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On the Existence of Spectrally Distinct Classes of Flavoprotein Semiquinones. A New Method for the Quantitative Production of Flavoprotein Semiquinones*

Vincent Massey and Graham Palmer†

ABSTRACT: A new method for the conversion of flavoproteins to the semiquinoid form is described. This consists of anaerobic photoirradiation in the presence of EDTA.

A survey of a number of simple flavoproteins with this technique leads to the conclusion that there exist two different classes of flavoprotein free radicals, which are very readily distinguished on the basis of their optical absorption properties. With one enzyme, glucose oxidase, both types of radical are found, the concentrations depending on the pH. These results suggest that the two different spectral species may be due to the neutral and anionic radical forms of the flavin coenzyme prosthetic groups.

Following the original observations of Beinert (1956) it has become generally accepted that the half-reduced or semiquinoid form of flavin coenzymes has an absorption maximum in the region 570–600 mμ. This fact has been amply documented by correlation of optical and epr[1] spectra (Ehrenberg, 1962; Gibson *et al.*, 1962), and quantitative estimates have shown that this absorption band has a considerable extinction coefficient, with values ranging between 3000 and 10000 l. mole^{-1} cm^{-1} (Beinert, 1960; Gibson *et al.*, 1962). On careful anaerobic titration with dithionite a similar absorption band has been observed with glucose oxidase (Massey and Gibson, 1964; Massey *et al.*, 1966) and *Azotobacter* flavoprotein (Beinert, 1965). Again this long wavelength absorption has been found to be well correlated with an epr-detectable free radical, and experimental extinction coefficients in the range 3000–5000 l. mole^{-1} cm^{-1} have been found at 570 mμ. In the case of two other flavoproteins, D-amino acid oxidase (Massey and Gibson, 1964; Massey *et al.*, 1966) and the Old Yellow Enzyme (Nakamura *et al.*, 1965), anaerobic titration with dithionite gave rise to absorption spectra which were very similar to each other, but which were completely different from those seen with glucose oxidase and *Azotobacter* flavoprotein. The D-amino acid oxidase and Old Yellow Enzyme semiquinone spectra are characterized by having very little absorption at 600 mμ, a well-marked peak at 480–490 mμ, and another peak with a very high extinction coefficient around 360–370 mμ. With D-amino acid oxidase a small but well-resolved peak was also observed at 400 mμ. In both cases, 80–100% yields of the free radical species were found by epr spectrometry. We were naturally very interested in determining whether this division of flavoprotein semiquinones into two categories (based on their spectral properties) was valid as a generalization, and

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[1] Abbreviations used: epr, electron paramagnetic resonance; FMN, riboflavin 5′-phosphate; TPNH, reduced triphosphopyridine nucleotide; FAD, FADH, FADH$_2$, oxidized, semiquinoid, and fully reduced flavin-adenine dinucleotide; TPN$^+$, oxidized triphosphopyridine nucleotide.

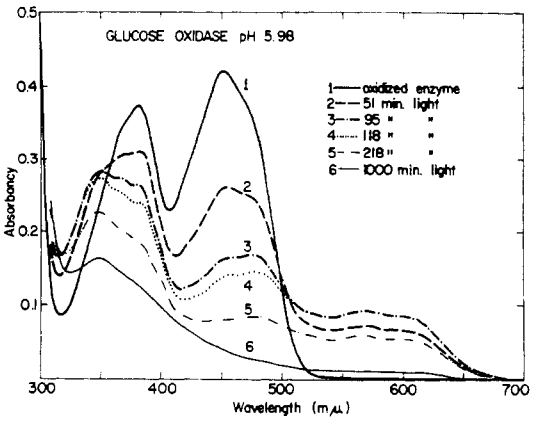

FIGURE 1: Effect of light on glucose oxidase under anaerobic conditions in the presence of 0.15 M phosphate and 0.05 M EDTA, pH 5.98. The sample was treated as described in the text for the times shown.

hence decided to survey a wider range of flavoprotein enzymes. Before embarking on this survey, however, we decided to explore the possibility of using reducing agents other than dithionite. An alternative method of reduction was desirable for many reasons. First, because of ready oxidation by air, dithionite titrations are very difficult technically. Secondly, as observed with glucose oxidase (Swoboda and Massey, 1966) and other flavoproteins (V. Massey, unpublished), an added complication is that sulfite or bisulfite ions, reaction products of dithionite oxidation, complex avidly with certain flavoproteins to produce a form spectroscopically similar to that of fully reduced flavin.

