84, 1345.
Mandelkern, L., and Stewart, W. R. (1964), *Biochemistry 3*, 1135.
Miller, G. L., and Miller, E. S. (1948), *Anal. Chem. 20*, 481.
Némethy, G. Steinberg, I. Z., and Scheraga, H. A. (1963), *Biopolymers 1*, 43.
Pauling, L. (1960), The Nature of the Chemical Bonds, 3rd ed, Ithaca, N. Y., Cornell Univ., pp 321, 465, 498.
Robinson, W., and Jencks, W. (1963), *J. Biol. Chem. 238*, 1558.
Robinson, W., and Jencks, W. (1965a), *J. Am. Chem. Soc. 87*, 2462.
Robinson, W., and Jencks, W. (1965b), *J. Am. Chem. Soc. 87*, 2470.
Robinson, R. A., and Stokes, R. H. (1959), Electrolyte Solutions, London, Butterworths, p 497.
Schellman, J. A. (1955), *Compt. Rend. Trav. Lab. Carlsberg 29*, 223.
Scheraga, H. (1963), *Proteins*, 535.
von Hippel, P. H., and Wong, K. Y. (1964), *Science 145*, 577.

Intramolecular Interactions in Flavin Nucleotides*

Irving Listowsky,† Sasha Englard, Joseph J. Betheil, and Sam Seifter

ABSTRACT: The intramolecular interactions of flavin–adenine dinucleotide (FAD) have been investigated using optical rotatory dispersion techniques. A Cotton effect with a trough at 395 mμ and a peak at 355 mμ was observed for flavin–adenine dinucleotide in aqueous solution. An equimolar mixture of its constituent mononucleotides, riboflavin 5′-phosphate and adenosine 5′-phosphate, exhibited a curve with no maxima or minima in these wavelength regions. Moreover, the shapes of this curve and that of FAD between 280 and 220 mμ were dissimilar, and the rotational magnitude near 260 mμ in the case of the dinucleotide was almost 10-fold greater than that observed for the combined mononucleotide components. These divergences were attributed to intramolecular interactions occurring in FAD. Environmental conditions that disrupt such interactions were then studied in relation to specific changes in optical rotatory dispersion properties. Several solvents of low dielectric constant were shown to diminish the amplitude of the Cotton effect of FAD in the region of 400–350 mμ and, in the case of riboflavin 5′-phosphate, induce a Cotton effect in this spectral region. In contrast to results obtained with water as solvent, FAD in certain nonaqueous solvents exhibited optical rotations equal to the added rotations of its component mononucleotides.

It is well established that flavin–adenine dinucleotide (FAD)[1] and nicotinamide–adenine dinucleotide (NADH) exist in folded conformations, with the two component ring systems of either compound occurring in close spatial juxtaposition. The internal complex formation, *i.e.*, interaction, between the adenylyl and isoalloxazine moieties of FAD results in marked quenching of the flavin fluorescence (Weber, 1950; Bessey *et al.*, 1949). Furthermore, the absorption spectra of the constituent mononucleotides of FAD are not additive in the dinucleotide spectrum (Warburg and Christian, 1933; Whitby, 1953). Some specific structural requirements for formation of intramolecular complexes of this type have been elucidated (Tsibris *et al.*, 1965; Chassey and McCormick, 1965). Following the action of nucleotide pyrophosphatase on NADH, increased absorption of light at 260 mμ was observed (Seigel *et al.*, 1959) together with disappearance of the fluorescence spectrum due to activation at 260 mμ (Kornberg and Pricer, 1950). One should note that the constituent mononucleotides of NADH are not fluorescent; adjacent to one another in the dinucleotide, however, they allow transfer of excitation energy from adenine to pyridine moieties (Weber, 1957, 1958). When the nucleotide coenzymes are bound to specific proteins, a wide variety of properties due to interactions occurs (Beinert, 1960; Kaplan, 1960).

Ultraviolet optical rotatory dispersion techniques have been applied to the study of the ordered stacking of the bases in nucleic acids (Samejima and Yang, 1964, 1965) and in polynucleotides (Sarker and Yang, 1965), and to the thermal disruption of these structures.

