Reduction, Oxidation, and Addition Reactions between Free Radicals and Flavins†

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ABSTRACT: Flavins and reduced flavins were reacted with a variety of free radicals produced in dilute aqueous solution at pH 7 ± 0.1 by radiation chemical methods. The radical $\cdot CH_2C(CH_3)_2OH$ and the aliphatic β, γ, and δ radicals of ethanol, 2-propanol, and 1-butanol added to the radical form of flavin adenine dinucleotide (FAD) (FH·) to yield products that could not be reoxidized to flavin by oxygen. The first radical also added to FAD but with a much lower efficiency. In contrast, the α-carbon radicals $\cdot CH(OH)CH_2OH$, $CH_3\dot{C}HOH$, and $(CH_3)_2\dot{C}OH$ appeared to undergo two reactions:

$$F + RR'\dot{C}OH \rightarrow FH\cdot + RR'C{=}O$$

$$FH\cdot + RR'\dot{C}OH \rightarrow FH^- + RR'C{=}O + H^+$$

The formate radical anion $\cdot CO_2^-$ reacted similarly, producing stoichiometric two-electron reduction of riboflavin and lumiflavin as well as FAD. While e_{aq}^- also seemed capable of this, it was found to react irreversibly with $FADH_2$, which makes it a poor reagent for producing the dihydroflavin. The dihydro form of FAD was reoxidized to FAD by the species RS· and $\cdot Br_2^-$. In contrast to FAD, the alloxazine lumichrome underwent only one-electron reduction and oxidation by $\cdot CO_2^-$ and $\cdot Br_2^-$, respectively.

Extensive electrochemical studies (Michaelis et al., 1936; Lowe & Clark, 1956; Draper & Ingraham, 1968) have demonstrated that flavins exist in oxidized (F),[1] radical (FH·), and two-electron reduced (FH_2) forms, which are interconvertible through reactions 1 and 2.

$$F + H^+ + e^- = FH\cdot \qquad E_1^{\circ\prime} = -0.23\ V \qquad (1)$$

$$pK \text{ of } FH\cdot \simeq 8.5$$

$$FH\cdot + e^- = FH^- \qquad E_2^{\circ\prime} = -0.17\ V \qquad (2)$$

$$pK \text{ of } FH_2 \simeq 6.5$$

The one-electron reduction of riboflavin by formate radical anions ($\cdot CO_2^-$) was reported by Land & Swallow in 1969:

$$F + \cdot CO_2^- + (H^+) \rightarrow FH\cdot + CO_2 \qquad (3)$$

Although $E^{\circ\prime}$ for $\cdot CO_2^-$ is ≤ -0.80 V (Anderson, 1976) and reaction 4 is clearly possible, systematic studies of *two-electron*

$$FH\cdot + \cdot CO_2^- \rightarrow FH^- + CO_2 \qquad (4)$$

reductions of flavins by $\cdot CO_2^-$ and other radicals of low $E^{\circ\prime}$ do not appear to have been made. Also, investigations of radical oxidations of FH_2 and FH· seem to be lacking. Research on such reactions is important for two reasons. In the first place, certain two-electron transfer reactions between flavins and organic molecules may occur through two one-electron steps, with a free-radical intermediate being involved (Williams & Bruice, 1976; Williams et al., 1977). Second, the advantages of pulsed techniques for free-radical production and the utility of certain radicals for probing redox states of short-lived species (Bielski & Gebicki, 1977; Adams & Wardman, 1977) have led to a current increase in their application to flavin systems (Goldberg et al., 1981; Anderson, 1976, 1981a,b).

This paper deals with the reactions of riboflavin (RF), flavin adenine dinucleotide (FAD), lumiflavin (LF), and lumichrome (LC) with $\cdot CO_2^-$ and a variety of aliphatic radicals (RH·). It also describes oxidations of the reduced flavins with $\cdot Br_2^-$ and thiyl (RS·) radicals. All radicals were produced by the absorption of ^{60}Co γ rays in situ in dilute buffered (pH ~7) aqueous solutions of the flavins. For these conditions, the primary action of the γ rays is described quantitatively by eq 5 (Draganić, I. G., & Draganić, 1971):

$$4.2H_2O \xrightarrow{^{60}Co\ \gamma} 2.7e_{aq}^- + 0.6H\cdot + 2.8\dot{O}H + 2.7H^+(aq) + 0.5H_2 + 0.7H_2O_2 \qquad (5)$$

Here, the coefficients are the yields of species in molecules per 100 eV of radiation energy absorbed.

