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Flavoenzyme Models. I. Flavin Free-Radical Formation in the Reduced Nicotinamide–Adenine Dinucleotide–Flavin Mononucleotide System*

J. Lawrence Fox† and Gordon Tollin

ABSTRACT: The mode of production of flavin free radical was investigated in a reduced pyridine nucleotide dehydrogenase model system. Reaction kinetics were followed optically at 340, 445, 570, and 900 nm and by electron paramagnetic resonance spectrometry, using an anaerobic stopped-flow apparatus. The identity of the 570-nm absorbing species and the flavin free radical is confirmed by kinetics and a molar absorption coefficient of 8000–10,000 cm^{-1} M^{-1} is obtained. A comparison of the various biological analogs of the coenzymes was also made. It was found that flavin–adenine dinucleotide (FAD) has an enhanced rate of flavin free-radical formation as compared with flavin mononucleotide (FMN). The direct formation of flavin free radical *via* a single electron transfer from reduced nicotinamide–adenine dinucleotide (NADH) to FMN is ruled out. A mechanism which satisfactorily accounts for all of the data is presented.

Flavoenzymes often function as mediators between one-electron- and two-electron-transfer processes. Free-radical signals have been observed in a number of flavoenzymes and the flavin free radical has been invoked as the species responsible (Beinert and Palmer, 1965). Inasmuch as the experimental difficulties encountered in attempts to elucidate the mechanism of reaction of flavoenzymes are formidable, it was felt that an examination of a model system might lead to new insight into mechanisms of reaction.

The model chosen for this study was a reaction system

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containing NADH[1] and FMN. One of the primary goals was to ascertain whether flavin free radical is formed directly from the reaction of NADH and FMN, or as a result of a series of steps. The oxidation of NADH can be followed spectrophotometrically at 340 nm. Reduction of FMN can be followed at 445 nm. The flavin free radical can be followed by epr and presumably also by absorption at 570 nm. A species absorbing at 900 nm has been assigned to either free-radical polymers (Beinert, 1956) or to an oxidized-reduced flavin charge-transfer complex (Gibson *et al.*, 1962). All of these various parameters were used to follow the course of the dark anaerobic reaction between NADH and FMN.

Several reports have appeared claiming that no reaction can take place in the dark between NADH and FMN (Haas, 1937; Frisell and Mackenzie, 1959; Kosower, 1962). However, other work is contrary to this. For example, Singer and Kearney (1950) demonstrated the nonenzymic dark reduction of O_2 and cytochrome *c* by NADH, NADPH, and NMNH mediated by various flavins. The reaction was followed at 340 nm and was found to be first order with respect to both riboflavin and NADH. Cytochrome *c* (2 moles) was reduced per mole of reduced flavin oxidized. When Commoner and Lippincott (1958) performed this reaction in an epr spectrometer, they observed flavin free-radical formation.

Suelter and Metzler (1960) followed the oxidation of reduced pyridine nucleotide analogs (principally 1-propyl-1,4-dihydronicotinamide) by FMN at 380 nm. Virtually no change in rate was noted from pH 5 to 9. The reaction was found to be first order with respect to reduced pyridine nucleotide analog and FMN, and to be second order over-all. An activation energy of 4–5 kcal/mole was determined. A kinetic isotope effect of 3.16 for the 4-deuterated analog was found. This evidence was interpreted as indicating a two-electron transfer with the hydride ion exchange being the rate-limiting step. However, hydrogen atom transfer, leading directly to free radicals, would also be consistent with the data.

Isenberg *et al.* (1961) examined anaerobic mixtures of NADH and FMN under strictly dark conditions. A flavin free radical was observed and a mechanism was proposed which involved an initial complex formation, followed by one-electron transfer, a rapid pyridine nucleotide radical disproportionation reaction, and a possible rapid flavin protonation step. No real evidence was provided for this, however.

Radda and Calvin (1964) made an extensive investigation of the chemical and photochemical reductions of flavins by NADH. The dark reaction was followed at 447 nm at 37° and was found to be first order with respect to both FMN and NADH and to give a second-order over-all rate constant of 0.159 M^{-1} sec^{-1}. The activation energy was determined as 8.3 kcal/mole. No evidence for a NADH–FMN charge-transfer complex was found when the optical absorption or fluorescence changes were extrapolated back to zero time.

