Hendra, P. J., & Loader, E. J. (1968) *Chem. Ind. (London)*, 718–719.
Hirakawa, A., & Tsuboi, M. (1975) *Science 188*, 359–361.
Kitagawa, T., Nishina, Y., Shiga, K., Matsumura, Y., & Yamano, T. (1978) in *Flavin and Flavoproteins* (Yagi, K., & Yamano, T., Eds.) (in press) Scientific Societies Press, Tokyo.
Lord, R. C., & Thomas, G. J., Jr. (1967) *Spectrochim. Acta, Part A 23*, 2551–2591.
Miles, H. T., Lewis, T. P., Becker, E. D., & Frazier, J. (1973) *J. Biol. Chem. 248*, 1115–1117.
Miyazawa, T. (1960) *J. Mol. Spectrosc. 4*, 155–167.
Miyazawa, T. (1964) *J. Mol. Spectrosc. 13*, 321–325.
Nishikimi, M., & Kyogoku, Y. (1973) *J. Biochem. (Tokyo) 73*, 1233–1242.
Nishina, Y., Horiike, K., Shiga, K., & Yamano, T. (1977) *J. Biochem. (Tokyo) 82*, 1715–1721.
Nishina, Y., Kitagawa, T., Shiga, K., Horiike, K., Matsumura, Y., Watari, H., & Yamano T. (1978) *J. Biochem. (Tokyo) 84*, 925–932.
Rhodes, M. B., Bennett, N., & Feeney, R. E. (1959) *J. Biol. Chem. 234*, 2054–2060.
Sun, M., Moore, T. A., & Song, P-S. (1972) *J. Am. Chem. Soc. 94*, 1730–1740.
Tsuboi, M. (1976) *Proc. Int. Conf. Raman Spectrosc., 5th*, 135–143.
Watenpaugh, K. D., Sieker, L. C., & Jensen, H. L. (1973) *Proc. Natl. Acad. Sci. U.S.A. 70*, 3857–3860.
Whitby, L. G. (1953) *Biochem. J. 54*, 437–442.
Yagi, K., Ohishi, N., Takai, A., Kawano, K., & Kyogoku, Y. (1976a) in *Flavin and Flavoprotein* (Singer, T. P., Ed.) pp 775–781, Elsevier, Amsterdam.
Yagi, K., Ohishi, N., Takai, A., Kawano, K., & Kyogoku, Y. (1976b) *Biochemistry 15*, 2877–2880.

Base Interactions in the Triplet States of NAD^+ and NADH†

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ABSTRACT: We report here the phosphorescence spectra, 77 K lifetimes, triplet state zero-field splittings (zfs), and optical detection of magnetic resonance (ODMR) linewidths of the coenzyme nicotinamide adenine dinucleotide. The triplet state parameters of both the oxidized and reduced coenzyme (NAD^+ and NADH, respectively) were compared with those of the model compounds ADP, ADP-ribose, and 1-methyl-3-carbamidopyridinium chloride, and it was found that the major phosphorescence of NAD^+ and NADH is due to the adenine base. In aqueous ethylene glycol glasses, we found that the adenine triplet state in the coenzyme was strongly perturbed. There were large shifts in the $|D - E|$ and $|D + E|$ transitions, considerable broadening of the zf resonances, and changes in the sign of the zf spin polarizations. Furthermore the zfs of neither NAD^+ nor NADH were additive ($|D - E| + |2E| \neq |D + E|$), in contrast to those of ADP and ADP-ribose. The discrepancy was approximately six times larger for NAD^+ (117 MHz) than for NADH (19 MHz); the discrepancy is expected to be not larger than about 10 MHz based on the precision of the experimental data. In nonaqueous propylene glycol, the zfs of NAD^+ and NADH were additive and similar to those of ADP-ribose. Consideration of all the triplet state data, including 77 K phosphorescence lifetimes and ODMR linewidths, suggests that, in frozen *aqueous* solution, 95–100% of the coenzyme molecules are folded, and more than one conformation is stable for NAD^+, whereas one conformation appears to predominate for NADH. In neutral propylene glycol, in the absence of water, the intramolecular association is weak.

Luminescence studies on nicotinamide adenine dinucleotide, an important coenzyme in biological oxidation–reduction reactions, have long focused on the fluorescence of the reduced form of the dinucleotide (NADH),[1] since the oxidized form (NAD^+), by comparison, is essentially nonfluorescent. The observed fluorescence of NADH is due to the dihydronicotinamide base; the fluorescence yield of adenine by itself is negligible.[2] Fluorescence excitation spectra of NADH in aqueous solutions show bands at 260 and 340 nm. Upon enzymatic cleavage of the diphosphate bridge joining the two nucleotides, the 260-nm peak in the excitation spectrum disappears and has therefore been attributed to energy transfer from adenine to the dehydronicotinamide base in a stacked conformation (Weber, 1957; Scott et al., 1970).