In nitrous oxide saturated solutions, the solvated electron is converted to $\dot{O}H$ on a time scale of about 5 ns via reaction 6. The radicals mentioned above are then produced through

$$e_{aq}^- + N_2O + H_2O \rightarrow N_2 + OH^- + \dot{O}H \qquad (6)$$

the following reactions by incorporating the appropriate solutes at concentrations sufficiently large to suppress direct reactions of $\dot{O}H$ with the flavins or FH_2:

$$\dot{O}H \text{ (or H·)} + HCO_2^- \rightarrow H_2O \text{ (or } H_2) + \cdot CO_2^- \qquad (7)$$

$$\dot{O}H \text{ (or H·)} + RH_2 \rightarrow H_2O \text{ (or } H_2) + RH\cdot \qquad (8)$$

$$\dot{O}H + 2Br^- \rightarrow OH^- + \cdot Br_2^- \qquad (9)$$

$$\dot{O}H \text{ (or H·)} + RSH \rightarrow H_2O \text{ (or } H_2) + RS\cdot \qquad (10)$$

The quantitative nature of eq 5 means that the number of equivalents of a radical created per unit radiation dose can be calculated. This feature made it possible to determine the uptake of reducing (or oxidizing) equivalents per mole of flavin (F or FH_2) when it was *titrated* with each radical species. Conversion of F to FH_2 or vice versa was followed by spectrophotometry. A preliminary report of some of this work was given elsewhere (Elliot et al., 1982).

Experimental Procedures

Materials

The flavins (RF, FAD, LF, and LC) were obtained from Sigma Chemical Co. The water for preparing solutions was

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[1] Abbreviations: F, oxidized flavin; FH·, flavin radical; FH_2, reduced flavin; RF, riboflavin; FAD, flavin adenine dinucleotide; LF, lumiflavin; LC, lumichrome; RH·, aliphatic free radical; RS·, thiyl radical.

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redistilled first from alkaline permanganate and then from acid dichromate.

Methods

All experiments were performed at room temperature (24 ± 1 °C) with solutions containing 5 mM phosphate buffer (pH 7.0).

Irradiations were performed in an AECL ^{60}Co Gammacell with steady dose rates of 15 or 1.4 Gy min^{-1} (i.e., 15 or 1.4 J kg^{-1} min^{-1}). The exact dose rates were checked periodically with the Fricke dosimeter. All solutions were irradiated in 1-cm path-length Suprasil optical cells, which either were fitted with air-tight stopcocks or could be sealed off with a glass-blowing torch after deaeration. Both techniques gave the same results. Before the cells were closed off, the air was removed by bubbling with N_2O or Ar, both of which were passed through a Ridox column (Fisher Scientific Co.). The spectra of solutions were recorded on a Cary 15 or Cary 219 UV–visible spectrometer.

Calculation of the Moles of Radicals Produced. As stated in the introduction, the yields per 100 eV in eq 5 refer to dilute solutions (~10^{-3} M) of reactive solutes. They must be adjusted for scavenging of ·OH, ·H, and e_{aq}^- in radiation spurs (Draganic, I. G., & Draganic, 1971) when concentrations appreciably exceed 10^{-3} M. Procedures for doing this from published data have been described (Draganic, Z. D., & Draganic, 1971, 1973). For typical concentrations of 2, 100, and 300 mM formate, the yields of free radicals, G_r, were 6.0, 7.3, and 7.6 molecules/100 eV, respectively, and those of H_2O_2, i.e., $G^M_{H_2O_2}$, were 0.58, 0.36, and 0.29 molecules/100 eV, respectively.

As shown in eq 5, hydrogen peroxide is always produced concomitantly with free radicals. A number of experiments were carried out with reduced FAD and LF to check the half-life of the thermal reaction between FH_2 and H_2O_2, viz.:

$$FH_2 + H_2O_2 \rightarrow F + 2H_2O \qquad (11)$$

Small (0.05–0.20 cm^3) quantities of deaerated 1×10^{-4} M H_2O_2 solutions were introduced under anaerobic conditions into cuvettes containing 20–50 μM FH_2. The stoichiometry and rate of reaction (eq 11) were followed spectrophotometrically, the cuvette being removed from the light beam except when measurements were taken. The kinetics were not examined in detail, but half-lives were in the range 4–10 min for conditions similar to those which would exist after a typical radiation dose ([FH_2] ~10–20 μM; [H_2O_2] ~1–3 μM). This meant that reaction 11 would take place on a time scale similar to the periods used for radiolysis (~2 min). Therefore, in the radiation chemical titrations, the cuvettes were allowed to stand for about 0.5 h to ensure that peroxide had reacted. Under these circumstances, if the total time of irradiation (t) is in minutes and the dose rate (D) is in grays per minute, the net moles of equivalents of reactive radicals produced per liter of solution is given by eq 12:

$$e_{red,ox} = [D\ (\text{J kg}^{-1}\ \text{min}^{-1}) \times (1.00\ \text{L kg}^{-1}) \times (G_r \pm 2G^M_{H_2O_2})(\text{molecules}/100\ \text{eV}) \times t\ (\text{min})]/[(1.602 \times 10^{-19}\ \text{J eV}^{-1}) \times (6.02 \times 10^{23}\ \text{molecules mol}^{-1}) \times 100]$$

$$e_{red,ox} = 1.04 \times 10^{-7} Dt(G_r \pm 2G^M_{H_2O_2}) \qquad (12)$$

The plus and minus signs apply for oxidizing and reducing species, respectively.

