[CONTRIBUTION FROM THE INSTITUTE FOR ENZYME RESEARCH, UNIVERSITY OF WISCONSIN]

# Spectral Characteristics of Flavins at the Semiquinoid Oxidation Level[1]

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Spectra of flavins were studied over the wave length range 230 to 1300 mμ. Families of curves were recorded during progressive oxidation of the reduced to the oxidized forms at various conditions of *p*H and concentration. Absorption bands were observed which are maximally developed at about 50% oxidation. These bands were assigned to semiquinoid intermediates in their monomeric and dimeric forms on the basis of concentration and temperature dependence. The monomeric semiquinone between *p*H 2 and 7 is characterized by a band with $\lambda_{max}$ 565 mμ and below *p*H 2 by a shift of the base of the main flavin peak ($\lambda_{max}$ 445 mμ for flavin mononucleotide at *p*H 6) toward longer wave lengths. The principal absorption of the dimeric forms occurs between 700 and 1100 mμ.

On the basis of potentiometric titrations, magnetic measurements and spectral observations, several investigators[2-8] concluded, 20 years ago, that riboflavin and related compounds are oxidized and reduced in two distinct one-electron steps and therefore pass through a true semiquinoid state of oxidation. The semiquinoid intermediates were found to have a considerable lifetime under certain conditions.

Recent studies on a group of enzymes which contain flavin as prosthetic group has provided strong evidence that stable semiquinoid intermediates of these prosthetic flavins may play a significant role in flavoprotein catalysis.[9] As this evidence rests mainly on spectral observations it was desirable to obtain more information of the spectral properties of the semiquinoid intermediates of simple flavin compounds. This communication contains a qualitative survey of the spectral characteristics of flavins at different levels of oxidation and under varying conditions of *p*H, concentration and temperature. The work on the flavoproteins will be described in a separate communication.

## Experimental

**Materials.**—Riboflavin was a product of Merck and Co. (U.S.P.), FMN[10] (sodium salt) of Hoffmann-LaRoche, Inc., and FAD[10] of the Sigma Chemical Co. Both FMN and FAD showed only insignificant spots characteristic of other flavins, when chromatographed in two solvent systems.[11] A molar extinction coefficient of $12.2 \times 10^6$ cm.$^2$ $\times$ mole$^{-1}$ was assigned to FMN and of $11.3 \times 10^6$ cm.$^2$ $\times$ mole$^{-1}$ to FAD at 450 mμ.[12,13] On the basis of these coefficients the samples of FMN and FAD used in these studies were estimated to contain 88 and 75%, respectively, of the pure nucleotides (free acid form). The dithionite used was of commercial grade (Mallinckrodt).

**Apparatus.**—For the initial exploratory work the rapid scanning spectrophotometer of the American Optical Company[14] was used. Because this instrument played a more decisive role in the study of the flavoproteins, its specific use will be described in a later communication. Under the conditions of the present experiments the lifetime of the intermediates was sufficiently long so that the spectra could be recorded with a Beckman automatic recording spectrophotometer, Model DK 1. This instrument was equipped for linear recording of absorbance and for temperature control of the cell compartment. The wave length scale was not linear. Assignment of wave lengths to maxima and minima was made according to Lewin and Fairbanks.[15] Occasional control measurements with a spectrophotometer, Model DU, were made when precise quantitative information was desired. Silica cells of varying light path from 0.5 to 10 cm. were used and quartz inserts for shorter light paths. The *p*H was routinely measured after each experiment with a Beckman *p*H meter Model G.

**Procedure.**—Since in a cuvette with a 1 cm. light path the concentration of total flavin has to be about $10^{-3}$ *M* for the characteristic bands of the intermediate states to be recognized, the use of riboflavin for routine work is ruled out because of its low solubility, except at alkaline *p*H. Most of the spectral studies were carried out therefore with FMN and FAD; 0.016 *M* stock solutions of these substances were kept frozen and protected from light. Aliquots of these solutions were diluted with appropriate buffers. For the study of the semiquinone band in the range above 500 mμ a concentration of flavin and a corresponding light path were chosen such that the product $c \times d$[16] was about $2 \times 10^{-3}$ mole $\times$ cm. per liter. At this concentration of flavin the principal absorption band at 450 mμ appears only as a steep end absorption at the left side of the records at about 500 mμ. In the spectral range below 500 mμ, flavin concentration and light path were chosen such that $c \times d$ was 2 to 3 $\times$ $10^{-5}$ mole $\times$ cm. per liter. The absorbance of the sample against an identical buffer solution devoid of flavin was determined at the starting wave length in a spectrophotometer (Beckman Model DU) and the recording instrument was set accordingly.