Supportive evidence for intramolecular association of the dinucleotide bases has been obtained from proton NMR studies. Early experiments at 60 MHz provided a picture in which the coenzyme exists in a rapid equilibrium between folded and unfolded conformers (Jardetsky & Wade-Jardetsky, 1966; Sarma et al., 1968). At 220 MHz the two C4 protons of the dihydronicotinamide ring in NADH were resolved. Their nonequivalence indicated two folded forms (Sarma &

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[1] Abbreviations used: NAD^+, nicotinamide adenine dinucleotide; NADH, dihydronicotinamide adenine dinucleotide; EGW, ethylene glycol–water; AMP, adenosine 5′-monophosphate; ADP, adenosine 5′-diphosphate; ADP-ribose, adenosine 5′-diphosphoribose; MeNCl, 1-methyl-3-carbamidopyridinium chloride; NMR, nuclear magnetic resonance; ODMR, optically detected magnetic resonance; zfs, zero-field splittings.

[2] Eastman & Rosa (1968) measured the fluorescence quantum yield of adenine in 30/70 (v/v) water–ethylene glycol as a function of temperature and obtained a value of less than 0.0005 at 298 K.

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Table I: Phosphorescence Maxima and Lifetimes of the Coenzyme and Model Compounds[a]

sample	lifetimes (s)	emission maxima (nm)		
NAD^+	2.61 ± 0.03	381.6	402.9	425.4
NADH	2.7 ± 0.1	381.3	402.5	425.6
ADP	2.66 ± 0.03	380.4	402.2	424.4
ADP-ribose	2.64 ± 0.03	380.6	402.5	425.0
MeNCl	0.5 ± 0.1		403.8	

[a] Spectra were measured at 1.3 K, with excitation at 280 nm and a 3-nm band-pass for both excitation and emission. The lifetimes were measured at 77 K with monitoring of the emission at 378 and 403 nm. Sample concentrations were in the range 10^{-4} to 10^{-3} M. Estimated precision of the emission maxima is ±0.5 nm.

Kaplan, 1969; Patel, 1969). But interpretation of the data in terms of models has been questioned (Jacobus, 1971). Reexamination of the solution structure of NADH by Oppenheimer et al. (1971, 1978) showed that one folded form predominates.

In this paper we compare the folding in NAD^+ and NADH on the basis of their triplet state properties. The phosphorescence, zero-field splittings (zfs), and optically detected magnetic resonance (ODMR) linewidths of the triplet states of oxidized and reduced nicotinamide adenine dinucleotide are reported here and examined in terms of the model compounds ADP, ADP-ribose, and *N*-methylnicotinamide chloride (MeNCl). These triplet state data indicate that the bases in the coenzyme strongly interact in frozen *aqueous* solution and much less so in frozen propylene glycol, an unfolding solvent (see Scott et al., 1970). Although one folded conformation appears to predominate in NADH, essentially in agreement with the results of Oppenheimer et al. (1971, 1978), more than one folded conformation exists in NAD^+, according to the zfs and ODMR linewidths.

Materials and Methods

β-NADH, β-NAD^+, ADP, and ADP-ribose were obtained from Sigma. MeNCl was a gift from Professor David A. Deranleau, Theodor Kocher Institut, Bern, Switzerland. Chromatoquality ethylene glycol was purchased from Matheson Coleman and Bell. 1,2-Propanediol (propylene glycol) was obtained from Eastman and was neutralized with $NaHCO_3$. All other chemicals, from various U.S. suppliers, were reagent grade and used without further purification.

The general optical and microwave configurations used for the ODMR experiment have been described previously (Ross et al., 1977). In the case of NADH, as will be discussed in the results, the triplet state zfs were obtained while the phosphorescence decay was monitored (delay ODMR) (Moore & Kwiram, 1974). All of the other ODMR measurements employed continuous optical excitation. Excitation of the samples at λ = 280 nm was provided by a mercury–xenon diffused-arc lamp (Canrad-Hanovia, Inc., Newark, NJ), and detection at λ' = 405 nm was aided by an 0-51 Corning filter set between the emission monochromator and the sample to minimize scattered exciting light. Samples were dissolved in 50% (v/v) ethylene glycol–water (EGW) buffered at pH 7.0 with 0.05 M K_xPO_4. Concentrations were determined by dry weight and checked by the absorbance spectrum of the sample in pH 7.0, 0.05 M K_xPO_4 buffer at room temperature.

During lifetime measurements, the photomultiplier output was monitored by using a logarithmic amplifier. Phosphorescence decays were followed over several decades of phototube current, and the experimental decay curve was fitted to a single exponential using both weighted and unweighted least-squares methods (Motten, 1978). The difference between the two procedures was within the quoted experimental error.