A reaction that has been known for some time with free flavins (Frisell *et al.*, 1959), but to our knowledge not tested with flavoproteins, is the photochemical reduction of flavins with potential electron donors such as EDTA. While the photoreduction of flavoproteins with EDTA has been found to be very much slower than with free flavins, the unexpected finding was made that the semiquinoid form of the flavoprotein accumulates in high yield. Apart from its intrinsic mechanistic interest, we believe this finding to be of great practical application in the ready production of flavoprotein semiquinones. This study shows that all simple flavoenzymes investigated have semiquinone spectra of one of either of the two types described above. Furthermore, it is found that the two types are also characterized by distinctly different epr spectra.

Materials and Methods

Purified Enzymes. Glucose oxidase from *Aspergillus niger* was prepared by the method of Swoboda and Massey (1965), D-amino acid oxidase from pig kidney was isolated by the method of Massey *et al.* (1961) as modified by Brumby and Massey (1966); L-amino acid oxidase was isolated from *Crotalus adamanteus* venom according to Wellner and Meister (1960). Lipoyl dehydrogenase from pig heart was isolated by the method of Massey (1960); yeast glutathione reductase was prepared as described by Massey and Williams (1965). Oxynitrilase (Becker *et al.*, 1963) was a gift from Dr. E. Pfeil and *Azotobacter* flavoprotein (Shethna *et al.*, 1966) a gift from Dr. H. Beinert. All reagents used were analytical grade and made up in glass-distilled water. A stock solution of 0.3 M EDTA was neutralized to pH 7.0 and diluted appropriately for the particular experiments.

Anaerobic Light Irradiation. The enzymes used were mixed with EDTA under the conditions described in the legends of the appropriate figures. The spectrum was recorded with a Cary Model 14 recording spectrophotometer. Specially constructed anaerobic cells were used as described previously (Massey *et al.*, 1960). The enzyme solution was then made carefully anaerobic by repeated degassing with a water pump and flushing with prepurified N_2 that was further purified by passage through Fieser's Solution. This cycle of degassing and flushing was repeated at least four times, the final phase being an evacuation. The anaerobic cuvet was then placed in a water bath at 12° approximately 6 in. from a 100-w tungsten light bulb and spectra were recorded at intervals.

Epr measurements were obtained with a Varian V4500-10A spectrometer using 100-kc/sec field modulation. Spectra were run at about 200°K in anaerobic epr tubes (Palmer, 1966). Quantitation was achieved by double integration of the spectra and comparison with a standard of 1.6×10^{-3} M copper in 1×10^{-2} M EDTA, pH 7.2. Some experiments were also performed at room temperature using the Varian quartz flat cell; under these conditions quantitation was made with reference to nitrosyl disulfonate. The results obtained at the two different temperatures were in excellent agreement.

Results

Optical Spectra (General). Figure 1 shows the changes in spectrum produced on light irradiation of glucose oxidase at pH 5.98 in the presence of EDTA. No changes were noted if the enzyme was kept under these conditions but in the absence of light, or if enzyme was light irradiated in the absence of EDTA. It is seen that there is a rapid development of a long wavelength absorption band very similar to that previously identified as the semiquinoid form of the enzyme by anaerobic dithionite titration (Massey *et al.*, 1966). This long wavelength band has a maximum at 570 mμ and a shoulder around 610 mμ and is qualitatively similar to that originally found by Beinert (1956) for the semiquinoid form of FMN. Prolonged light irradiation results in the gradual disappearance of the intermediate and the appearance of the spectrum of fully reduced enzyme. However, it should be noted that this phase of the reaction is much slower than the formation of the intermediate; whereas maximum production of the latter was found after about 100-min irradia-

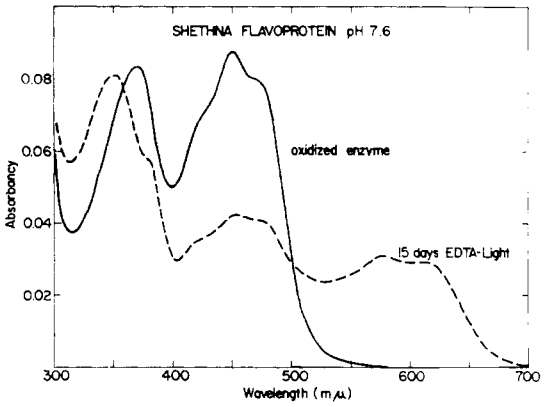

FIGURE 2: Effect of irradiation of *Azotobacter* flavoprotein for 15 days in the presence of 0.05 M phosphate and 0.05 M EDTA, pH 7.6. Approximately 90% conversion to the semiquinone was found on illumination for 5 days.

