Emission Properties of NADH. Studies of Fluorescence Lifetimes and Quantum Efficiencies of NADH, AcPyADH, and Simplified Synthetic Models[1]

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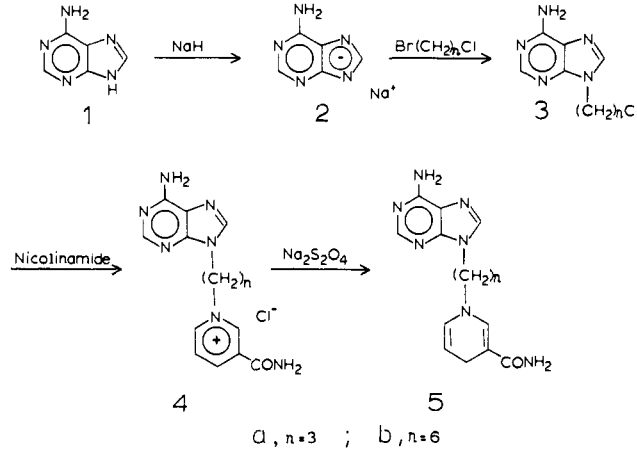
Abstract:[2] The fluorescence lifetimes τ and quantum efficiencies q of NADH, AcPyADH, and of the model compounds Ad-C_3-NicH and Ad-C_6-NicH have been measured in water and in 1,2-propanediol solution in the temperature range 0–30°. For Ad-C_3-NicH, NADH, and Ad-C_6-NicH at 25° in water the absolute quantum efficiencies are 0.035, 0.019, and 0.017, respectively, to a precision of a few per cent, and the lifetimes are 0.70, 0.40, and 0.28 ± 0.03 to 0.05 nsec The contribution of the ground to lowest singlet transition to the absorption spectrum has been evaluated by fluorescence polarization observations, and from these and the molecular fluorescence spectra, emissive lifetimes τ_e have been calculated by the equation of Strickler and Berg. For NADH and AcPyADH under various conditions, the relation $\tau = \tau_e \bar{q}$ is followed rigorously over a thirtyfold change in quantum efficiency. The absolute efficiency of quinine sulfate measured either by comparison with these derivatives or by the relation $\bar{q} = \tau/\tau_e$ turns out to be 0.70 ± 0.02 rather than the often quoted 0.55. The observed quantum efficiencies of energy transfer from the adenine to the dihydronicotinamide moiety were as follows for Ad-C_3-NicH, NADH, and Ad-C_6-NicH at 25° in aqueous solution: 0.44, 0.34, and 0.10, respectively. For these three compounds, comparison of the rates and of the energies of activation for radiationless transitions calculated from the temperature dependence of the lifetimes shows that the quenching processes are essentially identical in 1,2-propanediol but different in aqueous solution, indicating that interactions in water, but not in 1,2-propanediol, are characteristic and specific for each compound. The simplified spectroscopic models, Ad-C_3-NicH and Ad-C_6-NicH, which were designed to incorporate the absorption–emission chromophores of NADH by linking the adenyl and dihydronicotinamide moieties of NADH with triand hexamethylene chains, were prepared by dithionite reduction of the corresponding precursors possessing the nicotinamide ring in oxidized form.

Accumulated spectroscopic evidence[1a,3–18] indicates that coenzymes like NADH and FAD in aqueous solution tend to exist in "folded" or internally complexed forms (Figure 1) which resemble the "stacked" structures in the polynucleotides and nucleic acids. Because of the complicating features of the sugar and pyrophosphate moieties, full assessment of the factors that determine the folding can best be made by comparison with simpler models.[19] This paper describes the results of parallel observations of the fluorescence properties of NADH, AcPyADH, and two synthetic spectroscopic model compounds, Ad-C_3-NicH (**5a**) and Ad-C_6-NicH (**5b**).[2]



The reaction sequence for the synthesis of the model compounds is shown in formulas **1**–**5**. In brief, linkage of the adenyl and dihydronicotinamide moieties of NADH with tri- and hexamethylene chains was accomplished first by alkylation of sodium adenide (**2**) with

(1) (a) For the preceding paper on fluorescence lifetime studies, see R. D. Spencer and G. Weber, *Ann. N. Y. Acad. Sci.*, **158**, 361 (1969). (b) For the preceding paper (IV) in the series on "Synthetic Spectroscopic Models Related to Coenzymes and Base Pairs," see N. J. Leonard, J. Eisinger, and H. Iwamura, *Proc. Nat. Acad. Sci. U. S.*, **64**, 352 (1969). The present paper may be regarded as no. V in this series.

(2) Abbreviations used are as follows: Ad-C_3-NicH, 1-[3-(aden-9-yl)-propyl]-3-carbamoyl-1,4-dihydropyridine; Ad-C_6-NicH, 1-[6-(aden-9-yl)-hexyl]-3-carbamoyl-1,4-dihydropyridine; Ad-C_3-Nic$^+$, 1-[3-(aden-9-yl)-propyl]-3-carbamoylpyridinium chloride; Ad-C_6-Nic$^+$, 1-[6-(aden-9-yl)-hexyl]-3-carbamoyl pyridinium chloride; AcPyADH, reduced acetylpyridine-adenine dinucleotide; NADH may be represented as Ad-rib-pp-rib-NicH; P.G., propylene glycol = 1,2-propanediol.

(3) G. Weber, *Biochem. J.*, **47**, 114 (1950).
(4) G. Weber, *Nature*, **180**, 1409 (1957).
(5) G. Weber, *J. Chim. Phys.*, **55**, 878 (1958).
(6) S. F. Velick, *J. Biol. Chem.*, **233**, 1455 (1958).
(7) S. Shifrin and N. O. Kaplan, *Nature*, **183**, 1529 (1959).
(8) S. Shifrin and N. O. Kaplan, *Advan. Enzymol.*, **22**, 337 (1960).
(9) S. Shifrin and N. O. Kaplan in "Light and Life," W. D. McElroy and B. Glass, Ed., Johns Hopkins Press, Baltimore, Md., 1961, p 144.
(10) G. Czerlinski and F. Hommes, *Biochim. Biophys. Acta*, **79**, 46 (1964).
(11) S. F. Velick in "Light and Life," W. D. McElroy and B. Glass, Ed., Johns Hopkins Press, Baltimore, Md., 1961, p 108.
(12) O. Jardetzky and N. G. Wade-Jardetzky, *J. Biol. Chem.*, **241**, 85 (1966).
(13) K. Suzuki, H. Nakano, and S. Suzuki, *ibid.*, **242**, 3319 (1967).
(14) S. Freed, E. A. Neyfakh, and L. A. Tumerman, *Biochim. Biophys. Acta*, **143**, 432 (1967).
(15) D. W. Miles and D. W. Urry, *Biochemistry*, **7**, 2791 (1968).
(16) R. H. Sarma, V. Ross, and N. O. Kaplan, *ibid.*, **7**, 3052 (1968).
(17) R. H. Sarma and N. O. Kaplan, *J. Biol. Chem.*, **244**, 771 (1969).
(18) D. J. Patel, *Nature*, **221**, 1239 (1969).

(19) The value of simplified synthetic models, *e.g.*, Ad-C_n-Nic$^+$,[2] in the understanding of the electronic absorption of NAD$^+$ and of other *n*-methylene bridged models related to FAD and nucleic acid systems has been demonstrated in this Laboratory: (a) N. J. Leonard, T. G. Scott, and P. C. Huang, *J. Amer. Chem. Soc.*, **89**, 7137 (1967); (b) D. T. Browne, J. Eisinger, and N. J. Leonard, *ibid.*, **90**, 7302 (1968); (c) N. J. Leonard, K. Golankiewicz, R. S. McCredie, S. M. Johnson, and I. C. Paul, *ibid.*, **91**, 5855 (1969); (d) N. J. Leonard and R. F. Lambert, *J. Org. Chem.*, **34**, 3240 (1969). R. F. Lambert, Ph.D. Thesis, University of Illinois, 1967.