Samples in the cylindrical cuvettes of 5 and 10 cm. path length were gassed with helium while the smaller cuvettes were filled with helium above the liquid level and capped with a rubber or paraffin cap unless the quartz inserts were used. The spectrum of the oxidized form was then recorded. Thereafter, 10 to 20 μl. of a dithionite solution were added under gassing to reduce the flavin completely. The dithionite solution was prepared as follows: 0.5 g. of the anhydrous salt was dissolved in 4.5 ml. of a buffer solution 2 to 4 times more concentrated than that used in the experiment and 0.5 ml. of 6 *N* KOH was then added. The solution was cleared by centrifugation and kept well stoppered in ice. Such a solution is usable for at least one day and does not change the *p*H of the samples noticeably when added in the specified quantities. After dithionite addition the spectrum of the reduced form was recorded. Thereafter the sample was shaken gently and a few air bubbles were admitted when necessary by slightly lifting the cap. The characteristic color of the intermediate (see Table I) in most cases then became visible to the eye. Spectra were recorded in succession with intermittent agitation of the sample until the flavin was completely oxidized. Unless the samples were agitated there was rarely a significant change in absorption during a single scan of 2 to 3 minutes duration. This could be shown by repeated scanning without intermittent agitation.

(1) This work was supported by a grant (No. NSF-G1772) from the National Science Foundation

(2) R. Kuhn and T. Wagner-Jauregg, *Ber.*, **67**, 361 (1934).

(3) R. Kuhn and R. Ströbele, *ibid.*, **70**, 753 (1937).

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(5) L. Michaelis and G. Schwarzenbach, *ibid.*, **123**, 527 (1938).

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(7) K. G. Stern, *Biochem. J.*, **28**, 949 (1934).

(8) F. J. Stare, *J. Biol. Chem.*, **112**, 223 (1935).

(9) H. Beinert, *Biochim. Biophys. Acta*, **20**, 588 (1956).

(10) Abbreviations used: FMN, flavin mononucleotide; FAD, flavinadenine dinucleotide.

(11) F. L. Crane, S. Mii, J. G. Hauge, D. E. Green and H. Beinert, *J. Biol. Chem.*, **218**, 701 (1956).

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

(13) O. Warburg and W. Christian, *Biochem. Z.*, **298**, 150 (1938).

(14) American Optical Company, Instrument Division, Buffalo, New York.

(15) S. Z. Lewin and R. H. Fairbanks, *Anal. Chem.*, **22**, 2020 (1955).

(16) *c*, concentration of flavin in moles per liter; *d*, light path in cm.

TABLE I

ABSORPTION MAXIMA, MINIMA AND ISOSBESTIC POINTS OF FMN

| *p*H | 0 | 2.4 | 6.1 | 8.9 | 12.0 |
|---|---|---|---|---|---|
| Medium | 1 *M* HCl | 0.085 *M* phosphate | 0.25 *M* citrate | 0.10 *M* histidine | 0.125 *M* phosphate |
| Oxidized form $\lambda_{max}$ | 267, 385 | 267, 370, 445 | 267, 375, 445 | 267, 375, 447 | 269, 353, 450 |
| Oxidized form $\lambda_{min}$ | 238, 303 | 240, 303, 398 | 241, 303, 400 | 241, 303, 400 | 241, 295, 397 |
| Reduced form $\lambda_{max}$ | 250,[a] 317[a] | 248 | 251 | 256 | 256 |
| Isosbestic points | ?[a] | 233, 255, 289, 330 | 234, 255, 289, 330 | 234, 262, 289, 330 | 233, 264, 282, 320 |
| Semiquinoid form | | | | | |
| monomer $\lambda_{max}$ | 503[b] | 525[b] | 565 | ..... | ..... |
| dimer $\lambda_{max}$ | 830 (FAD 850) | 840 (FAD 850) | 880 (FAD 900) | 1010 (FAD 1040) | 770 (FAD 800) |
| visible color[c] | Brownish red | Greenish brown | Brownish green | Greenish yellow | Slightly greenish yellow |

[a] The spectrum of the 100% reduced form was not recorded, the values are estimated. The original spectrum of the oxidized form was different from that of the sample after final reoxidation (see Fig. 6); sharp isosbestic points were therefore not obtained. [b] Maxima of difference spectra: semiquinone form minus oxidized form. [c] The visible color is composed of contributions from the three components in equilibrium, R, S and T (*cf.* Scheme 1) and can therefore not be considered as the color of the semiquinone proper.

