On the Effect of Temperature on the Absorption Spectra of Free and Protein-Bound Flavines†

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ABSTRACT: The absorption spectra of free and protein bound flavines show small temperature dependent changes in the range 2–30°. These reversible effects were studied by measuring difference spectra between a solution of flavine at low temperature in the reference beam of a spectrophotometer and an identical solution at a higher temperature in the sample beam. With the free flavines, the changes observed above 10^{-4} M flavine are not linear with temperature and are probably influenced by intermolecular interactions. At 10^{-5} M flavine and below, the difference spectra are independent of concentration and they change linearly with temperature. The shape of the temperature difference spectrum, like the absolute spectrum, depends on solvent. The temperature difference spectrum of FAD in aqueous solution is different from those of other flavines, and is probably due largely to disruption of the intramolecular complex between adenine and isoalloxazine. In methyl Cellosolve which is known to disrupt the internal complex of FAD, the temperature difference spectra of FAD and tetraacetylriboflavine are very similar. The temperature difference spectra of flavoproteins are similar to those of the free flavines except that the peaks given by the proteins are generally better resolved. The changes are not due to dissociation of flavine from the apoproteins; they are reversible, linear with temperature and independent of concentration. The temperature difference spectra consist of three positive regions, which are ascribed to a broadening of the flavine absorption bands with increasing temperature, four negative peaks in the first absorption band, and three further negative peaks in the second band. We ascribe the positions of these seven negative changes to vibronic transitions in the two electronic absorption bands. Our interpretation is in accordance with published circular dichroism and low temperature light absorption data.

The visible and near-ultraviolet absorption spectra of naturally occurring flavines in aqueous solution have two featureless bands at about 450 and 375 nm. The shapes of these spectra can be altered in a number of ways. For example, in solvents less polar than water, the band at 375 nm is generally shifted to shorter wavelengths, while the visible band splits to give two extra bands which are thought to be associated with vibrational transitions in the first electronic absorption at about 450 nm (Harbury *et al.*, 1959; Weber, 1966). Addition of a variety of aromatic compounds such as caffeine and adenosine monophosphate to flavine mononucleotide, FMN,[1] or riboflavine in aqueous solution causes a decrease in the intensity of the absorption at 375 nm and a similar decrease accompanied by a broadening of the band at 450 nm (Weber, 1950, 1966). There is evidence that these changes are due to intermolecular complexes between the isoalloxazine ring and the aromatic ring of the added compound. The spectrum of flavine adenine dinucleotide (FAD) resembles the spectra of such intermolecular complexes of FMN and riboflavine, but in the case of FAD, an intramolecular complex between the isoalloxazine and adenine portions of the molecule has been proposed to explain the low extinction coefficients, decreased fluorescence (Weber, 1950), and altered circular dichroism (CD) spectrum (Miles and Urry, 1968).

Flavoproteins either resemble free FMN and FAD in aqueous solution in exhibiting a rather featureless absorption spectrum, or they show varying resolution of the band at about 450 nm. As with the free flavines, however, the spectrum of a particular flavoprotein depends on a number of factors. For example, the unresolved spectrum of D-amino acid oxidase is changed to a resolved type by addition of a variety of dicarboxylic acids (Massey and Ganther, 1965), and spectra of other flavoproteins are known to be influenced by pH (Schuman and Massey, 1971), the ionic composition of the solution (Schuman and Massey, 1971; Sullivan, 1971), or by the presence of small molecules which may be substrates (*e.g.*, Howell *et al.*, 1972), inhibitors (Massey and Ganther, 1965; Veeger *et al.*, 1966), or effectors (Howell *et al.*, 1972; White-Stevens and Kamin, 1972). These effects are not completely understood.

An additional factor which affects the absorption spectrum of D-amino acid oxidase was noted by Massey *et al.* (1966), who showed that the spectrum of this enzyme depends on temperature in the range of 0.5–20°. The data were used to support other indications that there is a temperature dependent conformational change in this enzyme. They led us to examine the influence of temperature on the spectra of other flavoproteins and also free flavines. Comparable effects were found with all free and protein-bound flavines that were examined.

Materials and Methods

Light absorption spectra were recorded with a Cary 17 spectrophotometer equipped with thermostatic control to the sample and reference compartments. Temperature difference spectra were recorded by placing solutions of enzyme

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[1] Abbreviations used are: FAD, flavine adenine dinucleotide; FMN, flavine mononucleotide; HIP, 7,8-dimethyl-*N*(10)(2′-hydroxyethyl)isoalloxazine 2′-phosphate; TDS, temperature difference spectrum.

or free flavine in matched cuvettes (light paths 0.45, 1.0, 5.0, or 10 cm) in the reference and sample beams of the spectrophotometer. Both cells were equilibrated at a constant temperature between 0.5 and 2°, and any differences between the cells were then canceled by adjusting the multipotentiometers. A base line was recorded. The temperature of the sample cuvette was then raised, and when the absorbance changes were complete, a difference spectrum was recorded. Except where noted, temperature difference spectra given in this paper have been corrected to a temperature difference of 20°, but they have not been corrected for the thermal expansion of the solvent. Where such a correction was applied (Figure 2, Table I) the values used for the solvent expansion between 2 and 22° were: water, 0.22%; acetonitrile, 2.67%; chloroform, 2.44%; formamide, 1.47%; ethanol, 2.08%; methyl Cellosolve, 1.12%. This expansion causes a decrease in the flavine concentration in the sample cuvette, and a corresponding decrease in absorbance. The absorbance decreases were calculated at 5-nm intervals from the absolute spectrum at 2° and added to the observed temperature difference spectrum.

Commercial *p*-dioxane was dried by boiling under reflux with metalic sodium and by subsequent distillation. Solutions of tetraacetylriboflavine in mixtures of *p*-dioxane and water were prepared by placing 0.8 ml of 10^{-3} M tetraacetylriboflavine dissolved in *p*-dioxane into a 10-ml volumetric flask, adding the required amount of water, and making the mixture up to 10 ml with *p*-dioxane A fresh solution was made in this way for each water concentration.