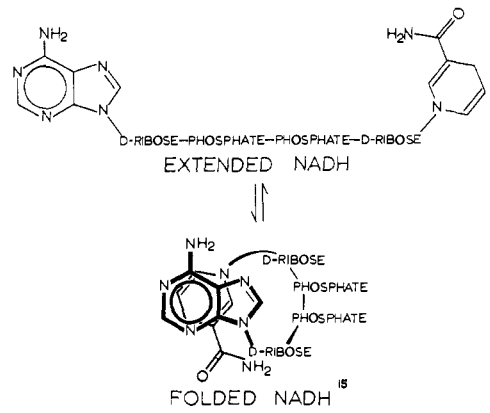


Figure 1. Limiting conformations for NADH.

the relevant 1-bromo-*n*-chloroalkane.[20,21] This was followed by treatment of the chloroalkylated product **3** with excess nicotinamide in a melt, giving Ad-C_n-Nic$^+$ (**4**). Dithionite reduction of **4** afforded Ad-C_n-NicH (**5**), spectroscopic models in which the absorption and emission properties can be directly related to 1:1 interactions between the heterocyclic rings when these properties are observed at concentrations sufficiently dilute to obviate intermolecular interactions.

The absolute fluorescence lifetimes and yields of the compounds mentioned above have been measured both in water and in 1,2-propanediol solutions, and, in the cases of NADH and AcPyADH, bound to the enzyme lactic dehydrogenase. The studies were made over a temperature range of 0–30° to obtain information on the absolute rate of radiationless transitions from the lowest singlet state. Apart from fixing the values of these and the lifetimes of the excited state with all the accuracy required for practical purposes, these studies have shed new light on the questions of absolute fluorescence efficiencies in solution and of the importance of water in the molecular complexes.

Experimental Section[22]

General. The previously described preparation of the intermediate 9-(ω-chloroalkyl)adenines (**4**) *via* the sodium adenide route was used.[20,21]

Preparation of Ad-C_n-Nic$^+$,[23,24] Model Compound Intermediates. The appropriate 9-(ω-chloroalkyl)adenine (2 mmol) was mixed thoroughly with nicotinamide (6 mmol). The system was flushed with nitrogen and sealed under vacuum. The reaction mixture was heated (*gradually*) to fusion in an oil bath, and was stirred for 2 hr at the temperature of fusion (generally between 120 and 140°). Upon cooling, an intractable solid was obtained, to which hot 95% alcohol was added to effect solution. The solution was evaporated at reduced pressure to a light yellow granular solid which was dissolved in 10 ml of water. The solution was applied to a 140 g Cellex-P (triethylammonium form) column (4 × 65 cm). The column was eluted with water (900 ml) and with 0.1 *M* triethylammonium bicarbonate (aqueous; pH 8.0). Twenty-milliliter fractions were collected. Those fractions with ultraviolet absorption maxima between 259 and 263 nm in acidic, neutral, and basic aqueous media with only minor changes in absorption coefficient were pooled, and the solvent was removed by rotary evaporation at a temperature maintained below 30°. The residue was dissolved in water (20 ml) and passed through a column (2 × 20 cm) of Dowex 1-X8 (Cl^-). The solution was treated with activated carbon, evaporated to dryness, and the product was recrystallized from water containing a few drops of methanol. Yields of analytically pure material were 26% for **5a** and 36% for **5b**.

1-[3-(Aden-9-yl)propyl]-3-carbamoylpyridinium Chloride (Ad-C_3-Nic$^+$, 4a): mp 255–256°; λ_{max}^{water} (pH 7.0) 261.5 nm (ε 15,900), (pH 1.0) 259.5 nm (ε 17,800), (pH 10.0) 261.5 nm (ε 15,900); nmr (0.8 *M* in D_2O, external TMS) τ 7.33 (p, 2, *J* = 7 Hz, C-CH_2-C), 5.70 (t, 2, *J* = 7 Hz, Ad-CH_2), 5.20 (t, 2, *J* = 7 Hz, Nic$^+$-CH_2), 213 (s, 1, adenine H-8), 2.01 (s, 1, adenine H-2), 1.95 (m, 1, *J* = 7 Hz, nicotinamide H-5), 1.17 (d, 1, *J* = 8 Hz, nicotinamide H-4), 1.03 (d, 1, *J* = 6 Hz, nicotinamide H-6), 0.83 (s, 1, nicotinamide H-2).

Anal. Calcd for $C_{14}H_{16}ClN_7O$:[25] C, 50.38; H, 4.83; N, 29.38. Found: C, 50.62; H, 5.00; N, 29.05. Calcd for $C_{14}H_{16}ClN_7O\cdot 3H_2O$: C, 43.35; H, 5.71; N, 25.28. Found: C, 43.22; H, 5.63; N, 25.27.

1-[6-(Aden-9-yl)hexyl]-3-carbamoylpyridinium Chloride (Ad-C_6-Nic$^+$, 4b): mp 233–235°; λ_{max}^{water} (pH 7.0) 263 nm (ε 17,700), (pH 1.0) 262, (pH 10.0) 263 (17,900); nmr (0.2 *M* in D_2O) τ 8.77 (m, 4, $-CH_2CH_2CH_2CH_2CH_2CH_2-$), τ 8.27 (m, 2, Ad-$CH_2CH_2-$), 7.97 (m, 2, Nic$^+$-$CH_2CH_2$), 5.97 (m, 2, Ad-C$H_2$-), 5.37 (m, 2, Nic$^+$-C$H_2$), 2.06 (s, 1), 2.03 (s, 1), 1.86 (q, 1), 1.17 (m, 1), 1.03 (m, 1), and 0.79 ppm (s, 1).

Anal. Calcd for $C_{17}H_{22}ClN_7O\cdot 2H_2O$: C, 49.57; H, 6.36; N, 23.81. Found: C, 49.67; H, 6.10; N, 24.01.

1-[3-(Aden-9-yl)propyl]-3-carbamoyl-1,4-dihydropyridine (Ad-C_3-NicH, 5a). To a saturated (at 0°) solution of sodium bicarbonate through which nitrogen was being bubbled, AdC_3Nic^+ (18 mg; 0.047 mmol based on trihydrate) was added. After 5 min sodium dithionite (13.3 mg, 0.076 mmol) was added,[26] and the system became yellow. The reaction was allowed to proceed at ambient temperature under a nitrogen atmosphere for 20 min, followed by 3 hr at 0°. Half-ml aliquots were removed and extracted with chloroform (3 × 5 ml). The organic phase was evaporated at diminished pressure at 0° and the light yellow product was stored at −5° under nitrogen; mass spectrum (70 eV) *m/e* (rel intensity) 299 [6, $(AdC_3NicH)^+$], 298 (2, AdC_3Nic^+), 254 [12, $(AdC_3Nic^+-CONH_2)$], 176 (38, $AdC_3H_7^+$), 149 (100, $AdCH_3^+$), 148 (60, $AdCH_2^+$), 135 (25, AdH^+), 122 (15, Nic^+), 44 [43, $(CONH_2)^+$].