Results

Reductions with ·CO_2^-. These radicals were produced through reaction 7 in N_2O-saturated solutions of formate at pH 6.9. Spectral changes resulting from the reduction of 25 μM riboflavin in 0.3 M sodium formate have been presented in Figure 1a. The initial spectrum of F, characterized by maxima at 266, 372, and 444 nm, was suppressed by each successive dose of radiation until finally after 6 min of total exposure there was no further change. As shown by the plots of ΔA, the change in absorbance, vs. $e_{red}/[F]_0$ in Figure 1b, the end point occurred at 2.1 reducing equiv per mol of riboflavin. This is consistent with the overall reaction (eq 13),

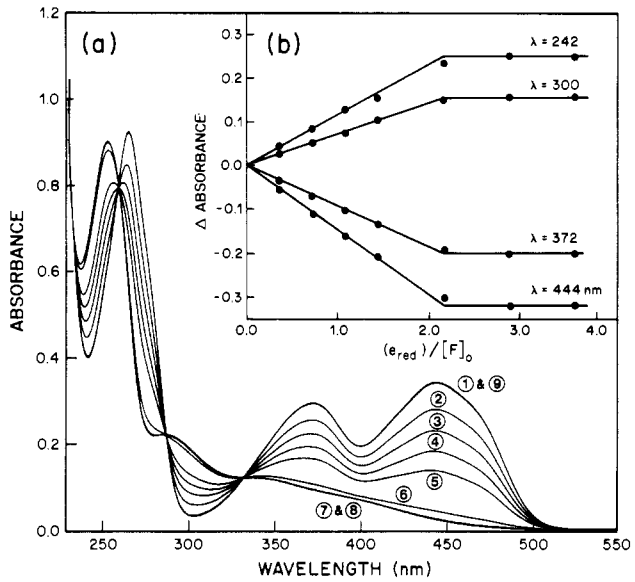


FIGURE 1: (a) Spectra obtained from an N_2O-saturated solution of 25 μM riboflavin in 0.3 M formate and 5 mM phosphate buffer before irradiation (1) and after 1 (2), 2 (3), 3 (4), 4 (5), 6 (6), 9 (7), and 10 min (8) of γ irradiation at 14 Gy min^{-1}. The spectrum after aeration of the irradiated solution is shown by (9). (b) Change in absorbance vs. number of equivalents of reducing species per mole of F initially present.

$$2{\cdot}CO_2^- + F + H^+ \rightarrow 2CO_2 + FH^- \qquad (13)$$

and the constancy of the spectrum beyond the equivalence point shows that ·CO_2^- does not react with FH_2 at a significant rate. The absence of other products was confirmed by the quantitative reoxidation to riboflavin (curve 9 in Figure 1a) on admission of air. Quantitatively reversible reductions with ·CO_2^- were also effected with FAD, LF, and LC at pH 7. For the former two, the reductions were overall two-electron processes, in accord with eq 13, but for LC, a one-electron equivalent process was observed. The difference between FAD and LC has been illustrated in Figure 2.

The magnitude of η, the moles of reducing equivalents taken up per mole of flavin at the end point, was found to be independent of formate concentration over the range 2 mM–0.3 M and of dose rate in the range 1.4–14 Gy min^{-1}. The average value of η for FAD is given in Table I. For LF, it was 2.09 and for LC, 1.1.

The effect of peroxide oxidation of FH_2 has been allowed for in eq 12. Evidence for its occurrence in 2 mM and 0.3 M formate solutions containing FAD has been presented in panels a and b of Figure 3, respectively. In each case, spectra were taken immediately after a 1-min radiolysis and then 0.5 h thereafter. The time course of a postirradiation reaction has been presented in Figure 3c. It has a half-life of about 6 min as expected for reaction 11 (see Methods).

Examples of hydrogen peroxide concentrations calculated from such changes in absorbance and by assuming the stoichiometry of reaction 11 were 0.8 and 2.6 μM for 1- and 4-min irradiations of 0.002 M formate solutions and 1.1 μM for a

Table I: Stoichiometry and Reversibility of Radical Reactions with FAD at pH 7.0

radical source	radical types	fractional yields	η	% return to FAD on admission of air
formate				
N_2O saturated	$\cdot CO_2^-$	1.00	2.1	100
Ar saturated	$\cdot CO_2^-$			
	e_{aq}^-		2.1	variable[a]
alcohols[b] (N_2O saturated)				
ethylene glycol	$\cdot CH(OH)CH_2OH$[c]	1.00	3.51[c]	100
ethanol	$CH_3\dot{C}HOH$	0.84	2.17	95
	$\cdot CH_2CH_2OH$	0.16		
2-propanol	$(CH_3)_2\dot{C}OH$	0.85	2.25	80
	$\cdot CH_2CH(CH_3)OH$	0.15		
1-butanol	n-$C_3H_7\dot{C}HOH$	0.41	2.04	0
	β, γ, and δ radicals	0.59		
2-methyl-2-propanol	$\cdot CH_2C(CH_3)_2OH$	1.0	5.0	0
2-methyl-2-propanol (Ar saturated)	$\cdot CH_2C(CH_3)_2OH$		3.7	0

[a] Depends on how long the system was irradiated beyond the equivalence point, due to reaction of e_{aq}^- and FH_2. [b] Radical types and fractional abundances for these radicals are from Asmus et al. (1973). [c] This radical produced only 60% reduction (i.e., $[FH^-]/[F] = 2.0$) when spectral changes ceased at a 14 Gy min^{-1} dose rate for a 20 μM solution. Also, the percentage reduction depended on the dose rate and initial concentration of flavin (see Discussion).