Lactate oxidase (Sullivan, 1968), glucose oxidase (Swoboda and Massey, 1965), flavodoxins (Mayhew and Massey, 1969; Mayhew, 1971a,b), and oxynitrilase (Becker and Pfeil, 1966) were prepared by published procedures. Shethna flavoprotein (*Azotobacter* flavoprotein) was a gift from Dr. H. Beinert. *Escherichia coli* thioredoxin reductase, pig heart and *E. coli* lipoyl dehydrogenase, and yeast glutathione reductase were gifts from Dr. C. H. Williams, Jr., and Mr. D. Arscott, and old yellow enzyme was a gift from Mr. A. S. Abramowitz.

FAD (Sigma) was purified with DEAE-cellulose as described by Massey and Swoboda (1963). FMN was prepared by enzymic hydrolysis of FAD followed by chromatography on DEAE-cellulose (Mayhew, 1971b). Tetraacetylriboflavine and *N*(3)-methyltetraacetylriboflavine were prepared according to the method of Hemmerich *et al.* (1960) and Hemmerich (1964). *S*-Methyl-2-thio- and 2-morpholino-2-desoxylumiflavine were prepared according to the method of Müller and Hemmerich (1966). *C*(7)-BrFMN and *C*(7,8)-Cl_2FMN were gifts from Dr. R. D. Andersen; *N*(3)-CH_3FMN and *N*(3)-CH_2COOHFMN were gifts from Dr. P. Hemmerich. HIP was synthesized from *N*(10)-(β-hydroxyethyl)lumiflavine (Fall and Petering, 1956) by suspension in water-treated $POCl_3$ according to the procedure of Flexser and Farkas (1952) for the preparation of FMN. HIP is readily separated from unreacted starting material by suspending the mixture in an aqueous solution at pH 7–8. The suspension is filtered and the filtrate acidified. This filtration and acidification was repeated twice to give a product which showed a single spot by thin-layer chromatography using plates of MN-Polygram SIL S-HR (binder-starch) from Macherey-Nagel and Company, Düren, Germany. The chromatograms were developed in the solvent mixture 1-butanol–water–2 N CH_3COOH, 6:2:2 (v/v).

Results

Studies with Free Flavines. Since the spectral changes induced in flavines by changes of temperature are small compared with their absolute spectra, we measured difference spectra between a solution maintained at a constant low temperature in the reference beam of the spectrophotometer, and an identical solution at a higher temperature in the sample beam. Examples of difference spectra produced by this means are shown in Figure 1. There was at least one isosbestic point between spectra at different temperatures with all of the flavines tested, and with certain exceptions which are described below, a linear relationship was found between absorbance change and temperature. The observed changes were also completely reversible.

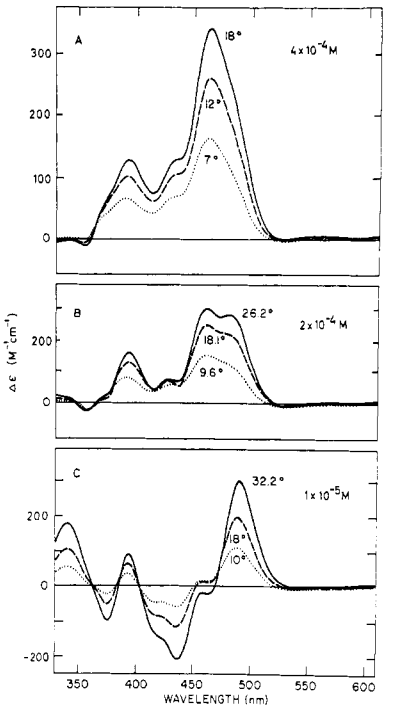


FIGURE 1: Concentration dependence of the temperature difference spectrum of HIP in 0.05 M postassium phosphate (pH 7): (A) 4×10^{-4} M, 0.45-cm light path; (B) 2×10^{-4} M, 0.45-cm light path; (C) 1×10^{-5} M, 10-cm light path. The temperature difference between sample and reference is shown for each curve.

HIP was used as a model for FMN since it is more readily obtained in high purity, it is much less photolabile than FMN, and the temperature difference spectra for these two compounds are almost identical. The shape of the TDS of HIP varies with concentration and with temperature. At concentrations of 4×10^{-4} M flavine and above, only positive changes occur and these show peaks at 465, 430, and 397 nm (Figure 1A). An extra peak (at 485 nm) is apparent when the flavine concentration is halved, but this peak is only clear when the temperature difference is greater than about 25° (Figure 1B); at smaller temperature differences, there is only a shoulder at 485 nm. When the temperature difference is increased to about 40°, the peak at 485 nm becomes more intense than the peak at 465 nm, and the peak at 430 nm declines. It is clear from these nonlinear changes that at this intermediate concentration more than one TDS is contributing to the observed changes. Further dilution of HIP to 1×10^{-5} M gives the temperature difference spectra of Figure 1C. These difference spectra show positive peaks at 490, 396, and 340 nm and negative peaks at 439, 420, and 377 nm, and the changes are now linear with temperature. This type of difference spectrum is obtained with HIP up to a concentration of about 1×10^{-4}

TABLE I: Light Absorption Characteristics of Temperature Difference Spectra of Free and Protein-Bound Flavines and Comparison with Circular Dichroism Data.