The reduction of flavin by addition of dihydrolipoic acid has been reported by Gascoigne and Radda (1965). The reaction was found to be first order with respect to flavin, dihydrolipoic acid, and hydroxyl ion and to show a strong pH dependence. The authors found evidence of flavin free-radical formation. They concluded that the mechanism was, in analogy to what had been proposed for the NADH and FMN system, a two-electron reduction followed by a disproportionation step to form flavin free radical. No direct evidence for this was given, however.

Materials and Methods

NADH and FMN were obtained from Calbiochem. The NADH was found to be 94–96% pure by spectral analysis; the FMN was found to be 97.1% pure. FAD was 90% pure and NADPH was chromatographically pure as supplied by Calbiochem. Potassium monohydrogen phosphate, potassium dihydrogen phosphate, and potassium iodide were Mallinckrodt analytical reagent grade. D_2O (99.5% plus pure) and research grade argon were obtained from Matheson Coleman and Bell. Fremy's salt, which was used to calibrate the epr spectrometer, was obtained from Alpha Inorganics.

Phosphate buffer (0.1 M, pH 6.8) was prepared from equimolar mixtures of potassium monohydrogen and dihydrogen phosphate and conductivity water. This latter was obtained from a Heraeus quartz Bi-distiller (resistivity of water, 10^6 ohms). This ensured the absence of metal ion effects.

Kinetic experiments were conducted with the use of an anaerobic stopped-flow apparatus specially designed for this study (Fox and Tollin, 1966a). The maximum oxygen concentration possible in this system was 10^{-9} M. This is at least three orders of magnitude below the detectable levels of reduced flavin, so that no observable oxygen effect on the initial rate measurements would be expected. A Cary 14M recording spectrophotometer and a Varian V-4501 electron paramagnetic resonance spectrometer equipped with Fieldial magnetic field regulation and 100-kcycles modulation were used for spectral measurements. Temperature measurement was made using a 36-gauge chromel-alumel thermocouple which was Teflon coated and connected to a Honeywell pyrometer for readings. Most of the kinetic experiments were conducted at 27°. Typical operating conditions are given in Fox and Tollin (1966a).

The possibility of the reaction being light catalyzed by the spectrometer or during sample handling was carefully excluded by various control experiments.

[1] Abbreviations used: epr, electron paramagnetic resonance; FMN, flavin mononucleotide; $FMNH_2$, fully reduced flavin mononucleotide; FMNH·, flavin mononucleotide free radical; FAD, flavin–adenine dinucleotide; NAD^+,ni cotinamide–adenine dinucleotide; NADH, reduced nicotinamide–adenine dinucleotide; NADPH, reduced nicotinamide–adenine dinucleotide phosphate; and NMNH, reduced nicotinamide mononucleotide.

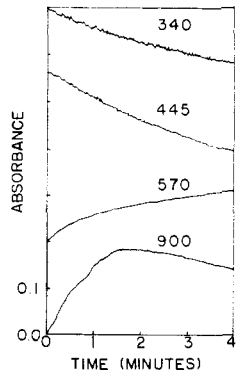

FIGURE 1: Reaction kinetics for a single sample at 340, 445, 570, and 900 nm. 2×10^{-3} M FMN, 7×10^{-3} M NADH. The kinetics at 340 and 445 nm were followed in a 0.223-mm path length cell and at 570 and 900 nm in a 1.087-cm path length cell.

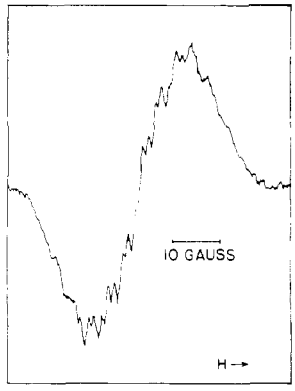

FIGURE 2: Epr hyperfine spectrum of FMN free radical at pH 6.8.

Samples were prepared in containers which were painted black and then covered with foil to totally exclude light. A series of experiments were performed wherein the light beam of the spectrophotometer was either not turned on or had been turned off for long periods of time following the initiation of the reaction. No differences in reaction rates were detected under these conditions.