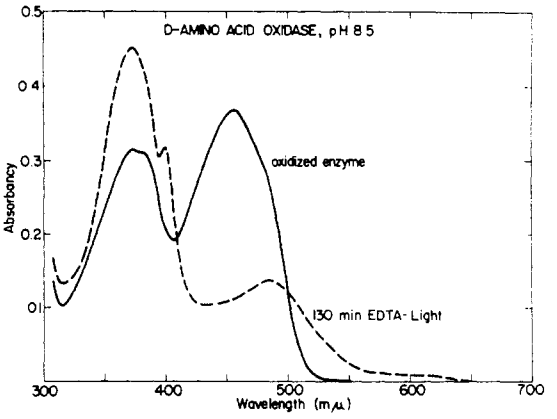

FIGURE 3: Effect of irradiation of D-amino acid oxidase for 130 min in the presence of 0.08 M pyrophosphate and 0.02 M EDTA, pH 8.5. A series of curves isosbestic at 409 and 500 mμ were obtained at shorter times of illumination.

tion there was still some long wavelength absorption present even after 1000-min illumination. On admitting air the spectrum returned immediately to that of the original oxidized enzyme and no loss in catalytic activity was observed as a result of the light irradiation. As detailed in a later section the spectral changes described above were correlated with the appearance of a free radical signal as determined by epr spectrometry, followed by the disappearance of this signal as the irradiation was continued for prolonged times.

Figure 2 shows the results obtained on EDTA light irradiation of the flavoprotein from *Azotobacter* commonly referred to as the Shethna flavoprotein (Shethna *et al.*, 1966). In this case prolonged illumination was required to produce the maximal development of the long wavelength absorbing species. It is evident that the absorption spectrum obtained is very similar to that described above for glucose oxidase, and to that described by Beinert (1965) for the semiquinoid form of this flavoprotein produced by dithionite. The characteristic long wavelength absorption of this semiquinoid species imparts a bright blue color to the solution when the yellow contribution of oxidized enzyme is removed. When partial reduction to the semiquinone is achieved and when a mixture of oxidized and semiquinoid enzyme is present olive green colors are observed. Almost identical spectral changes to those reported above in Figures 1 and 2 have also been observed on EDTA light irradiation of TPNH-thioredoxin reductase (G. Zanetti, C. H. Williams, and V. Massey, unpublished).

In contrast to the intense long wavelength absorption of the flavoprotein semiquinones just described, it was known (Massey and Gibson, 1964) that the semiquinoid form of D-amino acid oxidase had very little absorbancy at 600 mμ, but was characterized by absorption maxima at 490, 400, and 370 mμ. The lack of long wavelength absorption and the maximum at 490 mμ impart to this semiquinone species a distinct reddish

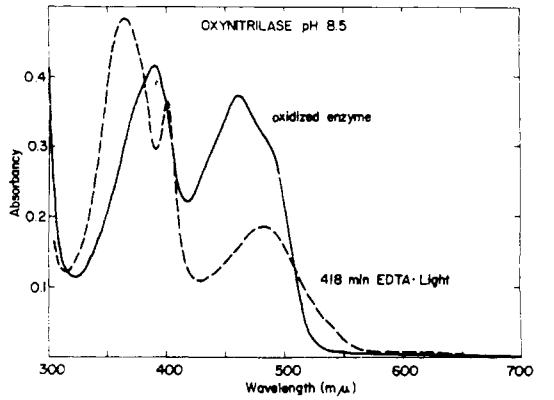

FIGURE 4: Effect of irradiation of oxynitrilase in the presence of 0.08 M pyrophosphate and 0.06 M EDTA, pH 8.5. A series of curves isosbestic at 381, 399, 406, and 508 mμ was obtained at shorter periods of illumination. Light irradiation for a further 2 days resulted in only 10% disappearance of the semiquinone spectrum shown.

color. Spectral changes identical with those previously reported with dithionite titration (Massey *et al.*, 1966) were obtained on EDTA light irradiation of D-amino acid oxidase (Figure 3). In this case, the subsequent reduction of the semiquinone to the fully reduced enzyme was even slower than that described in Figure 1 for glucose oxidase. Thus while complete formation of the semiquinoid form of D-amino acid oxidase under the conditions of Figure 3 occurred with about 2-hr illumination, very little further reduction had occurred with illumination continued for another 2 days, as judged by either visible or epr spectrometry.

