, except spectra are in the presence of NADH. The fairly poor spectral quality is due to the rapid decomposition of the doubly modified enzyme. The small upfield peak in the middle spectrum is due to incomplete saturation of the enzyme.

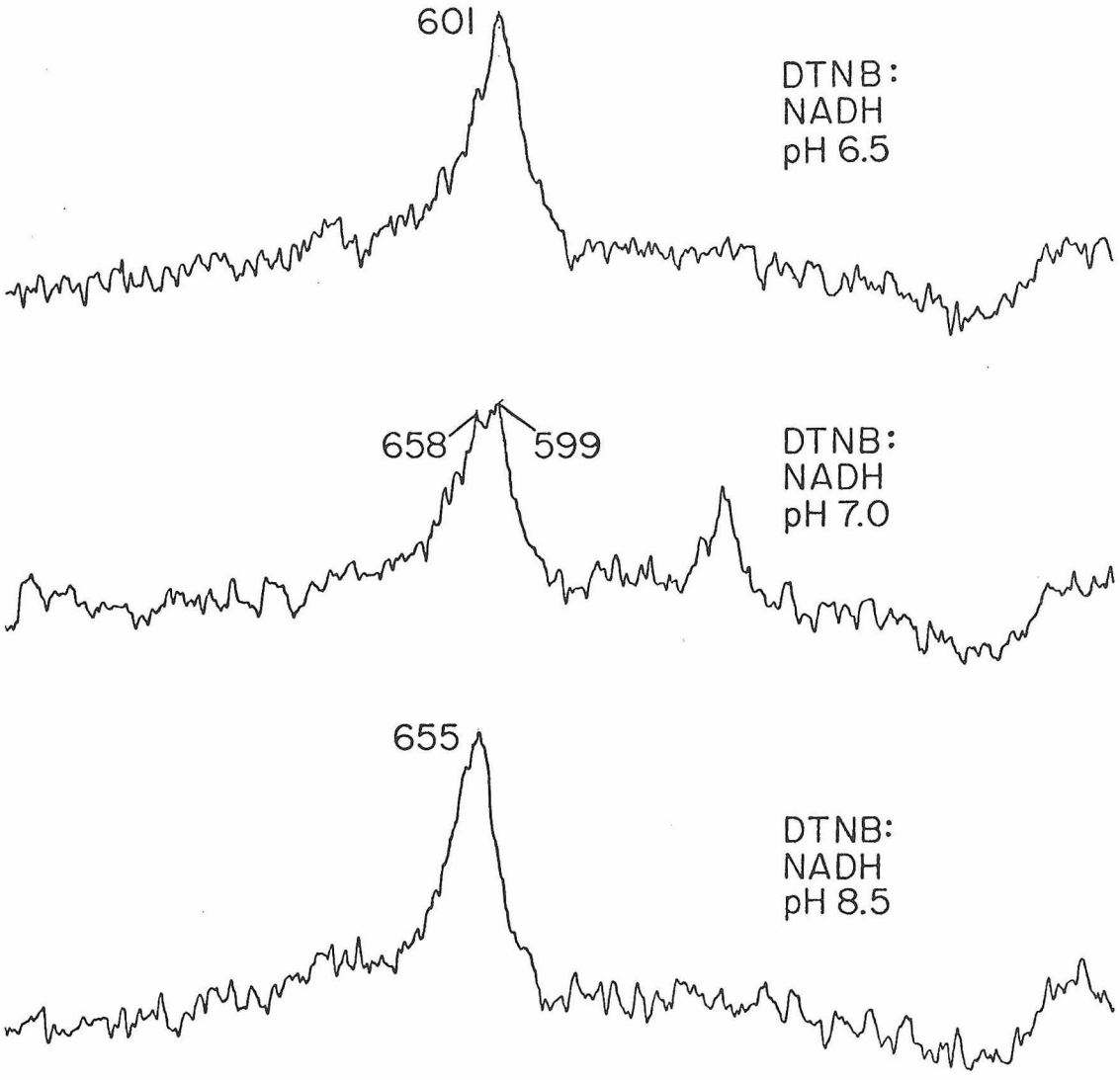

601
DTNB:
NADH
pH 6.5
658
599
DTNB:
NADH
pH 7.0
655
DTNB:
NADH
pH 8.5

Figure 8: Spectra of F_3-GPD saturated with NAD^+, in H_2O, 0.1 M KCl, 5 mm EDTA, pH 8.5. Each spectrum is the result of 4000 90° pulses. The acquisition time for all spectra was 0.04 sec. t represents the sum of the acquisition time plus the pulse delay, which was varied for each spectrum.

