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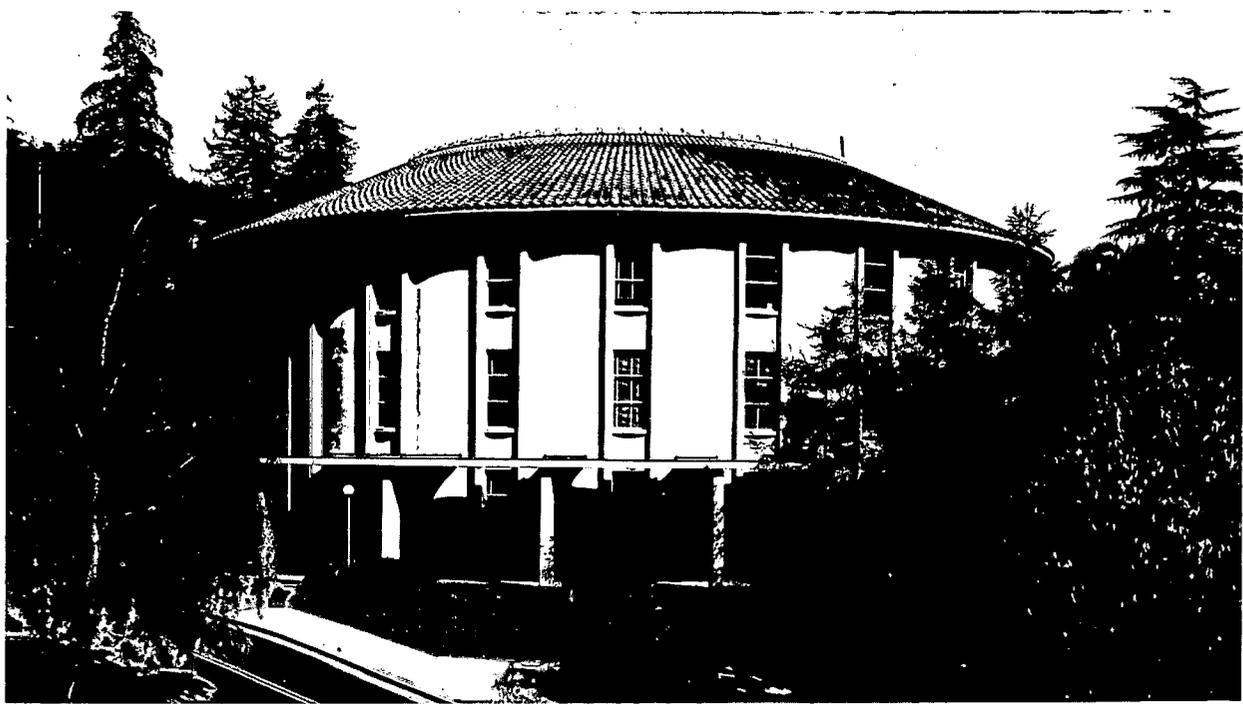## UNIVERSITY OF CALIFORNIA

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# Excited-State Dynamics of NADH and 1-N-propyl-1,4-dihydronicotinamide

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The reduced form of nicotinamide-adenine dinucleotide (NADH) is an important enzyme cofactor containing two chromophores - a dihydronicotinamide (lowest absorption band at 340 nm) and an adenine (absorption at 265 nm). Fluorescence of NADH has a λ_{max} at 460 nm and has been used for in vivo assays of NADH. The photophysics of NADH has been extensively studied [1-9]. In room temperature aqueous solution, the fluorescence quantum yield is 0.02 and the lifetime $\sim$ 0.40 ns [3-5]. Recently, biphotonic induced electron ejection by NADH has been reported [9]. NADH reportedly exists in aqueous solution in two conformations--extended and folded [3,5,10].

The present study reports time-resolved emission and transient absorption studies on the disodium salt of NADH and on a simple analog, 1-N-propyl-1,4-dihydronicotinamide (NPNH), (shown below) in several solvents.