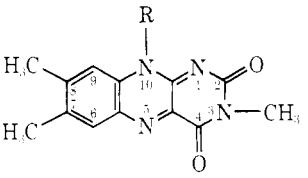
Compound	Solvent	Negative Peaks,[a] λ_{max} [$\Delta\epsilon$ (M^{-1} cm^{-1}), 20°][b]							Positive Peak[c]
Tetraacetylriboflavine[d]	0.1 M phosphate (pH 7)	335 (sh)	370 (110, sh)	380 (180)		424 (91)	444 (145)	475 (47)	498 (170)
	Formamide		362 (65, sh)	375 (122)		420 (60)	446 (148)	478 (140)	500 (145)
	Ethanol	334 (sh)	355 (65, sh)	364 (105)	400	420 (25)	447 (80)	477 (105)	500 (90)
	Acetonitrile		348 (80, sh)	362 (150)	396 (40)	414 (124)	439 (200)	470 (152)	490 (194)
	Methyl Cellosolve			368 (152)	400 (15, sh)	420 (90)	444 (150)	474 (150)	500 (88)
	Chloroform		362 (190, sh)	377 (550)	400 (sh)	428	455 (290)	485 (448)	510 (20)
HIP	0.85 M phosphate (pH 7)			380 (25)		420 (75, sh)	440 (92)	469	493 (200)
FMN	0.1 M phosphate (pH 7)	335 (sh)	362 (125, sh)	375 (167)		425 (110, sh)	440 (154)	465 (20, sh)	495 (165)
$N(3)$-CH_3FMN	0.1 M phosphate (pH 7.4)		364 (95, sh)	375 (135)		425 (82, sh)	442 (120)	470 (5)	497 (187)
FAD	0.05 M phosphate (pH 7)		365	381		422	451	480 (70)	505 (85)
	Methyl Cellosolve	335 (sh)		360 (200)		420 (105)	450 (176)	474 (178)	503 (85)
Flavodoxin	0.05 M phosphate (pH 7.4)	330 (sh)	364 (70, sh)	380 (175)		419 (95)	442 (175)	472 (79)	494 (314)
7-BrFMN flavodoxin			358 (40, sh)	373 (115)	400 (sh)	422 (45)	447 (118)	476 (77)	499 (185)
3-CH_3FMN flavodoxin			360 (94)	376 (94)		433	465 (15)	500 (132)	
3-CH_2COOHFMN flavodoxin			360 (142)		415 (sh)	432	465 (105)	497 (346)	
Shethna flavoprotein			365 (80, sh)	379 (120)	400 (sh)	420 (10)	452 (34)	479 (90)	500 (69)
Lactate oxidase	0.05 M phosphate (pH 7)			381 (170)	405	427 (45)	455 (150)	485 (182)	505 (100)
	0.1 M imidazole-HCl (pH 7)		368 (50, sh)	385 (370)		425	452 (65)	485 (100)	502 (90)
Old yellow enzyme	0.05 M phosphate (pH 7)	340	378 (40, sh)	393 (135)		440	470 (42)	503 (130)	
Lipoamide dehydrogenase									
Pig heart	0.1 M phosphate (pH 7)	338	355 (67)	375 (110)	405	429 (5)	458 (102)	491 (185)	510 (58)
E. coli		340 (35, sh)	354 (75)	372 (83)	410 (25, sh)	428 (100)	452 (155)	484 (95)	505 (230)
Thioredoxin reductase			372 (140)	388 (315)		430	459 (116)	490 (184)	510 (112)
Glutathione reductase		340 (sh)	367 (30)	385 (177)	420	434	465 (125)	498 (215)	517 (140)
Glucose oxidase			365 (100)	386 (123)		430 (sh)	459 (71)	490 (175)	516 (15)
Ferredoxin TPN reductase				375 (75)		435 (128)	455 (132)	489 (74)	510 (110)
Oxynitrilase	0.1 M phosphate (pH 7) plus 0.1 M benzoate		377 (160)	395 (260)		432 (75)	460 (120)	492 (105)	515 (85)
			380 (100, sh)	399 (184)		430	460 (60)	494 (110)	520 (35)

Compound	Solvent	Band (nm)						
3-Methyl-8α-morpholinolumiflavine[e]	Ethanol	324	340	356	395	420	442	470
Tetraacetyiriboflavine[f]	Carbon tetrachloride	330	350	380	405	422	450	480
FMN[g]	0.1 M phosphate (pH 7)	335	360	382		418	448	473
Flavodoxin[g]	0.025 M phosphate (pH 7)–0.05 M EDTA	332	359	378		423	442	471
Shethna flavoprotein[g]		341	368	378		423	446	475
Glucose oxidase[g]	0.1 M acetate (pH 6)–0.05 M EDTA	340	361	387		424	451	481

[a] Troughs in a positive part of the difference spectrum are included in the table but without values for $\Delta\epsilon$. [b] Values for $\Delta\epsilon$ are corrected for the thermal expansion of the solvent as described in Methods. [c] Only the positive peak near 500 nm is given. [d] Values given for the free flavines in aqueous solvents were obtained at a flavine concentration of 1×10^{-5} M. [e] From low temperature (100°K) light absorption data; personal communication of Dr. J. M. Lhoste. [f] From circular dichroism measurements (R. Gast, personal communication). Similar data have been reported by Scola-Nagelschneider and Hemmerich (1972). [g] Circular dichroism data taken from Edmondson and Tollin (1971).

M. We attribute these concentration effects to the intermolecular complexing of flavine which is known to occur at high concentrations (Gibson *et al.*, 1962). The effect of temperature on the spectrum of the complex is evidently different from the effect on the monomer.

A similar concentration dependence was observed with FMN, riboflavine, tetraacetylriboflavine, and lumiflavine. However, N(3)-alkylated flavines (I) gave the mixed type of difference spectrum of Figure 1B at concentrations as low as 5×10^{-3} M. A spectrum of the type shown in Figure 1C was only obtained at concentrations of 2×10^{-5} M and lower suggesting that formation of the complex is favored in N(3)-alkylated flavines. Further evidence that the N(3) position has an important influence in intermolecular complex formation was obtained from the effects of concentration on the temperature difference spectra of compound II. These



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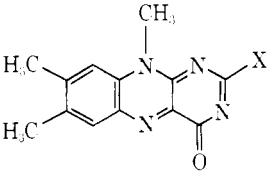

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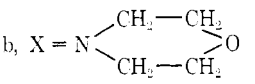
b, X = 

compounds, which also lack a proton at position N(3), show a concentration dependence similar to that of *N*(3)-methyltetraacetylriboflavine. It is possible that hydrophobic interactions are responsible for complex formation and that in the unmodified flavines such interactions are weakened by formation of a hydrogen bond between N(3)H and water. The temperature difference spectra of concentrated solutions of flavines show a shift to the monomer type at high temperature, suggesting that in fact the complex dissociates as the temperature is raised.