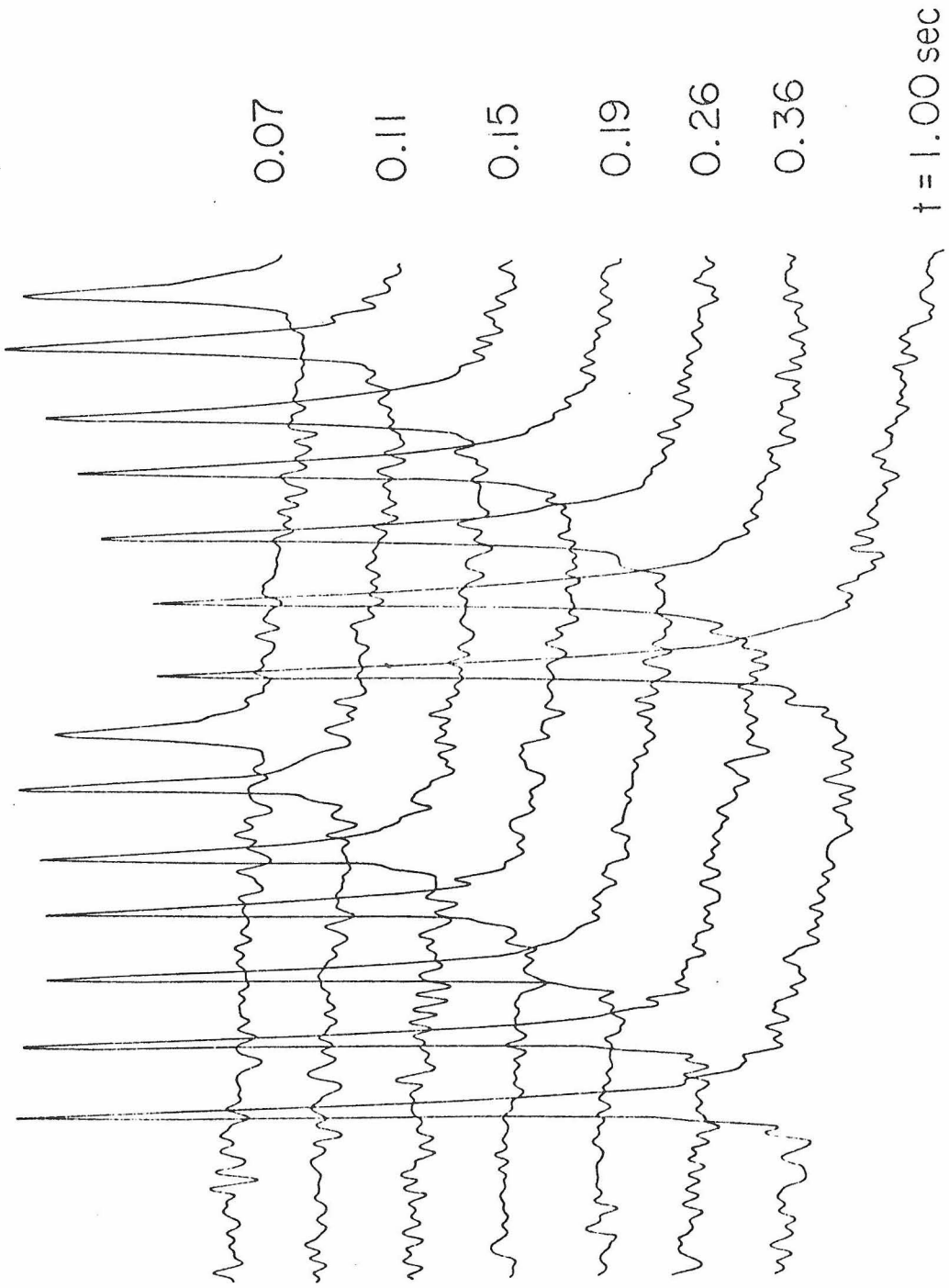