* From the Department of Biochemistry, Albert Einstein College of Medicine, Yeshiva University, New York, N. Y. 10461. *Received April 21, 1966.* This investigation was supported by Grants GM-04428 and GM-10878 from the National Institutes of Health, U. S. Public Health Service, and Grant GB-2536 from the National Science Foundation.

† Public Health Service Postdoctoral Fellow 5-F2-CA-19,958. National Cancer Institute.

[1] Abbreviations used in text: FAD, flavin-adenine dinucleotide; FMN, riboflavin 5′-phosphate; NAD^+ and NADH, oxidized and reduced nicotinamide-adenine dinucleotide; AMP, adenosine 5′-phosphate.

Curves exhibiting Cotton effects also have been described for mononucleosides and mononucleotides (Lin *et al.*, 1964; Ulbricht *et al.*, 1964; Emerson *et al.*, 1966). In the case of a simple dinucleotide, adenylyl-(3′→5′)-adenosine, the optical rotatory dispersion curve in the region of adenine absorption was found to display substantial differences from a curve obtained by addition of the curves of the constituent mononucleotides (Warshaw *et al.*, 1965; Holcomb and Tinoco, 1965). This result was ascribed to interactions between the two adenine rings in the dinucleotide leading to a splitting of the 260-mμ absorption band into two bands having Cotton effects of opposite sign. Base–base interactions in a trinucleotide also have been observed to induce optical rotatory dispersion properties different from those obtained by summation of parameters of the component bases observed individually (Vournakis *et al.*, 1966). Optical rotatory dispersion measurements have also been of great utility for the study of enzyme–coenzyme interactions (Vallee and Ulmer, 1965; Listowsky *et al.*, 1965); however relatively few studies have been made for the free coenzymes.

In this communication we report the optical rotatory dispersion properties arising from the intramolecular interactions in flavin–adenine dinucleotide and the rotational changes due to perturbations of the interactions. It will be shown that the rotatory measurements afford a method for detection of internal complexes in FAD and for study of their nature. While this manuscript was in preparation, a communication appeared (Simpson and Vallee, 1966) which includes a discussion of the optical rotatory dispersion curves for FMN and FAD. Data obtained by Simpson and Vallee agree closely with a portion of the results presented here.

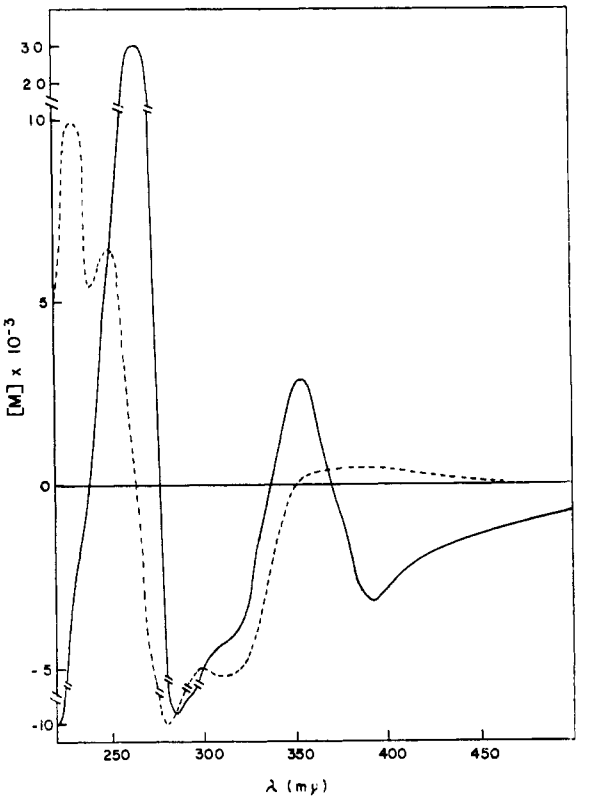

FIGURE 1: Optical rotatory dispersion curves of FAD (solid line) and the sum of AMP plus FMN (dashed line) in aqueous solution, pH 5. The flavin concentrations were 1.8×10^{-4} M for the measurements above 290 mμ, and 3.6×10^{-5} M for the measurements at lower wavelengths.

