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The Nature of Compounds Present in Mixtures of Oxidized and Reduced Flavin Mononucleotides

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Much recent work on flavin-containing enzymes has followed from the observations of Beinert (1956) on the spectral changes in solutions of reduced flavin mononucleotide during oxidation by molecular oxygen. In addition to the bands due to the oxidized and reduced flavins, bands due to two other compounds were seen, one with maximal extinction near 570 m μ and another with a peak at about 900 m μ (the exact position of the band varied with pH). As the oxidation of FMNH₂ progressed the two new bands increased in intensity to a maximum at about half oxidation and faded again as oxidation was completed. On the basis of concentration and temperature effects the 570 m μ band was attributed to the half-reduced (semiquinoid or free radical) form of the flavin and the 900 m μ band to the dimer of the semiquinone. The conclusion that a free radical species is present in mixtures of FMNH₂ and FMN has been confirmed by electron-spin-resonance spectrometry (Beinert & Sands, 1961), though no quantitative studies were made to relate spectroscopic appearances and electron-spin-resonance signals to the progress of the oxidation.

Massey & Palmer (1962) recently studied the spectra of the enzyme lipoyl dehydrogenase and of model systems and have concluded that both free and enzyme-bound flavin form charge-transfer complexes with oxidized nicotinamide nucleotides and their analogues. These complexes have extinc-

tion bands sufficiently similar to the band at 900 m μ seen by Beinert (1956) to suggest that the 900 m μ band might be due to a charge-transfer complex between FMN and FMNH₂ rather than to a dimer of the semiquinone. Further, if Beinert (1956) was correct in assigning the band at 570 m μ to a flavin semiquinone and the band at 900 m μ to its dimer, then the extinction at 900 m μ should be proportional to the square of the extinction at 570 m μ . Although Gibson & Hastings (1962) have found that this relation is roughly followed in dilute (< 0.5 mM) mixtures of FMN and FMNH₂, Massey & Palmer (1962) observed gross departures from it in more concentrated solutions.

In view of these difficulties and because of the wide use of spectroscopic findings on the model system FMN-FMNH₂ in interpreting the extinction bands found with flavoprotein enzymes, we have extended Beinert's (1956) work, collecting and correlating quantitative results by spectrophotometric, fluorescence and electron-spin-resonance methods.

MATERIALS AND METHODS

Chemicals. FMN was purchased either from The Sigma Chemical Co., St Louis, Mo., U.S.A., or from Nutritional Biochemicals Corp., Cleveland, Ohio, U.S.A. Dihydrolipoamide was prepared by the method of Reed, Koike, Levitch & Leach (1959). Sodium dithionite was British Drug Houses Ltd. laboratory reagent; the other chemicals

used were of Analytical grade. Sodium phosphate buffer, pH 6.3, was used throughout (molar ratio Na_2HPO_4 : NaH_2PO_4 , 22:78).

Preparation of FMN-FMNH₂ mixtures. Portions of a stock solution of FMN in the appropriate solvent and of a suitable concentration were transferred to two tonometers and thoroughly deoxygenated by repeated evacuation and flushing with O_2 -free N_2 . The contents of one tonometer were then titrated with approx. 0.5M- $\text{Na}_2\text{S}_2\text{O}_4$ until the flavin was just reduced. The end point of the reduction is very sharp and may be detected either by colour changes or by fluorescence (FMNH₂ does not fluoresce). The FMNH₂ solution was again evacuated and shaken and finally left under a positive pressure of N_2 . The volume of dithionite solution used as titrant was less than 1% of the volume of the flavin solution; therefore concentration changes on titration were negligible. Mixtures of known composition were then made by drawing off known volumes of FMN and FMNH₂ into a syringe, which was capped by modelling 'clay', and the contents were mixed by rotation. Samples of such mixtures were then injected into spectrophotometer tubes that had been made anaerobic by evacuation and flushing with O_2 -free N_2 , and into the Varian V-4548 aqueous solution sample cells for the electron-spin-resonance spectrometer. These tubes were fitted with a stopcock at one end as well as their usual ground-glass stopper at the other end. They were well flushed with O_2 -free N_2 before filling from the syringes and as an additional precaution against reoxidation of flavin several volumes of mixture were driven through in one continuous stream before the stoppers were closed. The mixtures for fluorescence experiments were made in the same way and injected into 1 mm. optical-path cells. It was not necessary to exclude O_2 from the surface, as the rate of diffusion of O_2 from the small exposed surface was so low that both fluorescence and extinction could be determined without detectable interference from reoxidation of FMNH₂.

Reduction of FMN by light or dihydrolipoamide. Mixtures of FMN and FMNH₂ were also obtained by reducing solutions of FMN in phosphate buffer either photochemically in the presence of 20 mM-EDTA or with 20 mM-dihydrolipoamide (Massey & Palmer, 1962). In the former case the solution was exposed, for periods varying from 1 min. to several hours, at a distance of 8 cm. from a 60 w tungsten lamp.

Measurements of extinction. These were made as described above, either with the anaerobic spectrophotometer cells previously described (Massey, Gibson & Veeger, 1960) or with 1 mm. cells. Readings were taken with a model DU Beckman spectrophotometer with the cells maintained at 25° by thermostat control.

Measurements of fluorescence. These were made with the experimental arrangement described by Weber & Teale (1958). Fluorescence was excited with light of wavelength 450 m μ from a xenon arc and Bausch and Lomb small-grating monochromator. The fluorescent light was measured at 530 m μ with a Bausch and Lomb large-grating monochromator, photomultiplier and galvanometer (Pye Ltd. Scalamp).

Measurements of electron-spin resonance. These spectra were taken with a Varian V-4501 100 kcy. spectrometer, with the Varian V-4548 aqueous solution sample cells. Examples of typical spectra are shown in Figs. 13 and 15.

All the spectra were run with a modulation amplitude

that did not broaden the observable hyperfine splittings, and at a microwave power level that did not give any detectable saturation. Under these conditions the area under the absorption curve is proportional to the number of radicals present, and relative concentrations can be measured by double integration of the derivative traces (Ingram, 1958). However, provided that the line shape remains constant, the amplitude of the derivative curve is a measure of the number of radicals; Fig. 13 shows that our spectra did not all show the same degree of resolution, but it was found that the amplitude of the peak marked with the arrow in Fig. 13 was always linearly proportional to the integrated area. Having established this correlation we were able to use the amplitude of the chosen peak, referred to constant instrumental conditions, as a measure of relative concentrations.