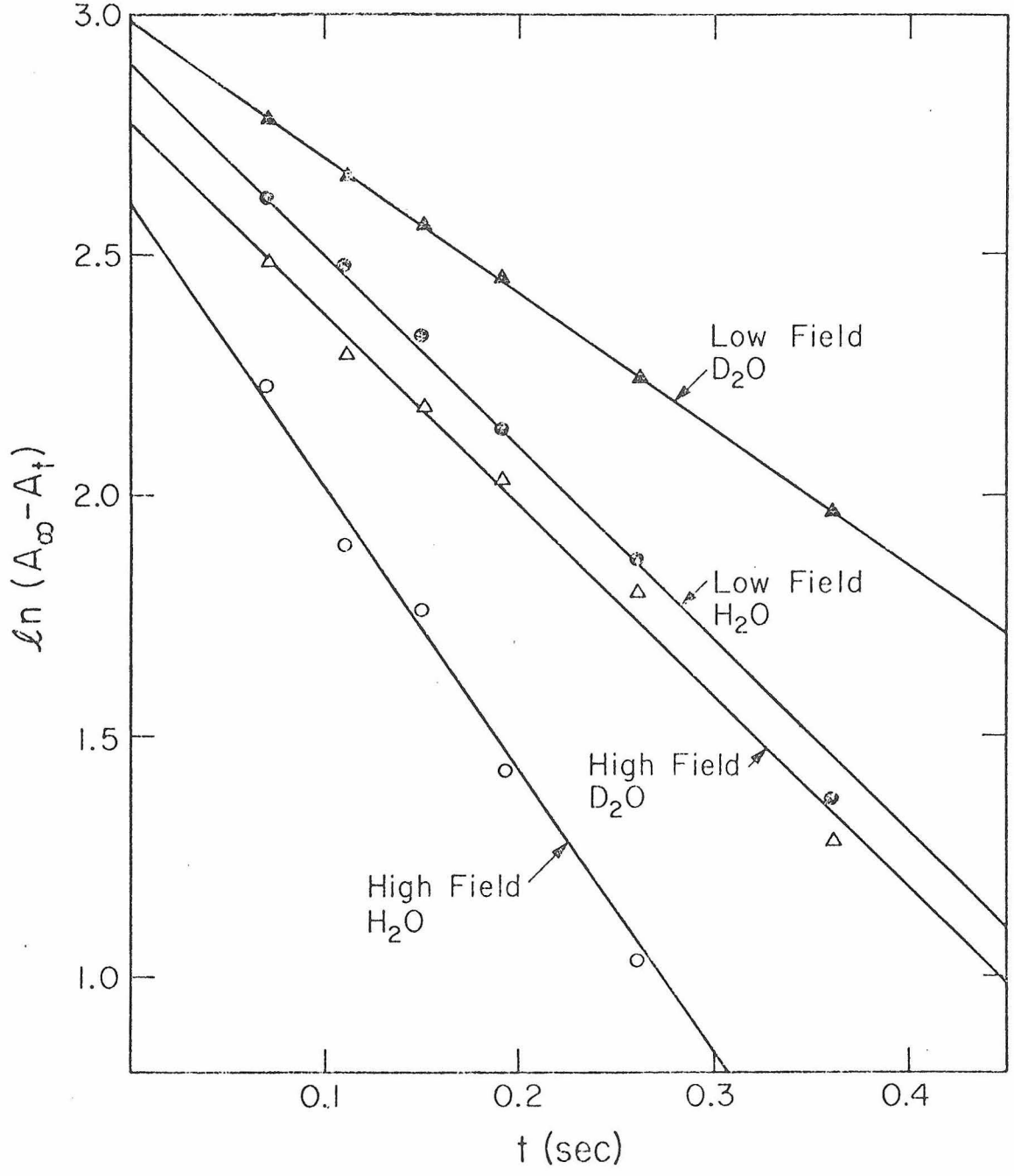