A spectrum closely similar to that of the semiquinone

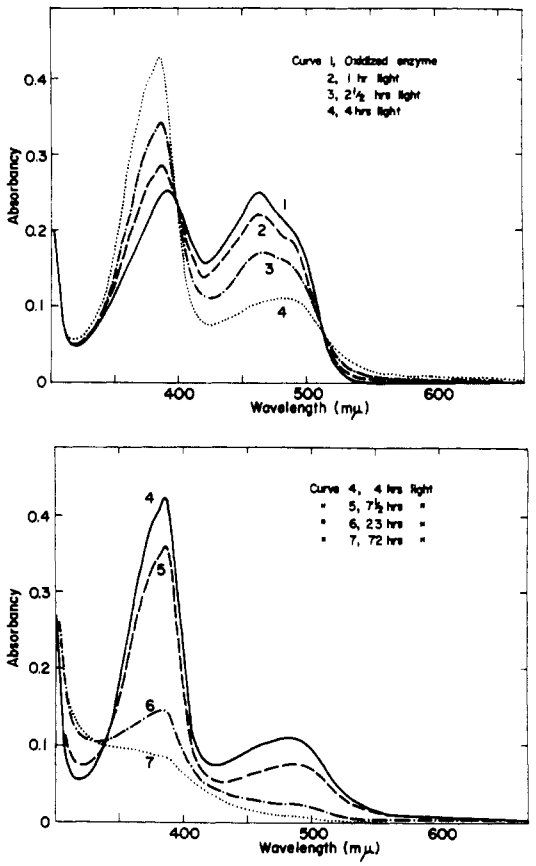

FIGURE 5: Effect of irradiation of L-amino acid oxidase in the presence of 0.06 M Tris and 0.09 M EDTA, pH 7.6. (a) Spectra during the conversion of oxidized to semiquinoid enzyme; (b) spectra during conversion of semiquinoid to fully reduced enzyme.

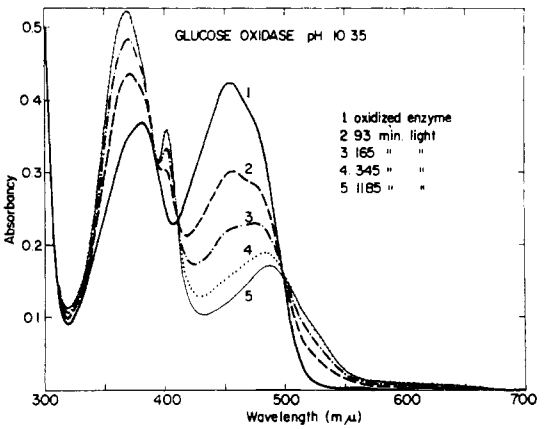

FIGURE 6: Effect of light irradiation of glucose oxidase in the presence of 0.07 M pyrophosphate and 0.06 M EDTA, pH 10.35.

just described was also found on EDTA light irradiation of oxynitrilase (Figure 4). The same characteristics, peaks at 370, 400, and 485 mμ, and a small flat absorption band between 550 and 650 mμ, are evident. Again it was found that while this spectral species was produced comparatively readily, further reduction occurred very slowly indeed. Thus, while the maximal development of the species shown in Figure 4 was obtained on illumination for 5 hr, only approximately 10% conversion to the fully reduced enzyme occurred on further illumination for another 3 days.

With L-amino acid oxidase from snake venom the semiquinoid enzyme produced by EDTA light irradiation is similar in many respects to those of D-amino acid oxidase and oxynitrilase (Figure 5a,b). A single but asymmetric peak at 385 mμ is found in place of the two peaks at 370 and 400 mμ. However the remaining characteristic features, a peak at 485 mμ and a weak flat band between 550 and 650 mμ, are evident. The absorption has again been shown by epr spectrometry to be correlated with a free radical species (see later section). While the ease of conversion to the semiquinone is again greater than that to the fully reduced enzyme, the latter reaction occurs much more readily than with either D-amino acid oxidase or oxynitrilase, as illustrated in Figure 5b.

Effect of pH on the Semiquinoid Spectra of Glucose Oxidase. When the EDTA light illumination with glucose oxidase is carried out at high pH values, the resulting spectral change is practically identical with that described above for the semiquinoid forms of oxynitrilase and D-amino acid oxidase. This is illustrated in Figure 6 at a pH of 10.35. [A similar effect has been noted previously on dithionite titration of the enzyme at pH 8.5 (Massey *et al.*, 1966)]. Again the spectral change was found to be correlated with the practically quantitative conversion to a free radical species. Similarly, as with D-amino acid oxidase and oxynitrilase, the conversion to the free radical occurred with relative ease but continued illumination for many days resulted in little further conversion to the fully reduced enzyme.

The dramatic change in spectral form of the radical species of the same enzyme as a function of pH obviously suggested that the two forms may represent two different ionic forms of the flavin coenzyme. At intermediate pH values spectral changes on EDTA light irradiation were clearly due to mixtures of the two species shown in Figures 1 and 6. The low pH form is most easily estimated by its high absorbancy at 600 mμ; the high pH form by its peak at 400 mμ. The observed extinction coefficients at these two wavelengths for the maximal radical produced in various experiments are shown in Figure 7. From these results it is seen that at pH values below 6 essentially only the blue radical is produced and that at pH values above 9 essentially only the red radical is formed. The midpoint of the transition between these two species is between pH 7.4 and 7.5 as estimated either by the extinction values at 600 or 400 mμ.

