STUDIES OF THE CONFORMATION OF PYRIDINE NUCLEOTIDE COENZYMES: I. ORD SPECTRA AND HYPOCHROMICITY OF NADH. *

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RUNNING TITLE: CONFORMATION OF NADH

FOOTNOTES

(1) ABBREVIATIONS USED IN THIS WORK:

NAD^+	Nicotinamide adenine dinucleotide
NADH	Reduced Nicotinamide adenine dinucleotide; 1, 4 dihydronicotinamide adenine dinucleotide
NMN	Nicotinamide mononucleotide
NMNH	Reduced Nicotinamide mononucleotide; 1, 4 dihydronicotinamide mononucleotide
AMP	Adenosine 5' monophosphate
ATP	Adenosine 5' triphosphate
ADPR	Adenosine 5' diphosphoribose
ApA	Adenylyl 3'5' Adenosine

All riboside linkages are in the β-configuration unless stated otherwise.

(2) The stereo projections of α and β NADH given in Velick (1961), which also appear in the review by Colowick et al., (1966), are incorrect. As drawn, the sugar residue attached to adenine is not ribose, and the nucleoside linkage is α rather than β. The diribose 5'5' pyrophosphate molecule does in fact contain an element of symmetry: the two ribose 5' phosphate units are

equivalent and are formally related by a two-fold axis. As drawn, they appear to be related by a plane of symmetry, which has inverted the configuration of all the asymmetric centers of the adenine-ribose unit.

ABSTRACT

The ORD, absorption and fluorescence spectra of NADH and its components have been examined to see if NADH shows the properties of a stacked system. The ORD spectrum of NADH is not the sum of the contributions of AMP and NMNH. The rotatory contribution resulting from intramolecular interaction between adenine and 1,4 dihydronicotinamide moieties is eliminated by pyrophosphatase digestion, heating or addition of certain organic solvents. The interactions giving rise to the ORD spectrum of NADH are complex. The system is non-conservative, indicating interaction of the 340 mμ dihydronicotinamide and the 260 mμ adenine transitions with unidentified higher energy transitions. These optical properties render unlikely a conformation in which the two bases are coplanar.

The measurement of hypochromicity of NADH by digestion with pyrophosphatase at 37° has been shown to be subject to errors due to the subsequent reaction of NMNH with water. Digestion at 22° is free from this artefact. It is found that both the 260 and 340 mμ bands are hypochromic relative to the separate chromophores, AMP and NMNH. The greater liability of NMNH to undergo addition reactions implies that the 5,6 double band of this moiety is protected in NADH.

The ORD and Hypochromic properties of NADH require that the two bases be in close proximity. This is most readily obtained by parallel stacking of the bases, with hydrophobic bonding as a determining force. Protection of the 5, 6 bond of nicotinamide in NADH is evidently a property of the stacked conformation.

INTRODUCTION

Weber (1957) first observed the transfer of electronic excitation energy between adenine and dihydronicotinamide in NADH.[1] In order to account for the efficiency of the process and the polarization of fluorescence, a conformation was proposed in which the two bases are stacked upon each other (Weber, 1958). In support of this inference, an apparent hypochromicity was reported. The value of the ratio OD_{260}/OD_{340} was observed to increase from 2.7 to 3.3 when the pyrophosphate bond was cleaved enzymatically at 37^{o}.

The effect of propylene glycol (Weber, 1958), methyl carbitol (Velick, 1961) upon fluorescence properties and the thermal relaxation properties, (Czerlinski and Hommes, 1964) have been explained in terms of the unstacking of NADH. The NMR spectrum of NADH (Meyer, et al., 1962, Jardetsky and Wade-Jardetsky, 1966) has been interpreted in terms of a conformation in which the parallel stacking of the planes of the bases causes an upfield shift in the resonance of the dihydronicotinamide protons.

The molecule of NADH contains two riboside linkages in the β-configuration, and the molecule is asymmetric. The ORD properties of molecules containing chromophores in an asymmetric stacked conformation are expected on theoretical grounds to exhibit characteristic striking features. A particularly simple case results

from the interaction of two identical chromophores. The degeneracy of the transitions is perturbed in a characterisitc way, leading to a readily recognizable pattern in the ORD, due to the close proximity of equal but oppositely-signed Cotton Effects. The resulting strong overlap has been termed a 'couplet', (Schellmann, 1968), and the system where rotational strengths sum to zero has been termed 'conservative', (Bush and Brahms, 1967). This pattern, resulting from the asymmetric interaction of two identical chromophores, is seen with ApA, (Van Holde et al., 1965; Holcomb and Tinoco, 1965; Warshaw et al., 1965), and certain dipeptides, (Bayley et al., 1968). In such systems, the resultant spectra are strictly dependent upon the relative orientation of the chromophores which should be restricted by covalent bonds or intramolecular forces.

In favorable cases, the ORD properties may be used to deduce information about orientations within a stacked structure. (Warshaw et al., 1965; Bush and Tinoco, 1967). The ORD properties of NADH have therefore been examined to see whether they reflect the properties of a stacked system.

In investigating the absorption and ORD spectra, it was found necessary to consider the problem of the hypochromicity of NADH. This represents a reinvestigation of the phenomenon reported by Weber (1957, 1958).

EXPERIMENTAL SECTION

MATERIALS

NADH was obtained from Boehringer and Soehn, Lot 6294563, (sodium salt, enzymically reduced) and from Sigma, Lot 127B-6350, Grade III. NAD, Lot 117B-9720, Grade III, NMN, Lot 54B-7310 and NMNH, Lot 64B-7340, Type III were also from Sigma. AMP, Lot 219, Crystalline, ATP, Lot 149A, Crystalline, and a sample of NADH, Lot 6505, Ion-exchange purified, were from P-L Biochemicals, Milwaukee.

The pyrophosphatase preparation was that of Kornberg and Pricer (1950) from potato, with activity 2200 units per mg.

Lactate dehydrogenase (rabbit muscle) was from Sigma, Lot 94B-1530, Type I, crystalline suspension in ammonium sulphate, 52 mg/ml, and was diluted directly before use.

Liver alcohol dehydrogenase was from Worthington, HLADHL 6KB (crystalline), 2.2 units per mg. 1 ml was dialysed at a concentration of 35 mg/ml for 48 hours versus four changes of 125 ml potassium phosphate, 0.1 M, pH 7.5, at 5°C.

The organic solvents were from Matheson Coleman and Bell, Baker, Eastman, Mallinckrodt, and Fisher, and were of spectroscopic grade where available. Ethanol was Gold Seal. All were used without further purification.

METHODS

All spectra were measured in 0.1 M Tris hydrochloride, pH 8.0 unless otherwise stated.

All measurements involving NADH were made on the Boehringer sample, unless specifically indicated otherwise.

ABSORPTION SPECTRA were recorded on the Cary 14 spectrophotometer using 1 cm cells. For temperatures other than 22° a thermostated cell block was used.

The spectral parameters, $R = OD_{260}/OD_{340}$ and $R' = OD_{290}/OD_{340}$ are defined for NADH.

ORD SPECTRA were recorded on the Cary 60 spectropolarimeter using 1 cm, 0.1 cm and 0.01 cm cells. A thermostated cell block was used for measurements at temperatures other than 27°. A small temperature-dependant correction was

necessary at elevated temperatures, to compensate for a drift in baseline.

Temperatures were measured in the ORD cells using a temperature probe, type 423 and telethermometer assembly 42SC Model A, from Yellow Springs Instrument Company, Ohio.

FLUORESCENCE SPECTRA were recorded using the apparatus as described previously (Stryer, 1965). For emission spectra, a mercury source was used. For excitation spectra, a xenon lamp was used. The filter on the emission side was a Corning 3-72, which transmits beyond 450 mμ. This apparatus permits the direct recording of the corrected excitation spectrum, thereby simplifying the evaluation of energy transfer efficiencies.

When excitation spectra were recorded for a digestion lasting several hours, the fluorimeter was calibrated periodically using a standard solution of 1-anilino-8-napthalene sulphonic acid, 1.0 μM in ethanol, excitation maximum 375 mμ, emission maximum 470 mμ.

The efficiency of energy transfer, T%, is evaluated from comparison of the excitation spectrum E of NADH, and the absorption spectra, ϵ of NADH and NMNH. Normalizing both E and the absorption

spectra to the same value of 6.2×10^3 at 340 mμ, T is evaluated from the values of E and ϵ at 260 as

$$T = 100 \times ({}^{E}\text{NADH} - {}^{\epsilon}\text{NMNH})/({}^{\epsilon}\text{NADH} - {}^{\epsilon}\text{NMNH}).$$

ENZYMIC DIGESTIONS with pyrophosphatase were carried out by two methods. Method (1) involved incubation of 0.5 ml of 5 mM NADH solution with 10 μl enzyme; aliquots were withdrawn and diluted to a 0.1 mM for measurement of the absorption spectrum over the range 400 to 220 mμ. Blanks without enzyme were treated similarly. Method (2) involved measurement of the spectrum of NADH (0.1 mM) against a blank containing buffer, aliquots of pyrophosphatase were added a solution and blank and the absorption at 260 mμ recorded as a function of time.

NUCLEOTIDE CONCENTRATIONS were estimated spectrophotometrically. ϵ_{340} for NADH at 22°C in 0.1 M tris hydrochloride , pH 8.0, was taken as 6.2×10^3, by comparison with the literature value under closely similar conditions, (Horecker and Kornberg, 1948).