0.07
0.11
0.15
0.19
0.26
0.36
t = 1.00 sec

Figure 9: Data from T_1 experiments on F_3-GPD saturated with NAD^+ at pH 8.5. The value of T_1 is equal to the negative reciprocal of the slope of each line. Note the higher T_1 values for the low-field peaks relative to the high field peaks, as well as the higher T_1 values in D_2O compared to H_2O.


3.0
2.5
2.0
1.5
1.0
ℓn (A∞-At)
0.1
0.2
0.3
0.4
t (sec)
Low Field
D2O
Low Field
H2O
High Field
D2O
High Field
H2O

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PROPOSITION I

It is proposed that the importance of the proline residue in oxytocin be studied by replacing this residue with different amino acids, via solid phase synthesis.

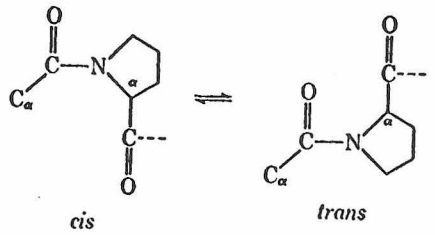
Oxytocin is a peptide hormone of the posterior pituitary gland causing uterus contraction and milk ejection. Its amino acid sequence is as follows:

It was first synthesized by du Vigneaud and co-workers in 1954 (du Vigneaud et al., 1954). More recently, oxytocin has been synthesized in the Merrifield solid phase technique (Manning, 1968).

Also, many analogues of oxytocin have been synthesized in which one or more of its residues have been modified or replaced by other amino acids. These studies have cast light on the importance of individual residues in the biological activity of oxytocin. For example, it has been found (Chan and Kelley, 1967) that removing the OH from tyrosine-2, or the carboxamide group from asparagine-5 or glycine amide-9 causes a large decrease in both the binding as well as the intrinsic activity of the hormone. Removing the carbonamide group of glutamine-4 decreases the binding of oxytocin but not its activity, while removing the amino group of cysteine-1 has virtually no effect on the hormone. Also, studies have shown (Jaquenod, 1965) that replacing leucine-8 with glycine leads to a large activity decrease,

but inserting alanine, valine, or isoleucine results in fairly high activity.

No replacement studies involving proline have been performed, but recent results indicate that this residue may be important in oxytocin's activity. For example, it has been shown (Brewster et al., 1972) that tocinamide, a compound consisting of the first five residues of oxytocin, has a three dimensional structure which differs from the structure of those same residues of oxytocin. Since toxinamide has very little biological activity, this result indicates the importance of the pro-leu-gly-NH_2 side chain. Also, nmr results have indicated that benzoxycarbonyl(z)-pro-leu-gly exhibits cis-trans isomerism about the z-pro bond as shown below (Hruby et al., 1971):



It is thus possible that this cis-trans isomerism helps to lock oxytocin into a conformation in which its interaction with its "receptor" is favorable.