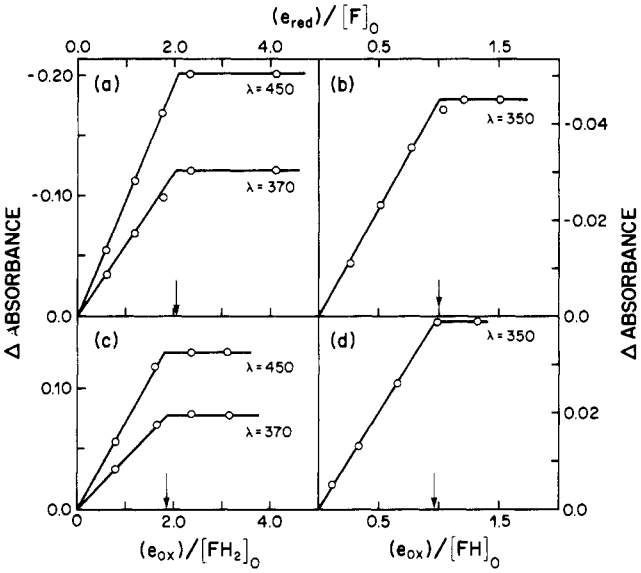


FIGURE 2: Change in absorbance vs. number of equivalents of reducing or oxidizing species reacted per mole of flavin present initially for (a) FAD + $\cdot CO_2^-$, (b) LC + $\cdot CO_2^-$, (c) reduced FAD + $\cdot Br_2^-$, and (d) reduced LC + $\cdot Br_2^-$.

4-min irradiation of a 0.3 M formate solution. These results may be compared with concentrations of 0.9, 3.8, and 1.9 μM calculated from $G^M_{H_2O_2}$ (see Methods). The tendency for the peroxide yield to fall below the calculated value at longer times was real. In fact, beyond the end point, there was no indication of any postirradiation oxidation at all (bottom line in Figure 3b). This feature can be attributed to reaction 14, which

$$H_2O_2 + \cdot CO_2^- = \cdot OH + OH^- + CO_2 \qquad (14)$$

should occur as soon as the oxidized flavin concentration has been depleted. The $\cdot CO_2^-$ lost in eq 14 would be replaced via eq 7, and there would be no net change in $[FH_2]$.

The pK of dihydroriboflavin is 6.3 so that at pH 7 it exists primarily in the anion form FH^-. The spectrum taken from Figure 1a is compared with that of dihydrotetraacetylriboflavin anion in Figure 4a. The resemblance is clear. The spectrum of the FH^- form of FAD determined after $\cdot CO_2^-$ reduction at pH 7.5 (pK = 6.7) was similar to that of riboflavin in Figure 4a. Thus, all of our observations are consistent with quantitative conversion of these flavins to the dihydro forms by $\cdot CO_2^-$.

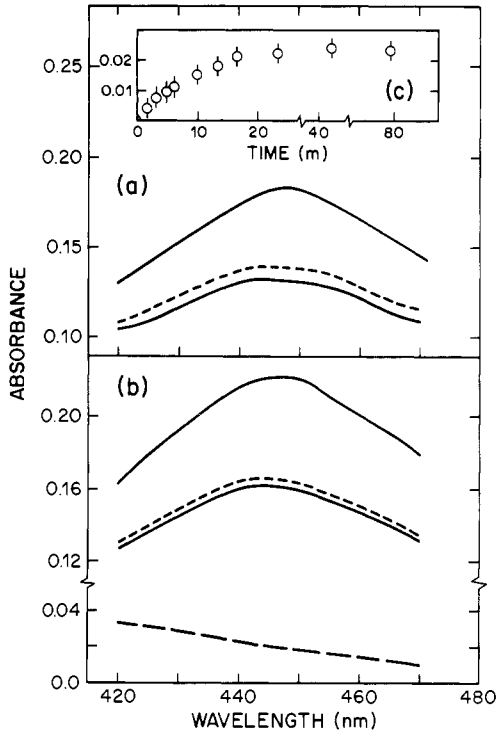


FIGURE 3: Spectra obtained from N_2O-saturated FAD solutions in sodium formate at pH 7 before (upper solid lines) and after 1-min irradiations (lower solid lines) and after 0.5 h standing to allow reaction of $FH_2 + H_2O_2$ (---). (a) 16 μM FAD in 2 mM $NaHCO_2$; (b) 19.5 μM FAD in 0.3 M $NaHCO_2$; note that after the equivalence point, the change in absorbance on standing was within the thickness of the line (--). (c) Plot of the change in absorbance at 450 nm vs. the time of standing after a 4-min γ radiolysis of 16 μM FAD in 2 mM $NaHCO_2$.