The temperature difference spectra of flavines, like their absolute spectra (Harbury *et al.*, 1959), depend on the solvent. We studied this solvent effect using tetraacetylriboflavine as a model because this flavine is soluble in a wide range of solvents. Due to solvent expansion, which causes a decrease of the flavine concentration in the sample cuvette, the experimental curves are distorted to an extent that depends on the solvent. In order to compare spectra in different solvents it was therefore necessary to correct for this expansion as described in the Methods Section. Experimental and corrected spectra for tetraacetylriboflavine in phosphate buffer, acetonitrile, and chloroform are shown in Figure 2, where it can be seen that the correction affects the intensities of the peaks in the TDS but causes little or no change in the positions of the peaks.

The TDS of tetraacetylriboflavine in phosphate buffer shows two positive peaks (499 and ~340 nm), four negative peaks (475, 445, 423, and 380 nm), and a shoulder at 370 nm. The difference spectra in ethanol, formamide, and methyl Cellosolve are similar, although the relative intensities of the peaks show some variation, and the spectra in ethanol and methyl Cellosolve have an additional peak around 400 nm. The TDS in acetonitrile again shows the same peaks, but in this case they are shifted to shorter wavelengths and in general they are more intense. The TDS of tetraacetylriboflavine in chloroform differs from the spectra in other solvents in showing only a very low positive peak above 500 nm, relatively

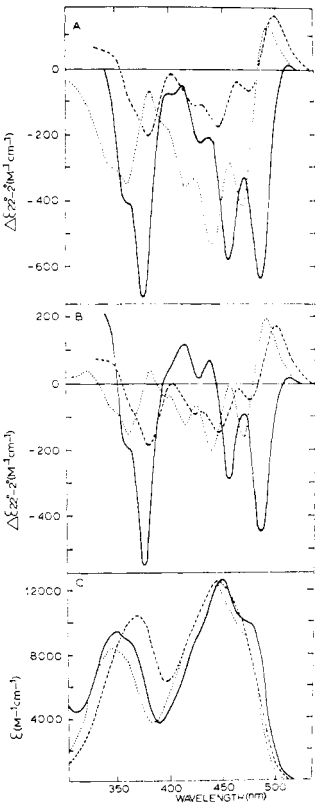


FIGURE 2: Effects of solvent on the absolute and temperature difference spectra of tetraacetylriboflavine: (A) uncorrected temperature difference spectra; (B) temperature difference spectra corrected for thermal expansion of the solvent as described in Methods; (C) absolute spectra at 22°; (——) chloroform; (·····) acetonitrile; (-----) 0.05 M phosphate buffer (pH 7).

intense negative peaks at 485 and 377 nm, and a shift of the peaks to longer wavelengths. The long-wavelength regions of these temperature difference spectra should be compared with the absolute spectra of tetraacetylriboflavine in different solvents, which show that the long-wavelength edge of the band at about 450 nm in the water spectrum is shifted to longer and shorter wavelengths in chloroform and acetonitrile, respectively. We did not observe any concentration effects on the TDS of flavines in organic solvents.[2] In addition, the temperature difference spectra of FAD, FMN, and tetraacetylriboflavine in methyl Cellosolve are only slightly different.

FAD in aqueous solution gives a TDS quite different from those observed with other flavines which lack the adenine of this molecule. The TDS of 1.48×10^{-4} M FAD shows a number of positive peaks (505, 465, 436, 403, ~370, and ~340 nm) but only one negative peak (485 nm) (Figure 3A). The spectrum of a tenfold lower concentration of FAD is similar, except for slight increases (505 and ~400 nm) and decreases (465, 436, and 403 nm) in some of the peaks (Figure 3B). No additional changes occur on further dilution. However, the spectrum is drastically altered when FAD is dissolved in guanidine hydrochloride or methyl Cellosolve (Figures 3C and D). The difference spectra become largely negative and similar to the spectrum of HIP in methyl Cellosolve. The effect of concentration on the TDS of FAD in water probably reflects intermolecular interaction between

[2] Qualitatively and quantitively the TDS of tetraacetylriboflavine and 3-methyltetraacetylriboflavine in chloroform were identical.

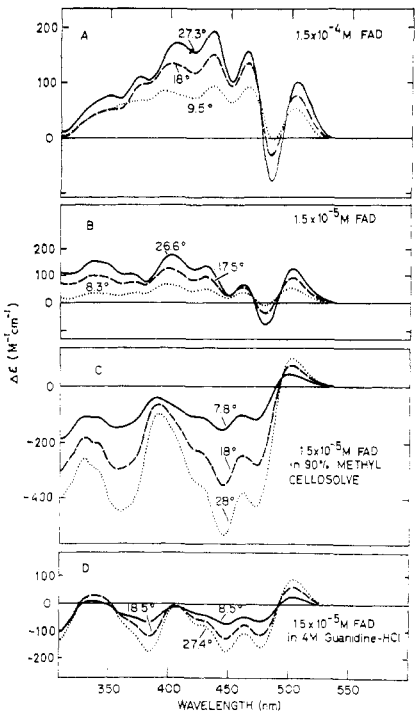


FIGURE 3: Concentration and solvent dependence of the temperature difference spectra of FAD: (A) 1.5×10^{-4} M FAD in 0.1 M potassium phosphate (pH 6.8), 1-cm light path; (B) 1.5×10^{-5} M FAD in 0.1 M potassium phosphate (pH 6.8), 5-cm light path; (C) 1.5×10^{-5} M FAD in 1 part 0.05 M potassium phosphate (pH 6.8) and 9 parts methyl Cellosolve, 5-cm light path; (D) 1.5×10^{-5} M FAD in 0.1 M potassium phosphate (pH 6.8) and 4 M guanidium chloride, 5-cm light path. The temperature difference between sample and reference is shown for each curve.