1-[6-(Aden-9-yl)hexyl]-3-carbamoyl-1,4-dihydropyridine (Ad-C_6-NicH, 5b). The preparation was carried out on a 0.05 millimolar scale following the above procedure for Ad-C_3-NicH except that methanol was used for the extracting solvent; mass spectrum (70 eV) *m/e* (rel intensity) 341 [3; M^+, $(AdC_6NicH)^+$], 296 [26, $(M-1-CONH_2)^+$], 219 (13, $AdC_6H_{13}^+$), 205 (10, $AdC_5H_{11}^+$), 190 (27, $AdC_4H_8^+$), 176 (15, $AdC_3H_6^+$), 162 (24, $AdC_2H_4^+$), 149 (32, $AdCH_3^+$), 135 (28, AdH^+), 122 (13, Nic^+), 44 [93, $(CONH_2)^+$ or $C_3H_8^+$].

When the dihydronicotinamide compounds were maintained under nitrogen at 0° and were used directly in the spectroscopic measurements, no appreciable decomposition was observed (by tlc and uv).

Coenzymes. The coenzyme NADH was obtained from Sigma Chemical Company. Reduced acetylpyridine-adenine dinucleotide (AcPyADH) from P-L Laboratories was purified on a DEAE cellulose column.[27]

Solutions. All aqueous solutions of the dihydronicotinamide compounds were prepared in glass-distilled water with 0.1 *M* tris(hydroxymethyl)aminomethane (purchased from Boehringer and Soehne, Mannheim, Germany, and recrystallized from aqueous ethanol), which was adjusted to pH 8.0 with acetic acid. All solu-

(20) N. J. Leonard, K. L. Carraway, and J. P. Helgeson, *J. Heterocycl. Chem.*, 2, 291 (1965).

(21) K. L. Carraway, P. C. Huang, and T. G. Scott in "Synthetic Procedures in Nucleic Acid Chemistry," Vol. I, W. W. Zorbach and R. S. Tipson, Ed., Interscience Publishers—John Wiley and Sons, Inc., New York, N. Y., 1968, p 3.

(22) Melting points are corrected. Electronic absorption spectra were recorded on a Cary 15 spectrophotometer. Nuclear magnetic resonance spectra were determined on a Varian A-60 or A-60A spectrometer. Mass spectra were determined by the direct inlet technique in an Atlas CH4 low resolution spectrometer.

(23) The lower representatives of this series (*n* = 2,3) have been reported in an earlier communication.[19a]

(24) P. C. Huang, Ph.D. Thesis, University of Illinois, 1966.

(25) The degree of hydration was dependent upon the drying technique employed: *e.g.*, the trihydrate was obtained simply by drying in a vacuum desiccator. The anhydrous material was obtained for microanalysis by warming the trihydrate for a brief period in the tared weighing boat on a hot plate prior to final weighing and combustion. We are grateful to G. Maciak of Midwest Microlab, Inc., Indianapolis, Ind., for the analysis of anhydrous **4a**.

(26) M. E. Pullman, A. San Pietro, and S. P. Colowick, *J. Biol. Chem.*, 206, 129 (1954).

(27) G. W. Rafter and S. P. Colowick, *Methods Enzymol.*, 3, 887 (1957).

Table I. Characteristics of Absorption and Emission Spectra[a]

	Absorption			Emission		
	Maximum, nm	Bandwidth[b] nm	Bandwidth[b] 10^{-3} cm^{-1}	Maximum, nm	Bandwidth[b] nm	Bandwidth[b] 10^{-3} cm^{-1}
NADH, in H_2O	340	310–368	5.1	470	426–540	4.9
NADH, in P.G.	340	310–368	5.1	450	412–520	5.0
NADH + LDH	335	308–364	5.0	440	402–506	5.1
NADH + LDH + Ox	340	310–368	5.1	430	392–482	4.8
Ad-C_3-NicH, in H_2O	360	329–388	4.6	465	428–532	4.6
Ad-C_3-NicH, in P.G.	355	324–384	4.8	455	418–520	4.7
Ad-C_6-NicH, in H_2O	360	330–391	4.7	475	432–545	4.8
Ad-C_6-NicH, in P.G.	355	325–386	4.9	455	420–518	4.5
PcPyADH	365	330–392	4.8	495	445–566	4.8
AcPyADH + LDH	355	325–380	4.5	455	418–520	4.7
AcPyADH + LDH + Ox	360	330–387	4.5	445	412–503	4.4
Quinine Sulfate				455	418–512	4.4

[a] Values quoted for 25°. [b] Bandwidth at half height.

tions in 1,2-propanediol (P.G.) were neutralized with 0.01 *M* sodium bicarbonate. All preparations of the coenzymes with the enzyme chicken heart lactic dehydrogenase (LDH) were prepared in 0.1 *M* phosphate buffer, pH 7.0, according to the techniques of Anderson and Weber.[28] The enzyme–coenzyme complexes were prepared with a molar ratio of 1:1 and a concentration of 1×10^{-5} *M* and are abbreviated in this paper as "LDH + AcPyADH" or "LDH + NADH." Those preparations containing a saturating level of 0.1 *M* oxalate are labeled "LDH + AcPyADH + Ox" or "LDH + NADH + Ox."

Instrumentation. The absorption spectra of NADH and the model compounds were determined on a Cary 15 spectrophotometer.

Technical spectra of the fluorescence emission were measured on a sophisticated digital spectrofluorometer. In this instrument, the ratio of the fluorescence intensity to the exciting light intensity was measured by a Dana ratio digital voltmeter and recorded on paper tape by a Tally perforator. An IBM 1800 computer averaged the data on the tapes and printed the technical spectra. The final molecular spectra were produced by an IBM 360 computer which was programmed to employ a modification of the technique of Teale and Weber[29] in correcting technical spectra for photomultiplier and optical responses as a function of wavelength. This digital data processing enabled the measurement of relative intensities across a technical spectrum within 0.003 volts of 10 volts full scale, or within 0.03% of the full scale of the instrument. Thereby, a precision of about 1% was achieved for relative quantum efficiencies from integrated molecular spectra which differed in intensity as much as tenfold.

Excitation polarization spectra of fluorescence were measured on an analog-digital version of the instrument described by Weber and Bablouzian.[30] The exciting light was monochromatic with a bandwidth of 2–3 nm. The fluorescence emission was filtered with a Corning filter CS 373 and a 2 *M* sodium nitrite solution.

Fluorescence lifetimes were determined from phase measurements on the cross correlation phase-modulation spectrofluorometer previously described.[1a,31] The exciting light was modulated at a frequency of 28.4 MHz. Such measurements have been shown to be accurate to at least $\pm 0.03 \times 10^{-9}$ seconds by optical delay calibrations. The wavelength of the exciting light with a 6-nm bandwidth was determined by a Jarrel-Ash (Model 82-410) monochromator. Fluorescence emission was selected by a Corning filter CS 373 and a 2 *M* sodium nitrite solution.

The extinction coefficients of Ad-C_3-NicH and Ad-C_6-NicH in aqueous solution were determined by oxidative titration with potassium ferricyanide.[5] For each compound, five different volumes of the dihydronicotinamide in pH 9.0 aqueous buffer were oxidized by addition of 3 ml of 10^{-3} *M* potassium ferricyanide. After equalizing the volumes with buffer, the solutions were incubated for one hour. The concentration of the unreduced ferricyanide was determined spectrophotometrically at 420 nm using a molar absorption coefficient of 1040 cm^2/m*M*. The slope of a plot of the OD_{420} *vs.* volume of nicotinamide solution added provided an accurate measure of the milliequivalents of ferricyanide consumed per milliliter of the dihydronicotinamide solution, and thus a measure of the actual concentration of the dihydronicotinamide. The extinction coefficient of the dihydronicotinamide was calculated from this experimental concentration and the measured optical density of the original nicotinamide solutions.