NADH   NPNH
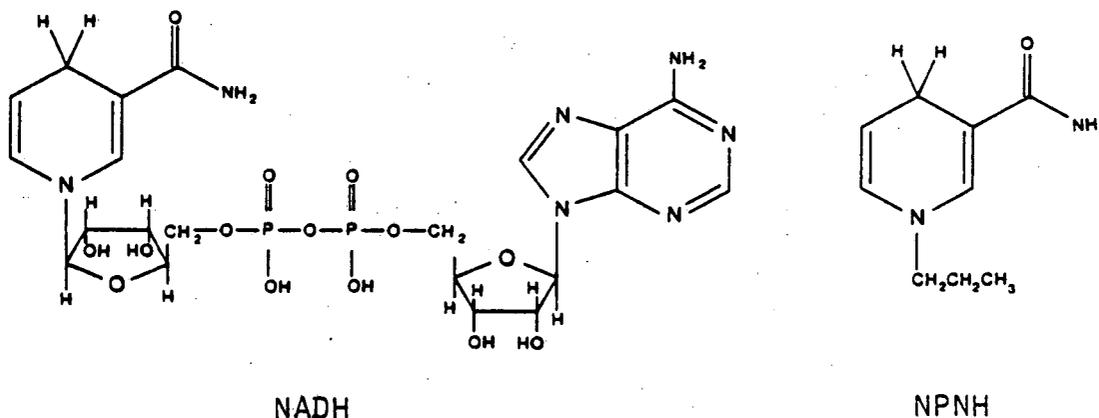
Purity of NADH (Boehringer-Mannheim) and of NPNH (prepared according to ANDERSON and BERKELHAMMER [11]) was tested using a reverse phase HPLC analysis, which showed for each a single peak with a homogeneous uv absorption spectrum. The photophysics of directly excited dihydronicotinamide was observed following excitation by a 355-nm pulse from a modelocked neodymium laser. Fluorescence decay kinetics were obtained using Nd:YAG laser excitation and 2-ps resolution streak camera detection [12]. Transient absorption spectra and kinetics were obtained using Nd:glass laser excitation and a picosecond continuum probe with polychromator/vidicon detection [13]. Energy transfer from adenine to dihydronicotinamide in NADH was studied by fluorescence kinetics after excitation at 266 nm.

Following excitation of NADH, the growth of a broad, unstructured absorption spectrum was observed (see Fig. 1). The similarity of these spectra to that of the solvated electron, e_{aq}^- [14], suggests this identification, but does not exclude a solvated electron-ion pair. A deconvolution analysis indicates a buildup time of 40 $\pm$ 10 ps for the transient
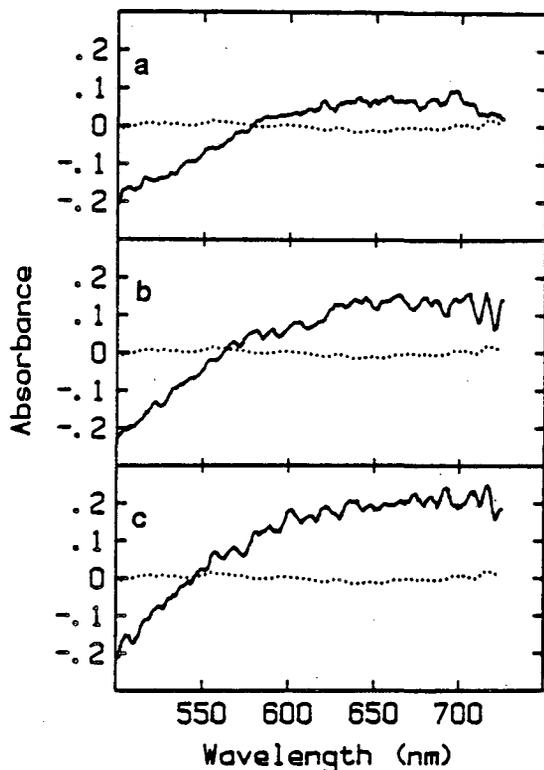
Figure 1. Transient absorption spectra of NADH in aqueous solution at room temperature taken at (a) 7 ps, (b) 20 ps, and (c) 376 ps after excitation at 355 nm. Apparent negative absorbance values at short wavelengths are due to sample fluorescence. Analysis shows that this spectrum grows linearly with intensity and not quadratically [9].

absorption spectrum. No perceptible decay occurs within 2 ns following excitation. Identical observations were made on NPNH. The spectral buildup rate is slower than electron solvation in water [14-16] and may be limited by electron ejection [17,18]. Since the electron ejection time does not match the known NADH fluorescence liftime [3-5], multiple species and decay routes must be involved. Using the known extinction coefficient for the solvated electron [19], the transient absorbance observed upon excitation of NADH with an actinometrically calibrated pulse gave a quantum yield for photon-induced electron ejection from NADH of ≈ 0.5. This yield is significantly higher than NADH photodecomposition [20], implying a high yield of ion recombination.

