One-Electron Reactions in Biochemical Systems as Studied by Pulse Radiolysis. II. Riboflavin[*]

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ABSTRACT: When riboflavin is subjected to high-energy irradiation in the presence of formate it becomes reduced to the semiquinone by hydrated electrons, CO_2^-, or COOH. Rate constants for the reactions are tabulated.

The semiquinone has pK's of 2.3 and 8.3. Improved spectra have been determined for the forms of the semiquinone present at pH −1.1, 5.1, and 11.4. The form present at pH −1.1 is stable, but the forms present at pH 5.1 and 11.4 disappear in second-order reactions with rate constants of $2k = 1.14 \times 10^9$ and 7×10^8 M^{-1}, respectively. The form present at pH 5.1 is uncharged. Electron transfer from the adenine ring to riboflavin is demonstrated. Riboflavin itself has been found to undergo a change in protonation at $H_0 \sim -8$.

It is well known that RF[1] can give rise to semiquinone free radicals by the action of one-electron reducing agents (Michaelis *et al.*, 1936). The properties of many different flavosemiquinones formed by chemical methods of reduction have been studied using electron spin resonance and electronic absorption spectroscopy (Beinert, 1956; Ehrenberg *et al.*, 1967). The spectral and acidic properties of the different flavosemiquinones, as of the flavins from which they are derived, tend to be very similar. Flavosemiquinones have also been observed after flash photolysis of riboflavin or derivatives (Holmström, 1962; Green and Tollin, 1968; Knowles and Roe, 1968). The photoreaction probably proceeds *via* the flavin triplet state and is different in the presence and in the absence of external reducing agents. The semiquinone intermediates for the two situations appear to have similar properties (Holmström, 1962). Riboflavin has been reduced to the semiquinone by irradiation with X-rays in acid solution in the presence of a high concentration of ethanol (Swallow, 1955). Under such conditions all the primary products of water radiolysis lead ultimately to reduction of riboflavin to the semiquinone.

The univalent reduction of riboflavin has now been studied using the technique of pulse radiolysis, which is the ionizing radiation equivalent of flash photolysis. In the high-energy irradiation of riboflavin in neutral aqueous solution in the presence of an excess of an organic substance such as formate, the various radical primary products of water radiolysis are quantitatively converted into the riboflavin semiquinone radical according to the following equations:

$$\text{H}_2\text{O} \rightsquigarrow e_{aq}^- \ (G = 2.60) + \text{H}(G = 0.55) + \text{OH}(G = 2.65) + \text{H}_2(G + 0.45) + \text{H}_2\text{O}_2(G = 0.7) \tag{1}$$

$$\text{OH} + \text{HCOO}^- \longrightarrow \text{COO}^- + \text{H}_2\text{O} \tag{2}$$

$$\text{H} + \text{HCOO}^- \longrightarrow \text{H}_2 + \text{COO}^- \tag{3}$$

$$e_{aq}^- + \text{RF} \longrightarrow \text{RF semiquinone} \tag{4}$$

$$\text{COO}^- + \text{RF} \longrightarrow \text{RF semiquinone} \tag{5}$$

$$2\text{RF semiquinone} \rightleftharpoons \text{RF} + \text{RF hydroquinone} \tag{6}$$

This mechanism is analogous to the corresponding reduction of NAD^+ (Land and Swallow, 1968). The side chain would not be expected to play a significant part in these reactions, so that conclusions reached for riboflavin should also apply to FMN and lumiflavin. The rate of reduction of riboflavin by e_{aq}^- and COO^- has now been determined and some of the spectroscopic and kinetic properties of the resulting semiquinones have been measured at various pH.

Materials and Methods

Riboflavin was used as supplied by B. D. H. or Koch-Light. The samples supplied by the two firms behaved identically. AMP was used as supplied by Koch-Light. All other chemicals were the purest available commercial products. Water was redistilled from alkaline permanganate in an atmosphere of nitrogen. Air was removed from solutions by bubbling with argon, stated to contain about 2 ppm of oxygen, obtained from Air Products. N_2O was medical grade from the British Oxygen Co. The buffers used (10^{-1}–10^{-2} M) were: HCOONa–HCOOH (pH 3–5), Na_2HPO_4–KH_2PO_4 (pH5–8), and H_3BO_3–NaOH (pH 8–10). The extremes of acidity or alkalinity were obtained using appropriate concentrations of H_2SO_4 or NaOH. A Pye pH meter was used to measure pH's in the range 12–1. For the more acid solutions, acidity functions of the various

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[1] Abbreviations used: RF, protonic form of riboflavin present in neutral solution; RFH_2^{2+}, RFH^+, and $RF(-H^+)^-$ are other protonic forms present at various pH; RFH_2^+, RFH, RF^-, and $RF(-H^+)^{2-}$ are different protonic forms of the semiquinone; AMP^-, semireduced form of adenosine 5′-monophosphate; e_{aq}^-, hydrated electron.

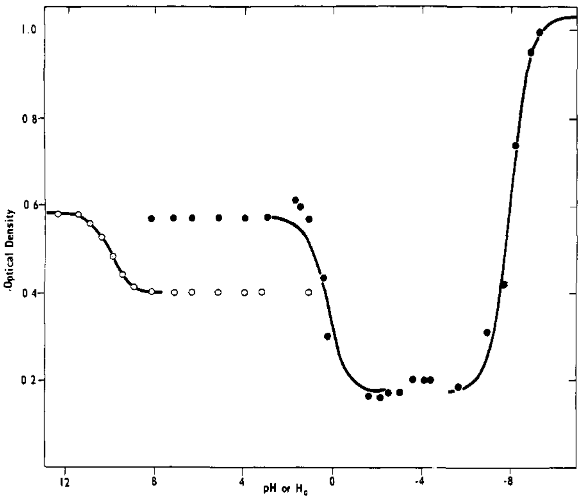


FIGURE 1: Riboflavin absorption changes as a function of solvent acidity. (○) 3500 and (●) 4400 Å. Optical path length 1 cm. Riboflavin concentration 5×10^{-5} M. The lines drawn in are theoretical equilibrium curves based on pK's of 10.05, 0.25, and −8.

concentrations of H_2SO_4 were taken from the literature (Paul and Long, 1957).