Results

In order to distinguish between the two possible types of reaction mechanisms for flavin-radical formation

$$\text{FMN} + \text{NADH} \dashrightarrow \text{FMNH}\cdot \qquad \text{(a)}$$

$$\text{FMN} + \text{NADH} \dashrightarrow \dashrightarrow \dashrightarrow \text{FMNH}\cdot \qquad \text{(b)}$$

the initial rate reaction orders for flavin radical with respect to NADH and FMN were desired. These were

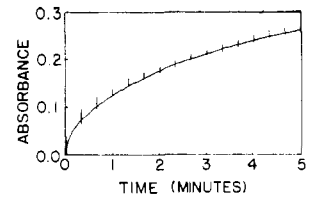

FIGURE 3: Identity of 570 nm and epr kinetic curves. The solid line represents the 570-nm kinetics. The vertical lines represent the epr kinetics after normalization at a point 5 min removed from the origin. The length of these lines is proportional to the error in the determination. 0.5×10^{-3} M FMN and NADH; 1.087-cm path length cell used for the optical measurements.

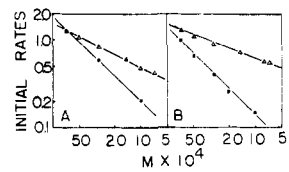

FIGURE 4: van't Hoff log–log reaction order plots for NADH and FMN at 570 nm and by epr. (A) epr, △ NADH, slope = 0.51; □ FMN, slope = 0.98; (B) 570 nm; △ NADH, slope = 0.45; □ FMN, slope = 0.99.

obtained through the use of van't Hoff log initial rate *vs.* log concentration plots (Frost and Pearson, 1961; Benson, 1960).

Figure 1 shows some typical kinetic data observed at 340, 445, 570, and 900 nm using the same sample. Initial rates were obtained from these using standard techniques. The rate curves at 340 and 445 nm are identical if correction is made at 340 nm for changes in absorption due to reduction of oxidized FMN. Using the molar absorption coefficients given by Whitby (1953) and Horecker and Kornberg (1948), a stoichiometric ratio FMN:NADH of 2:1 is obtained.

Figure 2 shows the epr spectrum obtained from a reaction mixture at pH 6.8. This is undoubtedly due to the flavin free radical (Ehrenberg, 1962). No evidence for a pyridine nucleotide radical was obtained.

Figure 3 shows the relationship between the 570 nm and epr kinetic data when they are normalized at a point 5 min from their origin. The two sets of data are seen to be superimposible within the various errors of superposition. This is a kinetic, rather than a stoichiometric (Gibson *et al.*, 1962), demonstration of the identity of the 570-nm species and the flavin free radical.

The kinetic reaction order plots for the flavin free radical with respect to both FMN and NADH are shown in Figure 4. In Table I, the reaction orders for all of the species are tabulated.

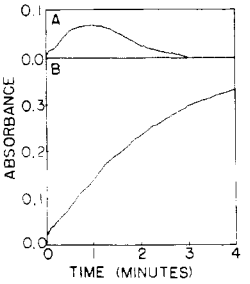

FIGURE 5: Kinetics (900 nm) at different NADH and FMN concentrations. (A) 1×10^{-3} M FMN, 7×10^{-3} M NADH. (B) 7×10^{-3} M FMN, 1×10^{-3} M NADH.

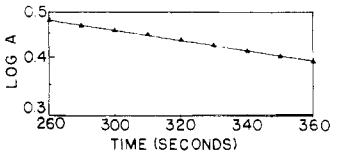

FIGURE 6: First-order plot of 900-nm absorption decay. 4×10^{-3} M FMN, 7×10^{-3} M NADH.

TABLE I: Initial Rate Reaction Orders for Flavin Free-Radical Formation.

Component Varied	Component Observed				
	340 nm	445 nm	570 nm	Epr	900 nm
FMN	1.00	1.00	1.01	0.98	0.82
NADH	1.00	1.00	0.45	0.51	0.84

Two conditions prevail at 900 nm at differing concentrations of NADH and FMN, as is seen in Figure 5. If the NADH concentration is greater than the FMN concentration, the absorption approaches a maximum and then falls off. The decay portion of this curve nicely fits a first-order plot as is shown in Figure 6. If the FMN concentration is greater than the NADH concentration, then a maximum is asymptotically approached. As the concentration of the reactants approaches each other, the kinetic curves approach an intermediate shape. The overshoot effect is apparently due to limiting quantities of FMN and to competing reactions for the 900-nm species.