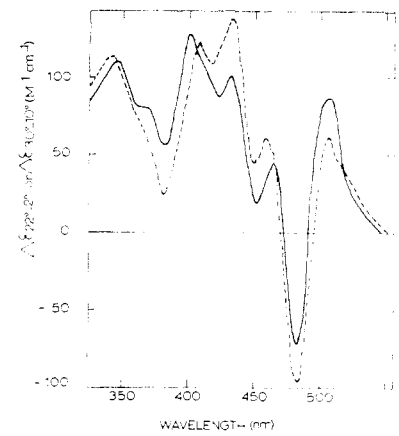


FIGURE 4: Comparison of the temperature difference spectra of FAD and the complex of HIP with caffeine: (——) 1.5×10^{-5} M FAD in 0.1 M potassium phosphate (pH 6.8). The sample and reference temperatures were 22 and 2°, respectively; (-----) 1.9×10^{-5} M HIP in 0.1 M potassium phosphate (pH 6.8) and 0.1 M caffeine. The sample and reference temperatures were 30 and 10°, respectively.

different molecules, and even at low concentrations, the spectrum is evidently complicated by intramolecular interaction of the isoalloxazine and adenine moieties. The intramolecular complex of FAD is known to be partly disrupted in guanidine hydrochloride and probably completely destroyed in methyl Cellosolve (Velick, 1961) changes which correspond with the observed changes in the TDS. The TDS of FAD in water appears to be the result of two effects; a large mainly positive change due to opening of the internal complex with increasing temperature (which causes a change to an FMN-like spectrum) and superimposed on this difference spectrum are the normal temperature induced changes seen with the simpler flavines. Further evidence that the intramolecular complex of FAD^+ could influence the shape of the TDS was obtained by measuring the TDS of HIP in the presence of 0.1 M caffeine, a compound which is known to complex weakly with flavine (determined dissociation constant with HIP = 0.13 M at 10° and 0.19 M at 30°). The TDS of this complex was found to be very similar to the TDS of FAD (Figure 4).

In seeking an explanation for these temperature effects, it became important to consider the possible interactions of water with flavine. When water (up to 6%) is added in increments to a solution of tetraacetylriboflavine in *p*-dioxane (see Methods Section), the absorption maxima at about 440 and 330 nm shift hypsochromically and bathochromically, respectively, and the 330-nm peak shows in addition a slight decrease in intensity (Figure 5). These changes are isosbestic (450, 385, and 340 nm). At water concentrations greater than 6%, the isosbestic points are lost, both absorption maxima shift bathochromically, and the intensity of the maximum at short wavelengths greatly increases. These results suggest that at low water concentrations there may be a specific interaction of water and flavine, possibly through a hydrogen bond(s). The subsequent large changes in the spectrum at higher concentrations of water may be due to other factors such as the increase in the dielectric constant of the solvent mixture.

Studies with Flavoproteins. It should be emphasized that the temperature difference spectra that we have measured for flavoproteins are all reversible and they are not caused by dissociation of the flavine from the protein. We used relatively high concentrations of protein well above the dissociation constants for the dissociation of holoprotein into apoprotein and coenzyme. In most flavoproteins, these dissociation constants are known to be low, and in all cases the TDS is quite different from the difference spectrum between free and protein-bound flavine. Dissociation of even small amounts of flavine could be readily recognized by a loss of the isosbestic points between spectra at different temperatures. In addition, if such a dissociation was induced by high temperature, the observed changes were generally not reversed when the temperature was lowered, and the resulting difference spectrum coincided with that expected for free *vs.* bound flavine.

The absolute spectra and temperature difference spectra of four FMN flavoproteins are compared in Figure 6. The peaks in the TDS of *P. elsdenii* flavodoxin coincide with those of such flavines as tetraacetylriboflavine and FMN in water, except that in the protein they are better resolved and generally of higher intensity. The temperature difference spectra of two other flavodoxins (from *Clostridium pasteurianum* and *Clostridium MP)* show peaks of identical shape and intensity but at very slightly different wavelengths. These spectra are independent of protein concentration in the range 1×10^{-5} to 4×10^{-4} M. The net effect of an increase of temperature is to cause a slight broadening of the absolute spectrum. The temperature difference spectra are clearly different from the spectrum of free FMN *vs.* protein-bound FMN which shows two positive peaks (438 and 367 nm) and shoulders (452 and 482 nm), and a negative peak at 515 nm.

The temperature difference spectra of lactate oxidase, and also the Shethna flavoprotein, show peaks similar to those of flavodoxin, but in general they are shifted 10–15 nm to longer wavelengths, and with the exception of the 384-nm

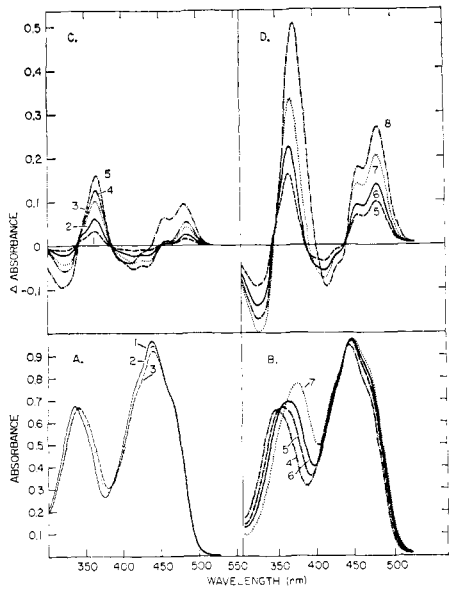


FIGURE 5: Effects of water on the spectrum of tetraacetylriboflavine in *p*-dioxane. Solutions of 8×10^{-5} M tetracetylriboflavine in mixtures of *p*-dioxane and water were prepared as described in the Methods Section. Absolute spectra: (A) curve 1, anhydrous *p*-dioxane; curves 2 and 3, plus 3 and 6% water, respectively; (B) curves 4–7, plus 10, 30, 50, and 92% water, respectively. Difference spectra: (C) curves 1–5; plus 1, 2, 4, 6, and 10% water, respectively; (D) curves 5–8; plus 10, 20, 40, and 92% water, respectively. The light path in all cases was 1-cm; temperature, 20°