NADH fluorescence kinetics data with detection at 380 nm and 460 nm show a previously unreported, fast decaying emission component on the blue edge of the spectrum (see Fig. 2). Fluorescence kinetics detected at 420 to 700 nm yielded only longer fluorescence lifetimes and no risetime (<10 ps). The decay time of the fast component (≈ 30 ps) is similar to the absorption buildup. Fluorescence lifetimes of NADH and NPNH are summarized in the Table.

The fluorescence kinetics show these features: (1) The kinetics are relatively independent of excitation wavelength or the presence of buffer. (2) In D_2O and ethanol lifetimes are longer than in H_2O. Since the fluorescence decay kinetics following excitation at 266 nm and at 355 nm are similar, this fluorescence is ascribed to a "folded" conformation of NADH, which allows energy transfer from the adenine [3] . Therefore, folded forms of NADH exist in H_2O and D_2O and contribute to the longer-lived fluorescence. The results on NPNH suggest that open forms of NADH also contribute to this longer-lived fluorescence.

Figure 2. Fluorescence kinetics of NADH in aqueous solution at room temperature using excitation at 355 nm. Detection was at the indicated wavelengths through 10 nm wide bandpass filters. Shortlived fluorescence at 380 nm was also observed for NPNH in water and for both NADH and NPNH in methanol.

Table. Lifetimes of the longer-lived fluorescence of NADH and NPNH at room temperature broad-band detected.

Solute[1]	Solvent[2]	λ_{exc}	λ_{detect}	τ (ps)
NADH	H_2O	266	> 345 nm	470 ± 50
NADH	D_2O	266	> 345 nm	720 ± 70
NADH	H_2O/Buffer	266	> 345 nm	520 ± 50
NADH	H_2O/Buffer	355	> 375 nm	440 ± 40
NADH	D_2O/Buffer	355	> 375 nm	620 ± 60
NADH	H_2O	355	> 375 nm	450 ± 40
NADH	EtOH/Buffer	355	> 375 nm	850 ± 80
NPNH	H_2O/Buffer	355	> 375 nm	320 ± 30
NPNH	D_2O/Buffer	355	> 375 nm	460 ± 50
NPNH	EtOH/Buffer	355	> 375 nm	940 ± 90

[1]NADH = Reduced form of nicotinamide-adenine dinucleotide, NPNH = 1-N-propyl-1,4-dihydronicotinamide, [2]Buffer = 10^{-2} M Tris (pH 8.6 in water)

Possible explanations of the observed photophysics of NADH and NPNH include (1) two different excited-state forms of reduced nicotinamide arising from two ground state conformers or (2) a branched excited state decay mechanism involving two fluorescent forms. For case (1), the two planar rotamers of reduced nicotinamide with different amide orientations were investigated by INDO/CI M.O. calculations, which indicated a small energy difference between these two forms (~ 3kJ/mole). For case (2), an excited state mechanism could include the following kinetic scheme:

$$S_0 \xrightarrow{h\nu} \{S_1\} \xrightarrow{k_1} [D^{\cdot +}\cdots e^-] \xrightarrow{k_2} D^{\cdot +} + e^-_{aq}$$

S_1 is the relaxed, fluorescent, excited singlet state of reduced nicotinamide, $[D^{\bullet+}...e^-]$ is a solvated, nonfluorescent ion pair, and $D^{\bullet+} + e_{aq}^-$ are a separated radical cation and a solvated electron. Initially fluorescence comes from "vertically" excited molecules, $\{S_1\}$, with, for example, ground-state solvent organization or hydrogen bonding. $\{S_1\}$ could exhibit a slightly blue-shifted fluorescence, relative to S_1, with a lifetime determined by the sum of k_1 and k_3. The ion pair and the solvated electron may well have similar absorption spectra. Whether the equilibrium with constant K would need to be established during the lifetimes of S_1 and $[D^{\bullet+}...e^-]$ is unclear. However, this could explain the complex multiexponential decay reported for the longer lived fluorescence [4, 5]. If the lifetime of S_1 were determined by k_2, then the rate of ion pair separation would have to depend on solvent deuteration (see table). A high probability of recombination of $D^{\bullet+}$ with e_{aq}^- is consistent with its low reactivity observed in the gas phase. NPNH has an ionization potential < 8 eV, and molecular ion dominates its mass spectrum at low ionization energies. In a Fourier Transform Mass Spectrometer, $NPNH^{\bullet+}$ does not react with neutral NPNH or with 10^{-6} torr of NH_3 on the 0.1 s timescale (during which $NPNH^{\bullet+}$ experiences ~ 50 collisions with NH_3). Further work to refine the kinetic model is in progress.

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