Experimental Procedure

Materials. FAD, FMN, AMP, and riboflavin were obtained from Sigma Chemical Corp. Concentrations of these substances in water were determined spectrally at the wavelengths of their respective absorption maxima using known molar extinction coefficients (Whitby, 1953). Unless indicated, solutions were not buffered. pH of solutions was adjusted by addition of NaOH or HCl. If necessary, organic solvents were purified by distillation

Methods. Optical rotatory dispersion measurements were made using a Cary Model 60 recording spectropolarimeter. A Cary Model 14 spectrophotometer was used for measurement of absorption of each solution over the spectral range studied in the spectropolarimetric experiments. Absorbancies were always below a value of 2.0, and concentrations of test substances were adjusted accordingly. The slit widths of the polarimeter were programmed to maintain a resolution of better than ±0.75 mμ at all wavelengths. The reproducibility of the observed rotations was within 5×10^{-4}°. A cell of 1-cm light path length was used routinely for the optical rotatory dispersion measurements except in the concentration dependence studies when a cell of 0.1-cm path was used. The temperature within the cell compartment was maintained at 27°.

Molar rotations ($[M]_\lambda$) were calculated from the specific rotations ($[\alpha]_\lambda$) by use of the following equation

$$[M]_\lambda = \frac{[\alpha]_\lambda MW}{100}$$

Corrections were not made for the variation of refractive index of the solutions as a function of wavelength and for the differences of refractive index of the various solvents, as these would not alter the conclusions made on the basis of the molar rotational calculations.

Results

The optical rotatory dispersion curve obtained for FAD in aqueous solution is shown in Figure 1. It is characterized by multiple Cotton effects in the spectral range from 450 to 210 mμ. A shoulder occurs in the region between 320 and 280 mμ. This shoulder is observed in the curve for FMN, shown in Figure 2, and indeed the rotations in this region are very similar to

TABLE I: Molar Rotational Values (in degrees) for Flavin Nucleotides at Selected Wavelengths.

Solvent and Conditions	FAD		FMN	
	$[M]_{355} - [M]_{395} \times 10^{-3}$	$[M]_{260} \times 10^{-3}$	$[M]_{355} - [M]_{395} \times 10^{-3}$	$[M]_{260} \times 10^{-3}$
Water, pH 5	+5.9 ± 0.2	+31.0 ± 0.9	+0.3	+3.1
Water, pH 10	+5.7	+23.0	−0.6	—
Water, pH 3	+3.6	+14.8	+0.3	—
90% (v/v) ethanol	+2.8	—	+2.7	—
90% (v/v) ethylene glycol	+3.2	+8.1	+3.0	+5.6
95% (v/v) 2-methoxyethanol	+4.6	+3.2	+4.4	+9.9
Potassium iodide (5 M)	+1.8	—	—	—
Caffeine (0.1 M)	+4.0	—	+0.3	—
5% phenol, pH 5.5	+3.1	—	—	—

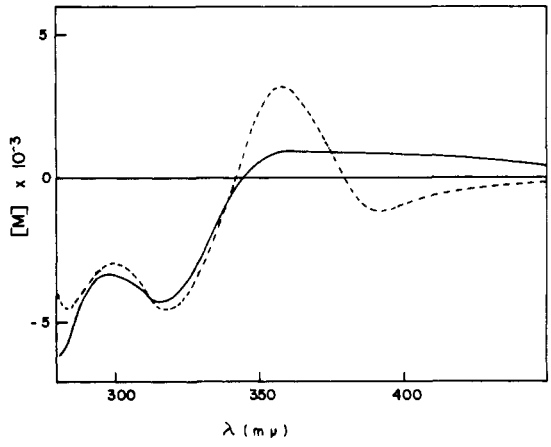

FIGURE 2: Optical rotatory dispersion curves of FMN in water, pH 5 (solid line), and in 2-methoxyethanol (dashed line). The concentrations were the same as in Figure 1.