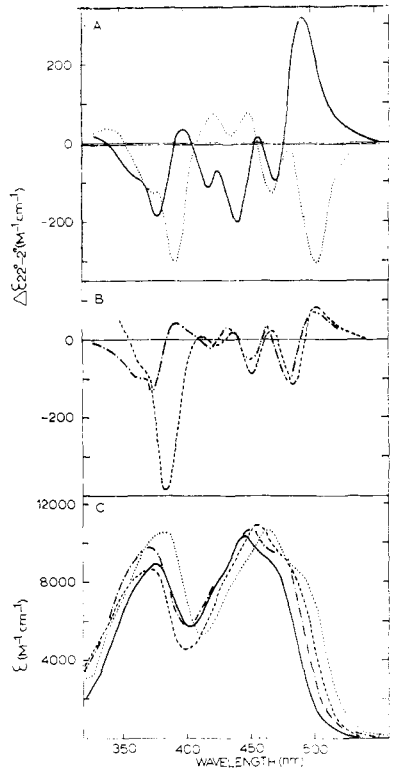


FIGURE 6: Absolute and temperature difference spectra of FMN proteins: (A) temperature difference spectra of flavodoxin (2×10^{-4} M) and old yellow enzyme (7×10^{-5} M); (B) temperature difference spectra of lactate oxidase (4×10^{-4} M) and the Shethna flavoprotein (7×10^{-5} M); (C) absolute spectra of flavodoxin, old yellow enzyme, lactate oxidase, and the Shethna flavoprotein at 22°; (——) flavodoxin; (·····) old yellow enzyme; (-----) lactate oxidase; (·-·-·) the Shethna flavoprotein. Samples were dissolved in 0.05 M potassium phosphate (pH 7) except for lactate oxidase which was dissolved in 0.1 M imidazole hydrochloride (pH 7).

peak from lactate oxidase which is more intense, they are of lower intensity. The spectra shown for lactate oxidase were determined in imidazole hydrochloride buffer (pH 7). Phosphate anions are known to bind to this enzyme and to cause marked changes in the flavine absorption spectrum; in the presence of phosphate, the visible spectrum tends to become more resolved (Sullivan, 1971). However, the TDS of lactate oxidase in phosphate buffer is similar to the spectrum in imidazole hydrochloride except for the intensities of the peaks; the 380–385-nm peak is much less intense, and the intensity of the remaining peaks is enhanced. Old yellow enzyme shows a TDS rather different from the other FMN proteins studies. In the long-wavelength region this enzyme shows an intense negative peak rather than a positive peak. It should be noted that in the absolute spectrum, the long-wavelength edge of the visible absorption peak is shifted relative to that of flavodoxin and FMN.

Since a stable apoprotein can be prepared from flavodoxin, it is possible to study the effects of modification to the FMN prosthetic group. The FMN derivatives *C*(7)-Br-, *C*(7,8)-Cl_2-, *N*(3)-CH_3-, and *N*(3)-CH_2COOHFMN, are all bound tightly to apoflavodoxin. The absolute spectra of these flavines and also their temperature difference spectra in water are similar to those of FMN at wavelengths greater than 400 nm. The absolute spectra and temperature difference spectra of *C*(7)-BrFMN and *C*(7,8)-Cl_2FMN bound to apoflavodoxin are also similar to native flavodoxin at these wavelengths. In contrast, the absolute spectra of flavines modified at N(3) show a large shift (to 458 nm) when they are bound to apoflavodoxin, and the temperature difference spectra of these complexes are different from those of the other flavodoxin derivatives (Figure 7). Thus the temperature difference spectra of *N*(3)-CH_3FMN flavodoxin and *N*(3)-CH_2COOHFMN flavodoxin show only a negative peak at 500 nm, similar to that in the TDS of old yellow enzyme.

All of the proteins discussed so far contain FMN as their prosthetic group. Figure 8 shows temperature difference spectra and absolute spectra for three FAD proteins, glucose oxidase, *E. coli* thioredoxin reductase, and yeast glutathionine reductase. It should be noted that the temperature difference spectra do not differ in any qualitative way from those seen with FMN-containing proteins, and are quite different to those found with free FAD.

Figure 9 shows absolute and temperature difference spectra of two more FAD-containing enzymes–lipoyl dehydrogenase from pig heart and *E. coli*. It should be noted that despite the near identity of the absolute spectra, marked quantitative differences are observed in the temperature difference spectra, especially with respect to the peak in the 505–510-nm region.

Discussion

Although the basis for temperature difference spectra is not clearly understood, it has been used often and proved useful as a tool for investigating small changes around the aromatic residues of proteins (Bello, 1969, 1970; Cane, 1969; Leach and Smith, 1972; Wetlaufer, 1962). We have applied the same technique to the study of the visible absorption spectra of flavines and flavoproteins, in an attempt to determine whether this method could give useful information about the active site environment of flavoproteins. Surprisingly detailed difference spectra are found, which may indeed provide information about the microenvironment of flavines not readily available by other experimental methods.