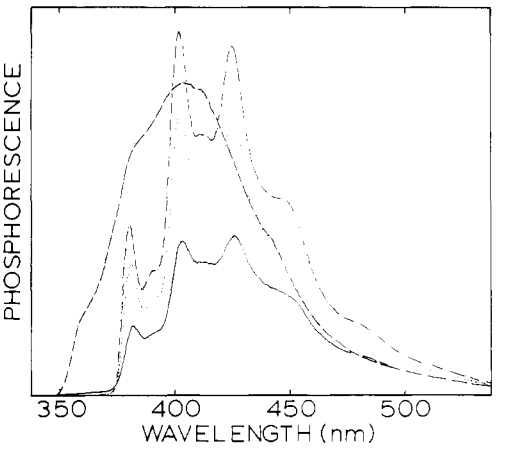

FIGURE 1: Phosphorescence spectra in arbitrary units with excitation at 280 nm of NAD^+ (—), ADP (—·—), ADP-ribose (···) at 2.5 × 10^{-4} M, and MeNCl (- - -) at 5.0 × 10^{-4} M in 50% (v/v) ethylene glycol–water buffered at pH 7.0 with 0.05 M K_xPO_4. Excitation and emission bandwidths set at 3 nm.

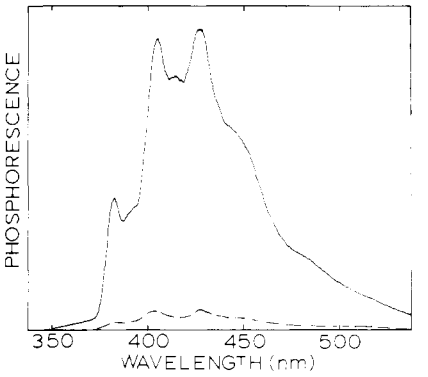

FIGURE 2: Phosphorescence spectra of NAD^+ (—) and NADH (—·—) at 2.5 × 10^{-4} M. Experimental conditions are the same as for Figure 1.

Results

Phosphorescence Spectra and Lifetimes. The phosphorescence spectra of NAD^+, ADP, ADP-ribose, and MeNCl in aqueous glass are shown in Figure 1, and their wavelength maxima are listed in Table I. Except for MeNCl, these spectra are qualitatively similar and reminiscent of the AMP spectrum reported by Bersohn & Isenberg (1963). As the figure illustrates, the phosphorescence yield (excitation at 280 nm) decreases in the order ADP > ADP-ribose > NAD^+; addition of the ribose group to ADP quenches the adenine phosphorescence by approximately 20–25%, and the addition of the nicotinamide ring to form the dinucleotide coenzyme quenches it by roughly another 40%. Reduction of the nicotinamide moiety dramatically decreases the adenine phosphorescence further (Figure 2) to a level about 5% of its value in the oxidized coenzyme. Close examination of the emission spectrum of NAD^+ (cf. Figures 1 and 2) shows that the blue edge of the NAD^+ emission coincides with that of MeNCl at 350 nm. Neither ADP nor ADP-ribose has significant phosphorescence in the wavelength region from 350 to 365 nm. The possibility of solvent or sample impurity luminescence contributing to the NAD^+ spectrum was considered. However, different batch lots of NAD^+ gave identical spectra at concentrations ranging from 10^{-5} to 10^{-3} M. Therefore, we concluded that the phosphorescence of the coenzyme below 370 nm is due to the oxidized nicotinamide ring. Based on the spectral distribution of the model MeNCl, nicotinamide

Table II: ODMR Transitions and Linewidths of the Coenzyme and Model Compounds[a]

sample	zfs parameters (GHz)[b]				linewidths (MHz)[c]		
	$\|2E\|$	$\|D-E\|$	$\|D+E\|$	Δ	$\|2E\|$	$\|D-E\|$	$\|D+E\|$
NAD^+	1.587	2.782	4.486	0.117	115 (+)	212 (+)	300 (+)
NADH	1.600	2.861	4.480	0.019			
ADP	1.617	2.902	4.520	0.001	75 (−)	195 (−)	206 (+)
ADP-ribose	1.621	2.898	4.520	0.001	83 (−)	191 (−)	178 (+)
MeNCl	0.74	3.483	(4.3)	(0.08)	205 (+)	257 (+)	>450 (−)

[a] Experimental conditions were the same as described in Table I, with phosphorescence detection at 405 nm. [b] The zfs reported are the average maximum position of magnetic resonance spectra (see Ross et al., 1977). The symbol Δ is the absolute difference between the experimental values $|D+E|$ and ($|2E| + |D-E|$) (i.e., $|\Delta| = |D+E| - (|2E| + |D-E|)$). Estimated precision of the zfs: ±5 MHz for NAD^+, ADP, and ADP-ribose; ±10 MHz for NADH; ±100 MHz for $|D+E|$; and ±15 MHz for $|2E|$ of MeNCl. [c] The (+) indicates an increase in the steady state optical emission during microwave resonance, and the (−) indicates a decrease in the emission. The ODMR linewidths of NADH are not given since the transitions could only be observed in the optical delay mode (delay ODMR). The linewidths are precise to within ±7%.

accounts for about 5% of the total oxidized coenzyme phosphorescence.