Oxidized lumichrome exists in the alloxazine form (see Discussion). As can be seen in Figure 4a,b, its spectrum is radically different from those of the flavins such as RF, which are isoalloxazines. The final spectrum observed when the one-electron reduction of LC was complete is given in Figure 4b.

Reactions with Alcohol Radicals. Free radicals were produced from a variety of alcohols by using reaction 8 in N_2O-saturated solutions. Spectra of 20.0 μM FAD solutions containing 0.02–0.2 M concentrations of these alcohols at pH 7 were taken after successive 1-min irradiations until no further change was observed. Following this, air was admitted to

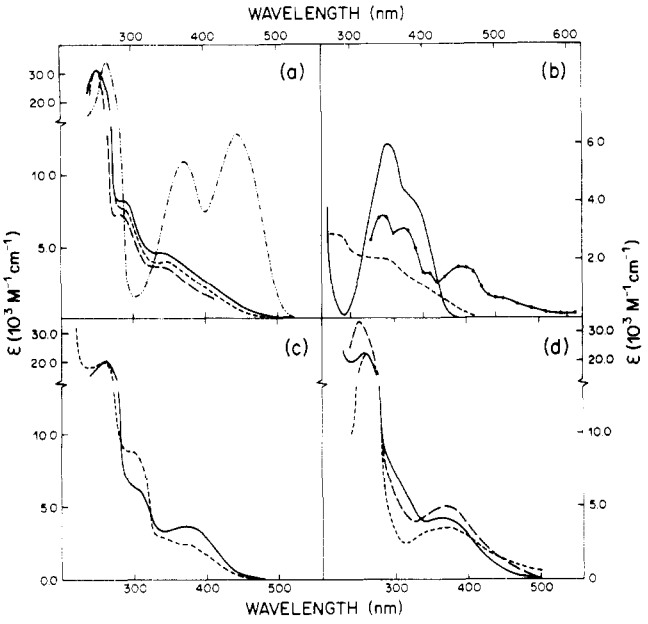


FIGURE 4: Spectra of flavins and products of flavin + radical reactions at pH 6.9: (a) (····) RF; (—) RF reduced with $\cdot CO_2^-$; (- - -) FMN anion (Ghisla et al., 1974); (– –) tetraacetyl RF anion (Dudley et al., 1964). (b) (—) LC; (●) LC radical from pulse radiolysis (A. J. Elliot et al., unpublished results); (- - -) LC reduced with $\cdot CO_2^-$. (c) Nonoxidizable products from reaction of FAD with $\cdot CH_2C(CH_3)_2OH$ radicals (—) and with a mixture of these radicals and e_{aq}^- (- - -). (d) Nonoxidizable products from reaction of FAD with radicals formed from ethanol (- - -), 2-propanol (– –), and 1-butanol (—).

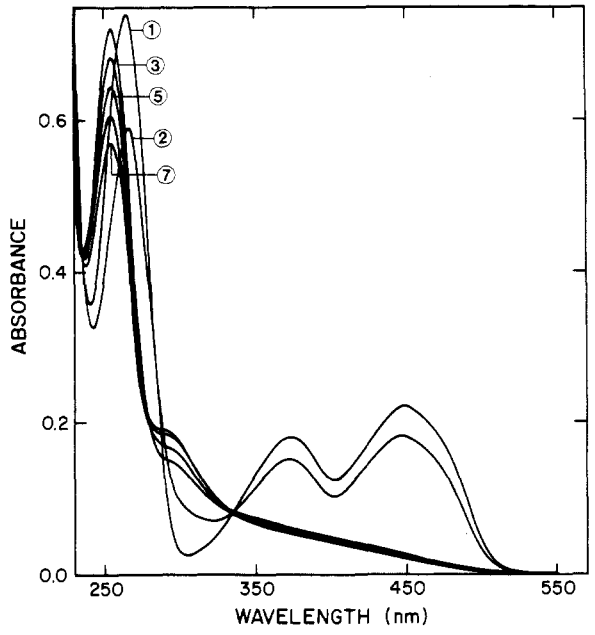


FIGURE 5: Absorption spectra of argon-saturated 19.7 μM FAD in 0.1 M $NaHCO_2$ at pH 7 before irradiation (1), after 6 (3), 15 (5), and 25 min of γ irradiation (7), and after aeration of the irradiated solution (2). Dose rate was 12.4 Gy min^{-1}.

reoxidize any dihydroflavin formed, and the spectrum was again recorded. From the change in absorbance at 450 nm, the concentration of re-formed FAD could be determined. This was used to calculate the percentage of reversible reduction, and the molarity of FH_2 formed.