It would thus be of interest to replace the proline, via solid phase synthesis, with both glycine and phenylalanine. Neither of

these residues would be expected to participate in cis-trans isomerism as does proline, and while glycine is a much smaller residue than proline, phenylanine is approximately the same size. Thus, if the biological activity of the two analogues were tested, and it was found that neither had activity, it would be a strong indication of the importance of cis-trans isomerism induced by proline. If only the phenylalanine analogue were active, it would indicate that proline's bulk was of importance, while if both derivatives had activity, it would indicate that the proline residue had no significance, except perhaps as a spacer.

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PROPOSITION II

It is proposed that the interaction of lactate dehydrogenase with NAD and related nucleotides be studied by ^{31}P nmr.

In Chapter I, Part A of this Thesis, studies involving ^{31}P nmr of pyridine coenzymes were described. It should prove fruitful to extend these studies to systems in which the coenzymes are complexed with enzyme molecules.

An enzyme particularly suitable for such studies is lactate dehydrogenase. The enzyme is involved in the glycolytic pathway, catalyzing the conversion of lactate to pyruvate. The enzyme has a molecular weight of 140,000 and is composed of four polypeptide chains, which are believed to have identical primary sequences (Allison et al., 1969). Its three dimensional structure has been determined by X-ray analysis (Adams et al., 1970) and the determination of its primary sequence is in its final states. Hopefully, it will therefore be able to relate effects on the ^{31}P spectrum of bound coenzymes to specific protein residues.

Some of the possible ^{31}P experiments which could be performed are the following:

Equivalence (or lack of it) of the coenzymes bound to different subunits:

If a titration of LDH with NAD^+ (or NADH) were performed, several different spectral patterns might appear. If the subunits

were equivalent at all stages of saturation only one line (or one AB pattern) would appear in the ^{31}P spectrum. However, nonequivalence of the subunits could give rise to a number of lines. One possible result would be that at low saturation, each subunit possessing bound coenzyme would be surrounded by three others containing no coenzyme, so the spectrum would consist of one line. At intermediate saturation levels, subunits with bound nucleotide would interact with subunits both with and without bound coenzyme, so the spectrum might have more than one line. At full saturation the spectrum might revert to that of a single resonance. Many other changes in the spectral pattern upon increasing coenzyme saturation are also of course possible.

Equivalence or nonequivalence of the two phosphorus atoms of NAD^+ (or NADH):

It was shown in Chapter I, Part A, that, in the absence of enzymes, the two phosphorus atoms of NAD^+ give rise to an AB pattern, while those of NADH cause a singlet. It is of interest to see if this also is true when the coenzymes are bound to LDH. It must be noted that the observation of the AB pattern would not necessarily require the nicotinamide-pyrophosphate to be present in the presence of LDH, since the nonequivalence of the two phosphates could be caused by interactions with the enzyme.

In order to differentiate between an AB pattern and a broad singlet, the resolution of the peaks must be fairly good. Fortunately, this would appear likely to be the case, as ^{31}P studies of phosphates

tightly bound to enzymes have reported linewidths of less than 5 cps (Huestis and Raftery, 1972).

Differentiation between an AB pattern and lines due to non-equivalent coenzymes on different subunits can be achieved through running the experiment at different frequencies. This is because the coupling constant (in Hz) of the AB pattern is independent of frequency, while chemical shift differences (in Hz) are directly proportional to frequency.

pH Titrations:

The performance of pH titrations may give indications of pK_a values of different groups on the enzyme, as well as indicate what conformational transitions the enzyme undergoes with varying pH.

It should be noted that the above experiments may be done in combination with each other, e.g., doing pH curves at different levels of saturation with coenzyme.

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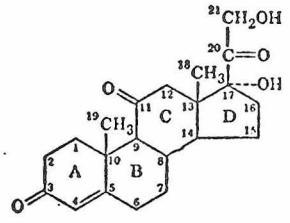
PROPOSITION III

It is proposed that the corticosteroid binding component of fibroblasts be isolated by means of affinity chromatography.