At *p*H levels above 8 reduced flavins are reoxidized readily and very gentle shaking and fast experimentation are therefore necessary. At *p*H levels below 6 the intermediate stages are quite stable. At *p*H levels below about 3 the basic procedure which has been described above fails because turbidity develops soon after addition of dithionite. This difficulty can be partly circumvented by reducing the flavin only partially so that no excess dithionite remains, by working rapidly and by measuring the spectrum at various phases of oxidation or reduction successively with several equal aliquots of the same original sample. As an alternative one may use reducing agents other than dithionite. Sodium amalgam (0.5%) was used successfully for this purpose as was also metallic zinc (below *p*H 1). With the amalgam, Dowex 50 ($H^+$) was added in small portions to control the *p*H. Strong buffers were used and the *p*H was frequently checked with strips of a narrow range indicator paper.[17] According to measurements with the glass electrode, the *p*H could thus be maintained within 0.2 unit of the *pK* of the buffering ion. However it was very difficult to reduce the flavin completely, clarify the solution and transfer it to a cuvette without partial reoxidation of the flavin. There was also a small unexplained loss of flavin under these conditions. Complete reduction with Zn is likewise difficult. $Zn^{++}$ ions may be removed with Dowex 50. This necessitates, however, clarification and transfer. It was therefore preferred in the present work to reduce the flavin in the cuvette by a piece of Zn suspended from a rubber band, remove the metal, leave $Zn^{++}$ ions in the solution and thus avoid a transfer.

When the spectrum below 400 mμ had to be recorded, special precautions had to be taken in the use of dithionite. Dithionite has a strong absorption band with $\lambda_{max}$ 313 mμ[18] at alkaline *p*H which is shifted to 316 mμ at *p*H 6.1. The molar extinction coefficient was found to be close to $6 \times 10^6$ cm.$^2 \times$ mole$^{-1}$ in agreement with Hellström.[18] This band will disappear completely on aeration and only a slight absorption will remain which rises exponentially toward shorter wave lengths. The residual absorption of a 0.1% solution of the dithionite used at a 1 cm. light path and *p*H 6.1 was 0.30, 0.11, 0.05, 0.02 and <0.01 at 240, 250, 260, 280 and 300 mμ, respectively. It is likely that this absorption will vary with the particular batch used. Dithionite can therefore be used safely for ultraviolet spectra of all oxidation states higher than that of full reduction, because only in the latter case can any excess dithionite be present. In order to obtain a correct spectrum of the reduced form of flavin, the flavin was reduced with a slight excess of dithionite and gently shaken with air until the dithionite band at 313 or 316 mμ was absent in the recorded spectrum. This relatively sharp band disappeared at the same time as the curve of the reduced flavin crossed for the first time the isosbestic points, which are also crossed by the curves of the subsequent stages of partial oxidation. A correction has to be applied for the remaining absorption of dithionite below 300 mμ. The fact that the curves obtained after reduction by dithionite and by sodium amalgam are identical seems to validate this procedure.

(17) "Lyphan," Medicina S. A., Vaduz, Liechtenstein, or "Oxyphen," obtainable through A. Daigger and Co., Chicago, Ill.

(18) H. Hellström, *Z. physiol. Chem.*, **246**, 155 (1937).

After each run the temperature and the *p*H of the sample were determined.

## Description of Spectra

**Basic Pattern.**—Figures 1 and 2 show records of oxidation levels of FMN from full reduction to full oxidation, which were taken at *p*H 6.1 in citrate buffer. FMN was present in a concentration of $4.2 \times 10^{-4}$ mole per liter. Between 230 and 580 mμ the light path was 0.05 cm. (Fig. 1) and between 500 and 1300 mμ (Fig. 2) the light path was 10 cm. Thus the intensity of the bands in the region between 500 and 1300 mμ in Fig. 2 is magnified 200 times over that for the 230 to 580 mμ region in Fig. 1. In order to correlate the spectra in the two regions which belonged to the same oxidation level, a sample of FMN which was $4.2 \times 10^{-4}$ *M* was examined in the Beckman Model DU spectrophotometer in a cell of 0.50 cm. light path. The absorption at the peaks at 445, 565 and 880 mμ was measured and the values were compared to the absorption values reached at these wave lengths by the curves of Figs. 1 and 2. Accordingly curves bearing the same numbers in Figs. 1 and 2 could be assigned to corresponding oxidation levels with a maximum deviation of 5%.

It is clear from this experiment that the bands with peaks at 565 and 880 mμ are at their maximum development when the absorption at the 445 mμ peak is about half-way between the oxidized and the reduced level (curve 5). This basic experiment shows clearly that the new bands belong to a transient intermediate which occurs during reoxidation of reduced FMN. The fact that these bands are observed during reoxidation of reduced flavin as well as during reduction of flavin with dithionite or sodium amalgam in a variety of buffers leaves little doubt that they are to be ascribed to an intermediate oxidation state of the flavin rather than to any complex of the flavin with other agents. We are therefore observing in the records of Figs. 1 and 2 the composite spectra of the components of the equilibrium system: R ↔ S ↔ T (Scheme 1) at various concentration levels of its components. R, S and T are the symbols introduced by Michaelis, *et al.*,[4] for the totally reduced, semiquinone and totally oxidized forms, respectively. In the concentration range of 40 to 60% R or T (R + S + T = 100%) the concentration of the intermediate S changes only to a small extent.