Absolute concentration of free radicals. Integrated areas were compared with those obtained from standard solutions of diphenyl picryl hydrazyl. The diphenyl picryl hydrazyl used was recrystallized from carbon disulphide to eliminate weighing errors that might arise through the formation of complexes with solvents such as chloroform or benzene (Lyon & Watson, 1955). Standard 50 μM solutions were made up in benzene. Spectra of these solutions were taken at the same modulation amplitude setting and power level as used for the FMN solutions, and the usual five-line spectrum was obtained. Under these conditions there was no modulation or saturation broadening of the five-line spectrum. The solutions were contained in the aqueous solution sample cells and were not rigorously degassed. Although separate experiments showed that with rigorous degassing and reduction of the modulation amplitude the proton hyperfine splittings could be resolved, the basing of our calibration on the five-line spectrum is probably not a serious source of error. More difficult to assess is the error introduced because the radical and diphenyl picryl hydrazyl spectra were taken in solutions of widely differing dielectric constant. This can cause the cavity Q to change considerably, and hence affect the spectrometer sensitivity. However, the Varian aqueous solution sample cells are designed to minimize damping by solvents of high dielectric constant, so the comparison should not be much affected by this: certainly only minor retuning was needed when an aqueous solution sample was replaced by one of diphenyl picryl hydrazyl in benzene. The other principal source of error is in the actual numerical integrations but these should be accurate to $\pm 5\%$. Taking all these factors into consideration we think that our estimates of absolute concentrations are accurate to $\pm 40\%$. With this calibration 370 arbitrary units of electron-spin-resonance amplitude are equivalent to a radical concentration of 0.1 mM.

Determinations of molecular weight. The molecular weights of FMN and FMNH₂ solutions were measured at 25° in 0.12M-phosphate, pH 6.3, by the use of the double-sector synthetic boundary cell of the Spinco analytical ultracentrifuge. The diffusion constant D and the sedimentation constant S were evaluated from the same experiment, as described by Schachman (1959). From these values the molecular weight was calculated by the relation

$$M = RTS/(1 - \bar{v}\rho)D,$$

where R is the gas constant, T the absolute temperature, $\bar{v}$ the partial specific volume of the solution and ρ the

density of the solvent. The partial specific volume was estimated by pycnometry, as described by Schachman (1957), and found to be 0.574.

RESULTS

Relation of extinction at 570 and at 900 m μ

Fig. 1 shows the lack of correlation between E at 900 m μ and the square of E at 570 m μ during re-oxidation of 3.33 mM-FMNH₂ by molecular O₂. The result shows clearly that the simple equilibria proposed by Beinert (1956) do not afford a complete description of the system in phosphate buffer, pH 6.3. Although there may be some overlap of the 900 m μ band into the 570 m μ region (see later sections of the paper), the two looped curves of Fig. 1 show that any correction for such an overlap only accentuates the divergences from the expected relation. A similar experiment carried out with the addition of 0.1 M-caffeine is also shown in Fig. 1. Here the expected relation between the two bands is observed (cf. below). Massey & Palmer (1962) have obtained results similar to these with dihydrolipoamide or light-energy to reduce FMN.

Spectral and electron-spin-resonance observations on known mixtures of FMN and FMNH₂. Fig. 2 shows the spectra of 4 mM-FMN at various stages of reduction. It is evident that the results shown in

Fig. 1 are due to E at 900 m μ reaching its maximum at 50 % reduction, whereas E at 570 m μ is maximal at 70 % reduction. The optical experiments at first

Fig. 1. Relation of E at 570 m μ to E at 900 m μ on re-oxidation of 3.33 mM-FMNH₂ in 0.12 M-phosphate, pH 6.3, at 25°. The FMN solution was reduced by a slight excess of Na₂S₂O₄ in an anaerobic spectrophotometer cell; small amounts of air were admitted and mixed and readings taken at 570 and 900 m μ : $\times$ and $\bullet$, in phosphate alone, without and with a correction to E_{570} of 10% of E_{900} (arrows indicate the direction of change after successive admissions of air); $\blacktriangle$ and $\circ$, in phosphate plus 0.1 M-caffeine; $\blacktriangle$, stages from FMNH₂ to half reoxidation; $\circ$, stages from half to full reoxidation.

Fig. 2. Spectra at various stages of reduction of 4 mM-FMN in 0.12 M-phosphate, pH 6.3, at 25°. Percentage reduction: $\blacktriangledown$, 10; $\times$, 90; $\bullet$, 20; ∇ , 80; $\blacktriangle$, 30; $\circ$, 70; broken line, 50 (observations were made at intervals of 10 m μ , but the experimental points have been omitted to reduce congestion at the short-wavelength end of the spectrum).

led us to doubt if E at 570 $m\mu$ was related to the free radical, but there is, in fact, a close relation between E and the electron-spin-resonance signal (Fig. 3). Both quantities are greatest at about 70% reduction and give a similar and markedly asymmetrical plot against percentage reduction. The radical is present only in small amounts, accounting at most for about 1% of the total flavin. The shape of the titration curve should be given by the equation

$$E_{570} = k(\% \text{ FMN})(100 - \% \text{ FMN}), \quad (1)$$

where k is an arbitrary constant. The broken line in Fig. 4 is the calculated curve, which has a rather flat maximum centred at 50% reduction. The

Fig. 3. Relation of E at 900 and 570 $m\mu$ to percentage reduction of flavin and electron-spin-resonance signal. Total flavin concentration 4 mM in 0.12M-phosphate, pH 6.3; temperature 23–25°. $\times$, E_{900} ; $\bullet$, E_{570} ; Δ , electron-spin-resonance amplitude.

Fig. 4. Correlation of electron-spin-resonance signal and E_{570} during reduction of 4 mM-FMN; temperature 23–25°. $\bullet$ and ∇ , Electron-spin resonance and E_{570} when the solvent was 0.12M-phosphate, pH 6.3; $\circ$ and $\blacktriangle$, electron-spin resonance and E_{570} when the solvent was 0.3M-phosphate, pH 6.3–formamide (3:22, v/v). The broken line shows the relative radical concentration expected if the only equilibrium existing was that expressed by equation (1).

experimental results from Figs. 2 and 3 are re-plotted in Fig. 4 to show the departure from the theoretical line. The Figure also contains the results obtained when the titration is carried out in formamide–0.3M-phosphate buffer, pH 6.3 (22:3, v/v). Under these conditions the amount of radical is much decreased (as is the amount of the intermediate absorbing at 900 $m\mu$ also) and a symmetrical titration curve agreeing well with equation (1) is obtained.

The marked asymmetry of the titration curve of E at 570 $m\mu$ and the electron-spin-resonance signal in phosphate buffer persists down to the lowest concentrations that can be studied by the electron-spin-resonance method [although Gibson & Hastings (1962) found from spectrophotometric measurements that it was much less marked in more dilute (less than 0.4 mM) solutions]. Three complete titration curves are given in Fig. 5 and in each the greatest amount of radical is found at about 70% reduction. This ratio of FMNH₂ to FMN was therefore chosen for a detailed study of the relation of electron-spin-resonance signal to total flavin concentration. The results (Fig. 6) show that the electron-spin-resonance signal does not increase linearly with total flavin concentration but falls off at the highest concentrations used. Over this more extended range of concentrations there is some breakdown in the correlation between E at 570 $m\mu$ and electron-spin-resonance signal, for, as Table 1 shows, E at 570 $m\mu$ increases linearly with flavin concentration over the whole range.