When the NADH concentration is nearly equivalent to the FMN concentration, the kinetic curves at 570 and 900 nm are essentially identical, as is seen in Figure 7A. However, at very low NADH concentration, a lag at 570 nm relative to the appearance of the 900-nm species is seen (Figure 7B). This is evidence that the 900-nm species preceeds the 570-nm species

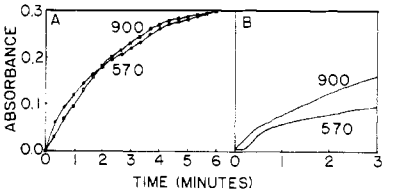

FIGURE 7: Kinetic (570 *vs.* 900 nm) behavior at different FMN and NADH concentrations. (A) 7×10^{-3} M FMN, 3×10^{-3} M NADH. Curve is normalized to 570-nm absorption kinetics at point 6 min from origin. (B) 7×10^{-3} M FMN, 7×10^{-4} M NADH; curves are not normalized, but are actual traces.

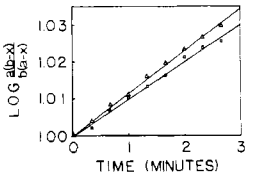

FIGURE 8: Over-all second-order plot (340 and 445 nm). □ 340 nm, $k_2 = 0.19$, △ 445, $k_2 = 0.23$. Graph represents first 15% of total theoretical reaction. 7×10^{-3} M FMN, 5×10^{-3} M NADH.

in time and is consistent with a sequential relationship between the two compounds.

The 340 and 445 absorption kinetics followed an over-all second-order plot (Figure 8). The slope gave a value of $k_2 = 0.23$ at 445 nm and 0.19 at 340 nm at 27° when determined from a plot of $1/(a - b)$ ln $b(a - x)/a(b - x) = kt$, where a and b are the initial reactant concentrations and x is the amount which has reacted. This is in good agreement with the data of Radda and Calvin (1964). Attempts to plot the 570,900 nm, and epr data as first-, three-halves-, and second-order plots were unsuccessful.

A quantitative comparison of the 570 nm and epr data for the same sample over a given period of time was made. Calibration of flavin free-radical spin concentration was made by the integration of absorption curves using Fremy's salt as a standard (reported to be an acceptable technique by Yamazaki *et al.*, 1960). Correlation of the 570-nm absorption change with the corresponding epr spin concentration gave 570-nm molar absorption coefficients ranging from 8000 to 10,000 cm^{-1} M^{-1}.

Measurements of the relative rates of reaction show that the velocity of formation of the species absorbing at 900 nm (using a molar absorption coefficient of 680 cm^{-1} M^{-1}; *cf.* Gibson *et al.*, 1962) is approximately equal to the rate of disappearance of FMN (see Table II). This indicates that very little free $FMNH_2$ is formed, in agreement with values calculated from the equilibrium constant reported by Gibson *et al.* (1962) and the rate constant for the appearance of

TABLE II: Initial Reaction Rates Measured at Various Wavelengths.[a]

Wavelength (nm)	445	570	900
Initial OD change/min	0.132 ± 0.002	0.125 ± 0.003	0.430 ± 0.005
Path length of cell (cm)	0.223	1.087	1.087
Molar absorption coefficient (cm^{-1} M^{-1})	12,500	10,000	680
Velocity (moles/min) × 10^{-4}	4.74	0.166	5.82

[a] 7×10^{-3} M FMN; 7×10^{-3} M NADH.

TABLE III: The Effect of D_2O and KI on Reaction Velocities.