NADH has a strong absorption band with a maximum at 340 nm in water or propylene glycol (Siegal et al., 1959; Scott et al., 1970). NAD^+ does not have a significant absorption in this region of the near-ultraviolet spectrum. Excitation in the 340-nm electronic band of NADH produces substantial fluorescence at room temperature (Weber, 1957). In frozen glasses, the NADH samples remain strongly fluorescent, but we observe no NADH *phosphorescence* when exciting at 313 nm or at longer wavelengths. Exciting at 280 nm, weak phosphorescence from the adenine moiety was observed as described above. The adenine phosphorescence intensity of NADH was considerably stronger in 100% propylene glycol than in ethylene glycol–water as was also the nicotinamide contribution to the spectrum of NAD^+, but the adenine phosphorescence emission maxima were not significantly shifted.

In neutral aqueous solvent, the 77 K phosphorescence lifetimes of all the adenine-containing compounds were about 2.6 s; the lifetime of MeNCl, corrected for solvent impurities, was a factor of five shorter, about 0.5 s (Table I). Because NADH has such a low adenine phosphorescence yield, it was impossible to follow the phototube current over more than three decades which made determination of the phosphorescence lifetime less accurate than for the other adenine nucleotides. At the leading edge of the NAD^+ decay, there was a fast component not observed in NADH, ADP, or ADP-ribose. Because the relative magnitude of this component was independent of coenzyme concentration, and had a lifetime similar to that of MeNCl, we attributed the fast decay component to emission from the oxidized nicotinamide base.

ODMR Spectra of Adenine in NADH and NAD^+. The most dramatic difference between the triplet state magnetic resonance transitions of the adenine ring in NAD^+, as compared with the same transitions in ADP or ADP-ribose, is the change in sign[3] (spin polarization) of the $|D-E|$ and $|2E|$ transitions.[4] In the dinucleotide, these transitions become positive (see Figure 3). The zfs of NADH could not be readily detected during steady-state optical excitation, perhaps because the strong fluorescence spectrum of the dihydronicotinamide group overlaps with the weak adenine phosphorescence of the reduced coenzyme and accounts for about 99% of the total detected luminescence even in the wavelength region where there is significant adenine phosphorescence. Therefore, the zfs reported were obtained by delay ODMR (Moore & Kwiram, 1974) which does not provide accurate linewidth information (Ross et al., 1977).

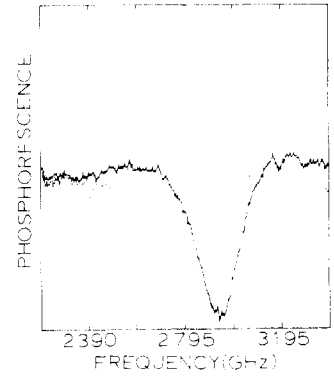

FIGURE 3: Variation in the spin polarization of the $|D-E|$ transition of ADP (—) and NAD^+ (···), monitoring of the phosphorescence at 405 nm. All other experimental parameters are the same as given in Figure 1.

The zfs and ODMR linewidths of the coenzyme and its model compounds are given in Table II. There is little that distinguishes the ODMR parameters of ADP from those of ADP-ribose. Except for the $|D+E|$ linewidth, all their parameters are identical within experimental error. Addition of the nicotinamide group, forming NAD^+, is accompanied by more than a 30-MHz decrease in both $|D+E|$ and $|2E|$ and by a 120-MHz decrease in $|D-E|$. Not only are there changes in the signs of the $|D-E|$ and $|2E|$ transitions of the adenine moiety, but also are there 50% increases in the $|2E|$ and $|D+E|$ resonance linewidths.

The transition frequencies of NADH are shifted from those of ADP and ADP-ribose, and, with the exception of $|D+E|$, they also differ from those of NAD^+. The $|2E|$ and $|D-E|$ values of the reduced coenzyme fall between those of the oxidized coenzyme and those of the mononucleotides. Relative to the zfs of ADP and ADP-ribose, there is about a 40-MHz decrease in $|D-E|$ and about a 20-MHz decrease in $|2E|$ of NADH. The difference in $|2E|$ between NADH and NAD^+ is nearly 15 MHz, and there is an 80-MHz increase in $|D-E|$ of NADH compared with NAD^+, which amounts to about 40% of the NAD^+ ODMR linewidth. The shifts are clearly significant since the estimated precision of the zfs is ±5 MHz for NAD^+, ADP, and ADP-ribose and ±10 MHz for NADH. Barring significant changes in the sublevel decay rate constants, the ODMR linewidths (except for NADH) can be directly compared since the microwave sweep rates were identical for measuring the triplet splittings in the different adenine nucleotides ($|2E|$, 40 MHz s^{-1}; $|D-E|$, 46 MHz s^{-1}; $|D+E|$, 50 MHz s^{-1}).