The pulse radiolysis equipment was basically that described by Keene (1964). Solutions contained in cells of optical path length 2.5 cm were irradiated with single 0.5- or 5-μsec pulses of 8–14 MeV electrons from a Vickers linear accelerator. The effect of the radiation on the optical transmission at selected wavelengths was observed as a function of time on a cathode ray oscilloscope. For the measurement of spectra, deflexions at a particular time after the pulse were noted. When kinetic data were required the whole trace was photographed using a Polaroid camera and accurate measurements made from the photograph. Radiation doses were measured either by modified ferrous sulfate (Fricke) dosimetry or using the absorption of the hydrated electron, taking $\epsilon(7200\ \text{Å}) = 1.8 \times 10^4\ \text{M}^{-1}\ \text{cm}^{-1}$ (Fielden and Hart, 1967) together with a $G(e_{aq}^-)$ value of 2.6 (Dorfman and Matheson, 1965). Absorption spectra of stable solutions were measured using a Bausch & Lomb Spectronic 505 recording spectrophotometer.

Since the deaerated riboflavin solutions used were very light sensitive, sample preparation was carried out in subdued light and container vessels were surrounded by black cloth. During the actual pulse radiolysis experiments, filters were used where possible to cut off those monitoring light wavelengths absorbed by riboflavin. Also the experimental cell was flushed out with fresh solution immediately before radiolysis.

Results

The optical densities at 4400 and 3500 Å of a 5×10^{-5} M riboflavin solution as a function of solvent acidity are shown in Figure 1. The pK occurring at 10.05 corresponds to the equilibrium $\text{RF} \rightleftharpoons \text{RF}(-\text{H}^+)^-$, and may be compared with estimates (for FMN) of 10.4 from redox titrations (Lowe and Clark, 1956) and of 9.65 from polarographic studies (Ke, 1957). The pK at 0.25 corresponds to the change $\text{RFH}^+ \rightleftharpoons \text{RF}$ which was found by Michaelis *et al.* (1936) to occur at −0.2. A further change in spectrum is now seen in very acid conditions around $H_0 \sim -8$ which probably corresponds to the equilibrium $\text{RFH}_2^{2+} \rightleftharpoons \text{RFH}^+$. The full spectra of all four protonic forms of riboflavin detected are shown in Figure 2.

In order to produce quantitatively the riboflavin semiquinone according to reactions 1–6, it is necessary that the formate concentration should be high enough to prevent irreversible attack on riboflavin by hydrogen atoms or hydroxyl radicals. Reversibility was tested by irradiating a N_2O-saturated riboflavin solution (5×10^{-5} M) containing formate (10^{-1} M) at pH 11.2, 5.1, 1.5, −1.0, −2.1, and −3.0, and admitting oxygen after the irradiation. At pH 11.2 and 5.1 the admission of oxygen restored the riboflavin absorption almost completely, so that any irreversible attack must have been less than about 10%. At the lower pH values reversibility was incomplete, but use of formate at a concentration of 1 M led to almost complete reversibility. These results are consistent with a decrease in the rate of reaction of H atoms on going from HCOO^- ($k = 2.5 \times 10^8\ \text{M}^{-1}\ \text{sec}^{-1}$) to HCOOH ($1.1 \times 10^6\ \text{M}^{-1}\ \text{sec}^{-1}$) (Rabani, 1962).

The rate constant for reaction of the hydrated electron with riboflavin, RF, in neutral solution was obtained by following the rate of disappearance of the e_{aq}^- absorption at 7000 Å in solutions containing various concentrations of riboflavin ($1\text{–}5 \times 10^{-5}$ M) in the presence of sodium formate (10^{-1} M). No buffer was present. The reaction of e_{aq}^- with formate is negligible under these conditions (Swallow, 1968). The corresponding rate of reaction of e_{aq}^- with $\text{RF}(-\text{H}^+)^-$ was obtained by following the disappearance of the electron absorption at 7000 Å for riboflavin in aqueous formate in the presence of 3×10^{-3} M NaOH. The results are given in Table I.

The disappearance of the hydrated electron absorption was found to be accompanied by a corresponding bleaching of the riboflavin absorption together with the formation of a new absorption in the visible which in neutral solution apparently occurred in the region 5000–6500 Å, where riboflavin semiquinone radicals are known to absorb (Holmström, 1962). There was an additional bleaching of riboflavin and increase in absorption around 5000–6500 Å after the electron had disappeared. This could be due to a protonation change of the riboflavin semiquinone, since it has been reported that the semiquinone has a pK around 6.5 (*e.g.*, Michaelis and Schwarzenbach, 1938). Or it could be due to a slow reaction of COO^- with riboflavin. For subsequent experiments buffer was added in order to ensure that any protonation step would occur rapidly. Under such conditions a slow bleaching of riboflavin still took place and there was still a build-up around 5000–6500 Å. That this was due to the reaction of COO^- was confirmed by saturating the solution with N_2O in order to convert hydrated electrons into additional COO^-.