System	Initial Rates (OD/min)		
	445 nm	570 nm	900 nm
7×10^{-3} M FMN; 2×10^{-3} M NADH	0.038 ± 0.001	0.34 ± 0.005	0.250 ± 0.006
7×10^{-3} M FMN; 2×10^{-3} M NADH + 5×10^{-2} M KI	0.037 ± 0.001	0.300 ± 0.004	0.142 ± 0.002
7×10^{-3} M FMN; 3×10^{-3} M NADH	0.055 ± 0.001	0.400 ± 0.003	0.370 ± 0.005
7×10^{-3} M FMN; 3×10^{-3} M NADH in D_2O	0.056 ± 0.001	0.380 ± 0.010	0.340 ± 0.008

charge-transfer complex as reported by Swinehart (1966). The rate of appearance at 570 nm was found to be only 4% of the rate of FMN disappearance using a molar absorption coefficient at 570 nm of 10,000 cm^{-1} M^{-1}. This value is in accord with the equilibrium radical concentration calculated with the equilibrium constant determined by Gibson *et al.* (1962) for oxidized and reduced flavin mixtures. The above relations argue for a rate-limiting initial step followed by rapid equilibration to form charge-transfer complex and flavin free radical.

Using a variation of temperature, an Arrhenius activation energy of 8.3 ± 0.5 kcal/mole was obtained from the 445-nm data. The plot of the respective rate constant *vs.* the reciprocal of temperature is given in Figure 9. This agrees closely with the value of 8.5 ± 0.5 kcal/mole reported by Radda and Calvin (1964).

An experiment was performed in D_2O to determine whether a proton exchange with the solvent might be involved in the rate-limiting process. No effect due to D_2O was observed on the rate of reaction at 445, 570, and 900 nm, as is seen in Table III.

When potassium iodide was added in concentrations considerably greater than those needed to efficiently quench the excited triplet state of flavin (Posthuma and Berends, 1966), no changes were noted in the rates at 445 and 570 nm (Table III). A small effect was noted at 900 nm. The lack of an observable effect under conditions where the triplet state is highly quenched argues against the proposals of a triplet state as an intermediate in the dark NADH and FMN reaction (Steele, 1963; McGlynn *et al.*, 1964).

The results obtained using the biological analogs

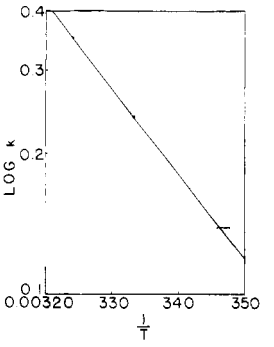

FIGURE 9: Arrhenius activation energy plot from 445-nm rate constants. 7×10^{-3} M FMN, 3×10^{-3} M NADH.

of FMN and NADH are given in Table IV. No correction was made for the differences in the FMN and FAD molar absorption coefficients at 445 nm. The added phosphate group in NADPH apparently slows all of the reactions by a like amount. The addition of an adenosine group to FMN leads to a decrease in the rate of reduction of nearly 35%. The rate of formation of free radical, however, increased, for the relative amount of reduction occurring, by nearly 100%. The charge-transfer complex rate decreased markedly in the FAD system.

Discussion

Mechanism. It was proposed above that a distinction between the two types of mechanisms

TABLE IV: Reaction Rates of Various Coenzyme Species.

System	Initial Rates (OD/min) 445 nm	570 nm	900 nm	Epr (arbitrary units)
7×10^{-3} M FMN, 2×10^{-3} M NADH	0.038 ± 0.001	0.340 ± 0.010	0.250 ± 0.004	
7×10^{-3} M FMN, 2×10^{-3} M NADPH	0.030 ± 0.001	0.300 ± 0.008	0.210 ± 0.004	
2×10^{-3} M FMN, 7×10^{-3} M NADH	0.050 ± 0.001	0.100 ± 0.003	0.235 ± 0.005	1.50 ± 0.10
2×10^{-3} M FAD, 7×10^{-3} M NADH	0.032 ± 0.001	0.220 ± 0.002	0.053 ± 0.004	2.80 ± 0.04

$$\text{FMN} + \text{NADH} \longrightarrow \text{FMNH}\cdot \qquad \text{(a)}$$

$$\text{FMN} + \text{NADH} \longrightarrow \longrightarrow \longrightarrow \text{FMNH}\cdot \qquad \text{(b)}$$

could be made by the use of kinetic reaction orders. If the flavin free radical were produced by mechanism a, then its appearance should be first order with respect to NADH and FMN. If the second mechanism is operative, the rate of appearance of flavin free radical could also be first order with respect to NADH and FMN as a limiting case, but it could also be of different order than first order with respect to each of these two moieties.