Columns four and five in Table I give η, the number of radicals produced per molecule of F when spectral changes ceased, and the percentage of reversible reduction, respectively. Except for ethylene glycol, which produced a completely reversible change like $\cdot CO_2^-$, all of the alcohol radicals formed a component product (or products), which could not be reoxidized to FAD. The spectra of these products were obtained by the following two methods from the known reversible reduction: (1) The absorbance of reoxidized FAD was subtracted from the spectrum after admission of air. (2) The absorbance of dihydroflavin was subtracted from the end-point spectrum before admission of air. In all cases, the two residual spectral agreed within experimental uncertainty. Some of them are plotted in Figure 4c,d.

Reaction with the Solvated Electron. Reaction 6 does not occur in argon-saturated solutions. Consequently, in the presence of formate one obtains a mixture of e_{aq}^- and $\cdot CO_2^-$ with yields close to 3.2/100 eV for each at our solute concentrations (Draganić, Z. D., & Draganić, 1973). Spectral changes occuring in irradiated dearated 0.10 M formate solutions containing 19.7 μM FAD are shown in Figure 5. Up to the equivalent point, which occurred at $\eta = 2.1$ (Table I), they were similar to those observed with $\cdot CO_2^-$ radicals alone. However, beyond that point, there is a steady bleaching of the FH_2 absorbance. This is particularly evident for the peak at 255 nm. Also, on addition of air, the initial spectrum of F was only 80% restored. These features indicate that, unlike $\cdot CO_2^-$, e_{aq}^- reacts with FH_2 and the reaction is irreversible.

Additional experiments were performed with 21 μM FAD at pH 7.0 in 0.10 M 2-methyl-2-propanol solutions which were purged with argon. These are the types of solutions frequently used for observing reactions of e_{aq}^- per se (Bielski & Gebicki, 1977; Adams & Wardman, 1977). However, in the present system, FAD is also reactive toward the $\cdot CH_2C(CH_3)_2OH$ radicals, as has been shown above. The reactions of FAD with the mixture of e_{aq}^- and $\cdot CH_2C(CH_3)_2OH$ radicals produced a spectrum which resembled that of FH^- above 300 nm, but the peak at 255 nm was much less prominent. Furthermore, there was no change on addition of oxygen. The value of η was 3.7. The spectrum is shown in Figure 4c.

Oxidations with Thiyl and $\cdot Br_2^-$. Following the two-electron reduction of 2.5 mL of a 16 μM FAD solution containing 2 mM sodium formate buffered at pH 7, 0.3 mL of thoroughly deaerated 3 M KBr was added anaerobically. The resulting bromide concentration was such that reaction 9 would suppress reaction 7 and produce only $\cdot Br_2^-$. The net yield of oxidizing equivalents per 100 eV in N_2O-saturated solutions on irradiation would then be $G_r - 2G_H + 2G^M_{H_2O_2}$. This was substituted for the term in parentheses in eq 12 in calculating e_{ox} for $\cdot Br_2^-$ titrations.

Radiolysis of reduced flavin solutions after addition of KBr as above produced spectral changes consistent with reoxidation of the flavins. Illustrative titration plots for FAD and LC are shown in panels c and d, respectively, of Figure 2. Beyond the end points, there were no significant spectral changes. Also, addition of air had no effect on the spectra, which were those of the initial flavin solutions with about 10% dilution due to addition of the KBr solution. The values of η for LF and FAD (cf. Figure 2c) were 2.0 and 1.9, respectively. These are in accord with

$$2\cdot Br_2^- + FH^- \rightarrow 4Br^- + F + H^+ \qquad (15)$$

as the titration reaction (from the known yields, the contribution of reaction 11 is only ~8%). Consistent with its one-electron reduction, LC required only 1 equiv of $\cdot Br_2^-$ for reoxidation (Figure 2d).

The same type of oxidation process was observed when 0.3 mL of deaerated 3 M $HO(CH_2)_2SH$ was added to the reduced FAD solution and irradiated under N_2O. In this case, H· and ·OH both produce RS· radicals (reaction 10) and expression 12 applies. The value of η for FAD was 2.1, which is consistent

with the following overall reaction:

$$2RS\cdot + FH^- + H^+ \rightarrow 2RSH + F \quad (16)$$

Discussion

The results with N_2O-saturated formate solutions have demonstrated the reversible two-electron reduction of RF, FAD, and LF by $\cdot CO_2^-$. The absence of a dependence of η on formate ion concentration (see Results) indicates that reaction 17 is unimportant, since it would have led to a chain

$$FH\cdot + HCO_2^- \rightarrow FH^- + H^+ + \cdot CO_2^- \quad (17)$$

reaction and a strong dependence of η on $[HCO_2^-]$. Similarly, the high efficiencies of reduction ($=2/\eta \times 100$) show that the reverse of eq 17 cannot be important. Finally, the efficiencies and absence of a dose-rate dependence of η for FAD demonstrate that the radical + radical reactions eq 18 and 19 are

$$FH\cdot + \cdot CO_2^- \rightarrow F + HCO_2^- \quad (18)$$

$$2\cdot CO_2^- \rightarrow \text{products} \quad (19)$$

insignificant up to the equivalence point, since their rates would have increased with dose rate, causing η to rise.