The sample of NAD was stated to be 98% pure. ϵ_{260} was therefore taken as 18.0×10^3, (Kornberg and Pricer, 1953).

The purity of samples of NMNH and NMN was given as 90%. NMNH was estimated using the same extinction coefficient as NADH. NMN was estimated using the value of 4.6×10^3 (Pabst Laboratories, 1961).

RESULTS

1. ORD MEASUREMENTS

THE ORD SPECTRA OF NADH AND ITS COMPONENTS

The ORD spectra of NADH, NMNH and AMP, and the sum of AMP and NMNH in the region of 260 mμ are given in FIGURE 1. The ORD spectrum of NADH also exhibits a weak negative effect in the region of 340 mμ. The apparent amplitude of the molecular rotation is 650 degree $cm^2/decimole$, between 365 mμ (minimum) and 322 mμ (maximum). This effect is difficult to quantitate, since it is partially obscured by the effect at 260 mμ.

It is not evident from inspection of the ORD spectrum of NADH alone that there is interaction between adenine and dihydronicotinamide. However, comparison of NADH with the sum (AMP +NMNH) shows that the ORD of NADH is greater than the sum of its parts, indicating the presence of some interaction.

The spectral properties of NADH, NMNH and AMP are summarized in TABLE I.

It is seen that the band at 340 mμ has an appreciable rotational strength: it is difficult to observe because of the characteristically wide bandwidth of the 340 mμ dihydronicotinamide absorption.

The possibility that the difference in rotatory properties between NADH and NMNH+AMP is due to intermolecular interactions of NADH was rendered unlikely by measurement of the ORD spectrum as a function of concentration. The spectra of NADH at 8.5 mM, 0.85 mM and 0.085 mM (in 0.01 cm, 0.1 cm and 1.0 cm cells, respectively), were identical to within experimental error (±5%). The value of R was constant at 2.7.

THE ORD SPECTRUM OF NADH AS A FUNCTION OF PYROPHOSPHATE DIGESTION

The effect of pyrophosphatase digestion at 22° upon the ORD spectrum of NADH is shown in FIGURE 2. The course of digestion was followed by absorption spectra, (see section on hypochromicity) and was essentially complete after 4 hours .

As a result of pyrophosphatase treatment, the ORD spectrum shows a significant decrease in amplitude at 260 mμ and a minor decrease at 340 mμ. The 4-hour spectrum approximates to that of a mixture of AMP+NMNH (FIGURE 1), showing that the intramolecular interaction between the bases does not persist after the pyrophosphate bond is cleaved.

THE ORD SPECTRUM OF NADH AS A FUNCTION OF TEMPERATURE

In order to minimize losses due to addition reactions at elevated temperatures, fresh solutions were made at the different temperatures and the spectrum scanned rapidly. The results are shown in FIGURE 3. It is evident that at high temperature, the spectrum of NADH shows weaker effects at 260 mμ. The effect at 340 mμ is obliterated. On cooling the final solution to 27°, the basic features of the NADH spectrum were restored. Some NADH was lost owing to addition reactions at 75°.

ORD OF NADH IN NON-AQUEOUS SOLVENTS

Previous work (Bayley, 1965, unpublished data) had shown a significant decrease in the amplitude of effects at 260 mμ for NADH in 8 M urea, pH 7.0. Measurement of the ORD of NADH (0.1 mM) in ethanol, and in propylene glycol, showed similar decreases. The extrema at 340 mμ were still visible: the amplitude of the 260 mμ effect was reduced to 50% of its value in aqueous solution.

2. HYPOCHROMICITY MEASUREMENTS

Pyrophosphatase digestion provides a convenient way of comparing the absorption of NADH with its components. The course of digestion is readily followed by measurement of the efficiency of energy transfer.

PYROPHOPHATASE DIGESTION OF NADH AT 25° AND 37°:

(a) FLUORESCENCE ENERGY TRANSFER

The effect of pyrophosphatase digestion upon the fluorescence energy transfer of NADH is shown in FIGURE 4. The efficiency of energy transfer, T_{25} and T_{37}, decreases steadily with time, and is effectively zero after about 2.5 hours. The same curve is obtained for the two temperatures. Doubling the amount of enzyme has compensated for the difference in temperature. In the absence of pyrophosphatase, the efficiency of energy transfer is constant over a period of 8 hours for NADH at 37°, (T_{37}, blank, FIGURE 4).

(b) ABSORPTION SPECTRUM

The spectral parameters R and R' are used to characterize the absorbances at 260 and 290 mμ relative to that at 340 mμ. FIGURE 4 shows R and R' as a function of the time of hydrolysis with pyrophosphatase at 37° and 25°.

At 37°, R increases significantly, passing through the value R = 3.3 at 2 hours (as previously observed by Weber, 1957). After 9 hours, R has further increased to 5.0, and there is no

sign of this increase reaching a plateau. In the absence of enzyme, the value of R at 37° remains constant throughout. In contrast to this, at 25° there is little change in R in the presence of pyrophosphatase over a period of 9 hours.

The data of FIGURE 4 also show that at 37°, R' shows a marked increase in step with R, while at 25°, it is effectively constant.

From the difference in behavior of R at 37° and 25°, the absence of a plateau, and the observation of increased absorption in the region of 290 mμ, it is clear that something more than the release of hypochromism of the 260 mμ band is occurring during hydrolysis at 37°. Apparently a new component is being formed.

The spectra after 4 and 9 hours digestion at 37° showed a decrease in extinction at 340 mμ, an increase at 260 mμ and the presence of a shoulder at 290 mμ. After subtraction of the absorbance of the residual NADH, estimated from the absorbance at 340 mμ, the spectrum of the new component was estimated from the spectra at 4 and 9 hours. The resulting spectra, shown in FIGURE 5, strongly resemble the spectrum of the primary acid addition product of NADH, formed rapidly at pH 3.5, (Stock et al., 1961). This has $\epsilon_{260} = 24 \times 10^3$, a shoulder at 290 mμ and zero absorption at 340 mμ.

From the data of FIGURE 4, it is clear that the formation of this component at neutrality at 37° is enhanced enormously by pyrophophatase hydrolysis. The stability of the hydrolysis produces were therefore examined. AMP is stable under these conditions. However, it is known that 1, 4 dihydronicotinamide derivatives are unstable (Karrer, et al., 1936; Weber, 1957) and undergo addition reactions. As with NADH, the reactions are accelerated by acid conditions: the site of addition for the primary acid addition product is the 5, 6 double-bond. (Diekmann et al., 1964a, 1964b). It was therefore of interest to examine the stability of the NMNH which is liberated from NADH by pyrophosphatase.

THE ABSORPTION SPECTRUM OF NMNH AT 37° AND 22°.

Samples of NMNH were maintained at 37° and 22°, and the spectrum recorded periodically. The results for 37° are shown in FIGURE 6. The spectra show an isosbestic system, suggesting the presence of NMNH and a single product. A small deviation from the isosbestic system was eventually observed. It is clear that NMNH is unstable under the conditions used for the hydrolysis at 37°.

The greater stability of NMNH at 22° is shown by the fact that, after 10 hours, the spectrum was identical with that of

curve 2, FIGURE 6, i.e., it had changed only 7% of the change at 37^{o}.

Assuming a 1:1 relationship between NMNH and product, the spectra at 37^{o} were plotted to give a first-order rate constant, $k_{37} = 1.8 \times 10^{-5}$ sec^{-1}, i.e., a half-life of 10.7 hours. From the limited data at the lower temperature, the initial rate is reduced by a factor of 13, giving an activation energy of 30 kcals.

ESTIMATION OF THE EXTINCTION COEFFICIENT OF NADH AT 260 mμ

The parameter R is evidently untrustworthy for detecting the possible occurrence of hypochromicity by stacking in NADH because it is sensitive to contributions from artefacts of the digestion procedure. A more direct approach is to measure the extinction coefficient for NADH at 260 mμ, and compare it with those for AMP and NMNH. The experimental millimolar extinction coefficients at 260 mμ is 16.8. While this is close to the sum of AMP plus NMNH, it is considerably in excess of the published values of 14.0 - 14.5 for NADH which had been prepared by enzymic reduction from carefully purified NAD (TABLE II). The difference is probably due to the presence of an adenine-containing impurity, as has been observed for NAD

and other NADH preparations (Dalziel, 1963; Silverstein, 1965). The presence of this impurity is indicated by a value of R greater than 2.3. The Boehringer sample (R = 2.7) had been stored at 0^{o} for several months. The P-L preparation had R = 2.5, and the most recent preparation, from Sigma, had R = 2.4.

The purity of the NADH was estimated by the method of Horecker and Kornberg (1948) in which the use of a pure substrate and dehydrogenase of high specificity enables the extinction coefficient of the coenzyme to be determined accurately in the presence of impurities which are inert in the oxidation-reduction reaction. It was established that the preparation of Boehringer NADH was 98% pure as judged by the residual extinction at 340 mμ after treatment with excess pyruvate and lactate dehydrogenase. At the same time it was observed that the difference in millimolar extinction between NAD and NADH was -3.5 at 260 mμ. This is in good agreement with the literature values in TABLE I.