those obtained with FAD. The prominent differences between the curves for FMN and FAD are in the spectral region between 400 and 350 mμ and at wavelengths below 280 mμ. The curve for FAD is distinguished by a trough at 395 mμ and a peak at 355 mμ with an inflection in the region of 375 mμ (Figure 1). In contrast, the curve for FMN in water has no significant maxima or minima in the region of these wavelengths (Figure 2). In addition, a maximum near 260 mμ is observed for FAD, and the rotation at this wavelength is approximately 10-fold that obtained for FMN. The curves for FMN and FAD also diverge greatly at wavelengths below 260 mμ. In other experiments, riboflavin was found to exhibit optical rotatory dispersion characteristics very similar to those of FMN.[2]

It is likely that the large rotational differences between FAD and its mononucleotide components examined separately are a consequence of the conformational changes induced in the molecule by interaction between the isoalloxazine and adenine rings. Accordingly, the amplitude of the Cotton effect in the vicinity of the 375-mμ absorption band of FAD and the optical rotation at 260 mμ may be convenient parameters for study of the intramolecular complexes. In this regard, then, the effects of various perturbing conditions on these parameters were studied, and some of the results are shown in Table I.

In the table one may note the influence of pH on the optical rotatory properties. The amplitude of the rotations in the region between 395 and 355 mμ is significantly diminished at pH 3, and the rotations at 260 mμ are also much lower than those at the higher pH values. In the far-ultraviolet region, the optical rotatory dispersion curves obtained at pH 5 and 10 are very similar ($[M]_{220}$ −11,000 and −13,200°, respectively), and distinct from the curve obtained at pH 3 ($[M]_{220}$ +22,800°).

When the concentration of FAD was varied between 10^{-4} and 10^{-3} M, no changes in the shape of the optical rotatory dispersion curve were encountered. Nor were changes in shape of the curve observed when measurements were made in 0.2 M phosphate buffer, pH 7, or in saturated NaCl solution. Thus the increased ionic strength in these instances was without effect.

Caffeine, phenol, and many purines and pyrimidines have been shown to interact intermolecularly with the isoalloxazine ring of flavins (Harbury and Foley, 1958) resulting in quenching of riboflavin fluorescence (Weber, 1958) in a manner analogous to the internal quenching by the adenine moiety of FAD. As seen in Table I,

[2] After this paper was submitted for publication, optical rotatory dispersion curves for FMN and FAD, similar to those described here and by Simpson and Vallee (1966), have been reported in a paper by Wellner (1966). The optical rotatory dispersion curve for FAD previously reported by Gascoigne and Radda (1965) differs markedly from the corresponding curve obtained in the present study. This discrepancy may be attributed to instrumentational artifacts (Resnik and Yamaoka, 1966) inherent in the spectropolarimeter used by Gascoigne and Radda (1965).

caffeine and phenol indeed decrease the amplitude of the 375-mμ Cotton effect. In addition, potassium iodide, which is considered to quench the fluorescence of riboflavin by a collision mechanism (Weber, 1958), sharply diminishes the amplitude of the FAD Cotton effect.

The effects of a hydrophobic environment on the rotatory properties of FAD were studied, and the results are shown in Table I. It may be seen that in ethanol, ethylene glycol, or 2-methoxyethanol, the rotations near 260 mμ are much lower than those at this wavelength for FAD in water. In all cases, however, the Cotton effects in the 375-mμ region are present, and have amplitudes intermediate between that of the Cotton effects of FAD and of the sum of FMN and AMP in aqueous solution (Figure 1). The optical rotatory properties of FMN in the same nonaqueous solvents were then investigated. Some of these results are also shown in Table I and in Figure 2. Although a smooth curve was obtained in the 400–350-mμ region for FMN in water, an apparent Cotton effect was induced in the nonaqueous media (Figure 2). Comparison of the $[M]_{355} - [M]_{395}$ values for FMN and FAD in the nonaqueous solvents shows them to be very similar (Table I). The rotations in the ultraviolet region below 280 mμ obtained for FMN or FAD in the several solvents do exhibit some variation, but all are quite distinct from those obtained for FAD in water.