The molar extinction coefficients of the compounds in other environments, such as in 1,2-propanediol or bound to the enzyme lactic dehydrogenase (LDH), were determined by measurements of the optical densities relative to the aqueous solutions. In the determinations for NADH, Ad-C_3-NicH, and Ad-C_6-NicH in 1,2-propanediol, a concentrated aqueous solution (pH 8.0) of the dihydronicotinamide was diluted volumetrically $^1/_{50}$ by 1,2-propanediol and also by aqueous buffer. Using this procedure, the optical density of NADH was observed to increase by a factor of 1.037 in 1,2-propanediol, and the optical densities of Ad-C_3-NicH and Ad-C_6-NicH increased by 1.08. When samples of Sigma Grade NADH were dissolved separately in aqueous buffer and in 1,2-propanediol, the measured optical density of NADH in 1,2-propanediol was 1.041 times that in water.

In all corrected fluorescence spectra the ordinates are proportional to quanta per unit wavelength interval.

Results and Discussion

Absorption and Emission Spectra. The details of the maxima and half-bandwidths of the absorption and emission spectra of the dihydronicotinamide coenzymes and models in water and in 1,2-propanediol are presented in Table I. The NADH spectra are shown in Figure 2, and the positions of the maxima and the relative fluorescence intensities for NADH, Ad-C_3-NicH, and Ad-C_6-NicH are compared in Figure 3. The extinction coefficients of Ad-C_3-NicH and Ad-C_6-NicH in aqueous solution were determined by oxidative titration with potassium ferricyanide. This technique has the advantage of obtaining the concentration of a dihydronicotinamide in the presence of nonoxidizable contaminants, such as the oxidized form of the nicotinamide (Nic^+). Furthermore, the oxidized form does not absorb in the 340–360 nm absorption region where NicH absorbs, so that the experimental molar absorption coefficient is reliable. The molar absorption coefficients of the dihydronicotinamide compounds are assembled in Table II.

Separation of the Lowest Singlet Transition in the Absorption Spectrum. Since the 350-nm electronic band of dihydronicotinamide must be integrated in emissive lifetime calculations, its absorption spectrum

(28) S. R. Anderson and G. Weber, *Biochemistry*, 4, 1948 (1965).
(29) F. W. J. Teale and G. Weber, *Biochem. J.*, 65, 476 (1957).
(30) G. Weber and B. Bablouzian, *J. Biol. Chem.*, 241, 2558 (1966).
(31) R. D. Spencer, W. M. Vaughn, and G. Weber in "Molecular Luminescence," E. C. Lim, Ed., W. A. Benjamin, Inc., New York, N. Y., 1969, p 607.

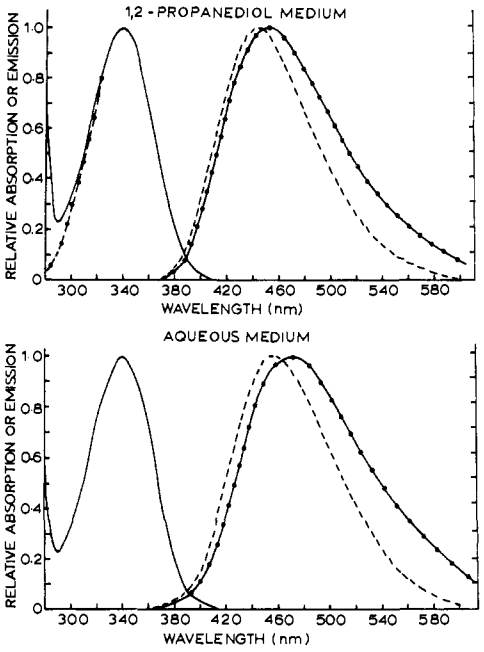


Figure 2. The absorption and emission spectra of NADH. Absorption spectrum (———); a technical emission spectrum (- - - - - - - - - -); corrected molecular emission spectrum (-●-●-●-●-); definition of absorption band of 340 nm transition from anisotropy measurements (—○—○—○—○—).

must be distinguished from absorption by other overlapping electronic bands. This distinction can be achieved by means of the absorption polarization spectrum of the fluorescence. This is the set of polarization values obtained when the excitation wavelength is

Table II. Molar Absorption Coefficients ($\epsilon \times 10^{-3}$)[a]

Solvent	NADH	AcPy-ADH	Ad-C_3-NicH	Ad-C_6-NicH
H_2O	6.2[b]	9.1[c]	6.3	7.6
1,2-Propanediol	6.4		6.8	8.2
LDH (1:1 Complex)	6.2	9.1		
LDH (1:1 Complex) + Oxalate	5.7	8.8		

[a] Values measured at the wavelengths of maximum absorption presented in Table I. [b] J. M. Siegal, G. A. Montgomery, and R. M. Bock, *Arch. Biochem. Biophys.*, **82**, 288 (1959). [c] N. O. Kaplan and M. M. Ciotti, *J. Biol. Chem.*, **221**, 823 (1956).

varied and a constant fluorescence band is observed. If this spectrum consists of regions of constant polarization joined by regions of rapidly changing polarization, it is reasonable to assume that the former correspond to a single electronic transition while the latter result from the overlap of contiguous transitions. Separation of these contiguous transitions may then be achieved by application of the additivity of the anisotropies of emission[32]

$$\bar{A} = \sum f_i A_i$$

where $\bar{A} = (1/\bar{P}_0 = -\frac{1}{3})^{-1}$ is the average or observed anisotropy, A_i the anisotropies of the components and f_i the relative contributions of the components to the total intensity of fluorescence. If the fraction of the

(32) G. Weber, *Biochem. J.*, **51**, 145 (1952).

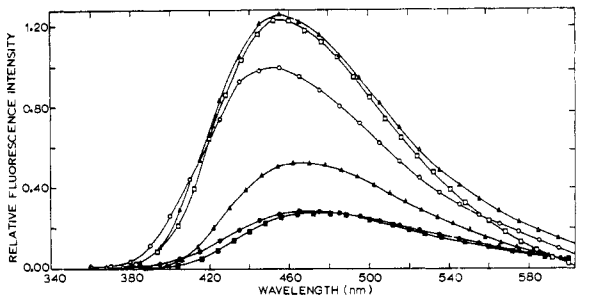


Figure 3. Fluorescence emission spectra of NADH and models. In aqueous buffer, Ad-C_3-NicH (—▲—▲—▲—▲—); NADH (—●—●—●—●—); Ad-C_6-NiCH (—■—■—■—■—). In P.G., Ad-C_3-NicH (—△—△—△—△—); NADH (—○—○—○—○—); Ad-C_6-NicH (—□—□—□—□—).

total exciting light absorbed by component i is Φ_i and the fraction of the absorptions converted to fluorescence emission is q_i, then $f_i = \Phi_i q_i / \Sigma \Phi_i q_i$ and

$$\bar{A} = \sum \Phi_i q_i A_i / \sum \Phi_i q_i$$

If two contiguous electronic bands a and b are responsible for the fluorescence,

$$\Phi_a + \Phi_b = 1$$

The average fluorescence yield $\langle q(\lambda) \rangle$ at any wavelength is

$$\langle q(\lambda) \rangle = \Phi_a q_a + \Phi_b q_b$$

and the last equation gives

$$\Phi_a = \frac{\bar{A}\,\langle q(\lambda) \rangle - A_b q_b}{A_a q_a - A_a q_b} \qquad (1)$$

In the case of most compounds in solution the fluorescence yield does not vary with wavelength so that eq 1 reduces to

$$\Phi_a = \frac{\bar{A} - A_b}{A_a - A_b} \qquad (2)$$

In our nicotinamide derivatives the simpler equation (2) is not valid. Electronic energy transfer from adenine to nicotinamide is 34% efficient in NADH in water and less or more efficient in the other compounds (see below). However, comparison of the results of eq 1 and 2 for these cases shows that the error introduced in using (2) is only a few per cent. This results from the reasonable separation already existing between the electronic bands. In cases of large overlap, the result would have been different.