The excess extinction of 16.8 - 14.5 = 2.3 is equivalent to 0.15 moles adenine per mole NADH, or a purity of 87%. Given the maximum value of 14.5 from TABLE II, and the sum of the values of AMP + NMNH as 16.4, it should be possible to observe an increase of 1.9 in an experimental value of 16.8, i.e., a hypochromicity of 11%.

THE RELEASE OF HYPOCHROMICITY IN NADH BY PYROPHOSPHATASE HYDROLYSIS

The optical density of a single sample of NADH was observed at 260 mμ as a function of time after the addition of a relatively large amount of pyrophosphatase. The hydrolysis was carried out at 22° to avoid complications due to the formation of addition products of NMNH. In the presence of 10 μl pyrophosphatase, the absorbance increased steadily from OD = 1.90 to a plateau of 2.06 after 4 hours, i.e., an increase of 8.4%. A second addition of enzyme had no further effect. At the same time it was observed that the peak at 340 mμ increased by about 4%. The value of R changed slightly from 2.70 to 2.83, and R' remained constant at 0.33 during the hydrolysis. It is clear that both the adenine and the dihydronicotinamide absorptions in NADH are hypochromic relative to AMP and NMNH respectively. Since R represents the absorbance at 260 mμ relative to that at 340 mμ it is relatively insensitive to the release of hypochromicity of NADH.

This direct observation of the change in extinction at 340 mμ allowed the spectra of FIGURE 5 to be expressed in terms of molar extinction coefficients, assuming a 1:1 relationship between NADH

and the addition product. It was assumed that the full hypochromicity had been released after 4 hours. Good agreement was then obtained in the extinction coefficient at 260 mμ for the product after 4 and 9 hours, as seen in FIGURE 5.

In order to exclude the possibility that there was a contribution to the change in extinction at 260 mμ on pyrophosphate hydrolysis from the change in charge from pyro- to ortho-phosphate, the experiment was repeated with ATP and ADPR. ATP is converted to AMP by pyrophosphatase (Kornberg and Pricer, 1950). There was no increase in extinction after addition of pyrophosphatase to ATP. This result is consistent with the constancy of the extinction coefficient of adenine in AMP, ADP and ATP, (Pabst Laboratories, 1956). Similarly, with ADPR, there was no change in extinction when treated with pyrophosphate. Thus the observed increase in 260 mμ extinction appears to be a genuine hypochromicity. It may be mentioned here, that similar effects have been observed with a variety of nicotinamide coenzyme analogues, both oxidised and reduced, and with model compounds related to NAD, (Bayley, to be published).

ABSORPTION AND FLUORESCENCE SPECTRA AS A FUNCTION OF SOLVENT

The effects of a range of organic solvents upon T%, the efficiency of energy transfer, and Q, the quantum yield of fluorescence at 340 mμ relative to that in water, have been examined.

Excitation spectra were recorded for solutions made from the addition of 5 μl NADH solution (4×10^{-3} M, in Tris, 0.1 M, pH 8.0) to 2.0 ml organic solvent. For absorption spectra, 50 μl NADH was added to 2.0 ml organic solvent. Solutions of NADH in dioxane and t-butanol were unstable: spectra were recorded immediately after addition to the organic solvent. The solvents n-butanol and sec-butanol could not be used, because of the insolubility of NADH. In dimethyl sulfoxide and trimethyl phosphate, the absorption spectra showed time-dependent changes, resulting in the appearance of increased absorbance at 290 mμ. This effect was more pronounced with trimethyl phosphate.

The results for NADH in the various solvents are given in TABLE III, arranged in order of increasing efficiency of energy transfer. Also tabulated are the values of R and R'. (Absorption spectra were not corrected for differences in refractive indices, since only spectral ratios are to be considered. The correction to the ratio is negligible.)

Some general patterns may be discerned in TABLE III. There is a general correlation between decreased efficiency of energy transfer and an enhancement in R. Such enhancement is not in general accompanied by an enhancement in R'. There is no correlation between T% and Q. Those organic solvents in which NADH is freely soluble at 10^{-4} M generally cause a decrease in T%. A few solvents show values of T% greater than that in water. These appear to be those in which NADH is on the verge of insolubility.

The fluorescence energy transfer in NADH was also examined in certain alcohol-water mixtures to see if effects in T (decreased in methanol and ethanol, and increased in t-butanol) follow a smooth or a discontinuous relationship with alcohol content. Solutions were made by the addition of 10 μl aliquots NADH solution (4×10^{-3} M) to 10 ml of alcohol-water mixture of varying volume percentage composition. The results are given in TABLE IV.

The general trend for alcohol water mixtures is of a continuous increase in the value of Q. The value of T% in general decreases smoothly. There are anomalous effects in 100% ethanol and t-butanol.

DISCUSSION

INTERPRETATION OF THE ORD SPECTRA

The evaluation of true optical rotatory parameters from extrema in the ORD spectrum requires that the extrema correspond to single transitions which are well separated in energy. The minimum condition to be met is that the effects are separated by at least the bandwidth of the broader transition. Even so, overlapping occurs, and the parameters of individual effects cannot be readily evaluated since the positions of the extrema no longer correspond to those of single effects. For separations less than the bandwidth the 'rotatory couple' can be evaluated, (Bayley et al., 1968).

In the case of NADH, an intermediate condition appears to hold. The identification of the effects with the two transitions at 340 mμ and 260 mμ seems reasonable both from the position of the extrema in the ORD curve of NADH, and from the comparison of bandwidths calculated from the absorption spectrum and from the extrema. There is no evidence for contributions from weak (electrically forbidden) transitions. The effects are separated from one another by more than the broader bandwidth, but yet not fully isolated from one another, nor from the higher energy transitions below 260 mμ. (See footnote to TABLE I). The para-

meters derived from the curves are therefore called 'apparent values'.

Since the effects at 340 mμ are obscured by the wide bandwidth of the transition, the interpretation is limited to the experimentally observable effects at 260 mμ. The evidence from ORD in favor of a stacked structure may be summarized as:

i) The ORD of NADH is not the sum of its components AMP+NMNH, but exhibits an enhanced amplitude in the region of 260 mμ.

ii) This amplitude is reduced to approximately that of AMP+NMNH by pyrophosphate digestion, heating, and by organic solvents.

iii) There is no evidence for intermolecular interactions; hence the effect is attributable to an intramolecular interaction between the adenine and dihydronicotinamide moieties.

iv) A structure in which the rings are coplanar would not contribute the necessary additional rotational strength from the interaction of the two lowest energy strong transitions, whereas a stacked structure in which the rings are restricted to a certain asymmetrical stack would provide increased rotational strength.

It is clear that the ORD spectrum of NADH does not resemble a simple couplet. The chromophores are not identical, and the

rotatory properties are associated with widely separated transitions at 260 mμ and 340 mμ. More important is the fact that both transitions are negative (TABLE I). Thus the system is non-conservative, taken over the range 260 mμ to 340 mμ. This indicates that the observed optical rotatory properties cannot be explained solely in terms of the interaction of the 260 mμ and 340 mμ transitions.

ABSORPTION AND FLUORESCENCE PROPERTIES

The use of pyrophosphatase in studying the spectral properties of pyridine nucleotide coenzymes was introduced by Weber, (1957, 1958). The method was applied to the coenzyme analogues by Siegel et al., (1959), who used pyrophosphatase from snake venom.

Potato pyrophosphatase has a wide substrate-specificity, splitting the pyrophosphate linkage of NAD, NADH, NADP, FAD, ADP, ATP and thiamine-pyrophosphate, (Kornberg and Pricer, 1950). The observation by Weber, that the cleavage of this linkage in NADH eliminates the transfer of fluorescence energy from adenine to dihydronicotinamide has been confirmed in this work. However, it is clear from FIGURE 4 that the energy transfer can be eliminated by pyrophosphate treatment without changing the value of R, contrary to what Weber had found. The cleavage of the pyrophosphate linkage does not change the relative

absorbancies at 260 and 340 mμ when the reaction is controlled so as to minimize the formation of addition products. The presence of hypochromicity at 260 mμ is inferred from the values of the extinction coefficients of the component parts, and this is demonstrated experimentally by the observation of the increase in the absolute absorption at both 260 and 340 mμ when treated at 22° with pyrophosphatase.

Both Weber (1957, 1958) and Siegel et al., (1959) had used pyrophosphate treatment of NADH to observe the hypochromicity. In both cases incubations were performed for 1 or 2 hours at 37° in Tris, pH 8. This study shows that enzymatic digestion under these conditions produces additional components which affect the measure of hypochromicity. An increase of R from 2.7 to 3.3 would imply a relative hypochromicity of 22%. Incubation of NMNH at 37° under these conditions causes a 5% decrease in absorbance at 340 mμ, and a 30% increase at 260 mμ. This latter is equivalent to an increase of 2% in OD_{260} for an equimolar mixture of AMP and NMNH. This should be compared with the increase of 6.6% reported by Siegel et al.

The lower temperatures used in this work exclude these artefacts. The observation of the increase of absorbance reaching a plateau indicates that the hypochromicity has been fully released. The magnitude of the hypochromicity observed at 260 mμ is in satisfactory agreement with that calculated from extinction coefficients.

This direct observation of the hypochromicity enforces the argument for a stacked structure. There have been recent reports, (Thomas and Kyogoku, 1967; Gratzer and McClare, 1967) that a hypochromic effect can result from a purine-pyrimidine pair, hydrogen bonded in a planar structure. Extreme hydrophobic solvents are required to demonstrate such effects. In aqueous solution, the properties of water as a hydrogen bond donor and acceptor would be expected to render such systems relatively unstable. It therefore seems more reasonable to attribute the hypochromicity of NADH to the stacking of the bases, by analogy with the dinucleoside phosphates and polynucleotides (see Michelson et al., 1967, for review).