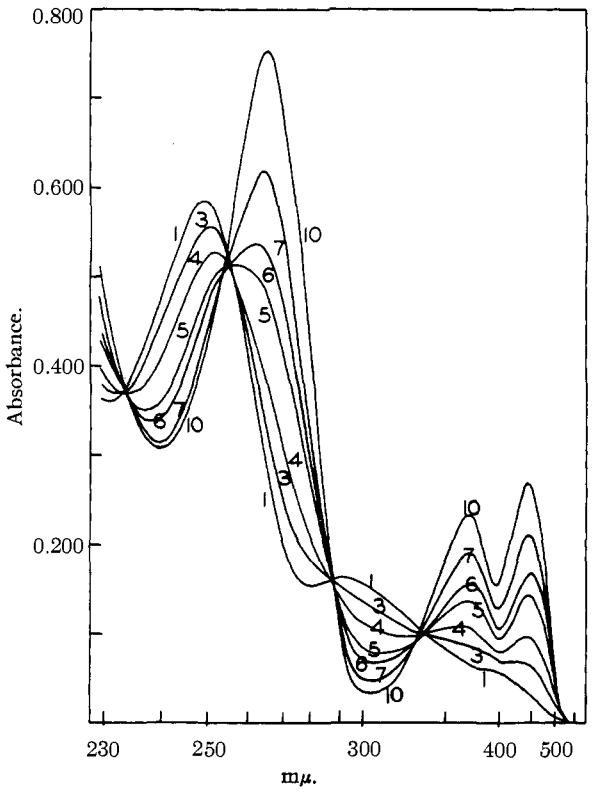


Fig. 1.—Absorption spectra of $4.2 \times 10^{-4}$ *M* FMN in 0.25 *M* citrate, *p*H 6.1, at successive oxidation states from full reduction (curve 1) to full oxidation (curve 10); light path 0.05 cm., temp. 31°, reduced with $Na_2S_2O_4$. The curves correspond to the same state of oxidation as those curves of Fig. 2, which bear the same number.

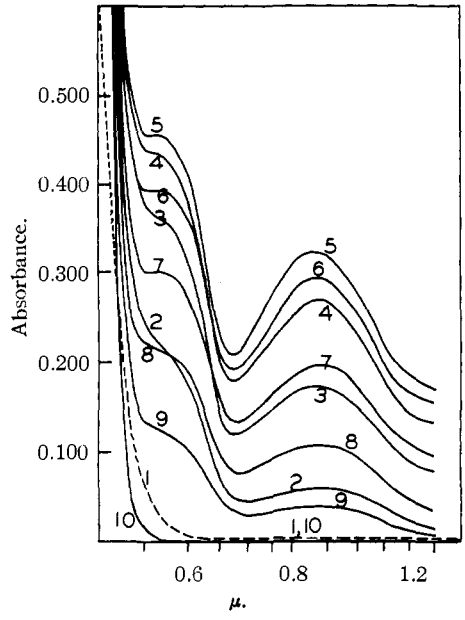


Fig. 2.—Absorption spectra of FMN, conditions as Fig. 1, but with a 10 cm. light path. The curves correspond to those of Fig. 1, as indicated by the numbers.

In the subsequent paragraphs the influence of various conditions on the observed bands will be examined. In every case a series of curves between the oxidized and the reduced level was recorded and only the situation at the maximum development of the bands corresponding to curve 5 will be considered.

**Concentration Dependence.**—Michaelis and Schwarzenbach[5] have pointed out that at concentration levels above $10^{-4}$ *M* dimerization of the monomeric semiquinoid intermediate may be significant. Elsewhere Michaelis[19] has discussed the effect of dilution on dimerization of monomeric semiquinones. A comparison of records at different concentrations of FMN and with a light path such that the product $c \times d$ was identical in all cases shows clearly that the band in the infrared region is very weak in dilute solutions (Fig. 3).