Figure 4 shows the spectra of the measured anisotropies for NADH, Ad-C_3-NicH, and Ad-C_6-NicH in 1,2-propanediol at 25°. The anisotropies at 350 nm and 260 nm where the values are relatively constant were used for A_a and A_b, respectively, in calculating Φ_a at wavelength increments across the absorption spectra. The corrected absorption spectrum for the 340 nm oscillator of NADH in 1,2-propanediol is included in Figure 2.

Emissive Lifetimes. Equation 3, which has been used by Perrin[33a] and by Förster[33b]

$$1/\tau_e = 2.88 \times 10^{-9} \eta^2 \bar{\nu}_a^2 \int \epsilon(\bar{\nu})\, d\bar{\nu} \qquad (3)$$

(33) (a) F. Perrin, *J. Phys.* (Paris), 7, 390 (1926); (b) T. Förster, "Fluorescenz organischer Verbindungen," Vandenhoeck and Ruprecht, Göttingen, 1951, p 154.

is known to provide a useful approximation for obtaining the emissive lifetimes. More recently, Strickler and Berg[34] have proposed a modification which should be considerably more accurate, namely

$$1/\tau_e = 2.88 \times 10^{-9}\eta^2\langle\nu_f^{-3}\rangle_{AV}^{-1}\int\epsilon(\bar{\nu})\,d\ln\bar{\nu} \quad (4)$$

In the above equations, η is the refractive index of the solvent, $\bar{\nu}_a$ is the wave number of maximum absorption, ϵ is the molar extinction coefficient, and $\langle\bar{\nu}_f^{-3}\rangle_{AV}$ is the mean value of $\bar{\nu}_f^{-3}$ in the fluorescence spectrum.

The spectra of the longest wavelength electronic transition (Figures 1 and 2), the determined molar extinction coefficients (Table II), and the corrected fluorescence spectra (Figure 1) were used to calculate the emissive lifetimes of the dihydronicotinamide compounds. Table III shows the results obtained by use of the Strickler and Berg equation. Calculations by eq 3 yield lifetime values which are nearly 40% shorter than those shown in the table. The difference results from the inclusion in the Strickler and Berg equation of the properties of the emission so that shifts in the fluorescence spectrum have a noticeable effect upon the calculated emissive lifetime.

Table III. Lifetimes and Absolute Quantum Efficiencies

Compound	Solvent	$\langle\tau_p\rangle$,[a] nsec	τ_e,[b] nsec	$\langle\tau_p\rangle/\tau_e$
Ad-C_3-NicH	H_2O	0.70	20.2	0.035
	P.G.	1.43	15.0	0.096
NADH	H_2O	0.40	20.6	0.019
	P.G.	1.24	15.6	0.080
Ad-C_6-NicH	H_2O	0.28	16.9	0.017
	P.G.	1.21	12.1	0.10
NADH + LDH		1.50	15.3	0.099
NADH + LDH + Ox		6.53	14.5	0.45
AcPyADH		0.21	16.2	0.013
AcPyADH + LDH		2.09	11.8	0.18
AcPyADH + LDH + Ox		5.19	11.6	0.45

[a] Values determined at 25°. [b] Calculated by the Strickler and Berg equation.[34]

Dynamic Quenching of Dihydronicotinamide. The observed yield $\bar{q}$ and the lifetime $\langle\tau_p\rangle$ calculated from the phase angle are average values that must be considered dependent, in principle, upon an unknown number of components present in solution. If the ith component present as a fraction f_i of the total excited population has rate of emission λ_i and overall rate of radiationless decay k_i, its quantum yield and lifetime are given by

$$q_i = \frac{\lambda_i}{\lambda_i + k_i} \text{ and } \tau_i = \frac{1}{\lambda_i + k_i} \quad (5)$$

The average value of the observed fluorescence yield is

$$\bar{q} = \sum f_i q_i$$

On the other hand, in the phase measurements the components are weighted according to the degree of modulation of each, and therefore according to the lifetime itself. This weighting factor in the phase measurements is

$$\eta_i = 1/(1 + \omega^2\tau_i^2)$$

where ω is the circular modulation frequency of the exciting light. Therefore, the lifetime calculated from phase angle is

$$\langle\tau_p\rangle = \frac{\sum f_i q_i \eta_i \tau_i}{\sum f_i q_i \eta_i} \quad (6)$$

Introducing (5) into (6) and setting $h_i = f_i\eta_i$ the mean lifetime is

$$\langle\tau_p\rangle = \frac{\sum h_i q_i^2/\lambda_i}{\sum h_i q_i} \quad (7)$$

The emissive lifetime, $\tau_{ei} = 1/\lambda_i$, of the ith component is directly dependent upon the oscillator strength of emission.[33b,34] Therefore, in a given medium, if absorption and fluorescence spectra of components contributing significantly to the fluorescence are identical, as can be verified by the rank analysis technique,[35] then the emissive lifetimes of the major components all equal $\tau_e = 1/\lambda$. Moreover, writing $q_i = \delta q_i + \bar{q}$ where δq_i is the deviation from the mean yield

$$\sum h_i q_i^2 = \sum h_i(\bar{q}^2 + 2\bar{q}\,\delta q_i + \delta q_i^2) = \bar{q}^2 + \sum h_i \delta q_i^2$$

and eq 7 becomes

$$\langle\tau_p\rangle = \tau_e\bar{q}\left(1 + \frac{\overline{\Delta q^2}}{\bar{q}^2}\right) \quad (8)$$

with $\overline{\Delta q^2} = \Sigma h_i \delta q_i^2$

The experimental verification of the relation

$$\langle\tau_p\rangle/\tau_e = \bar{q} \quad (9)$$

implies that the quantity $\overline{\Delta q^2}/\bar{q}^2$ is smaller than the standard deviation of the measurements and imposes an upper limit to the heterogeneity of the system.

It follows from this that if the fluorophor were examined in different solvents or placed in different environments, a plot of the absolute quantum efficiency measured from lifetimes *vs.* the relative fluorescence intensities measured from integrated emission should reveal static quenching.[36] Figure 5 shows such a graph

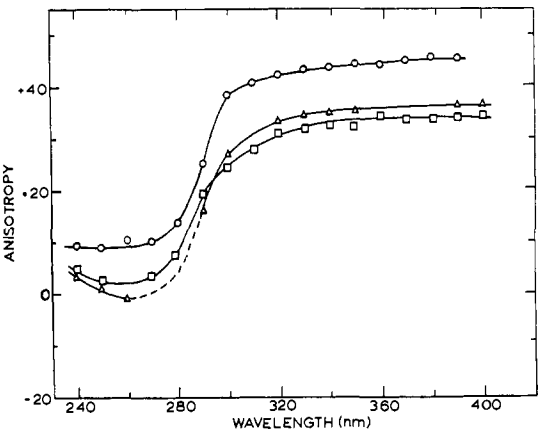


Figure 4. Absorption anisotropy spectrum of fluorescence for NADH (—○—○—○—○—), Ad-C_3-NicH (—△—△—△—△—), and Ad-C_6-NicH (—□—□—□—□—) at 20°.

(34) S. J. Strickler and R. Berg, *J. Chem. Phys.*, 37, 814 (1962).

(35) G. Weber, *Nature*, **190**, 27 (1961).