The observation of the occurrence of addition reactions of NMNH indicated the presence of artefacts in the hypochromicity measurements. However, this has important implications for the conformation of NADH. It is well established that NADH itself

undergoes addition reactions, particularly in acid solution, (see Stock et al., 1961, for review). Addition reactions occurring at neutrality have been observed for 1-methyl 1,4 dihydronicotinamide (Weber, 1958), 1-benzyl 1,4 dihydronicotinamide, (Anderson and Berkelhammer, 1958) and for NADH itself under special conditions, (Alivisatos et al., 1964 and 1965). These reactions are of considerable biological interest because of the possible relationship to intermediates in oxidative phosphorylation. The relative lability of NADH in phosphate as opposed to Tris buffers has been noted before, (Winer and Schwert, 1958). It is interesting that NADH undergoes a rapid decomposition in trimethyl phosphate.

The results of FIGURE 4 indicate a further important fact. Although NADH is unstable in acid solution, it is relatively stable at neutrality in Tris. The addition reactions described here with NMNH occur at neutrality, and are essentially dependent upon the prior cleavage of the pyrophosphate linkage. Thus, the dihydronicotinamide moiety in NADH is protected from addition reactions at neutrality in Tris. It was observed by Kornberg and Pricer (1950) that NADH is more stable than NMNH. This may now be rationalized in terms of the known addition reactions of the reduced nicotinamide. Alivisatos et al., (1965) observed a difference in rate constant between NMNH and NADH (0.04 and

0.02 M^{-1} min^{-1} respectively), for the addition reaction in 1.5 M phosphate, pH 6.62 at 34.4°. The rate for NMNH in 0.1 M Tris, pH 8.0, at 37° is 0.003 M^{-1} min^{-1}. Since this is a monobasic system, the results are not directly comparable with those of Alivisatos et al., (1965): assuming that protonated Tris is the proton donor, the rate constant would be 0.06 M^{-1} min^{-1}, i.e., of the same order as NMNH in phosphate from the comparison of the stability of NADH and NMNH at 37° in Tris pH 8.0, the protection afforded to NMNH in the coenzyme molecule reduces the rate constant for addition by an order of magnitude.

NADH and NMNH, both have an absorption maximum close to 340 mμ, and are 1,4 dihydro compounds. (See review by Chaykin, 1967.) The first acid addition product of NADH and N-dichlorobenzyl, 1,4 dihydronicotinamide has been identified by NMR as the 5,6 addition compound, with the acid anion in position 6, (Diekmann et al., 1964a). The similarity of the spectrum of NADH at pH 3.5 with that obtained by difference in FIGURE 2 indicates that the same type of substitution is involved in the product formed at

neutrality after pyrophosphatase digestion. Comparison of these spectra with those of known tetrahydro pyridine 3-carboxamide derivatives (Diekmann et al., 1964b; Stock et al., 1961) identifies the products as 1, 4, 5, 6 - rather than 1, 2, 3, 4-tetrahydro derivatives.

Thus the point of reaction for the first acid addition compound of NADH and the addition product of NMNH at neutrality in Tris appears to be the 5,6 double bond of the dihydronicotinamide ring, and it is this bond which exhibits decreased reactivity in NADH at neutrality. It is possible, but unlikely, that this difference is due to the different chemical character of the nicotinamide moiety - i.e., the difference between a 5' nucleotide, in NMNH and a 5'5' pyrophosphate in NADH. In view of the information about the stacked structure of NADH, it seems likely that this differential stability of NADH and NMNH to addition reactions is a reflection of the conformation of the coenzyme molecule.

The potential reactivity of the dihydronicotinamide at the 5,6 position has certain implications for the general properties of enzyme-coenzyme complexes containing NADH. It has been suggested from fluorescence excitation spectra of such complexes that the coenzyme may become unstacked on the enzyme, (Velick,

1961). The free energy of this process could presumably be afforded by a new stacking interaction with the protein. If unstacking does occur, the 5,6 bond is in danger of being exposed to addition reactions, unless it is specifically protected at the binding site. Such protection could be provided by the correct steric hindrance of the bond, or by transient covalent bond formation with, for example, an anionic group on the enzyme.

The fact that both A and B sides of the coenzyme are used by different dehydrogenases may seem to favor the unstacking hypothesis, but, as may be seen from atomic models, it is possible to construct different, but related stacked structures in which either side may be rendered more exposed at the 4 position.

The observation by Weber (1957) of fluorescence energy transfer, led to the proposal of the stacked form for NADH, by analogy with a similar model proposed to account for the fluorescence and absorption spectra of FAD, (Weber, 1950; Whitby, 1953). The value of the energy transfer reported here for NADH in aqueous solution is somewhat higher than that previously observed, (Weber, 1957; Velick, 1961). This is because of the more recent lower values of ϵ_{260} for NADH, TABLE IV, which is involved in computing T%. (See Methods.) Neither ADPR nor the first acid addition product, (the likeliest candidates for the impurity

which gives R = 2.7 rather than 2.3), is fluorescent in aqueous solution, and hence cannot contribute to T%.

The measurement of fluorescence energy transfer for NADH in organic solvents has previously been reported by Weber, (1957, 1958) and Velick, (1961), for propylene glycol and methyl carbitol, i.e., 2 (β-methoxyethoxy) ethanol, respectively. The organic solvents in which NADH is freely soluble in general show decreased energy transfer, and an increase in R which is not associated with formation of addition products, as judged by the constancy of R'.

The simplest conclusion is that deduced by the above authors, namely that such solvents decrease energy transfer by unstacking. An alternative explanation that these solvents have a direct effect upon the excited state of the energy donor, adenine, and parallel differential effect upon the absorbancies of adenine and dihydronicotinamide moieties, seems less likely, but cannot be excluded on the basis of the fluorescence results alone. As evidence of the complexity of effects to be expected in fluorescent systems in organic solvents, the discontinuous relationship between energy transfer (or quantum yield) and the alcohol content of aqueous mixtures should be noted. A similar result has recently been observed for NADH in methanol (Freed et al., 1967).

Since adenine exhibits a very low quantum yield of fluorescence in aqueous solution, good proximity is required between adenine and dihydronicotinamide for efficient energy transfer in NADH. Also, the orientation is likely to be important (Forster, 1948). The hypochromic properties will also be expected to depend on distance and orientation, but not necessarily in the same way as for energy transfer. Hence it is possible to have a system which exhibits hypochromism, but no energy transfer. This consideration becomes important for the α-isomer of NADH which exhibits low efficiency of energy transfer (Kaplan, 1960; Shifrin and Kaplan, 1961) but still exhibits hypochromicity of the same order as β-NADH, (Bayley, to be published).

CONFORMATION OF NADH

The evidence from the hypochromic studies in this work strongly suggests that NADH exists in a stacked conformation, but it is not clear from this data alone, that there is a single specific stacked conformation. The NMR evidence of Jardetzky and Wade-Jardetzky (1966), could not distinguish between a single specific conformation and several conformations undergoing rapid interchange. From model building[2] with the Corey-Pauling-Koltun models, to be

presented in detail elsewhere, it is evident that there are many possible rotameric conformations. Certain steric restrictions exist for the riboside linkages (e.g., those recently described by Sasiesekharan et al., (1967), for nucleosides). The di-ribose pyrophosphate unit introduces a large number of conformational possibilities for the molecule. However, only a limited number of these allow reasonable stacking. The properties of these conformations will be presented elsewhere. For the present argument, it suffices that there is no obvious single conformation in which to seek the rationalization of the optical rotatory properties.

Both the optical rotation and the hypochromic properties imply a proximity of the bases and a restriction of the conformation. The intramolecular interaction imposing this restriction can be overcome by organic solvents. This argues in favor of a hydrophobic interaction between the bases, which can be most easily achieved by parallel stacking. Alternative conformations in which rigidity of the two moieties is achieved by electrostatic interactions, including hydrogen bonding, e.g., analogous to the coplanar hydrogen bonded base-base interactions of nucleic acids, would appear to be less likely since these forces would be enhanced rather than decreased in organic solvents.

The interaction between the bases is evidently weak, since it is readily displaced by increasing temperature. It is possible to increase the magnitude of the extrema in the ORD spectrum by lowering the temperature, which apparently increases the extent of stacking. This situation is reminiscent of the stacking of oligoribonucleotides where it is difficult to achieve full stacking in order to evaluate the true magnitudes of the properties of the fully stacked state, and hence the thermodynamic parameters characterizing the unstacking (see Michelson et al., 1967, for review).