Dimerization of FMN and FMNH₂. In the course of the spectral studies it was noticed that the extinction coefficients of both FMN and FMNH₂ varied considerably with concentration and that the colour of their solutions changed with the

Fig. 5. Effect of concentration of flavin on the titration curves of free radical concentration against percentage reduction. Conditions: 0.12M-phosphate, pH 6.3; temperature 23–25°. Total flavin: $\blacktriangle$, 1.33 mM; $\times$, 4.0 mM; $\bullet$, 20 mM.

concentration of flavin and with the nature of the solvent. In dilute aqueous solutions (< 0.1 mM) FMN is light yellow and FMNH₂ a very pale yellow. In more concentrated solutions (> 10 mM) both are orange and FMNH₂ is now deeper in colour than FMN. In solvents such as formamide-0.3M-phosphate buffer (22:3, v/v) or methanol-0.24M-phosphate buffer (1:1, v/v), FMN is pure yellow even at the highest concentrations that can be reached, and FMNH₂ is much less strongly coloured than in aqueous solution. These colour changes may be due to dimerization, which, on theoretical grounds, would be expected to broaden spectral bands. The extinction coefficients of FMN and

FMNH₂ at 540 mμ in formamide and methanol were independent of concentration. The relations between extinction coefficients and concentration for FMN and FMNH₂ dissolved in 0.12M-phosphate buffer are shown in Fig. 7 and can be explained by the formation of dimers. From

where K_F and K_{FH_2} are given by $[\text{FMN}]^2/[(\text{FMN})_2]$ and $[\text{FMNH}_2]^2/[(\text{FMNH}_2)_2]$ respectively, if the molar extinction coefficients of the monomers and dimers are ϵ_1 and ϵ_2 and if the total flavin concn-

Fig. 6. Dependence of amplitude of electron-spin resonance on the total flavin concentration when the flavin is 70% reduced (0.12M-phosphate, pH 6.3; temperature 23–25°). ●, Experimental values; the continuous line is calculated from values of $K_F = K_{\text{FH}_2} = 10$ mM and $K_{\text{CT}} = 0.445$ mM.

Fig. 7. Variation of molar extinction coefficient at 540 mμ of FMN and FMNH₂ with flavin concentration in 0.12M-phosphate, pH 6.3, at 25°. ● and ○, Experimental results for FMNH₂ and FMN respectively; continuous lines are calculated as described in the text.

Table 1. Variation with total flavin concentration of the apparent extinction coefficient of the species absorbing at 570 mμ

Results were all obtained at 25° in 0.12M-phosphate, pH 6.3. Values shown were the maxima obtained in the particular experiments in cells of 1 cm. light-path. Methods of reduction: A, by light; B, by mixture of FMN and FMNH₂.

Total flavin concn. (mM)	Method of reduction	E_{570}		Corrected E_{570} /total flavin concn.
		Actual	Corrected for absorption of FMN and FMNH ₂	
1	A	0.108	0.105	105
1.33	B	0.105	0.144	108
2	A	0.220	0.213	107
3.33	A	0.365	0.351	105
3.33	Dihydrolipoamide	0.355	0.341	102
3.33	B	0.360	0.348	105
4	B	0.494	0.451	113
		0.462	0.432	108
5	A	0.550	0.529	106
6.67	B	0.780	0.747	112
10	A	1.035	0.984	98
10	B	1.225	1.154	115
20	B	2.47	2.33	116

tration expressed as monomer is x , the observed extinction coefficient ϵ_s is given by

$$\epsilon_s = \epsilon_2/2 + K(2\epsilon_1 - \epsilon_2)/[\sqrt{(8Kx + K^2)} - K] \quad (4)$$

the subscript of K varying according to the form of flavin being examined. As this expression contains three arbitrary constants several sets of values for the constants can be found to give a reasonable fit to any experimental curve. The choice can be much narrowed by taking ϵ_1 as the value found experimentally in aqueous formamide or aqueous methanol where, presumably, little dimerization occurs. This has been done in calculating the lines drawn in Fig. 7, where the values used are $K_F = K_{FH_2} = 10$ mM and ϵ_1 and ϵ_2 are 10 and 110 cm.²/m-mole for FMN and 20 and 150 cm.²/m-mole respectively for FMNH₂.

The formation of polymers of FMN and FMNH₂ has been confirmed by direct measurement in the ultracentrifuge (Table 2). The results for FMN are clear-cut and show that a dimer is the chief product (monomeric molecular weight: 514). The results with FMNH₂ have been less satisfactory, perhaps because of the difficulty of carrying out long runs under anaerobic conditions. When the FMN is reduced with an excess of dithionite the results suggest that a tetramer is the chief product, but if platinized asbestos and H₂ or Raney nickel catalyst and H₂ are used for reduction the values approach those expected for a dimer.

Effect of total flavin concentration on the species absorbing at 900 mμ. The concentration of the species absorbing at 900 mμ (CT) should increase as the square of the total flavin concentration, whether it is a dimer of the semiquinone (Beinert, 1956) or a charge-transfer complex between FMNH₂ and FMN as suggested by Massey & Palmer (1962). This is expressed formally by the identity in equations (5a) and (5b):

where K_{CT} represents either

$$[\text{FMN}][\text{FMNH}_2]/[(\text{FMNH}^*)_2]$$

or $[\text{FMN}][\text{FMNH}_2]/[(\text{FMNH}_2 \rightarrow \text{FMN})]$

It follows that if E at 900 mμ of 50% reduced FMN is expressed as an extinction coefficient (i.e. $E/\text{total flavin}$) its value should increase linearly with the concentration of flavin. This is true in aqueous formamide (lower continuous line in Fig. 8) but is far from being the case in aqueous solution (upper continuous curve in Fig. 8). A falling off from linearity would be expected because of dimerization of FMN and FMNH₂, but the calculated effect is not great enough to account

for the observations (broken curve in Fig. 8). An alternative explanation is that CT is an abundant species binding a high proportion of the total flavin in the system. The upper continuous curve in Fig. 8 has been calculated on this assumption from $K_F = K_{FH_2} = 10$ mM and $K_{CT} = 0.445$ mM. The curve shows that when the total flavin concentration is 5 mM about 60% is present in the form CT, which has a molar extinction coefficient at 900 mμ of 680 (or 340 in terms of monomeric flavin).