It has been demonstrated that mouse fibroblast growth is inhibited by small quantities of corticosteroids (Pratt and Aronow, 1966). Attempts at isolation of the component (from cultured cells) which interacts with the steroid have had some success (Hackney and Pratt, 1971) and the component believed to be responsible has been found to be a lipoprotein of molecular weight 600,000. However, extensive studies on the receptor have not been possible due to the poor yield obtained from the purification procedure (Hackney and Pratt, 1971). In particular the ammonium sulfate fractionation which is a key part of the isolation sequence is found to destroy much of the steroid binding capacity.

Recently, affinity chromatography has emerged as a highly successful method for isolating and purifying proteins (Cuatrecasas and Anfinsen, 1971). In general, affinity chromatography involves the covalent attachment of a particular ligand to a solid support, or resin. When a mixture containing several macromolecules is passed through a chromatography column containing this resin, only the macromolecule which interacts with this ligand will be retained on the column. After the other molecules have been washed through, various techniques would be used to elute the desired macromolecule.

In the present case one would wish to attach a corticosteroid, such as cortisone (shown below) to the resin. The resin most



commonly used for affinity chromatography is Sepharose, which consists of polydextron beads. Sepharose can be aminoethylated (Cuatrecasas, 1970) yielding a resin possessing free amino groups. One could then succinylate the primary hydroxyl group of cortisone (the tertiary hydroxyl is fairly unreactive, as evidenced by the formation of the monoester upon reaction with propionyl chloride (U.S. Pat. 2,746,978; 1956 to Upjohn). The aminoethylated sepharose could then be coupled to the succinylated cortisone by use of a carbodiomide mediated coupling (Cuatrecasas, 1970).

If the 105,000 g supernatant (where the corticosteroid binding component is located) of cultured cells were now passed through the column, the steroid binding component should be selectively retained. The component could be recovered by washing the column with a large

excess of cortisone phosphate (Cutler et al., 1958) (this is a fully active derivative of cortisone which is very water soluble). Hopefully, this would lead to the isolation of a quantity of cortisone binding protein which would be large enough for a variety of physical and biochemical studies.

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PROPOSITION IV

It is proposed that the binding of n-butyl isocyanide to hemoglobin be studied by observation of the contact shifted resonances in the ^1H nmr spectrum. These studies can be performed in H_2O, using Fourier transform nmr spectroscopy, making use of the technique recently introduced by Redfield (Redfield and Gupta, 1971).

Hemoglobin, the protein responsible for oxygen transport in blood, has probably been more extensively studied than any other macromolecule (Anfinsen et al., 1964; Antonini and Brunori, 1971; Perutz, 1970). The molecule has a molecular weight of 64,000, and consists of two pairs of identical polypeptide chains. It is thus designated an $\alpha^2\beta^2$ protein. One of the unsolved problems relating to hemoglobin is the order of ligation of the chains. Recently, two studies of the binding of n-butylisocyanide (BIC) to hemoglobin have reached opposite conclusions as to whether the ligand binds preferentially to the α or β chains (Lindstrom et al., 1971; Huestis and Raftery, 1972).

The first of these studies (Lindstrom et al., 1971) involved observation, by ^1H nmr, of the contacted shifted resonances arising from the heme groups of hemoglobin which are paramagnetic in the unliganded protein. In the absence of ligand, several peaks of equal intensity were observed, and from a study of various mutant hemoglobins, were assigned specifically to either α or β hemes. When

hemoglobin was partially saturated with BIC, it was found that the peaks due to the β chains became less intensive than those due to the α chains. This indicated that the β chains bound BIC preferentially, since liband binding causes hemoglobin to become diamagnetic, and therefore causes disappearance of the contact shifted resonances.