The observed reduction must be attributed to reactions 3 and 20, which from pulse radiolysis and flash photolysis studies

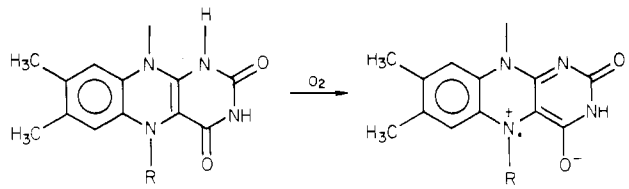
$$2FH\cdot \rightarrow F + FH_2 \quad (20)$$

have the following rate constants in units of 10^9 M^{-1} s^{-1}: $k_3 = 1.2 \pm 0.4$, 1.8 ± 0.4, and 3.6 ± 0.4 for FAD, LC, and LF, respectively (A. J. Elliot, L. McIntosh, R. Ahmad, and D. A. Armstrong, unpublished results); $k_3 = 3.6$ for RF (Land & Swallow, 1969); $2k_{20} = 0.26 \pm 0.06$ for FAD (A. J. Elliot et al., unpublished results), 2.3 for LF (3-methyl-LF; Hemmerich et al., 1980), and 1.1 for RF (Land & Swallow, 1969). Reaction 4 probably also contributes, but its rate has not thus far been studied by fast reaction techniques.

The demonstration of the reversible two-electron reduction of flavins by $\cdot CO_2^-$ is a further illustration of the potential for application of radiation chemical techniques to biological systems. On the other hand, the clear-cut behavior of $\cdot CO_2^-$ is somewhat unique. Thus, in the alcohol solute systems (see Table I), even the radicals generated from ethanol failed to give 100% reversible reduction. Also, while e_{aq}^- reduces F to FH^- (FH_2), there was a further reaction which destroyed FH_2 and was irreversible. Clearly therefore, caution must be exercised in the use of e_{aq}^- as a reductant, and in particular, two-electron reductions with e_{aq}^- should be avoided.

According to Asmus et al. (1973), the abstraction of hydrogen atoms from alcohols in reaction 8 produces radicals of the types and fractional abundances shown in columns two and three of Table I. The α-carbon radicals are generally good reducing agents, while β-carbon radicals are not. (Alkoxy radicals are strong oxidants, but these are formed in relatively small proportions.) From column five of Table I, the percentage of reversible reduction of FH^- (FH_2) is seen to fall drastically as the fractional yield of α-carbon radicals drops. Thus, the other types of radicals (i.e., $\cdot CH_2CH_2OH$, $\cdot CH_2CH(CH_3)OH$, $\cdot CH_2C(CH_3)_2OH$, etc.) appear to be responsible for the irreversible reactions. The spectra of these products in Figure 4c,d exhibit peaks in the region of 350–370 nm and evidence of a strong absorbance near 260 nm. These absorptions are similar to those of alkylated flavins (Dudley et al., 1964; Ghisla et al., 1974). It is therefore reasonable to propose that alkyl radical addition to the flavin rings occurred. The positions of the maxima at 370–380 nm in Figure 4c,d are closer to those of C4a-alkylated 4a,5-dihydroflavins (λ_{max} = 360–370 nm) than to N5-alkylated 1,5-dihydroflavins (λ_{max} = 327–355 nm) or N1-alkylated 1,5-dihydroflavins (λ_{max} = 340 nm) [see Table I of Ghisla et al. (1973) and Figure 4 of Ghisla et al. (1974)].[2]

For 2-methyl-2-propanol, where only β-carbon $\cdot CH_2C(CH_3)_2OH$ radicals are formed (Table I), η was 5.0, indicating a relatively low efficiency for addition. For ethanol, 2-propanol, and 1-butanol, η remained close to 2.0 despite the reduction of the yield of FH^- (FH_2). This feature coupled with the low efficiency for addition of the 2-methyl-2-propanol β radicals suggests that the reductive α-carbon radicals were rapidly consumed to produce FH·, which then reacted with the other types of radical present. The nonoxidizable products should therefore be mainly C4a-alkylated 4a,5-dihydroflavins. The absence of N5-alkylated 1*H*-hydroflavins (I) is consistent



I II

with the fact that the blue radical (II), which should be formed on oxidation of I and has $\lambda_{max} \simeq 580$ nm (Müller et al., 1970; Williams et al., 1977), was not observed.

The nonoxidizable product formed on reaction of FAD with a mixture of e_{aq}^- and $\cdot CH_2C(CH_3)_2OH$ may be explained on a similar basis, that is, a rapid reaction of e_{aq}^- to form FH· followed by addition of the alkyl radical. The spectrum is seen to be measurably different from that for reaction with the β-butyl radicals described alone (see Figure 4c).