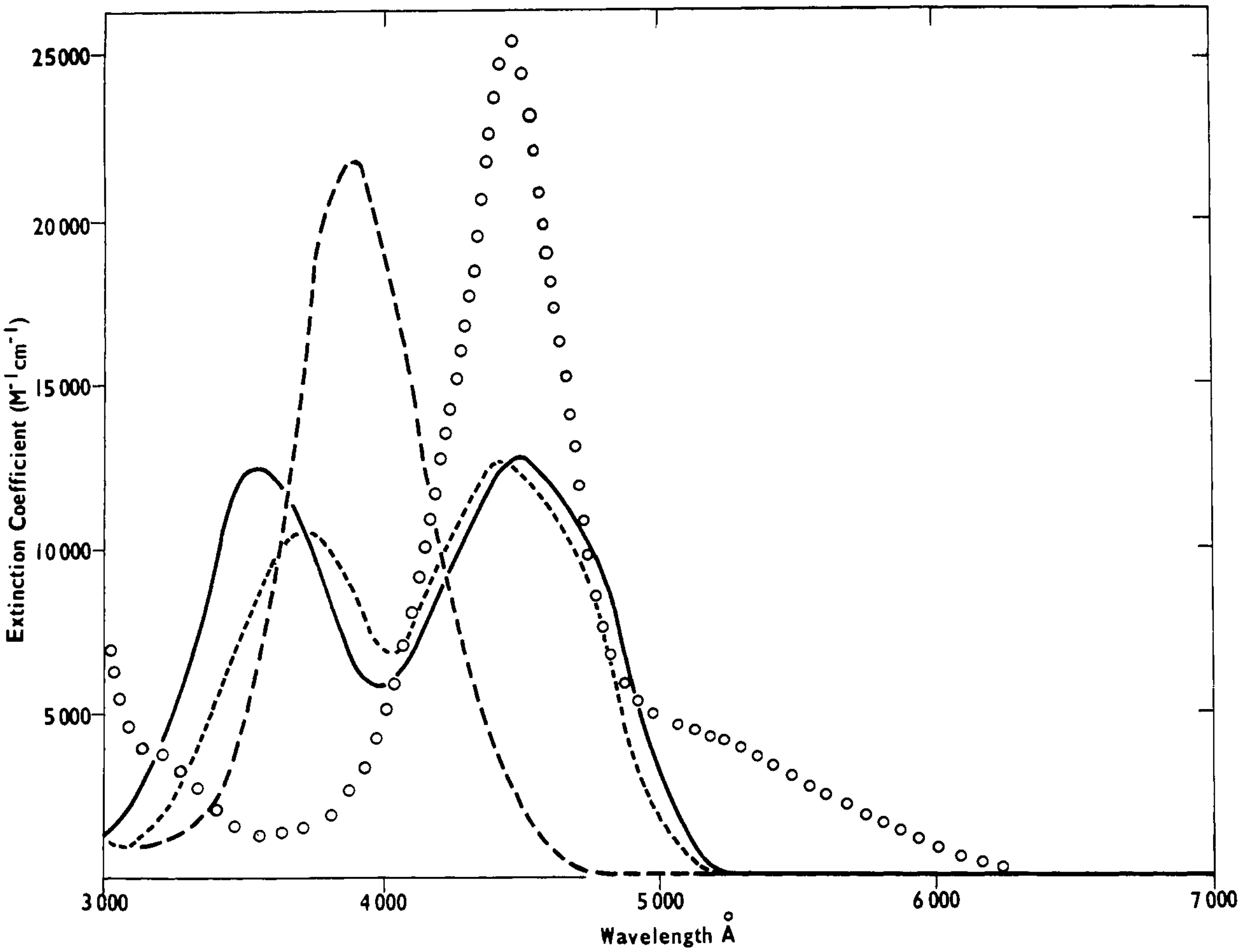


FIGURE 2: Absorption spectra of riboflavin at the acidities of predominance of the dication RFH_2^{2+}, $H_0 = -9$, ○○○○○; the mono cation RFH^+, $H_0 = -3.0$, — — —; the neutral molecule RF, pH 5.9, ---------; and the anion $RF(-H^+)^-$, 10^{-1} M NaOH, ———. Optical path length 1 cm, riboflavin concentration 3×10^{-5} M.

$$e_{aq}^- + N_2O \xrightarrow{H_2O} OH + N_2 + OH^- \qquad (9)$$

$$OH + HCOO^- \longrightarrow COO^- + H_2O \qquad (2)$$

Under such conditions (buffer present), the bleaching of neutral riboflavin at 4600 Å matched the build-up of semiquinone absorption at 5600 Å, the reaction following simple first-order kinetics from which the rate of RF + COO^- could be estimated. The corresponding rate for $RF(-H^+)^-$ was obtained from the rate of bleaching at 4600 Å of riboflavin in N_2O-saturated aqueous formate in the presence of 3×10^{-3} M NaOH. In such alkaline conditions very little semiquinone absorption occurs at 5600 Å. However, considerable semiquinone absorption occurs at 3600 Å, which again is formed at the same rate as the flavin is bleached.

At pH 3 the formate radical is present in the protonated form, COOH (Keene *et al.*, 1965b), and so the rate of bleaching of RF absorption at 4200 Å is now due to the reaction of COOH with RF. At such a pH, e_{aq}^- becomes converted into COOH *via* the recations

$$e_{aq}^- + H^+ \longrightarrow H \qquad (10)$$

$$H + HCOOH \longrightarrow H_2 + COOH \qquad (11)$$

without the mediation of N_2O. In more strongly acid conditions still, *e.g.*, at $H_0 = -2$, where riboflavin is monoprotonated, the reaction probably corresponds to COOH + RFH^+. It was found that at all acidities, in the presence of sufficient formate, buffer (pH 3–10), and nitrous oxide (pH 3.5–13), the kinetics of build-up of semiquinone absorption exactly matched the bleaching of riboflavin. The rates of reaction of COOH and COO^- with the various states of protonation of riboflavin are collected in Table I. It may be noted that all these rates lie in the range 1–4 × 10^9 M^{-1} sec^{-1}.

TABLE I: Reaction Rate Constants of e_{aq}^-, COO^-, and COOH with Various Protonic Forms of Riboflavin.