(36) If, predating the excitation, molecular complexes are formed with solvent or with another partner, and these complexes have negligible fluorescence efficiency because of a very rapid rate of deactivation compared to emission rate, the quenching has a static character. In static quenching the yield is determined at the instant of absorption by the degree of dissociation of the complexes. In dynamic quenching the excited molecules are all potentially equally capable of fluorescence, and

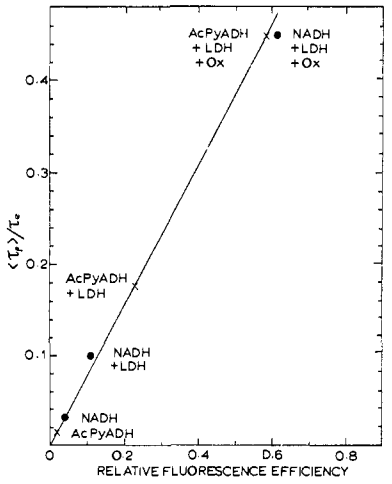


Figure 5. A comparison of absolute quantum efficiency calculated from the $<\tau_p>/\tau_e$ values of Table III with the measured relative quantum efficiencies determined from integrated molecular emission spectra for NADH and AcPyADH in various conditions: (1) free in solution, (2) bound to LDH, and (3) bound to LDH in the presence of a bound but unreactive substrate.

of the ratio $\langle\tau_p\rangle/\tau_e$ *vs.* the fluorescence intensity (relative to quinine sulfate fluorescence) for NADH and AcPyADH in aqueous solution, bound to the enzyme lactic dehydrogenase (LDH), and bound to LDH saturated with the nonreactive substrate oxalate. The points lie on a straight line, in agreement with eq 9. The linearity indicates that any static quenching of the dihydronicotinamide must occur in the same proportion in all three environments. Since it is highly unlikely that a "dark" state of the fluorophor would be the same in each environment, the conclusion follows that there is no static quenching of the dihydronicotinamide compounds.

A similar conclusion was reached in a solvent study of the compounds NADH, Ad-C_3-NicH, and Ad-C_6-NicH in aqueous buffer (pH 8.0) and in 1,2-propanediol. The intensities, relative to NADH in 1,2-propanediol = 1.00, obtained from the integrated corrected fluorescence spectra increased or decreased by nearly the same factor as the relative quantum efficiencies calculated from $\langle\tau_p\rangle/\tau_e$ values (Table IV).

Table IV. Relative Quantum Efficiencies[a]

Compound	Solvent	From lifetimes	From spectra
Ad-C_3-NicH	H_2O	0.44	0.47
	P.G.	1.20	1.20
NADH	H_2O	0.24	0.29
	P.G.	1.00	1.00
Ad-C_6-NicH	H_2O	0.21	0.24
	P.G.	1.26	1.18

[a] Relative to NADH in 1,2-propanediol.

In all studies a definite trend is that both lifetime and quantum efficiency change by the same factor with change in temperature. This finding may be quite important in the interpretation of kinetic studies of the dehydrogenases in which the temperature dependence of the fluorescence of NADH is measured, but where facilities for accurate measurement of short lifetimes are lacking.

The agreements of the changes in $\langle\tau_p\rangle/\tau_e$ values with relative fluorescence yields presented above indicate that the Strickler and Berg equation is accurate in predicting the emissive lifetimes of these coenzymes which have notably broad absorption and emission bands. In fact, large deviations from eq 9 are observed only if the measured lifetimes are compared with the relative fluorescence without the corrections for the change of τ_e in the different environments; *i.e.*, the *simple relation* of $\bar{q} \propto \langle\tau_p\rangle$ *is not observed*, while the *dynamic relation* $\bar{q} = \langle\tau_p\rangle/\tau_e$ *is accurately followed.*

Absolute Quantum Efficiency of Dihydronicotinamide Coenzymes and Models. Since the integrated fluorescence intensities of the coenzymes do adhere to the relation $\bar{q} = \langle\tau_p\rangle/\tau_e$, we may report with a degree of confidence the absolute fluorescence quantum efficiencies derived from lifetime measurements and spectral studies. These are listed in Table III in addition to the measured lifetimes $\langle\tau_p\rangle$ and calculated emissive lifetimes τ_e as described in the section on emissive lifetimes. The measured lifetimes are accurate to better than ±0.05 nsec, and the systematic errors in the calculated emissive lifetimes are less that 2–5%, based upon accuracies of absorption coefficients, isolation of the responsible absorption oscillator within the absorption spectra, and corrected fluorescence spectra.

Absolute Quantum Efficiency of Quinine Sulfate. Measurements of the absolute quantum efficiency of quinine sulfate have ranged from 0.52–0.57[37] to about 0.65.[38] Considering the technical difficulties in obtaining accurately corrected fluorescence spectra and absolute quantum measurements, large discrepancies in the direct measurements of quantum efficiencies are not surprising. However, since relative quantum efficiencies may be measured with great accuracy if the fluorescence spectra of the compounds compared are not very different, it was our purpose to calculate the absolute quantum efficiency of quinine sulfate from relative fluorescence yields and the determined absolute quantum efficiencies of the dihydronicotinamide derivatives. If the quantum efficiency of the dihydronicotinamide is $\bar{q} = \langle\tau_p\rangle/\tau_e$, and its total fluorescence relative to quinine sulfate emission is $R = \bar{q}(\text{NicH})/\bar{q}(\text{quinine sulfate})$, then the quantum efficiency of quinine sulfate (QS) may be calculated from the relation

$$\bar{q}(\text{QS}) = (\langle\tau_p\rangle/\tau_e)\left(\frac{1}{R}\right)$$

Applying this relation, the quantum efficiency of quinine sulfate at 25° was calculated from relative measurements with NADH, Ad-C_3-NicH, and Ad-C_6-NicH in 1,2-propanediol (R = 0.115, 0.138, and 0.137) to be 0.69, 0.70, and 0.73, respectively. The quantum efficiency may also be calculated from the slope of Figure 5, which embodies the results obtained with NADH and AcPyADH under three different sets of conditions, since the relative fluorescences of the coenzyme solutions were measured with quinine sulfate as a reference.

the quenching is always competitive with the emission. For discussion see: G. Weber, *Trans. Faraday Soc.*, **44**, 185 (1948); R. Epple and T. Förster, *Z. Electrochem.*, **58**, 783 (1954); K. Weber and M. Lokar, *Trans. Faraday Soc.*, **44**, 959 (1948); A. Weller, *Discussions Faraday Soc.*, 27, 28 (1959); G. Kortüm, H. Bauer, and G. Friedheim, *Z. Physik. Chem.*, **200**, 293 (1952); E. J. Bowen and E. Coates, *J. Chem. Soc.*, 105 (1947); W. M. Vaughan and G. Weber, *Biochem.*, in press.

(37) W. H. Melhuisch, *J. Phys. Chem.*, **65**, 229 (1961).
(38) J. A. Knopp, Ph.D. Thesis, University of Illinois, 1967.

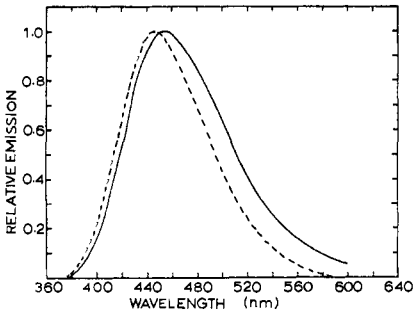


Figure 6. Technical emission spectrum (----------) and molecular emission spectrum (——————) of the fluorescence of quinine sulfate in water (0.1 N H_2SO_4).

The slope of the plot implies an efficiency of 0.70 ± 0.02 at 25° (0.73 at 15°), in excellent agreement with the figures given above.