Glucose oxidase is a particularly accommodating

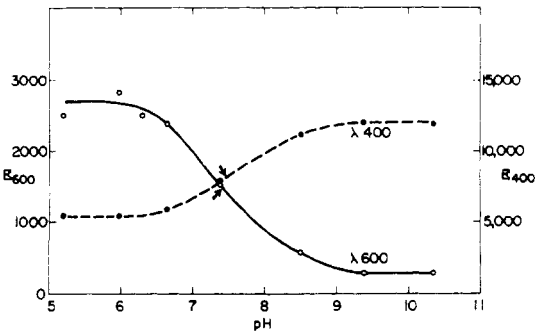

FIGURE 7: Effect of pH on the experimental molar extinction coefficients at 400 and 600 mμ achieved during EDTA light irradiation of glucose oxidase. Experiments were carried out as in Figures 1 and 6 and the values of absorbancy at 400 and 600 mμ reached at maximal conversion to semiquinone converted to molar extinction coefficients. The latter values were based on the previously estimated value of 1.41×10^4 l. $mole^{-1}$ cm^{-1} at 450 mμ of the oxidized enzyme (Swoboda and Massey, 1965). The pH values were measured at the end of each experiment after reoxidation with air.

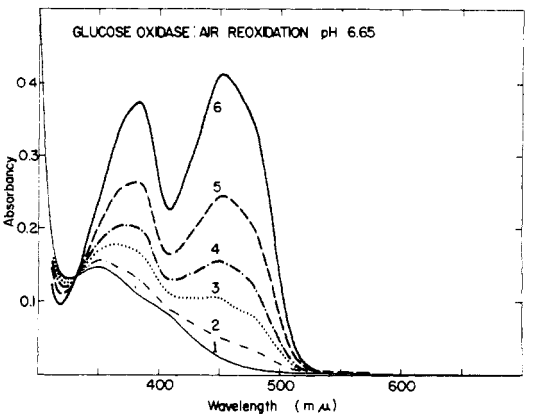

FIGURE 8: Spectra obtained on reoxidation of fully reduced glucose oxidase. The enzyme was converted to the fully reduced form by illumination for 40 hr in the presence of 0.15 M phosphate and 0.06 M EDTA, pH 6.65 (curve 1). Small amounts of air were then admitted to the evacuated anaerobic cuvet and spectra recorded (curves 2–5). Finally, the cuvet was mixed thoroughly with air; the resultant spectrum (curve 6) was identical with that of the original oxidized enzyme.

enzyme for a study of such pH effects as it can be maintained at pH values between 3.5 and 10.5 for long times without loss of activity. Such stability is not shown by all the flavoproteins studied. However, EDTA light irradiation of D-amino acid oxidase at pH 6.0 and oxynitrilase at pH 5.7 failed to show any evidence for the formation of the blue radical; spectral changes similar to those shown in Figures 3 and 4 were obtained.

Reoxidation of Fully Reduced Enzymes with the Exception of Azotobacter Flavoprotein, Where Reoxidation Was Slow. In all the cases studied the air reoxidation of either the semiquinoid or fully reduced enzymes was rapid and the original oxidized enzyme spectrum was regained. With the anaerobic spectrophotometer cells used, small amounts of air can be admitted and the reoxidation followed in detail. Such an experiment with glucose oxidase is shown in Figure 8. The enzyme had been allowed to become fully reduced by prolonged illumination in the presence of EDTA. On admitting air gradually the intermediate spectra shown were obtained. It can be seen that the results can be accounted for solely in terms of two species, fully reduced and fully oxidized, without any significant formation of the semiquinone. Furthermore enzyme could be left at a stage of reoxidation equivalent to curve 4 or 5 for several hours without the development of the absorption spectrum of the semiquinone. This experiment effectively rules out the possibility that the production of semiquinone by EDTA light irradiation is due to the primary reduction of enzyme to the fully reduced state followed by dismutation with oxidized enzyme to the radical (EFAD + $EFADH_2$ ⇆ 2EFADH). Such dismutation reactions have been shown to occur with L-amino acid oxidase and D-amino acid oxidase but they occur over a period of days rather than minutes.

EDTA Light Irradiation of Lipoyl Dehydrogenase and Glutathione Reductase. Previous studies (Massey and Veeger, 1961; Massey and Williams, 1965) have shown that these two enzymes possess an oxidation-reduction-active disulfide grouping in addition to the flavin prosthetic group, and that the half-reduced forms of these enzymes have spectra different from either of the two forms described above. Furthermore the spectra were identical whether produced by reduction by substrates or by dithionite; neither treatment resulted in the appearance of an epr-detectable free radical (Massey and Gibson, 1964). This spectral form has been ascribed to an interaction of a flavin radical and sulfur radical at the active center. Irradiation of these enzymes in the presence of EDTA resulted in the very slow production of the spectrum of half-reduced enzyme, identical with that seen previously with substrate or by partial reduction with dithionite. Again, no free radical could be detected by epr spectrometry.