	Rate Constant (M^{-1} sec^{-1})		
Protonic Form	e_{aq}^-	COO^-	COOH
$RF(-H^+)^-$	1.7×10^{10}	1.4×10^9	
RF	2.3×10^{10}	3.6×10^9	3.6×10^9
RFH^+			3.0×10^9

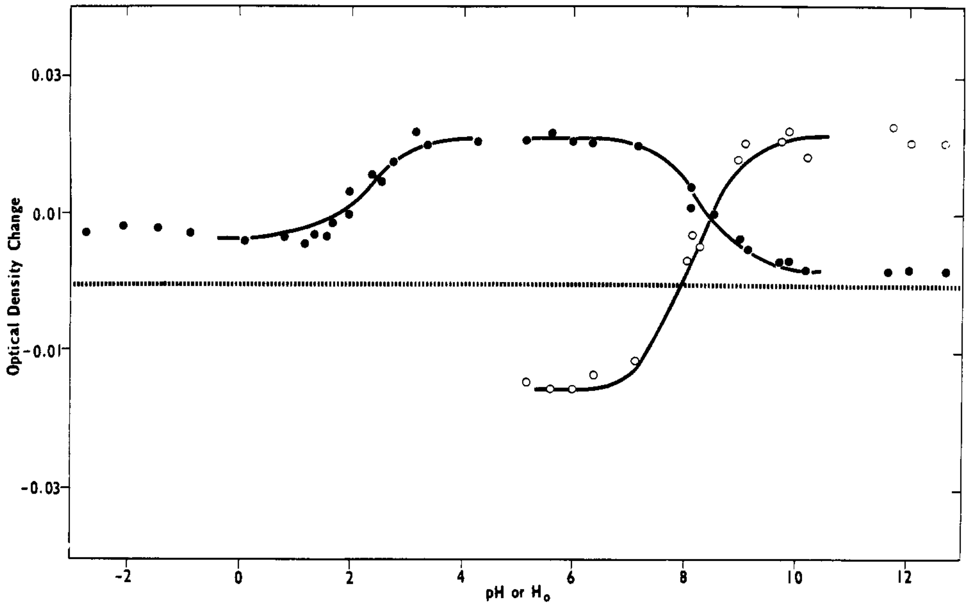
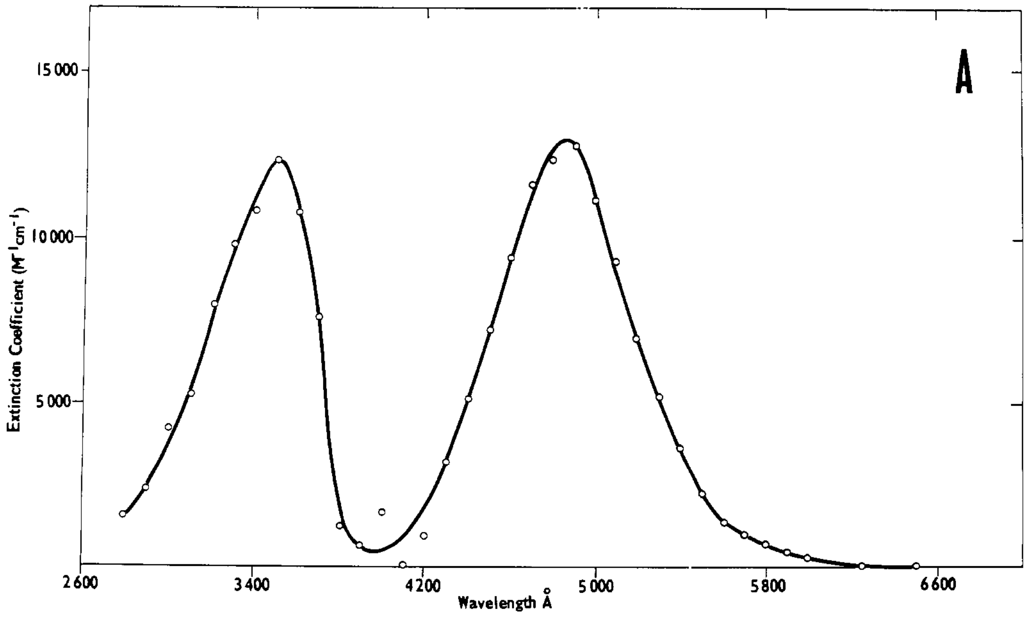


FIGURE 3: Absorption changes after pulse radiolysis as a function of solvent acidity. (○) 3600 and (●) 5600 Å. Optical path 2.5 cm, riboflavin concentration 5×10^{-5} M. Absorptions were measured ~30 μsec after a pulse of ~263 rads. Solutions were saturated with N_2O and contained 10^{-1}–1 M formate. The lines drawn in are theoretical equilibrium curves based on pK's of 8.3 and 2.3.

In order to find the pH's at which changes in the state of protonation of the semiquinone occur, the maximum change in optical density after a given dose was measured at two wavelengths, 3600 and 5600 Å, as a function of pH (Figure 3). The shorter wavelength is an isobestic point for the transition between RF and $RF(-H^+)^-$, so facilitating the separation of changes in semiquinone spectrum from changes in the state of protonation of fully oxidized riboflavin. At 5600 Å none of the relevant protonic forms of riboflavin itself absorb. The optical density changes were measured about 30 μsec after the pulse, at which time all the formate radicals had reacted with riboflavin but decay of the resulting semiquinone radicals was negligible. These data suggest that only two changes in protonation of the semiquinone occur in the pH range studied (13 to −3), namely, at pH 8.3 and 2.3.

The full spectra of the three protonic forms of semiquinone detected were obtained in the range 2800–7000 Å by measuring the change in spectrum as a function of wavelength at pH −1.1, 5.1, and 11.4 (Figure 4A–C). Allowance for loss of oxidized riboflavin absorption was made, assuming the mechanism postulated and using the primary radical yields already quoted (Dorfman and Matheson, 1965). Semiquinone spectra measured at $H_0 = 1.3$ and -3.0 were identical with that



obtained at pH −1.1; likewise the spectrum obtained at pH 13 was identical with the one found at pH 11.4.