These calculations indicate that the quantum efficiency of quinine sulfate in 0.1 N H_2SO_4 is larger by at least 20% than many direct attempts at quantum measurement have shown.[37] An explanation of our higher value is revealed in Figure 6, where the corrected and uncorrected fluorescence spectra of quinine sulfate are compared. From the technical spectrum, one would hardly expect significant fluorescence in the region beyond 550 nm, since typical recording spectrofluorometers have an accuracy of only about 1% of the full scale. However, our corrected spectrum reveals a fluorescence tail extending beyond 600 nm. The discrepancy arises because of the very poor red sensitivity of the high gain photomultiplier tubes in general use. We have partially overcome this problem by a digital-data acquisition system coupled with an IBM 1800 computer which provides detection of intensities that are a factor of 2×10^{-5} below the peak intensity. It is our contention that errors may have been unavoidable in previous quantum efficiency determinations due to the lack of red sensitivity, even though peak intensities could be accurately measured.

Energy Transfer. The quantum efficiencies of energy transfer from the adenine to the dihydronicotinamide moiety for Ad-C_3-NicH, NADH, and Ad-C_6-NicH are presented in Table V. The "experimental" results

Table V. Efficiency of Energy Transfer from Adenine to Dihydronicotinamide

Compound	Experimental ratio[a] H_2O	Experimental ratio[a] P.G.	Corrected ratio[b] H_2O	Corrected ratio[b] P.G.
Ad-C_3-NicH	0.50	0.32	0.44	0.26
NADH	0.42	0.08	0.34	0.0
Ad-C_6-NicH	0.16	0.06	0.10	0.0

[a] Ratios of fluorescence intensities resulting from excitation at 260 nm *vs.* 350 nm. [b] "Experimental" ratio corrected for direct absorption of dihydronicotinamide at 260 nm.

shown are the ratios of the fluorescence intensities of the compounds excited at 260 nm, where absorption is due mainly to adenine, to the intensities upon excitation at 350 nm, where absorption is due solely to dihydronicotinamide, and include corrections for differences in exciting light intensity, in absorption, and in optics. The "corrected" results take into account the direct absorption of 260 nm light by a dihydronicotinamide band extending into the far ultraviolet.[5] The temperature dependence of the quantum efficiency of energy transfer is shown in Figure 7.

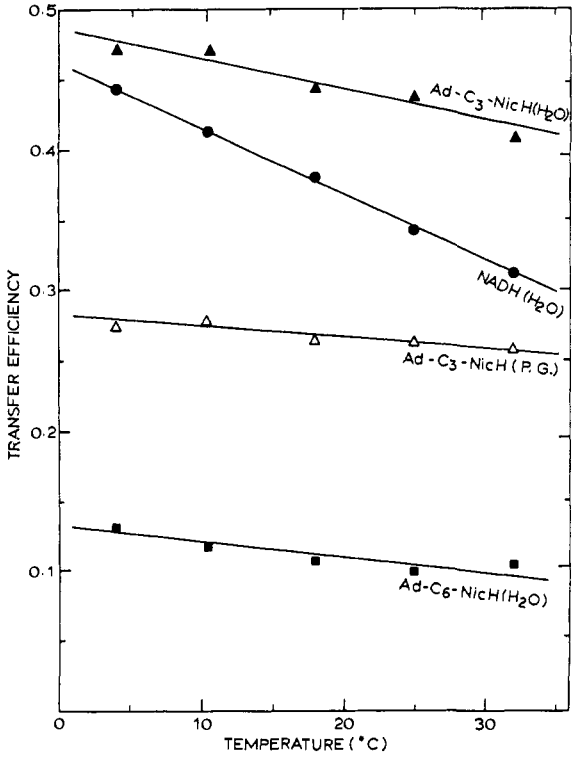


Figure 7. Temperature dependence of the quantum efficiency of the energy transfer from the adenyl moiety to the dihydronicotinamide moiety for Ad-C_3-NicH (—▲—▲—▲—▲—, water); (—△—△—△—△—, P.G.), NADH (—●—●—●—●—), and Ad-C_6-NicH (—■—■—■—■—).

In agreement with previous studies,[5,14] only in aqueous solution does appreciable energy transfer take place. Although Ad-C_3-NicH (**5a**) shows transfer to the extent of 26% in 1,2-propanediol because of the constraints to separation of the two heterocyclic rings, imposed by the short trimethylene chain (contrasted to the longer hexamethylene chain in **5b** and the ribose-phosphate-phosphate-ribose bridge in NADH), the efficiency in water is much greater (44%). Whatever the particular interactions that affect the transfer, the absolute values and the temperature dependences appear to be characteristic and specific for each compound in water solution. The total impact of the results is to support the proposal that only thermodynamically favored folded forms contribute to the singlet energy transfer efficiency. This conclusion is reached from the following considerations *inter alia*.

(1) The measured lifetime of NADH excited at 340 nm, where only dihydronicotinamide absorbs, is 0.40 ± 0.03 nsec in water solution. Excitation of NADH at 260 nm, where adenine mainly absorbs and transfers to the dihydronicotinamide with an efficiency of 34%, still shows a measured lifetime of 0.40 ± 0.05 nsec.[1a] Energy transfer from donor to acceptor would tend to lengthen the observed lifetime of the acceptor emission if the transfer rate were competitive with the acceptor's molecular lifetime; hence, the lack of extra time delay, attendant with high efficiency, for this transfer phenomenon indicates that the transfer rate must be faster than 1×10^{10} sec^{-1}.

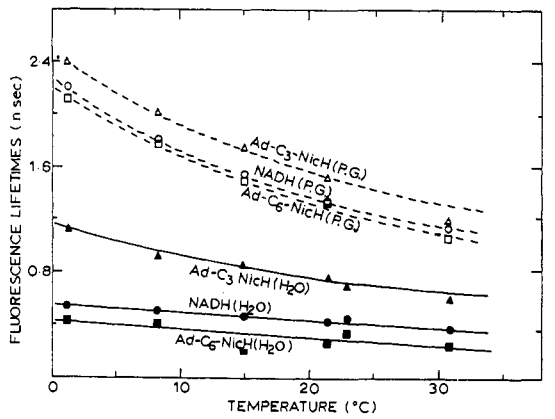


Figure 8. Temperature dependence of the fluorescence lifetimes for Ad-C_3-NicH, NADH, and Ad-C_6-NicH in aqueous buffer and in 1,2-propanediol.

(2) The fluorescence quantum efficiency, q, of adenine has been measured to be less than 0.0005 in 30/70 (v/v) water–ethylene glycol solution at 25°.[39] Assuming that adenine has an emissive lifetime, τ_e, of 10–50 nsec, then the actual lifetime may be approximated by the relation

$$\langle\tau_p\rangle = \bar{q}\tau_e = 3\text{–}15 \times 10^{-12} \text{ sec}$$

Therefore, the transfer rate must be of the order of 10^{11} sec^{-1} if it is to result in appreciable transfer. Since no appreciable diffusion of the parts of the molecules can take place in a time of 10^{11} sec only short range interactions can be considered in the energy transfer process.

(3) In aqueous solution, Ad-C_3-NicH, NADH, and Ad-C_6-NicH show transfer efficiencies in the general order (44, 34, and 10%, respectively) one might predict for the existence of folded forms. The trimethylene bridge in Ad-C_3-NicH readily permits the terminal rings to lie in parallel or near-parallel planes, with N-9 of adenine and N-1 of dihydronicotinamide in the *syn* relation to each other. For Ad-C_6-NicH, the tendency for intramolecular ring interaction may be balanced, or overbalanced, by the increased length of the chain. For the coenzyme NADH, there are more favorable conformations with the adenine and dihydronicotinamide rings in close proximity. It is the folded forms, in which the rings are in close proximity with favorable average orientations, that allow a 10^{10} sec^{-1} rate of transfer to occur. Extended conformations of these molecules would not partake in the transfer.