[3] The "sign" of a transition is used to denote an increase (positive sign) or a decrease (negative sign) in the phosphorescence intensity during microwave passage through resonance.

[4] The assignment of the zf transitions assumes $D > 3E > 0$.

Table III: ODMR Transitions and Linewidths of NAD+, NADH, and ADP-ribose in Propylene Glycol[a]

	zfs parameters (GHz)				linewidths (MHz)		
sample	$\|2E\|$	$\|D-E\|$	$\|D+E\|$	Δ	$\|2E\|$	$\|D-E\|$	$\|D+E\|$
NAD+	1.601	2.912	4.504	0.009	92 (+)		196 (+)
NADH	1.598	2.916	4.515	0.001			
ADP-ribose	1.605	2.927	4.524	0.008	86 (−)	205 (−)	150 (+)

[a] See footnotes *a*, *b*, and *c* to Table II for explanation of symbols and estimates of precision.

In general, it is expected that the frequencies of the $|2E|$ and $|D - E|$ transitions should sum to that of the $|D + E|$ transition within experimental error. However, only the zfs of ADP and ADP-ribose add up precisely in aqueous solvent. The absolute difference between $|D + E|$ and $|D - E| + |2E|$ (Δ in Table II) of NADH is just outside the limit of experimental error, and the Δ of NAD+ is an order of magnitude larger. This is a surprising result. On the other hand, the large Δ for MeNCl has little meaning since its $|2E|$ and $|D + E|$ transitions were weak, broad, and therefore difficult to assign accurately (indicated by parentheses in Table II).

As seen in Table III, propylene glycol shifts the $|D - E|$ and $|D + E|$ transitions of NAD+ and NADH dramatically, bringing them close to those of ADP-ribose, and the observed Δ values fall within the experimental error associated with the zfs. The $|D - E|$ transition of NAD+ could not be detected using steady-state optical excitation and was therefore measured by delay ODMR. As in ethylene glycol–water, the zfs of NADH in propylene glycol were obtained with delay ODMR.

No significant changes were observed in the $|2E|$ or $|D - E|$ linewidths of ADP-ribose or the $|2E|$ linewidth of NAD+. Although the $|D + E|$ linewidth of ADP-ribose decreased by about 15% compared with that measured in ethylene glycol–water, the $|D + E|$ linewidth of NAD+ decreased by more than 30%.

Discussion

The triplet state emission of NAD+ is primarily from the adenine ring. Although the phosphorescence is quite similar to that of ADP or ADP-ribose, the sublevel splittings of the NAD+ triplet state are substantially perturbed, and microwave passage through the $|D - E|$ and $|2E|$ resonances produces increases in the steady-state phosphorescence intensity rather than the phosphorescence decreases observed for these same transitions in ADP and ADP-ribose. The observations that the ODMR lines of adenine in NAD+ are broader than those of either of the two models and that the zfs of both NAD+ and NADH fail to sum ($|D - E| + |2E| \neq |D + E|$) provide evidence that more than one class of triplet states is being observed in the coenzyme. The simplest interpretation of these results is that we are observing the triplet states of more than one coenzyme conformer. Several considerations have led us to this conclusion.

First, we were concerned that the large value of Δ for the NAD+ zfs, as compared with ADP or ADP-ribose, meant that our coenzyme samples were impure. NAD+ is known to undergo hydrolysis to AMP, ADP, and ADP-ribose (Singer & Kearney, 1954). Since the phosphorescence and ODMR of the mononucleotide models are at similar frequencies and stronger than those of the coenzyme, hydrolysis products would contaminate the ODMR spectra. Since the negative transitions in the model compounds appear at higher microwave frequencies than the corresponding positive transitions in the coenzyme, any contribution from the former would require the appearance of a distortion on the high frequency side of the same two transitions in NAD+. No such distortions were observed.

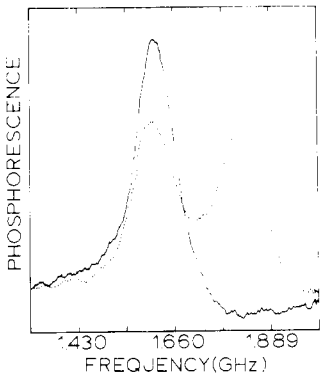

FIGURE 4: The $|2E|$ transition of NAD+ at 10^{-3} M (—) and 5×10^{-3} M (···) with monitoring of the phosphorescence at 405 nm. Otherwise, the experimental conditions are as given in Figure 1.