It is seen in TABLE I that the rotational strength of NADH at 260 mμ is twice that of AMP. It has been observed by Emerson et al., (1966) and Ulbricht et al., (1966), that the restriction to the anti-conformation of nucleosides leads to a doubling of the rotational strength. Such restrictions about the riboside bond are evidently possible in the stacked structure. However, the evidence of overall hypochromicity implies interaction of the transition dipole of one moiety with excited states of the other, and vice-versa. For this reason it seems necessary to search for an explanation of the rotational strengths in terms of the overall interactions of the 340 mμ band with several adenine

in fact NMN has a positive CD band at 260 mμ (Bayley, to be published). However, dihydronicotinamide in NMNH exhibits a negative CD band at 340 mμ. It seems unlikely that there is a drastic change in conformation in going from NMNH to NMN, although this process does involve the formation of the quaternary nitrogen. The planarity of the atoms 5, 6, 1, 2, 3 is apparently preserved in the 1,4 dihydronicotinamides, judging from the X-ray studies of N-benzyl 1,4 dihydronicotinamide, (Karle, 1961). The inversion of the sign of the rotational strength is therefore attributable to the creation of essentially a new chromophore with new directional properties to the transition moments. It is significant that the transition dipole for the 340 mμ transition of the 1,4 dihydronicotinamide chromophore calculated by Evleth (1967) is effectively perpendicular to that of the 260 mμ band of the pyridines (e.g., uracil; Clark and Tinoco, 1965).

Since an independent approach to the conformation of NADH is apparently not possible at this time, an alternative approach based upon NAD may be applied. The ORD curve of NAD exhibits strong overlap (Bayley, 1965, unpublished observations) and this system approaches more closely the requirement of being conservative. This is shown more directly in the CD spectrum of NAD^+,

(Bayley, to be published) and of di-adenosine 5'5' pyrophosphate, (Massoulie and Michelson, 1964; Bayley, 1968), and these systems are more amenable to a theoretical treatment.

This work has shown that the ORD properties of NADH are in fact consistent with a stacked structure as proposed by Weber, (1957). Analysis of the rotatory properties of stacked conformations of related coenzymes and analogues, in conjunction with examination of molecular models is in hand to obtain further information about relative orientations within the stack.

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REFERENCES

Alivisatos, S.G.A., Ungar, R., and Abraham, G., Nature, 203, 973, (1964).

Alivisatos, S.G.A., Ungar, F., and Abraham, G., Biochemistry, 6, 2616, (1965).

Anderson, A.G., Jr., and Berkelhammer, G., J. Am. Chem. Soc., 80, 992, (1958).

Bayley, P.M., Abstract, 5th International Symposium on the Chemistry of Natural Products, The Chemical Society, London, (in press), (1968).

Bayley, P.M., Nielsen, E. and Schellman, J.A., J. Phys. Chem., (in press), (1968).

Bush, C.A. and Brahms, J., J. Chem. Phys., 46, 79, (1967).

Bush, C.A. and Tinoco, I., Jr., J. Mol. Biol., 23, 601, (1967).

Chaykin, S., Ann. Rev. Biochem., 36, 149, (1967).

Clark, L.B. and Tinoco, I., Jr., J. Am. Chem. Soc., 87, 11, (1965).

Colowick, S.P., van Eys, J., and Park, J.H., Comprehensive Biochemistry, ed. Florkin, M. and Stotz, E.H., Vol. 14, p. 1, Elsevier, Amsterdam, (1966).

Czerlinski, G. and Hommes, F., Biochim. et Biophys. Acta, 79, 46, (1964).

Dalziel, K., J. Biol. Chem., 238, 1538, (1963).

Diekmann, H., Englert, G. and Wallenfels, K., Tetrahedron, 20, 281, (1964a).

Diekmann, H., Hofman, D. and Wallenfels, K., Ann. Chem., 674, 79, (1964b).

Emerson, T.R., Swan, R.J., and Ulbricht, T.L.V., Biochem. Biophys. Res. Comm., 22, 505, (1966).

Freed, S., Neyfakh, E.A. and Tumerman, L.A., Biochim. et Biophys. Acta, 143, 432, (1967).

Gratzer, W.B., and McClare, C.W.F., J. Am. Chem. Soc., 89, 4224, (1967).

Holcomb, D.N. and Tinoco, I., Jr., Biopolymers, 3, 121, (1965).

Horecker, B.C. and Kornberg, A., J. Biol. Chem., 175, 385, (1948).

Jardetzky, O. and Wade-Jardetzky, N.G., J. Biol. Chem., 241, 85, (1966).

Kaplan, N.O., The Enzymes, 3, 105, (1960).

Karle, J.L., Acta. Cryst., 14, 497, (1961).

Karrer, P., Schwartzenbach, G., Benz, F. and Solmssen, U., Helv. Chim. Acta, 19, 811, (1936).

Kornberg, A. and Pricer, W.E., Jr., J. Biol. Chem., 182, 763, (1950).

Kornberg, A. and Pricer, W.E., Jr., Biochem. Preparations, 3, 20, (1953).

Massoulie, J. and Michelson, A.M., C.R. Acad. Sci. Poris, 259, 2923, (1964).

Meyer, W.L., Mahler, H.R. and Baker, R.H., Biochim. et Biophys. Acta, 64, 353, (1962).

Michelson, A.M., Massoulie, J. and Guschlbauer, W., Prog. Nucleic Acid Research, 6, 83, (1967).

Moscowitz, A., in 'Optical Rotatory Dispersion,' by Djerassi, C., Chapter 12, McGraw-Hill, (1960).

Pabst Laboratories (now P-L Biochemicals, Inc.), Circular OR-10, (1956).

Pabst Laboratories, Circular OR-18, (1961).

Sasisekharan, V., Lakshminarananan, A.V. and Ramachandran, G.N., 'Conformation of Biopolymers,' ed. Ramachandran, G.N., Academic Press, New York, p. 641, (1967).

Schellman, J.A., Accounts of Chemical Research, 1, 144, (1968).

Schellman, J.A. and Schellman, C.G., The Proteins, 2, 1, (1965).

Shifrin, S. and Kaplan, N.O., Nature, 183, 1529, (1959).

Siegel, J.M., Montegomery, G.A. and Bock, R.N., Arch. Biochem. Biophys., 82, 288, (1959).

Silverstein, E., Anal. Biochem., 12, 199, (1965).

Stock, A., Sann, E., and Pfleiderer, G., Ann. Chem., 647, 188, (1961).

Stryer, L., J. Mol. Biol., 13, 482, (1965).

Thomas, G.J., Jr. and Kyogoku, Y., J. Am. Chem. Soc., 89, 4170, (1967).

Ulbricht, T.V.L., Jennings, J.P., Scopes, P.M., and Klyne, W., Tetrahedron Letters, 695, (1964).

Ulbricht, T.V.L., Emerson, T.R., and Swan, R.J., Tetrahedron Letters, 1561, (1966).

Van Holde, K.E., Brahms, J. and Michelson, A.M., J. Mol. Biol., 12, 726, (1965).

Velick, S.F., 'Light and Life,' ed. McElroy, W.D. and Glass, B., The Johns Hopkins Press, Baltimore, p. 108, (1961).

Warshaw, M.M., Bush, A.C. and Tinoco, I., Jr., Biochem. Biophys. Res. Comm., 18, 633, (1965).

Weber, G., Biochem. J., 47, 114, (1950).

Weber, G., Nature, 180, 1409, (1957).

Weber, G., J. Chim. Physique, 88, 878, (1958).

Whitby, L.G., Biochem. J., 54, 437, (1953).

Winer, A.D. and Schwert, G.W., J. Biol. Chem., 231, 1065, (1958).

Yang, J.T., Samejima, T. and Sarkar, P.K., Biopolymers, 4, 623, (1966).

TABLE I: SPECTRAL PROPERTIES OF NADH, NMNH AND AMP.

	NADH	NMNH	AMP
λ max	339 mμ	337 mμ	
Bandwidth, Δ	33 mu	35 mu	
[M] pk,tr	0.65×10^3	1.0×10^3	
[θ]	-0.53×10^3	-0.82×10^3	
R_{340}	0.007	0.011	
λ max	259 mμ		259 mμ
Bandwidth, Δ	18 mμ		15 mμ
Crossover (ORD)	258 mμ		258 mμ
[M] pk,tr	14.1×10^3		5.7×10^3
	(15.7×10^3)*		
[θ]	-11.6×10^3		-4.7×10^3
	(-12.9×10^3)*		
R_{260}	-0.12		-0.04

NOTE: [M] pk,tr represents the apparent amplitude in the 340 mμ or 260 mμ region and [θ], the associated maximum ellipticity, is given by Schellman and Shellman, (1965)as:

$$[\theta] = \pm\ 0.82\ [M]\ pk,tr$$

Units of [M] and [θ] are degree cm^2/decimole.

The Bandwidth, Δ, is evaluated from the long wavelength side of the absorption band. It may also be evaluated approximately from positions of extrema using:

$$\Delta = \frac{[\lambda pk - \lambda tr]}{1.86}$$

The true measure of rotational strength is not the amplitude, which is inversely proportional to the bandwidth. The rotational strength of the K^{th} band, R_K is given in Debye magnetons, by

$$R_K = \frac{[\theta_K] \cdot \Delta_K}{7514 \cdot \lambda_K}$$

assuming a Gaussian shape to the ellipticity (Moscowitz, 1960).

* This effect is unsymmetrical relative to the crossover. These figures represent a symmetrical effect based on twice the magnitude of the trough, 260 - 267 mμ.

TABLE II: EXTINCTION COEFFICIENTS OF PYRIDINE NUCLEOTIDE COENZYMES AND RELATED COMPOUNDS.