Whatever the proportion of CT formed, if E at 900 mμ is plotted against the percentage of reduced

Table 2. *Determinations of molecular weight of flavin mononucleotide and reduced flavin mononucleotide at high concentrations*

Values shown were determined as described in the Materials and Methods section. All determinations were made at 25° in 0.12M-phosphate, pH 6.3.

Conditions	S (s)	$10^6 D$ (cm. ² /sec.)	Mol. wt.
20 mM-FMN	0.50	2.7	1100
40 mM-FMN	0.48	2.86	1000
18 mM-FMNH ₂ reduced with 25 mM-Na ₂ S ₂ O ₄	0.66	2.26	1750
9 mM-FMNH ₂ reduced with 25 mM-Na ₂ S ₂ O ₄	0.77	2.20	2060
10 mM-FMNH ₂ reduced with H ₂ and Pt	0.73	3.47	1240
20 mM-FMNH ₂ reduced with H ₂ and Raney Ni	0.74	3.54	1200

Fig. 8. Variation of E_{900} with total flavin concentration at 50% reduction in 0.12M-phosphate, pH 6.3 (●) and in 0.24M-phosphate, pH 6.3-formamide, 3:17, v/v (■). The broken line is calculated by assuming that the dimers of FMN and FMNH₂ cannot form the 900 mμ band and that this species accounts for only a small proportion of total flavin. K_F and K_{FH_2} were assumed to be 10 mM. The upper continuous line is calculated on the assumption that large amounts of the original FMN and FMNH₂ are used in the formation of the 900 mμ species and that $K_{CT} = 0.445$ mM. The lower continuous line is calculated by assuming $K_{CT} = 27$ mM and $\epsilon_{900} = 680$ cm.²/m-mole. (If expressed in terms of the monomeric flavin concentration, $\epsilon_{900} = 340$ cm.²/m-mole.)

flavin, the resulting curve should be symmetrical about 50 %, as was found by Gibson & Hastings (1962) for dilute aqueous solutions, i.e. less than 0.5 mM. This is true under all the conditions used (see Figs. 3, 11 and 14).

Fluorescence-quenching studies. If solutions of high E (more than 5/cm. optical depth) at the wavelength of excitation are observed by front-face excitation and if all the absorbing molecules belong to an excitable species, the intensity of fluorescence is independent of the concentration of fluorescent substance, small geometrical effects being neglected. If a material that absorbs at the exciting wavelength but which does not fluoresce is also present, a proportion of the quanta that would otherwise have excited fluorescence will be absorbed by it and quenching of fluorescence will occur.

As FMN is fluorescent and dimers are non-fluorescent in solution at ordinary temperatures, measurements of fluorescence-quenching can be used to follow the dimerization of FMN. In addition to quenching by simple optical shielding, non-fluorescent molecules also quench by collision with and energy transfer from excited molecules of fluoracer in the interval between excitation and emission (see Weber, 1950). The quenching due to shielding will be given by:

$$F/F_0 = \epsilon_1 Y / (\epsilon_1 Y + \epsilon_2 Z) \quad (6)$$

where F is the fluorescent intensity in presence of quencher, F_0 the fluorescent intensity without quenching, Y the concentration of monomer, Z the concentration of dimer and ϵ_1 and ϵ_2 are the extinction coefficients of monomer and dimer respectively at the wavelength of excitation. Quenching by collision and energy-transfer may be regarded as a first-order process competing with deactivation by fluorescence. The contribution of this mechanism is

$$F/F_0 = k_f / (k_f + Ck_q) \quad (7)$$

where k_f is the lifetime of the excited state expressed as a rate constant, k_q the rate constant for the reaction between quencher and excited FMN molecule, and C the concentration of quencher. Combining (6) and (7), expressing Y and Z in terms of the total flavin (x), and replacing k_f by its reciprocal τ , gives:

$$\frac{F}{F_0} = \frac{4\epsilon_1 A}{[2\epsilon_1 A + \epsilon_2(x - A)][2 + k_q\tau(x - A)]} \quad (8)$$

where $A = [\sqrt{(8K_F x + K_F^2)} - K_F]/4$. To apply (8), K_F was taken as 10 mM as before, $\epsilon_2 = 2\epsilon_1$ (i.e. dimerization does not affect the absorption of FMN at 450 m μ) and $\tau = 5 \times 10^{-9}$ sec. (Dr G. Weber, personal communication). It remains only to fix a value for K_Q , which has been taken to be $1.5 \times 10^{10} \text{ M}^{-1} \text{ sec}^{-1}$. This value was obtained from measurements of the polarization of fluorescence of

solutions of FMN of varying concentrations made with the polarization photometer described by Weber (1956), using the theory developed by Weber (1950). With these values an agreement with experiment is found (upper curve in Fig. 9), which is regarded as excellent when the approximate nature of the treatment is taken into account. Thus the spectrophotometric and fluorescence methods are in good agreement for the dimerization of FMN.

If the 900 m μ -absorbing species CT absorbs strongly at a wavelength used for fluorescence excitation, it, too, should function as a quencher by optical shielding, and would also be expected to quench by deactivating excited FMN molecules. Thus strong fluorescence-quenching would be expected in mixtures of FMN and FMNH₂. This has been confirmed experimentally (lower two curves in Fig. 9). Further, the symmetry of the titration curve in Fig. 3 shows that the mixtures containing 7FMN + 3FMNH₂ and those containing 3FMN + 7FMNH₂, which were examined in the experiment of Fig. 9, must contain the same concentration of CT (at similar total flavin concentrations). If CT were a minor constituent of the solutions the quenching in 7FMN + 3FMNH₂ would be much the same as in 3FMN + 7FMNH₂, differing only because of the light-absorption of FMNH₂ (about one-tenth of that of FMN at 450 m μ) and because of reaction of the excited state with FMNH₂ and dimerization of FMN. In fact the

Fig. 9. Fluorescence of solutions of: ●, FMN; ▲, FMN-FMNH₂ mixtures (7:3); ■, FMN-FMNH₂ mixtures (3:7). The solutions, in 0.12M-phosphate buffer, pH 6.3, were examined at 22° by front-face excitation. The lines were calculated with the constants specified and by the methods described in the text.

quenching is much greater in solutions containing $3\text{FMN} + 7\text{FMNH}_2$ and is close to that predicted from $k_{\text{CT}} = 0.445 \text{ mM}$ (as in calculating the upper continuous curve of Fig. 8) and

$$K_F = K_{\text{FH}_2} = 10 \text{ mM},$$

together with the assumption that

$$\epsilon_{\text{CT}} = \epsilon_{\text{F}} + \epsilon_{\text{FH}_2}$$

at $450 \text{ m}\mu$. The two lower curves in the Figure were obtained by first calculating the composition in terms of FMN, $(\text{FMN})_2$, FMNH_2 , $(\text{FMNH}_2)_2$ and CT. The optical shielding was calculated by summing E values for the various species at $450 \text{ m}\mu$ and the collisional quenching by summing the concentrations of the quenching species. The values used were exactly as in equations (6) and (7) with the same values for K_Q and τ . The agreement with experiment leaves little doubt that large amounts of CT are present in strong solutions of mixtures of FMN and FMNH_2 .