The rates of decay of the various semiquinones were investigated at pH −1.1, 5.1, and 11.4. In the very acid conditions no decay occurred over several seconds after the pulse in agreement with the high stability of this semiquinone found by Swallow (1955) using X irradiation. At pH 5.1 the semiquinone decay at 5600 Å followed good second-order kinetics (Figure 5). The rate constant, k, for the reaction, defined by $-\mathrm{d}[\mathrm{S}]/\mathrm{d}t = 2k[\mathrm{S}]^2$, where S is the semiquinone species, was found from the slope of Figure 5, together with the extinction coefficient at 5600 Å, to be 5.7×10^8 M^{-1} sec^{-1}. At pH 11.4 the semiquinone absorbs only weakly at longer wavelengths than riboflavin itself. It was therefore necessary to measure the semiquinone decay at a wavelength where the riboflavin and semiquinone absorptions overlap. The rate of decay of absorption at 3600 Å was found to obey accurate second-order kinetics (Figure 6), a value of $2k = 8.13 \times 10^4(\epsilon_{\mathrm{S}} - (\epsilon_{\mathrm{RF}}/2))\ \text{M}^{-1}\ \text{sec}^{-1}$ being obtained, where ϵ_{S} and ϵ_{RF} are the extinction coefficients of the semiquinone and riboflavin itself, respectively. Use of the riboflavin and semiquinone extinction coefficients

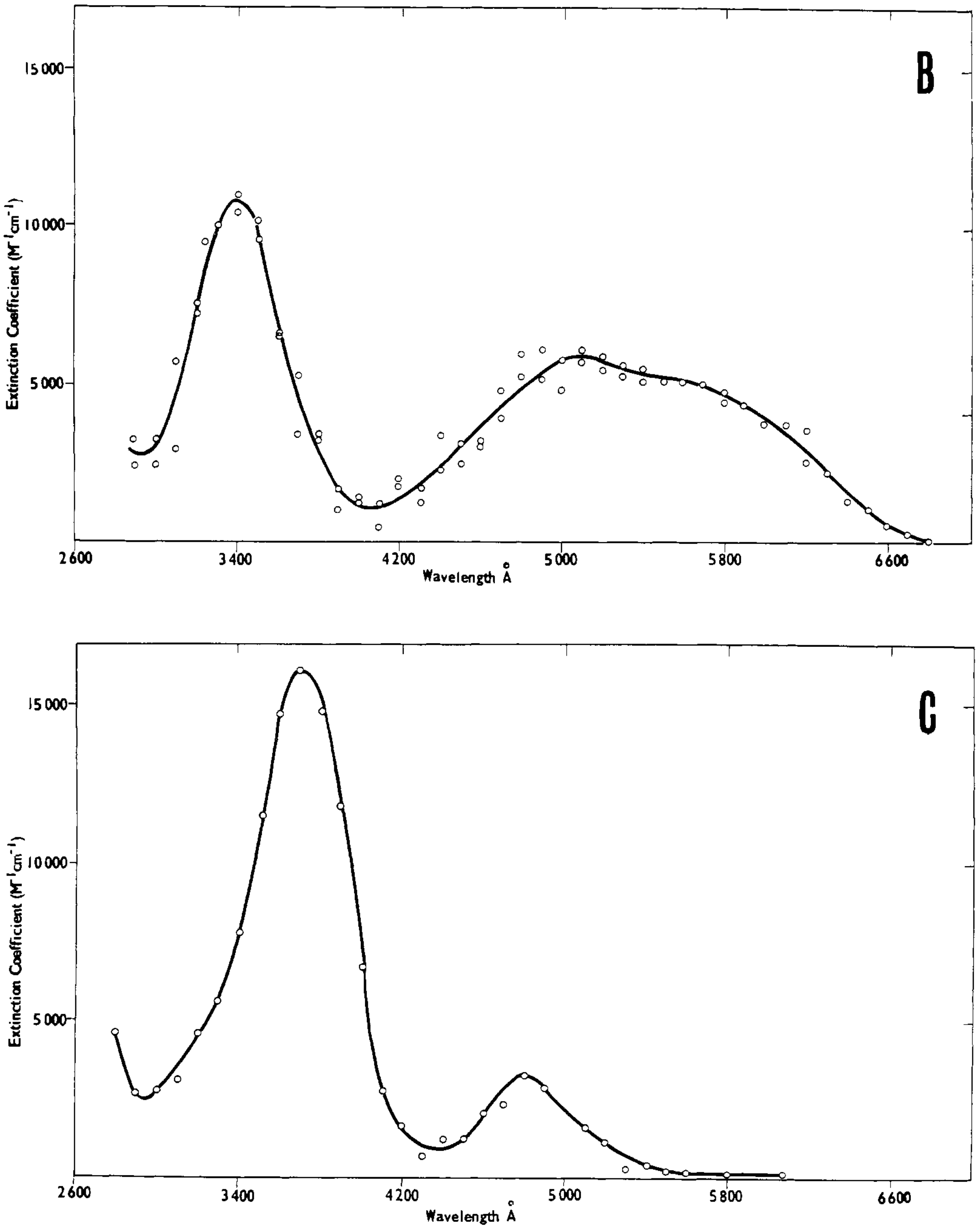


FIGURE 4: Absorption spectra of riboflavin semiquinone at the acidities of predominance of (A) the radical cation $\mathrm{RFH_2^+}$, $H_0 = -1.1$; (B) the neutral radical RFH, pH 5.1; and (C) the radical anion $\mathrm{RF^-}$, pH 11.4. Optical path 2.5 cm, riboflavin concentration 5×10^{-5} M. Absorptions were measured ~30 μsec after a pulse of ~393 rads. Solutions were saturated with $\mathrm{N_2O}$. For part A the formate concentration was 1 M, for parts B and C it was 10^{-1} M.

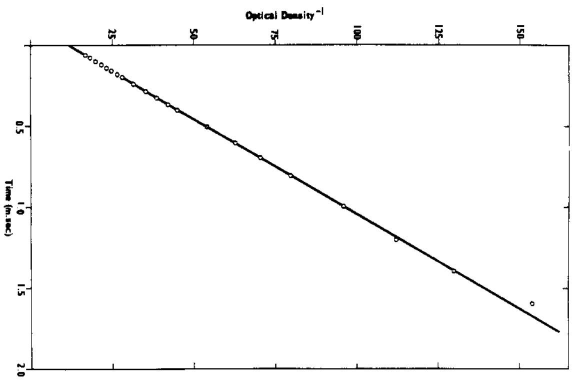


FIGURE 5: Second-order plot of decay of transient absorption at 5600 Å observed on pulse radiolysis of nitrous oxide saturated 5×10^{-5} M riboflavin in 10^{-1} M sodium formate (pH 5.1).