Experimentally, the reaction orders were found to be first order with respect to FMN and one-half order with respect to NADH, whether determined by epr spectroscopy or optically at 570 nm. The identity of the reaction kinetics and reaction orders by the two methods confirms the free-radical assignment to the 570-nm absorption band. The initial rate of radical appearance, based on published and calculated 570-nm molar absorption coefficients, did not exceed 4% of the rate of flavin reduction or of NADH oxidation. It is thus to be concluded that flavin free-radical formation is not an immediate consequence of the reaction between NADH and FMN, *i.e.*, that mechanism a is operative.

The only other species observed in this system was characterized by a broad optical absorption with a maximum around 900 nm. As this species is also present in oxidized and reduced flavin mixtures, it must be another flavin species. The likely candidates are flavin free-radical dimer (Beinert, 1956), or higher order species, or $FMNH_2$–FMN charge-transfer complex (Gibson *et al.*, 1962). If the first step of the reaction is assumed to be a two-electron-reduction step, then three modes of formation of the 900-nm species can be envisioned, assuming no other species are present (none were seen). In two of these, the 900-nm species is formed either in competition with the free radical, or from it.

$$H^+ + \text{NADH} + \text{FMN} \longrightarrow FMNH_2 + NAD^+$$

$$900 \xleftarrow{1} FMNH_2 \xrightarrow{1,2} \text{FMNH}\cdot \xrightarrow{2} 900$$

Using a steady-state assumption for the concentration of $FMNH_2$, the reaction orders with respect to both FMN and NADH for the 900- and 570-nm species are the same form for reaction pathway 1. Experimentally, however, they were first and one-half order, respectively, for NADH. In addition, the observed lag at 570 relative to 900 nm argues against pathway 2.

The third possibility is consistent with the kinetic data and is also consistent with what is known for the oxidized and reduced flavin mixtures

$$H^+ + \text{NADH} + \text{FMN} \longrightarrow FMNH_2 + \text{NADH}$$

$$FMNH_2 + \text{FMN} \xrightarrow{3} (FMNH_2\text{–FMN})\,(900\text{-nm species})$$

$$(FMNH_2\text{–FMN}) \longrightarrow 2\text{FMNH}\cdot$$

The mathematical expressions for the rates of free-radical and 900-nm species formation as a function of NADH and FMN concentrations are not soluble in convenient form. It is quite unlikely that strictly first-order dependencies would be expected, however. Due to the great disparity in the reaction rates between the first reaction step and subsequent steps, representing a range of at least ten orders of magnitude, a computer simulation of the proposed mechanism was precluded. Neither digital nor analog techniques could be used here.

Several of the other experimental observations corroborate mechanism 3 as proposed above. These are: the identity of initial rates of appearance at 900 nm and of disappearance at 445 nm; the rise and first-order decay at 900 nm; and the apparent lag at 570-nm relative to the 900-nm kinetic curve. All of these argue for the 900-nm species being a kinetic intermediate in the formation of flavin radical. The most logical structure for this species from what is known of oxidized and reduced flavin mixtures is a reduced–oxidized flavin charge-transfer complex.

The requirement of FMN:NADH (2:1) supports this type of mechanism, though not conclusively. The results also suggest that the initial two-electron reduction is the rate-limiting step. The absence of an effect when the reaction was performed in D_2O argues against

a solvent proton exchange being involved in the rate-limiting step. When coupled with the kinetic isotope effect of 3.16 seen for NADH analogs by Suelter and Metzler (1960), it can be concluded that the initial step must involve direct hydrogen transfer concomitant with a two-electron transfer. Reduction of oxidized flavin thus occurs *via* a hydride ion transfer, or an indistinguishable equivalent.