The case of 1-butanol is particularly interesting. Although 41% of the radicals produced were α-carbon radicals, no reversible reduction was observed. This suggests a high efficiency for the combination of FH· with $CH_3CH_2\dot{C}HCH_2OH$, $CH_3\dot{C}HCH_2CH_2OH$, and $\cdot CH_2(CH_2)_2CH_2OH$. At the same time reactions of the type

$$R\dot{C}H\text{–}CH_2R' + F \rightarrow RCH{=}CHR' + FH\cdot \quad (21)$$

and

$$R\dot{C}H\text{–}CH_2R' + FH\cdot \rightarrow RCH{=}CHR' + FH^- + H^+ \quad (22)$$

must be much slower.

The fact that the η values for ethanol and 2-propanol were close to 2.0 is also evidence that close to 100% of $RR'\dot{C}OH$ radicals undergo reaction 23 or 24. Reaction 24 has been

$$F + RR'\dot{C}OH \rightarrow FH\cdot + RR'C{=}O \quad (23)$$

$$FH\cdot + RR'\dot{C}OH \rightarrow FH^- + RR'C{=}O + H^+ \quad (24)$$

postulated as a possible step in the reversible two-electron oxidation of alcohols by flavins (Bruice & Taulane, 1976; Bruice, 1976) and has been justified largely on thermodynamic and mechanistic grounds.

The α-carbon radical from ethylene glycol was unique in that it was the only radical which produced partial yet completely reversible reduction (Table I, footnote *c*). The reversibility indicates that the radicals from ethylene glycol did not add to flavin, while the partial reduction suggests that oxidative and reductive processes were in competition when the final ratio of $[FH^-]/[F]$ was attained. Actually the $\cdot CH(OH)CH_2OH$ radical is known to eliminate water to produce the formylmethyl radical, $\cdot CH_2CHO$, and this is an oxidizing radical (Bansal et al., 1973). Thus, while eq 25 and 26 must

[2] We are grateful to one of the referees for bringing some of the earlier data on alkylated flavins to our attention.

$$\cdot CH(OH)CH_2OH + F \rightarrow CHOCH_2OH + FH\cdot \quad (25)$$

$$\cdot CH(OH)CH_2OH + FH\cdot \rightarrow CHOCH_2OH + FH^- + H^+ \quad (26)$$

clearly dominate in the early stages of the reduction, it appears that, as [F] was depleted, reaction 27 became important and was followed by reaction 28. Since reactions 25 and 26

$$\cdot CH(OH)CH_2OH \rightarrow CHOCH_2\cdot + H_2O \quad (27)$$

$$FH^- + H^+ + CHOCH_2\cdot \rightarrow FH\cdot + CHOCH_3 \quad (28)$$

depend on the flavin concentration, while eq 27 does not, the final $[FH^-]/[F]$ ratio should also depend on total flavin present. Such a dependence was indeed observed.

Our present experiments with $\cdot Br_2^-$ and RS· appear to be the first observations of radical oxidations of reduced flavins. For FAD and LF, the two-electron oxidations must obviously proceed through two one-electron steps, because both $\cdot Br_2^-$ and RS· are one-electron oxidants. For RS·, these would be

$$RS\cdot + FH^- + H^+ \rightarrow RSH + FH\cdot \quad (29)$$

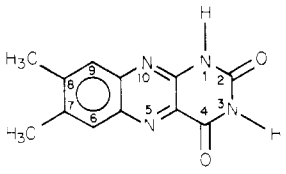
$$RS\cdot + FH\cdot \rightarrow RSH + F \quad (30)$$

Clearly, the two-electron reduction of flavin in the reverse sequence of eq 29 and 30 would not occur, and the well-known reduction of flavins by sulfhydryls (Loechler & Hollocher, 1975; Desa, 1976; Bruice, 1976) could not take place via the reverse sequence, followed by dimerization of RS·. In fact, the postulated mechanism involves formation of a C4a adduct of RS^-, followed by attack by a second RS^- and elimination of RSSR (Loechler & Hollocher, 1975; Bruice, 1976). The ability of RS· to oxidize FH· and FH^- stems from the fact that its reduction potential is much higher than that of RSSR.

One further point worth noting is that RS· radicals evidently do not add efficiently to F or FH· as do the aliphatic alcohol radicals.

The spectrum of the one-electron reduced form of lumichrome produced in our titrations may be compared with the spectrum of the one-electron reduced radical observed in pulse radiolysis (A. J. Elliot et al., unpublished results) in Figure 4b. Obviously, the two species are not the same. In pulse radiolysis, the radical is seen to decay by a second-order process. It is logical therefore to suggest that the long-lived species observed here is a dimer.

Unlike the flavins, which are isoalloxazines, lumichrome has the structure of an alloxazine (III) with p*K*s of 8.4 and ≥12



III

(Lasser & Fietelson, 1977). Dimerization by single bonds between the N10 or N5 positions is therefore possible.

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