A study was then made of the effect of varying the proportion of water to 2-methoxyethanol on the rotatory dispersion properties of FMN and FAD. The results are plotted in Figure 3. For FMN, the amplitude of the Cotton effect increases from a value of almost zero in water to +4400° in 95% (v/v) 2-methoxyethanol. In the case of FAD, the amplitude in water decreases from a value of +5900° through a minimum of +3500° in 50% 2-methoxyethanol and increases to a final value of +4600° in 95% 2-methoxyethanol. Figure 3 also shows that the difference between the curves for FMN and FAD decreases almost linearly with the increase of concentration of 2-methoxyethanol. It is thus apparent that the Cotton effect in the region of 375 mμ observed for FAD in the nonaqueous solutions is associated with the riboflavin phosphate moiety and, contrary to appearances, is not indicative of a residual intramolecular interaction.

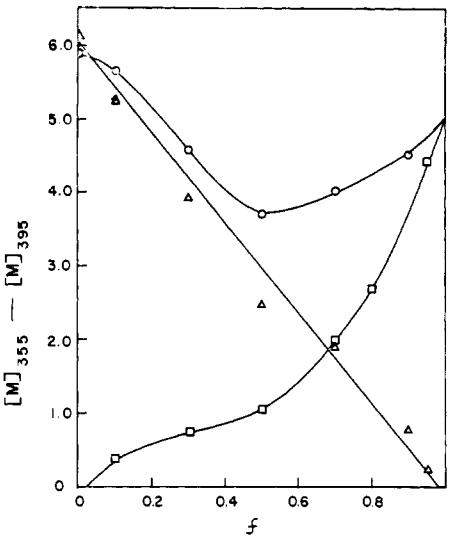

FIGURE 3: Molar rotational difference between the rotations at the peak at 355 mμ and those at the trough at 395 mμ as a function of the fraction of 2-methoxyethanol (v/v). Circles represent the values for FAD; Squares represent the values for FMN; and triangles represent the differences between the FMN and FAD values.

Discussion

Since a folded conformation restricts the free rotation about the bonds at or near the asymmetric centers of FAD, the stereochemistry about the optically active chromophores is modified, resulting in changes in the optical rotatory dispersion and spectral properties of the molecule. The hypochromicity of the isoalloxazine absorption band at 375 mμ, observed for FAD in comparison with FMN, appears to be associated with the formation of a new optically active absorption band. In FAD, this would account for the Cotton effect with a trough at 395 mμ and a peak at 355 mμ; this is not observed for FMN or riboflavin. In addition, the rotations in the region of the 260-mμ absorption band for FAD in the intramolecularly complexed form are considerably greater than those for FMN. Thus the optical rotatory dispersion curve for FAD cannot be constructed by addition of the separate curves for FMN and AMP, its constituent nucleotides. These results are analogous to those obtained with adenylyladenosine and adenylyladenylylcytosine (Warshaw *et al.*, 1965; Vournakis *et al.*, 1966). Conditions or substances that disrupt the intramolecular complex in FAD may be characterized by their influence on the amplitude of the Cotton effect in the region of 375 mμ and the peak near 260 mμ.

In aqueous solution, riboflavin and FMN, compared to FAD, exhibit relatively smooth curves in the region of 350–400 mμ and much lower rotations near 260 mμ. In solvents of lower dielectric constant, however, riboflavin and FMN exhibit curves with a trough at 395 mμ and a peak at 355 mμ, but the amplitude is smaller than that observed with FAD in water. The rotations in the region of 260 mμ, as in the aqueous situation, are much lower than those for FAD in the same region. In the nonaqueous solvents, the amplitude of the 375-mμ Cotton effect observed for FAD is less than that for FAD in water, but almost identical with that for FMN in the same nonaqueous solvents. Also, the 260-mμ peak for FAD in the nonaqueous solvents is greatly decreased in rotational strength. Thus with regard to the rotatory properties of FAD in the nonaqueous solvents, two opposing tendencies would appear to operate: (a) because the intramolecular interaction between the constituent ring systems is modified, the 375-mμ Cotton effect observed in water

is absent; (b) because of the effect of the nonaqueous solvent on the isoalloxazine chromophore, a new Cotton effect of similar properties appears. The resultant is expressed as a diminished 375-mμ Cotton effect.