Our second consideration was intermolecular association since previously we found that aggregation can cause ODMR line broadening of, for example, tryptophan zf transitions in polypeptides (Ross et al., 1977). Therefore, we examined the ODMR of NAD+ as a function of concentration. The triplet state parameters of the coenzyme were independent of concentration from 10^{-5} to 10^{-3} M, which led us to the conclusion that the line broadening, the shift in the zfs, the large Δ, and the sign of the phosphorescence response during resonance were characteristic of the oxidized monomer dinucleotide. The effect of aggregation was made evident at concentrations higher than 10^{-3} M by the appearance of a second set of resonances at about 1.81, 2.4, and 4.41 GHz (cf. Figure 4); the 2.4-GHz resonance was poorly resolved and appeared as a decrease rather than an increase in the phosphorescence. The line shapes and linewidths of the other two transitions were similar to those of NAD+ at concentrations of 10^{-3} M and below. At high concentrations of NAD+, we detected an additional two resonances at about 0.53 and 0.80 GHz by using delay ODMR. It is possible that these two transitions are associated with the nicotinamide ring in both monomer and aggregated dinucleotide. (The $|2E|$ transition of MeNCl occurs at 0.74 GHz.) Similar shifts due to aggregation are observed for the adenine zfs in the coenzyme. Further experiments are in progress to investigate and characterize the high concentration ODMR lines in NAD+.

The third possibility was that a fraction of the coenzyme molecules were unfolded in frozen aqueous (EGW) glass. It is well established in the literature that the intramolecular association between the coenzyme bases becomes stronger as the temperature is decreased (see Jardetsky & Wade-Jardetsky, 1966; Reisbig & Woody, 1978; Oppenheimer et al., 1978). We found that there was a contribution from the nicotinamide base to the phosphorescence lifetime and spectrum of the oxidized coenzyme. If it is assumed that the phosphorescence quantum yields of MeNCl and the nicotinamide base in unfolded NAD+ are similar, and if the nicotinamide phosphorescence is fully quenched in the folded

coenzyme, then we would conclude that about 5% of the NAD^+ molecules are unfolded in frozen solution. And in this regard, it is interesting to note that the adenine phosphorescence in NADH was about 5% that of the oxidized coenzyme (see Figure 2). According to Weber (1957) and Scott et al. (1970), very little singlet energy transfer from adenine to dihydronicotinamide takes place in NADH unfolded in propylene glycol; they estimate that the rate of fluorescence transfer is greater than 10^{10} s^{-1} in the folded dinucleotide. However, even if all the molecules are folded, an intersystem crossing rate of 10^8 to 10^9 s^{-1} would be sufficient to explain the few percent adenine phosphorescence in the aqueous glass. Similarly in NAD^+, if folded, intersystem crossing from the nicotinamide singlet excited state must be competitive with its intramolecular quenching and, thereby, account for the nicotinamide contribution to the phosphorescence.

The broad ODMR lines of dilute NAD^+ (Table II) in ethylene glycol–water most likely reflect both the range of nicotinamide–adenine base interactions and the contributions from solvent. The large value of Δ could be due to differences in the spin polarization and/or sublevel lifetimes of the adenine triplet state for unique monomer configurations of the oxidized dinucleotide; that is, each population of a particular folded configuration would give rise to a different unique set of nonhomogeneously broadened triplet state lines. Reisbig & Woody (1978) have investigated the absorption and circular dichroism of NAD^+ extending above 300 nm and have interpreted the spectra as being due to a charge-transfer complex (adenine as the donor and nicotinamide as the acceptor). Invoking a charge-transfer complex provides a possible interpretation for the shifted zfs, ODMR linewidths, and zfs spin polarizations. However, ODMR studies of tetracyanobenzene charge-transfer complexes with benzene and hexamethylbenzene by Yagi et al. (1976) show that, *as the degree of charge transfer in the excited state increases, the overall phosphorescence lifetime and zfs decrease.* ODMR studies of paracyclophanes by Schweitzer et al. (1976) again show a decrease by a factor of two or more in the magnitude of the zfs for the charge-transfer complex when compared with the monomer(s). The zfs of NAD^+ decrease by only 5% or so (Table II) and, since the 77 K lifetimes of NAD^+, NADH, and the adenine-containing models are virtually identical (Table I), the radiative and nonradiative processes from the triplet state are also not significantly perturbed by the intramolecular association of adenine with either nicotinamide or dihydronicotinamide. These results do not seem to support a substantial charge-transfer contribution in the triplet state.