Quantitative Determination of Radical Concentration formed by EDTA Light irradiation. In separate experiments the appearance of epr-detectable free radicals was monitored by light irradiation of enzymes in anaerobic cylindrical quartz cells. These experiments are difficult to compare directly with the optical spectral experiments already described, since the conditions of enzyme concentration and light irradiation were necessarily different. However, in all cases the appearance and disappearance of free radical during the process

TABLE I: Quantitative Determination of Free-Radical Concentration of Flavoproteins during EDTA Light Irradiation.[a]

Enzyme (M)	Flavin Concn (M)	Free-Radical Concn by Epr Spectrometry (M)	
Glucose oxidase, citrate (0.055), phosphate (0.13), EDTA (0.025), pH 5.3	1.42×10^{-4}	35 min[b]	1.05×10^{-4}
		50	9.4×10^{-5}
		65	6.6×10^{-5}
Glucose oxidase, pyrophosphate (0.038), EDTA (0.034), pH 9.3	1.42×10^{-4}	15	6.3×10^{-5}
		30	1.10×10^{-4}
		50	1.28×10^{-4}
		80	1.50×10^{-4}
		240	1.44×10^{-4}
D-Amino acid oxidase, pyrophosphate (0.08), EDTA (0.033), pH 8.5	1.55×10^{-4}	68	9.3×10^{-5}
		93	1.10×10^{-5}
Oxynitrilase, phosphate (0.033), EDTA (0.1), pH 8.0	2.5×10^{-4}	72 hr	2.0×10^{-4}
L-Amino acid oxidase, Tris (0.14), EDTA (0.085), pH 7.4	4.7×10^{-5}	6	1.5×10^{-5}
		12	5.0×10^{-5}
		16	5.0×10^{-5}
		30	8.5×10^{-6}
Azotobacter flavoprotein, phosphate (0.055), EDTA (0.12), pH 7.3	2.7×10^{-4}	10 days	2.4×10^{-4}

[a] For conditions of illumination and epr spectrometry see Materials and Methods section and text. [b] Duration of light irradiation.

of continued illumination was readily correlated with the visible greenish-blue or red colors produced. Furthermore it can be seen from the results presented in Table I that in many cases quantitative or almost quantitative conversion to the semiquinone occurred as a result of irradiation, and that those enzymes which were not too difficult to reduce to the fully reduced form (glucose oxidase at low pH, L-amino acid oxidase) were also the ones in which the maximal radical concentration was found to develop at short times of illumination and then disappear on prolonged illumination. It is further evident that the enzymes which it was found difficult to reduce beyond the semiquinoid level in the optical spectral experiments were also the ones which maintained high levels of free radical in the epr experiments. Comparison of epr spectra of radicals produced with glucose oxidase and D-amino acid oxidase by either EDTA light irradiation or by partial reduction with dithionite showed that the epr spectra were closely similar with respect to line shape and power saturation behavior. These studies will be published in detail separately (G. Palmer and V. Massey, manuscript in preparation).

Discussion

It is clear from the results presented here that the spectra of flavoprotein semiquinones so far examined fall into either of two classes. The first of these, which we shall refer to as the blue radical, has a spectrum similar to that originally described by Beinert (1956) for the semiquinoid form of FMN. The second class, which we shall refer to as the red radical, has very characteristic absorption properties, with very little absorption at 600 mμ, a peak at 485 mμ, and another peak with very high extinction coefficient in the region of 370 mμ.[2] Most of the red radicals have a third rather sharp absorption band at 400 mμ. An exception is the radical form of L-amino acid oxidase which lacks the 400-mμ peak. However in this case the "370-mμ peak" is located at 386 mμ; the possibility exists that the 400-mμ band in this case is hidden in the intense absorption of the shorter wavelength band. With glucose oxidase both types of absorption spectrum are found, depending on the pH. The results shown in Figure 7 give the midpoint of the transition as pH 7.4–7.5. It is tempting to ascribe this effect to the ionization of the isoalloxazine ring system of the flavin. From potentiometric studies Michaelis and Schwarzenbach (1938) estimated a pK of 6.5 for the conversion of neutral flavin semiquinone to the anionic species. It

[2] We have previously referred to these two spectra as types A and B, respectively (Palmer and Massey, 1966), but for simplicity refer to them here by their visible colors.