Compound	Wavelength	ϵ	Reference
	mμ	$x10^{-3}$	
NADH	340	6.2	Horecker and Kornberg, 1948
	260	14.4	Siegel, et al., 1959
		14.0	Silverstein, 1965
NAD	260	18.0	Kornberg and Pricer, 1953
		17.8	Siegel, et al., 1959
		17.6	Dalziel, 1963
		17.4	Silverstein, 1965
AMP	260	15.4	Pabst Laboratories, 1956
NMN	260	4.6	Pabst Laboratories, 1961
NMNH	340	(6.2)	See text of this work
	260	1.0	See text of this work

TABLE III: FLUORESCENCE AND ABSORPTION PROPERTIES OF NADH IN ORGANIC SOLVENTS.

Solvent	T%	Q	R	R'
Polyethylene glycol	0	0.7	2.99	0.34
2-Methoxyethanol	0	1.8	2.86	0.32
2-Ethoxyethanol	4	1.4	2.86	0.34
2-Butoxyethanol	4	1.6	3.08	0.36
Methanol	10	1.8	2.80	0.30
Propylene glycol	10	0.9	2.74	0.31
Ethylene glycol	11	2.5	2.86	0.44
Ethanol	14	1.3	2.86	0.31
Glycerol	19	2.2	2.70	0.33
n-Propanol	37	1.5	2.73	0.54
Water	67	(1.0)	2.70	0.33
Dioxane	84	2.1	2.40*	0.83*
t-Butanol	97	1.8	2.69*	0.67*
sec-Propanol	100	1.6	2.68	0.66

*NOTE: Solutions unstable at 10^{-4} M.

TABLE IV: FLUORESCENCE PROPERTIES OF NADH IN WATER - ALCOHOL MIXTURES.

Alcohol	Methanol		Ethanol		t-Butanol	
% by Volume	T%	Q	T%	Q	T%	Q
0	67	1.0	67	1.0	67	1.0
20	51	1.1	55	1.1	38	1.2
40	32	1.2	29	1.3	29	1.3
60	23	1.4	20	1.4	25	1.3
80	14	1.5	15	1.5	15	1.5
100	10	1.8	14	1.3	97	1.8

FIGURE LEGENDS

FIGURE 1 ORD spectra of NADH, AMP, NMNH, and the sum, AMP+NMNH at 27°. Concentaration: 0.12 mM, in Tris, 0.1 M, pH 8.0. Path length = 1 cm. Scale: 2 Millidegrees = molecular rotation of 1.6×10^3 degree $cm^2/_{decimole}$. Trough at 273 mμ = 7.8×10^3 degree $cm^2/_{decimole}$. The arrow on the baseline indicates the maximum noise-level encountered.

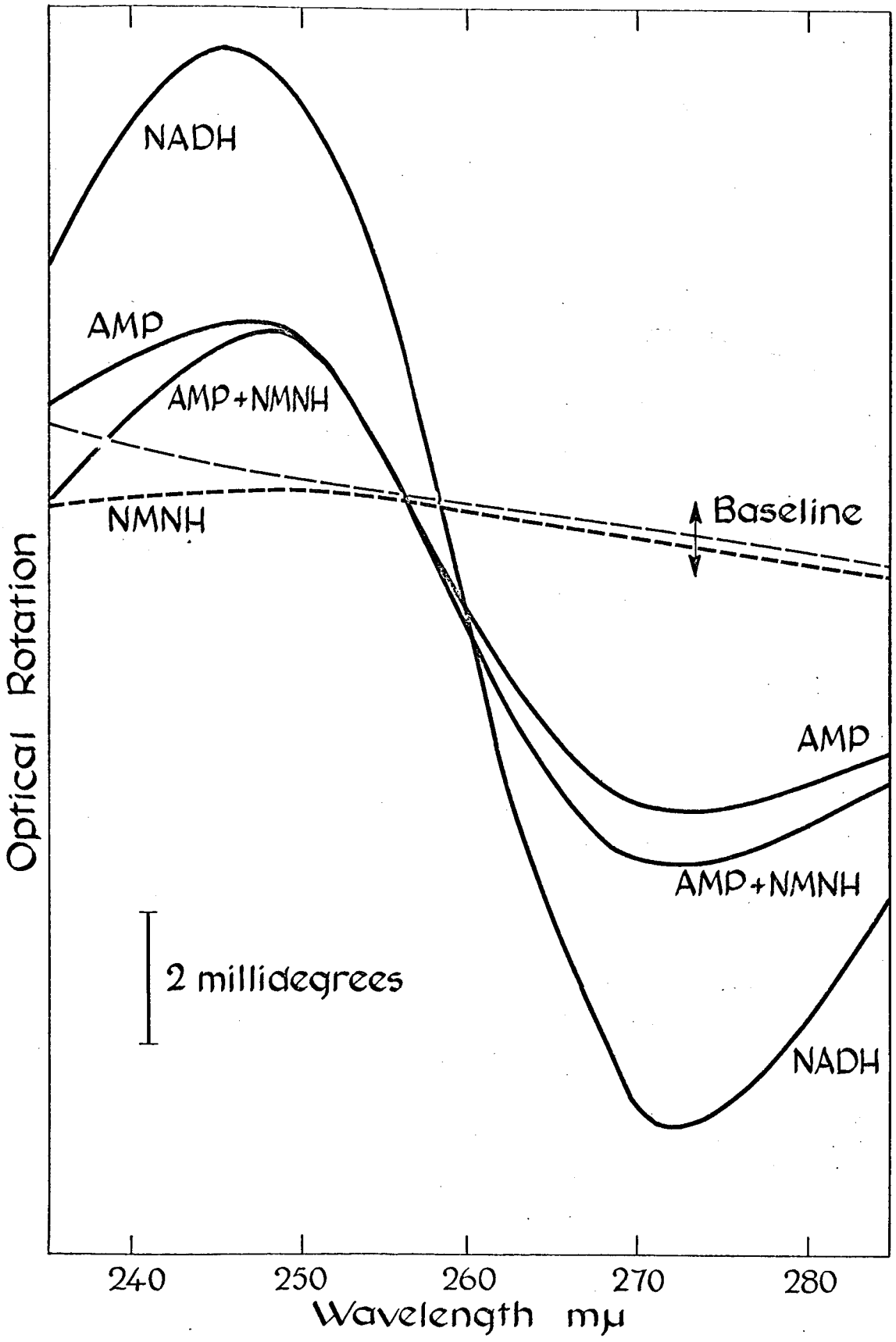
FIGURE 2 Effect of pyrophosphatase digestion at 22° on the ORD spectrum of NADH, 0.11 mM, in Tris, 0.1 M, pH 8.0. Elapsed time: 1,0'; 2,60'; 3,140'; 4,250'. The broken line, 5, is after standing until 450'.

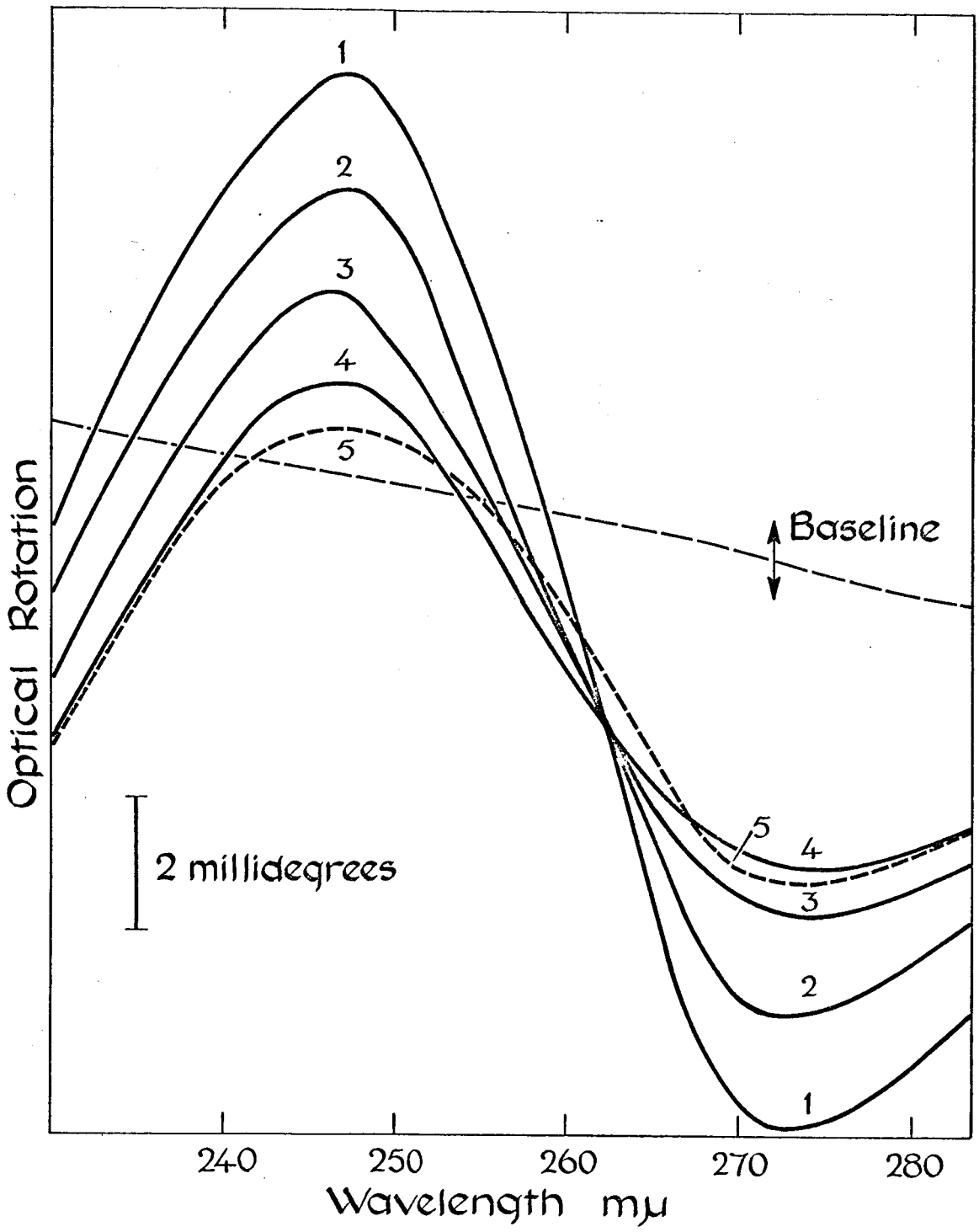
FIGURE 3 The ORD spectrum of NADH at different temperatures. Concentration: 0.085 mM. Path length = 1 cm.