The change in sign of the steady-state NAD^+ ODMR transitions of adenine can be reasonably explained by triplet–triplet energy transfer from the nicotinamide ring. Triplet–triplet energy transfer involves exchange between three donor and three acceptor spin levels, and the probability of exchange depends upon the projection of the donor spin axes upon those of the acceptor (El-Sayed, 1971). Such exchange would provide a mechanism for changing the relative triplet sublevel populating rates of adenine and account for the change in spin polarization of its triplet state in the oxidized coenzyme, without changing its radiative rate constants. Each possible folded conformer will have a unique projection of donor and acceptor spin axes. Therefore, not only will each configuration give rise to separate adenine zfs but also will the relative spin polarization for a given transition vary from one conformer to another. The ODMR spectra from such a distribution of folded molecules will exhibit broadened lines with a large value of Δ, as is observed for NAD^+. Thus triplet–triplet energy transfer provides a plausible explanation: a large charge-transfer interaction is not supported by the data. On the other hand, it is difficult to exclude a relatively small charge-transfer interaction in the triplet state. This issue merits further study.

The most convincing argument supporting the notion that the base interactions are responsible for the broad ODMR lines and large Δ in NAD^+ is that, when the coenzyme is unfolded in propylene glycol, both the adenine zfs and linewidths approach those of ADP-ribose and Δ becomes insignificant (less than 10 MHz; see Table III). The somewhat broader ODMR lines of NAD^+, compared with ADP-ribose in propylene glycol, can be ascribed to the adenine base in the dinucleotide experiencing not only a similar range of solvent interactions as adenine in ADP-ribose but also interactions which involve near approaches of the nicotinamide ring, with concomitant reorientation or exclusion of solvent molecules.

Unfolding will change the probability for triplet–triplet energy transfer in the oxidized coenzyme. Although the intensities of the ODMR lines change significantly (reflecting changes in the spin polarization), the signs remain unchanged in propylene glycol. (The signs of the ADP-ribose magnetic resonance transitions are unchanged in going from aqueous solvent to propylene glycol.) This suggests that triplet–triplet transfer still occurs in the unfolded state. ODMR studies by Rousslang & Kwiram (1976) on the tryptophyltyrosinate dipeptide provide independent evidence for triplet–triplet energy transfer in such systems. This hypothesis is consistent with the observation of Das & Longworth (1975) that the nicotinamide base in $NADP^+$ had a shortened lifetime under destacking conditions (dimethyl sulfoxide/ethylene glycol). Both their results and ours are reasonable because, considering the long lifetime of the triplet state, the possibility for triplet–triplet energy transfer still persists in the unfolded coenzyme. The effect on the populating rates for the acceptor triplet sublevels would then reflect the possible random orientations of the bases selected by the freezing process.

Since one folded conformation predominates for NADH in water (Oppenheimer et al., 1971, 1978), it is not surprising that its value of Δ, although significant, is small compared with that of aqueous NAD^+. It is unlikely that the observed adenine phosphorescence of NADH in ethylene glycol–water is due to a few percent of unfolded molecules because its adenine zfs are substantially different from those of ADP and ADP-ribose. In 100% propylene glycol the zfs of NADH are nearly the same as those of ADP-ribose, and Δ becomes insignificant, as would be expected if the coenzyme is truly unfolded.

In conclusion, the intramolecular association between the bases of NAD^+, at low temperatures (77–1.3 K) in frozen neutral aqueous (EGW) solution, is close to 100%. The triplet state data are consistent with a model in which more than one folded conformation is stable for NAD^+. By contrast, in NADH the population of alternate conformers is small. Finally, now that the triplet states of nicotinamide adenine dinucleotide have been described, luminescence and magnetic resonance studies on the interactions of both the oxidized and reduced forms of the coenzyme with the dehydrogenases can be made by using the coenzyme as a natural probe.

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References

Bersohn, R., & Isenberg, I. (1963) *Biochem. Biophys. Res. Commun. 13*, 205–208.