would be expected that such a pK value may be affected considerably by the influence of ionized residues of the specific protein to which the flavin is bound. Such a phenomenon has in fact been described in the case of D-amino acid oxidase for the oxidized enzyme where the pK was determined as 9.50 compared to the value of 10.5 found for the free coenzyme (Massey and Ganther, 1965). Thus it is feasible that the two spectral species described may be due simply to ionization of the isoalloxazine nucleus, the neutral flavin radical having a strong absorbancy at 600 mμ and appearing blue–green in color, the anionic radical having very little absorbancy at 600 mμ and appearing reddish in color. In this connection it is interesting to note that a spectrum has not yet been ascribed to the anionic radical species of free flavin coenzymes or simpler derivatives. Significantly, it is stated by Ehrenberg *et al.* (1965) that although higher concentrations of free radicals were obtained in mixtures of oxidized and reduced flavins at high pH values than at neutral pH, considerably less long wavelength absorption was found. This is indeed suggestive that the anionic form of flavin radicals may have only weak absorption in the wavelength region hitherto considered typical of the semiquinone. While this explanation of the two different types of semiquinone spectra is thus very plausible, it must remain tentative until other flavoproteins can be found which exhibit the same phenomenon as glucose oxidase.[3]

Epr studies have also been used under various conditions (room temperature frozen samples; 9- and 35-Gc field modulation) with all the flavoproteins listed in Table I. The results of this study, to be published separately (G. Palmer and V. Massey, manuscript in preparation), suggest that the two classes of semiquinones differentiated on the basis of their optical spectra can be similarly classified into two categories on the basis of the line shape and saturation behavior of their epr spectra, the epr spectrum of the red species having a peak-to-peak width some 4 gauss less than the blue species.

The results presented here have interesting implications concerning the mechanism of the photochemical reduction reaction with EDTA and possibly other potential electron donors. With free flavins this reaction results in the rapid conversion to fully reduced flavin. The appearance of free radical in such reactions (Massey and Palmer, 1962) could be readily accounted for by the known dismutation reaction between oxidized and reduced flavin ($F + FH_2 \leftrightarrows 2F\dot{H}$). Thus with the study of model systems it was not possible to determine if the reduction reaction occurred through a flavin semiquinone intermediate or whether the reduction was a two-electron process. However in the present study of the reaction with flavoproteins it seems inescapable that the reduction proceeds by a photoinduced free radical mechanism, since the rate of formation of the flavoprotein semiquinones is considerably greater on illumination in the presence of EDTA than it is from a mixture of oxidized and reduced flavoprotein.

Finally, we wish to draw attention to the fact that the classification of flavoprotein semiquinones into the blue and red radical species is not limited to the enzymes we have studied here. Table II includes a list of enzymes whose semiquinone spectra have also been reported and which from published epr data we may reasonably conclude to be true semiquinones. This table also includes information on whether the optical spectral changes occurring on the addition of reducing substrates are the same as the semiquinone produced by dithionite titration or illumination in the presence of EDTA. A knowledge of the spectral characteristics of the true semiquinoid forms of glucose oxidase and D-amino acid oxidase has previously permitted the exclusion of these forms from a role in the catalytic reaction mechanism of these enzymes, since in neither case was the semiquinoid form reactive with substrate (Massey and Gibson, 1964; Massey *et al.*, 1965). Similarly recent work from this laboratory (V. Massey and B. Curti, unpublished) has shown that the semiquinoid form of L-amino acid oxidase cannot be involved in catalysis, since this form does not react with L-amino acids. Thus the reaction mechanism previously suggested by Wellner and Meister (1961) for this enzyme, which involves the participation of the uncomplexed free radical species, cannot be correct. The reported spectra of substrate- and dithionite-produced intermediates with the Old Yellow Enzyme (Nakamura *et al.*, 1965) suggest strongly that both substrate and dithionite give rise to the same absorption spectrum, which is very similar to that found for the red semiquinoid form of several enzymes in the present study. This suggests that the reaction mechanism of the Old Yellow Enzyme may indeed involve an uncomplexed semiquinoid form of the flavin in catalysis. A similar conclusion might be reached concerning the electron-transferring flavoprotein, which from published spectra has elements of the characteristic absorption properties of the red radical (Beinert and Frisell, 1962). While much further work remains to be done to clarify the issue, it would appear that the blue radicals produced by substrate with TPNH-cytochrome *c* reductase (Masters *et al.*, 1965), ferredoxin-TPN^+ reductase (G. Foust, V. Massey, and G. Palmer, unpublished), and TPNH-thioredoxin reductase (G. Zanetti, C. H. Williams and V. Massey, unpublished) may also be involved in enzyme catalysis. The two cases of enzymes in which the same intermediate spectrum is produced whether the reducing agent be dithionite, EDTA–light, or substrate, and which show no epr signal (lipoyl dehydrogenase and glutathione reductase) have clearly different spectra than either of those found with the more simple flavoproteins. This phenomenon is entirely in keeping with the fact that they contain a second prosthetic group (an oxidation–reduction-active disulfide) which is intimately involved in the spectra of the half-reduced intermediates found with these two enzymes.

[3] Added in proof: We have recently learned (F. Mueller and A. Ehrenberg, personal communication) that a spectrum very similar to that of the red radical described in this paper has been found for the anionic radical of a simple derivative of lumiflavin.