(4) In 1,2-propanediol, the population of molecules of NADH or Ad-C_6-NicH is dynamically distributed among an indefinite number of conformations within the steric limitations applied by the bridging groups. Little energy transfer occurs because the probability is negligible for adenine and nicotinamide to be close enough for the transfer rate to be competitive with the rate of radiationless transitions from the adenine singlet. The transfer efficiency of 26% for AdC_3NicH in 1,2-propanediol is unique among the compounds studied, as has been mentioned earlier, and is due to the close proximity of the Ad- and NicH-moieties imposed by the short trimethylene bridge.

(39) J. W. Eastman and E. J. Rosa, *Photochem. Photobiol.*, 7, 189 (1968).

Arrhenius Analysis of Lifetime Measurements. The measurement of the fluorescence lifetime, τ, gives directly the total number of transitions per second which depopulate the excited state. Adenine does not seem to quench the emission of dihydronicotinamide competitively or statically,[5] so that the measured lifetime of the fluorophor must reflect only the effects of the rates of fluorescence emission, λ, and competitive quenching, k_s, according to the expression

$$\langle\tau_p\rangle = \frac{1}{\lambda + k_s} \tag{10}$$

Since the rate of fluorescence emission, λ, is the reciprocal of the emissive lifetime, τ_e, the rate of competitive solvent quenching is

$$k_s = \frac{1}{\langle\tau_p\rangle} - \frac{1}{\tau_e} \tag{11}$$

The expression for the temperature dependence of the dynamic quenching rate is given by the Arrhenius equation

$$k_s = A^{-\epsilon_a/RT} = A\,10^{-\epsilon_a/2.3RT} \tag{12}$$

where, as usual, A is the temperature independent frequency factor in sec^{-1}; ϵ_a is the Arrhenius activation energy in kcal/mole; R is the molar gas constant; and T is the temperature in degrees Kelvin. Therefore, we may obtain the absolute rates and energies of activation for radiationless transitions calculated from the temperature dependence of the lifetimes.

Figure 8 shows the temperature dependence of the lifetimes obtained for NADH and the Ad-C_3-NicH and Ad-C_6-NicH models. The Arrhenius plots of eq 12 are presented in Figures 9 and 10 for the compounds in water and 1,2-propanediol, respectively, and the Arrhenius parameters are summarized in Table VI.

Table VI. Arrhenius Determinations for NADH and Model Compounds[a]

Compound	Solvent	k_s[b], 10^8 sec^{-1}	A, 10^{11} sec^{-1}	ϵ_a, kcal $mole^{-1}$
Ad-C_3-NicH	P.G.	5.9	7.2	4.2
NADH	P.G.	6.7	8.2	4.2
Ad-C_6-NicH	P.G.	6.7	8.2	4.2
Ad-C_3-NicH	H_2O	12.7	3.5	3.3
NADH	H_2O	24.0	1.2	2.3
Ad-C_6-NicH	H_2O	39.4	15.9	3.6

[a] Calculated at 21.5°. [b] Calculated from the expression $k_s = \frac{1}{\langle\tau_p\rangle} - \frac{1}{\tau_e} = A^{-\epsilon_a/RT}$.

In 1,2-propanediol where there is little evidence of interaction between the rings in energy transfer studies, both frequency factors, A, and energies of activation, ϵ, are remarkably uniform: approximately 8×10^{11} sec and 4.2 kcal/mole, respectively. In aqueous solutions, in which additional interactions occur, the A values in these three samples range over a factor of ten, and the energies of activation, over a factor of two. Clearly, specific effects are introduced by the presence of water, so that the adenine and nicotinamide not only interact much more strongly in this solvent but the participation of the different molecular groups between

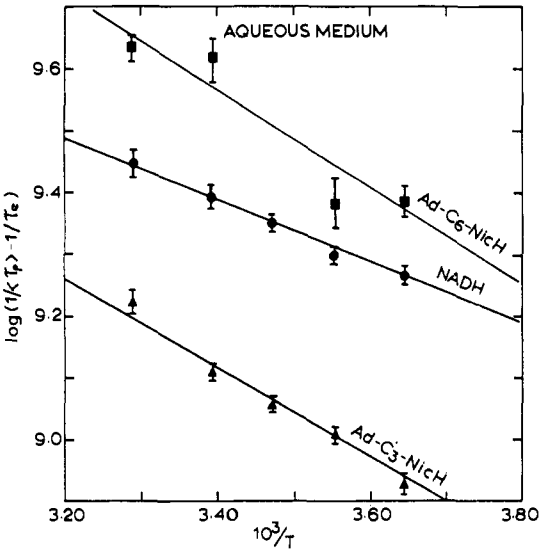


Figure 9. The Arrhenius plots of the rates of radiationless transitions, k_s (eq 11), *in aqueous solution* for Ad-C_6-NicH (-■—■—■—■-) NADH (—●—●—●—●—), and Ad-C_3-NicH (-▲—▲—▲—▲-).

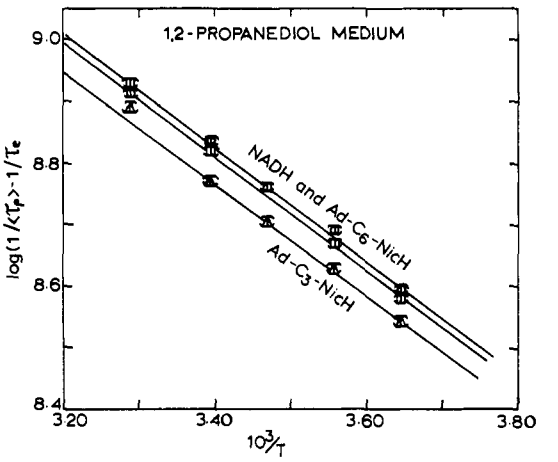


Figure 10. The Arrhenius plots of the rates of radiationless transitions, k_s (eq 11), in *1,2-propanediol* for Ad-C_6-NicH (—□—□—□—□—), NADH (—○—○—○—○—), and Ad-C_3-NicH (—△—△—△—△—).

the terminal rings in the interactions must be weighted differently in the three cases.

Conclusions. We wish to stress the usefulness of the simplified model compounds for (a) evaluating the emission properties of NADH and (b) appraising the nature of adenine–dihydronicotinamide interactions. In particular, observations on the model compounds have been integral in drawing the following conclusions.

(1) The process *itself* of electronic energy transfer from adenine to dihydronicotinamide *is not dependent* upon any unique solvent characteristic in a simple one-to-one relationship; rather, it *is dependent* on the effect of the gross solvent characteristics and their effect on the proximity of the interacting chromophores *via* favoring either extended or folded dynamic conformations.

(2) For NADH-type compounds in solvents which promote extended dynamic conformations, both the probability and the energy of activation for radiationless transitions are the same.

(3) With changes in the molecular environment upon enzymatic binding or upon changes of temperature or solvent, we have observed that the lifetime and quantum efficiency change by the same factor. Thus, we conclude that *in NADH systems there are no significant static modes of quenching of dihydronicotinamide fluorescence.* As a corollary we conclude that these studies have direct relevance to the interpretation of kinetic studies of the dehydrogenases where the temperature dependence of the fluorescence of NADH is measured but where facilities for accurate measurement of short lifetimes are not available.

(4) We conclude that *in water* NADH behaves like a molecule in which adenine and dihydronicotinamide are *proximate* (Ad-C_3-NicH), whereas *in nonaqueous media* NADH resembles a model in which the terminal heterocycles are *remote* (Ad-C_6-NicH).

Finally, these studies have fixed the values of the absolute quantum efficiency of NADH and the lifetime of the NADH excited state with all the accuracy required for practical purposes. Beyond the value of these studies for future experimental investigation of NADH–dehydrogenase systems, they have shed new light on the question of absolute fluorescence efficiencies in solution and have reaffirmed the importance of water in the dynamic states of biological systems.

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