The assumption that at $450 \text{ m}\mu$ $\epsilon_{\text{CT}} = \epsilon_{\text{F}} + \epsilon_{\text{FH}_2}$ is supported by direct determinations, which, up to the highest concentration that could be measured in 1 mm . path cells (about 5 mM for 70% FMNH_2), showed that E is equal, within experimental error, to the sum of E values for the FMN and FMNH_2 present. It is further supported by determinations of the excitation spectrum of (i) 10 mM -FMN, (ii) 10 mM -FMN + 10 mM - FMNH_2 . When the front-face-excitation method is used with solutions containing only molecules that can be excited the intensity of fluorescence observed when the wavelength of the exciting light is varied is proportional only to the numbers of quanta used for excitation at the different wavelengths. If, in addition, a non-fluorescent absorbing species is present the fluorescence will be quenched by optical shielding and the relations $f/f_0 = \epsilon_f C_f / (\epsilon_f C_f + \epsilon_{nf} C_{nf})$ will hold, where f is the fluorescence of the mixture, f_0 the fluorescence of the pure substance, ϵ_f and ϵ_{nf} the extinction coefficients of the fluorescent and non-fluorescent substances at the wavelength being considered and C_f and C_{nf} are their concentrations. If f/f_0 remains constant as the wavelength of the exciting light is varied the extinction spectrum of the fluorescent and non-fluorescent species must be identical in form. Between 480 and $340 \text{ m}\mu$ CT has a spectrum closely similar to that of a mixture of FMN and FMNH_2 , whereas between 340 and $300 \text{ m}\mu$ E of CT is lower than that of the sum of its constituents.

The correctness of these conclusions is further supported by studies with formamide- 0.12M -phosphate buffer, pH 6.3 (85:15, v/v). In this solvent dimerization is much reduced; correspondingly the fluorescence quenching at high concentrations of FMN is also very much less than

in phosphate alone (Fig. 10). The upper curve in Fig. 10 leads to a dimerization constant of 120 mM , a 12-fold decrease in affinity compared with that in phosphate buffer alone. The large effect of formamide on the formation of CT is also illustrated in Fig. 10, where the two lower sets of points illustrate experiments analogous to those of the corresponding sets in Fig. 9. There is again much less quenching, the solid curves having been calculated with a value for K_{CT} of 27 mM , a decrease in affinity of 60-fold as compared with buffer alone.

Thus the optical and fluorescence data are consistent in indicating that in mixtures of FMN and FMNH_2 at concentrations greater than 1 mM a large proportion of the total flavin is in the form of the species giving an absorption band at $900 \text{ m}\mu$. Fig. 11 shows the proportion of the total flavin in the form of CT at a total flavin concentration of 4 mM together with the relative values of E observed at $900 \text{ m}\mu$ at various stages of reduction.

Nature of the species absorbing at $570 \text{ m}\mu$. The close relation between the electron-spin-resonance signal and E at $570 \text{ m}\mu$ leaves little doubt that the latter is due either to the flavin semiquinone or to an immediate precursor of it. The asymmetry of the plot of electron-spin-resonance signal against the extent of reduction of FMN was left unexplained. It cannot be explained by the dimerization of FMN and FMNH_2 and the only satisfactory suggestion so far found is that E at $570 \text{ m}\mu$ is due

Fig. 10. Fluorescence of solutions in 0.12M -phosphate buffer, pH 6.3-formamide (15:85, v/v) of: ●, FMN; ▲, FMN- FMNH_2 mixtures (7:3); ■, FMN- FMNH_2 mixtures (3:7). Experimental conditions and method of calculation are specified in the text.

to a mixture of two substances with unpaired electrons, the monomeric semiquinone,

$$K_{\text{FMNH}\cdot} = [\text{FMN}][\text{FMNH}_2]/[\text{FMNH}\cdot]^2 \quad (9)$$

and a complex of the semiquinone with FMNH₂

$$K_{(\text{FMNH}_2\cdot\text{FMNH}\cdot)} = [\text{FMNH}_2][\text{FMNH}\cdot]/[\text{FMNH}_2\cdot\text{FMNH}\cdot] \quad (10)$$

These two equations allow the asymmetric titration curves to be fitted satisfactorily, as is shown by the example given in Fig. 12. The line shown was derived with the values of K_F , K_{FMH_2} and K_{CT} already determined and assuming in addition that (i) FMNH $\cdot$ and (FMNH₂·FMNH $\cdot$) do not represent a large part of the total flavin, (ii) (FMNH₂·FMNH $\cdot$) contributes 30 % of the total E at 570 m μ in a mixture of 8 parts of FMNH₂ with 2 parts of FMN at a total flavin concentration of 4 mm.

Convincing evidence for the existence of (FMNH₂·FMNH $\cdot$) comes from examination of the details of the electron-spin-resonance spectra at various stages of reduction of FMN. In the

Fig. 11. Proportion of the total flavin in the form of the 900 m μ -absorbing species (CT), during reduction of 4 mM-FMN in 0.12M-phosphate, pH 6.3, at 25°. The continuous line is calculated as described in the text; ●, experimental values.

Fig. 12. Comparison of experimental results (●) and calculated values (continuous line) of E_{570} during reduction of 4 mM-FMN in 0.12 M-phosphate, pH 6.3. The line is calculated as described in the text.

example shown in Fig. 13 it will be noted that whereas the resolution is relatively good below 50 % reduction it deteriorates as reduction progresses. This result is in good accord with the existence of the complex proposed (see Discussion).

Effect of caffeine on the electron-spin-resonance signal and on E at 570 m μ . Caffeine is known to be a potent complexing agent for flavins (Weber, 1950). It was therefore of interest to examine its effect on the titration curves of mixtures of FMN and FMNH₂ as it would be expected to compete effectively with the dimerization of FMN, FMNH₂, the formation of CT and the formation of the radical complex FMNH₂·FMNH $\cdot$. The results of an experiment carried out with 0.12M-phosphate buffer + 0.1M-caffeine are given in Fig. 14. The asymmetry of the curve found in phosphate alone is now completely abolished, the experimental points lying close to the curve expected for the formation of semiquinone according to equation (1). The absorption at 900 m μ is also considerably reduced as compared with that found in phosphate alone (cf. Fig. 3).

If the asymmetry of the radical titration curve found in phosphate buffer alone is due to the production of a complex between FMNH₂ and FMNH $\cdot$,

Fig. 13. Electron-spin-resonance spectra at various stages in the reduction (shown as percentages) of 4 mM-FMN in 0.12M-phosphate, pH 6.3. All spectra were taken at the same instrumental setting and therefore radical concentration is directly proportional to the amplitude of the peak marked with the arrows (see Materials and Methods section). The total spread of the spectra between the peaks marked with arrows is 17.8 ± 0.5 g.