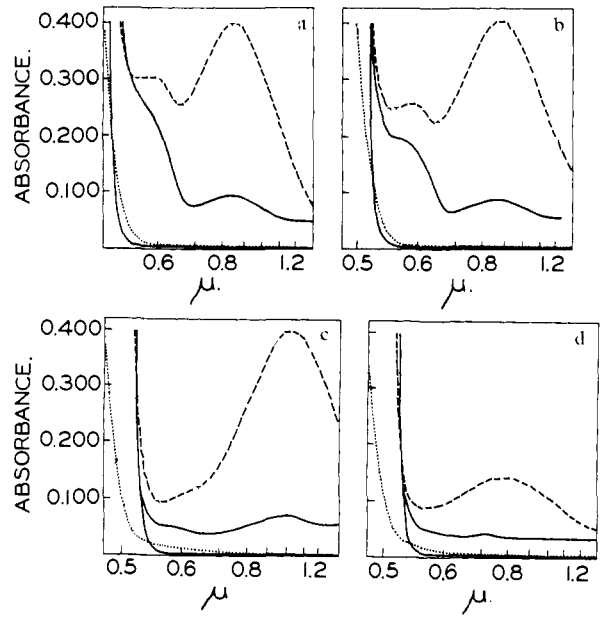


Fig. 3.—Absorption spectra of FMN at the semiquinoid oxidation level, reduced with $Na_2S_2O_4$, temp. 28 to 30°. Upper solid line, $2 \times 10^{-4}$ *M* FMN with a 10 cm. light path; dash line, $4 \times 10^{-3}$ *M* FMN with a 0.50 cm. light path, both at semiquinoid oxidation level. Lower solid line, oxidized form; dotted line, reduced form. (a) in 0.085 *M* phosphate, *p*H 2.4; (b) in 0.17 *M* citrate, *p*H 6.1; (c) in 0.1 *M* histidine, *p*H 8.9; and (d) in 0.085 *M* phosphate, *p*H 11.8.

The band at 565 mμ appears only slightly decreased. There is some overlapping of the two bands with $\lambda_{max}$ 565 and 880 mμ, respectively. In an attempt to correct for this overlapping, it was assumed that the bands are symmetrical when absorbance is plotted *versus* wave number. When a correction is applied accordingly, the 565 mμ band appears essentially unchanged on dilution, provided $c \times d$ remains constant. Since the amount of a monomeric free radical formed should depend only on the amount of parent compound present and not on its concentration we may assign the band with $\lambda_{max}$ 565 mμ to the semiquinone form. The concentration dependent band with $\lambda_{max}$ 880 mμ has never been observed without the simultaneous appearance of the semiquinone band ($\lambda_{max}$ 565 mμ). Because of this relationship to the semiquinone and

(19) L. Michaelis, THIS JOURNAL, **58**, 873 (1936).

because of its dependence on concentration and temperature (see below) the 880 mμ band most likely belongs to a bimolecular (or higher molecular) complex of semiquinone molecules with each other or with other components in solution. These other components could be either the buffer salts used or trace metal impurities. It is unlikely that the salts used as buffers are involved as the near infrared bands have been observed in a great variety of buffer salts with organic and inorganic constituents. The intensity of the bands has, however, occasionally been found to depend on the kind of buffer used. It is also unlikely that trace metal impurities contribute to the near infrared absorption bands, because these bands were observed in the same location and with the same intensity for two FMN preparations of different origin and with two FAD preparations of 75 and 90% purity, respectively. No significant difference of the infrared bands was observed when $4 \times 10^{-3}$ *M* FMN was examined in 0.17 *M* citrate, 0.075 *M* Versene and 0.015 *M* succinate buffer at *p*H 6. The lack of any influence of Versene on the infrared band at such a high concentration suggests strongly that the infrared bands indicate indeed a dimer (or less likely a higher polymer) of the semiquinone itself. Although the assignment of the observed bands to the monomeric and dimeric forms of the semiquinone is not considered established all evidence so far available is consistent with such an assignment and the bands will be designated accordingly in the subsequent discussion.

It may be seen from the relative intensities of the 565 and 880 mμ bands in the successive curves of Fig. 2 that the formation of the dimer from the monomer is a slow process. For instance, curve 6 which was recorded about 20 minutes later than curve 4 has a stronger dimer-band and a weaker monomer-band than curve 4. The same is true for the curve pairs 2, 8 and 3, 7. At concentrations of about $2 \times 10^{-2}$ *M* in the *p*H range 3 to 6 a green flocculent precipitate appears on partial reduction of FMN or FAD which can be readily sedimented by centrifugation, dried with acetone, and redissolved to a deep brownish green solution in fresh solvent. Cooling facilitates the formation of the precipitate and warming its dissolution. According to this behavior it is assumed that the precipitated material is the quinhydrone-like dimer.

**Temperature Dependence.**—All records discussed so far were taken at about 30°. When the temperature was varied, it was observed that the intensity of the band in the near infrared showed considerable temperature dependence (Fig. 4). The reversibility of these intensity changes could be shown. A solution of FMN under the conditions used for Fig. 3b but at the oxidation level of curve 6 (Fig. 2) was chosen because at this level any slight autoöxidation of the flavin would only lead to a decrease in intensity. When such a solution was cooled from 40 to 6° the intensity of the infrared band was increased 1.7-fold. On subsequent warming the intensity reverted to its original level and repeated cooling intensified the band as before. This behavior is also in agreement with the assignment of the band to the dimer as suggested above.