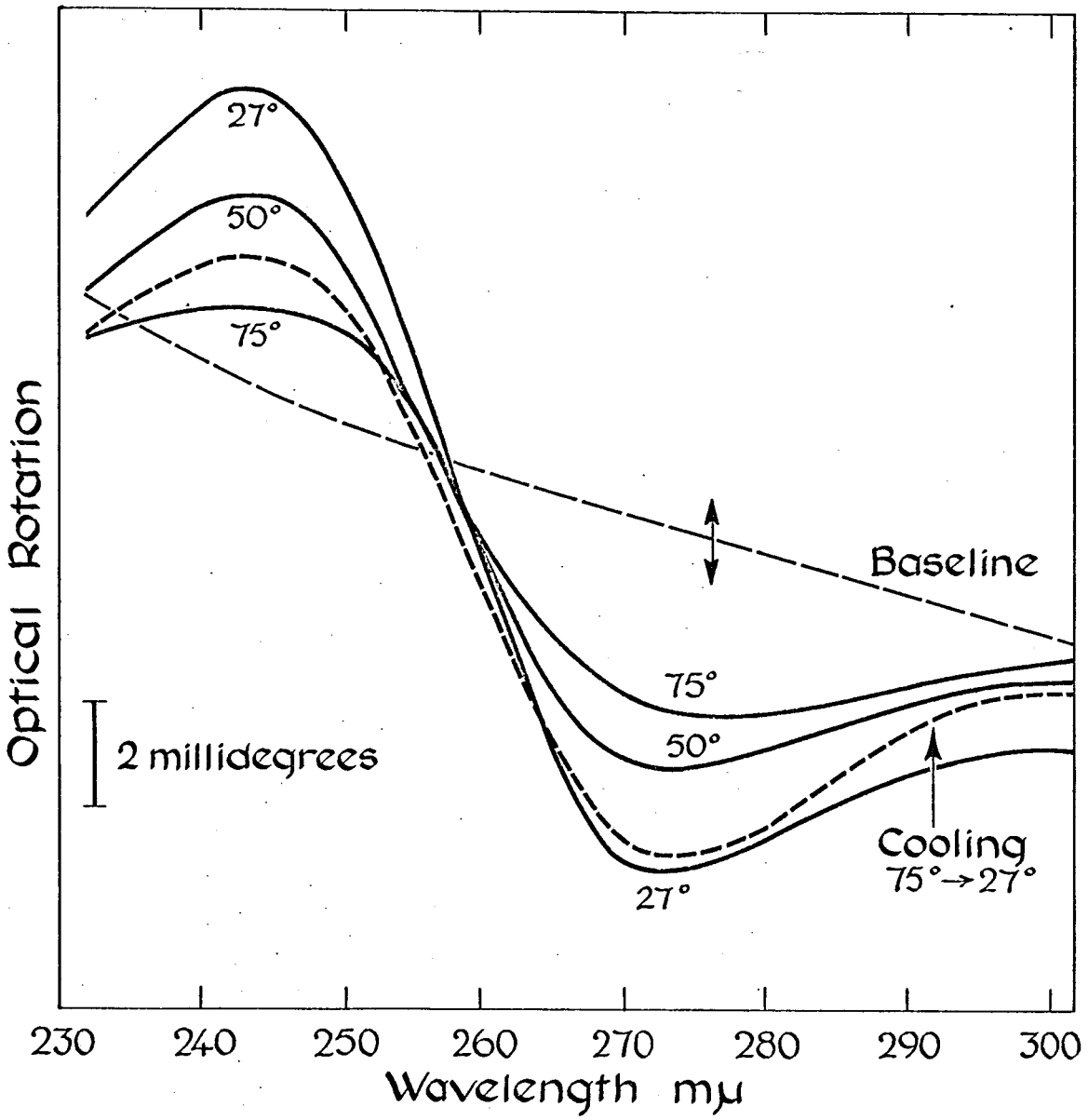
FIGURE 4 Effect of pyrophosphatase hydrolysis at 37° and 25° on the efficiency of energy transfer, T%, and the absorption spectrum, $R = OD_{260}/OD_{340}$, $R' = OD_{290}/OD_{340}$. To 0.5 ml 5.0 μM NADH was added 10 μl (37°) or 20 μl (25°) pyrophosphatase. Aliquots were withdrawn at various times and diluted in 0.1 M Tris pH 8.0, 50-fold for OD and 500-fold for fluorescence measurements. Blanks were performed similarly without enzyme.

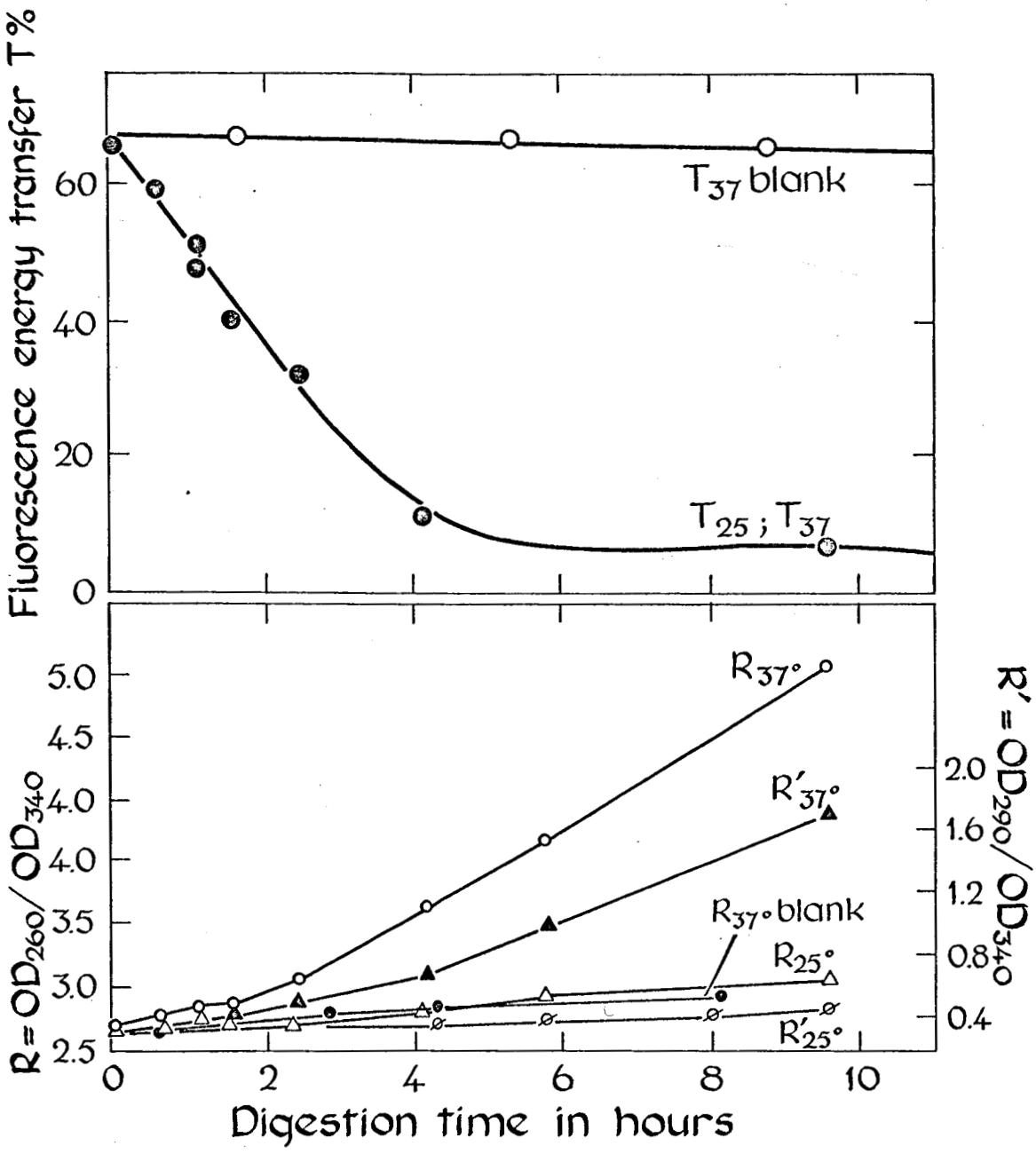
FIGURE 5 Absorption spectra of the addition product formed during pyrophosphatase digestion at 37°, pH 8.0.