Molar Absorption Coefficient at 570 nm. The molar absorption coefficients previously calculated at 570 nm fall into two distinct categories dependent upon their origin. The strictly spectroscopic studies, *e.g.*, Holmström (1964), have given values from 700 to 3,050 cm^{-1} M^{-1}; Swinehart (1966) has obtained reasonable results for some of his calculations only by using a value of 500 cm^{-1} M^{-1}. On the other hand, the present work and the work of Gibson *et al.* (1962), where correlations were made between 570 nm absorption spectra and epr spectra following calibration with some standard (Fremy's salt used here; Gibson used diphenylpicrylhydrazyl), gave molar absorption coefficients in the range of 8000–13,600 cm^{-1} M^{-1}. A value of 5440 cm^{-1} M^{-1} has been assigned using a flavoprotein isolated as a free radical from *Azotobacter* (J. W. Hinkson, personal communication, 1966). The reported spectroscopic determinations depend solely upon extrapolative techniques with no absolute concentration reference point. Complex formation could conceivably lower the epr-detectable radical concentration, but not the optical absorption intensity, leading to high values. However, it is difficult to believe that this could be large enough to completely account for the discrepancy. Errors are present in both techniques, so that the true molar absorption coefficient may lie somewhere between these two extremes, as suggested by the unpublished results of J. W. Hinkson.

Triplet Mechanism. It has been suggested by Steele (1963) and McGlynn *et al.* (1964) that the dark reaction between NADH and flavodehydrogenases, or FMN here, may pass through an excited triplet state in analogy to the finding for the photochemical reactions (Holmström and Oster, 1961). Using potassium iodide at a concentration an order of magnitude greater than that shown to quench 40% of the flavin phosphorescence (Posthuma and Berends, 1966), no effect on the rate of reaction between FMN and NADH was observed. NADH is not believed to complex with FMN at room temperature to any extent that is measurable (Radda and Calvin, 1964). Potassium iodide is known to effectively interact with the triplet state (Shiga and Piette, 1965; Tegner and Holmström, 1966; Posthuma and Berends, 1966). The rate constant for the interaction of potassium iodide and the triplet state of flavin has been determined in phosphate buffer, pH 6.5, to be 0.7×10^{10} M^{-1} sec^{-1} by Tegner and Holmström (1966). It follows that the flavin triplet state is not involved as an intermediate in the dark reaction. The finding of Radda and Calvin (1964) that serotonin inhibits the photoreduction of FMN by NADH, but has no effect on the dark reaction, also favors the lack of analogy between the triplet-mediated photoreduction and the dark reaction. The effect of potassium iodide on the 900-nm rate is probably due to the formation of a complex with FMN which structurally, and/or electronically, interferes with charge-transfer complex formation.

Analogs. It was noted that NADPH functions at similarly reduced rates in all determinations as compared to NADH. This is consistent with the first step being rate limiting and steric or electrostatic repulsion effects from the added phosphate group slowing the rate of reaction for the initial step.

FAD functioned with a decreased rate of reduction and of charge-transfer complex formation. The steric and/or electrostatic interaction between the adenosine and isoalloxazine moieties could account for this. A relative increase in the rate of free-radical production is quite evident. The adenine apparently plays a role in stabilizing the flavin free radical. A less marked, but analogous, effect was seen in tertiary systems containing adenine derivatives (Fox and Tollin, 1966b). The increased stability of the flavin free radical in FAD may be the evolutionary function of the addition of the adenine nucleotide to FMN.

Flavoenzyme Mechanisms. Extrapolation of the present mechanism of flavin free-radical formation involving a flavin–flavin disproportionation reaction to biological systems is tenuous. Current structural information is not sufficient to locate the relative positions of flavin prosthetic groups to one another, but Åkeson *et al.* (1963) found no kinetic indications that the two FMN's per molecule of old yellow enzyme interacted with each other. Miller and Massey (1965) reported that they were unable to detect any kinetic differences between the FMN and FAD moieties in dihydroorotic acid dehydrogenase, indicating that they were reduced simultaneously. Lipoyl dehydrogenase (Massey, 1963) and NADH cytochrome b_5 reductase (Strittmatter, 1965), on the other hand are believed to possess only one flavin per molecule, so flavin–flavin interactions are quite unlikely in these systems. However, most flavoenzymes appear to possess two flavins per molecule. Most of the nonmetal containing flavoenzymes appear to function as two-electron donors (Mahler and Glenn, 1956), though. The mechanism would be envisioned as two-electron reduction followed by two-electron reoxidation in these systems. This will be discussed more fully in paper II (Fox and Tollin, 1966b).

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