***p*H Dependence.**—Flavins have several dissociations over the *p*H range and one would therefore expect a dependence of the spectral curve type on *p*H. The basic structures of the R, S and T forms of riboflavin which may exist at the various *p*H levels have been discussed by Michaelis, *et al.*[4,5] Michaelis and his collaborators also have derived *pK* values for these forms of riboflavin from their titration curves. They arrived at values of 1.3 and 6.5 for the semiquinone.[5]

Figure 3 shows records of the spectrum for the maximally developed intermediate oxidation level of FMN over the range from 500 to 1300 mμ and at *p*H 2.4, 6.1, 8.9 and 11.8. The concentration of FMN was varied by a factor of 20 and the light path was chosen correspondingly such that $c \times d$ was constant. The curves for $2 \times 10^{-4}$ *M* FMN are represented by solid lines, those for $4 \times 10^{-3}$ *M* FMN by dash lines. The lowermost solid curve represents the oxidized and the dotted line the reduced form of FMN. As expected from the *pK* values suggested by Michaelis and Schwarzenbach we find that the basic spectral type at *p*H 2.4 and 6.1 is similar. At *p*H 2.4 the absorption rises more steeply toward shorter wave lengths. This indicates that at this *p*H a measurable amount of another form is present, which is typical for *p*H values below 1. This form will be described below. At *p*H 8.9 the characteristic band at 565 mμ is almost absent and the dimer band has shifted to a new maximum from 880 to 1010 mμ.[20] At *p*H 11.8 the 565 mμ band is absent and the dimer band is relatively weak and again shifted to a different maximum, now at 770 mμ. We may conclude from this shift of the dimer band that there is a dissociation of FMN involved in addition to those of the semiquinone considered by Michaelis. The maximum shift of curve type occurs between *p*H 10.5 and 11.2.

Corresponding curves for the region below 500 mμ revealed no essential characteristics of the intermediate. Records for the successive stages at *p*H 12.0 (Fig. 5) and at *p*H 0 (Fig. 6) are shown for the region 230 to 580 mμ, which are comparable to the corresponding record in Fig. 1. All these curves show a continuous transition from the reduced to the oxidized pattern. The characteristic features are summarized in Table I and will be briefly discussed here

***p*H 6.1 (Fig. 1).**—There are 4 isosbestic points at 234, 255, 289 and 330 mμ. The ultraviolet peak of the reduced form at 251 mμ is gradually replaced by the 267 mμ peak of the oxidized form as oxidation proceeds. The ultraviolet band has its greatest width and its lowest intensity when oxidized and reduced forms make an about equal contribution (curve 5). This oxidation state coincides with the state when the semiquinone band is maximally developed. There is a flat maximum of the reduced form at about 295 mμ which drops to a minimum as oxidation proceeds. There is no essential deviation from this pattern at *p*H 2.4 and 8.9.

***p*H 12.0 (Fig. 5).**—A change is apparent at this *p*H. The isosbestic points are now at 233, 264, 282 and 320 mμ. The two main flavin peaks which

(20) The infrared maxima are slightly displaced to longer wave lengths for FAD (see Table I), but the general curve types are the same as for FMN.

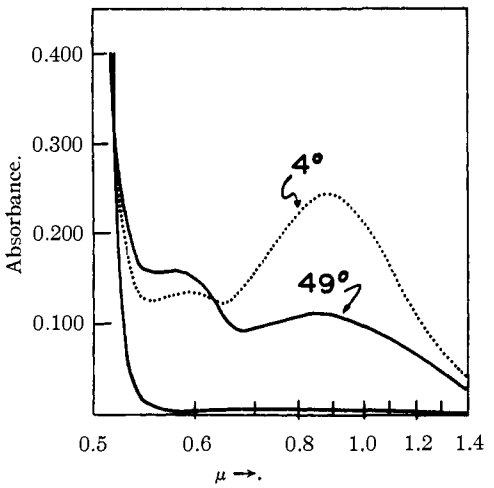


Fig. 4.—Absorption spectra of $1.1 \times 10^{-3}$ *M* FMN at the semiquinoid oxidation level in 0.17 *M* citrate, *p*H 6.1; light path 1.00 cm., temp. 4 and 49° as indicated, reduced with $Na_2S_2O_4$. Lower solid line, oxidized form at 4 and 49°.