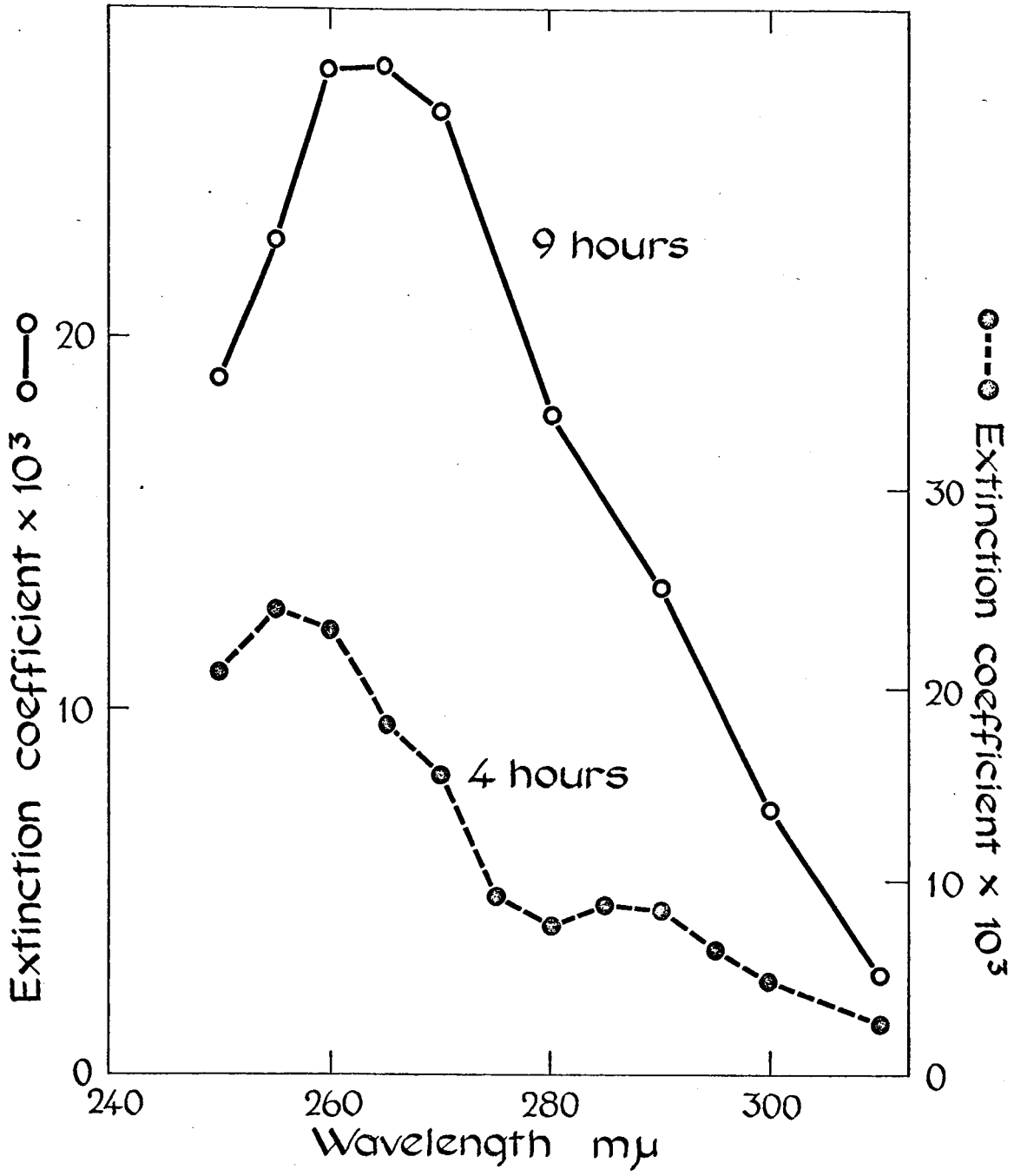
FIGURE 6 Absorption spectrum of NMNH, 0.083 mM, in Tris 0.1 M, pH 8.0, at 37°. Elapsed time: 1, 0'; 2, 50'; 3, 140'; 4, 260'; 5, 380'; 6, 620'.

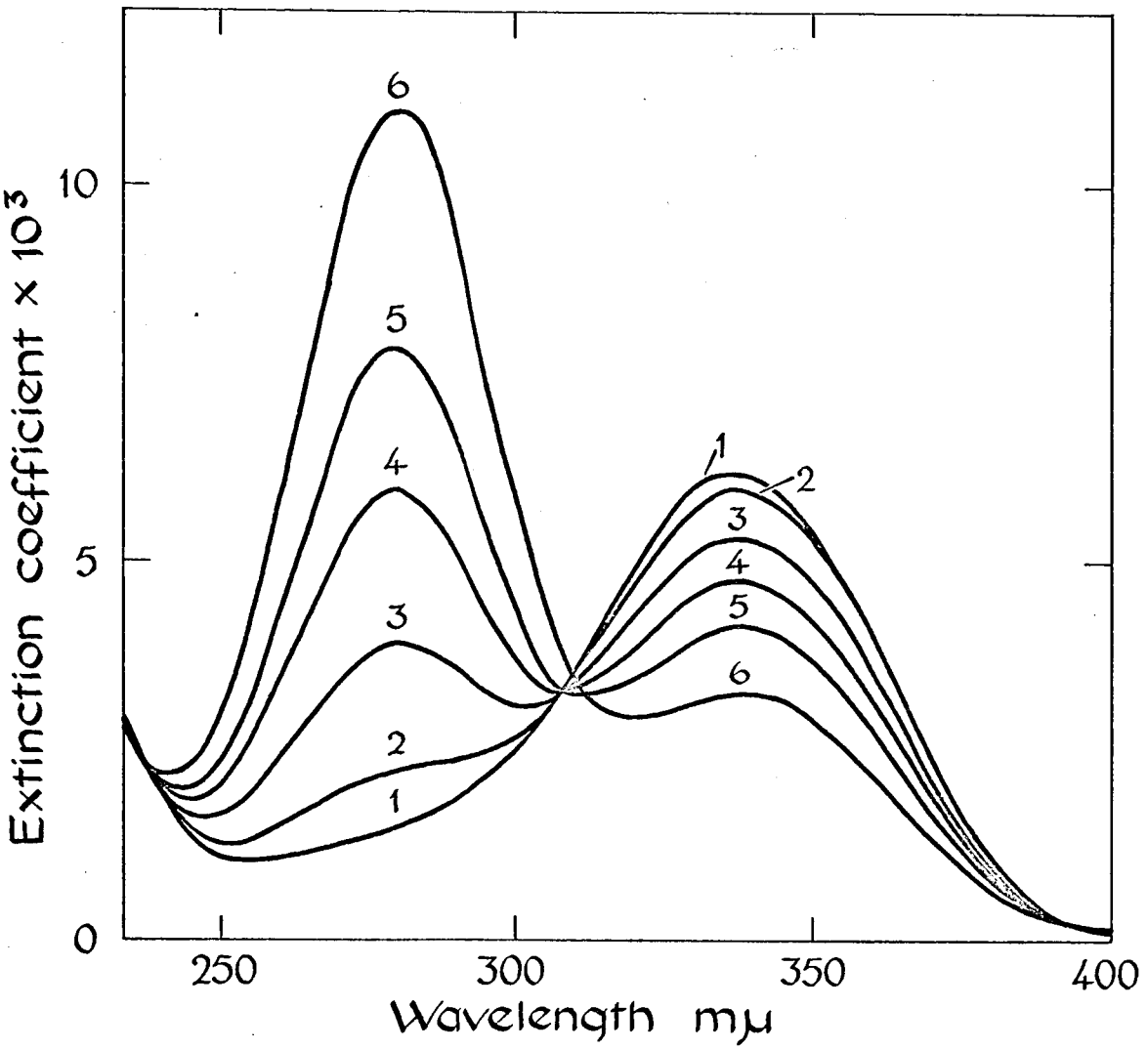

NADH
AMP
AMP+NMNH
NMNH
Baseline
AMP
AMP+NMNH
NADH
2 millidegrees
Optical Rotation
240
250
260
270
280
Wavelength mμ


1
2
3
4
5
Baseline
Optical Rotation
2 millidegrees
5
4
3
2
1
240
250
260
270
280
Wavelength mμ


27°
50°
75°
Baseline
75°
50°
27°
Cooling
75°→27°
2 millidegrees
Optical Rotation
230
240
250
260
270
280
290
300
Wavelength mμ


Fluorescence energy transfer T%
60
40
20
0
T_{37} blank
T_{25} ; T_{37}
$R = OD_{260}/OD_{340}$
5.0
4.5
4.0
3.5
3.0
2.5
$R_{37°}$
$R'_{37°}$
$R_{37°}$ blank
$R_{25°}$
$R'_{25°}$
$R' = OD_{290}/OD_{340}$
2.0
1.6
1.2
0.8
0.4
0
2
4
6
8
10
Digestion time in hours


9 hours
4 hours
Extinction coefficient × 10³
Extinction coefficient × 10³
0
10
20
0
10
20
30
240
260
280
300
Wavelength mμ


Extinction coefficient × 10³
10
5
0
250
300
350
400
Wavelength mμ
6
5
4
3
2
1
1
2
3
4
5
6