Fig. 14. Effect of 0.1M-caffeine on the relation between percentage reduction of 4 mM-FMN at 23–25° in 0.12M-phosphate, pH 6.3, and E_{900} (●), E_{570} (×) and amplitude of electron-spin resonance (△). The broken line is calculated on the basis of equation (1) or equation (9).

then, from Fig. 14, this complex should not be formed in the presence of caffeine. The electron-spin-resonance spectra support this argument. Fig. 15 shows the electron-spin-resonance spectra at 10 and 90% reduction of 4 mM-FMN in 0.12M-phosphate and in 0.12M-phosphate + 0.1M-caffeine. It is clear that the loss of resolution at 90% reduction seen in phosphate buffer does not occur at all in the presence of caffeine. The optical spectra taken during the experiment shown in Fig. 15 are given in Fig. 16. In caffeine the spectra at 10 and 90% reduction are effectively superimposable. In the absence of caffeine the bands around 900 m μ are closely similar at this percentage reduction but the bands at 570 m μ differ widely. The experiments with caffeine thus give strong support to the suggestion that there are two distinct compounds with unpaired electrons and with E at 570 m μ .

Effect of solvent on the relation of the bands at 570 and 900 m μ . The lack of correlation between

Fig. 15. Electron-spin-resonance spectra at 10 and 90% reduction of 4 mM-FMN in 0.12M-phosphate, pH 6.3 (a and b) and in 0.12M-phosphate, pH 6.3, plus 0.1M-caffeine (c and d). The spectra shown were taken at the same instrumental setting.

Fig. 16. Spectral changes produced when reduction of 4 mM-FMN is carried out in 0.12M-phosphate, pH 6.3, with and without 0.1M-caffeine. Temperature, 25°. At 10% (x) and 90% (O) reduction in 0.12M-phosphate alone, and at 10% (●) and at 90% (Δ) reduction in phosphate + caffeine.

Table 3. *Effect of solvent on the wavelength of the absorption band near 900 mμ*

FMN at a total concentration of 3.33–4.0 mM was either mixed with FMNH₂ as in Figs. 2, 3, 4 and 14 or was reduced with Na₂S₂O₄ and then reoxidized with air as described in Fig. 1.

Solvent	Wavelength max. (mμ)
0.12M-phosphate, pH 6.3	890
0.12M-phosphate, pH 6.3, + 0.1M-caffeine	930–950
Formamide-0.3M phosphate, pH 6.3 (22:3, v/v)	900–930
Propylene glycol-0.24M-phosphate, pH 6.3 (1:1, v/v)	890
0.12M-phosphate, pH 6.3, + 7.2M-urea	890

the bands at 570 and 900 mμ during reduction of FMN, as demonstrated in experiments of the type shown in Figs. 1 or 3, has been seen only in aqueous solutions buffered with phosphate. When the mixtures of FMN and FMNH₂ are prepared in the presence of urea, caffeine, methanol, propylene glycol, dimethyl sulphoxide or formamide this effect is abolished and the square of E at 570 mμ is directly proportional to E at 900 mμ throughout the whole range of mixtures studied. There is, moreover, a constant relation between these quantities that is independent of the nature of the solvent, even though widely differing absolute values for E are found at these wavelengths in

Fig. 17. Effect of solvent on the relation between E_{570} and E_{900} . The solutions were of the following composition: ▲, 0.40 mM-FMN and 0.93 mM-FMNH₂, 1.0 mM-FMN and 2.33 mM-FMNH₂, 1.2 mM-FMN and 2.8 mM-FMNH₂, all in 0.12M-phosphate, pH 6.3. In the remaining solutions FMNH₂ and FMN were present in equal concentrations dissolved in the following solvents: O, formamide-0.3M-phosphate, pH 6.3 (22:3, v/v), total flavin 3.4 mM; □, propylene glycol-0.24M-phosphate, pH 6.3 (1:1, v/v), total flavin, 3.33 mM; ■, methanol-0.24M-phosphate, pH 6.3 (1:1, v/v), total flavin 4 mM; ▼, 0.12M-phosphate, pH 6.3, plus 0.1M-caffeine, total flavin 3.33 and 4.0 mM; ●, 0.12M-phosphate, pH 6.3, plus 7.2M-urea, total flavin 3.33 mM; Δ, dimethyl sulphoxide-0.3M-phosphate, pH 6.3 (67:33, v/v), total flavin 3.33 mM.

Table 4. *Effect of solvent on experimental constants involving the 570 and 900 m μ absorbing species*

$$K_{\text{FMNH}^\cdot} = \frac{[\text{FMN}][\text{FMNH}_2]}{[\text{FMNH}^\cdot]^2}; \quad K_{\text{CT}} = \frac{[\text{FMN}][\text{FMNH}_2]}{[\text{CT}]}; \quad K_x = \frac{[\text{CT}]}{[\text{FMNH}^\cdot]^2}; \quad K_y = \frac{[\text{FMNH}^\cdot]^2}{[\text{CT}]} = \frac{1}{K_x} = \frac{K_{\text{CT}}}{K_{\text{FMNH}^\cdot}}$$

Solvent	$10^{-3} K_{\text{FMNH}^\cdot}$	$10^3 K_{\text{CT}}$ (M)	$10^6 K_y$ (M)	$10^{-5} K_x$ (M ⁻¹)
Formamide-0.3M-phosphate, pH 6.3 (22:3, v/v)	27	56	2.1	4.7
Dimethyl sulphoxide-0.3M-phosphate, pH 6.3 (67:33, v/v)	6.7	14	2.0	4.9
Propylene glycol-0.24M-phosphate, pH 6.3 (1:1, v/v)	5.3	12	2.3	4.4
Methanol-0.24M-phosphate, pH 6.3 (1:1, v/v)	5.2	11	2.1	4.7
0.12M-Phosphate, pH 6.3, + 0.1M-caffeine	2.6	6.8	2.7	3.7
0.12M-Phosphate, pH 6.3, + 7.2M-urea	1.7	5.7	3.2	3.1

different solvents. These results, which are illustrated in Fig. 17, bear closely on the relation between the compounds and are dealt with further in the Discussion. The solvent also has an effect on the position of the maximum of the long-wavelength band. This may be seen most clearly in Fig. 16, where the wavelength of maximum absorption in 0.12M-phosphate buffer is 890 m μ , whereas in 0.12M-phosphate buffer + 0.1M-caffeine the maximum is at 930–950 m μ . The effect of solvent on the position of the long-wavelength band is summarized in Table 3. The results give further support for the suggestion that the compound absorbing at 900 m μ is a charge-transfer complex between FMNH₂ and FMN.