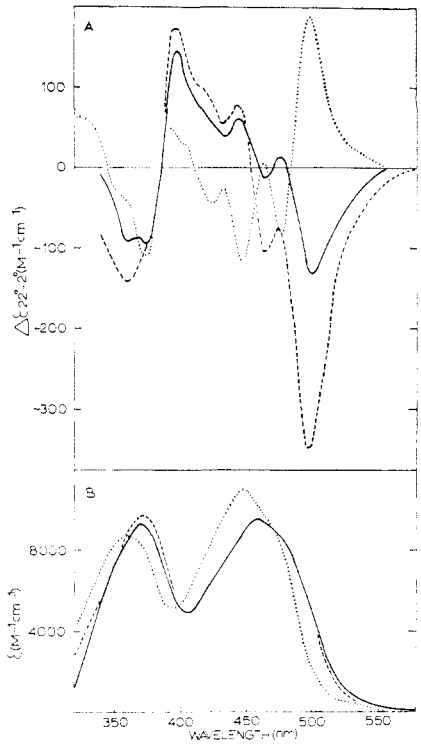


FIGURE 7: Absolute and temperature difference spectra of flavodoxin derivatives: (A) temperature difference spectra; (B) absolute spectra (22°) of 7-BrFMN (.....), 3-CH_3FMN (-------), and 3-CH_2COOH-FMN (——) flavodoxin. Samples were dissolved in 0.05 M potassium phosphate (pH 7.4) and the protein concentrations were 1.2×10^{-4} M.

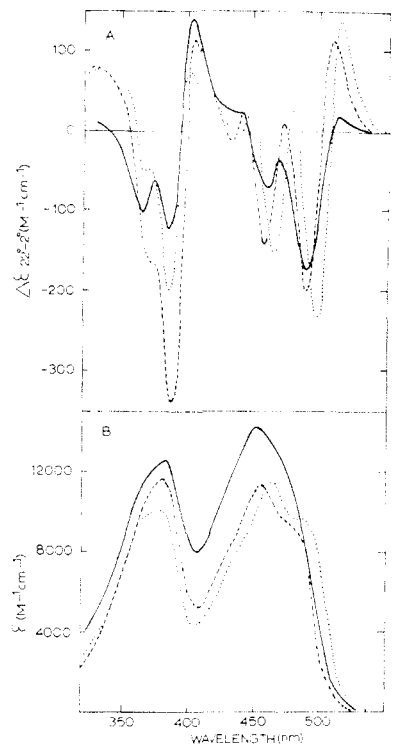
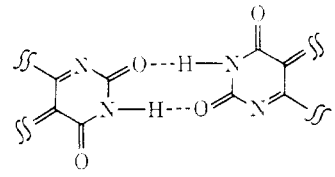


FIGURE 8: Absolute and temperature difference spectra of the FAD flavoproteins glucose oxidase (——), thioredoxin reductase (-------), and yeast glutathione reductase (.....): (A) temperature difference spectra recorded with 5×10^{-5} M glucose oxidase, 7×10^{-5} M thioredoxin reductase, and 9×10^{-5} M yeast glutathione reductase; (B) absolute spectra (22°). Samples were dissolved in 0.1 M potassium phosphate (pH 7).

In aqueous solution, all flavines studied (with the exception of FAD) showed concentration dependent difference spectra. This phenomenon is illustrated in Figure 1 for the case of HIP. At high concentrations (4×10^{-4} M) the TDS is markedly different from that observed at low concentrations (1×10^{-5} M). At intermediate concentrations (*e.g.*, 2×10^{-4} M) a TDS containing features of both high and low concentration types is evident. This phenomenon is probably due to complex formation between isoalloxazine ring systems as has been described previously for FMN^+ (Gibson *et al.*, 1962). In support of this interpretation, concentration effects are not observed in the presence of organic solvents such as methyl Cellosolve or chloroform. It should be noted that the temperature difference spectra of the flavoproteins studied were in no case dependent on the flavoprotein concentration. A comparison of the temperature difference spectra of model flavines in aqueous solution, as detailed in the Results Section, indicates that the presence of a proton at the N(3) position is an important factor in complex formation. When the N(3) position is alkylated, or compounds lacking any proton at N(3) are studied, the tendency to form complexes is enhanced.

Nuclear magnetic resonance studies (Sarma *et al.*, 1968; Kotowycz *et al.*, 1969; Kainosho and Kyogoku, 1972) indicate that in intermolecular complexes of FMN^- the isoalloxazine rings are stacked so that the benzene rings of two adjacent molecules are juxtaposed, but that the molecules are inverted with respect to each other. Our results indicate that the N(3) position influences the intermolecular complex. In model compounds such as I and II which lack a proton, the interactions between the flavines in aqueous solution are stronger. The weaker complex in flavines which have a proton at N(3) may be due to a decrease in hydrophobic interactions and/or hydrogen bonding with water molecules. A different kind of interaction of N(3)H is possible in solvents of low polarity. This interaction involves hydrogen bonding with a carbonyl group (C(2) or C(4)) of another flavine molecule (structure III) (Kyogoku and Yu, 1968; Lauterwein, 1971).



III

However, because of the effective isolation of the two isoalloxazines and the lack of conjugation in the pyrimidine rings, this kind of interaction would not have any significant influence on the visible absorption spectrum.

With FAD in aqueous solution only small concentration effects are observed in the TDS. As shown in Figure 3, such temperature difference spectra are qualitatively very different from those found with other flavines. However, on dissolving FAD in methyl Cellosolve the TDS became very similar to that of other flavines in the same solvent. Methyl Cellosolve is known to disrupt the internal complex between the isoalloxazine and adenine ring systems of FAD (Velick, 1961); hence the TDS of FAD in aqueous solvent must be typical of the internal complex. The results shown in Figure 3 demonstrate the temperature dependence of this internal interaction. In contrast to simpler flavines, where the TDS is a linear function of temperature change below concentrations where stacking formation is favored, the TDS of FAD shows nonlinear changes with temperature. At higher temperatures,

features characteristic of the TDS of simpler flavines are evident, indicating that the internal complex is disrupted at elevated temperature. It should be noted that in all FAD proteins studied the temperature difference spectra do not differ in any qualitative way from those seen with FMN-containing proteins, and are quite different from that of free FAD in water. Thus it would appear that on binding of FAD to these proteins the internal complex between the isoalloxazine and adenine ring systems is either abolished or it is stabilized to such an extent that it is not disrupted in the temperature range that we have used. The first of these interpretations is favored by the CD studies of Edmondson and Tollin (1971).