Das, S. K., & Longworth, J. W. (1975) *Fed. Proc., Fed. Am. Soc. Exp. Biol. 34*, 691 (Abstr. 2679).
Eastman, J. W., & Rosa, E. J. (1968) *Photochem. Photobiol. 7*, 189–201.
El-Sayed, M. A. (1971) *J. Chem. Phys. 54*, 680–691.
Jacobus, J. (1971) *Biochemistry 10*, 161–164.
Jardetsky, O., & Wade-Jardetsky, N. G. (1966) *J. Biol. Chem. 241*, 85–91.
Moore, T. A., & Kwiram, A. L. (1974) *Biochemistry 13*, 5403–5407.
Motten, A. G. (1978) Dissertation, University of Washington, Seattle, WA.
Oppenheimer, N. J., Arnold, L. J., & Kaplan, N. O. (1971) *Proc. Natl. Acad. Sci. U.S.A. 68*, 3200–3205.
Oppenheimer, N. J., Arnold, L. J., & Kaplan, N. O. (1978) *Biochemistry 17*, 2613–2619.
Patel, D. T. (1969) *Nature (London) 221*, 1329–1341.
Reisbig, R. R., & Woody, R. W. (1978) *Biochemistry 17*, 1974–1984.
Ross, J. B. A., Rousslang, K. W., Deranleau, D. A., & Kwiram, A. L. (1977) *Biochemistry 16*, 5398–5402.
Rousslang, K. W., & Kwiram, A. L. (1976) *Chem. Phys. Lett. 39*, 226–230, 231–235.
Sarma, R. H., & Kaplan, N. O. (1969) *J. Biol. Chem. 244*, 771–779.
Sarma, R. H., Ross, V., & Kaplan, N. O. (1968) *Biochemistry 7*, 3052–3062.
Schweitzer, D., Hausser, K. H., Taglieber, V., & Staab, H. A. (1976) *Chem. Phys. 14*, 183–187.
Scott, T. G., Spencer, R. D., Leonard, N. J., & Weber, G. (1970) *J. Am. Chem. Soc. 92*, 687–695.
Siegel, J. M., Montgomery, G. A., & Bock, R. M. (1959) *Arch. Biochem. Biophys. 82*, 288–299.
Singer, T. P., & Kearney, E. G. (1954) *Adv. Enzymol. 15*, 79–139.
Weber, G. (1957) *Nature (London) 160*, 1409.
Yagi, M., Nlshi, N., Kinoshita, M., & Nagakura, S. (1976) *Chem. Phys. Lett. 39*, 240–244.

Purification and Partial Characterization of a Stimulatory Factor for Lamb Thymus RNA Polymerase II†

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ABSTRACT: A heat-stable protein (HSF) that stimulates the activity of lamb thymus RNA polymerase II has been purified 2500-fold and partially characterized. This factor stimulates the activity of RNA polymerase II up to 13 times and retains complete activity when heated at 90 °C for 5 min. Stimulation is observed only in the presence of RNA polymerase II and requires native DNA as template. The stimulatory factor has a sedimentation coefficient of 2.7 S, a diffusion coefficient of 9.55×10^{-7} cm^2/s, and an isoelectric point of 8.0. Calculated from the sedimentation and diffusion data, the factor has a molecular weight of about 24000. Electrophoresis of the purified factor on polyacrylamide gels in the presence of sodium dodecyl sulfate results in a single band corresponding to a molecular weight of 25000. The number-average length of the RNA synthesized by RNA polymerase II is increased in the presence of the factor. Sedimentation velocity and exclusion chromatography experiments suggest that the stimulatory factor interacts with RNA polymerase II. These results suggest that the factor stimulates RNA synthesis through a direct interaction with RNA polymerase II. The stoichiometry of the HSF–RNA polymerase binding appears to be about 1:1. HSF is located in the nucleus, as determined by cell fractionation studies.

Eucaryotic cells contain three distinct classes of DNA-dependent RNA polymerases, designated I, II, and III, as determined by their order of elution from DEAE-Sephadex and by their sensitivity to α-amanitin [for reviews see Chambon (1974) and Roeder (1976)]. Since RNA polymerase II appears to be involved in the production of hnRNA and mRNA (Roeder, 1976), an understanding of its structure and function is central to an understanding of the mechanism of gene regulation. RNA polymerase II does not appear to transcribe DNA in vitro asymmetrically or with high fidelity (Mandel & Chambon, 1974; Honjo & Reeder, 1974). In attempts to resolve this question, proteins have been described that stimulate the activity of RNA polymerase II in vitro (Stein & Hausen, 1970; Seifart, 1970; Lentfer & Lezius, 1972; Mondal et al., 1972; Lee & Dahmus, 1973; Sugden & Keller, 1973; Chuang & Chuang, 1975; Sekimizu et al., 1976; Benson et al., 1978). Based on ease of denaturation, these stimulatory factors can be separated into those that are stable to heat treatment at 90 °C for 5 min (Stein & Hausen, 1970; Lee & Dahmus, 1973; Stein et al., 1973; Seifart et al., 1973; Chuang & Chuang, 1975) and those that are heat labile (Lentfer & Lezius, 1972; Lee & Dahmus, 1973; Sugden & Keller, 1973; Dahmus, 1976; Sekimizu et al., 1976). This paper reports the purification and partial characterization of a heat-stable factor, designated HSF, from lamb thymus. Stein et al. (1973) and Seifart et al. (1973) have extensively purified factors that have small molecular weights and stimulate the elongation reaction of RNA polymerase II. HSF, a protein that appears to be similar to these proteins, has been characterized with respect

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