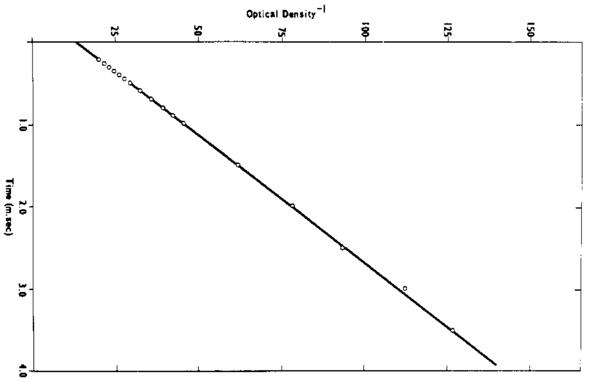


FIGURE 6: Second-order plot of decay of transient absorption at 3600 Å observed on pulse radiolysis of nitrous oxide saturated 5×10^{-5} M riboflavin in 10^{-1} M sodium formate (pH 11.4).

given in Figures 2 and 4C leads to a rate constant, k, for decay of the semiquinone at pH 11.4 of 3.5×10^8 M^{-1} sec^{-1}.

Information concerning the state of protonation of the riboflavin semiquinone at pH 5 was provided by salt effect studies. The decay of this semiquinone at 5600 Å obtained from nitrous oxide saturated RF (5×10^{-5} M) was measured as a function of ionic strength using $NaClO_4$ as added salt. Formate could not be used as reducing agent since it contributes too much ionic strength. However it is known that ethanol radicals, like formate radicals, reduce riboflavin to the semiquinone (Swallow, 1955), so that ethanol (10^{-1} M) was used in these experiments. Perchloric acid was added to bring the pH to 5.1. It was found that there was no significant alteration in second-order decay constant, k, on varying the sodium perchlorate concentration in the range 0–4 $\times 10^{-2}$ M. The Brønsted–Bjerrum relationship ($\log k_\mu/k_0 = 1.02 Z_aZ_b(\mu/(1 + \mu))$, where μ is the ionic strength, Z is the charge on the decaying ion, k_0 is the rate constant at zero ionic strength, and k_μ is the value for a given ionic strength) predicts that such a change in salt concentration would lead to a k_μ/k_0 ratio of 1.6, for a reaction between two species carrying unit charge. The lack of a detectable ionic strength effect is therefore consistent with the semiquinone present at pH 5.1 being uncharged or in a zwitterion form.

In the first paper of this series (Land and Swallow, 1968) evidence was presented for a rapid intramolecular transfer of electrons from the adenine ring of NAD to the nicotinamide ring. Rapid intermolecular electron transfer from AMP to NAD$^+$ was also found. In order to find out if a similar transfer of electrons can occur between the adenine and flavin rings of FAD, solutions containing 10^{-3} M AMP, 10^{-1} M HCOONa, and 10^{-4} M NaOH were pulsed in the presence of 1×10^{-5}, 2×10^{-5}, and 4×10^{-5} M riboflavin. In these solutions all the hydrated electrons would react with AMP to give AMP$^-$ and all the COO$^-$ radicals would react with riboflavin. The absorption of AMP$^-$ (Land and Swallow, 1968) is at all wavelengths much smaller than that of the riboflavin semiquinone at this pH, so that it was not possible to follow directly the concentration of AMP$^-$ in the mixture. However at 3800 Å the absorption of the semiquinone is very large compared with that of AMP$^-$, and at this wavelength the absorption obtained showed that all the hydrated electrons which had initially reacted with AMP must have ended up by reducing riboflavin. Furthermore it was found that saturation of the solutions with N_2O had no detectable effect upon the build-up rate of the flavin semiquinone absorption at 3800 Å. This result means that transfer of electrons from AMP$^-$ to riboflavin must take place, and at a rate about equal to the rate of reaction of COO$^-$ with riboflavin. The first-order build-up of semiquinone in N_2O-saturated solutions increased in proportion to the riboflavin concentration and the kinetics lead to a rate constant for the reaction of COO$^-$, and AMP$^-$, with riboflavin at this pH of 2.3×10^9 M^{-1} sec^{-1}. This is consistent with the rates already found for the reaction of COO$^-$ with RF and RF($-$H$^+$)$^-$ (Table I) since at pH 10 there would be about equal amounts of the two forms of riboflavin present, and the expected rate constant, k', for the COO$^-$ reaction would be

$$k'[\text{flavin}] = k_{(\text{COO}^- + \text{RF})}\left[\frac{\text{flavin}}{2}\right] + k_{(\text{COO}^- + \text{RF}(-\text{H}^+)^-)}\left[\frac{\text{flavin}}{2}\right]$$

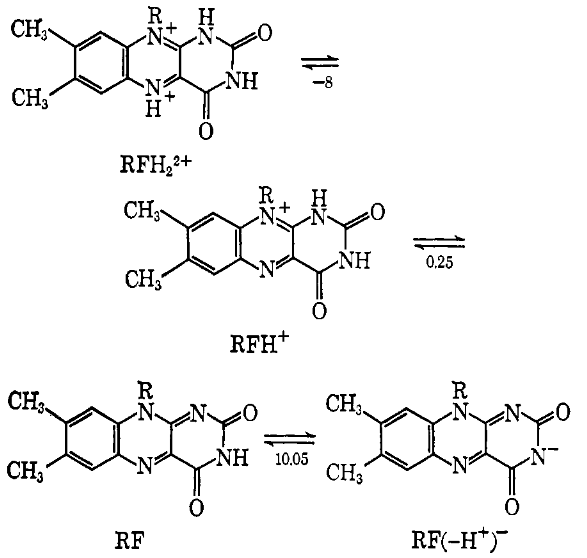
so that

$$k' = \frac{3.6 \times 10^9 + 1.4 \times 10^9}{2} = 2.5 \times 10^9 \text{ M}^{-1} \text{ sec}^{-1}$$

in reasonable agreement with the experimental value of 2.3×10^9 M^{-1} sec^{-1}.