TABLE II: Spectral Characteristics of Flavoprotein Semiquinones.

Enzyme	$Na_2S_2O_4$ or EDTA–Light Produced Intermediate[a]	Substrate-Produced Intermediate
Glucose oxidase, low pH	Blue[a–c] (epr+)	None seen[d,e]
Glucose oxidase, high pH	Red[a,c] (epr+)	
D-Amino acid oxidase	Red[a–c] (epr+)	Different (epr−)[b]
L-Amino acid oxidase	Red[a] (epr+)	Different[f]
Oxynitrilase	Red[a] (epr+)	
Azotobacter flavoprotein	Blue[a,g] (epr+)	
TPNH-thioredoxin reductase	Blue[h]	Blue[h]
TPN-ferredoxin reductase	Blue[i]	Blue (epr+)[i]
Old Yellow Enzyme	Red[j] (epr+)	Red[j] (epr+)
Acyl-CoA dehydrogenases	Blue[k] (epr+)	Different[k] (epr−)
Electron-transferring flavoprotein	—	Probably red[l]
TPNH-cytochrome *c* reductase	—	Blue (epr+)[m]
Lipoyl dehydrogenase	High absorbancy at 450 mμ, long wavelength shoulder (epr−)[a–c]	Identical with $Na_2S_2O_4$ intermediate (epr−)[a–c]
Glutathione reductase	Very similar to lipoyl dehydrogenase[a,c,n]	Identical with EDTA light intermediate[a,c,n]

[a] This paper. [b] Massey and Gibson (1964). [c] Massey *et al.* (1966). [d] Gibson *et al.* (1964). [e] Nakamura and Ogura (1962). [f] V. Massey and B. Curti, unpublished. [g] Beinert (1965). [h] G. Zanetti, C. H. Williams, and V. Massey, unpublished. [i] G. Foust, V. Massey, and G. Palmer, unpublished. [j] Nakamura *et al.* (1965). [k] Beinert and Sands (1961). [l] Beinert and Frisell (1962). [m] Masters *et al.* (1965). [n] Massey and Williams (1965). [o] The blue species is characterized by high absorption at 570–600 mμ (*cf.* Figures 1 and 2). The red species is characterized by very little absorption at 600 mμ and peaks at 485 mμ and in the vicinity of 400 and 370 mμ (*cf.* Figures 3–5). The spectra of the half-reduced forms of lipoyl dehydrogenase and glutathione reductase are very similar, but quite distinct from the other enzymes listed (*cf.* footnotes *b*, *c*, and *n*).

It is clear that a convenient method for the quantitative conversion of flavoproteins to the free radical form would aid greatly in mechanistic studies of this group of enzymes. The method of photoirradiation described in this paper appears to us to offer many advantages over those previously employed. The only disadvantage that the method appears to have is that certain enzymes such as *Azotobacter* flavoprotein are reduced very slowly. However the possibility should be explored that other potential electron donors may be even more efficient than EDTA in photochemical reactions.

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The Kinetics of Thiocyanate Binding to Aquocobalamin*

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ABSTRACT: The apparent binding and dissociation rate constants for the thiocyanate–aquocobalamin reaction are determined over the pH range 2.7–10.0. The rate of thiocyanate binding to hydroxocobalamin is found to be negligible compared to the rate of binding to aquocobalamin.

The kinetic data are interpreted in terms of two mechanisms corresponding to the SN2 and SN1 mechanisms considered for simpler Co(III) complexes. Both mechanisms can fit the kinetic data.

Several features of the structure of aquocobalamin make a study of the kinetics of ligand binding to the Co(III) atom in this molecule of particular interest. (1) The three-dimensional structure of aquocobalamin is known. The vitamin B_{12} structure has been determined (Hodgkin, 1958), and aquocobalamin presumably differs only in having a water molecule instead of a cyanide ion coordinated to the Co(III) atom (Kaczka *et al.*, 1951). (2) The corrin ring–Co(III) complex in aquocobalamin is not surrounded by other portions of a larger molecule as is the porphyrin ring–Fe(III) complex in metmyoglobin and methemoglobin. (3) The planar, tetradentate corrin ring prevents any *cis–trans* isomerization of the remaining two ligands. (4) The benzimidazole group bound to the fifth site on the Co(III) atom prevents any mechanism involving backside attack. (5) Many ligands displace the water molecule on the sixth coordination site (George *et al.*, 1960). The reactions are rapid and easily followed spectrophotometrically. (6) A comparison can be made with the kinetics of binding of various anions to simpler Co(III) complexes.

Hydroxocobalamin is formed by the ionization of the coordinated water molecule of aquocobalamin (Buhs *et al.*, 1951). This transition of aquocobalamin to hydroxocobalamin will introduce a pH dependence

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