The observation that the rotational amplitude of the 375-mμ Cotton effect for FAD is equal to that for FMN in the nonaqueous solvents is additional evidence that the adenylyl–isoalloxazine interaction is abolished. This is consistent with the observation that the fluorescent properties of FMN and FAD in 2-methoxyethanol are identical (Velick, 1961). The hydrophobic interaction of the riboflavin moiety with the solvent in these instances appears to dominate its hydrophobic interaction with the adenine moiety. The induced 375-mμ Cotton effect for FMN in the organic solvents might possibly be related to the tendency of this molecule to dimerize (Gibson *et al.*, 1962; Radda and Calvin, 1964). Attempts to study the concentration dependence of this phenomenon were not conclusive, and the nature of the responsible molecular modifications remains unresolved.

At pH values below 1, both FMN and FAD do not fluoresce. In the region of pH 3, FAD exhibits a maximum fluorescence, and above pH 3, the fluorescence is progressively quenched internally. The protonation of the amino group of adenine at pH 3 results in changes in the rotatory properties consistent with a probable alteration of the hydrophobic adenine–isoalloxazine interaction. The spectral properties of FAD at wavelengths below 250 mμ are different at pH 3 from those at high pH values. The changes in spectral absorption are accompanied by large differences in the optical rotatory dispersion curve.

The results with FAD again demonstrate the utility of the techniques of optical rotatory dispersion for study of conformational changes associated with intramolecular interactions. It should be apparent that similar studies with flavoproteins might give useful information concerning the conformational aspects of the flavin binding.

References

Beinert, H. (1960), *Enzymes 2*, 339.

Bessey, O. A., Lowry, O. H., and Love, R. H. (1949), *J. Biol. Chem. 180*, 755.

Chassey, B. M., and McCormick, D. B. (1965), *Biochemistry 4*, 2612.

Emerson, T. R., Swan, R. J., and Ulbricht, T. L. V. (1966), *Biochem. Biophys. Res. Commun. 5*, 505.

Gascoigne, I. M., and Radda, G. K. (1965), *Chem. Commun. 21*, 533.

Gibson, Q. H., Massey, V., and Atherton, N. M. (1962), *Biochem. J. 85*, 369.

Harbury, H. A., and Foley, K. A. (1958), *Proc. Natl. Acad. Sci. U. S. 44*, 662.

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

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

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

Lin, C. Y., Urry, D. W., and Eyring, H. (1964), *Biochem. Biophys. Res. Commun. 17*, 642.

Listowsky, I., Furfine, C. S., Betheil, J. J., and Englard, S. (1965), *J. Biol. Chem. 240*, 4253.

Radda, G. K., and Calvin, M. (1964), *Biochemistry 3*, 384.

Resnik, R. A., and Yamaoka, K. (1966), *Biopolymers 4*, 242.

Samejima, T., and Yang, J. T. (1964), *Biochemistry 3*, 613.

Samejima, T., and Yang, J. T. (1965), *J. Biol. Chem. 240*, 2094.

Sarker, P. K., and Yang, J. T. (1965), *J. Biol. Chem. 240*, 2088.

Seigel, J. M., Montgomery, G. A., and Bock, R. M. (1959), *Arch. Biochim. Biopohys. 82*, 288.

Simpson, R. T., and Vallee, B. L. (1966), *Biochem. Biophys. Res. Commun. 22*, 712.

Tsibris, J. C. M., McCormick, D. B., and Wright, L. D. (1965), *Biochemistry 4*, 504.

Ulbricht, T. L. V., Jennings, J. P., Scopes, P. M., and Klyne, W. (1964), *Tetrahedron Letters, 13*, 695.

Vallee, B. L., and Ulmer, D. D. (1965), Symposium on Non Heme Iron Proteins, San Pietro, A., Ed., Yellow Springs, Ohio, Antioch, p 43.

Velick, S. F. (1961), Light and Life, McElroy, W. D., and Glass, B., Ed., Baltimore, Md., Johns Hopkins, p 108.

Vournakis, J. N., Scheraga, H. A., Rushinsky, G. W., and Sober, H. A. (1966), *Biopolymers 4*, 33.

Warburg, O., and Christian, W. (1933), *Biochem. Z. 266*, 377.

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

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

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

Weber, G. (1958), *J. Chim. Phys. 55*, 878.

Wellner, D. (1966), *Biochemistry 5*, 1585.

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