Discussion

Various states of protonation of riboflavin are illustrated as

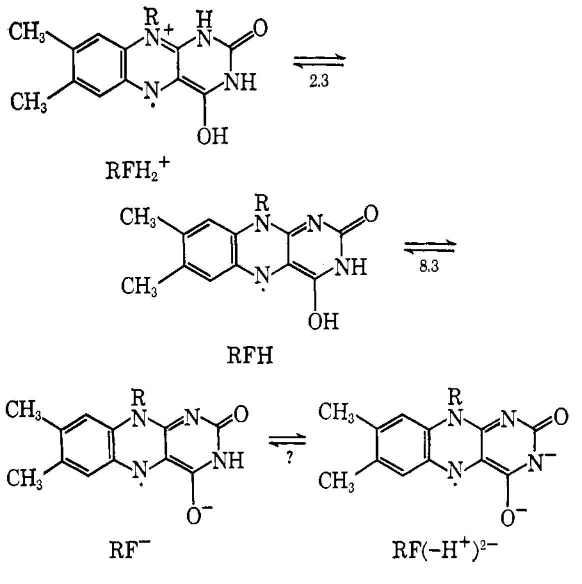


The position of the protons in RF and RFH$^+$ are those suggested by Dudley *et al.* (1964). The position of the further proton in RFH$_2$$^{2+}$, on the 5 N atom, is tentative. Our work provides no information about the location of the charges in the molecules; in the case of RFH$^+$ recent work by Tollin (1968) indicates that the positive charge is located on the 10 N atom.

The p*K*'s found at 10.05 and 0.25 are in reasonable agreement with previous estimates by other techniques. The further p*K* detected at $H_0 \sim -8$ has not been previously reported as far as the authors are aware. At such acidities the color of riboflavin changes from pale yellow to red.

Hydrated electrons react very rapidly both with RF and RF($-$H$^+$)$^-$ (Table I). The reaction is probably diffusion controlled. The slightly slower rate obtained with RF($-$H$^+$)$^-$ is presumably caused by the negative charge. The formate radical rates are approximately one order of magnitude slower than the electron rates, as previously observed for methylene blue (Keene *et al.*, 1965a) and NAD$^+$ (Land and Swallow, 1968). This is probably mainly because of a lower rate of diffusion of the radicals derived from formate as compared with hydrated electons. As with e_{aq}^-, COO$^-$ reacts slightly more slowly with RF($-$H$^+$)$^-$ than with RF. The transition COO$^-$ $\rightleftharpoons$ COOH appears to have no effect upon the rate of reaction of formate radicals with RF.

Various possible states of protonation of the riboflavin semiquinone radical are illustrated as



The position of the protons in RF$^-$ and RFH are as given by Ehrenberg *et al.* (1967) and Dudley *et al.* (1964), respectively. The position of the further proton in RFH$_2$$^+$ is tentatively assigned to the N atom in position 1. Our work provides no evidence about the location of the charges within the structure, and in the case of RFH the radical could be in a zwitterion form.

In our experiments two changes in the state of protonation have been found in the pH range 13 to -3, corresponding to p*K*'s at 8.3 and 2.3. Protonation changes have been found previously in both these regions. The value of 8.3 is rather higher than the previous estimate of 6.5 (Michaelis *et al.*, 1936) and the value 7.3 for radicals derived from FMN (Holmström, 1964), but it agrees with recent estimates of 8.27 for radicals from riboflavin and 8.55 for radicals from FMN (Draper and Ingraham, 1968). It is also similar to the value of 8.4 obtained by Ehrenberg *et al.* (1967) for lumiflavin-3-CH$_2$COO$^-$. The experimental points used by Holmström to obtain the value 7.3 could be made to fit a value around 8 if the FMN radical anion has an extinction at 5600 Å of much less than the 1300 M^{-1} cm^{-1} used. An ϵ (5600 Å) value of $\sim$170 M^{-1} was found for RF$^-$ in the present study. The value of 2.3 found for the protonation change at lower pH values is higher than the previous estimate of 1.3 given by Michaelis *et al.* (1936).

Detailed electron spin resonance studies of various flavin semiquinone radicals have been interpreted in terms of the monoanion RF$^-$ being the stable form in alkaline solution (Ehrenberg, 1962). The absorption spectra of such species (Ehrenberg *et al.*, 1967) are very similar to the spectrum of the riboflavin semiquinone observed above pH 9 in the present studies. The lack of a salt effect upon the decay of the riboflavin semiquinone at pH 5.1 is consistent with the p*K* at 8.3 corresponding to equilibrium between an uncharged and a monoanionic species, supporting the above assignment. No change in absorption spectrum occurred in the range 9–13 which could be associated with deprotonation from the nitrogen at position 3. This accords with esr interpretations in this pH range, although it must be pointed out that since the spin density is low at this part of the flavin semiquinone (Ehrenberg, 1962), deprotonation here would little affect the esr spectrum. Moreover since the difference between the light absorptions of RF and $RF(-H^+)^-$ is rather small, the difference between RF^- and $RF(-H^+)^{2-}$ may likewise be small. Therefore deprotonation of RF^- somewhere in the pH range 9–13 cannot be ruled out from the present studies.