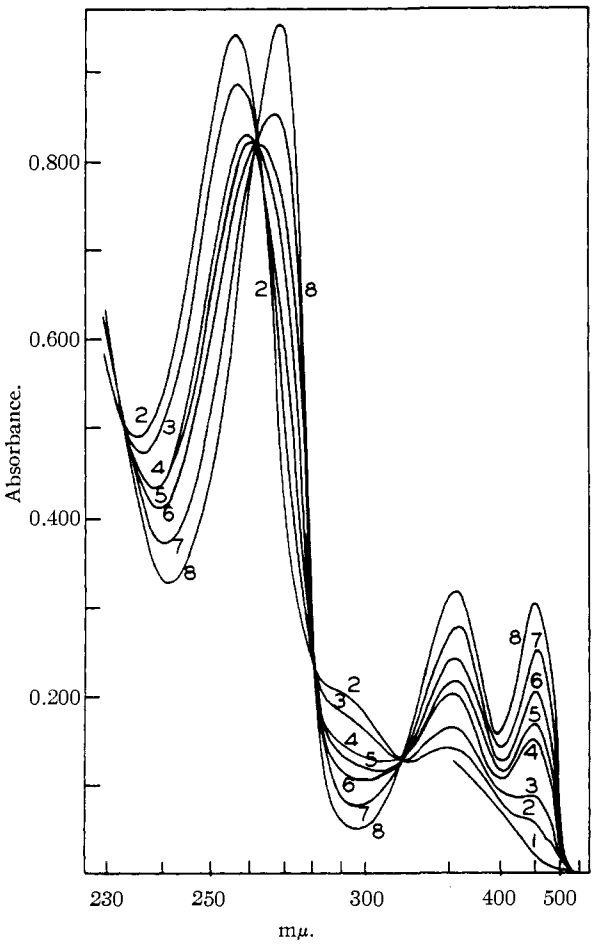


Fig. 5.—Absorption spectra of $5.1 \times 10^{-4}$ *M* FMN in 0.125 *M* phosphate, *p*H 12.0, at successive oxidation states from full reduction (curve 1) to full oxidation (curve 8); light path 0.05 cm., temp. 27°, reduced with $Na_2S_2O_4$. A complete curve of the fully reduced state was not obtained.

at *p*H 6.1 were located at 375 and 445 mμ are now moved apart to 353 and 450 mμ and are of about

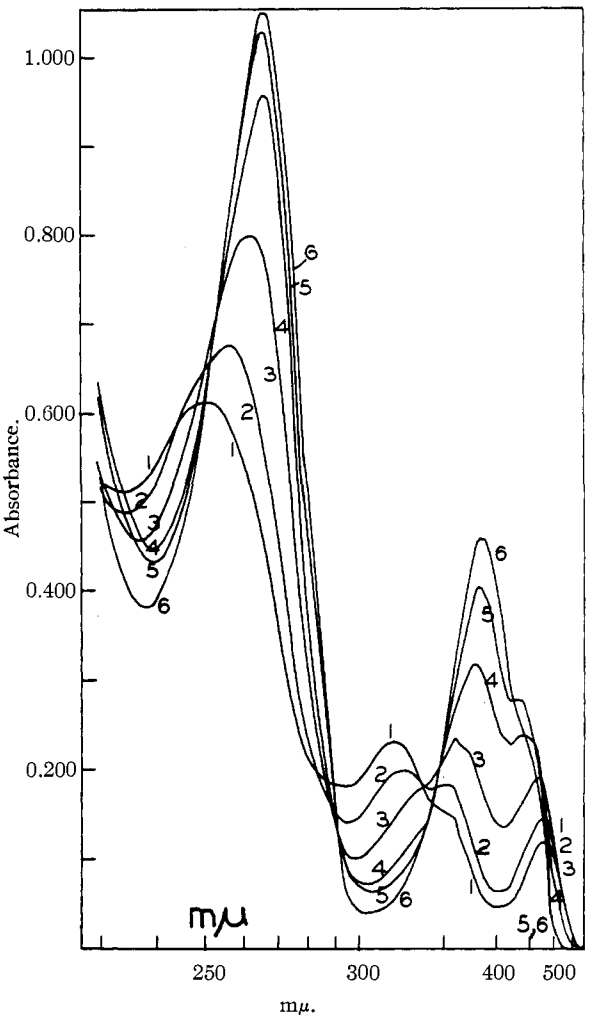


Fig. 6.—Absorption spectra of $6.4 \times 10^{-5}$ *M* FMN in ~1 *N* HCl at successive oxidation states from about 75% reduction (curve 1) to full oxidation (curve 5); light path 0.50 cm., temp. 28°; reduced with metallic Zn. It was not possible to obtain a spectrum of the fully reduced material. It should also be noted that the spectrum of the original solution in the oxidized state (curve 6) is not identical with the spectrum of the reoxidized material (curve 5) after previous reduction with Zn.

equal height. The minimum which separates them has shifted from 400 to 397 mμ and has become more pronounced.

*p*H 0 (Fig. 6).—At this *p*H the interpretation of the changes is somewhat obscured by the fact that the curve of the original oxidized form is not identical with that obtained at the end of the run with the reoxidized flavin. There remains a shoulder at 440 mμ which is more pronounced than it was in the original spectrum and the height of the 385 mμ peak is decreased. By addition of Dowex 50 ($H^+$) to remove $Zn^{++}$ ions the original shape of the curve can be restored, but not the full height of the peaks. The change of the curve shape after complete reoxidation can therefore be attributed to the $Zn^{++}$ ions which are present in solution after reduction. It was observed that even well dried Dowex 50, when added to a solution of oxidized FMN under

the conditions of the experiment, leads to a general decrease in absorption of the flavin, which is best explained by a removal of flavin by the resin. This observation would account for the fact that the height of the 385 mμ peak cannot be fully restored after removal of $Zn^{++}$ ions.