The temperature difference spectra show three regions where there are marked positive changes and in general these occur at the long-wavelength edges of the bands in the absolute spectrum. The spectra also showed at least five well-defined negative bands; two further negative bands (*ca.* 400 and 340 nm) that can be seen clearly in some spectra are in others either obscured by the large positive changes that also occur at these wavelengths or appear at troughs in the positive region of the spectrum. It is known that the resolution of a light absorption band depends on its vibrational components. These components are in a rather complicated way influenced by such factors as temperature, polarity, and viscosity of the solvent. Thus, in general, better resolved spectra are obtained at low temperature or in solvents of high viscosity or low polarity due to a decrease in the fluctuations of local electric fields.

The absolute spectra (20°) of favorable flavine models in apolar solvents show partial resolution as evidenced by several inflections on the two electronic transitions in the visible and near-uv spectra (Figure 2; Harbury *et al.*, 1959). This resolution is not seen in polar solvents. In comparison, the negative peaks in the temperature difference spectra of tetraacetylriboflavine are more intense and better resolved in chloroform than in water. The positions of the negative peaks in the temperature difference spectra roughly coincide with the peaks and inflections of the absolute spectra. We find that the negative peaks associated with each electronic transition are separated by about 1300 cm^{-1}. This suggests that they are due to vibrational progressions. Similar values are found for the ^1L_a transition of polycyclic hydrocarbons (Jaffé and Orchin, 1962). The positive peaks, on the other hand, are due to broadening of the vibrational bands. This broadening is most evident near 500 nm.

In general, the temperature difference spectra of flavoproteins are even better resolved than the temperature difference spectra of free flavines, indicating that the vibronic transitions are more discrete. This is clearly due to the binding of the flavine to the protein, which reduces the rotational freedom of the flavine and possibly introduces some strain on the bound coenzyme. A particularly instructive example of the TDS technique with a flavoprotein is that illustrated in Figure 7 for flavodoxin. The apoprotein of flavodoxin is readily made and can bind a large number of different FMN derivatives (Mayhew, 1971a,b). Figures 6 and 7 show absolute spectra of FMN, 3-CH_3-FMN, 3-CH_2COOHFMN and 7-BrFMN bound to apoflavodoxin. It should be noted that in free solution all of these derivatives have similar absorption spectra, with peaks at 445 and 360–375 nm. On binding to apoflavodoxin very pronounced shifts to longer wavelength are observed in the case of 3-CH_3FMN and 3-CH_2COOHFMN. The temperature difference spectra of these two derivatives are abnormal and show intensely negative peaks around 500 nm. This phenomenon indicates that in these two derivatives the protein isoalloxazine interactions must be altered in such a way that the vibronic transitions in the first electronic band are affected preferentially. The same effect is seen with old yellow enzyme showing that this phenomenon is not due *per se* to a substituent on the N(3) position but relies on the specific interactions (for example, interaction with a charged group or stacking with an aromatic residue) between the flavine and the particular apoprotein to which it is bound.

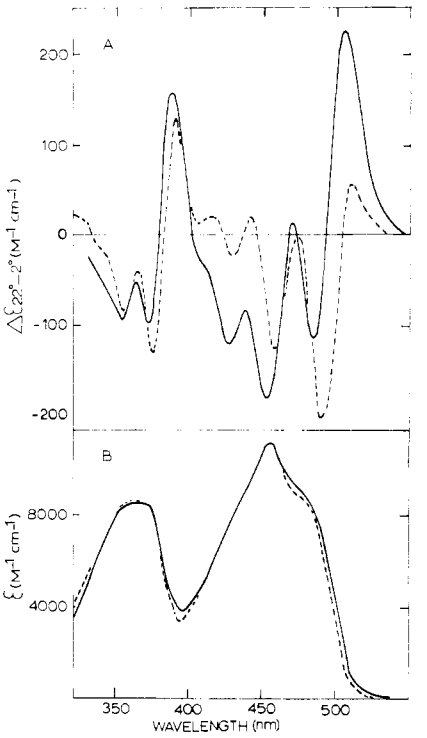


FIGURE 9: Absolute (B) and temperature difference spectra (A) of lipoamide dehydrogenases from *E. coli* (——) and pig heart (-------). Samples were dissolved in 0.1 M potassium phosphate (pH 7). Temperature difference spectra were recorded with 9.8×10^{-5} and 1.2×10^{-4} M lipoamide dehydrogenase from pig heart and *E. coli*, respectively.

Measurements of fluorescence polarization have shown (Weber, 1950) that there are only two electronic transitions associated with the first two light absorption bonds of the flavine spectrum. Our assignment of the negative peaks in temperature difference spectra to the various vibronic transitions in these two first electronic bands is supported by the CD studies of Edmondson and Tollin (1971) and Scola-Nagelschneider and Hemmerich (1972) and also by theoretical calculations of Song (1969). The first authors proposed that the visible and near-uv CD spectra of free flavines and certain flavoproteins could be accounted for in terms of six vibrational levels. Scola-Nagelschneider and Hemmerich (1972) obtained better resolved spectra for free flavines in several aprotic solvents, and their spectra show clearly seven vibrational levels, four associated with the longest wavelength transition and three with the near-uv transition. The negative peaks in our temperature difference spectra of both free and protein bound flavines correspond closely with the positions of the CD bands. This correlation is documented in Table I. The estimates of the vibronic transitions quoted by Edmondson and Tollin (1971) were derived by curve fitting procedures; those quoted by Scola-Nagelschneider and Hemmerich (1972) were correlated with low temperature (100°K) light absorption spectra determined by Lhoste (1971).

While our understanding of the nature of temperature difference spectra with flavines and flavoproteins is obviously still very incomplete, it would appear likely that a detailed study of this technique could provide a very sensitive and useful new method for studying the flavine chromophore.

Acknowledgments

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