The shape of the spectrum obtained for the cationic semiquinone RFH_2^+ which is stable in strong acid (Figure 4A) agrees well with a previous determination by Ogura *et al.* (1963). In the earlier study, the oxidized flavin was titrated against the flavin hydroquinone and at pH −0.4 the extinction coefficients at 3500 and 4900 Å were 9800 and 9600 M^{-1} cm^{-1}, respectively. The corresponding extinctions found in the present study are 13,000 and 12,300 M^{-1} cm^{-1}, respectively. The full spectrum of the neutral radical RFH (Figure 4B) has not been determined previously. However the spectrum of the corresponding neutral lumiflavin semiquinone obtained by Knowles and Roe (1968) is quite similar to that shown in Figure 4B, except that the minimum around 4000 Å is much more pronounced in the present spectrum. The extinction coefficient of neutral flavosemiquinones around 5500–5600 Å, where the semiquinones are free from overlap due to the parent flavin absorption, has been estimated on several previous occasions, the following recent values (in M^{-1} cm^{-1}) having been obtained, for FMN: 8,000–13,600 (Gibson *et al.*, 1962), 3050 (Holmström, 1964), 500 (Swinehart, 1966), 8,000–10,000 (Fox and Tollin, 1966); and for lumiflavin: 3400 (Knowles and Roe, 1968). The present estimate involved converting all the primary radicals formed on water radiolysis into flavin semiquinone radicals. Taking into account possible errors in dosimetry, primary G values and optical density measurements, this method should lead to an extinction coefficient correct to ±15%. Our value is thus 5130 M^{-1} cm^{-1} ±15% at 5600 Å. The spectrum obtained for RF^- (Figure 4C) is quite close to that determined for the semiquinone anion for lumiflavin-3-$CH_2COOC_2H_5$ (Ehrenberg *et al.*, 1967), except that the longest wavelength band, peaking around 4800 Å, is much weaker in the present case. An advantage of the present method of determination is that no interference due to dimer formation occurs since the spectra are measured a few tens of microseconds after formation, before the radicals have time to dimerize.

The rate constant for disappearance of the neutral riboflavin semiquinone radical RFH ($2k = 1.14 \times 10^9$ M^{-1} sec^{-1}) is very similar to the previous estimates of 0.75 and 1.00×10^9 for the corresponding neutral lumiflavin semiquinone (Knowles and Roe, 1968). Holmström (1964) obtained a value of $3\text{–}5 \times 10^8$ M^{-1} sec^{-1} for the neutral semiquinone derived from FMN, which would be increased to $\sim 7 \times 10^8$ M^{-1} sec^{-1} if the extinction coefficient found in the present study were to be applied. There thus appears to be no serious disagreement between these different measurements. The rate of decay of the semiquinone anion RF^- ($2k = 7.0 \times 10^8$ M^{-1} sec^{-1}) is again faster than the value found by Holmström (1964) for the corresponding radical anion from FMN (1×10^8 M^{-1} sec^{-1}). The discrepancy would be even more if the present value for the extinction of the radical anion at 5600 Å ($\epsilon \sim 170$ M^{-1} cm^{-1}) were applied to the results with FMN. Since the FMN radical decay was measured at pH 8.3 it seems likely in view of the present results that at this pH it is still mainly the neutral radical being observed. Neutral radical–radical anion reactions are also possible at pH 8.3. Hence the value obtained in the present study seems to be the more reliable. Very little decay of the radical cation RFH_2^+ occurred below pH 0 since in this case the equilibrium between flavin and flavin hydroquinone and the radical cation lies very much on the radical side. No disproportionation rate could therefore be obtained for RFH_2^+.

At natural pH (~6.5) the initial semiquinone observed by reaction of e_{aq}^- or COO^- with riboflavin is the radical anion RF^-, although the equilibrium between RFH and RF^- lies well on the side of RFH at this pH. Thus in the absence of buffer the anion RF^- is seen initially, which protonates slowly over several tens of microseconds to form RFH. At pH > 11 e_{aq}^- and COO^- react with $RF(-H^+)^-$ to form $RF(-H^+)^{2-}$ which since it does not appear to be observed probably rapidly protonates in <1 μsec by reaction with water to form RF^-.

The fact that electron transfer from AMP^- to riboflavin was observed makes it likely that in FAD any electrons reacting with the adenine ring would be very rapidly transferred to the flavin ring. Such an intramolecular transfer would be facilitated by the close proximity of the rings in the folded configuration of FAD present in aqueous solution (Gascoigne and Radda, 1965). This reaction is similar to the intramolecular electron transfer found in NAD (Land and Swallow, 1968).

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Structural Characterization of Hemoglobin Tacoma*

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ABSTRACT: Chemical characterization of hemoglobin Tacoma demonstrates a single substitution of a seryl residue for the arginyl residue number 30 of the β chain. Because this appears to be the only substitution in the abnormal hemoglobin, the structural formula is $\alpha_2\beta_2{}^{30\mathrm{Ser}}$. This substitution occurs in a region of residues 28–42 which is one of the relatively invariant parts of the β chain of normal animal hemoglobins.

Baur and Motulsky (1965) have described a new inherited hemoglobin variant, Hb Tacoma, which they discovered in three healthy members of a family of European origin. The proportion of the abnormal hemoglobin in the heterozygote was found to be 43% of the total hemoglobin. Clear separation of Hb Tacoma from Hb A has been achieved only by starch grain electrophoresis, where Hb Tacoma moves more rapidly towards the anode. Hemoglobin Tacoma is less heat resistant and is more rapidly denatured by 8 M urea than Hb A, Hb C, or Hb S. Hybridization experiments by Baur and Motulsky (1965) indicated the presence of a structural abnormality in the β chain and a normal α chain in Hb Tacoma.

The subject of this paper is the chemical study of the amino acid substitution in the abnormal β chain of Hb Tacoma.

Experimental Procedure

Isolation of Hb Tacoma from Hb A was by repeated starch block electrophoresis as described previously by Baur and Motulsky (1965). A small amount of the purified abnormal hemoglobin was hydrolyzed with trypsin according to the procedure of Ingram (1958). Approximately 2–3 mg of a freeze-dried preparation of this tryptic digest dissolved in 20 µl of 0.001 N HCl was applied to Whatman No. 3MM paper. Electrophoresis was carried out in a pyridine–acetic acid buffer of pH 6.5 for 2 hr at 2000 V (Baglioni, 1961). Ascending chromatography was effected with a solvent mixture of pyridine–3-methyl-1-butanol–water (7:7:6, v/v). The peptide patterns were reacted with ninhydrin and specific stains for arginine, tyrosine, histidine, and tryptophan residues (Lehmann and Huntsman, 1966).

Globin was prepared from most of the purified hemoglobin by removing the heme with cold acid acetone ac-

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