The lack of sharp isosbestic points in Fig. 6 indicates also that FMN is undergoing some reaction in addition to oxidation–reduction. When spectra recorded after reduction with Zn and with dithionite, respectively, are compared the essential features of the pattern at *p*H 0 are easily recognized. The most pronounced change in the flavin spectrum, which becomes apparent at this *p*H, concerns again the main flavin peaks (375 and 445 mμ at *p*H 6.1). These peaks appear at about 350 and 480 mμ as FMN first becomes partly oxidized and then grow together into a composite peak at 385 mμ as oxidation proceeds. The peak which first emerges at 480 mμ is shifted to shorter wave lengths during oxidation and finally appears only as a shoulder at 440 mμ in the composite peak of the totally oxidized form. It does not seem to be justified to conclude that the peak which emerges at 480 mμ is *per se* a characteristic feature of the semiquinone at this *p*H, because in all other spectra this peak is characteristic of the oxidized form of flavin. The only feature which is definitely characteristic of the intermediate oxidation level is the shift of the base of this peak from about 500 mμ to about 550 mμ at the flavin concentration used. This shift accounts for the well known red color of the semiquinone at this *p*H.[2] In experiments with dithionite as reducing agent it was possible to obtain the completely reduced form, which is not shown in Fig. 6. The reduced form has no absorption in this spectral region. The maximum of the difference spectrum: semiquinoid minus oxidized form is at 503 mμ. In Fig. 3a, recorded at *p*H 2.4, the presence of some of the red semiquinone is already apparent from the steep rise of the absorption at about 560 mμ. The wave length region of 500 to 1300 mμ is not shown for *p*H 0. There are no characteristic features except for a weak dimer band and a steep rise of the absorption from about 650 mμ toward shorter wave lengths at the concentration of FMN used in the measurements recorded in Fig. 3.

Through the kind collaboration of Drs. H. S. Gutowsky and R. L. Rutledge of the University of Illinois it was possible to examine the red intermediate for paramagnetic resonance absorption with a microwave spectrometer operating in the X-band. A signal characteristic of a free radical was observed when a $3 \times 10^{-3}$ *M* solution of FMN in 50% aqueous ethanol and 1 *N* with respect to HCl was reduced with Zn. This finding adds to the evidence adduced by others[2-8] and in the present paper that the intermediates formed on partial reduction of flavins are indeed free radicals.

## Discussion

It is apparent from Figs. 1, 5 and 6 that no single curve can be identified with the spectrum of the intermediate proper as is sometimes done.[21] We will always deal with the equilibrium system of scheme 1. The observed spectra will therefore be composite spectra of the species involved and will vary with the relative contribution of the components. It is only possible to search for characteristic features in the consecutive spectra, which are most pronounced halfway between full reduction and full oxidation. If such features cannot be explained as resulting from a mere transition of bands from the reduced to the oxidized type, then they can be assigned to the semiquinoid intermediate. As an example of the type of feature which may be ascribed to a mere transition from reduced to oxidized pattern we may cite the behavior of the ultraviolet peak which during reoxidation moves from 251 to 267 mμ (*p*H 6.1) and passes through a state of minimum development at 255 mμ just at maximum development of the semiquinone. As an example of a feature definitely characteristic of the semiquinone one can cite the appearance of the band at 565 mμ in the *p*H range 2 to 7 and the shift of the base of the 445 mμ peak from 500 to 550 mμ below *p*H 2 (Fig. 6). If the dimer is considered as a derivative of the radical, then the bands in the near infrared can also be considered characteristic of semiquinone formation.

The fact that these characteristic features are found on reduction of oxidized flavin with several reducing agents and also on reoxidation of reduced flavin with oxygen, rules out the possibility that these phenomena can be attributable to any substance in addition to flavin.

It was mentioned above that the dimeric forms of the semiquinone of FAD and FMN, respectively, show slightly different maxima for the main absorption bands. The dimeric form of the semiquinone of riboflavin deviates in this respect as well. Furthermore, it was observed that there may be an influence of the ions present in solution on the extent of dimerization. There is also evidence that above *p*H 11 the intensity of the dimer band of FAD is much lower than that of either riboflavin or FMN under the same conditions. This could possibly be explained by an interference of the adenylic acid moiety of FAD with dimerization. High concentrations of adenylic acid were indeed able to depress dimerization in the case of the semiquinone of riboflavin. These quantitative aspects of the dimerization of the semiquinone were not further studied.

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