DISCUSSION

Nature of the species absorbing at 570 m μ

The results shown in Figs. 3, 4 and 14 reveal a close correlation of *E* at 570 m μ and the concentration of free radical as measured by electron-spin-resonance spectrometry. This correlation extends over a variety of solvents and over widely different ratios of FMN:FMNH₂. It is possible, however, that at the highest concentrations in aqueous buffer solution *E* at 570 m μ is somewhat greater than would be expected from the electron-spin-resonance signal. Our results in general give quantitative support for Beinert's (1956) suggestion that this absorption band is due to the flavin semiquinone. It is necessary to postulate, however, that in aqueous phosphate buffer solution there is more than one radical species. In the presence of 0.1M-caffeine or in 88% formamide-buffer mixtures accurately symmetrical titration curves are obtained on progressive reduction of FMN in terms both of electron-spin-resonance signal and *E* at 570 m μ as is required by equation (9), but the asymmetrical titration curves found in phosphate buffer alone cannot be explained in this simple way. Further, although the absorption bands of the 900 m μ -absorbing species may extend to 570 m μ , corrections for such an overlap, as shown in Fig. 1,

serve only to accentuate the asymmetry of the curves. It is also impossible to explain the results in phosphate buffer by differences in dimerization of FMN and FMNH₂, as the dimerization constants for both compounds are similar. The curves (Figs. 5 and 12) can be fitted if it is supposed that an additional species with *E* at 570 m μ and with an unpaired electron is formed by the association of FMNH₂ with the semiquinone. This is borne out by an analysis of the way in which the resolution of the electron-spin-resonance spectra varies in the course of a titration. It is observed that in all experiments where the titration curve is asymmetric the resolution worsens on going beyond 50% reduction. This is seen in Figs. 13 and 15. The effect is not due to the increase in radical concentration (concentration broadening) because the effect is more evident at 90% reduction than at 70%, although the radical concentration is less at 90% reduction. The spectra of Fig. 13 are for the most concentrated solution we have used (20 mM-FMN) and our absolute-concentration measurements indicate that we cannot have observed a radical concentration of more than 0.2 mM. Past experience with electron-spin-resonance spectra of radicals in solution suggests that concentration broadening should be negligible at such a concentration. The more dilute FMN solutions were correspondingly poorer in radical, but the broadening effects were noted there also. We believe therefore that the effect is a true property of the system and is one of 'lifetime broadening'. The effect on the electron-spin-resonance spectrum of transferring the centre of free radical between different environments has been summarized by Weissman (1960). For example, if there were a transfer reaction of the type $\text{FMNH}^\cdot + \text{FMNH}_2 \rightleftharpoons \text{FMNH}_2 + \text{FMNH}^\cdot$, and this were proceeding at a rate comparable with the inverse of the hyperfine line-width (typically 10⁻⁶ cyc./sec. for a free radical in solution), then broadening of the hyperfine components would be expected to occur. For a partially resolved spectrum and a small amount of broadening this would have just the effect that we observe. The

effect is a manifestation of the uncertainty principle: the lifetime of a state, i.e. of a particular FMN molecule existing as a free radical, being limited by the transfer reaction.

The spectroscopic data indicate the formation of some sort of complex between radical and FMNH₂, and within such a pair an exchange reaction of the type indicated above would be expected to occur quite readily. This should perhaps be written $(\text{FMNH}^\cdot, \text{FMNH}_2) \rightleftharpoons (\text{FMNH}_2, \text{FMNH}^\cdot)$. The formation of such a complex would, of course, be much more important when the solution was more than 50 % reduced. It would exist in solution together with radical not in a complex so the net broadened spectrum we observe would result from the superposition of spectra from the radical not in a complex and the exchange-broadened spectrum. Since we cannot estimate the line-widths and the extent of line-broadening it is not possible to be precise about the rate of the transfer reaction within the complex. Its upper and lower limits, however, are set by the total width of the spectrum and a typical hyperfine component width for a radical in solution. These limits yield a unimolecular exchange rate within the complex in the range 10^5 – 10^9 sec.⁻¹, whereas the optical-spectral data suggest that the species in a complex, i.e. the association of a particular radical and a particular molecule of FMNH₂, persists over several such exchanges.

The possibility remains that the electron-spin-resonance spectra of the radicals not in complexes may be broadened by exchange reactions occurring on collision with other molecules. Such reactions could occur with FMN or with the charge-transfer complex, for example, and may contribute to the rather poor resolution observed in the electron-spin-resonance spectra generally, although this may well arise from the sheer complexity of the hyperfine pattern. The absence of extra broadening in solutions more than 50 % reduced in solvents such as formamide or in the presence of added solutes such as caffeine is consistent with the suggestion that such components tend to inhibit the formation of loose molecular complexes.

As mentioned earlier, the correlation between amplitude of electron-spin-resonance signal and E at 570 m μ , although good at low concentration (less than 4 mm-flavin), is less precise at higher concentrations. If there were neither dimerization of FMN nor formation of a charge-transfer complex the electron-spin-resonance signal should increase linearly with total flavin concentration. It does not in fact do so, and the departure from linearity is in agreement with calculations taking into account dimerization and formation of a charge-transfer complex. E at 570 m μ , however, does increase linearly with flavin concentration over the whole range studied and may possibly be due to the

formation of yet another species: the dimer of the semiquinone. Such a compound would be expected to absorb in the same general region of the spectrum as the monomer and might or might not have an electron-spin-resonance signal depending on whether the two odd electrons had paired or unpaired spins. In the former case the discrepancy between the electron-spin-resonance signal and E at 570 m μ at high concentrations would be explained.

Concentration of free radical in solution and the extinction coefficient of the radical