In the second study (Huestis and Raftery, 1972), cysteine 93 of the β chains was modified with trifluorobromacetone. It was shown that the position of the ^{19}F resonance of this modified hemoglobin was dependent on whether or not the β chains were liganded. Also, the visible spectrum of hemoglobin was used as a measure of the ligation of the entire molecule. It was found that upon titration with BIC, the change in the nmr spectrum lagged behind the charge in the visible spectrum, indicating that binding to the β chains lagged behind binding to the entire molecule, i.e., the ligand bound preferentially to the α chain.

The ^{1}H study was performed by continuous wave nmr, and due to the large linewidths ($\sim$75 cps), the spectral quality was exceedingly poor. Also, while the ^{19}F experiments were performed in H_2O, the ^{1}H experiments were done in D_2O.

In general it is impossible to get meaningful ^{1}H FT spectra in H_2O. This is because the water peak is so intense that other peaks are scaled down to a level below the lower limit of detection. Redfield, however, has developed a technique which overcomes this problem (Redfield and Gupta, 1971). After an FT pulse, each frequency receives a different amount of power, depending on its frequency

separation from the frequency of the rf pulse. At a certain point, there is a null, i.e., a certain frequency receives no power, and this frequency is dependent on the position and duration of the peak. If one sets up one's rf peaks so that this null occurs at the frequency of the H_2O peak, this peak (and any side bands which it may generate) disappears, and weak peaks may be detected. Since the null (or at least a severely decreased power level) extends over a range of a few hundred cps the technique can only be applied when observing resonances considerably removed from the H_2O peak. It is ideally suited for studying the contact shifted resonances of hemoglobin, which are 10-15 ppm downfield of the H_2O peak.

By employing FT nmr, as well as performing the experiment in H_2O, it should be possible to tell whether the ^1H experiment truly indicates that BIC binds preferentially to the β chains of hemoglobin, or whether this conslution was erroneous and was due to poor spectral quality and the deuterated solvent.

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PROPOSITION V

It is proposed that the binding of NAD^+ and NADH to yeast GPD, in which cys 148 has been alkylated, be studied via equilibrium dialysis.

Work presented in Chapter II of this Thesis, as well as elsewhere (Smith and Velick, 1972; Kirtley and Koshland, 1970) has demonstrated that the reaction of cysteine 148 of rabbit muscle GPD causes a marked decrease in the enzyme's affinity for NAD^+, while leaving NADH binding virtually unaffected. Also, the effect on coenzyme binding is the same, whether the cysteine reacts to form a thioester (Smith and Velick, 1972), as occurs during normal catalysis, or a thioether (Kirtley and Koshland, 1970), which is formed upon reaction with iodoacetate and similar compounds.

Yeast GPD does not possess an amino acid sequence which is identical to that of the rabbit muscle enzyme, but its active site residue is also cys 148, and this residue can be specifically alkylated (Perham and Harris, 1963). The binding of NAD^+ to the native enzymes from the two sources is considerably different, with the rabbit muscle enzyme possessing a much higher affinity for the coenzyme as well as eisplaying negative cooperativity (Conway and Koshland, 1968), while the binding to the yeast enzyme is noncooperative (von Ellenreider et al., 1972). The binding of NADH to the two enzymes is fairly similar (this thesis; von Ellenreider et al., 1972).

Thus the binding of coenzymes to yeast GPD resembles more closely the binding to modified rabbit GPD than to native enzyme from the latter source.

As of yet, no studies of coenzyme binding to modified (either acylated or alkylated) yeast GPD have been performed. The easiest way to do such experiments would be to use the alkylated enzyme, since it is more stable than the acyl derivatives. Binding constants to coenzymes could be determined through equilibrium dialysis, by using radioactive coenzymes.

The binding studies on rabbit GPD indicate that the enzyme undergoes a conformation change upon reaction of cys 148. The studies with yeast GPD would indicate whether it too undergoes such a conformation change upon alkylation.

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