The concentration of radical may be obtained from comparison with calibration experiments (see Materials and Methods section) by using known radical concentrations. This method is subject to large uncertainties (± 40 %) when used for determining the absolute concentration of radical. Relative measurements are much more accurate and are correct within about ± 5 %. Although the estimate of the absolute concentration of radical is subject to large uncertainty it is still worth while working out the extinction coefficient of the radical. This should be best obtained from experiments with conditions where formation of the complex FMNH₂·FMNH[·] or of the dimer is least, i.e. in aqueous formamide or in the presence of caffeine. At a total flavin concentration of 4 mM it can be estimated from Figs. 4 and 14 that the maximum radical concentrations were 8.6 μM and 35 μM respectively. The values of E at 570 m μ were 0.117 and 0.328 respectively, giving ϵ_{570} for the radical of 13600 and 9400 cm.²/m-mole. In phosphate buffer alone, at the same total flavin concentration and at 70 % reduction, the radical concentration was 38 μM with E at 570 m μ of 0.451, giving ϵ_{570} 11900 cm.²/m-mole. The first two points of Fig. 6 and the data for E in Table 1 yield ϵ_{570} 8000 cm.²/m-mole. These results compare interestingly with values for E of bands in the 500–600 m μ region produced in various flavoprotein enzymes under the influence of their specific substrates. It is often difficult to assign these bands to a free radical form of the flavoprotein (cf. Beinert & Sands, 1961), but in most cases the changes in E are in the region of 2000–4000 cm.²/m-mole, which suggests that if they are indeed free radical forms they must be produced in significant amounts rather than in the small quantities found in the free FMN system, where the greatest radical concentration is about 1 % of the total flavin concentration. From the values of $K_{\text{CT}} = 0.445$, $K_{\text{F}} = K_{\text{FH}_2} = 10$ mM and the variation of radical concentration with concentration shown in Fig. 6, numerical values of $K_{\text{FMNH}^\cdot}$ and $K_{\text{FMNH}_2, \text{FMNH}^\cdot}$ as defined in equations (9) and (10) are immediately obtained. In phosphate buffer the values are 4.8×10^3 and 5.6 mM respectively.

*Nature of the species absorbing at 900 mμ
and its relation to the semiquinone*

Beinert (1956) has proposed that the species absorbing at 900 mμ is the dimer of the semiquinone, whereas Massey & Palmer (1962) believe it is more likely to be a charge-transfer complex between FMNH₂ and FMN. Beinert's scheme requires that dimerization of the semiquinone be associated with a shift in the long-wavelength absorption band from 570 to 900 mμ. This shift is larger than is usually found on dimerization, and is out of keeping with the shifts that have been observed on the dimerization of FMN and FMNH₂. As with most organic compounds, dimerization of FMN and FMNH₂ is associated only with slight broadening of the principal absorption bands. On the other hand, the appearance of a broad and featureless band at 900 mμ, so far removed from the principal absorption bands of FMN and FMNH₂, is typical of a charge-transfer process. This is further supported by our failure to find a significant difference in *E* at 450 mμ between optical and physical mixtures of FMN and FMNH₂, even under conditions where it was known that the greater part of the total flavin had been converted into the species absorbing at 900 mμ. The effects of solvents on the wavelength maximum of this species, limited though they are, are also in keeping with the behaviour expected of a charge-transfer complex.

The two alternative schemes relating the semiquinone and species absorbing at 900 mμ are:

(i) FMN + FMNH₂

(ii) FMN + FMNH₂

These schemes lead to identical mathematical relations between *E* at 570 mμ and *E* at 900 mμ (provided that the experimental conditions exclude the formation of the FMNH₂.FMNH' complex), and it is formally impossible to distinguish between them. The evidence derived from change of solvent summarized in Fig. 17 and Table 4, however, argues strongly for scheme (ii). Dimerization or formation of a charge-transfer complex are processes associated with small energy changes and may be expected to be influenced by changes in solvent, whereas the formation of a semiquinone involves a definite chemical rearrangement and should be less affected by solvent. Each scheme contains a dimerization step and a radical formation step, but in inverse order. If scheme (i) were

correct we should expect *K*_{FMNH}· to show little change, but *K*_y to vary widely with solvent. If scheme (ii) is correct *K*_{CT} should vary widely and *K*_x remain substantially constant. As Table 4 shows, *K*_x and *K*_y show small changes of about 50 %, whereas *K*_{CT} and *K*_{FMNH}· change tenfold. (The four constants are not independent.) We therefore propose that the 900 mμ-absorbing species is a charge-transfer complex between FMNH₂ and FMN, and is an intermediate in the formation of the semiquinone rather than its dimer.

SUMMARY

1. The composition of the equilibrium mixtures obtained by mixing known amounts of FMN and FMNH₂ have been studied by spectrophotometry, fluorimetry and electron-spin-resonance spectrometry.

2. The major component of the mixtures containing more than 2 mM total flavin is a species absorbing at 900 mμ which we regard as a charge-transfer complex between FMNH₂ and FMN.

3. Comparison of the spectrophotometric and electron-spin-resonance data show a close correlation between the extinction at 570 mμ and the concentration of free radical.

4. When the radical concentration is plotted against total percentage of reduced flavin at constant flavin concentration, a strongly asymmetric curve is obtained with a maximum at about 70 % reduction. Evidence is presented that this asymmetry is due to the presence of another radical species, a complex between the semiquinone and FMNH₂.

5. The formation of the complex between semiquinone and FMNH₂ is inhibited in the presence of solutes such as caffeine and urea and in various organic solvents.

6. In aqueous solution (0.12 M-phosphate, pH 6.3) our data are suitably fitted by the following equilibria:

7. The effect of changing the solvent on the relation between the extinction at 570 mμ and that at 900 mμ leads us to the conclusion that the charge-transfer complex (900 mμ band) is a precursor of the free semiquinone.

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The Interaction of Glycosides and Saccharides with Antibody to the Corresponding Phenylazo Glycosides

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Previous study of the interaction of a glycosphingolipid, cytolin H, with anti-tissue antibody showed that this interaction could be inhibited preferentially by lactose in comparison with the other saccharides tested, indicating that a lactosyl residue was present in this molecule, and suggesting that this residue may be responsible for most of the observed interaction (Rapport, Graf & Yariv, 1961). The reactions of antibody directed against various glycosidic residues (lactose, glucose, galactose) thus became relevant for the study of other glycolipids.

Although methods for the preparation of anti-glycoside antibody are available (Avery & Goebel, 1929; Goebel, Avery & Babers, 1934), the estimation of the antibody present in such antisera is difficult because their antibody content is low and a high concentration of precipitating antigen, which is mainly protein, must be used. We therefore developed protein-free precipitating antigens for several of these systems by incorporating phenyl glycosides into a simple polyhaptenic molecule

[1,3,5-tri-(*p*-glycosyloxyphenylazo)-2,4,6-trihydroxybenzene]. These precipitating antigens were effective in low concentrations relative to that of antibody, permitting ready measurement of the content of antibody in the precipitate.

The inhibition of these antigen-antibody systems by simple saccharides gave results that could not easily be reconciled with the accepted concept that the reactivity of a free hexose, corresponding to the terminal glycosyl radical of the immunogenic determinant, will account for the interaction of antibody with this residue in the antigen. These findings are discussed and a possible explanation is offered.

MATERIALS AND METHODS

p-Nitrophenyl β -glycosides. *p*-Nitrophenyl β -D-galactoside was prepared from acetobromogalactose (Fischer, 1910) via the *p*-nitrophenyl tetra-acetyl β -galactoside obtained according to the method of Latham, May & Mossetig (1950) as adapted by Beiser, Burke & Tanenbaum (1960). Deacetylation with barium methoxide in hot methanol gave the required product which, after two recrystallizations from methanol, had m.p. 180–181° (decomp.).

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